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Peripheral, but not central nervous system, type I interferon expression in mice in response to intranasal vesicular stomatitis virus infection

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Type I interferon (IFN) is critical for resistance of mice to infection with vesicular stomatitis virus (VSV). Wild type (wt) VSV infection did not induce type I IFN production *in vitro* or in the central nervous system (CNS) of mice; however IFN-β was detected in lungs, spleen, and serum within 24 h. The M protein mutant VSV, T1026R1 (also referred to as M51R), induced type I IFN production *in vitro* and in the CNS, with poor expression in spleens. In addition, VSV T1026R1 was not pathogenic to mice after intranasal infection, illustrating the importance of IFN in controlling VSV replication in the CNS. Experiments with chemical sympathectomy, sRAGE, and neutralizing antibody to VSV were performed to investigate the mechanism(s) utilized for induction of peripheral IFN; neither sRAGE infusion nor chemical sympathectomy had an effect on peripheral IFN production. In contrast, administration of neutralizing antibody (Ab) readily blocked the response. Infectious VSV was transiently present in lungs and spleens at 24 h post infection. The results are consistent with VSV traffic from the olfactory neuroepithelium to peripheral lymphoid organs hematogenously or via lymphatic circulation. These results suggest that VSV replicates to high titers in the brains of mice because of the lack of IFN production in the CNS after intranasal VSV infection. In contrast, replication of VSV in peripheral organs is controlled by the production of large amounts of IFN. *Journal of NeuroVirology* (2007) **13,** 433–445.

Keywords: dendritic cells; iron production; viral encephalitis

Introduction

The rhabdovirus vesicular stomatitis virus (VSV) causes severe central nervous system (CNS) pathology when administered to mice intranasally (i.n.) (Huneycutt *et al*, 1994; Sabin and Olitsky, 1938). Immunocompetent mice exhibit high morbidity and mortality at low doses of virus, succumbing to infection between 6 and 11 days post infection (p.i.). In contrast, inoculation of immunocompetent mice with high doses of VSV by the intramuscular, subcutaneous, or intraperitoneal routes generally leads to limited viral replication and no apparent disease (Reiss and Aoki, 1994). Similarly, intravenous (i.v.) inoculation of mice with high doses of VSV leads to limited viral replication in the periphery, but can cause CNS pathology if virus gains access to the brain. One of the major factors controlling VSV replication

in peripheral organs is interferon (IFN); type I IFN receptor (IFNAR1)-deficient mice succumb rapidly to i.v. infection by VSV (100% mortality within 3 to 6 days p.i.), with high viral titers in peripheral organs (Muller *et al*, 1994). Similarly, STAT1-deficient mice succumb very rapidly to i.v. infection with VSV, with 100% mortality occurring within 2 days p.i. and high viral titers observed in the liver (Durbin *et al*, 1996).

The ability of VSV to replicate in the mouse CNS to high titers while replication is controlled in the periphery suggests that either IFN is not produced in the CNS or neurons are not sensitive to IFN-mediated inhibition of VSV. Neurons are exquisitely sensitive to the antiviral effects of type I IFN, as both cultured lines and primary mouse neurons restrict VSV replication after pretreatment with IFN-β (Trottier *et al*, 2005). Such results suggest a lack of IFN induction in the mouse brain may contribute to the high pathogenicity of VSV in neural tissues.

The VSV M protein has been shown to play an important role in suppressing IFN induction by VSV infection of L929, HeLa, and Baby Hampster Kidney (BHK) cells *in vitro* (Ahmed *et al*, 2003; Stojdl *et al*, 2003). VSV T1026R1 has been reported as an IFN-inducing mutant of VSV due to a mutation in the M protein (methionine to arginine in amino acid 51; M51R) (Francoeur *et al*, 1980, 1987). Subsequent studies using the cloned M gene from wild-type (wt) and T1026R1 virus have shown that wt M inhibits transcription of cellular genes including IFN-β, whereas M from T1026R1 does not (Ferran and Lucas-Lenard, 1997). wt M has also been shown to be able to block nucleocytoplasmic transport, in contrast to the M51R mutant (Petersen *et al*, 2000), and to be associated with induction of apoptosis (Gaddy and Lyles, 2005).

Host induction of type I IFNs during innate immune responses to antigens, viral, fungal, or bacterial challenge, to single-stranded RNA (ssRNA), double-stranded (dsRNA), and CpG in DNA has been associated with the triggering of a family of receptors termed Toll-like receptors (TLRs) for their sequence similarity to Toll, which was first identified in the fruit fly (reviewed in Boehme and Compton, 2004; Conzelmann, 2005; Kawai and Akira, 2005; Yang *et al*, 2005). Although many cell types are able to express some TLRs, research has indicated that the plasmacytoid dendritic cell (pDC) maintains a central role in the TLR-triggered IFN response (reviewed in Malmgaard, 2005). With viral challenges, type 1 IFN not only regulates host responses, but also can suppress viral replication. ssRNA viruses, like VSV, have been shown to elicit pDC activation through TLR7 and TLR8 (Diebold *et al*, 2004; Heil *et al*, 2004; Lund *et al*, 2004; Malmgaard, 2005), which signal through the MyD88 pathway (reviewed in Crozat and Beutler, 2004). A role for TLR 4 in VSV activation of pDCs has been suggested (Georgel *et al*, 2007; Jiang *et al*, 2005), as has the requirement for MyD88 signaling in VSV infection of the neuroepithelium (Zhou *et al*, 2007).

In the case of VSV, activation of IFN production by pDCs has been demonstrated *in vitro* and *in vivo* following peripheral inoculation (Barchet *et al*, 2002; Lund *et al*, 2004).

The sympathetic nervous system (SNS) (Blalock, 1989; Madden, 2003; Weihe *et al*, 1991) and the hypothalamic-pituitary-adrenal axis (reviewed by Bailey *et al*, 2003; McEwen *et al*, 1997; Silverman *et al*, 2005) regulate many peripheral immune functions in health and disease. Thus, VSV infection of the CNS might activate host responses through the SNS. A common way to evaluate the contribution of the SNS to host immune responses is by chemical sympathectomy with 6-hydroxydopamine (6-OHDA). In excitotoxicity or cerebrovascular ischemic events, traumatic insults to the brain, the host is alerted by a number of pathways, including the release of S100B by astrocytes (Herrmann and Ehrenreich, 2003). The receptor for S100B (and also for many other proinflammatory molecules including High Mobility Group B-1 (HMGB-1). (Chen *et al*, 2004) is RAGE (Hofmann *et al*, 1999), originally characterized as the receptor for advanced glycation endproducts in hyperglycemia (Neeper *et al*, 1992). Schmidt's group has elucidated many proinflammatory pathways and diseases in which RAGE contributes by the use of the soluble receptor, sRAGE (Lalla *et al*, 2000; Liliensiek *et al*, 2004; Yan *et al*, 2003).

In this report, we analyze the ability of VSV to induce IFN synthesis in the periphery and CNS. Infection of NB41A3 neuroblastoma cells by VSV did not result in IFN production, whereas infection by Newcastle disease virus (NDV) did, suggesting that neuronal cells were capable of producing type I IFN, but not in response to wt VSV infection. No IFN mRNA could be detected in the brains of VSV-infected mice as late as 6 days p.i. In contrast, i.n. infection by VSV induces high levels of type I IFN mRNA in spleens and lungs, and protein detected by enzyme-linked immunosorbent assay (ELISA) and bioassay in the serum of mice 24 h p.i. To determine the mechanism of IFN induction in the spleen, we examined two well-characterized pathways of inflammatory signaling between the CNS and the immune system, the SNS and RAGE; neither was found to contribute to the production of IFN by the spleen. Infectious VSV was detectable in lungs and spleens of VSV-infected mice at 24 h p.i., but little was found in serum. When we used a virus that was mutated in the M protein (T1026R1), IFN mRNA was induced *in vitro* and also in the CNS; T1026R1 virus infection of mice showed low pathogenicity, suggesting that the viral M protein is critical for evasion of host responses. IFN production required hematogenous or lymphatic transfer of infectious virus from the nasal neuroepithelium, because it could be prevented by passive immunization with neutralizing antibody (Ab). The results suggest that the M protein of VSV blocks IFN synthesis in neurons and other infected CNS cells, whereas peripheral IFN production

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is not antagonized by the viral M protein. The failure to elicit IFN in the CNS may be attributable to the lack of expression of TLR7 by neurons, and contributes to the disease pathogenesis (Trottier *et al*, in preparation).

Results

VSV does not induce type I IFN in infected neuroblastoma cells

NB41A3 cells, a neuroblastoma cell line, have been shown to have many properties of neurons, including the ability to form neurotubules and neurofilaments and in the storage and release of catecholamines (Breakfield *et al*, 1975; Schubert *et al*, 1969); they constitutively express neuronal nitric oxide synthase (NOS-1), IFN-γ receptor (IFN-γR), interleukin-12 receptor (IL-12R), and the *N*-methyl-D-aspartate (NMDA) receptor (Bi and Reiss, 1995; Ireland and Reiss, 2004; Komatsu *et al*, 1996). The effects of type I and II IFN on these cells have also been studied; the inhibitory effects of these cytokines on VSV replication are similar in these cells and in primary murine neurons (Chesler *et al*, 2003; Trottier *et al*, 2005).

To determine if NB41A3 cells were able to synthesize type I IFN in response to virus infection, NB41A3 cells were infected with either VSV or NDV at various multiplicities of infection (m.o.i.). At 12 and 24 h p.i., cell supernatants were harvested and assayed for type I IFN by bioassay. IFN bioassays take advantage of the stability of type I IFN to both ultraviolet (UV) irradiation and to acid pH. Test samples were treated with HCl to pH 2 and then neutralized, or (in the case of small volumes) with UV light. Treated samples were then applied to an indicator cell line, in this case NB41A3 cells. If samples contained IFN, the cells were able to enter an antiviral state, and thus resist subsequent challenge with virus. Thus results of the IFN bioassay were expressed as plaque-forming units (PFU)/ml or log PFU/ml. In the absence of IFN, the sample would yield a high viral titer, whereas if IFN were present in the serum or cell culture supernatant tested, the indicator cell line would be protected from infection, and a low viral titer would be apparent. Supernatants from NDV-infected NB41A3 cells infected at a m.o.i. = 10 had antiviral activity in IFN bioassays when diluted up to 20-fold (Figure 1). At lower moi, IFN production was at or below the level of detection of the assay (~1 unit/ml; not shown) (Figure 1). In contrast, VSV infection of NB41A3 cells did not result in detectable IFN production at 12 and 24 h p.i. (Figure 1). VSV infection of NB41A3 cells resulted in death of all cells within 12 to 16 h (not shown), indicating that further incubation of these cells would not increase the yield of IFN production.

VSV infection of neuroblastoma cells activates IRF-3 signaling pathway

To determine whether neuroblastoma cells sense VSV infection, the ability of VSV to induce IFN
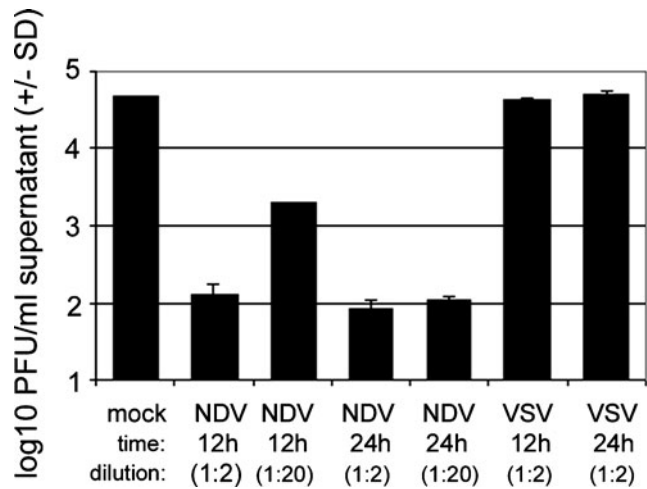

Figure 1 NDV but not VSV induces type I IFN production from neuroblastoma cells *in vitro*. Approximately 2×10^5 NB41A3 cells were infected with NDV or VSV (m.o.i. = 10) or mock-infected with medium for 12 or 24 h. Supernatants were assayed for the presence of type I IFN bioactivity by IFN bioassay. The presence of IFN in supernatants was indicated by inhibition of VSV replication, shown as a reduction of viral titers in supernatants. Dilution represents fold dilution (from 1 ml) of supernatant added to NB41A3 cells.

regulatory factor-3 (IRF-3) nuclear localization was tested. NB41A3 cells were infected for 4 h with wt VSV and assayed by immunofluorescence. Infection with VSV induced nuclear localization of IRF-3 (Figure 2), indicating that VSV infection was recognized by neuroblastoma cells, leading to phosphorylation and nuclear translocation of IRF-3. Both
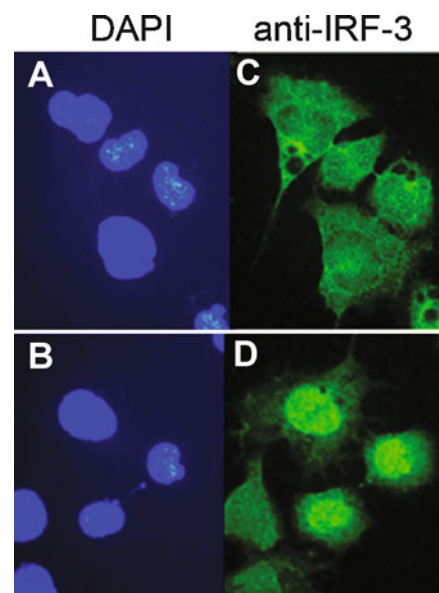

Figure 2 VSV infection of neuroblastoma cells results in nuclear localization of IRF-3. Approximately 2×10^5 NB41A3 cells were mock-infected (**A, C**) or infected with VSV (**B, D**) at an m.o.i. of 10. After 4 h, cells were fixed and nuclear localization of IRF-3 was determined using immunofluorescence (**C, D**). Cells were stained with DAPI (**A, B**) to visualize nuclei.

defective-interfering VSV particles (DI-VSV) and UV-inactivated VSV (UV-VSV) also promoted IRF-3 nuclear localization after infection of NB41A3 cells (not shown). We interpret these experiments to be compatible with an early cellular event during VSV infection leading to activation of IRF-3 signaling; however, IFN-β production is blocked after nuclear translocation of IRF-3 in neuronal cells.

VSV infection does not induce IFN mRNA synthesis in mouse brains

VSV infection of mice after i.n. inoculation results in rapid spread to the brain and resulting encephalitis. To determine whether i.n. VSV infection induced a type I IFN response in mouse brains, male BALB/c mice were infected intranasally with VSV. Brains from VSV-infected mice were harvested on days 1, 3, and 6 p.i. and analyzed for the presence of type I IFN mRNA. No IFN-β mRNA was detected in VSV-infected mouse brains (Figure 3A), even at times when mice were exhibiting signs of morbidity (weight loss, lack of grooming, reduced activity, and hind-limb paralysis in some mice) due to CNS infection (day 3) and when mice first began to succumb to disease (day 6). Similar results were observed with IFN-α mRNA (not shown). The results are consistent

with a failure of CNS parenchymal tissue to synthesize IFN mRNA.

Intranasal VSV infection induces splenic and serum IFN

To analyze the peripheral IFN response to VSV infection, mice were infected i.n. with VSV and spleens and serum harvested at 24 h p.i. reverse transcriptase–polymerase chain reaction (RT-PCR) assays performed on spleen total RNA revealed the presence of IFN-β mRNA at 24 h p.i. (Figure 3B). Similar results were observed for IFN-α mRNA (not shown). Analysis of mouse serum by bioassay revealed that IFN was present in blood at 24 h p.i. (Figure 3C, *top panel*); quantification of IFN in serum showed that IFN bioactivity corresponded to a concentration of approximately 200 to 1000 units per ml of serum (Figure 3C, *bottom panel*) (Trottier *et al*, 2005). In contrast, mock-infected animals showed no detectable expression of IFN mRNA in the spleen and no IFN bioactivity in serum.

Mutant VSV replicates and induces IFN in neuronal cells in vitro and in vivo

VSV T1026R1 has been reported as an IFN-inducing mutant of VSV due to the M51R mutation in the M protein (Francoeur *et al*, 1980, 1987). To test the ability of this virus to replicate in neuronal cells, NB41A3 cells were infected with wt VSV or VSV T1026R1 at m.o.i. = 0.1. At 8, 24, 32, and 36 h p.i., supernatants were harvested and subjected to plaque assay to determine viral titers. VSV T1026R1 replicated to high titers in NB41A3 cells, similar to that observed for wild type VSV (Figure 4A). Cells infected with wt virus appeared apoptotic by 14 to 18 h p.i., whereas cells infected with T1026R1 virus showed significant cytopathic effect at 24 h p.i. and were dead by 28 h p.i. (data not shown). These results indicate that VSV T1026R1 can replicate to high titers in neuronal cells *in vitro*.

To determine the ability of mutant VSV to induce IFN production by neuronal cells, NB41A3 cells were infected with VSV, NDV, or VSV T1026R1 for 24 h at various m.o.i. and cell supernatants were assayed for the presence of IFN (Figure 4B). NDV induced type I IFN production from NB41A3 cells at an m.o.i. = 10, while wt VSV did not induce detectable IFN bioactivity. Mutant VSV T1026R1 induced type I IFN bioactivity, even at m.o.i. as low as 0.1 (Figure 4B). These results indicated that mutant VSV T1026R1 was able to induce type I IFN production by neuronal cells and are consistent with wt VSV M protein blockade of cellular transcription and nuclear transport, as has been described for non-neuronal host cells (Ahmed *et al*, 2003; Ferran and Lucas-Lenard, 1997; Petersen *et al*, 2000; Stojdl *et al*, 2003).

To investigate the pathogenicity of and IFN induction by VSV T1026R1 *in vivo*, groups of 10 male BALB/c mice were infected i.n. with wt or T1026R1 VSV. wt VSV induced severe morbidity (Figure 4D)
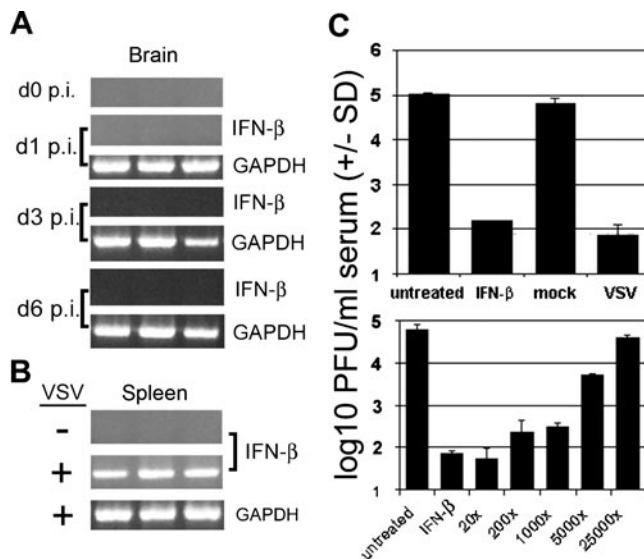

Figure 3 Intranasal infection of mice with VSV induces no detectable IFN mRNA expression in the brain but high levels of type I IFN mRNA in the spleen and IFN bioactivity in serum. Mice were mock-infected (receiving PBS) or infected with 1×10^4 PFU VSV i.n. and sacrificed at various times p.i. Total RNA was isolated from brains and subjected to RT-PCR to detect IFN-β mRNA at days 1, 3, or 6 p.i. (**A**). RT-PCR to detect IFN-β mRNA was also performed on total RNA from mock- or VSV-infected mouse spleens taken at 24 h p.i. (**B**). Mouse sera collected from mock- and VSV-infected mice was subjected to IFN bioassay to detect the presence of type I IFN (**C**, *top panel*). Serial dilutions of serum were analyzed to semiquantify IFN levels in VSV-infected mouse sera (**C**, *bottom panel*). IFN controls in bioassays were used at a concentration of 100 units/ml. GAPDH served as housekeeping gene control for RT-PCR reactions.
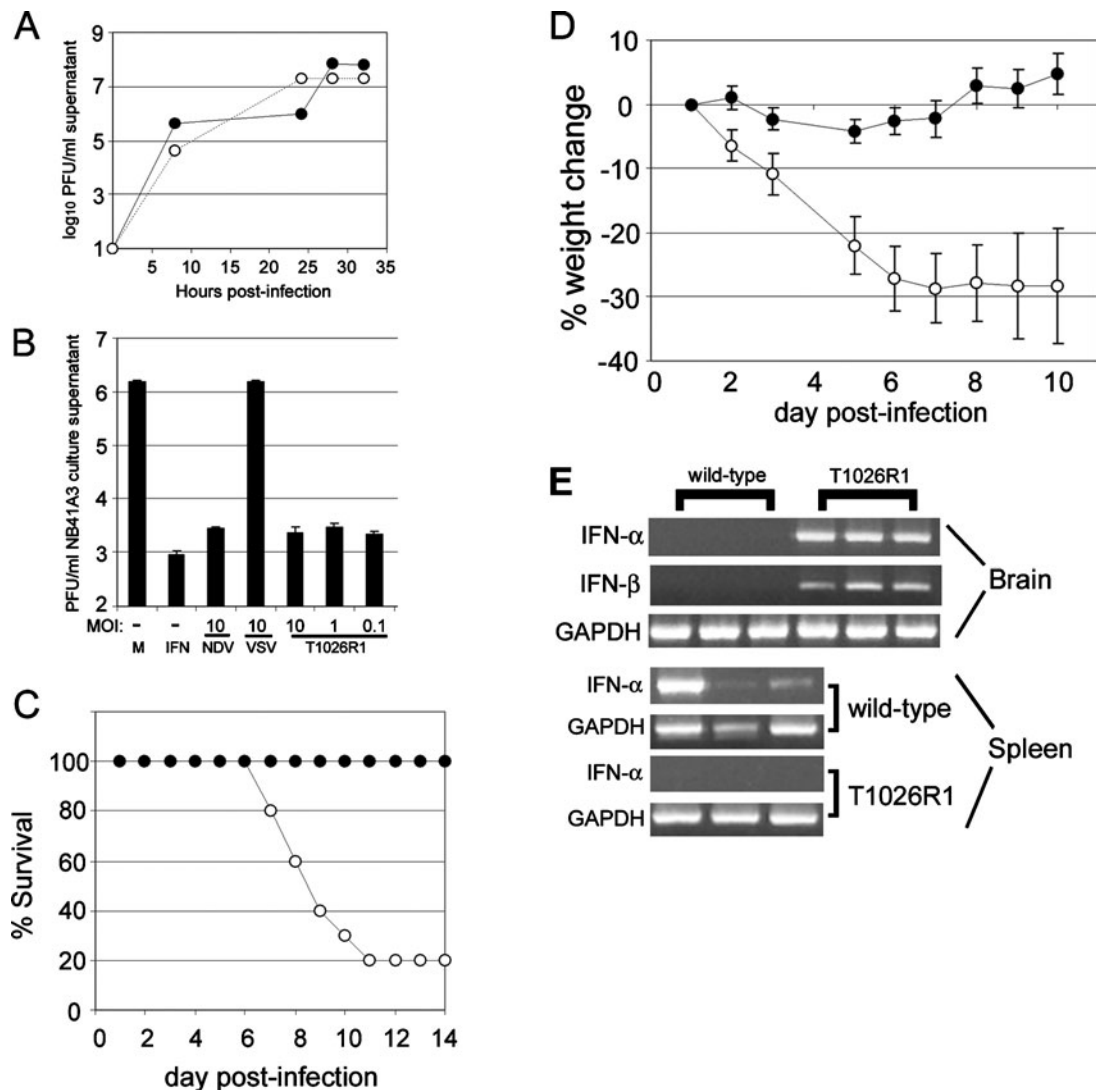
Figure 4 Pathogenicity of mutant VSV inversely correlates with ability to induce type I IFN. (**A**) NB41A3 cells were infected with wild type VSV (*open circles*) or VSV T1026R1 (*filled circles*) and supernatants were analyzed for viral titer by plaque assay at various times post-infection. (**B**) Approximately 2×10^5 NB41A3 cells were also mock-infected (M), or infected with NDV, wild-type VSV, or VSV T1026R1 at various m.o.i. for 24 h. Supernatants were subjected to IFN bioassay to determine presence of type I IFN. For *in vivo* studies, mice ($n = 10$ per group) were infected intranasally with VSV-Indiana (100 PFU) or VSV T1026R1 ($4 * 10^6$ PFU) and monitored for weight loss (**D**) and mortality (**C**). (**E**) VSV T1026R1 was apathogenic in mice infected i.n. Some animals were sacrificed at 24 h p.i. and tissues were harvested. RT-PCR was performed on total RNA from brains (*top panel*) and spleens (*bottom panel*) to detect IFN-β and -α mRNA in wt VSV and VSV T1026R1 infected mice. Data shown are from three individual mice, representative of two experiments.

and 80% mortality (Figure 4C) upon intranasal infection of mice with 100 PFU. In contrast, VSV T1026R1 induced virtually no morbidity and no mortality after inoculation with 4×10^6 PFU (Figures 4D and 3C). Intranasal VSV T1026R1 infection induced both IFN-α and IFN-β mRNA production in the brain at 24 h p.i., in contrast to wt VSV (Figure 4E, *top panel*). These results suggest that IFN induction in the brain prevents VSV induced encephalitis. Interestingly, no detectable IFN mRNA was detected in spleens of VSVT1026R1 infected mice, despite the high dose given (Figure 4E, *bottom panel*). ELISA analysis of IFN-β in serum indicated that T1026R1 infection induced 170.8 ± 5.71 pg/ml IFN-β, whereas wt VSV

elicited 1283 ± 21.6 pg/ml at 24 h p.i., or a 7.5-fold difference. Thus, the M protein mutation permitted IFN production in neurons *in vitro* and *in vivo*.

How does the periphery know that there is an intranasal infection?
In order to determine how the spleen "learned" that the nasal neuroepithelium and, after 6 to12 h, the olfactory bulb were infected, we investigated the possibility that the SNS was involved. Every lymphoid organ is innervated by sympathetic neurons (Blalock, 1989; Madden, 2003; Weihe *et al*, 1991). Mice were treated with 6-OHDA to establish a chemical sympathectomy and then challenged with VSV i.n. Serum
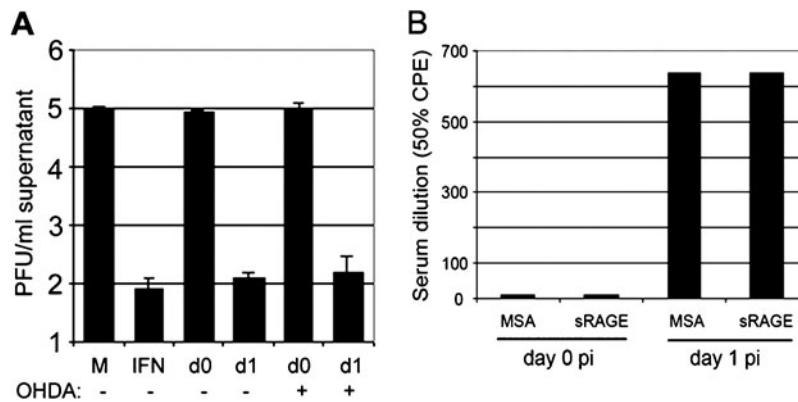
Figure 5   Chemical sympathectomy and infusion of sRAGE. (**A**) Three male BALB/c mice were treated daily with 100 mg/kg/day 6-OHDA and two mice were injected with PBS for 10 days. One control mouse and 2 sympathectomized mice were infected i.n. with 1×10^4 PFU VSV, the remaining mice were mock-infected with PBS. Spleens were assayed for mRNA specific for IFN-βsing IFN bioassay. "M," referring to mock-treated NB41A3 cells and "IFN," referring to IFN-β (100 U/ml)-treated NB41A3 cells, are IFN bioassay controls. "d0" refers to mock-infected mouse serum treated NB41A3 cells, and "d1" refers to VSV-infected mouse serum (taken at 24 h p.i.)-treated NB41A3 cells. There was no influence of sympathetic innervation on the IFN-β response. (**B**) sRAGE, the soluble form of the receptor for S100B, was infused into mice to determine if this proinflammatory pathway contributed to the IFN-β response. Mice were injected with 100 μg mouse serum albumin (MSA) in saline or 100 μg sRAGE immediately prior to i.n. infection with 1×10^4 PFU VSV. At 24 h p.i. blood was obtained from individuals by cardiac puncture, and then serum was analyzed for type I IFN by bioassay. There was no influence of RAGE on the IFN-β response.

was harvested at 24 h p.i. and presence of type I IFN was determined by bioassay. There was no difference between control and 6-OHDA–treated mice in the level of type I IFN present in serum (Figure 5A), or in the level of type I IFN mRNA detected in spleens from these mice (data not shown), indicating that the spleen was not alerted by the SNS.

If the infection in the nasal neuroepithelium and olfactory bulb were perceived of as traumatic by astrocytes, it was possible that S100B would be released (Herrmann and Ehrenreich, 2003). Previous studies in our laboratory had indicated that glial fibrillary acidic protein (GFAP) is readily expressed by astrocytes in the olfactory bulb as early as when viral antigens are first detected in the CNS (Barna *et al*, 1996b), which would be consistent with astrocytic responsiveness to the viral infection. To assess whether S100B was released and could activate splenic macrophages or plasmacytoid dendritic cells through the receptor, the soluble receptor, sRAGE, was obtained from Ann-Marie Schmidt (Columbia University) and administered to mice which were infected with VSV i.n. As shown in Figure 5B, there was no difference between controls (albumin-treated) and sRAGE-treated mice in the production of splenic IFN-βRNA.

Intranasal VSV-infected mice produce IFN mRNA in the lung
Another possibility for how i.n. infection of mice leads to peripheral IFN is via replication of VSV in the lungs. With delivery of large volumes of inoculum, and high titers of virus, VSV can be inhaled into the lung; however, we use 5 μl, a volume essentially restricted to the nasal turbinates. Alterna-

tively, infection may lead to enhanced secretion of fluid by Bowman's glands in the neuroepithelial tissue, which could result in virus being carried into the lower respiratory tract. RT-PCR analysis of lung total RNA revealed the presence of type I IFN mRNA in lungs of infected animals at day 1 p.i. (Figure 6A), suggesting that VSV gains access to the lungs after intranasal administration.

VSV replicates in lung and spleen at 24 h p.i.
To determine if VSV is inducing peripheral IFN synthesis by infecting peripheral tissues, male BALB/c mice were infected i.n. with 1×10^4 PFU VSV and brains, lungs, spleens, and serum were harvested. Tissues homogenates were subjected to plaque assays to determine the presence of infectious VSV. As expected, infectious VSV was detectable in the brain at 24 h p.i. both by plaque assay and by RT-PCR for VSV M RNA (Figures 6B, 7A). VSV was also found in the lungs, albeit at lower levels (Figure 6B), indicating that i.n. infection leads to infection of the respiratory tract. Surprisingly, at 24 h post i.n. application, VSV was found in mouse spleens, at titers similar to that observed in the brain (Figure 6B). Virus was rapidly cleared from the spleen (data not shown). These results suggest that viral replication in peripheral organs leads to induction of IFN. In keeping with this finding, neither UV-VSV nor DI-VSV induced IFN production in the spleen or serum upon i.n. administration (data not shown).

How does VSV get from the neuroepithelium to the spleen?
Although it is possible that VSV is passively carried by fluid in the nasal turbinates to the lung, the
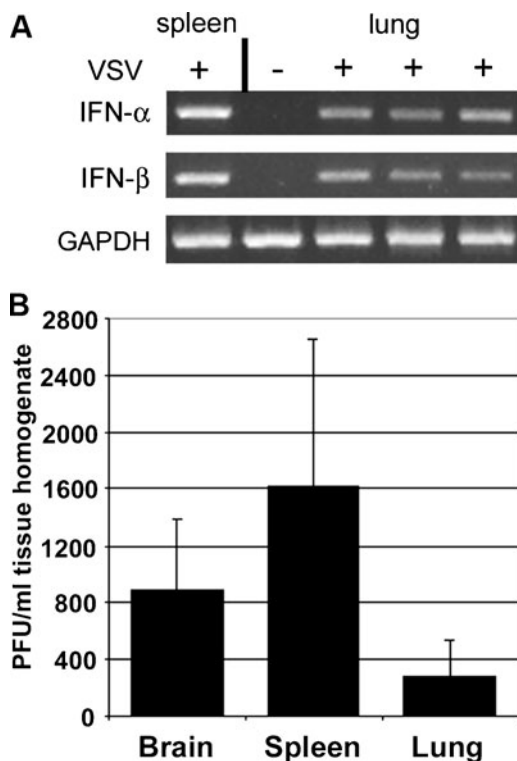
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Figure 6 Intrnasal infection of mice with wt VSV induces type I IFN expression in mouse lungs and results in replication of VSV in mouse brains, spleens and lungs. Mice were infected i.n. with wt VSV (1×10^4 PFU) or mock-infected and sacrificed 24 h p.i. RT-PCR was used to detect IFN-α and IFN-β mRNA expression in the lungs of 3 infected and 1 mock-infected mice (**A**). GAPDH was used as housekeeping gene control. Homogenates from mouse brains, spleens and lungs taken at day 1 p.i. were subjected to plaque assays to detect the presence of VSV in these tissues (**B**; $n = 3$ per group).

mechanism of transport of infectious virus from the nasal neuroepithlium to the spleen was investigated. One possibility we evaluated was the hematogenous route or via lymphatic drainage. If VSV were cell associated, it would be inaccessible to neutralizing Ab. To determine if free (that is, cell-free) virus were transmitted in either plasma or lymphatic fluid, groups of BALB/c mice were injected intraperitoneally (i.p.) with neutralizing sheep anti-VSV Ab or normal sheep serum either 2 h before the i.n. inoculation of VSV or 6 h thereafter. Spleens were harvested at 24 h p.i. and assessed for expression of VSV M and IFN-β mRNA. As shown in Figure 7, prior administration of anti-VSV Ab prevented infection of the spleen and prevented the expression of IFN-β mRNA at 24 h p.i. However, delay of 6 h p.i. prior to treatment with Ab was sufficient to permit a measurable host IFN response. VSV mRNA was not found in all brain mRNA samples tested, which we attribute to the large amount of RNA harvested from whole brains, compared to the low level of viral infection, localized to the olfactory bulb, at 24 h p.i. In more sensitive assays, such as viral replication, infectious virus could

be detected by plaque assay in all samples (data not shown). We interpret these data to be consistent with circulation of VSV by blood or lymph from the neuroepithelium almost immediately after initial inoculation of the virus.

Discussion

Previous studies have revealed the critical importance of the type I IFN pathway in control of VSV replication in mice. IFNAR1 $-/-$ mice and STAT1 $-/-$ mice are highly susceptible to lethal VSV infection, showing high viral titers in organs not normally infected productively in wt mice (Durbin *et al*, 1996; Muller *et al*, 1994). Similarly, dsRNA–dependent protein kinase (PKR) $-/-$ mice were shown to be highly susceptible to lethal infection with VSV administered i.n. (Durbin *et al*, 2002; Stojdl *et al*, 2000). Thus, pathways involving type I IFN are crucial for control of VSV in peripheral organs. In contrast, wt mice are highly susceptible to viral encephalitis following i.n. inoculation, which allows VSV efficient access to the brain through olfactory receptor neurons (Plakhov *et al*, 1995b). In this study we tested the hypothesis that IFN rapidly controls peripheral infections but fails to suppress VSV infection of the CNS, and investigated the mechanisms that contribute to this innate immune failure.

Of note, Stojdl *et al* (2000) showed low mortality in wild-type BALB/c mice infected i.n. with VSV. These results conflict with the severe mortality observed in wt VSV infected mice in our study (see Figure 4). The discrepancy in the susceptibility of BALB/c mice to i.n. VSV infection can be attributed in part to the age of the mice used: 5 to 7 weeks of age in this work and 8 to 10 weeks of age in Durbin *et al* (2002). We have found that mice quickly lose susceptibility to VSV i.n. infection at ages greater than 8 weeks (unpublished results) (Sabin and Olitsky, 1938). In addition, Durbin *et al* used female mice (2002), which are less susceptible than male mice to VSV-induced encephalitis (Barna *et al*, 1996a).

We have shown previously that VSV replication in neurons is strongly inhibited by type I IFN *in vitro* (Trottier *et al*, 2005), suggesting that if IFN was produced in the CNS in response to VSV infection, neurons could be protected from VSV infection. In this study we investigated that possibility, showing that type I IFN was detected in mouse spleens, lungs, and serum of i.n. infected mice at 24 h p.i., but no IFN mRNA was found in brain tissue homogenates as late as day 6 p.i (Figure 3). Similarly, VSV infection of neuronal cells *in vitro* did not induce type I IFN synthesis (Figure 1), an observation that has been made with VSV infection of a number of cell lines of different tissue origin in past studies (Marcus *et al*, 1998). Importantly, VSV infection of neuronal cells induced the nuclear translocation of IRF-3, indicating that these cells sensed VSV infection and activated IFN
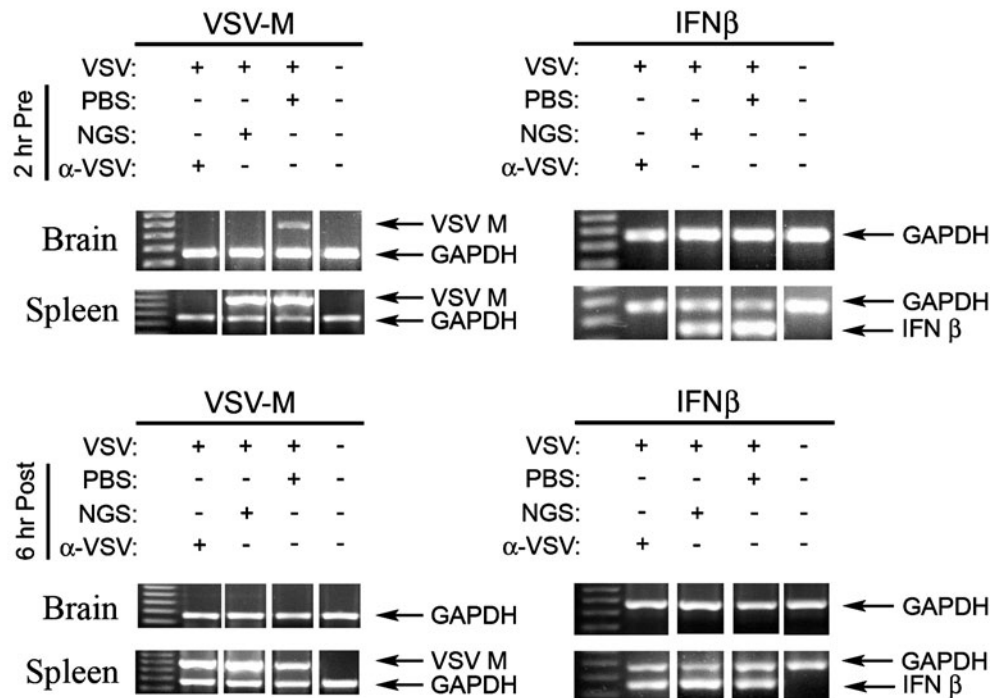
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Figure 7 Administration of neutralizing antibody to VSV prevents splenic IFN-β mRNA production. Anti-VSV polyclonal Ab was administered i.p. either 2 h prior to infection (*top panels*) or 6 h after i.n. application of 1×10^4 PFU virus (*bottom panels*) to groups of three BALB/c mice. Controls included i.n. mock-infection with PBS and i.p. injection of normal goat serum. mRNA was harvested at 24 h. p.i. from brains or spleens and RT-PCR was performed for amplification of VSV M protein, IFN-β or GAPDH message. Where viral gene expression was observed, IFN-β mRNA was produced. Anti-VSV Ab neutralized virus and prevented splenic mRNA production if present before infection.

signaling pathways. The wt VSV M protein blocks nucleocytoplasmic transport, thereby inhibiting IFN production from infected cells (Ahmed *et al*, 2003; Stojdl *et al*, 2003). In contrast, neuronal cells were able to produce type I IFN in response to infection by NDV (Figure 1) and the M protein mutant VSV T1026R1 infection *in vitro* (Figure 5B), indicating that neurons can respond to and produce type I IFN in response to viral infection, and when VSV M is inactivated, produce IFN in response to VSV infection. The mutant VSV T1026R1 also replicated to high levels in neuronal cells indicating that reduced replicative capacity of T1026R1 was not the reason these cells were able to produce IFN (Figure 4A). Together, the results suggest that wt VSV is able to replicate to high levels in the CNS of mice because of a lack of local induction of type I IFN. The lack of IFN induction is likely caused by the ability of VSV to block IFN production by infected cells. This point is emphasized by IFN-inducing mutant VSV T1026R1, which induces strong IFN mRNA expression in the CNS at 24 h p.i., and causes no apparent disease in mice (Figure 4C and D). Thus, it appears that blockade of IFN production by infected cells is critical for VSV replication in the CNS.

Astrocytes respond to i.n. VSV infection as early as 24 h by the production of GFAP and NOS-3

(Barna *et al*, 1996b). Similarly, Campbell and colleagues have observed that the liver rapidly responds to CNS injury, triggering leukocyte recruitment and chemokine production (Campbell *et al*, 2005). To determine if there were a contribution of either S100B release by astrocytes reacting to infection of adjacent neurons, or the SNS, to the induction of the peripheral IFN in response to i.n. VSV infection, we administered the soluble receptor for S100B, sRAGE, and performed a chemical sympathectomy, respectively. Neither treatment abrogated the production of IFN in the spleen or serum (Figure 5A and B).

Examination of lung and spleen for infectious VSV or VSV RNA confirmed that virus had rapidly entered these organs and replicated (Figures 6 and 7). To determine whether the virus traveled to the spleen in a cell-associated or free form, mice were injected with neuralizing Ab to VSV and the presence of VSV RNA and IFN-β mRNA was determined at 24 h p.i. Prior administration of Ab prevented infection from spreading and abrogated the IFN response of the spleen, but if Ab treatments were delayed 6 h, a modest response was observed (Figure 7). These experiments indicated that virus rapidly spreads free in plasma or lymphatic fluid to the spleen. VSV replication in peripheral organs after i.n. infection has been reported (Publicover *et al*, 2006; Ramsburg *et al*,

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2005); however, those studies inoculated mice with much higher titers and in a larger volume than in our work, which can have dramatic effects on the ability of viruses to replicate in the lung (Yetter *et al*, 1980). Despite the small dose of virus given in our work (as low as 100 PFU in 5 μl), VSV was still able to productively infect peripheral tissues.

The relatively high VSV titer in mouse spleens at 24 h p.i. suggests that transient viral replication in the spleen leads to IFN induction. Indeed, i.n. application of UV-inactivated VSV does not induce peripheral IFN production indicating a need for viral replication. Thus, it appears that VSV gains access to either blood or lymphatic circulation and then travels to the lung and spleen. Analysis of mouse blood by plaque assay has shown no evidence of infectious VSV after our inoculum with low dose (data not shown); however, VSV in serum has been described after mice were infected with higher titers (Publicover *et al*, 2005). Administration of neutralizing Ab to VSV 2 h before, but not 6 h after, i.n. infection abrogated the IFN response in the spleen at 24 h p.i; this was also associated with inhibition of infection of the spleen when Ab was present before i.n. application of virus (Figure 6).

Although the mutant virus T1026R1 replicated in the brains of mice at 24 h post i.n. infection, no virus was detected in the lung or spleen. In spite of this, 4 of 4 mice infected with T1026R1 virus produced detectable amounts of IFN-β in blood. The ELISA titers of IFN-β were substantially lower, however, than those obtained in mice administered wt VSV. In contrast to mice infected with wt VSV, IFN-β was found in the brains of T1026R1-infected mice. It is likely that the rapid production of IFN by this mutant virus profoundly limited its replication and spread *in vivo*, by eliciting a protective antiviral state in adjoining cells.

The lack of IFN production in the CNS after VSV infection does not appear to be a universal phenomenon with viruses. Type I IFN is strongly induced, early, in brains of MHV-JHM infected mice, and IFN production was sustained for at least 7 days (Rempel *et al*, 2004). Similarly, the GDVII strain of Theiler's murine encephalomyelitis virus (TMEV) induces type I IFN in the CNS, and this IFN is produced by neurons (Delhaye *et al*, 2006). Interestingly, both of these viruses cause severe, acute encephalitis similar to that observed with VSV. In contrast, studies with lymphocytic choriomeningitis virus (LCMV) and the BeAn strain of TMEV have shown a lack IFN production after early viral replication in the brain (Sandberg *et al*, 1994; Trottier *et al*, 2004). Similar to our findings, LCMV induced peripheral, but not CNS, production of IFN (Sandberg *et al*, 1994).

One question in this viral encephalitis model is why a type I IFN response is not rapidly induced in the brains of wt VSV-infected mice whereas it was in the spleen. There are abundant pDCs in the spleen and lymph nodes, which are absent in the resting CNS, and pDCs monitor for pathogen exposure and produce IFN. It is clear that wt VSV blocks IFN synthesis in infected cells via the biological effects of M protein (Ahmed *et al*, 2003; Ferran and Lucas-Lenard, 1997; Francoeur *et al*, 1980, 1987; Petersen *et al*, 2000; Stojdl *et al*, 2003). Any VSV-infected neuronal or extraneuronal cell may have IFN gene expression limited by the activity of VSV M protein. In contrast, pDCs are poised to recognize Pathogen associated molecular pattern (PAMPs), through receptors including TLRs, and rapidly produce and secrete IFNs, despite the evasive pathways developed by pathogens. The cell type and critical molecules involved in this system are currently under investigation.

Although many peripheral cell types infiltrate the CNS during the course of VSV encephalitis, starting with neutrophils as early as 24 to 36 h p.i. (Ireland and Reiss, 2006), followed by NK cells at 3 days p.i. and both CD4 and CD8 cells as well as macrophages around day 7 p.i. (Christian *et al*, 1996), we have found no evidence for the presence of plasmacytoid dendritic cells during the first 14 days after VSV infection (Palian *et al*, unpublished). Indeed, pDCs are reportedly absent from the brain parenchyma, but have been found in brain during chronic inflammation (Ambrosini *et al*, 2005; Bailey *et al*, 2007; Matyszak and Perry, 1997; Newman *et al*, 2005; Rosicarelli *et al*, 2005; Serafini *et al*, 2006; Zozulya *et al*, 2007). IFN-producing cells do not enter the CNS at early times after infection by wt VSV, and peripheral IFN does not cross the blood-brain barrier efficiently (Dafny and Yang, 2005). Thus it appears the lack of pDCs in the brain and the inability of infected neurons to produce IFN provides VSV with an ideal site for replication.

Materials and methods

Cells, animals, and viruses
Mouse neuroblastoma (NB41A3) cells were purchased from ATCC and maintained in F12K medium with 15% horse serum, 2.5% fetal bovine serum, 1% penicillin/streptomycin. Chinese hamster ovarian (CHO) cells were a gift of Alice Huang and were maintained in Cellgro Free medium with 10% fetal bovine serum, 1 mM L-glutamine, and 1% penicillin/streptomycin.

BALB/c and C57BL/6 mice were obtained from Taconic Laboratories (Germantown, NY). All mice were maintained in specific pathogen-free facilities at New York University in accordance with University Animal Welfare Committee (UAWC) guidelines.

VSV (Indiana strain, San Juan serotype; originally obtained from Alice S. Huang) and Newcastle disease virus (NDV strain B1; generously provided by Peter Palese, Mt Sinai School of Medicine) were used for virus infections. UV-VSV and DI particles were prepared as previously described (Bi *et al*, 1995; Browning *et al*, 1991; Plakhov *et al*, 1995a, 1995b). VSV T1026R1 was isolated from the HR strain was generously provided by Laurent Poliquin (University of

Quebec); the virus has been shown to have a mutation in the M protein and to induce IFN in several cell types including mouse L cells (Francoeur *et al*, 1980, 1987).

Immunofluorescence studies

NB41A3 cells were grown on 4-well chamber slides and infected with either VSV or NDV at a multiplicity of infection (m.o.i.) = 10. At various times p.i., cells were washed and treated with 200 μl 10% buffered formalin in phosphate-buffered saline (PBS) for 5 min at 4°C, washed again, and then permeablized with 1% Triton X-100 in PBS at 4°C for 10 min. After washing, slides were incubated overnight at 4°C with 5% goat serum, 1% bovine serum albumin (BSA) in PBS, then incubated for 2 h at 20°C (room temperature [rt]) with rabbit anti-IRF-3 (Zymed, San Francisco, CA) at a concentration of 0.75 μg/ml in 1% BSA-PBS. Cells were washed with PBS and incubated with goat anti-rabbit Alexafluor 488–conjugated secondary antibody (Molecular Probes) for 1 h at rt. Slides were washed before mounting in Permount containing Pro-Long antifade reagent (Molecular Probes) and 4′-6′-Diamidino-2-phenylindole (DAPI) (0.5 mg/ml).

ther 100 μg mouse serum albumin (Sigma) diluted in saline or 100 μg sRAGE (generously provided by Ann-Marie Schmidt, College of Physicians and Surgeons, Columbia University) and immediately infected i.n. with 100 PFU VSV or mock-infected with PBS. Blood and organs were sampled as described above at 24 h p.i.

Normal serum (Sigma) or sheep neutralizing polyclonal anti-VSV antibody (0.5 ml) were administered i.p. to groups of 10 mice either 2 h prior to or 6 h post i.n. infection with 30 PFU VSV. Tissues were harvested at 24 h p.i. and processed as above.

RT-PCR

Total RNA was isolated from mouse tissues using Trizol Reagent (Invitrogen). Total RNA was treated with DNase I (DNA-free kit, Ambion) to remove chromosomal DNA contamination. One microgram of total RNA was reverse transcribed using Omniscript RT kit (Qiagen) in a volume of 20 μl. Two μl cDNA was used in PCR reactions using the GeneAmp Gold RNA-PCR kit (Applied Biosystems), with PCR conditions being 95°C 10 s followed by 35 cycles of 94°C

	Sense 5′ → 3′	Antisense 5′ → 3′	Product sizes
IFN-α4	ATGGCTAGRCTCTGTGCTTTCCT	AGGGCTCTCCAGAYTTCTGCTCTG	511bp
IFN-β	CATCAACTATAAGCAGCTCCA	TTCAAGTGGAGAGCAGTTGAG	353bp
GAPDH	ATTCAACGGCACAGTCAAGG	TGGATGCAGGGATGATGTTC	467bp

Experimental infection and treatment of mice

Male BALB/c mice (6 to 7 weeks of age) were anesthetized with isofluorane and infected i.n. with various doses of VSV ranging from 30 to 1×10^4 PFU in PBS with a dose of 5 μl per nostril; mock-infected mice were inoculated with the same volume of PBS, i.n. Mice were treated with UV-VSV at a dose 5×10^6 PFU. At various times p.i., mice were anesthetized with Avertin. Blood was obtained from animals by cardiac puncture into Eppendorf tubes and stored at 4°C overnight for clotting, and subsequent collection of serum. Spleens, lungs, and brains were aseptically removed, divided in half and then immediately homogenized using Potter-Elvijem tubes either in PBS (for plaque assays) or in Trizol (Invitrogen) for isolation of total RNA.

Mutant VSV T1026R1 was also used for intranasal infection at a dose of 4×10^6 PFU, and mice were processed as above.

Chemical sympathectomy was performed by i.p. injection of groups of five male BALB/c mice with 100 mg/kg/day 6-hydroxydopamine (6-OHDA, Sigma) or media for 10 days prior to i.n. infection with VSV as described above.

To assess the possible role of S100B release, groups of five male BALB/c mice were injected i.p. with ei-

30 s, 55°C 30 s, 72°C 1 min. PCR products were visualized by agarose gel electrophoresis. Primers for IFN-β and IFN-α were as reported previously (Marie *et al*, 1998). Primers and the size of the products follow.

IFN bioassays

IFN bioassays were used to estimate the amount of type I IFN in mouse serum or cell culture supernatants. Mouse serum was irradiated for 3 min in a 50-μl volume on ice using a Stratalinker (Stratagene). Tissue culture supernatants from NB41A3 cells (1 ml) were adjusted to pH 2 with 0.25 N HCl and incubated at 4°C for 24 h, after which the pH was adjusted to 7 with 0.25 N NaOH. Irradiated serum (50 μl) or acid-treated supernatants (500 μl) were added to NB41A3 monolayers and allowed to incubate 18 to 24 h to allow cells to achieve an antiviral state. After incubation, monolayers were challenged with VSV at m.o.i. = 0.1 for 30 min in 200 μl F12K medium, after which virus was removed and fresh media added. After 7 h, cell supernatants were harvested and serially diluted before adsorption on CHO cell monolayers. Following overnight incubation in 0.9% agar-containing medium, the monolayers were

fixed and stained with methylene blue and plaques were counted. The presence of IFN in either serum or culture supernatant resulted in a 500- to 1000-fold decrease in plaques in the treated monolayers. Alternatively, to semiquantify the amount of IFN in serum samples, serial dilutions were added to NB41A3 cells and allowed to incubate for 18 h. Cells were challenged with VSV at m.o.i. = 0.01. After 24 h, cells were fixed with 10% buffered formalin and stained with methylene blue. Preserved monolayers stained blue after 24-h infection indicated the presence of

IFN. Mouse IFN-β (R&D Systems; 100 U/ml) was used as positive control.

IFN ELISA assays

ELISA assays to determine concentration of murine IFN-β in plasma samples was performed according to manufacturer's directions (PBL Medical Laboratories). Mouse IFN-β (R&D Systems) was used as positive control. Normal mouse serum was used as a negative control.

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