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Targeted foreign gene expression in spinal cord neurons using poliovirus replicons

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A hallmark of poliovirus is the propensity to infect and replicate in spinal cord neurons of the central nervous system. Previously, we characterized a poliovirus self-replicating RNA genome (replicon), which encodes firefly luciferase in place of the capsid genes. This replicon is encapsidated into an authentic poliovirion by providing the poliovirus capsid protein *in trans*. The amount of enzymatically active luciferase in cells infected with this replicon correlated with the infectious dose. To begin to characterize the *in vivo* infectious potential of replicons, we have inoculated mice transgenic for the human receptor for poliovirus (PVR), either intracranially or intraspinally, with the replicon encoding luciferase. Wild-type poliovirus delivered to PVR mice via intracranial or intraspinal routes resulted in paralysis and death. Replicon preparations were shown by a sensitive biological assay to be free of infectious poliovirus. Neither intracranial nor intraspinal inoculation of the replicon encoding luciferase resulted in any obvious paralysis or disease symptoms. Following intraspinal inoculation with replicons encoding luciferase, luciferase enzyme activity was detected at 4 h post-inoculation, with peak activity at approximately 8 h post-inoculation; by 48–72 h, the luciferase activity had returned to background levels. Luciferase activity was detected in spinal cord predominantly near the site of inoculation, although activity was detected anterior and posterior to the site of inoculation, indicating that replicons undergo limited movement within the CNS presumably via the cerebrospinal fluid. In stark contrast to poliovirus though, inoculation of replicons into the spinal cords of PVR mice did not result in noticeable pathogenesis. Using immunofluorescence with antibodies to double-stain for replicons and neurons, we determined that replicons exclusively infect the neurons of the spinal cord, with the expression of the luciferase and replicon proteins confined to the cytoplasm of the infected cells. Replicons, then, possess the identical capacity for infection of spinal cord neurons *in vivo* as poliovirus. The lack of discernible neuronal destruction following replicon inoculation into the spinal cord suggests that some of the pathogenesis observed during a poliovirus infection might not be due entirely to primary infection of neurons. Finally, the results of this study point to future use of replicons as a means to target recombinant protein expression to neurons in the spinal cord. *Journal of NeuroVirology* (2000) **6,** 95–105.

Keywords: poliovirus replications; spinal cord; neurons; viral vector

Introduction

The major features of the pathogenesis of poliovirus infection have been known for some time. Poliovirus spreads to the central nervous system (CNS) in approximately 1% of all natural infections. Once the virus invades the CNS, poliovirus primarily infects neurons, resulting in cytological changes, which are manifested as dissolution of cytoplasmic (Nissl) bodies. Progression of the Nissl body disappearance leads to changes in the nucleus and shrinkage of the cytoplasm. The neuronal changes are accompanied by a substantial influx of inflammatory cells, including polymorphonuclear leukocytes, microglia and mononuclear leukocytes

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(Blondel *et al*, 1998; Bodian, 1949). The necrotic neurons are thought to be removed by inflammatory cells, such as leukocytes or macrophages (Blondel *et al*, 1998; Bodian, 1949). Neuronal death as a result of poliovirus is typified by 'lesions' in the tissue, mainly confined to the hindbrain and spinal cord (Bodian, 1949).

One of the striking features of poliovirus tissue tropism is that the infection is confined almost exclusively to the neurons of the CNS. Although the expression of the poliovirus receptor (PVR) on the cell surface is thought to be a major determinant of tissue tropism (Holland, 1961; Blondel *et al*, 1998), recent studies have revealed the existence of tissues which express the receptor, but are resistant to poliovirus infection, suggesting that expression of the PVR may not be sufficient to confer susceptibility to poliovirus (Blondel *et al*, 1998; Freistadt, 1994; Freistadt *et al*, 1990; Mendolsohn *et al*, 1989; Ren *et al*, 1990; Ren and Racaniello, 1992). Some of the new insights into poliovirus pathogenesis have come from studies using a transgenic mouse model expressing the poliovirus receptor (Koike *et al*, 1991; Ren *et al*, 1990). Consistent with studies from human and nonhuman primate tissues, poliovirus antigens and RNA have been detected in the neurons of the CNS of infected transgenic mice, but not in the glial or vascular endothelial cells (Blondel *et al*, 1998; Hashimoto *et al*, 1984; Ren and Racaniello, 1992). The neuronal damage in the brain stem and spinal cord of these animals resembles the pattern of lesions seen in humans and primates with poliomyelitis (Abe *et al*, 1995; Bodian, 1949; Ren *et al*, 1990; Ren and Racaniello, 1992).

Previous studies from this laboratory have described the construction and characterization of poliovirus RNA genomes (replicons) encoding foreign proteins (Porter *et al*, 1993, 1995, 1998). The foreign genes are substituted for the genes encoding the capsid proteins, VP2, VP3, and VP1. *In vitro* transcription of cDNAs of replicons generates RNA molecules which, upon transfection into HeLa cells, result in replication of the RNA and expression of the foreign protein. Previous studies have also demonstrated that replicons that do not amplify their genomes (replicate) are not encapsidated; foreign proteins, then, are not expressed from replicons in the absence of replication (Choi *et al*, 1991; Porter *et al*, 1993). The replicons are encapsidated by providng P1 *in trans* using a recombinant vaccinia virus (VV-P1) which encodes the capsid precursor proteins from poliovirus Type 1 Mahoney. Replicons are serially passaged in the presence of VV-P1, resulting in stocks of encapsidated virus particles containing replicon genomes (Ansardi *et al*, 1993; Porter *et al*, 1993). Since the replicons do not encode the capsid proteins, they only undergo one round of infection (Ansardi *et al*, 1993; Porter *et al*, 1993, 1998). Recently, replicons have been generated which encode the gene for firefly luciferase (Porter *et al*, 1998). Infection of cells with this replicon results in production of enzymatically active luciferase protein. The amount of luciferase detected from cells infected with the encapsidated replicon correlates with the infectious dose used for infection. Luciferase enzyme activity was first detected at 6 h and peaked at 12 h post-infection (Porter *et al*, 1998). In the present study, we have utilized this replicon to examine the *in vivo* characteristics of replicon infection in the CNS of PVR mice. In contrast to infection with wild-type poliovirus, inoculation with the replicon by either intracranial or intraspinal routes resulted in no detectable paralysis or observed pathogenesis. Immunohistochemical analysis demonstrated that neurons in the spinal cord were infected with the replicons, without indication of gross neuronal damage, as seen with a poliovirus infection. Abundant luciferase activity was detected in extracts from the CNS for up to 24 h post-administration of the replicons. The results of these studies are discussed with respect to poliovirus pathogenesis and the future use of replicons as a means to express foreign proteins in the spinal cord of the CNS.

Results

Pathogenesis from intracranial or intraspinal administration of replicons

The tissue tropism and pathogenesis of poliovirus in the PVR mice following either intracranial or intraspinal inoculation into the CNS have been documented (Ren and Racaniello, 1992). To establish the parameters for replicons, 12 PVR transgenic mice were inoculated intracranially with 10^6 p.f.u. of poliovirus Type 1 Mahoney. Four of these mice were euthanized on day 1 and the skulls and spines were extracted for analysis. By 2 days post-inoculation, one of the eight remaining mice exhibited hind-limb paralysis and breathing difficulties, while on day 3, this mouse was dead and two other mice were showing symptoms of poliomyelitis (Table 1). Tissues from these three mice were collected and processed for histochemistry. By day 5, one of the remaining four mice was showing symptoms of disease. Tissues from the remaining four mice were collected at this point, since previous studies have shown the normal course for viral infection and manifestation of neuronal pathogenesis in these mice is 2–3 days (Ren *et al*, 1990).

Given the extreme sensitivity of the transgenic mice to poliovirus infection when inoculated intraspinally, it was important to be assured of the lack of detectable infectious virus in the replicon preparations. To test for poliovirus, we performed a biological assay for the presence of poliovirus by serial passage of replicon preparations on HeLa

Table 1 Morbidity following intracranial inoculation of wild-type poliovirus or replicons into PVR mice

Dose	*# mice inoculated*	*Days*[a]	*# mice showing signs of poliomyelitis*[b,c]
10^6 p.f.u. poliovirus	12	1	0
		2	1
		3	2
		5	1
10^6 i.u. replicon	5	1	0
		2	0
		3	0
		5	0
		60	0

[a]Days post-inoculation; mice were inoculated on Day 0. [b]As indicated by paralysis and breathing difficulties. [c]Mice were euthanized when they exhibited severe breathing problems.

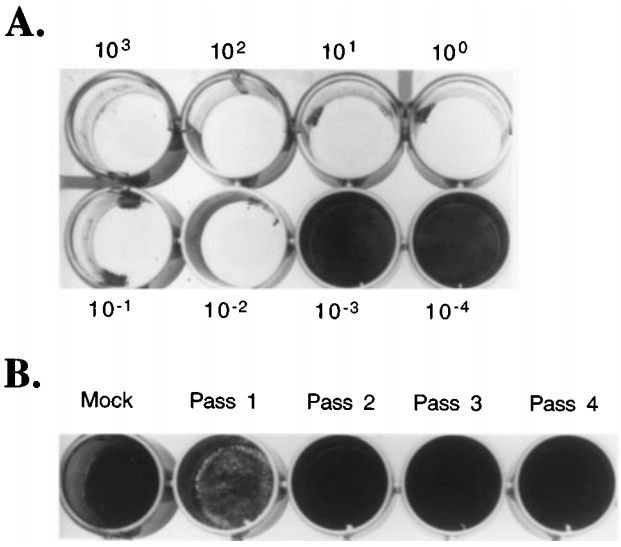

Figure 1 Biological assay for presence of infectious poliovirus in replicon preparations. HeLa H1 cells were infected with (**A**) decreasing amounts of poliovirus Type 1 Mahoney, ranging from 10^3 p.f.u./well to 10^{-4} p.f.u./well or (**B**) 10^6 infectious units/well of replicons expressing firefly luciferase. Cells were incubated for 48 h post-infection, fixed with 5% trichloroacetic acid (TCA) and stained with Coomassie Blue.

cells (Figure 1). The initial infection of HeLa H1 cells by replicons resulted in a cytopathic effect. This was likely due to the expression of P2 proteins, such as $2A^{pro}$, which results in shut-off of host cell translation (Joachims *et al*, 1999). Since replicons do not encode capsid proteins, they do not possess the genetic capacity to spread from cell to cell. Passage of the supernate from the primary replicon infection onto new HeLa H1 cells did not result in a cytopathic effect; subsequent passage of the supernate onto HeLa H1 cells also did not result in a cytopathic effect. If the replicon preparations had been contaminated with poliovirus, the serial passage would have amplified the poliovirus, resulting in a clear cytopathic effect even with very low amounts of wild type poliovirus (Figure 1A). To further confirm the replicon preparations were devoid of poliovirus, we radiolabeled replicon-infected cells, followed by immunoprecipitation with anti-capsid antibodies. No capsid proteins were immunoprecipitated (data not shown). From the results of these studies, we concluded the replicon preparations do not contain detectable amounts of infectious poliovirus.

To determine the *in vivo* effects of the replicon on the PVR mice, 10^6 infectious units of the replicon were inoculated intracranially into five PVR mice and observed for symptoms of poliomyelitis. In contrast to the infection with wild-type poliovirus, none of the five mice developed disease by 60 days post-inoculation, at which time they were euthanized (Table 1). This experiment has been repeated an additional three times, with the same results each time, demonstrating that intracranial administration of replicons does not result in obvious disease.

Direct intraspinal inoculation of poliovirus into the transgenic mice results in animals exhibiting classic symptoms of poliomyelitis (Ren *et al*, 1990). To determine the sensitivity of transgenic mice to this route of inoculation under our experimental conditions, four mice per dose were given poliovirus Type 1 Mahoney intraspinally (Table 2). Three of the four mice inoculated with 10^4 plaque forming units (p.f.u.) of poliovirus were dead by day 2 post-inoculation, with the remaining mouse exhibiting hind-limb paralysis and breathing difficulties consistent with poliomyelitis. Similarly, three of the four mice inoculated with 10^5 p.f.u. of the virus were dead by day 2; the remaining mouse was dead by day 3 post-inoculation. Three of the four mice inoculated with 10^6 p.f.u. of virus were dead on day 2, with the fourth mouse exhibiting symptoms of poliomyelitis. In a subsequent experiment, mice were inoculated intraspinally with doses of poliovirus Type 1 Mahoney ranging from 10^3 p.f.u. to 10 p.f.u. per animal. All of the mice which received either 10^2 or 10^3 p.f.u. of poliovirus died within 3 days post-inoculation, while one out of four mice inoculated intraspinally with 10 p.f.u. of virus developed disease (Table 2).

To determine whether intraspinal administration of the replicons under these same conditions would result in any obvious signs of poliomyelitis, five mice were inoculated intraspinally with 10^6 infectious units of the luciferase replicon and observed for symptoms of disease. None of the mice exhibited symptoms of poliomyelitis, including paralysis or difficulty in breathing at any time during the post-inoculation observation period of 60 days (Table 2). This study has been repeated three times and in no instance did we observe symptoms of disease in animals inoculated intrasp-

Table 2 Morbidity following intraspinal inoculation of wild-type poliovirus or replicons into PVR mice

Inoculum	*Dose*	*Days*[a]	*# mice showing signs of poliomyelitis*[b,c]	*# mice dead*
Wild-type poliovirus (four mice/dose)	10 p.f.u	3	1[c]	N/A[d]
	10^2 p.f.u.	3	2	0
		4	1	2
	10^3 p.f.u.	3	N/A	4
	10^4 p.f.u.	2	1	3
	10^5 p.f.u.	2	0	3
		3	N/A	1
	10^6 p.f.u.	2	1	3
Replicon (five mice)	10^6 i.u.	1	0	0
		2	0	0
		3	0	0
		5	0	0
		60	0	0

[a]Days post-inoculation; mice were inoculated on Day 0. [b]As indicated by paralysis and breathing difficulties. [c]Mice were euthanized when they exhibited severe breathing problems. [d]N/A denotes 'not applicable'.

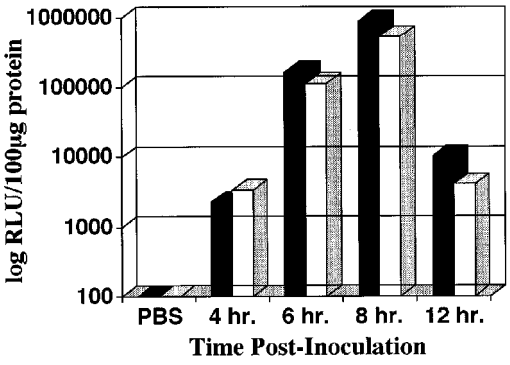

Figure 2 Luciferase enzyme activity in the spinal cords of PVR mice inoculated intraspinally with replicons encoding luciferase. Tissues at and around the injection site were extracted at specified times post-inoculation, homogenized and analyzed for luciferase activity. Samples were standardized for protein amount (100 μg total). Each bar represents a single mouse. RLU=relative light units.

inally with replicons. The results of these studies, then, demonstrate a lack of overt disease following inoculation of replicons into the CNS.

Luciferase expression following replicon inoculation in the spinal cord

A previous study from this laboratory found that the amount of enzymatically active luciferase correlated with the infectious potential of the replicon (Porter *et al*, 1998). To determine the extent that the replicons could infect the spinal cord cells of the CNS, we assayed for the expression of luciferase following intraspinal inoculation with the replicon. Eight mice were inoculated intraspinally with 10^6 infectious units each of the luciferase replicon. At specified times post-inoculation, two mice per time point were euthanized and the spinal cords at and around the injection site were extracted. The tissues were homogenized and lysed and luciferase enzyme activity was determined, with mice inoculated with PBS serving as controls (Figure 2). Luciferase activity was detected in extracts from the spinal cords by 4 h, with peak activity at approximately 8 h post-inoculation. By 12 h post-inoculation, luciferase expression decreased, returning to near background levels by 72 h. A similar time course for the expression of luciferase was found following *in vitro* infection of HeLa cells with this replicon (Porter *et al*, 1998).

Distribution of replicons on the CNS following intraspinal administration

To further characterize the infection of neurons within the CNS by replicons, we examined the distribution of luciferase following intraspinal inoculation. Mice were inoculated intraspinally with 10^6 infectious units of the replicon encoding luciferase. At each of the indicated time points post-inoculation, the mice were euthanized and the brains and spinal cords were removed. The tissues were divided into the following regions: forebrain (FB); hindbrain (HB); SC1, the area of the spinal cord anterior to the injection site; SC2, the injection site; SC3, the area posterior to the injection site. The tissues were processed and enzyme activity was determined. Luciferase activity was detected at the site of inoculation and throughout the spinal cord, both anterior and posterior to the site of injection (Figure 3). No luciferase activity was detected in the brain tissue analyzed from these animals. Thus, replicons show some movement in the spinal cord from the site of injection. Since replicons have the capacity to undergo only a single round of infection, the movement from the site of inoculation is probably facilitated by the cerebrospinal fluid to transport replicons to neurons anterior and posterior to the injection site.

Histochemical analysis of CNS following intraspinal administration of replicons

The neuropathogenesis of poliovirus has been known for some time (Blondel *et al*, 1998; Bodian, 1949). To investigate the pathogenesis of replicon infection in the CNS, serial sections from replicon-infected or, as a control, poliovirus-infected animals, were analyzed first by using a hematoxylin/eosin stain (Figure 4). As expected, tissues from mice inoculated intraspinally with 10^4-10^6 p.f.u. of poliovirus Type 1 Mahoney exhibited considerable neuronal destruction. The few neurons which could be identified following poliovirus infection had clear damage reflecting possible necrosis (Bodian, 1949). In stark contrast, in the tissues from the replicon-inoculated animals, the neurons, even at the site of injection, appeared normal. There was no

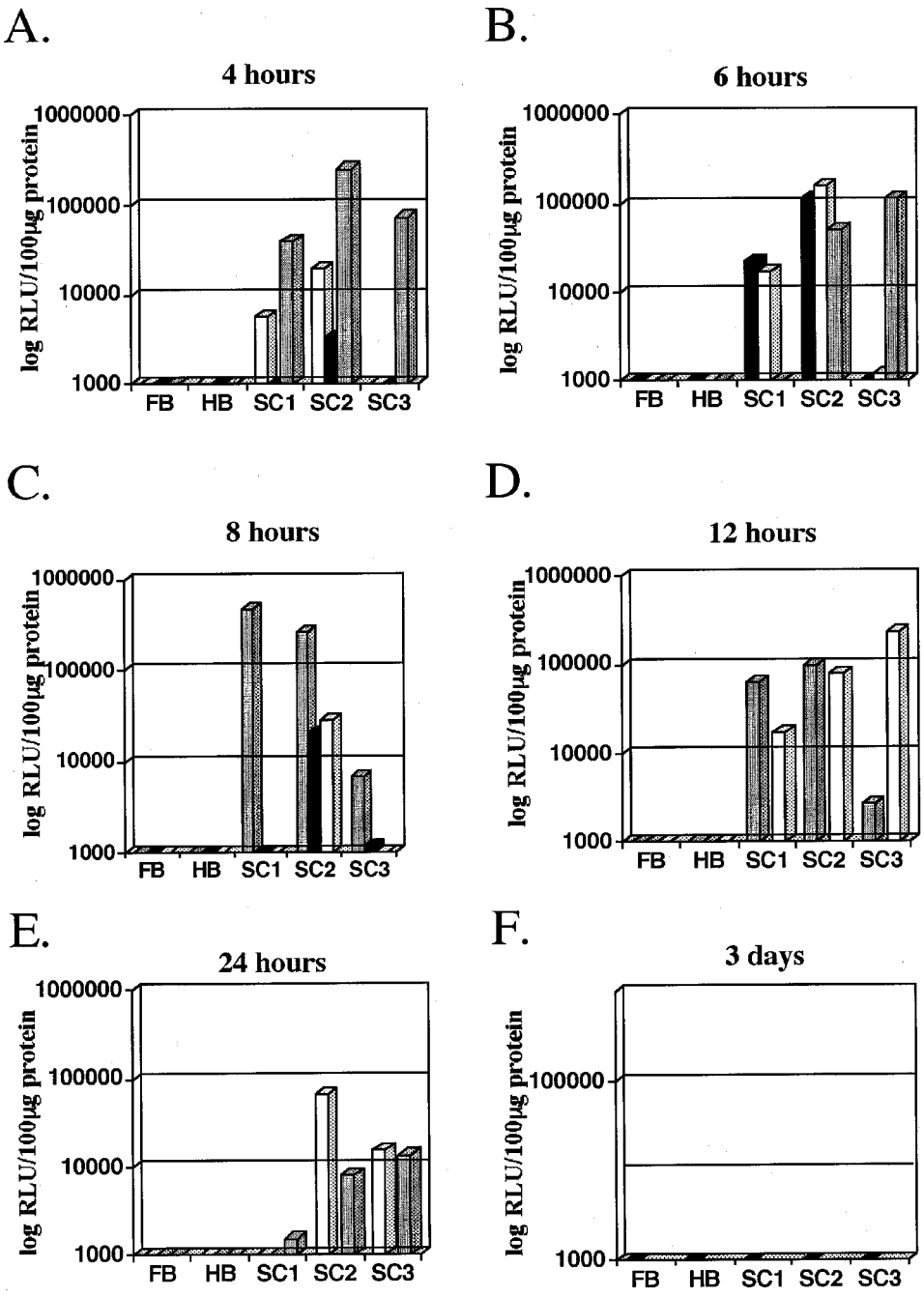

Figure 3 Luciferase enzyme activity in different sections of the spinal cords of PVR mice inoculated intraspinally with replicons encoding firefly luciferase. At specified times post-inoculation, the spinal cords were extracted and divided into the following regions: FB=forebrain; HB=hindbrain; SC1=area anterior to the injection site; SC2=the injection site; SC3=area of the spinal cord posterior to the injection site. The tissues were homogenized and assayed for luciferase activity; samples were normalized to 100 μg total protein. Each bar pattern represents a single mouse. Luciferase values from the brains and spinal cords of PBS-inoculated mice ranged from 62 to 129 RLU/100 μg protein.

evidence of neuronal damage or necrosis in sections examined from the spinal cords of any of the mice given replicons.

To establish the identity of the cells of the spinal cord infected by the replicon, mice were inoculated intraspinally with the luciferase replicon, PBS, or wild-type poliovirus. At various time points, the spinal cords were fixed, paraffin embedded, sectioned and immunostained using antibodies to the poliovirus $3D^{pol}$ RNA-dependent RNA polymerase (Figure 5). The fluorescence was restricted to the cytoplasm of the cells, which is consistent with the

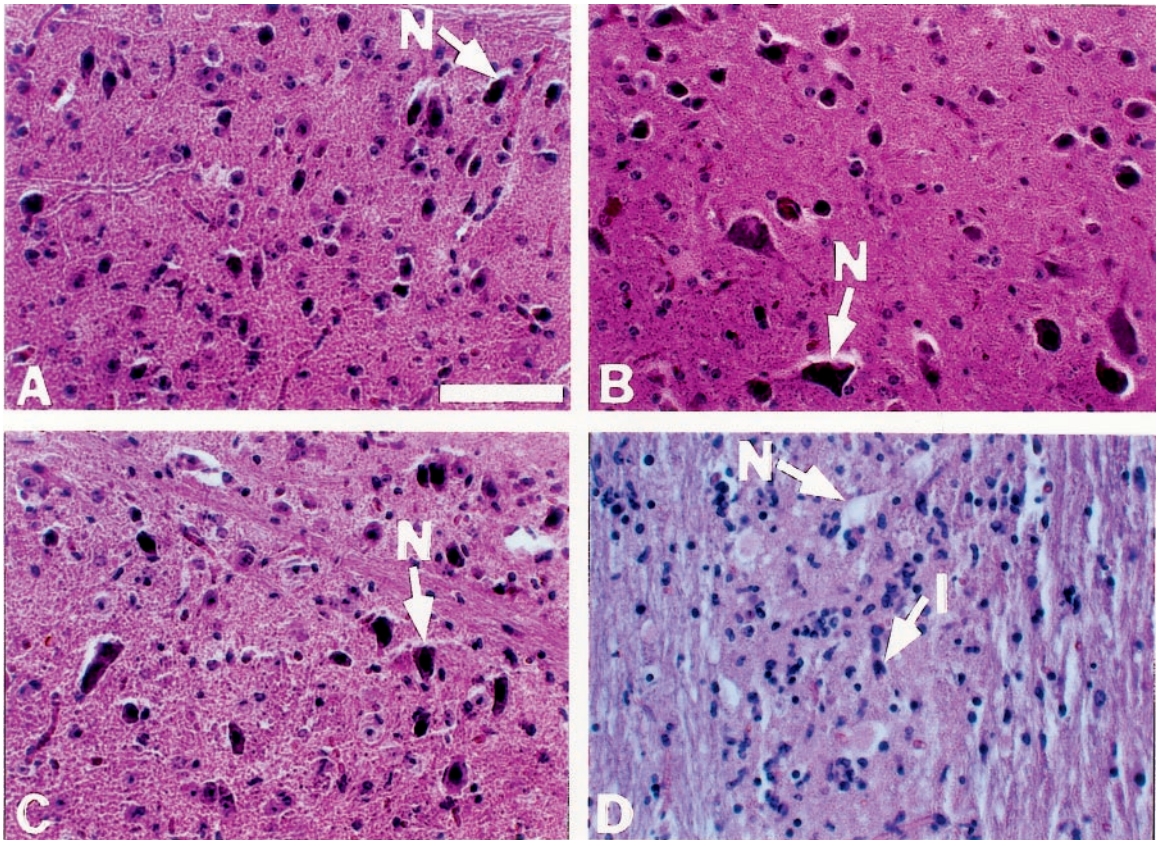

Figure 4 Analysis of CNS following intraspinal inoculation of replicons. Hematoxylin and eosin stains of spinal cords inoculated intraspinally with (**A**) PBS; (**B**) replicons encoding firefly luciferase 8 h post-inoculation; (**C**) replicons encoding luciferase 3 days post-inoculation; (**D**) poliovirus Type 1 Mahoney 2 days post-inoculation. The photographs are of the injection site and all were taken at the same magnification. N=neuron; I=inflammatory cell. Scale bar=500 μm.

known cytoplasmic location of the viral proteins involved in poliovirus replication (Koch and Koch, 1985). The expression of the $3D^{pol}$ proteins encoded in the replicons correlated with the kinetics of luciferase activity detected in spinal cord tissues (Figures 2 and 3). The greatest number of immunostaining cells were found at 8 h post-inoculation, with very few, if any, cells staining for $3D^{pol}$ by 3 days post-inoculation (data not shown). Serial sections of the tissues collected at 8 h post-inoculation were simultaneously stained with an antibody to luciferase and an antibody to NeuN (Figure 5G–I). The immunofluorescence using anti-luciferase antibodies co-localized with the immunofluorescence using the neuron-specific antibody demonstrating replicons had exclusively infected the neurons of the spinal cord. Analysis of multiple tissue sections and numerous fields under the microscope revealed no evidence of replicon proteins in cells other than neurons of the anterior horn of the spinal cord. Spinal cords from mice inoculated with PBS or wild-type poliovirus served as controls; background staining was seen in the PBS tissue using anti-$3D^{pol}$ antibodies, while immunostained neurons from poliovirus-infected mice were readily evident. The tissue inoculated with PBS was immunostained with the NeuN antibody to demonstrate the region of the spinal cord shown in the photographs was similar to that shown for replicon or poliovirus-infected mice (the anterior horn; Figure 5B).

Discussion

In this study, we have demonstrated for the first time that RNA replicons based on poliovirus target gene expression to spinal cord neurons. Direct intracranial or intraspinal injection of replicons into mice transgenic for the human poliovirus receptor did not result in paralysis or overt symptoms of poliomyelitis. No significant luciferase activity was detected in the brain following either intraspinal or intracranial inoculation of replicons, suggesting low level (if any) replicon infection of the brain. Inoculation of the spinal cord with replicons encoding luciferase resulted in luciferase activity both anterior and posterior to the site of inoculation. Histochemical analysis at the site of inoculation revealed that replicons caused little or

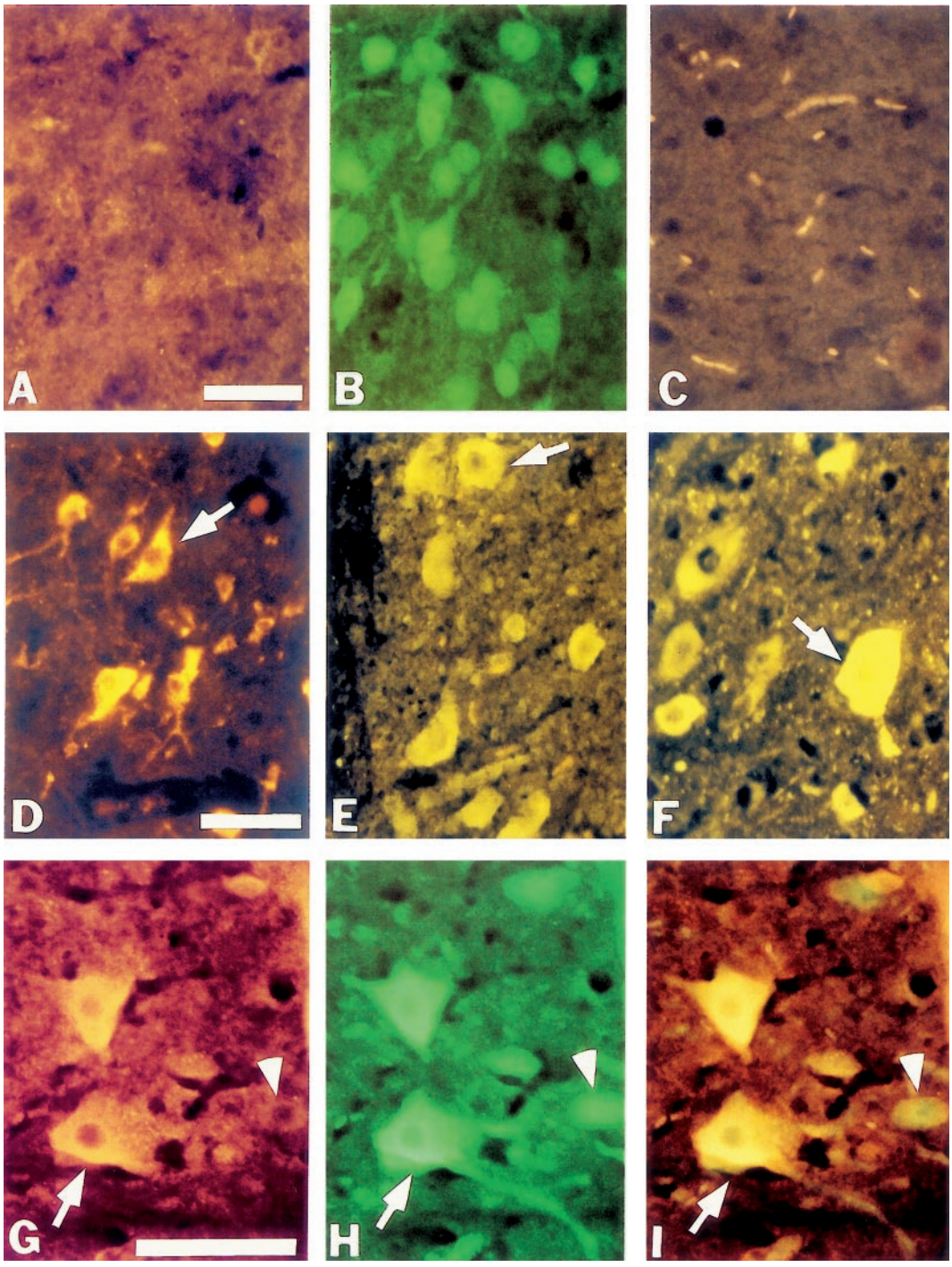

Figure 5 Analysis of replicon-infected cells following intraspinal inoculation. Immunofluorescence of spinal cord tissues at the anterior horn. PVR mice were inoculated intraspinally with PBS (**A** and **B**), the replicon encoding luciferase (**C**–**E**, **G**–**I**), or wild-type poliovirus (**F**). Panels (**C**, **D**) and (**G**–**I**) show the replicon-inoculated tissues at 8 h post-inoculation. Panel (**E**) shows the replicon-inoculated spinal cord at 24 h post-inoculation. Panel (**F**) shows spinal cord tissues inoculated with poliovirus Type 1 Mahoney at 24 h post-inoculation. Panels (**A**, **D**–**I**) were immunostained using an anti-$3D^{pol}$ antibody. Panel (**E**) was stained with an anti-NeuN (neuronal marker) antibody. Panel (**C**), which was incubated without a primary antibody, serves as a control. Panels (**G**–**I**) were double-stained with an anti-luciferase antibody and the anti-NeuN antibody. Photographs of panels (**G**–**I**) were taken with the following filters: rhodamine (**G**); FITC (**H**) or a double cube containing both the rhodamine and the FITC filters (**I**). White arrows: neurons staining with anti-$3D^{pol}$ antibody (**A**–**F**) or with anti-luciferase antibody (**G**–**I**); white arrowheads: neurons staining with anti-NeuN antibody, but not with anti-luciferase antibody (**G**–**I**). Scale bars=500 μm. Photographs of panels (**A**–**F**) were taken at the same magnification; photographs of panels (**G**–**I**) were taken at a higher magnification.

no pathogenesis within the spinal cord. Using antibodies specific for a replicon protein and a neuronal marker, we demonstrated by immunofluorescence that replicons exclusively infected neurons within the spinal cord.

The basic elements of the pathogenesis in the CNS following poliovirus infection have been known for some time (Bodian, 1949). Analysis of the brains from patients with poliomyelitis obtained at autopsy revealed lesions within localized areas consisting of the cerebral cortex including the precentral gyrus and the roof nuclei of the cerebellum (Bodian, 1949). Lesions within the brain stem were also prominent. At the cellular level, it has been known for some time that, within the CNS, poliovirus primarily infects neurons, with a specific predilection for motor neurons (Bodian, 1949). The neuropathogenesis of poliovirus in transgenic animals which express the PVR parallels that are seen for poliomyelitis in humans. The PVR transgenic mice are susceptible to all three serotypes of poliovirus, as indicated by the development of flaccid hind limb paralysis following administration by several different routes (Ren *et al*, 1990; Ren and Racaniello, 1992). In this study, we have confirmed that under our experimental conditions, direct intracranial or intraspinal inoculation of these animals with poliovirus Type 1 Mahoney resulted in clinical symptoms similar to that observed for poliomyelitis. Consistent with these symptoms, histochemical analysis of the CNS revealed inflammatory infiltrates, as well as a clear loss of neurons at and around the site of inoculation. Recent studies have suggested that the observed neuronal death may be due to poliovirus-induced apoptosis (Girard *et al*, 1999). Since poliovirus is known to cause such neuronal destruction, it was surprising that direct inoculation of replicons encoding luciferase intracranially or intraspinally into the transgenic animals did not result in obvious disease. Histochemical analysis confirmed a lack of obvious pathogenesis, as evidenced by the normal appearance of neurons at or near the site of inoculation in the spinal cord. In contrast to poliovirus infected animals, no significant inflammatory cell infiltration was found in the spinal cord tissues of replicon-inoculated animals. One question that remains unclear is whether replicon infection results in neuronal cell death *in vivo*. Replicon infection of HeLa cells *in vitro* results in cell death (Figure 1; Porter *et al*, 1998). Our studies using immunofluorescence indicate that a substantial number of cells were infected with the replicon at 8 h post-inoculation. If all of the infected cells died due to the infection of the replicon, one would have expected to see a substantial loss of neurons in the spinal cord tissues upon analysis by hematoxylin and eosin staining, as is seen with poliovirus-infected tissues. We did not observe any obvious loss of neurons in replicon-infected tissues. Since replicons do not encode capsid proteins and are unable to spread to adjoining cells following the initial infection, replicon infection may result in less cellular disruption and consequently, less recruitment of inflammatory cells. An alternative, less likely explanation is that the poliovirus capsid proteins themselves contribute to the pathogenesis possibly via interaction with the PVR. Further studies will be required to resolve the issue of why poliovirus infection, but not replicon infection, results in substantial neuropathogenesis.

The results of our study using replicons encoding luciferase clearly establish that replicons can be used to target expression of foreign proteins to the neurons in the spinal cord. A striking feature of the kinetics of luciferase expression *in vivo* was that it paralleled what we have observed in our *in vitro* cultures (Porter *et al*, 1998). The peak levels of luciferase obtained within the spinal cord following intraspinal administration of replicons were similar to that observed following *in vitro* infection of highly susceptible HeLa H1 cells. Furthermore, the duration of recombinant protein expression was also similar to that found for our *in vitro* infections. The level of expression, coupled with the similar kinetics of expression *in vitro* and *in vivo*, supports the contention that the neurons of the spinal cord are exquisitely susceptible to infection by replicons. This point was further highlighted by analysis of the spinal cord and brain following intraspinal and intracranial inoculation. The expression of luciferase following intraspinal administration was not confined to the inoculation site, but rather was detected throughout the spinal cord. In contrast, little luciferase activity was detected within the brain following either intraspinal or intracranial inoculation. Analysis of the spinal cord following intracranial inoculation revealed increasing luciferase activity at later time points post-administration (24–48 h; data not shown). The levels of luciferase were considerably lower than that following intraspinal inoculation of replicons. Thus, replicons, like poliovirus, do not possess an inherent capacity to infect cells within the brain or non-neuronal cells (i.e. astrocytes, oligodendrocytes) of the spinal cord.

Finally, the ability of the replicons to infect and express recombinant proteins in the spinal cord points to the further development of the replicon as a vector for delivery of recombinant proteins to the CNS. As replicons express foreign proteins for a limited duration, they may be useful in expressing biological response modifiers, such as cytokines, which if expressed chronically could result in inflammation and disease. One potential application would be to use replicons for expression of anti-inflammatory cytokines, which when transiently expressed, could prove to be useful in reducing the sequelae associated with spinal cord injury. Previous studies have shown that cytokine modulation is an important component in the

body's natural response to trauma, including spinal cord injury (Bartholdi and Schwab, 1997; Xing *et al*, 1998). Replicons might also have an application for the delivery of neurotrophins as well as other growth factors, which have been shown to be useful in stimulating neuron regeneration, thus improving recovery (Blesch and Tuszynski, 1997; Kim *et al*, 1996). Studies are currently underway to pursue the use of replicons to deliver biologically active molecules to the neurons of the spinal cord.

Materials and methods

Tissue culture cells and viruses

HeLa H1 cells were grown in Dulbecco's Modified Eagle Medium (GIBCO BRL, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (GIBCO BRL, Gaithersburg, MD, USA) and 1% antibiotic/antimycotic (GIBCO BRL, Gaithersburg, MD, USA). The recombinant vaccinia virus, VV-P1, which expresses the poliovirus P1 capsid precursor protein was prepared as previously described (Ansardi *et al*, 1991). Poliovirus Type 1 Mahoney was grown in HeLa H1 cells and purified through a cesium chloride gradient. Briefly, the virus was concentrated by ultracentrifugation over a sucrose cushion (30% sucrose; 30 mM Tris-HCl pH 7.0; 15 mM $MgCl_2$; 150 mM NaCl) at 28 000 r.p.m., 4°C, overnight. The pellet was resuspended in PBS and microcentrifuged at maximum speed 20 min to remove insoluble material. The supernate was removed and cesium chloride was added to a solution density of 1.33 g/ml, plus 0.8% Triton X-100. The gradient was ultracentrifuged at 45 000 r.p.m., 20°C, overnight. Fractions were collected and assayed on a SDS-10% polyacrylamide gel for presence of the virus. The gel was silverstained to visualize the capsid proteins of the virus. Peak fractions were pooled and dialyzed against PBS. The virus was titered by plaque assay on HeLa H1 cells and stored at −70°C.

Preparation of replicons

Replicons encoding firefly luciferase were constructed and prepared as previously described (Porter *et al*, 1998). Briefly, replicons were concentrated by ultracentrifugation (SW28 rotor at 28 000 r.p.m., 4°C overnight) extracted one time with chloroform, followed by a second concentration by ultracentrifugation (SW55 rotor at 55 000 r.p.m., 4°C, 90 min). The replicons were titered by infection of HeLa H1 cells, followed by metabolic labeling and immunoprecipitation with anti-$3CD^{pol}$ antibodies, as previously described (Jablonski *et al*, 1991). The levels of 3CD immunoprecipitated were compared to that immunoprecipitated from cells infected with known amounts of poliovirus. The titers of replicons are presented in infectious units (i.u.), which correspond directly with plaque forming units of poliovirus.

To assay for poliovirus in replicon preparations, HeLa H1 cells were plated in 6-well tissue culture plates and infected 24 h later with 10^6 infectious units of the luciferase replicon. Two hours later the inoculum was removed and the cells were washed twice. Complete media was added to the wells and cells were incubated for 48 h. The cells were lysed by three freeze/thaw cycles, after which the cell debris was pelleted. The supernates were used to reinfect HeLa H1 cells in 6-well tissue culture plates. The process was continued for three serial passages. The supernates from each passage were used to infect HeLa H1 cells plated in 24-well tissue culture plates. In parallel, 1 : 10 serial dilutions of poliovirus Type 1 Mahoney (starting with 10^3 p.f.u./well) were used to infect HeLa H1 cells, to establish a minimum amount of virus needed to result in 100% cell death after 48 h. The cells were fixed with 5% TCA, stained with Coomassie Blue and photographed.

Animals

Transgenic mice, TgPVR1-27, 6–8 weeks of age were used for all animal experiments (Ren *et al*, 1990). The mice were obtained from Lederle-Praxis Laboratories (Deatly *et al*, 1998).

Intracranial administration

Mice were anesthetized with 20 mg/ml ketamine plus 0.30 mg/ml xylazine in saline administered intraperitoneally at a dose of 0.07 ml/10 g body weight (Chambers *et al*, 1995) into PVR transgenic mice (Deatly *et al*, 1998; Koike *et al*, 1991; Ren *et al*, 1990). A 0.5–1 mm midline incision was made in the skin and a 1 mm burr hole was made in the skull, 1.5 mm to the right of midline and 0.5–1.0 mm anterior to the coronal suture. The virus was loaded into a 250 μl Hamilton syringe and mounted in a stereotaxic holder. A 30-gauge needle was inserted vertically through the burr hole to a depth of 2.5 mm. Two 5 μl injections of virus (30 s apart) were made into the caudate nucleus; the needle was removed after 2 min (Chambers *et al*, 1995). The incision was closed with sterile 9 mm wound clips, applied with a wound clip applier (Fisher Scientific, St. Louis, MO, USA).

Intraspinal administration

Mice were anesthetized by metofane inhalation (Pittmann Moore, IL, USA). Intraspinal inoculations were performed as described by Abe *et al* (1995). Briefly, the back of each mouse was disinfected with ethanol and a 2–3 cm incision was made lengthwise in the skin in the lumbar region. The mouse was placed over a tube (as illustrated in Abe *et al*, 1995) and a 30-gauge needle was inserted between the spinous processes at the top of the curved thoracolumbar region. Jerking of the hind-limbs or tail was a sign of correct needle position. For injections, virus was loaded into a 250 μl Hamilton

syringe, fitted with a 30-gauge needle attached to a repeating dispenser; one 5 μl injection of virus was administered per mouse. The skin incision was closed with sterile wound clips (Fisher Scientific, St. Louis, MO, USA).

Luciferase enzyme assays
Mice were euthanized by CO_2 inhalation and spinal cords (and/or brains) around the injection site were dissected out, placed in microcentrifuge tubes and frozen at -70°C overnight. The tissues were lysed with 1× luciferase lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM DTT, 2 mM 1,2,diaminocyclohexane-*N*,*N*,*N*′*N*′-tetraacetic acid, 10% glycerol, and 1% Triton X-100), vortexed and sonicated (Heat Systems, Inc., Farmingdale, NY, USA) at maximum setting (in ice water) until tissue was lysed completely (approximately 3 min/tissue). Spinal cords were lysed in 150 μl lysis buffer; brains in 500 μl lysis buffer. Samples were microcentrifuged 20 min at 4°C to remove cell debris. Supernatants were used for luciferase assays (Promega), as described previously (Porter *et al*, 1998). Briefly, 50 μl of each lysate was added to 100 μl of luciferase substrate reagent (20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2{\cdot}5H_2O$; 2.67 mM $MgSO_4$; 0.1 mM EDTA; 33.3 mM DTT; 270 μM coenzyme A, 470 μM luciferin; 530 μM ATP, pH 7.8); 100 μl of that mixture was assayed. Protein content for each sample was determined (Pierce). The luciferase activity was normalized to 100 μg protein for each sample.

Tissue preparation and histopathology analysis
The PVR transgenic mice were euthanized by CO_2 inhalation. The skulls and spines from each animal were removed and fixed in 4% paraformaldehyde at 4°C for at least 24 h. The brains and spinal cords were harvested, paraffin-embedded and serially sectioned at 10 μm intervals. Sections were deparaffinized in xylene and rehydrated through two successive incubations in each of the following: absolute ethanol, 95% ethanol, 70% ethanol and murine-PBS (m-PBS; 200 mM NaCl, 10 mM $NaH_2PO_4{\cdot}H_2O$) and allowed to air dry.

For hematoxylin and eosin staining assays, tissues were fixed, sectioned, deparaffinized and rehydrated as stated above and then were incubated in hematoxylin plus 4% glacial acetic acid for 60 s. The sections were drained and stained with one to two drops of alcohol eosin, rinsed for 5 s with 95% ethanol, agitated in 100% ethanol and dipped in xylene. Coverslips were mounted on sections and slides were allowed to air dry for 24 h. The slides were examined using a microscope and photographed.

For immunofluorescence, sections were rehydrated in m-PBS for 10 min at room temperature. Slides were then microwaved for 10 min at high power in citrate buffer (1.8 mM citric acid; 8.2 mM sodium citrate; pH 6.0) for antigen retrieval. Sections were washed with H_2O, followed by m-PBS. The sections were incubated at 4°C overnight with the appropriate primary antibody: a polyclonal rabbit antibody to poliovirus $3D^{pol}$ (Jablonski *et al*, 1991), a rabbit polyclonal antibody to luciferase (Promega) or a mouse monoclonal antibody to the neuronal marker, NeuN, (Chemicon International, Inc., Temecula, CA, USA). Sections were washed three times with m-PBS and then incubated for 2 h at room temperature with a secondary antibody. Tissues that were stained for $3D^{pol}$ were incubated with a rhodamine-conjugated goat-α-rabbit secondary antibody; tissues which were double-stained for luciferase and for NeuN were incubated with a cocktail of the rhodamine-conjugated goat-α-rabbit secondary antibody and a FITC-conjugated goat-α-mouse secondary antibody. Slides were again washed three times and allowed to dry at room temperature (about 15–20 min). Coverslips were mounted over sections. The slides were examined using a fluorescent microscope and photographed.

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