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VIRUS-CELL INTERACTIONS

Chikungunya, Influenza, Nipah, and Semliki Forest Chimeric Viruses with Vesicular Stomatitis Virus: Actions in the Brain

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ABSTRACT Recombinant vesicular stomatitis virus (VSV)-based chimeric viruses that include genes from other viruses show promise as vaccines and oncolytic viruses. However, the critical safety concern is the neurotropic nature conveyed by the VSV glycoprotein. VSVs that include the VSV glycoprotein (G) gene, even in most recombinant attenuated strains, can still show substantial adverse or lethal actions in the brain. Here, we test 4 chimeric viruses in the brain, including those in which glycoprotein genes from Nipah, chikungunya (CHIKV), and influenza H5N1 viruses were substituted for the VSV glycoprotein gene. We also test a virus-like vesicle (VLV) in which the VSV glycoprotein gene is expressed from a replicon encoding the nonstructural proteins of Semliki Forest virus. VSVΔG-CHIKV, VSVΔG-H5N1, and VLV were all safe in the adult mouse brain, as were VSVΔG viruses expressing either the Nipah F or G glycoprotein. In contrast, a complementing pair of VSVΔG viruses expressing Nipah G and F glycoproteins were lethal within the brain within a surprisingly short time frame of 2 days. Intranasal inoculation in postnatal day 14 mice with VSVΔG-CHIKV or VLV evoked no adverse response, whereas VSVΔG-H5N1 by this route was lethal in most mice. A key immune mechanism underlying the safety of VSVΔG-CHIKV, VSVΔG-H5N1, and VLV in the adult brain was the type I interferon response; all three viruses were lethal in the brains of adult mice lacking the interferon receptor, suggesting that the viruses can infect and replicate and spread in brain cells if not blocked by interferon-stimulated genes within the brain.

IMPORTANCE Vesicular stomatitis virus (VSV) shows considerable promise both as a vaccine vector and as an oncolytic virus. The greatest limitation of VSV is that it is highly neurotropic and can be lethal within the brain. The neurotropism can be mostly attributed to the VSV G glycoprotein. Here, we test 4 chimeric viruses of VSV with glycoprotein genes from Nipah, chikungunya, and influenza viruses and nonstructural genes from Semliki Forest virus. Two of the four, VSVΔG-CHIKV and VLV, show substantially attenuated neurotropism and were safe in the healthy adult mouse brain. VSVΔG-H5N1 was safe in the adult brain but lethal in the younger brain. VSVΔG Nipah F+G was even more neurotropic than wild-type VSV, evoking a rapid lethal response in the adult brain. These results suggest that while chimeric VSVs show promise, each must be tested with both intranasal and intracranial administration to ensure the absence of lethal neurotropism.

KEYWORDS blood-brain barrier, brain, chikungunya, influenza virus, neurotropic viruses, Nipah virus, vesicular stomatitis virus, viral vaccine

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Vesicular stomatitis virus (VSV) is an enveloped, negative-sense, single-stranded RNA virus. It has been studied extensively for its potential as a vaccine vector, and in laboratory studies has shown efficacy against a large number of microbes, including HIV, the henipaviruses Nipah virus and Hendra virus, Marburg virus, Lassa fever virus (LASV), influenza virus, *Yersinia pestis* (bubonic plague), and a wide variety of other human and animal pathogens (1–4). A chimeric VSV effectively protects nonhuman primates against Ebola virus (5–9) and has been employed recently in human vaccination trials in Africa, where it protected against Ebola virus (10).

In addition, VSV shows potential as an oncolytic virus that can target a number of different animal and human cancers. It is currently in clinical trials for targeting liver cancer, employing a VSV that also carries an interferon (IFN) gene that can serve to improve the selectivity of the virus for cancer cells (11, 12). A primary mechanism underlying VSV targeting cancer cells is the reduced innate immunity reported in up to 80% of human cancer cells from the NCI60 panel (13, 14). In addition to directly evoking cytolysis, VSV can also upregulate an attack by the immune system on different types of infected cancer cells, and this immune attack generalizes to noninfected cancer cells of the same type (15, 16).

The primary limitation of VSV in clinical use is that it is neurotropic, and if it enters the brain, it can cause adverse neurological responses, including death (17–19). VSV generally does not enter the healthy brain, and the blood-brain barrier keeps the virus out. However, the virus can cross the blood-brain barrier in the region of brain tumors to infect the tumor. This is due, in part, to the weakened blood-brain barrier near tumors (20). The virus can also enter the brain if peripheral cancer cell metastases migrate to the brain meninges, where the metastasis can serve as a bridge for VSV from the periphery into the brain (21), potentially resulting in substantive adverse effects.

A number of approaches have been tested to attenuate the virulence of VSV to improve its safety. They include mutations of the M protein (22, 23), insertion of genes into either the first or fifth position of the genome to attenuate expression of downstream genes (24–27), truncation of the VSV G (glycoprotein) cytoplasmic domain (28), shuffling of the normal VSV gene order (29–31), and a combination of VSV G cytoplasmic domain truncation and gene shuffling (31), which can all attenuate the neurotropic actions of VSV within the brain but, with rare exceptions (25), generally do not completely block the potential lethal effects in the central nervous system (CNS).

Here, we examine experimental chimeric-VSV-based vaccines against several viruses, including chikungunya (CHIKV), influenza H5N1, and Nipah viruses; in each vaccine candidate, the VSV G gene has been replaced by the glycoprotein gene from another virus. First identified in Africa, chikungunya virus is a positive-sense single-stranded RNA alphavirus that arrived in South and North America in late 2013. Symptoms include high fever and joint pain that may be long lasting. Infections are associated with substantive morbidity, and the virus had spread to most of the Americas by 2016 (32). Influenza virus contains 7 or 8 segments of negative-sense single-stranded RNA and causes fever, headache, and coughing (33, 34). Nipah virus is a negative-sense single-stranded RNA virus from the genus *Henipavirus* that can cause lung and neurological problems, and it has a high fatality rate in humans. Nipah virus may be spread by large bats (flying foxes) that can infect pigs, secondarily leading to human infection (35). Infection has been restricted to southern Asia.

Semliki Forest virus (SFV) is a positive-sense single-stranded RNA alphavirus of African origin; it can cause encephalitis in rodents, but symptoms in humans are normally mild (36). The virus-like vesicle (VLV) tested here encodes the VSV glycoprotein gene, together with genes coding for the nonstructural proteins of SFV (37–39). As such, it is not intended to combat SFV, but rather, to serve as a possible vehicle for a vaccine candidate for microbes whose glycoprotein genes can be substituted for or added to the VSV G protein gene.

Because the VSV glycoprotein conveys neurotropism and enhances infection of neurons in the brain, addition of genes to the wild-type VSV genome does not generally eliminate its neurotoxic effect. On the other hand, substitution of glycoprotein genes

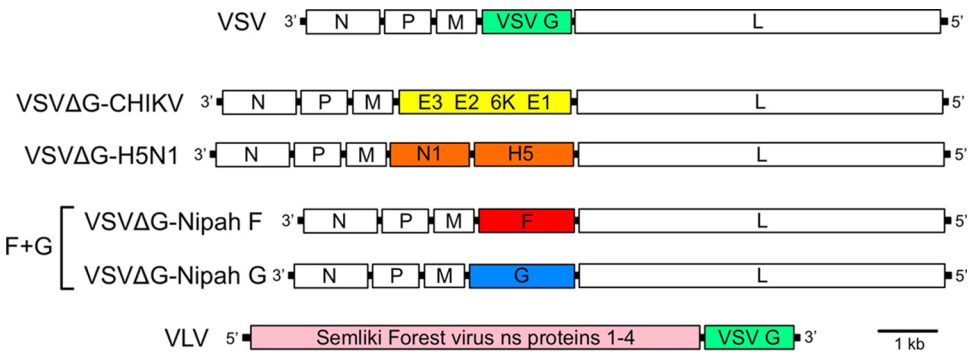

FIG 1 Schematic diagram of recombinant chimeric VSVs. The top diagram, VSV, shows the gene sequence and relative gene size of normal VSV. Below that are schematics of the chimeric virus genomes with the VSV glycoprotein gene (green) deleted and replaced with the glycoprotein genes from chikungunya virus (yellow), influenza virus H5N1 (orange), and Nipah virus F (red) and G (blue) and VLV, in which only the VSV glycoprotein gene is used and all other VSV genes are deleted and replaced by the Semliki Forest virus nonstructural protein genes (pink). VSVΔG-Nipah F+G is the combination of VSVΔG-Nipah F and VSVΔG-Nipah G.

from other viruses that are not neuroselective can provide a more substantial level of safety within the brain. We recently showed that substituting the glycoprotein genes from Ebola or Lassa fever virus for the VSV glycoprotein gene appeared to eliminate the neurotropic actions of the virus in mice and rats (40). Similarly, substitution of the lymphocytic choriomeningitis virus (LCMV) glycoprotein for VSV glycoprotein also showed safety within the brain (41). VSV with the Ebola glycoprotein substituted for the VSV glycoprotein was found to be safe in the brains of nonhuman primates (8).

Remarkably, even substitution of the deadly rabies virus glycoprotein for the VSV glycoprotein resulted in a virus that still infected neurons and moved by axonal transport to other neurons away from an injection site but was no longer lethal if injected into the brains of rodents (42, 43). Thus, a number of independent lines of experimentation suggest that elimination of the VSV glycoprotein and replacement with glycoprotein genes from other viruses, even from potentially more dangerous viruses, appears to result in recombinant chimeric viruses that show a safer profile within the brain.

Here, we study several chimeric viruses that have not previously been tested in the brain, including VSVs lacking the G gene (VSVΔG) expressing influenza, chikungunya, and Nipah virus glycoproteins, as well as an SFV RNA replicon expressing VSV G (Fig. 1). Each may have potential as a vaccine candidate. We find that, similar to previously tested chimeric viruses, VSVΔG-influenza virus, -chikungunya virus, and -VLV did appear to be safe when administered directly into the adult mouse brain. Similarly, substitution of VSV G for either Nipah virus F or G was also safe. However, a complementing pair of VSV recombinants separately expressing the Nipah virus F and G glycoproteins in place of the VSV G protein were not only lethal in the brain, but evoked a lethal response even more rapid than that of wild-type VSV. VSVΔG-influenza virus appeared to be safe in the adult brain, but not in the immature brain. A key mechanism of brain protection was dependent on type I IFN. All viruses were lethal in adult mouse brains lacking type I IFN receptors.

RESULTS

We examined the actions of five chimeric viruses in the brain. The gene orders and substitutions of the recombinant viruses are shown in Fig. 1. The VSV glycoprotein gene was deleted (VSVΔG) in four of the viruses, VSVΔG-CHIKV, VSVΔG-H5N1, VSVΔG-Nipah F, and VSVΔG-Nipah G. VLV was generated from the Semliki Forest virus RNA replicon encoding VSV G and the four nonstructural proteins of SFV but none of the SFV structural proteins (37, 39). All four recombinants have been tested as possible vaccine vectors (38, 44–49). Three additional recombinant VSVs were used for control purposes; all three have been previously used to test intracranial safety (18, 41, 50). They included

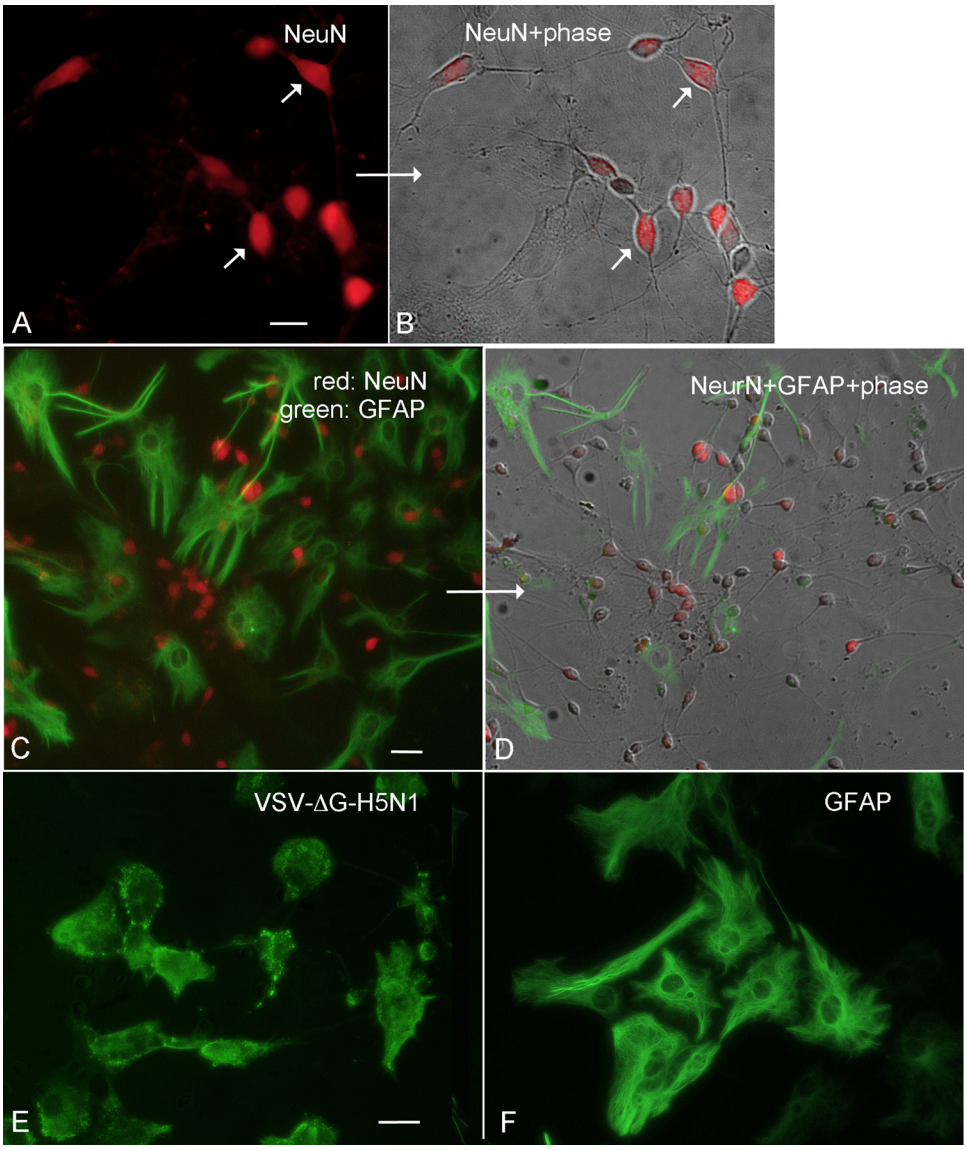

FIG 2 Identification of neurons and astrocytes. (A) Neuronal nuclei are immunostained red for the neuronal antigen NeuN (short arrows). (B) Neurons with 2 to 4 thin dendritic or axonal processes contain NeuN-positive nuclei. (A and B) Bar, 20 μm. (C) Neurons are labeled by NeuN immunostaining (red), and astrocytes are labeled green by GFAP immunostaining. (D) In the same field shown in panel C, phase contrast shows that phase-bright neurons and astrocyte immunostaining overlap. (C and D) Bar, 40 μm. (E) Astrocytes 2 days after inoculation with VSVΔG-H5N1. Astrocyte processes have withdrawn, and GFAP immunoreactivity is no longer fiber-like, as seen in normal astrocytes (C and F), but has become more globular, indicating cell cytolysis. (F) Normal astrocytes immunolabeled with GFAP show long, thin GFAP staining. (E and F) Bar, 40 μm.

a wild-type-like VSV-G/GFP, in which the green fluorescent protein (GFP) gene was expressed fused to a second copy of the VSV G gene (18, 51); VSV-LCMV-GFP, which had the LCMV glycoprotein substituted for the VSV glycoprotein (41); and VSV-IFN-β, which expressed mouse beta-interferon (11, 21).

***In vitro* infection of brain cells.** To study the relative infection of neurons and glia *in vitro*, embryonic day 18 mouse brains were cultured, and 8 days later, virus (multiplicity of infection [MOI], 0.5) was added. Infections were detected by immunostaining for VSV proteins. All the cultures contained both neurons and glial cells. To corroborate the cell type as determined by morphological differences in shape and size, we first used antisera against the neuronal nuclear antigen NeuN and the astrocyte antigen glial fibrillary acidic protein (GFAP). Figure 2A and B shows cultured neurons that express NeuN; the NeuN labeling is primarily nuclear within a small cell body and 3 or

4 thin dendritic or axonal processes extending from the perikaryon. Figure 2C and D shows red NeuN labeling neurons and green immunofluorescence labeling GFAP in astrocytes; glial cells show a typical large flattened cell body, often difficult to detect in phase contrast with thin GFAP labeling stretching from the area of the nucleus to the cell perimeter. When cells were infected by the chimeric VSVs, they generally withdrew processes and showed cytolytic responses to the virus, as shown by the astrocytes infected by VSVΔG-H5N at 2 days postinfection (dpi); whereas normal astrocytes in culture showed radial expression of GFAP and processes that reached out to contact other cells or the substrate (Fig. 2F), infected astrocytes showed that the processes had retracted and GFAP immunostaining had changed to a more globular vesicular structure.

None of the viruses showed an absolute preference for a single cell type. VSVΔG-CHIKV tended to infect cells with a morphological profile typical of astrocytes, with a thin sheet-like cell body and a large diameter (Fig. 3C); VSVΔG-CHIKV also infected cells with a neuronal morphology, consisting of two or three dendritic processes and a long, thin axon. VSVΔG-H5N1 (Fig. 3B) infected mostly small neuron-like cells, and also some glial cells. VSVΔG-Nipah F+G infected small cells but also showed some infection of larger glial cells (Fig. 3A). VLV immunostaining showed infection mostly of small neurons (Fig. 3D), as well as a smaller proportion of astrocytes. Control cultures with no virus inoculation showed no immunostaining with VSV antiserum (Fig. 3E).

Infection and survival after intranasal inoculation. Intranasal inoculation is often used to estimate the adverse neurotropic effects of VSV-related viruses, generally based on infection of immature, 2- to 3-week-old mice (17, 18, 52), which are more sensitive to VSV than adult mice. Neurotropic viruses often infect olfactory receptor neurons in the olfactory mucosa in the nasal cavity and then move along the olfactory nerve into the rostral part of the brain, the olfactory bulb. As expected, when inoculated intranasally (15 μl in each naris at postnatal day 14 [P14]), VSV-G/GFP had a lethal effect on all the mice tested ($n = 11$) (Fig. 4). In contrast, the same intranasal inoculation with VSVΔG-CHIKV ($n = 8$) and VLV ($n = 6$) showed complete safety, with all mice surviving to the end of the experiment 42 days postinoculation. Although VSVΔG-H5N1 is nonpathogenic in adult mice outside the brain (45, 46), here, we found it to be lethal after intranasal inoculation of P14 mice, with only a single survivor out of a large cohort ($n = 23$) and almost all the mice succumbing to the virus within 4 to 10 days of inoculation (Fig. 4). We also examined histological sections of the brains of infected mice. VSVΔG-H5N1 infection in the brain was substantial. Figure 4B and C shows robust cytolytic VSVΔG-H5N1 within the brain at 5 dpi. As VSVΔG-H5N1 spread caudally from the olfactory system into the brain, the sites of infection did not appear random, as would occur from long-distance diffusion of the virus. For instance, Fig. 4B shows strong infection of the left but not the right midline cortex, and Fig. 4C shows strong infection of the hypothalamus but no infection in the hippocampus (Fig. 4D) in the same brain sections. To confirm that the critical tissue infected was the brain, we also examined the lungs of the same mice that showed strong brain infection. Little substantive infection was found in the lungs (Fig. 4E).

VSVΔG-Nipah F+G was lethal to 5 of the 8 mice tested intranasally by day 6 postinoculation (Fig. 4). The remaining 3 mice survived. In additional P14 mice inoculated with VSVΔG-Nipah F+G, strong infection was detected in the olfactory bulb. In contrast to wild-type VSV, which shows strong infection of GABAergic granule and periglomerular cells with little infection of the excitatory mitral cells, VSVΔG-Nipah F+G showed strong infection of all cell types, including granule, periglomerular, and mitral cells (Fig. 4F).

Together, these data suggest that intranasal inoculation with VSVΔG-CHIKV and VLV was completely safe, whereas VSVΔG-H5N1 and VSVΔG-Nipah F+G were not safe after intranasal inoculation in P14 mice.

VSVΔG-LCMV was used as a control for brain safety, and similar to a previous report (41), evoked no adverse effect in the P14 mice here after intranasal inoculation. We also

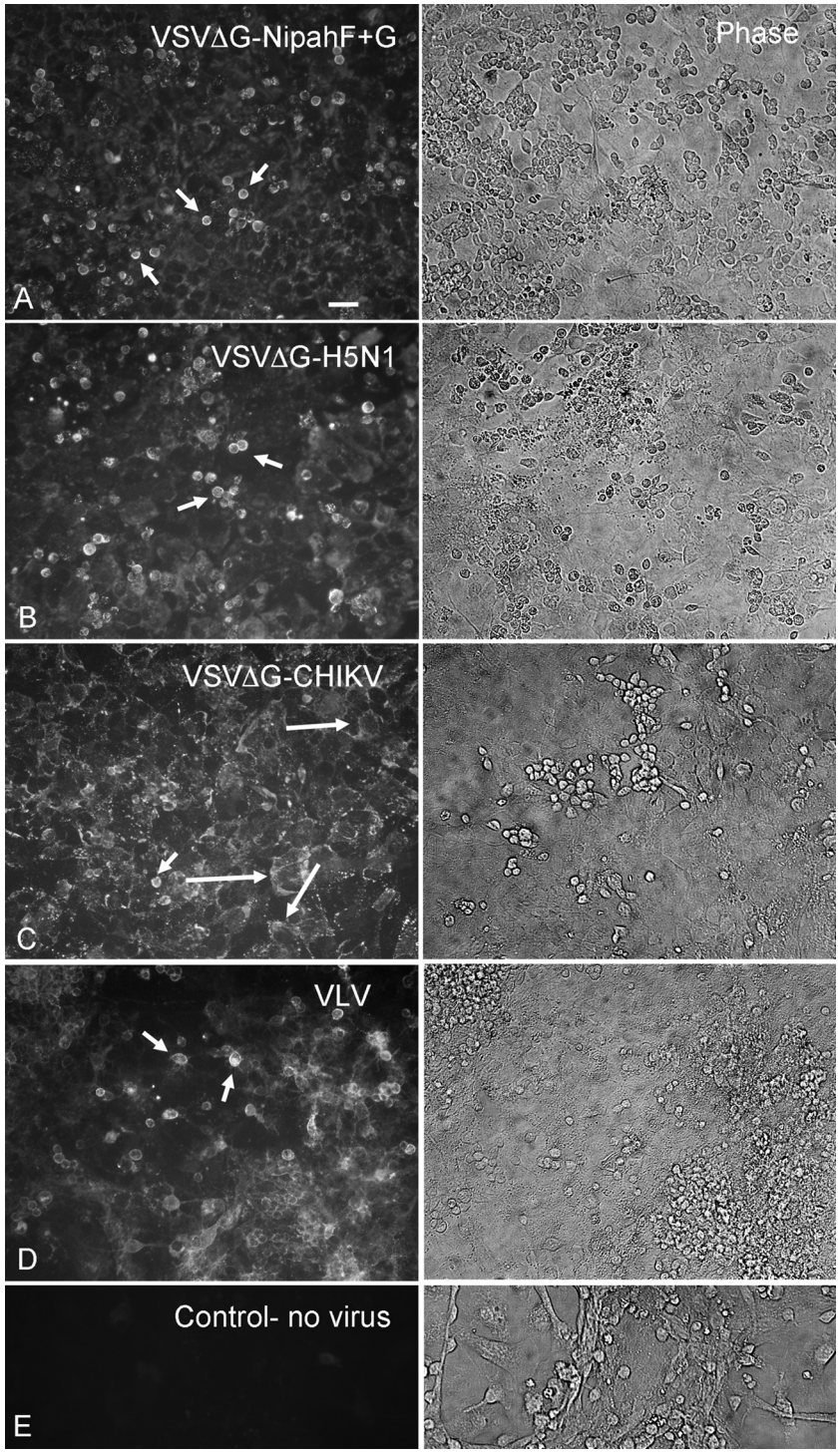

FIG 3 Immunostaining for chimeric VSVs in mouse brain cultures containing neurons and glia. (A and B) VSVΔG-Nipah F+G and VSVΔG-H5N1 showed immunostaining in small neurons (arrows). Scale bar, 30 μm (all panels). (C) VSVΔG-CHIKV showed immunoreactivity in the plasma membranes of large astrocytes (long arrows) and also staining in some small neuron-like cells (short arrows). (D) VLV showed staining in neurons (arrows). (E) Control cultures with no virus showed no immunoreactivity.

tested VSV-IFN-β (11). VSV-IFN-β shows an attenuated phenotype *in vitro* and *in vivo* outside the brain, although it can be lethal when injected directly into the mouse brain (50). In contrast to wild-type VSV, VSV-IFN-β was substantially attenuated when given by the intranasal route and evoked no adverse effect, consistent with an attenuated phenotype previously described (11, 12), and safer than wild-type VSV.

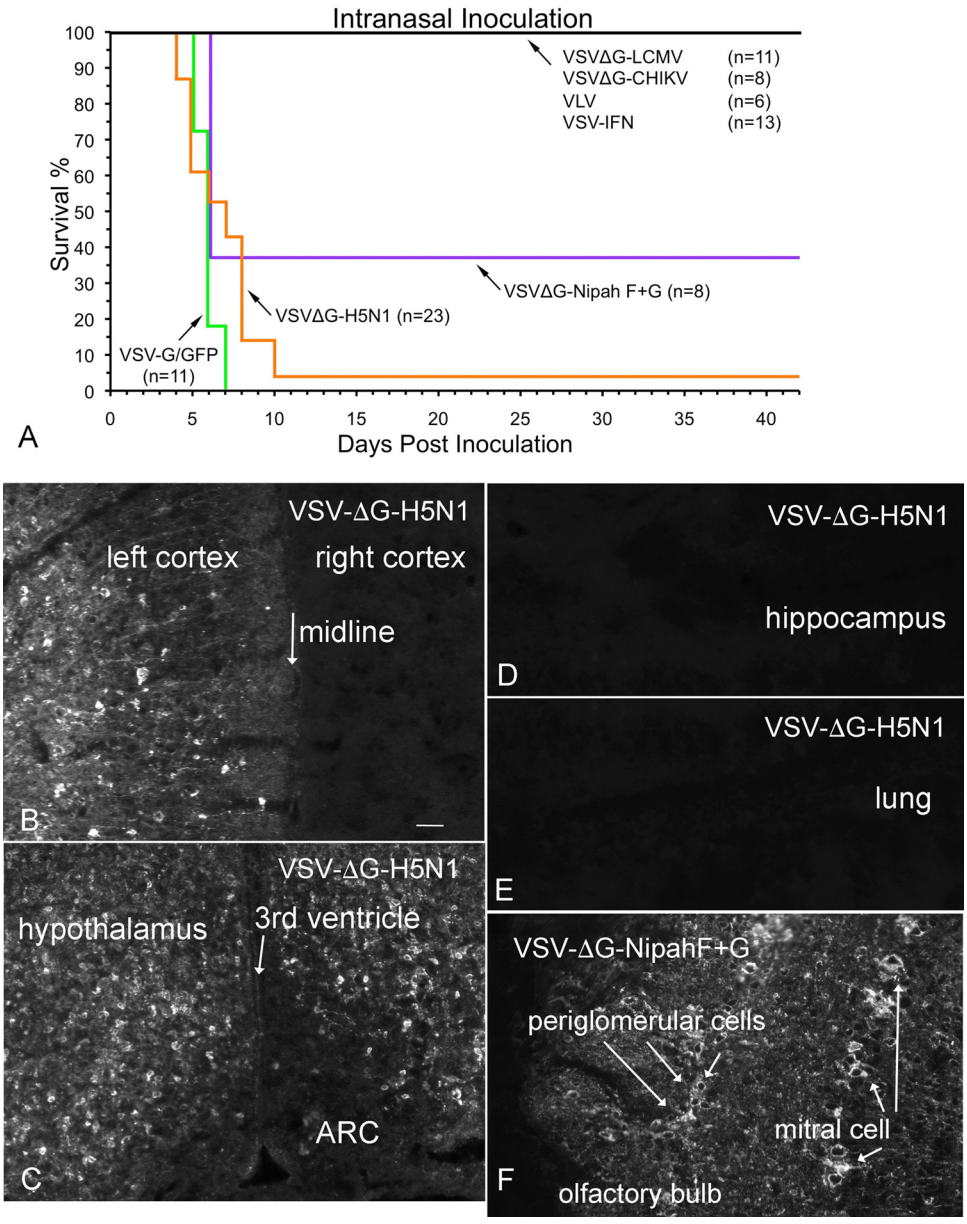

FIG 4 Survival and infection after intranasal inoculation with chimeric VSVs. (A) In postnatal day 14 mice, viruses were applied to the external nares. VSV-G/GFP was lethal, as previously reported. VSVΔG-CHIKV and VLV were without lethal effect. VSV-IFN showed no adverse effect. Similarly, VSVΔG-LCMV, used as a negative control for safety, showed no lethal effect. In contrast, VSVΔG-Nipah F+G was lethal in many mice. VSVΔG-H5N1 showed a very strong lethal effect, resulting in a fatal response in almost all of the 23 mice tested. (B) VSVΔG-H5N1 at 5 dpi showed strong infection of the left cortex with minimal infection of the adjacent right cortex. (C) VSVΔG-H5N1 in the same brain strongly infected cells of the hypothalamus in the region of the arcuate nucleus (ARC). (D and E) Hippocampus (D) and lungs (E) showed no detectable infection in the same mouse. (F) VSVΔG-Nipah F+G showed strong infection of periglomerular cells and mitral cells in the olfactory bulb at 3 dpi.

Intracranial inoculation. When VSV-G/GFP was injected intracranially, mice succumbed to the virus within a week, as expected (18). Intracranial injection of VSVΔG-CHIKV appeared completely safe, and no lethal responses or adverse effects were detected (Fig. 5A and 6). At the site of injection into the brain, both infected neurons and astrocytes were found, but the infection showed little spread, and the contralateral brain showed no infection (Fig. 6B). Similarly, injection of VLV into the brain also appeared safe, and no adverse neurological symptoms or lethal effects were detected (Fig. 5A and 7). Similar to VSVΔG-CHIKV, both infected neurons and astrocytes were

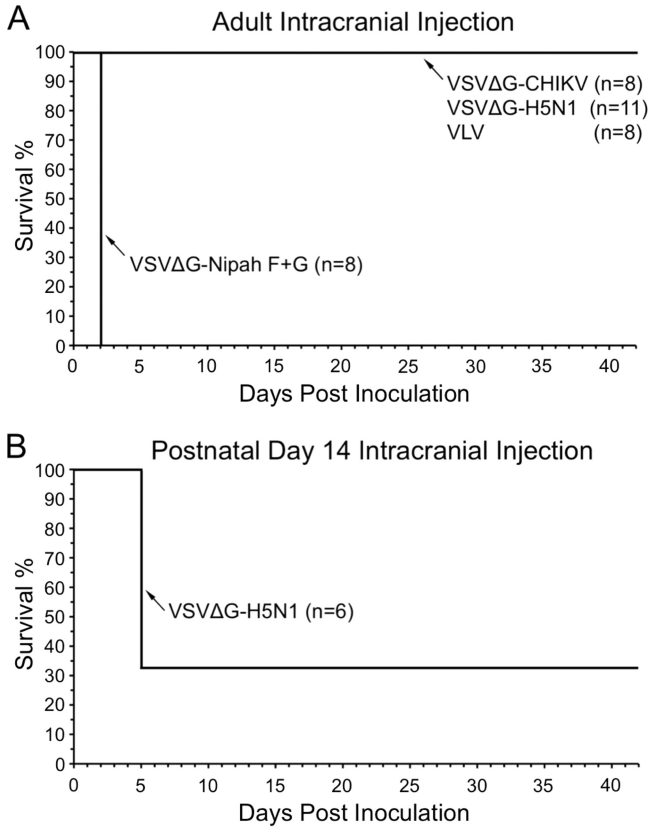

FIG 5 VSVΔG-CHIKV, VSVΔG-H5N1, and VLV are safe in the adult brain, whereas VSVΔG-Nipah F+G is lethal. (A) Of the 4 viruses tested, only VSVΔG-Nipah F+G was rapidly lethal when injected into the adult mouse brain. VSVΔG-CHIKV, VSVΔG-H5N1, and VLV were all completely without lethal effect in the adult brain. (B) In contrast to its lack of adverse action in the adult brain, VSVΔG-H5N1 showed lethal actions when injected into the brains of younger, postnatal day 14 mice.

detected at the site of injection (Fig. 7A and B), similar to infection of both cell types *in vitro* (Fig. 7C). The infection showed little spread from the site of inoculation.

The high lethality of VSVΔG-H5N1 administered intranasally at postnatal day 14 suggested that injection directly into the brain in adults would also be lethal. Surprisingly, VSVΔG-H5N1 appeared to be completely safe in the brains of adult mice, with no adverse neurological symptoms (Fig. 5A and 8A and B). Both neurons and glia were infected *in vivo*, as well as *in vitro* (Fig. 8C). However, although VSVΔG-H5N1 infected both neurons and glia after intracranial injection, minimal spread of the virus to other brain regions was detected in the adult; this is in striking contrast to the strong spread of VSVΔG-H5N1 within the P14 brain after intranasal inoculation, as described above (Fig. 4A, B, and C).

To determine whether the adverse response of P14 mice inoculated intranasally was due to neurological dysfunction rather than some undetermined non-CNS peripheral action, VSVΔG-H5N1 was injected into the brains of P14 mice. These injections evoked a lethal consequence in 4 of 6 mice tested (Fig. 5B). Together, these data indicate a developmental shift in the potential of VSVΔG-H5N1 to infect brain cells with spreading lethal infection at P14, but with no substantive spread or adverse effect in the adult brain.

Of all the viruses tested in the present set of experiments, VSVΔG-Nipah F+G showed the most rapid lethal consequences when injected into the brain. The viruses spread to nearby local cells rapidly and then showed an expanding pattern of infection that involved mostly neurons (Fig. 9A to D). Although many astrocytes were interspersed among the neurons of the cortex and striatum near the site of inoculation, neurons were much more commonly infected than glia (Fig. 9). The virus caused rapid

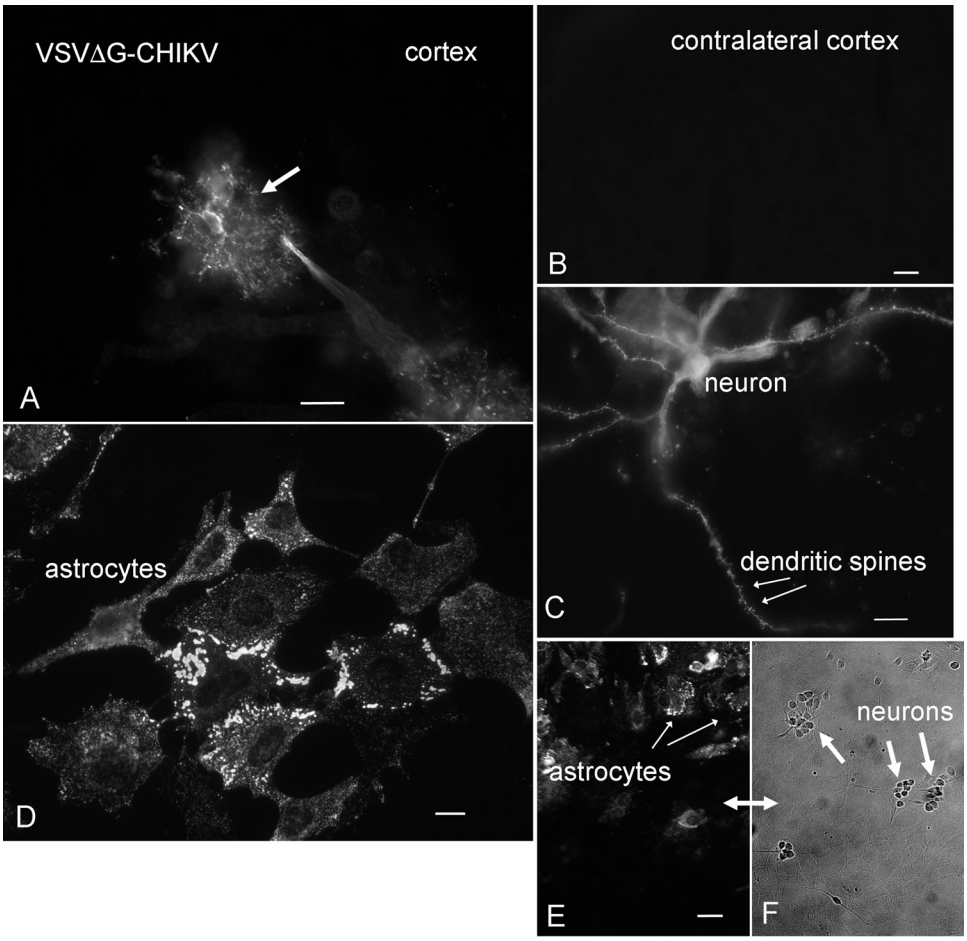

FIG 6 VSVΔG-CHIKV in brain and culture. (A) Injection of VSVΔG-CHIKV into the brain infected both neurons and astrocytes at 3 dpi. The arrow points to an astrocyte with many radiating processes. Bar, 35 μm. (B) No infection was seen in the contralateral cortex. Bar, 25 μm. (C) Some neurons also showed VSVΔG-CHIKV infection. Bar, 25 μm. (D) In culture, large, flat astrocytes are preferentially infected at 1 dpi. (E) Astrocytes are infected *in vitro* at 1 dpi. Bar, 12 μm. (F) The arrows indicate cells with neuronal morphology in the same field shown in panel E. Whereas many glial cells were infected, neurons were not infected. Bar, 30 μm.

neurological deterioration, leading to reduced mobility within 1 day and to death within 2 days, faster even than the neurological deterioration found with any other VSV variants or wild-type VSV. Irrespective of where in the brain VSVΔG-Nipah F+G was injected, combined infection evoked lethal consequences.

We also tested VSVΔG-Nipah G and VSVΔG-Nipah F individually. Because neither can infect cells without both Nipah glycoproteins, VSVΔG-Nipah was grown in cells that expressed the VSV glycoprotein, allowing pseudotyping of the initial virions. This pseudotyping allowed initial infection and replication in normal cells, but the progeny virions were postulated to be noninfectious to additional cells. In contrast to the lethal actions of VSVΔG-Nipah F+G, when we used either VSVΔG-Nipah F ($n = 4$) or VSVΔG-Nipah G ($n = 4$) alone with direct injection into the brain, all the mice survived with no apparent adverse effect and no detectable symptoms during the 42-day period of observation. On day 42 postinoculation, the mice were euthanized by anesthetic overdose.

Type I interferon is critical for protection of the brain. Here, we asked the role of type I interferon in protecting the brain against the three viruses that did not evoke a lethal response in the healthy brain. First, we examined the ability of IFN to attenuate each of the chimeric viruses *in vitro* in human brain cells. Each of the viruses, VSVΔG-CHIKV, VSVΔG-H5N1, and VSVΔG-Nipah F+G, did infect the cells in the absence of IFN (Fig. 10A and B), with both VSVΔG-CHIKV and VSVΔG-Nipah F+G showing infection of

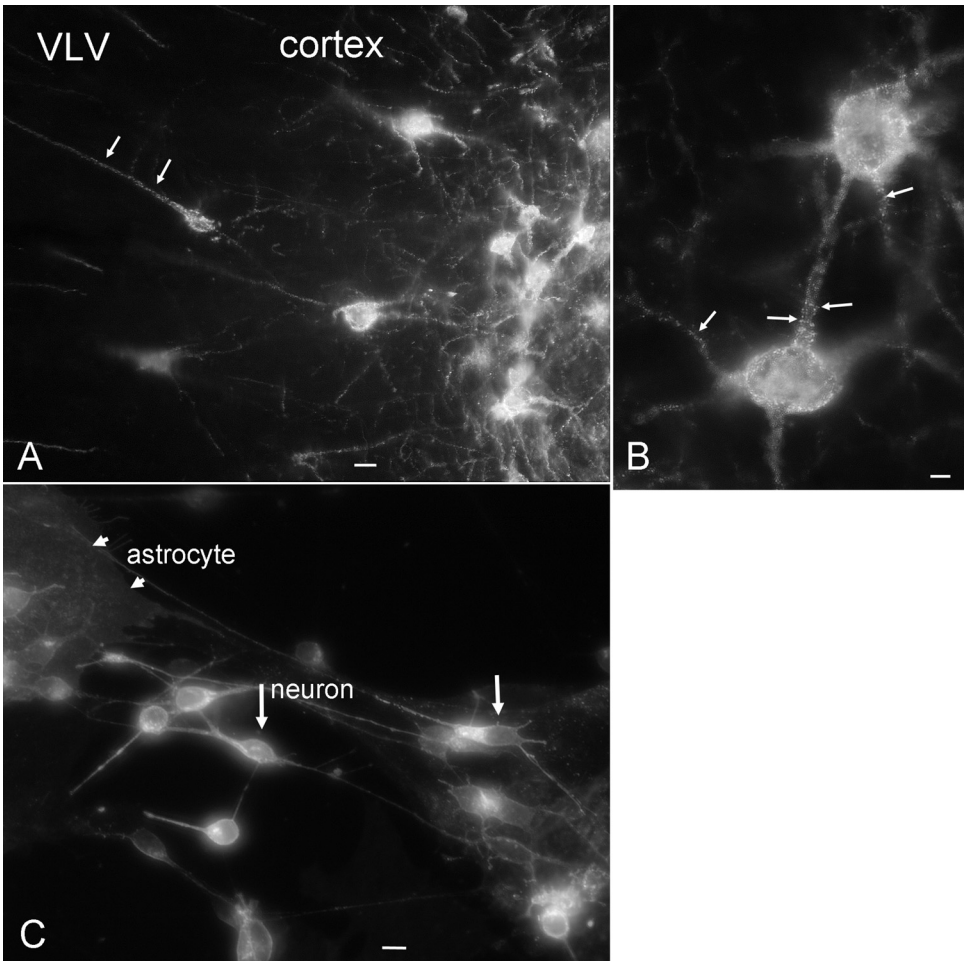

FIG 7 VLV in brain and culture. (A) After intracranial inoculation, VLV infects mostly neurons. The arrows show a dendrite stretching toward the outer cortex filled with small vesicles, a typical sign of early infection. Bar, 10 μm. (B) Two infected cells in the cortex, with vesicle-like labeling of the cell body and processes (arrows). Bar, 4 μm. (C) *In vitro*, both astrocytes and neurons are infected by VLV. Bar, 9 μm.

almost all the cells. In contrast, preincubation of cells in 1 and 10 U/ml IFN completely blocked subsequent infection by all 3 viruses (Fig. 10A and B). The cells looked healthy and showed no sign of VSV immunoreactivity after IFN treatment.

Viruses that showed no adverse effect in the brains of healthy adult mice were microinjected into the brains of adult mice in which the type I interferon receptor gene was absent (IFNR-KO [knockout]). All three viruses, i.e., VSVΔG-CHIKV, VSVΔG-H5N1, and VLV, were fatal to IFNR-KO adult mice ($n = 4$ in each of the 3 groups) that received intracranial injection (Fig. 10C). Most of the lethal effects occurred within a week. Together, these results suggest that the type I IFN response is critical for protection of the brain against all 3 of the chimeric viruses that were safe in the healthy, immunocompetent brain.

DISCUSSION

Here, we examined the actions in the brain of 4 VSV-related chimeric viruses that show potential as vaccine immunogens. A large number of previous studies have shown efficacy of VSV-related antigen vaccines (1–9), with the primary concern being the potential adverse or even lethal response if the virus enters the brain. Although the blood-brain barrier generally blocks VSV from entering the CNS, brain entry can still occur infrequently, either in early development through the olfactory nerve, with injury to the blood-brain barrier, or in situations where metastases from peripheral cancers form an infectible cellular bridge across the meninges and into the brain (21).

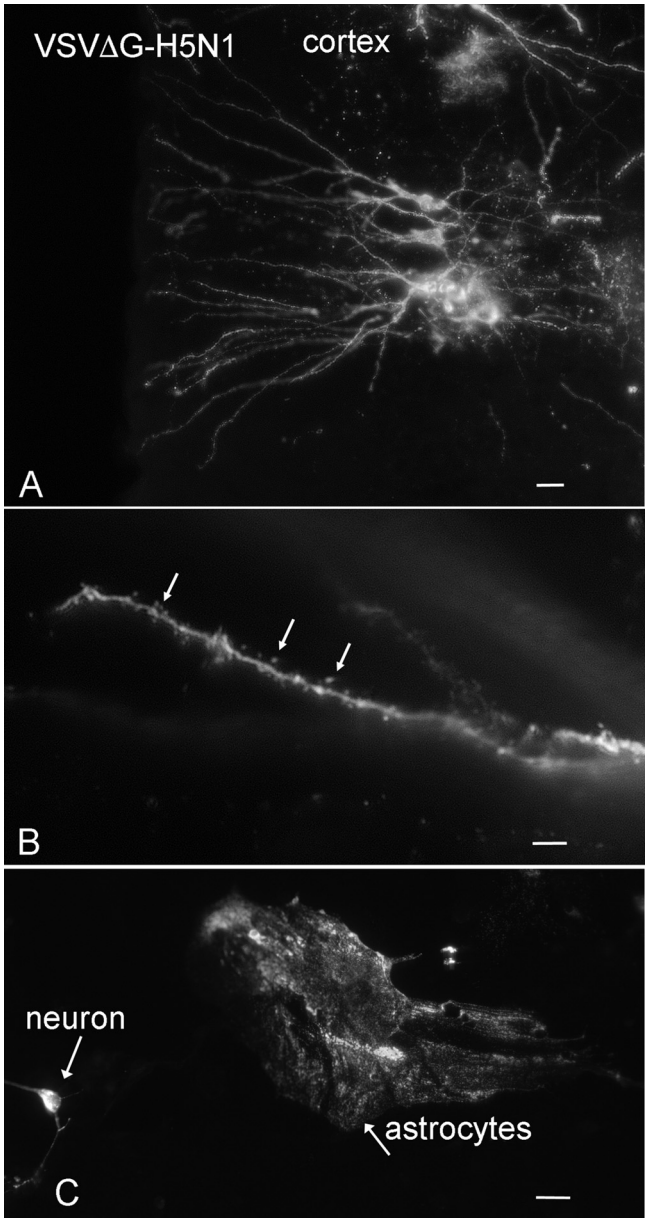

FIG 8 VSVΔG-H5N1 in the brain. (A) Cortical neurons were infected by VSVΔG-H5N1 at 3 dpi in the adult mouse brain. Bar, 18 μm. (B) High magnification of a neuronal dendrite with dendritic spines (arrows). Bar, 4 μm. Although the cells were infected at the injection site, the virus showed little spread. (C) *In vitro*, neurons and astrocytes show infection. Bar, 14 μm.

An intranasal route of administration can constitute an effective and simple accessible site for vaccination with VSV vectors (19, 28). In addition, the intranasal route of virus administration is often used to provide a rough estimate of the neurotropism of the virus, based on the working assumption that a neurotropic virus will travel along the olfactory nerve into the brain (17, 18, 52). Our data from mouse studies suggest that VSVΔG-Nipah F+G and VSVΔG-H5N1 may not be safe for intranasal inoculation and would require additional safety studies. We did not study the response of mice younger than P14, but we expect that, given their immature immune status, they would be more vulnerable to these chimeric viruses, consistent with the lethal responses of mice lacking the type I IFN receptor.

VSVΔG-H5N1 is unusual in that it is lethal by intranasal inoculation at P14 or by direct intracranial injection at P14, but when injected directly into the brains of adult

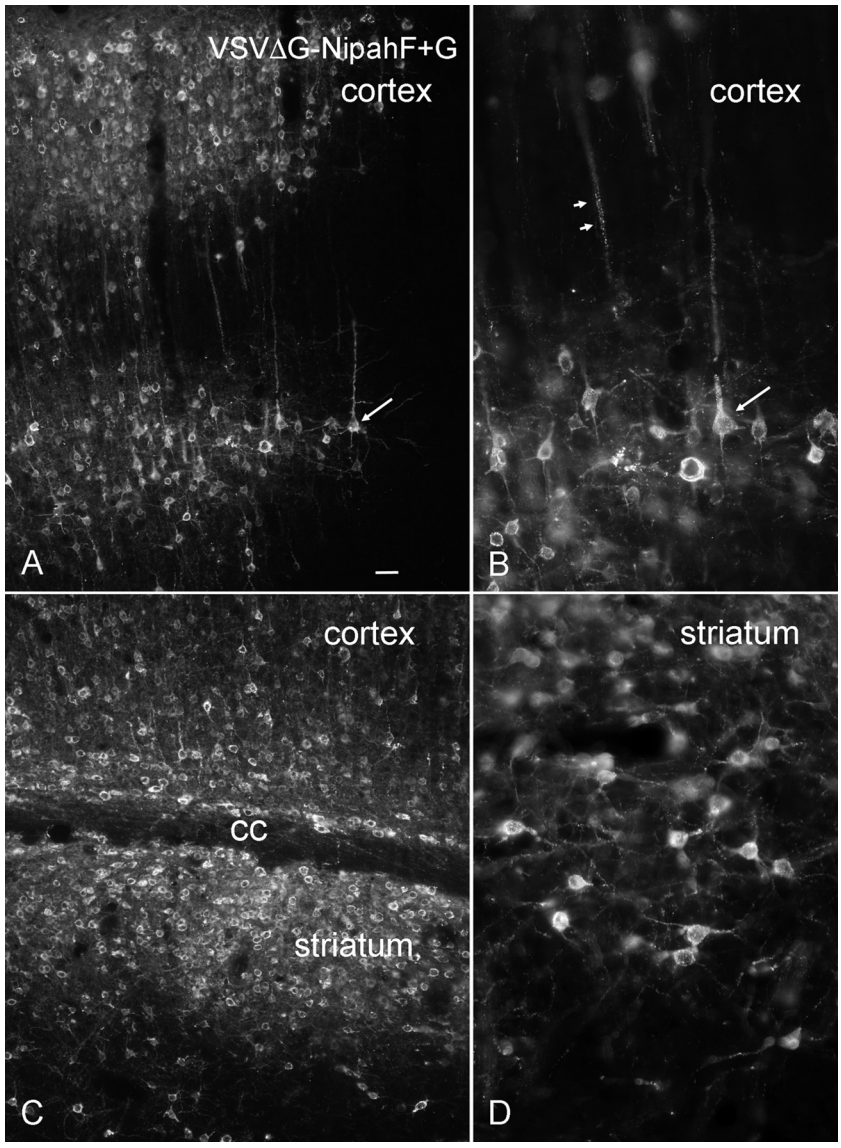

FIG 9 VSVΔG-Nipah F+G in the brain. (A) At 1 day after a small, 0.4-μl intracortical injection, VSVΔG-Nipah F+G had spread to a large number of neurons. The arrow indicates a neuron with pyramidal cell morphology. Bar, 22 μm. (B) Higher magnification of panel A. Arrows point to the same neuron in panels A and B. (C) Virus spread across the corpus callosum (cc) into the striatum. (D) Higher magnification of infected striatal cells. In both the striatum, as shown, and the cortex (B), most infected cells had the morphology of neurons. No infection was seen in the contralateral cortex.

mice, it shows no adverse action. Most VSV-related viruses are more lethal when administered directly into the brain rather than by intranasal inoculation, even when done at a younger age. The most probable explanation for our finding is that VSVΔG-H5N1 is able to overpower brain cells during the period of development when innate immunity is still maturing (53, 54). Thus, the virus is able to enter and replicate and disseminate within the brains of younger mice, but virus spread is blocked in the mature brain.

Of all the viruses tested here, including wild-type VSV-G/GFP, the complementing pair of VSVΔG-Nipah F+G viruses is the most aggressive and lethal in the brain. Mice that received an intracranial injection of the VSVΔG-Nipah F+G pair all succumbed to the virus within 2 days, a surprisingly rapid lethal response. Having both Nipah F and G expressed by a single VSV inoculum is therefore likely to be of limited vaccine value, due to the potential to be rapidly lethal. Having different VSV recombinants each

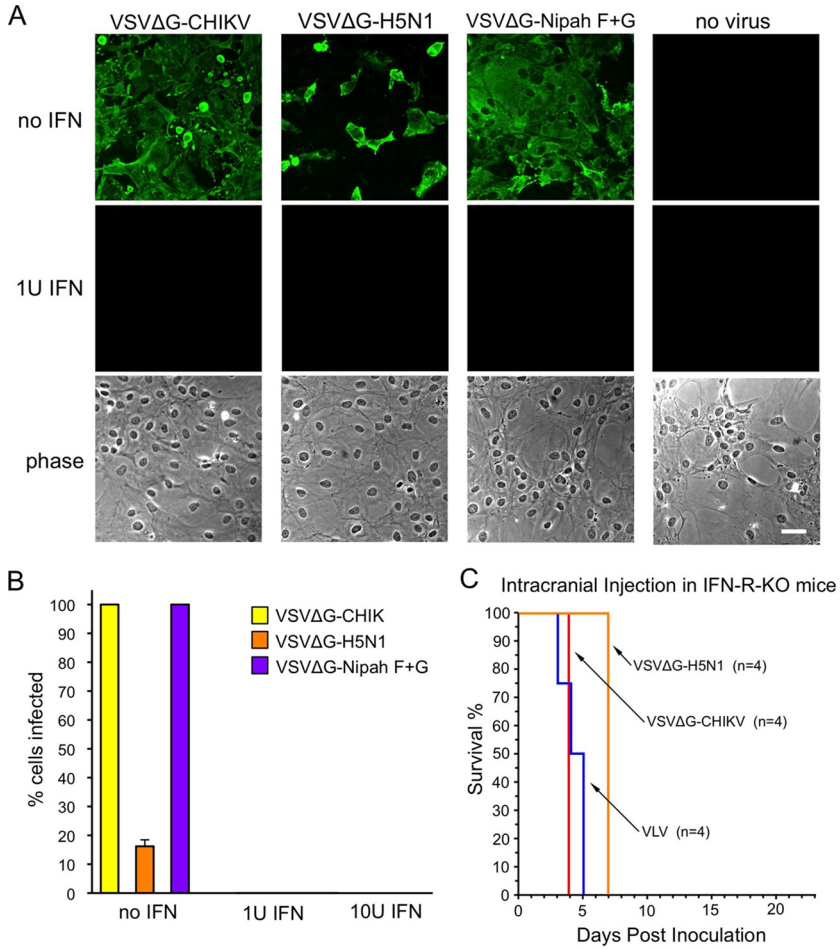

FIG 10 Type I IFN blocks chimeric VSVs in human brain cultures. (A) Primary cultures of human brain astrocytes were pretreated with 0 (control), 1, or 10 U/ml of type I IFN for 18 h and then inoculated with the indicated chimeric VSVs (final concentration, 2×10^5 PFU/ml) or medium control and allowed to incubate for a further 24 h. The cells were then fixed and immunostained. The phase contrast images show typical cell density. The VSV-immunostained cultures show virus infection. Scale bar, 50 μm. (B) Percent infected cells in cultures without IFN pretreatment or with 1 U/ml IFN; 1 U/ml IFN was sufficient to completely block chimeric VSV infection, and 10 U/ml IFN (not shown) also completely blocked infection. The error bars indicate standard errors of the mean (SEM) (n = 3). (C) Three viruses, VSVΔG-H5N1, VSVΔG-CHIKV, and VLV, were injected intracranially into adult mice lacking type I IFN receptors. All three viruses were lethal within 1 week in all IFNR-KO mice tested.

expressing only one Nipah glycoprotein, however, appears safe. Nonspreading VSVΔG-Nipah F or VSVΔG-Nipah G, used separately, generated a protective immune response against Nipah virus (47, 48) and in the absence of ongoing replication would be substantially safer and more viable than the combined VSVΔG-Nipah F+G.

VSVΔG-Nipah F+G is lethal with surprising speed. The virus infects in a rapidly expanding pattern around the injection site. After cortical microinjections of a relatively small volume (500 nl), the virus infected all layers of the cortex in the area in which it was injected and then spread across the corpus callosum into the nearby ipsilateral striatum. Although glial cells were initially infected at the injection site, cells at the periphery of the expanding pattern were mostly neurons. Release of progeny virus from long dendrites that may extend several hundred micrometers away from the cell body may contribute to the rapid expansion of infection (55, 56). It is interesting that the substitution of the glycoprotein from another neurotropic virus, rabies virus, ameliorates the resulting chimeric VSV, and interestingly, it is no longer lethal when injected into the brain (42, 57), whereas substitution of Nipah F + G for VSV-G shows enhanced

lethality. *A priori*, the different CNS responses to chimeric VSVs with rabies G or Nipah F+G would have been difficult to predict in the absence of CNS testing.

Of the viruses tested, VSVΔG-H5N1, VSVΔG-CHIKV, and VLV all appeared safe when injected directly into the healthy adult mouse brain. All adult mice that received intracranial inoculations survived with no obvious adverse effect. A key mechanism underlying the immunological protection against these three viruses within the brain is the type I IFN response. All three viruses that were found to be safe in the healthy immunocompetent adult brain were lethal in adult mice lacking the type I IFN receptor. This is consistent with our recent work showing that VSV-LASV-GPC, consisting of VSV with the VSV glycoprotein replaced with the Lassa fever virus glycoprotein, GPC, was completely safe in the brains of healthy mice, SCID mice, and rats but was lethal in the brains of mice lacking a type I IFN response (40). The fact that the lack of a serious adverse effect in the healthy brain in the present study was dependent on type I interferon suggests that each of the three viruses has the potential to infect and to replicate and spread within the brain, leading to death, except that such spread is successfully prevented by the innate immune blockade of virus dispersion. IFN can act in the brain both locally (58) and far from the initial site of inoculation. VSV and an unrelated DNA virus, cytomegalovirus, both induced IFN-mediated signaling, not just in cells near the local site of infection, but also in distant parts of the brain in which no virus was detected, serving to attenuate virus infections both locally and at remote sites within the brain (59).

Here, we focused on the actions of the above-mentioned chimeric viruses in the brain. The reason for this focus was that the brain appears to be the most problematic organ for many recombinant VSVs. With the exception of analysis of the lung after intranasal VSVΔG-H5N1 inoculation, where we did not detect substantive infection, we did not study other organs in detail here, as they have not been shown to constitute a substantial problem for wild-type VSV. Nonetheless, given the differential tissue targeting of the different virus glycoproteins, future analyses may profit from additional inspection of infection of other organs by chimeric VSVs expressing glycoproteins other than that of VSV.

All the chimeric viruses discussed here have shown efficacy in acting in a vaccine capacity to protect against the respective virus antigens, including Nipah virus, chikungunya virus, and influenza virus (44, 46–49). By virtue of their safety in both adult and developing brains, VSVΔG-CHIKV and VLV showed the least adverse neurotropism among the chimeric viruses tested; these viruses merit further investigation for potential applications, including vaccination. Whereas VSVΔG-CHIKV itself may be an effective vaccine against CHIKV, the addition of other antigen-coding regions to the chimeric genome or to the VLV genome could potentially create a safe and effective vaccine against a variety of pathogens, as shown for a vaccine against hepatitis B virus using VLV as a hepatitis B virus immunogen (49). In general, substitution of glycoprotein genes from other viruses appears to be a viable approach to addressing the problem of the neurotropism and potential adverse action within the brain associated with the VSV glycoprotein, with the caveat that substitution alone may not generate the desired attenuated phenotype, as seen from the neurotropic effects we observed with VSVΔG-Nipah F+G in the adult brain and VSVΔG-H5N1 in the developing brain. Furthermore, although the VSV glycoprotein has been shown to be neurotropic and therefore problematic in the brain (17, 18), the glycoprotein alone may not lead to lethality, as shown by the highly attenuated VLV, which, although it employs the VSV glycoprotein and does infect neurons, shows highly attenuated replication that does not lead to lethal actions in the healthy adult brain.

MATERIALS AND METHODS

Viruses used. VSV-G/GFP has been described previously and expresses a GFP reporter fused to a second copy of VSV-G (18, 51). VSVΔG-LCMV has been described previously (42) and was a kind gift from S. Whelan (Harvard University). VSV-IFN-β has been described previously (11) and was a kind gift from Glen Barber (University of Florida).

VSVΔG-CHIKV was generated by replacing the VSV G gene with the entire CHIKV envelope polyprotein (E3-E2-6K-E1). The chimera incorporated functional CHIKV glycoproteins into the viral envelope in place of VSV G (44). VSVΔG-H5N1 has the VSV G gene deleted, and in its place were substituted the two influenza virus genes, H5 and N1, as described in detail previously (45, 46). Genomic diagrams of the recombinant chimeric viruses are shown in Fig. 1.

VSVΔG-Nipah F and VSVΔG-Nipah G have the Nipah F or G glycoprotein substituted for the VSV G protein and are both replication incompetent. VSVΔG-Nipah F+G is a combination of the two replication-incompetent VSVs that each express a single Nipah glycoprotein, F or G. This was done by adding together the two replication-incompetent viruses (VSVΔG-Nipah F plus VSVΔG-Nipah G). Whereas each separately does not propagate, together the two recombinants complement each other and generate a replication-competent chimeric virus pair that expresses both VSV F and G on the virus surface (47, 48) when both viruses infect the same cell. Each of the two chimeric viruses was initially pseudotyped with the VSV glycoprotein by expanding the respective virus in cells that express the VSV glycoprotein on the surface.

The VLVs are replication-competent virus-like vesicles derived from a Semliki Forest virus RNA replicon expressing VSV glycoprotein G in place of the Semliki Forest virus structural proteins but lacking a nucleocapsid protein (37) and expressing the Semliki Forest nonstructural proteins. The VLVs used in this study were evolved through extensive passaging to generate high titers (39).

***In vivo* studies.** P14 male and female Swiss Webster mice were used for intranasal inoculations. Fifteen microliters of virus (1×10^6 PFU/ml for VSVΔG-Nipah F+G; 2×10^7 PFU/ml for all others) was administered into the left and right nares. For intracranial administration, a small (0.5-μl) volume (the same concentration mentioned above) of sterile saline containing virus was injected by microsyringe into the striatum on one side of the adult mouse brain using a mouse stereotactic device during deep anesthesia. Additional experiments examined the response of P14 mice to intracranial virus injection. In some experiments, adult mice lacking the type I interferon receptor (IFNR-KO) were used to test the safety of viruses administered into the brain in animals lacking a type I IFN response.

All animal experiments were approved by the institutional animal care and use committee (IACUC). If any mouse showed substantial disabling health deterioration, it was euthanized by anesthetic overdose, as required by the IACUC, and recorded as showing a lethal response to the virus tested. Similarly, if any mouse's body weight showed a decrease of 25%, it was euthanized, as mandated by the IACUC.

***In vitro* studies.** Mouse brain cultures were generated by removing the brains from embryonic day 18 mice and disaggregated with papain (20 U/ml; Worthington Biochemical: LS003126) in phosphate-buffered saline (PBS) to allow generation of multiple isolated cells not attached to others. The cells were plated on poly-D-lysine (Sigma; P6407)-coated plastic or glass bottom culture wells. The cultures contained both neurons and astrocytes. Cultures were grown in minimal essential medium supplemented with 10% fetal bovine serum (Gibco). Eight days later, the cultures were inoculated at an MOI of 0.5 with the different viruses tested; 16 h later, the cultures were washed with PBS and fixed with 4% paraformaldehyde. The cells were permeabilized with 0.3% Triton X-100 in a PBS blocking solution containing 1% bovine serum albumin and 2% normal horse serum (Vector Laboratories; S-2000). A primary rabbit anti-wild-type VSV antibody was used to immunostain the cultures (60); the antibody bound to multiple VSV proteins, allowing detection of chimeric viruses that expressed only part of the VSV genome. The antibody reacted with all the viruses used in the present study. A secondary goat anti-rabbit antibody conjugated to a green fluorescent molecule (Alexa Fluor 488; A11008; Invitrogen) was used to localize the virus in infected neurons and glia.

Interferon response experiments were done *in vitro* using cultures of human brain primary astrocytes (20). Briefly, nearly confluent cultures were grown in 24-well plates and pretreated for 18 h with type I IFN-αA/D (Sigma; I4401) at the indicated concentrations. After IFN pretreatment, the cultures were inoculated with virus (final concentration, 2×10^5 PFU/ml) or medium (control) and allowed to incubate for 24 h. The cells were then fixed and immunocytochemically labeled using the primary antibody described above (overnight; dilution, 1:5,000) and the secondary goat anti-rabbit antibody (1 h; dilution, 1:500) also described above. Infected cells were counted in triplicate wells for each condition.

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