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Neurokinin-1 enables measles virus trans-synaptic spread in neurons

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Abstract

Measles virus (MV), a morbillivirus that remains a significant human pathogen, can infect the central nervous system, resulting in rare but often fatal diseases, such as subacute sclerosing panencephalitis. Previous work demonstrated that MV was transmitted trans-synaptically and that, while a cellular receptor for the hemagglutinin (H) protein was required for MV entry, it was dispensable for subsequent cell-to-cell spread. Here, we explored what role the other envelope protein, fusion (F), played in trans-synaptic transport. We made the following observations: (1) MV-F expression in infected neurons was similar to that seen in infected fibroblasts; (2) fusion inhibitory peptide (FIP), an inhibitor of MV fusion, prevented both infection and spread in primary neurons; (3) Substance P, a neurotransmitter with the same active site as FIP, also blocked neuronal MV spread; and (4) both genetic deletion and pharmacological inhibition of the Substance P receptor, neurokinin-1 (NK-1), reduced infection of susceptible mice. Together, these data implicate a role for NK-1 in MV CNS infection and spread, perhaps serving as an MV-F receptor or co-receptor on neurons.

Keywords: Measles; Fusion inhibitory peptide; Substance P; Neuron; Subacute sclerosing panencephalitis; Central nervous system; Fusion; CD46; Spread; Neurokinin-1

Introduction

In rare cases, MV can invade and persist within the human central nervous system (CNS), leading to progressive and fatal neurological diseases, including subacute sclerosing panencephalitis (SSPE) (Anlar, 1997; Griffin and Bellini, 1996; Schneider-Schaulies et al., 1999). Two hallmarks of SSPE are the protracted period between initial infection and signs of disease (often years), and the inability to recover infectious virus from affected brain tissues, despite the extensive presence of MV RNA and protein (Katz, 1995; Payne et al., 1969). This is in contrast to the rapid and highly productive infections that occur in non-CNS tissues. The apparent differences in MV replication and spread within the human CNS implied that viral neurotransmission was substantively less cytopathic and productive than in non-neuronal tissues.

Mice are normally non-permissive for MV; thus, to study MV spread and pathogenesis in the CNS, a transgenic mouse model of neuronal infection was developed. These transgenic mice expressed CD46, the first identified human MV receptor (Dorig et al., 1993; Naniche et al., 1993), now thought to be the primary receptor for vaccine strains (Cattaneo, 2004), under the transcriptional control of the neuron-specific promoter, neuron-specific enolase (NSE) (Rall et al., 1997). Subsequent challenge of NSE-CD46 transgenic mice with the vaccine strain, MV-Edmonston, by either the intracerebral or intranasal route resulted in infection in all transgenic mice. While adult immunocompetent mice resolved the infection, both $CD46^{+}$ neonates and immunodeficient

Abbreviations: MV, measles virus; CNS, central nervous system; dpi, days post-infection; hpi, hours post-infection; NK-1, neurokinin-1; F, fusion; H, hemagglutinin; NSE, neuron-specific enolase.

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(e.g., RAG knockout (KO)) CD46$^+$ adults developed CNS disease resulting from unrestricted neuronal infection (Lawrence et al., 1999; Rall et al., 1997).

Using primary hippocampal neurons obtained from NSE-CD46$^+$ embryos, we showed that MV neuronal infection was noncytopathic and did not result in release of extracellular progeny, despite extensive viral spread (Lawrence et al., 2000). Interestingly, neuron-to-neuron spread of MV occurred exclusively at the synapse and did not require expression of CD46 (Lawrence et al., 2000; Oldstone et al., 1999; Rall et al., 1997), indicating that a hemagglutinin (H)–receptor interaction may not be required at the synaptic interface (Lawrence et al., 2000).

Based on this observation, we next wanted to establish what role, if any, the viral fusion protein played in trans-synaptic transport. While it is generally believed that H and F have interaction domains that coordinate receptor binding and fusion (Hu et al., 1992), work by other laboratories has suggested that F may act independently of H and may even govern target cell specificity. For example, in simian virus 5 (SV5), F promotes fusion in the absence of the hemagglutinin-neuraminidase (HN) protein, albeit less efficiently than when HN is present (Horvath et al., 1992; Ito et al., 1997). Moreover, SV5 F protein mutants that require SV5 HN for fusion at 37 °C can fuse in an HN-independent manner at higher temperatures. Thus, fusion may require surmounting a thermodynamic threshold, which can be accomplished by a conformational change in the HN protein, increased temperature, or perhaps interaction of the F protein with its own cellular receptor (Dutch et al., 2001; Paterson et al., 2000). This final possibility has support in the literature since the F_2 fusion protein of respiratory syncytial virus (RSV), a *Pneumovirus* within the *Paramyxovirus* family, determines host cell tropism (Schendler et al., 2003), and recombinant RSV particles lacking the G protein remained viable and able to spread (Techaarpornkul et al., 2001).

In this study, an inhibitor of paramyxovirus fusion, fusion inhibitory peptide (FIP) was used (Richardson and Choppin, 1983; Richardson et al., 1980). The tripeptide sequence of FIP (Phe-Phe-Gly) is identical to the active site of the neurotransmitter Substance P (Fig. 4A), which can also inhibit MV spread in non-neuronal cultures (Harrowe et al., 1990; Schroeder, 1986). Substance P transduces signals to target cells by engagement of the neurotachykinin family of G protein-coupled neurotransmitter receptors, including neurokinin-1 (NK-1) (Gerard et al., 1991; Schaffer et al., 1998), a highly conserved protein expressed on diverse mammalian cells, including neurons, lymphocytes, smooth muscle cells and vascular endothelium (Pennefather et al., 2004). NK-1 expression is not ubiquitous within the CNS, though most central structures display some expression (Mantyh, 2002). In neurons, NK-1 is broadly distributed on the plasma membrane, though concentrated at the synapse (Bozic et al., 1996). Here, we show that both FIP and Substance P prevent MV spread in neurons and that both genetic deletion and pharmacological inhibition of NK-1 also reduce infection of permissive mice. Together, these data indicate that fusion is a required step for viral transport across the synaptic cleft and suggest that NK-1 plays a key role in this process, perhaps serving as an MV-F receptor.

Results

Expression and distribution of the fusion protein in MV-infected primary neurons

We previously showed that inter-neuronal transmission of MV was trans-synaptic and CD46-independent (Lawrence et al., 2000). Because CD46-negative neurons were non-permissive for infection by extracellular virus, but could support infection when co-cultured with infected CD46$^+$ neurons, we hypothesized that the MV-H protein, though needed for initial MV entry, may be dispensable for neuron-to-neuron trans-synaptic spread of MV.

Because both MV-H and F are typically involved in viral entry and spread, we next asked whether MV-F was translated and processed in infected neurons. Lysates were prepared from MV-infected or mock-infected neurons and Vero cells. Western blots using a rabbit antibody raised against the F carboxy terminus (Cathomen et al., 1998) were then performed on protein lysates equivalent to 10^5 infected cells. For the mock-infected controls, an amount of lysate corresponding to 10^5 cells was used. MV-F is synthesized as a precursor (F_0) that is post-translationally cleaved into two subunits, F_1 and F_2. As shown in Fig. 1, equivalent numbers of infected Vero fibroblasts and CD46$^+$ neurons yielded both F_0 and F_1 fragments, though the amount of each present in neurons was consistently less. Despite slight differences in expression levels, cleavage of the F_0 protein was efficient in both cell types. Note that the F_2 fragment is not recognized by this antibody.

Fusion inhibitory peptide blocks MV spread in primary neurons

Fusion inhibitory peptide (FIP) is a synthetic tripeptide (z-D-Phe-L-Phe-Gly) that prevents fusion by multiple viruses, though its strongest activity is against MV (Norrby, 1971; Richardson and Choppin, 1983; Richardson et al., 1980). To determine if FIP blocked MV spread in neurons, primary neurons obtained from NSE-CD46$^+$ embryonic hippocampi, and control Vero fibroblasts were infected with MV-Ed using a

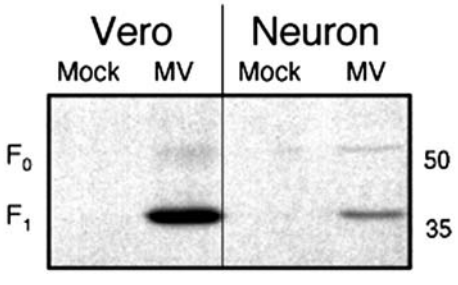

Fig. 1. MV F expression in infected neurons. 48 hpi with MV-Ed (MOI=3), primary CD46$^+$ neurons and Vero cells were fixed for immunostaining or solubilized in protein sample buffer as described. The percent of infected cells was determined by immunochemistry, after which the protein lysate volume equivalent to 10^5 infected cells was then subjected to Western blot analysis using an anti-F antibody. Size standards are indicated at the right. Shown is one representative experiment of three.

range of MOIs from 0.1 to 3, followed by addition of FIP at 6 h post-infection (hpi). Cells were then collected 1–3 dpi and immunostained for the presence of MV antigens. One representative experiment of three using an MOI=0.1 and FIP at 200 μM is shown in Fig. 2. In Vero cells without FIP, MV spread rapidly, resulting in extensive infection and syncytia (Fig. 2A). As expected, addition of FIP after infection prevented viral spread and syncytia formation, and the monolayer remained intact (Fig. 2B). Because FIP was added 6 hpi, individual MV-positive cells were expected.

Similarly, addition of FIP after primary infection blocked the trans-synaptic neuronal transmission of MV (Fig. 2D) that was otherwise observed in untreated cultures (Fig. 2C). Identical results were obtained when infection and FIP addition were separated by 1 h, or when other MOIs were used (data not shown). As shown in Table 1, the effect of FIP addition on MV spread was dose-dependent for both cell types, with >50% inhibition occurring at 50 μM. The inhibitory effect of FIP was specific for MV as identical experiments with two other neurotropic RNA viruses, lymphocytic choriomeningitis virus (LCMV) and poliovirus (PV), showed no differences in infection levels in the presence of FIP (data not shown).

FIP blocks MV infection of primary neurons

The initial characterization of paramyxovirus hydrophobic fusion inhibitors showed that both *de novo* infection and subsequent cell-to-cell spread were prevented (Norrby, 1971; Richardson and Choppin, 1983; Richardson et al., 1980). In our hands, however, addition of FIP prior to viral challenge did not prevent infection of Vero cells (Fig. 3D), though subsequent viral spread was blocked (Figs. 3E and F). The suppressive effect of FIP was reversible: when FIP was washed out of the cultures at 3 dpi, MV rebounded rapidly, resulting in the same cytopathicity and syncytia formation as observed in untreated cultures (compare Figs. 3G and C). In contrast, pretreatment of CD46$^+$ neurons with FIP completely blocked infection (Figs. 3K–M), and, as expected, FIP washout did not result in MV re-emergence (Fig. 3N). The inability of FIP to block infection of Veros could be due to cell-specific differences in receptor density, distribution (or utilization) of putative fusion receptors (discussed below) or access of FIP to cell surfaces. Taken together, these data indicate that FIP can prevent both infection and spread in primary CD46$^+$ mouse hippocampal neurons.

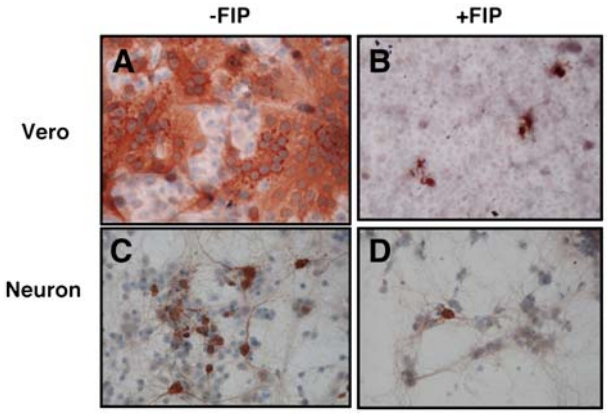

Fig. 2. Fusion inhibitory peptide prevents MV spread in primary neurons. Vero cells and CD46$^+$ neurons were plated onto coverslips as described and infected with MV-Ed, MOI=0.1. Six hpi, FIP was added to achieve a final concentration of 200 μM, and slips were fixed and immunostained 3 days thereafter. (A and B) Vero cells; (C and D) neurons. (A and C) DMSO added; (B and D) FIP added. Original magnification=400×. Shown are fields from one representative experiment of three at this virus and dose concentration. No differences were noted using MOIs ranging from 0.1 to 3.

Table 1
The ability of FIP to prevent MV spread is dose-dependent[a]

FIP concentration (μM)	MV infection (%)[b]	
	Veros	Neurons
200	12 (±8)	4 (±3)
100	17 (±11)	9 (±5)
50	42 (±18)	14 (±4)
5	92 (±6)	15 (±5)
DMSO	87 (±11)	28 (±10)

[a] Veros and CD46$^+$ primary neuron cultures were infected with MV-Ed at an MOI=3. FIP (at the indicated concentrations) or the diluent, DMSO, was then added 6 hpi, and cells were collected 2 dpi, immunostained and counted.
[b] Values represent the percent of infected cells per field, viewed at 100× magnification, ±standard deviation. A total of 10 random fields were counted from each of 3 independent experiments.

Inhibition of measles virus spread by neurotransmitters

The Phe-X-Gly sequence of FIP is also the active site of the neurotachykinin family of peptide neurotransmitters that include Substance P, Neurokinin A and Neurokinin B (Fig. 4A). For Substance P, the central residue of the motif is a phenylalanine (as in FIP), while for Neurokinin A and B it is a valine. In addition, for FIP, the central Phe residue is the L-isomer, whereas for the neurokinins, it is the D-isomer.

It was previously shown that Substance P could inhibit MV spread in non-neuronal cultures (Harrowe et al., 1990; Schroeder, 1986). To determine if the neurotachykinins block MV trans-neuronal spread in our model system, we performed inhibition experiments identical to those described above with FIP, using the three neurotachykinins, two other non-neurotachykinin peptide transmitters (bradykinin and opioid receptor antagonist peptide) and a Substance P antagonist, spantide, which does not contain the Phe-X-Gly motif. Peptides were added at the indicated concentrations 6 hpi. Cells were collected at 3 dpi and immunostained for MV. The number of infected cells was expressed relative to DMSO treated cultures, which were normalized to 100%. One representative experiment of five is shown in Fig. 4B. Note that, as in Fig. 2, the neuropeptides were added after infection, so that some infected cells were expected in all cultures. The infection baseline at 1 dpi is indicated by the solid line, approximately 20%. For these studies, virus yields were not used as a measure of activity as no extracellular virus is released from infected primary neurons (Lawrence et al., 2000).

All members of the neurotachykinin family inhibited MV transmission in a dose-dependent manner, although Substance P

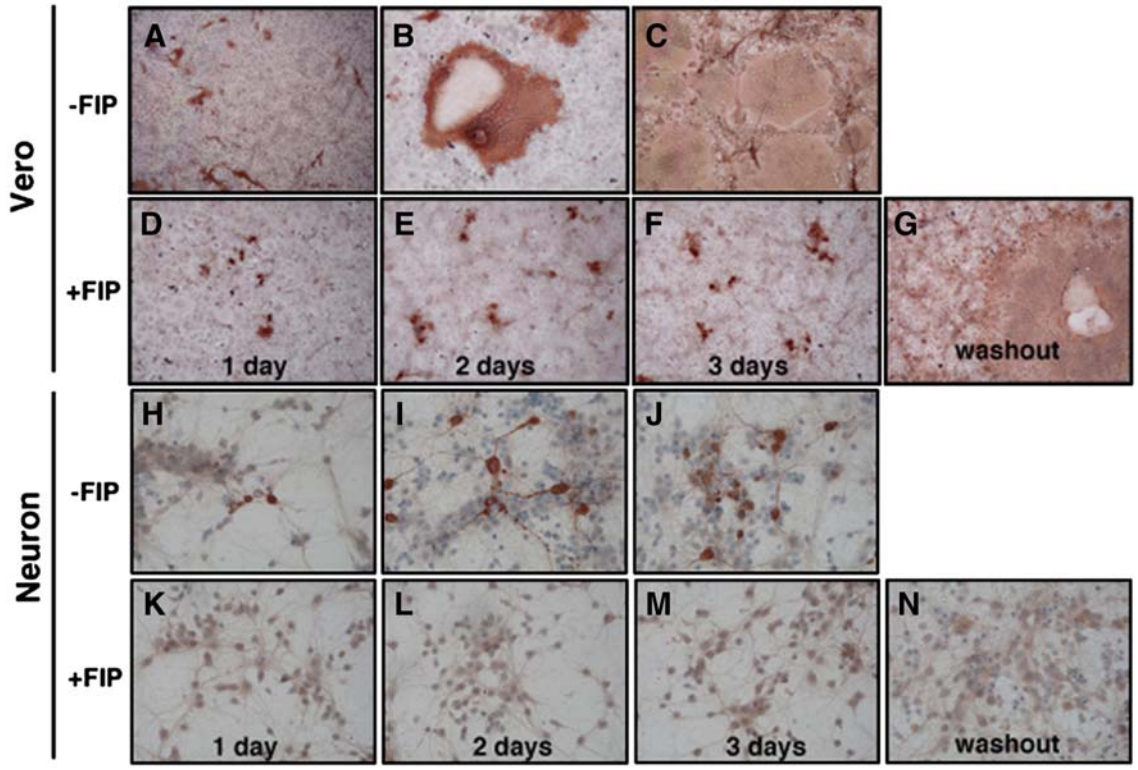

Fig. 3. FIP prevents *de novo* infection in neurons. Vero cells (A–G) and primary CD46$^+$ neurons (H–N) were plated on coverslips as described. FIP (200 μM) (D–G; K–N), or an equivalent volume of DMSO (A–C; H–J) were added and, after a 1 h incubation, cells were infected with MV-Ed, MOI = 3. Coverslips were fixed and immunostained 1–3 dpi. For the washout experiments (G and N), at 3 dpi the cells were washed and replaced with fresh media without FIP. Samples were then collected 48 h post-FIP removal, equivalent to 5 dpi. Representative fields from one of four independent experiments are shown. Original magnification = 100×.

showed greater potency than NK-A or NK-B at the same concentrations (Fig. 4B, and data not shown). Non-neurotachykinin peptides had no effect on MV transmission. Moreover, spantide, a Substance P antagonist that lacked the Phe-Phe-Gly motif and that presumably does not bind to the same site on NK-1 as Substance P, also had no effect on MV spread, underscoring the potential importance of this motif in MV-mediated fusion.

The Substance P receptor, neurokinin-1, contributes to MV trans-neuronal spread

We next asked if the cellular receptors for the neurotachykinin peptides were required for MV spread, with particular emphasis on NK-1, the predominant Substance P receptor. Two strategies were used: genetic knockout mice (Fig. 5) and pharmacological NK-1 inhibitors (Fig. 6).

NK-1 knockout (NK-1 KO) mice (Bozic et al., 1996) were intercrossed with NSE-CD46$^+$ homozygous mice to obtain NSE-CD46$^+$/NK-1 KO mice. As reported in the original characterization, NK-1 KO mice show no apparent developmental abnormalities with regard to lifespan, fertility, fecundity or brain anatomy (Bozic et al., 1996). All mice were genotyped for CD46 and NK-1 status, as described in Materials and methods. Because neonatal CD46$^+$ mice (that are also NK-1$^+$) succumb to MV-mediated CNS disease following viral challenge (Lawrence et al., 1999; Rall et al., 1997), we asked what impact genetic deletion of NK-1 would have on MV neuronal infection and resultant pathogenesis in the neonatal model. As previously shown, MV-infected control CD46$^+$/NK-1$^+$ neonates (black triangles) developed symptoms within 4–8 dpi and died 1–2 days thereafter (Fig. 5A). In contrast, CD46$^+$/NK-1 KO neonates (black circles) either showed no signs of illness through adulthood, or developed illness at a much later time (18 dpi in 35% of CD46$^+$/NK-1 KO neonates vs. 5–9 dpi in 100% of CD46$^+$/NK-1$^+$ neonates) (Fig. 5A). None of the surviving CD46$^+$/NK-1 KO mice showed evidence of MV CNS infection by RT-PCR analysis; in contrast, MV antigens were observed in all moribund CD46$^+$/NK-1 KO mice collected at 18 dpi (data not shown). As expected, CD46$^-$/NK-1$^+$ mice (white circles) were non-permissive for infection and showed no signs of illness or infection at any timepoint, indicating that NK-1 alone cannot mediate MV entry.

To ascertain the extent of MV infection in NK-1 expressing and non-expressing neonates collected at the same timepoint post-infection, neonatal CD46$^+$/NK-1$^+$ and CD46$^+$/NK-1 KO mice were challenged as described, and brains from all mice were collected 6 dpi, the approximate peak of infection within the CNS (Lawrence et al., 1999). Detection of the MV-N gene by RT-PCR analysis of total brain RNA showed evidence of replication in 4/4 (100%) CD46$^+$/NK-1$^+$ mice, whereas only 2/11 (18%) CD46$^+$/NK-1 KO mice were positive (Fig. 5B). These data suggest that, even when CD46 is present, expression of NK-1 is required for infection in the majority of mice. However, while NK-1 influences both the number of mice infected and the kinetics of infection, it is not obligatory

A)

FIP	z-D-Phe-L-Phe-Gly
Neurotachykinins	
Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly- Leu-Met-NH_2
Neurokinin A	His-Lys-Thr-Asp-Ser-Phe-Val-Gly- Leu-Met-NH_2
Neurokinin B	Asp-Met-His-Asp-Phe-Phe-Val-Gly- Leu-Met-NH_2
Other neuropeptides	
Bradykinin	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Opioid peptide	Tyr-Tic-NH_2
Spantide	Arg-Pro-Lys-Pro-Gln-Gln-D-Trp-Phe-D-Trp-Leu-Leu

B)

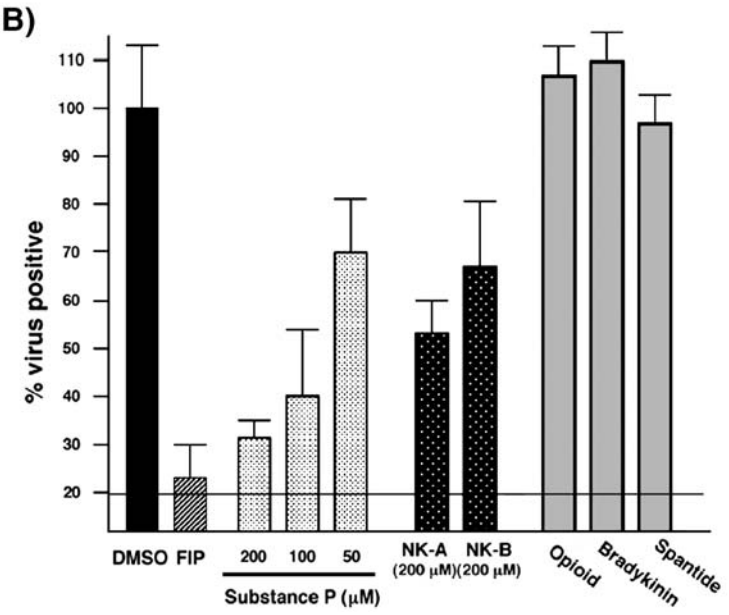

Fig. 4. Effect of neuropeptide transmitters on MV spread. (A) Amino acid sequences of neuropeptides used. Gray boxes indicate active sites of the neurotachykinins and homology with FIP. (B) Inhibitory effect of each neuropeptide on MV spread. Neurons were infected with MV-Ed, and neuropeptides were added to a final concentration of 200 μM; for Substance P, three doses were tested: 200, 100 and 50 μM. Samples were collected 3 dpi and counted; values reflect the percent infection as compared with the DMSO control. The horizontal line indicates the initial level of infection after ~ 1 round of replication (24 hpi). Because the peptides were added after infection, this represents the maximal achievable inhibitory effect. Shown is one representative experiment of five.

as some NK-1 KO neonates were susceptible to infection and neuropathogenesis.

As an alternative strategy to ascertain what role NK-1 played in MV infection of neurons, the high-affinity pharmacological NK-1 antagonist, aprepitant, currently used clinically as an anti-emetic (Emend®), was employed. Unlike the genetic knockout studies described above, neonates could not be used for the aprepitant studies because of the technical obstacle of oral drug delivery to neonatal mice. Thus, $CD46^+$ adult mice on a RAG-2 KO background were used: these mice, which lack mature T and B cells, cannot resolve a MV challenge and virtually 100% become moribund between 10 and 14 dpi (Lawrence et al., 1999). As shown in Fig. 6A, all MV-inoculated $CD46^+$/RAG-2 KO mice that did not receive aprepitant showed an average weight loss of 15% and an average illness score of 3.4 (obtained between day 6 and 10) prior to sacrifice. In contrast, aprepitant-treated $CD46^+$/RAG-2 KO mice segregated into two populations: those that developed neuropathology, and those that did not. In the representative experiment shown, 14/25 mice developed a milder illness than non-aprepitant-treated controls (2.1 vs. 3.4), and weight loss in these mice was less, averaging 5%. Importantly, 11/25 MV-challenged mice treated with aprepitant showed no signs of illness or weight loss. RT-PCR analysis of purified total brain RNA confirmed the absence of MV-N RNA in brains of healthy aprepitant-treated mice (data not shown).

When brains were collected from aprepitant-treated and untreated, MV-infected $CD46^+$/RAG-2 KO mice between 10 and 12 dpi and immunostained, mice without aprepitant showed

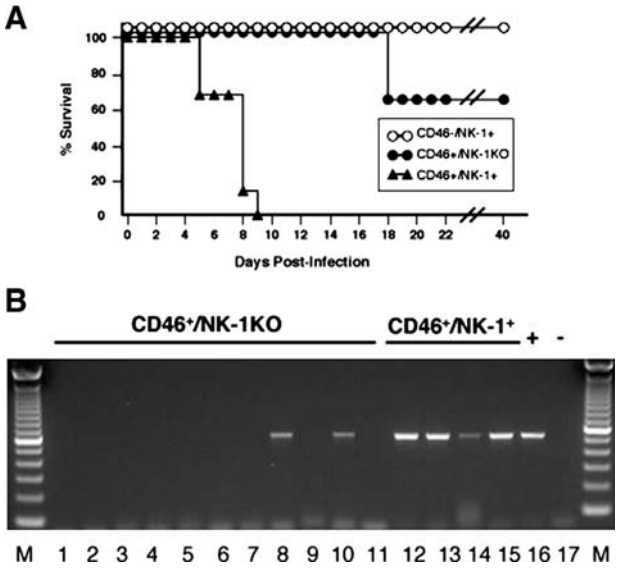

Fig. 5. Infection in neurokinin-1 knockout neonatal mice. (A) Survival curves following MV challenge in CD46$^+$/NK-1$^+$ neonates (black triangles, $n=11$); CD46$^-$/NK-1$^+$ neonates (white circles, $n=20$); and CD46$^+$/NK-1 KO neonates (black circles, $n=12$). Shown is one representative experiment of three. NSE-CD46$^+$ neonates were chosen because these mice are permissive for MV infection and develop CNS disease within 1 week post-challenge (Lawrence et al., 1999). (B) RT-PCR analysis for MV-nucleoprotein of total brain RNA, harvested from neonatal CD46$^+$/NK-1$^+$ and CD46$^+$/NK-1 KO mice at 6 dpi with MV-Ed, as described in Materials and methods.

characteristic extensive and unrestricted infection of the CNS (Fig. 6B(a–c)), whereas sick mice that were treated with aprepitant had less viral infection that seemed to be more focally restricted within the parenchyma (Fig. 6B(d–f)). No evidence of MV infection within the CNS was observed in healthy mice that received aprepitant (Fig. 6B(g–i)). Taken together, the results from both the drug and knockout experiments indicate that NK-1, a receptor for the neurotransmitter Substance P, plays a crucial role in MV spread. We hypothesize that NK-1 may be a docking receptor for the MV-F protein, facilitating cell-to-cell MV transmission.

Discussion

Our previous studies demonstrated that the mechanism of MV transmission in neurons differed dramatically from that in non-neuronal cells (Lawrence et al., 2000). In fibroblasts, MV was highly cytolytic and resulted in robust release of infectious particles. In contrast, no virus-mediated lysis of infected primary hippocampal neurons was detected, nor could extracellular virus be recovered, despite extensive viral replication and spread. Electron microscopy studies revealed both a block in the ability of MV to bud from the neuronal plasma membrane as well as the presence of viral RNPs at synaptic junctions, indicating that MV adopts a trans-synaptic mode of spread in neurons (Lawrence et al., 2000). Interestingly, trans-synaptic transmission was CD46-independent, a characteristic that appears to be unique to neurons, as CD46 was required for viral spread in fibroblast co-cultures (Makhortova et al., manuscript in press).

A synthetic tripeptide, FIP, that blocks MV fusion (Norrby, 1971; Payan et al., 1984; Richardson and Choppin, 1983), inhibited both MV infection and spread in primary neurons expressing one of the human receptors, CD46. The mechanism by which FIP prevents fusion is not known, but may either be due to direct interference of the amino terminus of the F1 subunit with the target cell membrane, or to preventing the exposure of the fusogenic peptide (Norrby, 1971; Richardson and Choppin, 1983; Kelsey et al., 1991).

The similarity of sequence between FIP and the neurotransmitter Substance P, coupled with previous reports that Substance P could interfere with MV replication in non-neuronal cells (Schroeder, 1986; Richardson et al., 1980), prompted us to ask whether Substance P and other members of the neurotachykinin family could prevent viral translocation across the synaptic cleft. Indeed, all neurotachykinins blocked spread, a process that appeared to depend, at least in part, on the presence of the neurotachykinin receptor, NK-1. The majority of NK-1 deficient neonates, as well as NK-1$^+$ adults mice treated with the specific antagonist, aprepitant, survived viral challenge without any symptoms of disease or viral replication in the CNS. Thus, we speculate that NK-1 may promote MV entry into CD46$^+$ neurons by serving as a docking receptor for the MV-F protein and that the ability of FIP and Substance P to block infection and spread may be due to competitive inhibition with the NK-1 receptor, a hypothesis currently under study in our laboratory.

The apparent ability of NK-1 to mediate viral spread at the neuronal synapse in the absence of an H receptor may be a unique attribute of this specialized membrane. MV-Ed trans-synaptic spread can occur in the absence of CD46, and primary mouse neurons do not express the other identified MV receptor, SLAM/CD150w. While these data indicate that trans-synaptic spread may not require a receptor for the H protein, we cannot rule out the possibility that a protein expressed at the post-synaptic membrane may serve as a unique H-receptor in neurons, specifically facilitating neuron-to-neuron transport across synapses. However, given the similarity in sequence and activity of Substance P and the fusion inhibitor FIP, we favor the idea that F, through binding to neurokinin receptors, facilitates transport of MV RNPs across the synapse in the absence of a need for H. Importantly, NK-1 is expressed in all mammals and on virtually all cells, including the Vero fibroblasts used as controls in our experiments (data not shown). Thus, we are also currently testing whether NK-1 is a necessary co-factor for MV entry into all permissive cells. Moreover, given that these studies were performed with MV-Edmonston, a vaccine strain, it will be important to address what role NK-1 plays in entry and spread of wild type isolates.

While our data implicate an important role for NK-1 in MV spread in neurons, some infected mice with deleted or impaired NK-1 did become infected, and neither FIP nor Substance P was able to fully ablate MV spread in primary neurons. This inability could be explained by: a) an insufficient concentration of these inhibitors at the synaptic membrane; b) a rapid off-rate of Substance P from NK-1 (Sarntinoranont et al., 2003); and/or c) the potential for MV-F

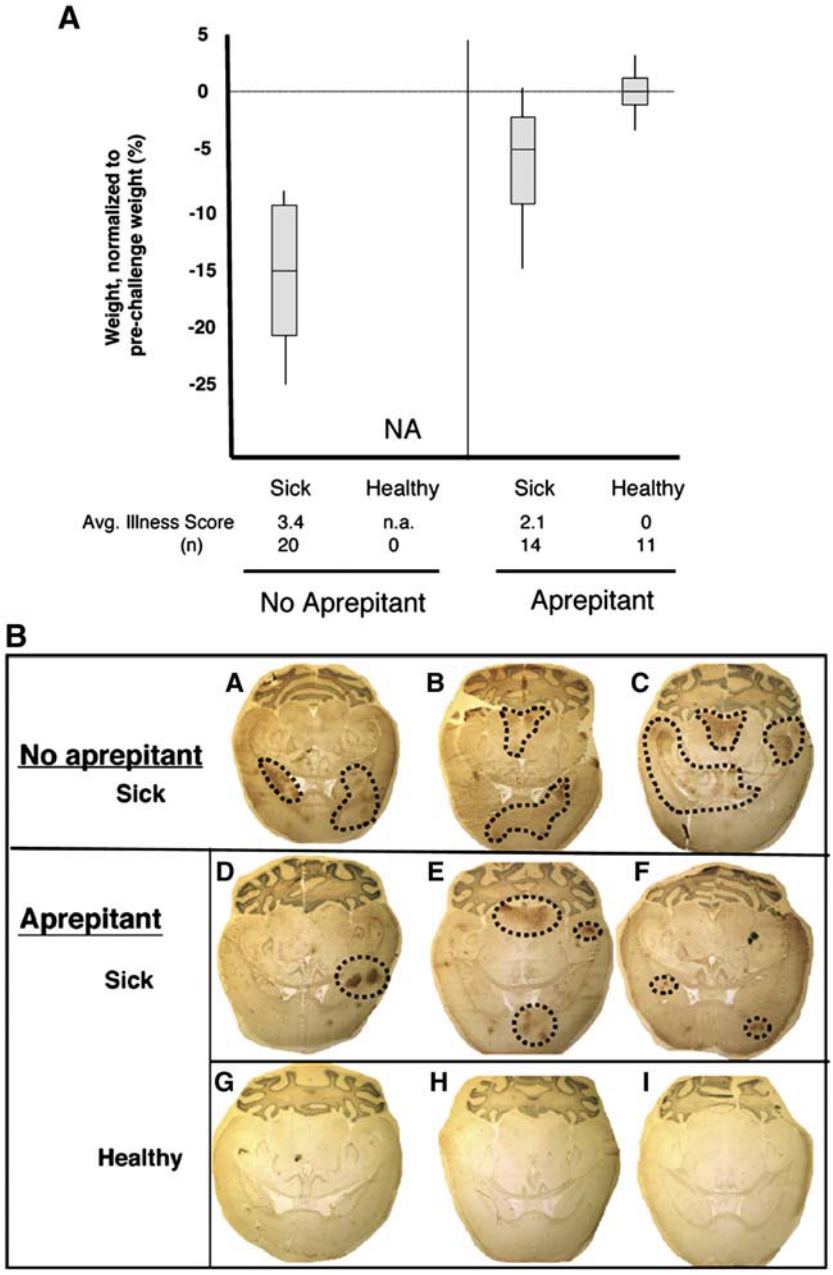

Fig. 6. Weight loss, morbidity and MV infection levels in untreated and aprepitant-treated NSE-CD46^{+}/RAG-2 KO mice. (A) Mice were infected as described and administered aprepitant orally 1 day prior to infection (double dose), 1 day after infection, and every other day thereafter. Mice were monitored daily and scored from 0 to 4, based on the defined criteria (see Materials and methods). Average values (obtained between days 6 and 10) are shown. Mice were also weighed on the day before infection to establish individual baselines, and every other day thereafter. Maximal weight changes are shown as percent weight loss or gain. (B) Mice were infected as described and collected between 10 and 12 days post-infection. Shown are immunohistochemical results from untreated (a–c) and aprepitant-treated (d–i) mice. (d–f) Sick animals at collection; (g–i) healthy animals at collection. Original magnification=20×.

to utilize multiple neurokinin receptors, including NK-2 and NK-3, for entry. Because all three neurotachykinins inhibited MV spread to some degree (Fig. 4B), and each serves as the ligand for three different neurokinin receptors, it remains possible that the other neurokinin receptors, NK-2 and NK-3, may serve as surrogates for MV entry under circumstances in which NK-1 is not present or active.

A recent paper provides additional support for the fusion–tachykinin relationship. The F protein of bovine respiratory syncytial virus (BRSV), a member of the *Paramyxovirus* family, is post-translationally cleaved twice, unlike MV-F, which is cleaved once. The small peptide that is released from BRSV-F cleavage is converted to a biologically active tachykinin, called virokinin (Zimmer et al., 2003). This soluble, virus-encoded peptide interacts with NK-1 and triggers the same downstream pathways as the *bona fide* ligand, Substance P. Thus, fusion proteins or post-translationally cleaved products derived from the fusion proteins of multiple *Paramyxoviruses* appear to interact with neurokinin receptors, though the benefit such an interaction confers to the virus is not yet understood.

Finally, these data may have implications for MV pathogenesis in the CNS. In the NSE-CD46 mouse model of neuronal MV infection, CNS disease occurs in infected, immunodeficient animals in the absence of neuronal death (Patterson et al., 2002), suggesting that CNS disease in this mouse model is due to neuronal dysfunction rather than overt cell loss. We hypothesize that MV-mediated fusion of neuronal synapses could promote neuropathology through a number of possible mechanisms, including constitutive neurotransmitter signaling or loss of synaptic integrity. Continued use of this model system will allow us to determine how receptor interactions may contribute to neuropathology and may identify novel candidates for antiviral therapies to prevent or limit MV-induced neuropathology in humans.

Materials and methods

Cells and virus

Vero fibroblasts were maintained in Dulbecco's modified Eagle medium (DMEM) (Life Technologies), supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, and 100 ng/ml streptomycin. Primary hippocampal neurons were prepared from day e15–e16 embryonic mice, as described (Banker and Goslin, 1991; Pasick et al., 1994; Rall et al., 1995), except that the cells were maintained in Neurobasal medium (Life Technologies), containing 4 μg/ml glutamate, in the absence of an astrocyte feeder layer. All neurons were obtained from NSE-CD46$^+$ embryonic brain tissues, except for the control poliovirus study, in which neurons were isolated from PVR$^+$ transgenic mice (a gift from Raul Andino, UCSF). All cells were maintained at 37 °C in a humidified incubator with 5% CO_2.

Measles virus (MV; Edmonston strain) was purchased from American Type Cell Collection (ATCC) and was passaged and titered in Vero fibroblasts, as was poliovirus (PV; Mahoney strain), a gift from Luis Sigal (Fox Chase Cancer Center). Lymphocytic choriomeningitis virus (LCMV; Armstrong strain), a gift from Michael Oldstone (The Scripps Research Institute, La Jolla, CA), was passaged on BHK-21 fibroblasts and titered on Vero fibroblasts.

Western blot analysis

Hippocampal neurons were plated onto poly-lysine coated 6-well tissue culture dishes and were infected 72 h post-plating with MV-Ed at a multiplicity of infection (MOI)= 0.1–3. Vero fibroblasts (4 h post-plating) were infected identically. Three days later, infected and mock-infected cell lysates were collected by scraping cells in 1× protein buffer, followed by a 20 s sonication. Samples were stored at −70 °C and boiled for 5 min prior to loading. Proteins were separated using 8% SDS–PAGE and transferred to a nylon membrane. Rabbit polyclonal antiserum directed against the MV-F cytoplasmic tail (a gift from Roberto Cattaneo, Mayo Clinic, Rochester, MN; (Cathomen et al., 1998)) was used as the primary antibody (diluted 1:1000 in 10% milk) followed by incubation with a peroxidase-conjugated anti-rabbit IgG secondary antibody (diluted 1:2000; Vector Laboratories). Bands were visualized using chemiluminescence (ECL; Dupont NEN).

Immunohistochemistry

Coverslip-bound cells or cryosectioned, 10 μm-thick horizontal brain sections were fixed with ice-cold methanol/acetone (1:1) for 10 min, dried and rehydrated in PBS. Samples were blocked with 1% (vol/vol) normal goat serum in PBS, followed by avidin and biotin blocking (Vector Laboratories). A human antiserum (Keller, a gift from Michael Oldstone) was used at a dilution of 1:2000. Appropriate secondary antibodies conjugated to biotin were used at a 1:300 dilution. A streptavidin–peroxidase conjugate (ABC Elite; Vector) was then added followed by visualization with diaminobenzidene (DAB; 0.7 mg/ml in 60 mM Tris; Sigma) and H_2O_2 (1.6 mg/ml). Cells and sections were counterstained with hematoxylin and mounted with an aqueous mounting medium. As controls, uninfected cells or brains, samples from which the primary antibody was omitted, or samples using an irrelevant, isotype-matched primary antibody were included.

Inhibitor studies in cell culture

To determine the impact of various reagents on viral infection, primary neurons or control Vero cells were first treated with the reagents at the indicated concentrations for 1 h followed by infection in the presence of the putative inhibitor. Following a 1 h incubation with virus, the inoculum was removed, and the cells were gently washed with warmed PBS (pH 7.4). Thereafter, the appropriate medium, containing fresh inhibitor, was added. Coverslips were then fixed at the indicated day post-infection (dpi).

To explore the impact of these reagents on viral spread, the process was reversed: cells were infected for either 1 or 6 h followed by addition of the reagents thereafter at the indicated concentrations. All reagents were obtained from Bachem and were dissolved in DMSO or 1 N acetic acid. Reagents used include the following: fusion inhibitory peptide (FIP; z-D-Phe-L-Phe-Gly-OH); the neurotachykinins Substance P (H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2), neurokinin A (H-His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2) and neurokinin B (H-Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2); bradykinin (H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH); the opioid receptor antagonist, H-Tyr-L-1,2,3,4-tetrahydroisoquinoline-3-carboxamide (H-Tyr-Tic-NH_2); and the Substance P antagonist, spantide (H-D-Arg-Pro-Lys-Pro-Gln-Gln-D-Trp-Phe-D-Trp-Leu-Leu-NH_2). Control cultures were incubated with an equivalent volume of DMSO or 1 N acetic acid and coverslips were harvested and stained for MV as indicated above.

Challenge experiments in mice

Adult mice between 6 and 10 weeks of age and neonatal mice between 2 and 3 days of age were used. Neurokinin-1

knockout (KO) mice (H-2^b) were obtained from Norma Gerard (Harvard University) (Bozic et al., 1996) and intercrossed for 8 generations with NSE-CD46 transgenic mice (H-2^b). For all mice, genotype verification for CD46, NK-1 and haplotype was performed on DNA isolated from tail biopsies or on blood samples collected from the retro-orbital sinus. To confirm CD46 status, standard PCR (30 cycles) was performed using the following primers: upstream: CGGTC GCTAC CATTA CCAGT; downstream: CCCCC TGAAC CTGAA ACATA. To confirm NK-1 KO status, two primer pairs were used, one pair to verify the absence of the targeted region of the NK-1 gene (upstream: TTGAG TCCTG CTACT CAGGA; downstream: ACTTT TACTA GCTGC GCTGC), and the other to confirm the presence of the neomycin resistance gene (upstream: TCAGC GCAGG GGCGC CCGGT TCTTT; downstream: ATCGA CAAGA CCGGC TTCCA TCCGA).

Neonatal $CD46^+$/NK-1 KO mice (less than 3 days of age) were challenged intracerebrally with 10^4 PFU MV-Ed, delivered in a volume of 10 μl along the midline using a 27-gauge needle. Infected pups were monitored daily, and animals showing signs of virus-induced illness (maternal rejection, absence of weight gain) were euthanized.

For the aprepitant (Emend®, Merck Pharmaceuticals) studies in adult mice, the same dose recommended for humans (80 mg/kg), adjusted for body weight, was given to adult $CD46^+$ mice on a RAG-2 KO background (Lawrence et al., 1999). The drug was suspended in PBS and delivered in a volume of 200 μl via oral gavage every other day following viral challenge. The day prior to infection, a double dose of the drug was administered. Animal health was monitored daily, and mice were weighed and assessed every other day following viral challenge. For the health assessment, mice were scored based on the following subjective scale: 0=healthy; 1=slight ruffling, hunching, or lack of mobility; 2=any two of these symptoms or severe appearance of one; 3=all three symptoms, or severe incidence of two; 4=moribund. All mouse experiments were reviewed and approved by the Fox Chase Cancer Center IACUC.

Tissue harvesting, histology and RNA isolation and analysis

From some mice, brain tissues were collected for either IHC or RNA analysis. For IHC studies, tissues were immersed in OCT embedding compound, quick-frozen in a dry ice/isopentane bath and stored at −80 °C. Thereafter, 10 μm cryosections were obtained and stored at −80 °C. Sections were thawed, fixed in ice-cold 95% ethanol and immunostained as described above.

To isolate RNA from infected tissues, brains were snap-frozen in liquid nitrogen and homogenized in TriReagent (Sigma). Thereafter, RNA was purified and quality-tested by gel electrophoresis. RNA was then subjected to RT-PCR using SuperScript II reverse transcriptase (Invitrogen) followed by amplification using MV-specific primers for nucleoprotein (upstream: ACTTA GGAGC AAAGT GATTG CCT; downstream: AACAA CACGG AACCT CTGCG G). Amplicons were visualized by electrophoresis on a 1.5% agarose gel.

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