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West Nile virus preferentially transports along motor neuron axons after sciatic nerve injection of hamsters

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Prior findings led us to hypothesize that West Nile virus (WNV) preferentially transports along motor axons instead of sensory axons. WNV is known to undergo axonal transport in cell culture and in infected hamsters to infect motor neurons in the spinal cord. To investigate this hypothesis, WNV was injected directly into the left sciatic nerve of hamsters. WNV envelope-staining in these hamsters was only observed in motor neurons of the ipsilateral ventral horn of the spinal cord, but not in the dorsal root ganglion (DRG). To evaluate the consequence of motor neuron infection by WNV, the authors inoculated wheat germ agglutinin–horseradish peroxidase (WGA-HRP) 9 days after WNV sciatic nerve injection, and stained the spinal cord and the DRG for HRP activity 3 days later. The degree of HRP-staining in DRG was the same in WNV- and sham-infected animals, but the HRP-staining in the motor neuron in the ventral horn was considerably less for WNV-infected hamsters. To investigate the mechanism of WNV transport, hamsters were treated with colchicine, an inhibitor of membranous microtubule-mediated transport. The intensity of the WNV-stained area in the spinal cord of colchicine-treated hamsters at 6 days after WNV infection were significantly reduced ($P \leq .05$) compared to the placebo-treated hamsters. These data suggest that WNV is preferentially transported through the motor axons, but not the sensory axons, to subsequently infect motor neurons and cause motor weakness and paralysis. *Journal of NeuroVirology* (2009) **15,** 293–299.

Keywords: anterograde; axon; infection; motor neuron; retrograde; West Nile virus

Introduction

Axonal transport was first described as an ''axoplasmic flow'' in 1948 (Weiss *et al,* 2004). It can be classified as anterograde or retrograde, and fast or slow transport, according to the direction of afferent or efferent transport and kinetic components. The axonal transport provides access to the central nerve system (CNS) for various molecules (e.g., toxins) (Griffin *et al,* 1978; Oztas, 2003), and viruses of the *Herpesviridae* (Oztas, 2003), *Rhabdoviridae* (Tsing *et al,* 1989), and *Picornaviridae* (Ohka *et al,* 1998) families. West Nile virus (WNV), a neurotropic member of the family *Flaviviridae*, also undergoes anterograde and retrograde axonal transport, as recently established in cell culture and in WNV-infected hamsters (Samuel *et al,* 2007b). A compartmentalized culture system in that study consisted of a soma chamber separated from a neurite chamber by a methylcellulose barrier from which axons pass between the two chambers. Virus could infect one chamber from inoculation in the other chamber in either the retrograde or anterograde direction by axonal transport, whereas interchamber infection was prevented by cutting axons within the methylcellulose barrier. When the sciatic nerve was injected with WNV, paralysis would occur on the ipsilateral hind limbs, but not the contralateral

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limbs. These experiments established axonal transport of WNV.

Because WNV predominantly infects motor neurons within the ventral horn of the spinal cord, experiments herein address the question of whether axonal spread also occurs predominantly in motor axons or also occurs via sensory axons.

Results

We tested the hypothesis that WNV undergoes axonal transport primarily through axons originating from motor neurons by injecting the virus directly into the sciatic nerve and then staining for WNV envelope protein in the lumbosacral spinal cord (Figure 1A, B) and dorsal root ganglion (DRG) of L5 (Figure 1C, D). When virus was injected into the sciatic nerve on one side of the animal, but not the other, motor neurons in the ventral horn on the same (ipsilateral) side (Figure 1A, B, arrow) were stained for WNV, whereas motor neurons on the contralateral side were not stained. The cytoplasms of the sensory neurons in the DRG of the same animals were not stained for WNV (Figure 1C, D). When the cell bodies in the DRG were directly exposed to WNV by injecting the DRG with virus (Figure 1E), the neuronal cytoplasms were positively stained with WNV. Therefore, dorsal horn cells were capable of being infected by WNV, but were not infected if exposure to the virus was dependent on axonal transport by sensory axons.

The status of motor or sensory axonal transport was evaluated by measuring the transport of wheat agglutinin–horseradish peroxidase (WGA-HRP) from the sciatic nerve into the lumbosacral spinal cord and DRG of L5. Nine days after intrasciatic nerve injection of WNV, the ipsilateral side sciatic nerve was injected with WGA-HRP. Three days after WGA-HRP injection, sections of the DRG and horizontal sections of the lumbosacral spinal cord were processed for WGA-HRP enzyme activity. Less HRP-staining was apparent in the ventral horns of WNV-infected hamsters (Figure 2B) as compared to sham-infected hamsters (Figure 2A), but there were no differences in the level of staining in the DRGs of WNV- (Figure 2D) compared to sham-infected hamsters (Figure 2C), which suggested that motor neurons, but not sensory neurons, were adversely affected in WNV-infected animals. These differences were quantified by counting the number of HRP-stained cell bodies (Figure 2). There was no statistically significant difference in the HRP-staining in the DRG of infected hamsters compared to sham-infected hamsters, but the difference in the spinal cord staining between the WNV-infected and sham-infected hamsters was significantly different ($P \leq .05$).

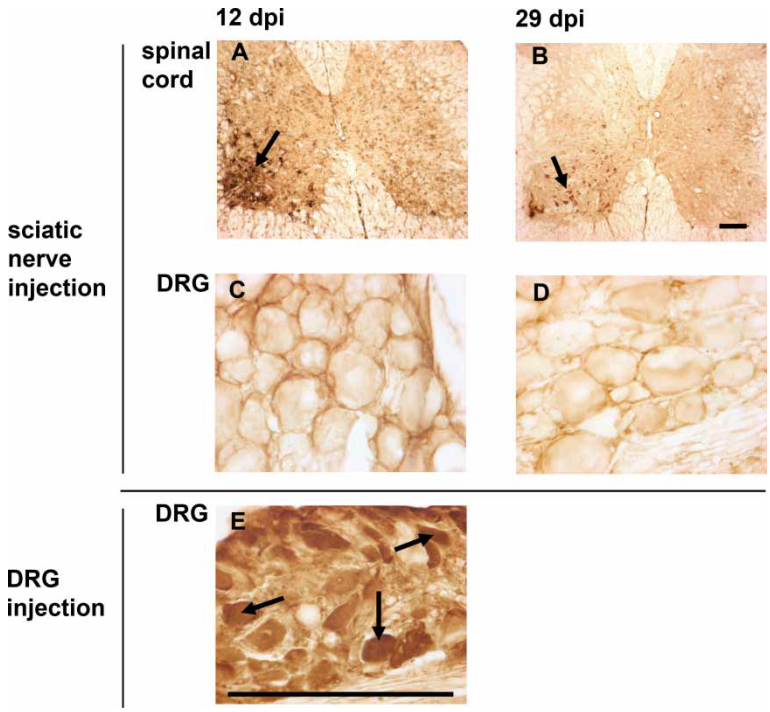

Figure 1 WNV envelop staining form the lumbar spinal cord at (**A**) 12 and (**B**) 29 days post viral injection (d.p.i.), and ipsilateral DRG of L5 at (**C**) 12 and (**D**) 29 days d.p.i. (**A**–**D**) from a hamsters injected in the sciatic nerve with WNV. (**E**) A DRG from a separate hamster injected directly into the DRG with WNV. Arrows indicate positively stained areas. (**A, B**) Scale: small-sized bar = 250 μm; (**C**–**E**) scale: large-sized bar = 250 μm.

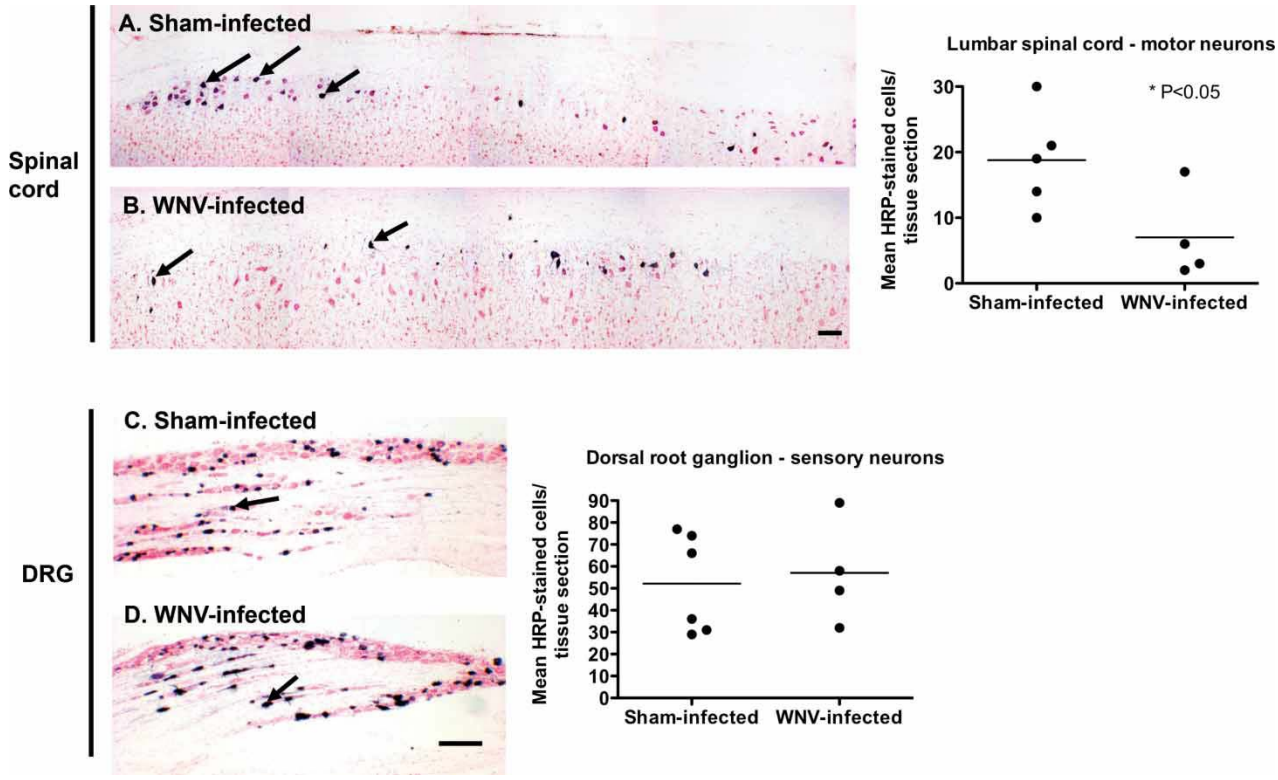

Figure 2 WGA-HRP–labeled motor neurons in lumbosacral cord (**A, B**) and sensory neurons in DRG (**C, D**). Lumbosacral spinal cord from (**A**) sham-infected hamster and (**B**) WNV-infected hamster. DRG of L5 from (**C**) sham-infected hamster and (**D**) WNV-infected hamster. Hamsters were injected in the sciatic nerve with WNV, followed by injection in the sciatic nerve with WGA-HRP 9 days later, and necropsied 3 days later (12 days after WNV infection) for WGA-HRP staining of the lumbosacral spinal cord and the ipsilateral DRG. Graphs are the respective quantification of the number of motor neurons labeled with WGA-HRP in lumbosacral spinal cord and sensory neurons in DRG. The numbers of WNV-infected hamsters were fewer than the sham-infected hamsters, because not all WNV-infected hamsters survived for analysis. Scale bar = 100 μm.

To evaluate the kinetics of retrograde transport from the sciatic nerve to the ventral horn, the apparent velocity was calculated by dividing the distance from the point of injection to the lumbosacral cord, by the time required for infection of the neurons in the cord. The time required for infection was 5 days (Figure 3) and the distance was 5 cm; therefore, the velocity was calculated as 1 cm/day.

Because this velocity was considerably lower than the known velocities of other viruses, such as the rabies virus (10 to 40 cm/day) in rats (Tsiang *et al,* 1989) or poliovirus (16 cm/day) in mice (Ohka *et al,* 1998), the transport mechanism was investigated by treating infected animals with an inhibitor (colchicine) of membranous microtubule-mediated axonal transport (Chen *et al,* 2007). Hamsters were treated once with colchicine 12 h before sciatic nerve injection of WNV. Tissues were collected on day 6 after WNV exposure and processed for WNV envelope staining (Figure 4). Subjectively, the number of positively stained motor neurons (Figure 4A, D), interneuron-like cells (Figure 4B, E), and glial-like cells (Figure 4C, F) were reduced in the colchicine-treated animals as compared to PBS-treated animals. Quantitatively, the percentage area of staining as determined by NIH ImageJ (Noguchi *et al,* 2003) was significantly reduced ($P \leq .05$) in colchicine-treated animals.

Discussion

Data from this study indicate that WNV can undergo axonal transport preferentially in the motor axons, but not sensory axons, of the sciatic nerve. When the sciatic nerve of hamsters was injected with WNV, motor neuron cell bodies were stained for WNV in the spinal cord on days 5 to 9, 12, and 29. The DRG cell bodies of the same animals were not stained for WNV at any of these time points. Moreover, the infection of motor neurons in the spinal cord resulted in reduced transport of WGA-HRP to motor neurons, but not to DRG neurons. This preferentially reduced transport to motor neurons may have been due to reduced metabolism or to dysfunction of transport mechanisms as a consequence of WNV infection of the motor neurons. Earlier studies also demonstrate that WNV-infected neurons possess apoptotic signals (TUNEL staining) (Morrey *et al,* 2007, 2008; Samuel *et al,* 2007a). Even though direct

Days after sciatic nerve WNV injection	Lumbosacral cord	Dorsal root ganglion
4	0/3	0/3
5	3/3	0/3
6	3/3	0/3
7	3/3	0/3
8	3/3	0/3
9	3/3	0/3
12	3/3	0/3
29	2/2	0/2

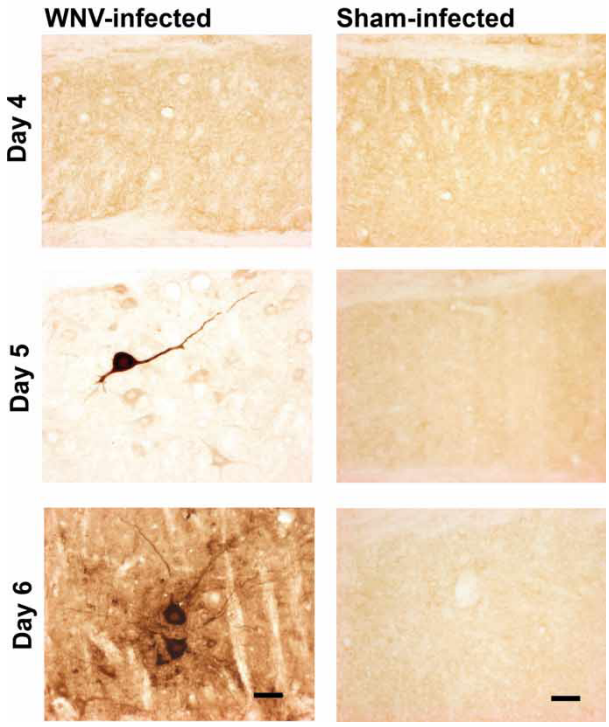

Figure 3 WNV envelope-antigen immunostaining of lumbosacral spinal cord and dorsal root ganglion of hamsters injected with WNV in the sciatic nerve. Images are examples of staining from horizontal sections of the lumbosacral spinal cord. Scale bar = 50 μm.

injection of the sciatic nerve probably affected the axons, it is unlikely that the direct inject would have affected only the motor axons and not the sensory axons; therefore, the reduced transport of the WGA-HRP was likely due to WNV infection of motor neurons. When the virus was injected directly into the DRG, the DRG neurons became infected by the virus; but when the virus was injected into the sciatic nerve, the DRG neurons were not infected. Other studies have reported the staining of WNV in the DRG of mice (Hunsperger and Roehrig, 2005, 2006), but the lack of DRG staining in this study may be explained by the use of direct sciatic nerve injection as compared to subcutaneous injection. These data indicated that WNV preferentially transports along motor axons to infect motor neurons, but that nonmotor neurons can be infected if directly exposed to the virus.

There are several potential reasons for preferential transport of the virus along motor axons. Hypothetically, a trivial reason is that the 32-gauge needle mechanically cuts only the motor axons, but this is not a reasonable explanation. Also, more time may be required for transport of WNV into the DRG, but the distance to the DRG is actually shorter than to the ventral horn, and retrograde transport to the DRG is functional as evidenced by the WGA-HRP experiment described above. Another possible explanation for WNV infecting the motor neurons in the ventral horn of the spinal cord instead of the DRG is because there may be more motor axons in the sciatic nerve than sensory axons for WNV to infect. This possibility is not likely because WGA-HRP injected into the sciatic nerve is detected both in the spinal cord and DRG. Another reason is that WNV transport mechanisms are more specific to motor axons, and not sensory axons. Exploring these reasons await further investigations.

The preferential transport of the virus along selective axons, such as motor neuron axons, may explain why the WNV preferentially infects motor neurons throughout the spinal cord, which leads to poliomyelitis-like disease. Once motor neurons become infected, the virus may selectively spreads along motor neuron axons within the spinal cord to infect other motor neurons. For the WNV to spread throughout the spinal cord, it would either need to spread randomly from cell-free particles in the cerebrospinal fluid (CSF) into cells, or by directional axon-to-cell spreading. Evidence suggests that the virus can spread from cell to cell, possibly through the synaptic gaps. In a cell culture system used to demonstrate transaxonal transport, WNV could spread from neurons to indicator cells, wherein the viral particles were seen in the apical ends of neuritis (Samuel *et al,* 2007b). The location of the virus at the apical end of the neurite may indicate the propensity for the virus to spread via synaptic gaps similar to poliovirus (Ohka *et al,* 2004). Moreover, when WNV was injected directly into hamster spinal cords at T8, viral staining was not only observed at the injection site in cell bodies of both the ventral motor and dorsal sensory horns, but staining was only observed in the ventral motor horns containing motor neurons rostral or caudal to the injection site (data not shown). These data suggest that WNV can spread to and throughout the spinal cord along motor axons to infect other motor neuron.

Poliovirus (Ohka *et al,* 2004) and WNV (Samuel *et al,* 2007b) share the ability to undergo axonal transport to infect motor neurons in the spinal cord to cause a poliomyelitis (Ohka *et al,* 2004). The

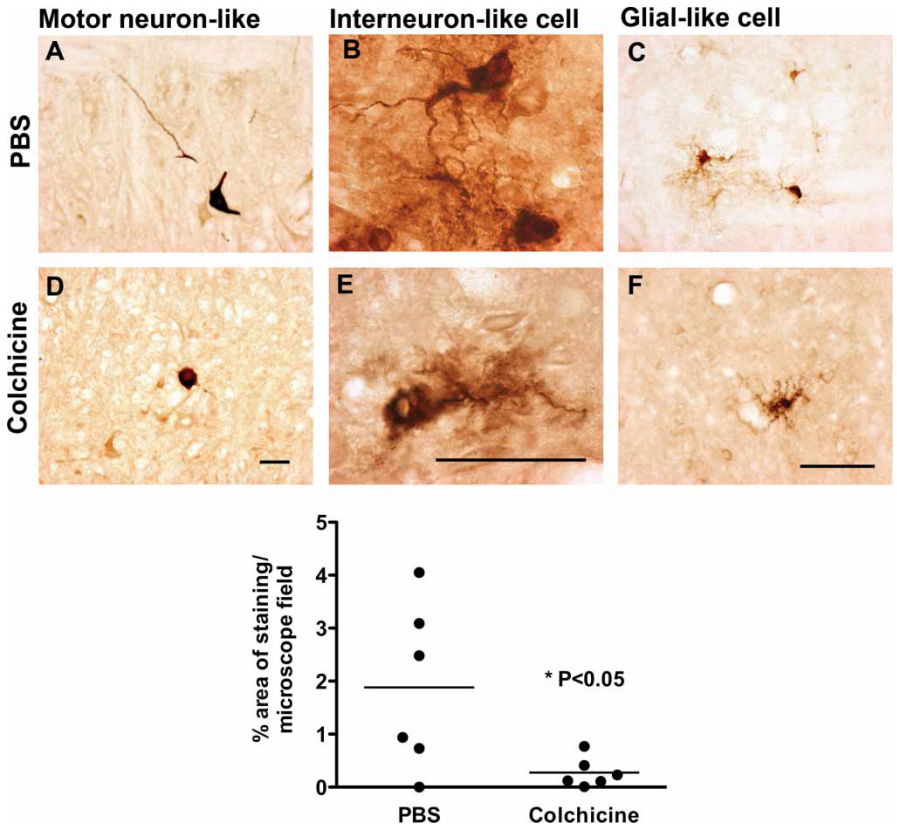

Figure 4 WNV envelope-antigen staining on 6 d.p.i. of (**A, D**) neurons, (**B, E**) interneuron-like cells, and (**C, F**) glial-like cells in the lumbosacral cord sections from hamsters injected in the sciatic nerve and treated with (**A, B, C**) placebo or (**D, E, F**) colchicine. Cochicine (2 mg/kg) or PBS was injected intramuscularly into the hind limb of hamsters 12 h before the intrasciatic nerve injection of WNV. Graph is the quantification of the average percent WNV positively stained area (μm^2) per microscope field. A one-tail Mann-Whitney nonparametric test was used. Scale bar = 50 μm.

dynein light chain, Tctex-1, interacts with the cytoplasmic domain of the human poliovirus receptor to probably facilitate transport of the virus. This mechanism involves microtubules because an anti-microtubule agent, vinblatine, inhibits the transport of poliovirus to the spinal cord. To investigate the involvement of microtubules in the axonal transport of WNV, hamsters were treated with colchincine, a microtubule inhibitor, and then infected with WNV by direct injection of the sciatic nerve. The average percent of WNV-stained area per microscope field in the lumbosacral spinal cord of colchicine-treated hamsters at 6 days after WNV infection were significantly reduced compared to the placebo-treated hamsters. Moreover, in an analogous experiment, an inhibitor of slow axonal transport of neurofilaments, 3,3'-iminodipropionitrile (IDPN) (Llorens *et al,* 1993), did not affect WNV-axonal transport (data not shown). These data suggested that WNV axonal transport involves membranous microtubule-mediated transport similar to poliovirus (Ohka *et al,* 2004).

Despite the apparent similarity of WNV and poliovirus to utilize microtubule-mediated transport, the velocities of these two viruses differed as calculated by the distance of the viral injection site to the lumbar spinal cord, divided by the time for the appearance of stained cells in the spinal cord. Poliovirus has an axonal velocity of 16 cm/day in mice (Ohka *et al,* 1998), whereas WNV velocity was estimated in this study to be 1 cm/day. The reason for the apparent slow transport of WNV may be attributed to other rate-limiting steps of infection other than the axonal transport from the sciatic nerve to the spinal cord, which could be investigated more accurately in cell culture in the future.

These studies establish that WNV axonal transport under the conditions of the study preferentially occurs in sciatic nerve motor axons to infect motor neurons in the spinal cord, as compared to lack of transport in the sensory axons. The mechanism of transport likely involves membranous microtubule-mediated transport, similar to the poliovirus mechanism of transport, because chemical inhibitors of this transport mechanism delayed the arrival of the virus into the spinal cord. The relevance of these findings in relation to the natural infection and disease awaits further investigation.

Materials and methods

Animals and virus
Adult female Syrian golden hamsters greater than 7 weeks of age were used (Charles River Laboratories). Animals were randomized to treatment groups. This study was conducted in accordance with the approval of the Institutional Animal Care and Use Committee of Utah State University. A New York isolate of WNV (NY WNV) (Lanciotti *et al,* 2002; Lanciotti and Kerst, 2001) from crow brain (Davis *et al,* 2004) were used. Hamsters were injected with $10^{1.8}$ plaque-forming units (PFU) of WNV into the sciatic nerve in a volume of 1 μl (Samuel *et al,* 2007b). The virus was propagated one time in MA-104 cells, which yielded high levels of virus, and diluted 1/10 in minimal essential medium (MEM) immediately prior to injection in the sciatic nerve.

Sciatic nerve and dorsal root ganglion (DRG) injection
Injection of the sciatic nerve was described previously (Morrey *et al,* 2008). The sciatic nerve was injected 1 mm deep at ~30-degree angle with 1 μl of WNV ($10^{1.8}$ PFU) using a Hamilton syringe and a 32-gauge needle. To expose the DRG at L4, a laminectomy surgical procedure was used (Marrey *et al,* 2008). A volume of 0.5 μl of WNV was injected into the DRG using a Stereotaxic apparatus (David Kopf Instruments, Model 900). The MEM diluent of the virus was used for the sham-injection control.

Immunohistochemistry for WNV envelope
The hamsters were injected with euthasol (100mg/kg, intraperitoneal [i.p.]) and cardiac perfused with 0.01 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. The lumbar cord, maintained in 30% sucrose for overnight, was cut into coronal sections (18 μm) by a cryostat instrument (Leica CM 1900) and permeabilized in 0.5% triton X-100, blocked with 5% horse serum in 0.1% bovine serum albumin (BSA), incubated with 7H2 anti-WNV mouse monoclonal antibody (mAb) (BioReliance; Invitrogen Bioservices, Rockville, MD) at 4°C overnight, incubated with horse anti-mouse immunoglobulin G (IgG) (1:200; BA-2000; Vector Lab) for 2 h at room temperature, and developed with avidin-biotin-peroxidase complex, ABC, detection system (1:100; Vectastain ABC kit; Vector Lab) for 30 min, employing 0.3% hydrogen peroxide and 3,3′-diaminobenzidine (DAB). The samples were washed between each step with 0.01 M PBS.

Hamster studies with WGA-HRP intrasciatic nerve injection
A total of 25 hamsters were inoculated in the sciatic nerve with WNV, and 6 animals were injected with sham. Nine days after the sciatic nerve injection, the sciatic nerve in the great sciatic notch was surgically exposed and injected with 1 μl of 5% wheat germ agglutinin–horseradish peroxidase (WGA-HRP) (Sigma-Aldrich, L7017) (Liu *et al,* 199511). The sciatic nerve in the great sciatic notch was injected, because the nerve is much larger at this anatomical location, and there are larger numbers of sensory and motor axons than in the caudal portion of the nerve. Histochemistry for HRP was performed in the lumbosacral spinal cord 72 h later on the four surviving WNV-injected hamsters and the six sham-injected hamsters (Romero *et al,* 1999).

Histochemical reaction for HRP
Every third horizontally cut cryostat section (18 μm) of the lumbosacral spinal cord was mounted on a slide coated with poly-lysine (Sigma-Aldrich, P8920). Then the slides were incubated with the HRP substrate 3,3′,5,5′,-tetramethylbenzidine (TMB; Sigma-Aldrich, 860336) reaction solution for 20 min. The slides were incubated with a concentration of 0.3% hydrogen peroxide for 20 min, and transferred to stabilizer solution for 20 min at 0°C to 4°C. Neutral red was used as a counterstain. The slides were dehydrated in a series of alcohols and cleared in xylene (Morrell *et al,* 1981).

Colchicine study
Hamsters were inoculated in the sciatic nerve with WNV or PBS. Twelve hours before intrasciatic nerve injection of WNV, hamsters were intramuscularly injection with colchicine (1.5 mg/kg; Sigma-Aldrich, C3915) or PBS (Chen *et al,* 2007; Karlsson *et al,* 1971), and necropsies were performed at day 6 after sciatic nerve injection of WNV. The horizontal sections of lumbosacral spinal cord were stained with WNV envelope.

Quantification of HRP and WNV histological staining
The HRP-labeled neurons containing blue granules in their cytoplasms (400 ×) were counted in every other third horizontal section of lumbosacral spinal cord and every other section of DRG from L5. The average labeled neurons per section from 11 sections of lumbosacral spinal cord (Darman *et al,* 2004) and 15 sections DRG (Hippenmeyer *et al,* 2005) for each animal were determined. To determine the average percent WNV-stained area per microscope field, the most intensely stained area of every fifth section of each animal were analyzed by using NIH Image J system with a fixed threshold (Noguchi *et al,* 2003) for a total 30 images for each treatment group. A one-tailed Mann-Whitney nonparametric test was used for HRP- and WNV-stained data.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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