Published in final edited form as:

Exp Neurol. 2007 February ; 203(2): 381–393.

The HSV-2 Protein ICP10PK Prevents Neuronal Apoptosis and Loss of Function in an *In Vivo* Model of Neurodegeneration Associated with Glutamate Excitotoxicity

Erin K. Golembewski^a, Samantha Q. Wales^a, Laure Aurelian^a, and Paul J. Yarowsky^{a,b,*}

^aDepartment of Pharmacology and Experimental Therapeutics, University of Maryland, Baltimore, MD

^b VA Maryland Healthcare System, Baltimore, MD

Abstract

Excessive glutamate receptor activation results in neuronal death, a process known as excitotoxicity. Intrastriatal injection of N-methyl-D-aspartate (NMDA) is a model of excitotoxicity. We used this model to examine whether excitotoxic injury is inhibited by the anti-apoptotic herpes simplex virus type 2 (HSV-2) protein, ICP10PK, delivered by the replication incompetent HSV-2 vector, Δ RR. Intrastriatal Δ RR administration (2500 plaque forming units) was nontoxic and did not induce microglial activation five days after injection. Intrastriatal injection of Δ RR with NMDA or four hours after NMDA injection showed increased neuronal survival and decreased mitochondrial damage compared to injection of NMDA alone. Neuroprotection was due to the inhibition of NMDA-induced apoptosis through ERK activation. Δ RR treated mice did not develop NMDA-associated behavioral deficits. The data suggests that Δ RR is a promising platform for treatment of acute neuronal injury.

Keywords

Apoptosis; ERK activation; excitotoxicity; glutamate; HSV-2; ICP10PK; neurodegeneration

INTRODUCTION

In the central nervous system (CNS), an acute injury, such as ischemia, hypoxia, hypoglycemia, infection or trauma causes excessive neuronal death and results in devastating and sometimes fatal symptoms. Ischemic brain injury, or stroke, is the third most common killer of Americans and the leading cause of severe, long-term disability (American Heart Association, 2005). An embolus blocking the flow of blood containing oxygen and nutrients causes neuronal death due to excessive activation of glutamate receptors, a process known as excitotoxicity. Excitotoxicity is accompanied by the generation of intracellular free radicals, mitochondrial damage and induction of programmed cell death (apoptosis). Effective treatments remain elusive due to the difficulty in targeting the brain, safety concerns and the failure to override apoptosis. Molecular therapy strategies have not been proven effective in clinical situations. Identification of the relevant target genes and delivery platforms are a clinical challenge.

* **Corresponding Author:** Paul J. Yarowsky 655 West Baltimore Street 4-002 Bressler Research Building Baltimore, MD 21201-1509 pyarowsk@umaryland.edu 410-706-3134 (telephone) 410-706-0032 (fax)

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Viruses are particularly promising vectors, for gene delivery as they demonstrate cell-type specificity and can be genetically modified to deliver the genes of interest. They also encode anti-apoptotic genes that ensure prolonged cell survival, which is required for virus replication and evasion of normal host cell constraints (Aurelian, 2005). HSV-based vectors are likely candidates for gene therapy of CNS disorders due to their inherent neurotropism. Indeed, HSV can cross synapses infecting each neuron in a pathway (Geller et al., 1991; Glorioso et al., 1994). Recent attempts to minimize ischemic damage have used HSV-1 based vectors to deliver genes encoding a variety of anti-apoptotic proteins such as Bcl-2, glial-derived neurotrophic factor (GDNF) or heat shock protein 72 (Hsp72) (Linnik et al., 1995; Lawrence et al., 1996; Fink et al., 1997; Wang et al., 1997; Yenari et al., 1998; Antonawich et al., 1999; Harvey et al., 2003). However, safety concerns linger because HSV-1 has pro-apoptotic activity in the CNS and can cause encephalitis in immunocompetent human adults (Perkins, 2002; Perkins et al., 2003). In some cases, HSV-1 based vectors have been shown to induce inflammation and neurotoxicity (Monville et al., 2004).

One strategy to overcome vector toxicity while retaining neurotropism is to develop vectors based on HSV-2, which does not cause neuronal apoptosis nor encephalitis in adult humans (Harvey et al., 2003). We have recently described the construction of a replication incompetent HSV-2-based vector (Δ RR) and showed that it confers neuroprotection at relatively low doses (Laing et al., 2006). Neuroprotection is mediated by the anti-apoptotic HSV-2 gene ICP10PK that appears to function at reactivation from HSV-2 latency (Gober et al., 2005) and protects neurons from apoptotic stimuli such as viral infection, treatment with a protein kinase C inhibitor, disruption of osmolar environment or growth factor withdraw (Smith et al., 1997; Perkins et al., 2002). In the virus infected neurons, anti-apoptotic activity is through activation of the Ras/Raf-1/MEK/ERK pathway, that up-regulates and/or stabilizes the anti-apoptotic proteins Bag-1 and Bcl-2 (Smith et al., 1994; Perkins et al., 2003).

The studies described in this report examined the neuroprotective potential of Δ RR in an *in vivo* model of excitotoxicity in which mice were intrastrially injected with the excitotoxin N-methyl-D-aspartate (NMDA). The data indicate that Δ RR prevented NMDA-induced neurotoxicity and the surviving animals were protected from behavioral damage.

MATERIALS AND METHODS

Cells and Viruses

Vero (African green monkey kidney) cells were grown in minimal essential medium with 10% fetal bovine serum (FBS) and 100 U of penicillin-streptomycin (Invitrogen, Grand Island, NY) per ml, as described (Smith et al., 1994; Smith et al., 1997; Smith et al., 1998; Smith et al., 2000). Herpes simplex virus type 2 (HSV-2) (strain G) and the mutants Δ PK and Δ RR established from HSV-2 (G) were previously described (Smith et al., 1998; Laing et al., 2006). Briefly, the large subunit of the HSV-2 ribonucleotide reductase (R1 also known as ICP10) is a chimera that consists of an amino terminal domain with serine/threonine protein kinase (PK) activity and a carboxy terminal domain with R1 activity that function independently of each other (Chung et al., 1989; Chung et al., 1991; Smith et al., 1994). In the Δ RR mutant, the R1 domain was replaced with the *lacZ* gene. In the Δ PK mutant, the PK domain was deleted. Both viruses retain the amino-terminal residues 13-26, which include the LA-1 epitope that is specifically recognized by the ICP10 antibody (Aurelian et al., 1989). The mutant viruses, Δ RR and Δ PK, are grown in Vero cells and titrated by plaque assay in medium containing 10% serum, as described (Smith et al., 1998). They are replication incompetent in neurons (Smith et al., 1998; Perkins et al., 2002; Perkins et al., 2003; Laing et al., 2006).

Reagents and Antibodies

Apomorphine, 2-methyl butane, hematoxylin, thionin, cytochrome c oxidase, diaminobenzidine (DAB), sucrose, nickel ammonium sulfate, H₂O₂, MAP-2 antibody (2a+2b) (1:50) and NMDA were purchased from Sigma-Aldrich (St. Louis, MO). Eosin Y was purchased from Baker (Phillipsburg, NJ). The *In Situ* Cell Death Detection Kit, Fluorescein [with Fluorescein (FITC) labeled dUTP] was purchased from Roche (Indianapolis, IN). The NeuN antibody (1:100) was purchased from Chemicon (Temecula, California), cleaved caspase-3 antibody (1:200) from Cell Signaling (Beverly, MA), pERK1/2 antibody (1:100) from Promega (Madison, WI), CD11b (1:100) antibody from Leinco Technologies. TNF α antibody (1:100) from R&D Systems (Minneapolis, MN) and F4/80 (1:200) antibody from Serotec (Raleigh, NC). VP5 antibody (1:200) was purchased from Virusys (Sykesville, MD). The ICP10 antibody (1:100) was raised in rabbits using a synthetic peptide consisting of amino acid residues 13 to 26 (Aurelian et al., 1989). It recognizes ICP10 and the p95 and p175 proteins, respectively expressed by Δ PK and Δ RR, (Smith et al., 1998; Aurelian et al., 2000). Alexa Fluor 546-conjugated anti-rabbit and Alexa Fluor 594-conjugated anti-goat antibodies were purchased from Molecular Probes (Eugene, OR). Texas red-conjugated anti-rat and FITC-conjugated anti-mouse antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). The LSAB horseradish peroxidase secondary antibody kit (DAKO Corporation; Carpinteria, CA) was used for immunoperoxidase staining.

Animals

Adult male 129S6 SV/EV mice (Taconic; Germantown, NY) were housed in a light (12 h light/12 h dark) and temperature controlled environment with both food and water present *ad libitum*. All experiments were performed in compliance with institutional guidelines for humane and ethical treatment of animals (IACUC).

Intrastriatal Injection (ISI)

Mice were anesthetized by intraperitoneal injection of 1.25% Avertin (0.025 ml/g body weight) and placed in a rodent stereotaxic frame (David Kopf; Tujunga, CA) for intrastriatal injection, as previously described (Black et al., 1994; Ayata et al., 1997; Yenari et al., 1998; Kitagawa et al., 1999). The scalp was opened and the connective tissue was dissolved with 30% H₂O₂. A cannula (33 gauge) containing the solution (0.5 μ l) to be delivered was lowered through the dura mater 3.0 mm via a small hole drilled 1.0 mm anterior and 2.5 mm lateral to the bregma. A microinjection pump (CMA/100; Carnegie Medicin; Solna, Sweden) regulated the solution flow rate (0.1 μ l/min) over a period of 5.0 min; the cannula remained in place for an additional 3.0 min to permit diffusion. Afterwards, the cannula was removed and the incision was closed. Animals awoke in approximately one hour after injection and were observed for 2-100 days. The concentration of NMDA was 40.5 nmole/0.5 μ l. The concentration of the HSV-2 and Δ RR viruses was 2500 pfu. The concentration of Δ PK virus was 4200 pfu.

Cytochrome c oxidase staining

Mice were sacrificed by cervical dislocation. After decapitation, brains were extracted and immersed in a 30% sucrose solution at 4 °C until they completely sank. They were frozen in 2-methyl butane at -60 °C and embedded in Optimal Cutting Temperature (OCT) compound. The embedded brains were sliced into 10-30 μ m sections using a cryostat (Leica; Wetzlar, Germany) and each section was mounted on superfrost plus slides. Serial sections were stained with metal-enhanced cytochrome c oxidase (mitochondria) as described (Poeggeler et al., 1998). For cytochrome c oxidase staining, brain sections were incubated for 90 min at 25 °C in HEPES buffer containing 0.02% cytochrome c oxidase, 0.01% diaminobenzidine (DAB) 4.5% sucrose and 0.001% nickel ammonium sulfate. Tissue sections were then fixed for 15 min in 4% paraformaldehyde. The slides were dehydrated in a series of ethanol baths of

increasing concentration (50%-100%), covered with cytooseal (xylene based mounting media) coverslipped and analyzed by light microscopy. Lesion area was determined for each slice and multiplied by the slice thickness to calculate slice volume, as described (Guidetti et al., 2003). Total lesion volume for each animal was calculated by adding all the individual slices lesion volume using the formula

$$\text{Total lesion volume} = (\text{area} \times \text{thickness})_1 + (\text{area} \times \text{thickness})_2 + \dots + (\text{area} \times \text{thickness})_n$$

where n = total number of slices through the lesion.

Thionin staining

Thionin staining was used to detect Nissl substance in the cytoplasm. Sections were post-fixed in 4% paraformaldehyde for 15 min, and incubated for 45 min at 25 °C in a solution containing 0.06% thionin, 0.3% acetic acid, and 0.3% sodium acetate. Sections were dehydrated and prepared for light microscopy as described above.

TUNEL

The *In Situ* Cell Death (TUNEL) Detection Kit, Fluorescein (containing FITC labeled dUTP) was used according to manufacturer's instructions. Briefly, brain sections were fixed in 4% paraformaldehyde, permeabilized in a solution of 0.1% Triton X and 0.1% sodium citrate and incubated with the TUNEL reaction mixture containing terminal transferase (TdT) and FITC-dUTP for 60 min at 37 °C. The slides were covered in mounting media containing DAPI (to label nuclei of all cells) (Vector Labs; Burlingame, CA), coverslipped and examined by fluorescent microscopy using a Nikon Eclipse E400 microscope and Spot Advanced digital camera and software. The total number of cells (DAPI+) was counted in 5 randomly selected fields (>250 cells each). The number of TUNEL+ cells was counted in each of these fields. The percent of TUNEL+ cells was calculated relative to total cell number (DAPI+).

Immunohistochemistry and Immunofluorescence

Immunohistochemistry was performed as we have previously described (Chung et al., 1989; Perkins et al., 2002). Briefly, brain sections were incubated overnight (4 °C) with primary antibodies; immunolabeled cells were subsequently detected with the DAKO streptavidin-biotin method as recommend by the manufacturer or fluorescently-conjugated secondary antibodies (30 minutes at 25 °C). Fluorescently labeled tissue was mounted as described above and examined by fluorescent microscopy. Peroxidase labeled tissue was mounted in glycerol and examined by light microscopy.

Apomorphine behavioral test

Two or five days after intrastriatal injection, mice were given an apomorphine test to monitor behavioral changes indicative of neuronal function, as previously described (Mason et al., 1978; Schwarcz et al., 1979). Briefly, animals were acclimated for 10 min to the open field environment apparatus and then briefly removed and subcutaneously injected with the apomorphine solution (1 mg/kg body weight). Following drug administration, mice were re-acclimated in the cage for three minutes. The locomotor response, direction and number of 360° rotations, was recorded for 10 min. Rotational preference is reported as the difference between ipsilateral (right) and contralateral (left) rotations.

Statistical analysis

Statistical analysis between treatment groups was done by a one-way ANOVA with Tukey-Kramer post-test.

RESULTS

Intrastrially injected Δ RR is not toxic and does not induce inflammation

HSV-1 and HSV-2 have established neurovirulence particularly after intracranial delivery and growth compromised HSV-1-based vectors have also been associated with brain toxicity and deleterious inflammatory response (Monville et al., 2004). Although Δ RR is growth compromised in neurons (Laing et al., 2006), we wanted to know whether it lost toxicity when delivered intrastrially. Mice (n=5) were injected with 2500 plaque forming units (pfu) of Δ RR or HSV-2 in the right striata and observed for 100 days after injection. Mice injected with HSV-2 were lethargic on day 2 and died on days 5, 10, and 11 after injection. Mice injected with Δ RR lived for the 100 days of observation without displaying any untoward symptoms. The data indicate that Δ RR is not toxic and does not cause any symptoms of infection after intrastriatal inoculation, at least at the dose used in these experiments

To examine the propensity of Δ RR to elicit undesirable inflammatory responses in the brain, brain tissues from animals infected with HSV-2 or Δ RR (2500 pfu) were collected 5 days later, when the blood brain barrier failure caused by the injection itself had resolved (Olschowka et al., 2003) and stained with antibodies to TNF α or F4/80 (markers for microglial activation/macrophage infiltration). Infiltration with systemic lymphocytes was examined by staining with antibody to CD8+ T cells. Mice injected with HSV-2 were positive for all markers, indicating that infection induces both inflammation and lymphocytic infiltration. By contrast staining was not seen in mice given Δ RR (Fig. 1). Consistent with a previous report (Laing et al., 2006), the absence of inflammation correlates with the failure of Δ RR to replicate in the brain. Indeed in HSV-2 infected mice but not in Δ RR infected mice the major capsid protein VP5 a DNA replication marker (Roizman et al., 1996), was found in axons and varicosities (Fig. 1b) as has been reported for the infected peripheral nervous system (Saksena et al., 2006). Collectively, the data indicate that Δ RR does not replicate nor induce inflammatory responses in intrastrially injected mice. All of the Δ RR treated mice remained free of disease for at least 100 days.

Δ RR reduces the volume of the excitotoxic NMDA lesion after intrastriatal injection

During an ischemic event, energy deprivation triggers increased release of the excitatory amino acid glutamate from neurons. Glutamate over-stimulates the NMDA receptor to neurotoxic level (Lipton, 2004) and Ca²⁺ influx through the NMDA receptors causes neuronal death by necrosis and apoptosis (Ankarcrona et al., 1995). Intrastriatal injection of NMDA is used as an ischemic model, where necrotic cell death induces an ischemic core, surrounded by an apoptotic penumbra (Black et al., 1994; Hara et al., 1997; Ruocco et al., 1999; Kanimatsu et al., 2001). To examine whether Δ RR can inhibit neuronal death caused by excitotoxic injury, mice were given NMDA, alone or NMDA with Δ RR, as described in Materials and Methods. Controls were given NMDA together with Δ PK, an HSV-2 mutant deleted in ICP10PK that is also replication incompetent in neurons (Laing et al., 2006). Specifically, animals were divided into four treatment groups (A-D) and there were 5 animals per group (summarized in Table 1). Group A received PBS in both striata. Group B received NMDA in the right striatum and PBS in the left striatum. Group C received NMDA combined with Δ RR in the right striatum and PBS in the left striatum and Group D received NMDA combined with Δ PK in the right striatum and PBS in the left striatum. Brains were removed on days 2 and 5 after treatment and examined for neuronal damage by cytochrome c oxidase staining. Tissue containing live mitochondria stained black while tissue that appears pale or white does not contain live mitochondria. Small circular striosomes, which are white matter, remain unstained in all treatments (Silverman et al., 1987). A characteristic lesion (white tissue) was observed in the striata injected with NMDA or with NMDA+ Δ PK, but it was minimal in striata injected with

NMDA+ Δ RR (Fig. 2) suggesting that ICP10PK has neuroprotective activity in this model of excitotoxicity.

To quantitate the neuroprotective activity of ICP10PK, total lesion volume was measured as described in Material and Methods. The total volume of the focal lesion generated by NMDA alone was $0.038 \pm 0.002 \text{ mm}^3$. The combination of NMDA+ Δ PK, caused a lesion of similar size [$0.035 \pm 0.001 \text{ mm}^3$ ($p > 0.10$)]. By contrast, the focal lesion seen in animals given NMDA + Δ RR was significantly ($**p < 0.01$) smaller [$0.007 \pm 0.002 \text{ mm}^3$] (Fig. 2) suggesting that ICP10PK has neuroprotective activity in this model of excitotoxicity. In another series of experiments, Δ RR (2500 pfu) was given 4 hrs after the standard NMDA injection. Δ RR caused a significant reduction ($0.018 \pm 0.002 \text{ mm}^3$; 53%; $*p < 0.05$) in the lesion volume as compared to NMDA alone (Fig. 2), supporting the interpretation that ICP10PK has neuroprotective activity, even when given after NMDA.

The failure of Δ PK to prevent NMDA induced lesions is not due to improper cellular penetration, because striatal sections from animals given NMDA+ Δ PK stained with ICP10 antibody showed the same pattern of immunostaining as those immunostained from animals given NMDA+ Δ RR (Fig. 3). Mice given NMDA+ Δ RR had $57 \pm 2\%$ ICP10+ cells, those given NMDA+ Δ PK had $62 \pm 4\%$ ICP10+ cells and mice given PBS or NMDA had no ICP10 labeled cells. ($***p < 0.001$). Staining was specific and it was not seen in the uninjected lateral striata and brain sections given NMDA alone or PBS instead of vector (data not shown). This is consistent with the biology of HSV infection.

Δ RR increases neuronal survival after NMDA administration

To examine whether the decrease in the striatal volume of the NMDA lesion in animals given Δ RR is due to increased cell survival, serial sections of the brains from the same animal were stained with thionin which recognizes the Nissl substance of live neurons. Survival was significantly decreased with NMDA or NMDA+ Δ PK treatments, but not with NMDA+ Δ RR treatment (Fig. 3). Indeed, when striatal neurons were counted and the results expressed as percent survival normalized to PBS-treated tissue, we found that in mice given NMDA+ Δ RR survival was 83.0% ($p > 0.10$ relative to PBS-treated mice). This is in contrast to NMDA or NMDA+ Δ PK treated tissue that had only 16.1% and 21.7% cell survival respectively ($**p < 0.01$) (Fig. 3). Together with the observation that Δ RR, but not Δ PK, causes a significant reduction in the NMDA lesion volume these findings indicate that ICP10PK prevents NMDA induced excitotoxic injury.

Δ RR neuroprotection is due to ICP10PK anti-apoptotic activity

To determine whether Δ RR-mediated neuroprotection is due to apoptosis inhibition, striatal sections from animals in Groups A-D (Table 1) were examined for apoptosis by TUNEL. TUNEL+ cells were counted and the percentage calculated relative to total (DAPI+) cells in the field as described in Materials and Methods. As shown in Fig. 4, merged images indicate that mice treated with NMDA or NMDA+ Δ PK had more TUNEL+ cells than those given PBS or NMDA+ Δ RR at 2 days post-injection. The percentage of TUNEL+ cells was $41.4 \pm 6.5\%$ and $46.8 \pm 1.0\%$ for NMDA and NMDA+ Δ PK respectively, as compared to $2.3 \pm 1.0\%$ and $1.3 \pm 0.6\%$ for PBS or NMDA+ Δ RR, respectively. To determine when the TUNEL+ cells started to appear striatal sections from animals in Groups A-D (Table 1) were examined at 8, 12, 24 and 48h post-injection. The difference in the percentage of TUNEL+ cells was first seen at 24 hrs and continued to 48 hrs after injection (Fig. 5). Because TUNEL has come under scrutiny in terms of its ability to detect apoptosis in neurons, serial sections of the same brains were examined for caspase-3 activation by double immunofluorescence with antibodies to activated caspase-3 (the p20 cleavage product) and NeuN (a neuronal marker). Doubly labeled cells were counted and the % of stained cells was calculated relative to DAPI+ cells. Significantly fewer

cells positive for both caspase-3p20 and NeuN were seen in mice given NMDA+ Δ RR than NMDA alone (Fig. 6). At 48 h post-injection, $81.4 \pm 8.1\%$ of striatal neurons from mice treated with NMDA were caspase-3p20+. The addition of Δ RR decreased the percentage of caspase-3p20+ neurons to $15.3 \pm 1.3\%$, similar to that seen in PBS treated tissues ($12.2 \pm 1.1\%$; $p > 0.10$). Collectively the data indicate that ICP10PK protects neurons from NMDA-induced apoptosis.

Δ RR anti-apoptotic activity is through ERK activation

ICP10PK protects hippocampal cultures from apoptotic insult via activation of the ERK pathway (Perkins et al., 2002). To examine whether ERK is activated *in vivo* in Δ RR treated striata, serial striatal sections studied for TUNEL were stained with antibodies to pERK and MAP2, a neuronal dendritic marker (Caceres et al., 1983) as described in Materials and Methods. pERK staining was seen in Δ RR, but not in PBS treated tissue (Fig. 7) Striata of mice given Δ RR had $83.3 \pm 6\%$ pERK+ neurons as compared to mice given PBS which only had $14.2 \pm 4.8\%$ pERK+ neurons ($***p < 0.001$). To confirm the role of pERK in protection, animals were given a combination of NMDA+ Δ RR together with the MEK inhibitor U0126 and the brain tissues were examined for lesion volume. We conclude that ERK activation is involved in Δ RR-mediated neuroprotection, because the lesion volumes for animals given NMDA + Δ RR together with U0126 were similar to those seen in mice given NMDA alone, as illustrated by cytochrome c histochemistry (Fig. 8).

Δ RR decreased NMDA-induced behavioral damage

Having seen that Δ RR protects from NMDA-induced neuronal death, we wanted to know whether the rescued neurons continue to function effectively. To address this question we used a rotational motor test in which animals are given a subcutaneous apomorphine injection 2 or 5 days after intrastriatal injection. Apomorphine will cause a mouse with unequally functioning striata to rotate towards the side of reduced dopamine input (i.e. excitotoxic lesion) (Schwarcz et al., 1979). A normal mouse with equal dopamine innervation from two fully functioning striata will not rotate preferentially. Mice were divided into seven treatment groups (groups A-G) for intrastriatal injection, with 5 animals per group (Table 2). Data are presented as preferential rotation which represents the difference between ipsilateral (right) and contralateral (left) rotation. PBS-treated control animals did not show a rotational preference (Fig. 9). At 2 days post-injection, animals given NMDA alone rotated a net average of 19.6 times toward the side of the NMDA lesion (ipsilateral side). Mice treated with NMDA+ Δ RR rotated 5.3 times towards the side of the lesion, a 72.8% decrease compared to NMDA-only ($**p < 0.01$), and a non-significant increase over the uninjected controls ($p > 0.10$). Animals injected with NMDA+ Δ PK rotated an average of 21.0 times towards the ipsilateral side, which was equal to those injected with NMDA-only ($p > 0.10$). Mice treated with NMDA on one side and NMDA+ Δ RR on the contralateral side, rotated an average of 9.4 times towards the NMDA only side. Similar to PBS treated control animals, neither mice injected with Δ RR unilaterally nor with NMDA bilaterally rotated preferentially to either side ($p > 0.10$). At day 5 after mice were given NMDA+ Δ RR in the right striatum, they rotated an average of 7.4 times towards the side of injection, which is statistically similar to NMDA+ Δ RR mice at 2 days after injection ($p > 0.10$). These data indicate that the co-injection of Δ RR with NMDA diminishes behavior patterns associated with an excitotoxic lesion (loss of neuronal function) at least from day 2 to 5 post-injection.

DISCUSSION

Ischemic brain injury, is the third most common killer of Americans and the leading cause of severe, long-term disability (American Heart Association, 2005). Excessive NMDA receptor activation, excitotoxicity, causes neuronal injury/death in stroke victims, limiting a patient's

cognition, communication skills, mobility and independence. Strategies to attenuate the neurological symptoms of acute injury are of great clinical importance and their development is the subject of major research interest (Harvey et al., 2003). Intrastriatal delivery of Bcl-2, GDNF or Hsp72 with HSV-1 based vectors was previously shown to protect a small percentage of neurons from ischemic death (Linnik et al., 1995; Lawrence et al., 1996; Fink et al., 1997; Wang et al., 1997; Yenari et al., 1998; Antonawich et al., 1999; Harvey et al., 2003), but at least one such vector, caused inflammation and neurotoxicity (Monville et al., 2004). Genome-free HSV-based vectors were constructed (amplicons), and their safety improved by the development of a helper-free delivery system that supports the replication and packaging of co-transfected amplicon DNA (Olschowka et al., 2003). However, they did not demonstrate significant neuroprotection. Improved efficiency was achieved when using extremely high vector doses, but these were associated with toxicity (Olschowka et al., 2003). Moreover, as these vectors lost the immune evasion properties characteristic of HSV, they induced specific immune responses which are likely to interfere with repeated treatment, should it be required. Most importantly, for therapy to be effective, the surviving neurons must retain normal function (Dumas et al., 2001). In most of the aforementioned studies, neuronal function was not examined (Linnik et al., 1995; Lawrence et al., 1996; Fink et al., 1997; Wang et al., 1997; Yenari et al., 1998; Antonawich et al., 1999), and when examined, neurons were found to be dysfunctional (Harvey et al., 2003).

Δ RR is based on HSV-2, which is less virulent in the CNS than HSV-1 (Bergstrom et al., 1991). HSV-2 does not trigger apoptosis in CNS neurons (Perkins et al., 2002) nor cause encephalitis in immunocompetent adult humans (Natsume et al., 2001). Nonetheless, we used an in vivo model (intracerebral injection) that is very stringent with respect to toxicity assessment, in order to examine whether Δ RR is safe. Although intracranial delivery is generally associated with HSV toxicity, Δ RR injected mice lived for at least the 100 days of observation, without striatal inflammation, infiltration by systemic lymphocytes or viral replication. While we cannot exclude the possibility that this reflects the relatively low virus dose used in these studies (2500 pfu), all the mice given a similar dose of HSV-2 died between 5-11 days post-injection and death was associated with virus replication, as determined by VP5 expression and inflammatory and immune responses.

Previous studies had shown that NMDA causes neuronal cell death with features suggestive of apoptosis (a model of ischemia). Indeed, while the irreversible component of death in the first few hours after insult are attributed to necrosis, the histological and biochemical features seen at later times after excitotoxic insult (more than eight hours) are typical of apoptosis (Ankarcrona et al., 1995; Ayata et al., 1997). Our studies were focused on neuronal cell death seen at 48 hrs post-injection. At this time, histological examination by both cytochrome c oxidase and thionin staining showed that Δ RR decreased neuronal cell loss by approximately 80%. Protection was due to ICP10PK, because the ICP10PK deleted virus, Δ PK, did not decrease NMDA induced neurodegeneration. Δ PK is a particularly stringent control for Δ RR because: (i) it was constructed from the same HSV-2 strain, (ii) the PK-deleted ICP10 protein is driven by the same promoter and is expressed as well as the RR deleted protein in Δ RR, and (iii) the virus is also replication incompetent in infected primary hippocampal neurons and organotypic hippocampal cultures (Smith et al., 1998; Perkins et al., 2002; Laing et al., 2006).

The magnitude of neuroprotection conferred by Δ RR delivered ICP10PK is unprecedented compared to protection imparted by other vector/target gene systems, particularly as observed at very low doses (2500 pfu) of Δ RR. We conclude that protection is due to inhibition of NMDA-induced apoptosis, because both TUNEL and caspase-3 activation were inhibited by Δ RR, but not Δ PK. Protection appears to be mediated by activation of the ERK pathway, although we do not exclude the possibility that mechanisms other than ERK activation may contribute to neuroprotection. Our findings are consistent with previous reports about the anti-

apoptotic activity of ICP10PK in neurons and the ability of intranasally delivered Δ RR to protect from kainic acid induced seizures (Perkins et al., 2003; Laing et al., 2006).

A foremost aim for the treatment of acute ischemia is to reduce behavioral deficits. We show that the co-injection of Δ RR with NMDA corrected the rotational behavior induced by apomorphine test by 72.8% as compared to NMDA alone. Δ PK with NMDA did not afford a similar protection from behavioral deficits, indicating that striata expressing ICP10PK retained normal function. Survival, together with this extent of functional protection, distinguishes ICP10PK from other anti-apoptotic genes and identifies it as a promising gene therapy candidate. Ongoing studies are designed to further elucidate the mechanism of anti-apoptotic activity in the NMDA model and define the therapeutic window.

ACKNOWLEDGEMENT

These studies were supported by NINDS, National Institutes of Health, NS45169 (L.A.) and the Veterans Administration Merit Award (P.Y.). We thank Dr. R. Schwarcz for comments and equipment.

REFERENCES

- American Heart Association. Heart Disease and Stroke Statistics - 2005 Update. American Heart Association; Dallas: 2005. 2005
- Ankarcrona M, Dypbukt JM, Bonfoco E, Zhivotovsky B, Orrenius S, Lipton SA, Nicotera P. Glutamate-induced neuronal death: A succession of necrosis or apoptosis depending on mitochondrial function. *Neuron* 1995;15(4):961–973. [PubMed: 7576644]
- Antonawich FJ, Federoff HJ, Davis JN. BCL-2 Transduction, Using a Herpes Simplex Virus Amplicon, Protects Hippocampal Neurons from Transient Global Ischemia. *Experimental Neurology* 1999;156(1):130–137. [PubMed: 10192784]
- Aurelian L. HSV-Induced Apoptosis in Herpes Encephalitis. *Curr. Top. Microbiol. Immunol* 2005;289:79–1122. [PubMed: 15791952]
- Aurelian L, Smith C. Herpes simplex virus type 2 growth and latency reactivation by cocultivation are inhibited with antisense oligonucleotides complementary to the translation initiation site of the large subunit of ribonucleotide reductase (RR1). *Antisense Nucleic Acid Drug Dev* 2000;10(2):77–85. [PubMed: 10805158]
- Aurelian L, Terzano P, Smith CC, Chung T, Shamsuddin A, Costa S, Orlandi C. Amino-terminal Epitope of Herpes Simplex Virus Type 2 ICP10 Protein as a Molecular Diagnostic Marker for Cervical Intraepithelial Neoplasia. *Cancer Cells* 1989;7:187–191.
- Ayata C, Ayata G, Hara H, Matthews RT, Beal MF, Ferrante RJ, Endres M, Kim A, Christie RH, Waerber C, Huang PL, Hyman BT, Moskowitz MA. Mechanisms of Reduced Striatal NMDA Excitotoxicity in Type I Nitric Oxide Synthase Knock-Out Mice. *J. Neurosci* 1997;17(18):6908–6917. [PubMed: 9278526]
- Bergstrom T, Svennerholm B, Conradi N, Horal P, Vahlne A. Discrimination of herpes simplex virus types 1 and 2 cerebral infections in a rat model. *Acta Neuropathol. (Berl)* 1991;82(5):395–401. [PubMed: 1662855]
- Black MD, Crossman AR, Hayes AG, Elliott PJ. Acute and chronic behavioural sequelae resulting from intrastriatal injection of an NMDA agonist. *Pharmacol. Biochem. Behav* 1994;48(2):441–446. [PubMed: 8090812]
- Caceres A, Payne MR, Binder LI, Steward O. Immunocytochemical Localization of Actin and Microtubule-Associated Protein MAP2 in Dendritic Spines. *PNAS* 1983;80(6):1738–1742. [PubMed: 6572937]
- Chung T, Luo J, Wymer J, Smith C, Aurelian L. Leucine repeats in the large subunit of herpes simplex virus type 2 ribonucleotide reductase (RR; ICP10) are involved in RR activity and subunit complex formation. *J. Gen. Virol* 1991;72(Pt 5):1139–1144. [PubMed: 1851814]
- Chung T, P WJ, Smith CC, Kulka M, Aurelian L. Protein kinase activity associated with the large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10). *J. Virol* 1989;63:3389–3398. [PubMed: 2545912]

- Dumas TC, Sapolsky RM. Gene therapy against neurological insults: sparing neurons versus sparing function. *Trends in Neurosciences* 2001;24(12):695–700. [PubMed: 11718873]
- Fink SL, Chang LK, Ho DY, Sapolsky RM. Defective Herpes Simplex Virus Vectors Expressing the Rat Brain Stress-Inducible Heat Shock Protein 72 Protect Cultured Neurons from Severe Heat Shock. *J. Neurochem* 1997;68(3):961–969. [PubMed: 9048741]
- Geller A, During M, Neve R. Molecular analysis of neuronal physiology by gene transfer into neurons with herpes simplex virus vectors. *Trends Neurosci* 1991;14(10):428–432. [PubMed: 1722360]
- Glorioso J, Goins W, Meaney C, Fink D, DeLuca N. Gene transfer to brain using herpes simplex virus vectors. *Ann Neurol* 1994;35(Suppl):S28–34. [PubMed: 8185293]
- Gober M, Wales S, Hunter J, Sharma B, Aurelian L. Stress up-regulates neuronal expression of the herpes simplex virus type 2 large subunit of ribonucleotide reductase (R1; ICP10) by activating activator protein 1. *Journal of NeuroVirology* 2005;11(4):329–336. [PubMed: 16162476]
- Guidetti P, Schwarcz R. 3-Hydroxykynurenine and quinolinate: pathogenic synergism in early grade Huntington's disease? *Adv. Exp. Med. Biol* 2003;527:137–145. [PubMed: 15206726]
- Hara H, Ayata C, Huang P, Waeber C, Ayata G, Fujii M, Moskowitz M. [3H]L-NG-nitroarginine binding after transient focal ischemia and NMDA-induced excitotoxicity in type I and type III nitric oxide synthase null mice. *J. Cereb. Blood Flow Metab* 1997;17(5):515–526. [PubMed: 9183289]
- Harvey BK, Chang CF, Chiang YH, Bowers WJ, Morales M, Hoffer BJ, Wang Y, Federoff HJ. HSV amplicon delivery of glial cell line-derived neurotrophic factor is neuroprotective against ischemic injury. *Exp. Neurol* 2003;183(1):47–55. [PubMed: 12957487]
- Kitagawa H, Sasaki C, Sakai K, Mori A, Mitsumoto Y, Mori T, Fukuchi Y, Setoguchi Y, Abe K. Adenovirus-Mediated Gene Transfer of Glial Cell Line-Derived Neurotrophic Factor Prevents Ischemic Brain Injury After Transient Middle Cerebral Artery Occlusion in Rats. *Journal of Cerebral Blood Flow & Metabolism* 1999;19(12):1336–1344. [PubMed: 10598938]
- Kunimatsu T, Asai S, Kanematsu K, Kohno T, Misaki T, Ishikawa K. Effects of glutamate receptor agonist on extracellular glutamate dynamics during moderate cerebral ischemia. *Brain Research* 2001;923(12):178–186. [PubMed: 11743986]
- Laing JM, Gober MD, Golembewski EK, Thompson SM, Gyure KA, Yarowsky P, Aurelian L. Intranasal Administration of the Growth-Compromised HSV-2 Vector [Delta]RR Prevents Kainate-Induced Seizures and Neuronal Loss in Rats and Mice. *Mol. Ther* 2006;13(5):870–881. [PubMed: 16500153]
- Lawrence MS, Ho DY, Sun GH, Steinberg GK, Sapolsky RM. Overexpression of Bcl-2 with herpes simplex virus vectors protects CNS neurons against neurological insults in vitro and in vivo. *J. Neurosci* 1996;16(2):486–496. [PubMed: 8551333]
- Linnik MD, Zahos P, Geschwind MD, Federoff HJ. Expression of Bcl-2 From a Defective Herpes Simplex Virus-1 Vector Limits Neuronal Death in Focal Cerebral Ischemia. *Stroke* 1995;26(9):1670–1675. [PubMed: 7544927]
- Lipton SA. Failures and Successes of NMDA Receptor Antagonists: Molecular Basis for the Use of Open-Channel Blockers like Memantine in the Treatment of Acute and Chronic Neurologic Insults. *NeuroRx* 2004;1(1):101–110. [PubMed: 15717010]
- Mason ST, H.C. F. Kainic acid lesions of the striatum: behavioural sequelae similar to Huntington's chorea. *Brain Research* 1978;155(2):313–329. [PubMed: 28817]
- Monville C, Torres E, Thomas E, Scarpini CG, Muhith J, Lewis J, Finn J, Smith C, Cai S, Efsthathiou S. HSV vector-delivery of GDNF in a rat model of PD: partial efficacy obscured by vector toxicity. *Brain Research* 2004;1024(12):1–15. [PubMed: 15451362]
- Natsume A, Mata M, Goss J, Huang S, Wolfe D, Oligino T, Glorioso J, Fink DJ. Bcl-2 and GDNF Delivered by HSV-Mediated Gene Transfer Act Additively to Protect Dopaminergic Neurons from 6-OHDA-Induced Degeneration. *Exp. Neurol* 2001;169(2):231–238. [PubMed: 11358438]
- Olschowka JA, Bowers WJ, Hurley SD, Mastrangelo MA, Federoff HJ. Helper-free HSV-1 amplicons elicit a markedly less robust innate immune response in the CNS. *Mol. Ther* 2003;7(2):218–227. [PubMed: 12597910]
- Perkins D. Targeting apoptosis in neurological disease using the herpes simplex virus. *J. Cell Mol. Med* 2002;6(3):341–56. [PubMed: 12417051]

- Perkins D, Gyure KA, Pereira EF, Aurelian L. Herpes simplex virus type 1-induced encephalitis has an apoptotic component associated with activation of c-Jun N-terminal kinase. *J. Neurovirol* 2003;9(1):101–11. [PubMed: 12587073]
- Perkins D, Pereira EFR, Aurelian L. The Herpes Simplex Virus Type 2 R1 Protein Kinase (ICP10 PK) Functions as a Dominant Regulator of Apoptosis in Hippocampal Neurons Involving Activation of the ERK Survival Pathway and Upregulation of the Antiapoptotic Protein Bag-1. *J. Virol* 2003;77(2):1292–1305. [PubMed: 12502846]
- Perkins D, Pereira EFR, Gober M, Yarowsky PJ, Aurelian L. The Herpes Simplex Virus Type 2 R1 Protein Kinase (ICP10 PK) Blocks Apoptosis in Hippocampal Neurons, Involving Activation of the MEK/MAPK Survival Pathway. *J. Virol* 2002;76(3):1435–1449. [PubMed: 11773417]
- Poeggeler B, Rassoulpour A, Guidetti P, Wu H-Q, Schwarcz R. Dopaminergic Control of Kynurenate Levels and N-Methyl-D-Aspartate Toxicity in the Developing Rat Striatum. *Developmental Neuroscience* 1998;20(23):146–153. [PubMed: 9691189]
- Roizman, B.; Sears, AE. Herpes simplex viruses and their replication in Fields Virology. In: Fields, BN.; Knipe, DM.; Howley, PM., editors. Lippincott–Raven Publishers; Philadelphia: 1996.
- Ruocco A, Nicole O, Docagne F, Ali C, Chazalviel L, Komesli S, Yablonsky F, Roussel S, MacKenzie E, Vivien D, Buisson A. A transforming growth factor-beta antagonist unmasks the neuroprotective role of this endogenous cytokine in excitotoxic and ischemic brain injury. *J. Cereb. Blood Flow Metab* 1999;19(12):1345–1353. [PubMed: 10598939]
- Saksena MM, Wakisaka H, Tijono B, Boadle RA, Rixon F, Takahashi H, Cunningham AL. Herpes simplex virus type 1 accumulation, envelopment, and exit in growth cones and varicosities in mid-distal regions of axons. *J. Virol* 2006;80(7):3592–3606. [PubMed: 16537627]
- Schwarcz R, Fuxe K, Agnati LF, Hokfelt T, Coyle JT. Rotational behaviour in rats with unilateral striatal kainic acid lesions: a behavioural model for studies on intact dopamine receptors. *Brain Research* 1979;170(3):485–495. [PubMed: 37986]
- Silverman M, Tootell R. Modified technique for cytochrome oxidase histochemistry: increased staining intensity and compatibility with 2-deoxyglucose autoradiography. *J. Neurosci. Methods* 1987;19(1):1–10. [PubMed: 2434810]
- Smith CC, Aurelian L. The Large Subunit of Herpes Simplex Virus Type 2 Ribonucleotide Reductase (ICP10) Is Associated with the Virion Tegument and Has PK Activity. *Virology* 1997;234(2):235–242. [PubMed: 9268154]
- Smith CC, Luo JH, Hunter JCR, Ordonez JV, Aurelian L. The Transmembrane Domain of the Large Subunit of HSV-2 Ribonucleotide Reductase (ICP10) Is Required for Protein Kinase Activity and Transformation-Related Signaling Pathways That Result in ras Activation. *Virology* 1994;200(2 SU):598–612. [PubMed: 8178446]
- Smith CC, Nelson J, Aurelian L, Gober M, Goswami BB. Ras-GAP Binding and Phosphorylation by Herpes Simplex Virus Type 2 RR1 PK (ICP10) and Activation of the Ras/MEK/MAPK Mitogenic Pathway Are Required for Timely Onset of Virus Growth. *J. Virol* 2000;74(22):10417–10429. [PubMed: 11044086]
- Smith CC, Peng T, Kulka M, Aurelian L. The PK Domain of the Large Subunit of Herpes Simplex Virus Type 2 Ribonucleotide Reductase (ICP10) Is Required for Immediate-Early Gene Expression and Virus Growth. *J. Virol* 1998;72(11):9131–9141. [PubMed: 9765459]
- Wang Y, Lin S-Z, Chiou A-L, Williams LR, Hoffer BJ. Glial Cell Line-Derived Neurotrophic Factor Protects against Ischemia-Induced Injury in the Cerebral Cortex. *J. Neurosci* 1997;17(11):4341–4348. [PubMed: 9151750]
- Yenari MA, Fink SL, Sun GH, Chang LK, Patel MK, Kunis DM, Onley D, Ho DY, Sapolsky RM, Steinberg GK. Gene therapy with HSP72 is neuroprotective in rat models of stroke and epilepsy. *Annals of Neurology* 1998;44(4):584–591. [PubMed: 9778256]

Fig 1.

HSV-2 but not Δ RR induces an inflammatory response in the striatum. Mice were injected with 2500 pfu of either HSV-2 or Δ RR in the striata. At day 5 post-injection, striatal-containing tissue sections were stained with antibodies to microglial proteins, TNF α , F4/80 or antibody to CD8+ T cells (a). Duplicate sections of tissues in (a) were stained with VP5 and the neuronal specific antibody β III tubulin (b). Yellow stain in merged panel indicates VP5 and β III tubulin co-localization.

Fig 2.

Δ RR reduces the excitotoxic lesion volume after intrastriatal injection. Mice were intrastriatally injected with PBS (n=5), NMDA (n=10), NMDA+ Δ RR (n=5) or NMDA+ Δ PK (n=5). After four hours, mice previously given NMDA were injected with Δ RR (n=5). At day 2 post-injection, striatal tissue sections were incubated with cytochrome c oxidase solution (a) and lesion volume was calculated as described in Materials and Methods. Average total lesion volume $\pm$ s.e.m. is graphed. (b). **p<0.01, *p<0.05 for lesion volume compared to NMDA.

Fig 3.

Δ RR and Δ PK distribution is similar after intrastriatal injection but only Δ RR increases survival. Mice were injected with PBS, NMDA, NMDA+ Δ RR or NMDA+ Δ PK (n=5 for each treatment). Striatal tissue sections collected 2 days later, as in Fig. 2, were stained with ICP10 antibody or thionin (evaluates neuronal cell loss) (a). ICP10+ cells and thionin stained neurons were counted in 5 randomly selected fields in a 3 mm² area surrounding the lesion (at least 250 cells each) and the averages $\pm$ s.e.m. are graphed as percent survival normalized to PBS-treated tissue and % ICP10+ cells, respectively (b). ***p<0.001, **p<0.01, *p<0.05.

Fig 4.

Δ RR decreases the number of TUNEL+ striatal cells after NMDA injection. Mice were intrastrially injected with PBS, NMDA NMDA+ Δ RR or NMDA+ Δ PK. At day 2 post-injection, striatal tissue sections were incubated with TUNEL (FITC-labeled) to label fragmented DNA and mounted in DAPI media to label nuclei. Merged images are shown. Red arrows indicate cells co-labeled with TUNEL+ and DAPI. TUNEL+ cells were counted in 5 randomly selected fields, (at least 250 cells each) and the % positive cells was calculated relative to total (DAPI+) cells. The average %TUNEL+ cells $\pm$ s.e.m. are graphed. ** $p < 0.01$.

Fig5.

Timecourse of striatal apoptosis following NMDA injection. Mice were intrastriatally injected with NMDA or NMDA+ΔRR. At 8, 12, 24 and 48 hours post-injection, striatal tissue sections were incubated with TUNEL (FITC-labeled) to label fragmented DNA and mounted in DAPI media to label nuclei. Merged images are shown. TUNEL+ striatal cells first appear in the striatum 24 h post-injection. Red arrows indicate cells co-labeled with TUNEL+ and DAPI. TUNEL+ cells were counted in 5 randomly selected fields, (at least 250 cells each) and the % positive cells were calculated relative to total (DAPI+) cells. The average %TUNEL+ cells $\pm$ s.e.m. are graphed. *** $p < 0.001$ for NMDA compared to NMDA+ΔRR at 24h and 48h.

Fig 6.

ΔRR decreases NMDA-induced caspase-3 activation.. Mice were intrastrially injected with PBS, NMDA and NMDA+ΔRR (n=5 for each treatment). At day 2 post-injection, striatal tissue sections were incubated with antibodies to NeuN to label neurons and activated caspase-3 (p20 cleavage product), and DAPI. Merged images are shown. Cells positive for activated caspase-3 cells were counted in 5 randomly selected fields in a 3 mm² area surrounding the lesion, (at least 250 cells each) and the % positive cells were calculated relative to total neurons (NeuN +). Averages ± s.e.m are graphed. ***p<0.001.

Fig 7.

Δ RR decreases apoptosis through ERK activation. Mice were intrastrially injected with PBS or Δ RR. At day 2 post-injection, striatal tissue sections were stained with antibodies to pERK and MAP2ab and mounted in DAPI to label nuclei. Cells positive for pERK cells were counted in 5 randomly selected fields in a 3 mm² area surrounding the lesion, (at least 250 cells each) and the % positive cells was calculated relative to total MAP2ab+ neurons. Averages $\pm$ s.e.m. are graphed. *** $p < 0.001$.

Fig 8.

MEK inhibition prevents Δ RR neuroprotection. Mice were intrastriatally injected with NMDA + Δ RR (n=5) or with U0126 (U, n = 5) 30 min prior to NMDA+ Δ RR co-injection. At day 2 post-injection, striatal tissue sections were incubated with cytochrome c oxidase solution and lesion volume was calculated as described in Materials and Methods. Averages $\pm$ s.e.m. are graphed. **p<0.01.

Fig 9.

ΔRR reduces NMDA lesion related behavior. Mice intrastrially injected with PBS, NMDA NMDA+ΔRR, NMDA+ΔPK (n=5 for each treatment) in the ipsilateral or contralateral striatum (Table 2) were challenged with apomorphine at day 2 post-injection. The number of contralateral turns was subtracted from the number of ipsilateral rotations. The average net number of turns for five animals in each treatment group ± s.e.m. is graphed here. P values are compared to PBS/PBS ***p<0.001, **p<0.01, *p<0.05.

Table 1 Description of injections for histochemical, immunohistochemical and TUNEL experiments. ¹

Treatment Group	Group A	Group B	Group C	Group D
Ipsilateral (Right)	PBS	NMDA	NMDA+ΔRR	NMDA+ΔPK
Contralateral (Left)	PBS	PBS	PBS	PBS

¹ In all cases, injection volume was 0.5 μl. The concentration of NMDA was 40.5 nmole/0.5 μl. The concentration of ΔRR was 2500 pfu/0.5 μl. The concentration of ΔPK was 4200 pfu/0.5 μl. The solution flow rate was 0.1 μl/min, total injection time was 5.0 min.

Table 2 Description of injections for the behavioral experiments.¹

Treatment Group	Group A	Group B	Group C	Group D	Group E	Group F	Group G
Ipsilateral (Right)	PBS	NMDA	NMDA + Δ RR	NMDA + Δ PK	NMDA	NMDA	Δ RR
Contralateral (Left)	PBS	PBS	PBS	PBS	NMDA	NMDA + Δ RR	PBS

¹ In all cases, injection volume was 0.5 μ l. The concentration of NMDA was 40.5 nmole/0.5 μ l. The concentration of Δ RR was 2500 pfu/0.5 μ l. The concentration of Δ PK was 4200 pfu/0.5 μ l. The solution flow rate was 0.1 μ l/min, total injection time was 5.0 min.