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

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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, \( \Delta RR \). Intrastriatal \( \Delta RR \) administration (2500 plaque forming units) was nontoxic and did not induce microglial activation five days after injection. Intrastriatal injection of \( \Delta RR \) 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. \( \Delta RR \) treated mice did not develop NMDA-associated behavioral deficits. The data suggests that \( \Delta RR \) 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.

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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 (ΔRR) 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 ΔRR 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 ΔRR 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 ΔPK and ΔRR 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 ΔRR mutant, the R1 domain was replaced with the lacZ gene. In the ΔPK 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, ΔRR and ΔPK, 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, H$_2$O$_2$, 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. TNFα 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 ΔPK and ΔRR, (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; Tunjunga, 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% H$_2$O$_2$. A cannula (33 gauge) containing the solution (0.5 μl) 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 μl/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 μl. The concentration of the HSV-2 and ΔRR viruses was 2500 pfu. The concentration of ΔPK 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 μm 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...

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increasing concentration (50%-100%), covered with cytoseal (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

\[
\text{Total lesion volume} = (\text{area} \times \text{thickness})_1 + (\text{area} \times \text{thickness})_2 + \ldots + (\text{area} \times \text{thickness})_n
\]

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 intrastratial injection, mice were given an apomorphine test to monitor behavioral changes indicative of neuronal function, as previously described (Mason et al., 1978; Schwarz 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

Intrastriatally injected ΔRR 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 ΔRR is growth compromised in neurons (Laing et al., 2006), we wanted to know whether it lost toxicity when delivered intrastriatally. Mice (n=5) were injected with 2500 plaque forming units (pfu) of ΔRR 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 ΔRR lived for the 100 days of observation without displaying any untoward symptoms. The data indicate that ΔRR 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 ΔRR to elicit undesirable inflammatory responses in the brain, brain tissues from animals infected with HSV-2 or ΔRR (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 TNFα 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 ΔRR (Fig. 1). Consistent with a previous report (Laing et al., 2006), the absence of inflammation correlates with the failure of ΔRR to replicate in the brain. Indeed in HSV-2 infected mice but not in ΔRR 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 ΔRR does not replicate nor induce inflammatory responses in intrastriatally injected mice. All of the ΔRR treated mice remained free of disease for at least 100 days.

ΔRR 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 Ca⁺⁺ 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;Kunimatsu et al., 2001). To examine whether ΔRR can inhibit neuronal death caused by excitotoxic injury, mice were given NMDA, alone or NMDA with ΔRR, as described in Materials and Methods. Controls were given NMDA together with ΔPK, 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 ΔRR in the right striatum and PBS in the left striatum and Group D received NMDA combined with ΔPK 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+ΔPK, but it was minimal in striata injected with ΔRR.
NMDA+ΔRR (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±0.002 mm$^3$. The combination of NMDA+ΔPK, caused a lesion of similar size [0.035±0.001 mm$^3$ (p>0.10)]. By contrast, the focal lesion seen in animals given NMDA +ΔRR was significantly (**p<0.01) smaller [0.007±0.002 mm$^3$] (Fig. 2) suggesting that ICP10PK has neuroprotective activity in this model of excitotoxicity. In another series of experiments, ΔRR (2500 pfu) was given 4 hrs after the standard NMDA injection. ΔRR caused a significant reduction (0.018±0.002 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 ΔPK to prevent NMDA induced lesions is not due to improper cellular penetration, because striatal sections from animals given NMDA+ΔPK stained with ICP10 antibody showed the same pattern of immunostaining as those immunostained from animals given NMDA+ΔRR (Fig. 3). Mice given NMDA+ΔRR had 57±2% ICP10+ cells, those given NMDA+ΔPK had 62±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.

ΔRR increases neuronal survival after NMDA administration

To examine whether the decrease in the striatal volume of the NMDA lesion in animals given ΔRR 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+ΔPK treatments, but not with NMDA+ΔRR 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+ΔRR survival was 83.0% (p>0.10 relative to PBS-treated mice). This is in contrast to NMDA or NMDA+ΔPK treated tissue that had only 16.1% and 21.7% cell survival respectively (**p<0.01) (Fig. 3). Together with the observation that ΔRR, but not ΔPK, causes a significant reduction in the NMDA lesion volume these findings indicate that ICP10PK prevents NMDA induced excitotoxic injury.

ΔRR neuroprotection is due to ICP10PK anti-apoptotic activity

To determine whether ΔRR-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+ΔPK had more TUNEL+ cells than those given PBS or NMDA+ΔRR at 2 days post-injection. The percentage of TUNEL+ cells was 41.4±6.5% and 46.8±1.0% for NMDA and NMDA+ΔPK respectively, as compared to 2.3±1.0% and 1.3±0.6% for PBS or NMDA+ΔRR, 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+ΔRR than NMDA alone (Fig. 6). At 48 h post-injection, 81.4±8.1% of striatal neurons from mice treated with NMDA were caspase-3p20+. The addition of ΔRR decreased the percentage of caspase-3p20+ neurons to 15.3±1.3%, similar to that seen in PBS treated tissues (12.2±1.1%; p>0.10). Collectively the data indicate that ICP10PK protects neurons from NMDA-induced apoptosis.

**ΔRR 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 ΔRR 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 ΔRR, but not in PBS treated tissue (Fig. 7) Striata of mice given ΔRR had 83.3±6% pERK+ neurons as compared to mice given PBS which only had 14.2±4.8% pERK+ neurons (***p<0.001). To confirm the role of pERK in protection, animals were given a combination of NMDA+ΔRR together with the MEK inhibitor U0126 and the brain tissues were examined for lesion volume. We conclude that ERK activation is involved in ΔRR-mediated neuroprotection, because the lesion volumes for animals given NMDA+ΔRR together with U0126 were similar to those seen in mice given NMDA alone, as illustrated by cytochrome c histochemistry (Fig. 8).

**ΔRR decreased NMDA-induced behavioral damage**

Having seen that ΔRR 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+ΔRR 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+ΔPK 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+ΔRR 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 ΔRR unilaterally nor with NMDA bilaterally rotated preferentially to either side (p>0.10). At day 5 after mice were given NMDA+ΔRR in the right striatum, they rotated an average of 7.4 times towards the side of injection, which is statistically similar to NMDA+ΔRR mice at 2 days after injection (p>0.10). These data indicate that the co-injection of ΔRR 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).

ΔRR 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 ΔRR is safe. Although intracranial delivery is generally associated with HSV toxicity, ΔRR 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 ΔRR decreased neuronal cell loss by approximately 80%. Protection was due to ICP10PK, because the ICP10PK deleted virus, ΔPK, did not decrease NMDA induced neurodegeneration. ΔPK is a particularly stringent control for ΔRR 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 ΔRR, 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 ΔRR delivered ICP10PK is unprecedented compared to protection imparted by other vector/target gene systems, particularly as observed at very low doses (2500 pfu) of ΔRR. We conclude that protection is due to inhibition of NMDA-induced apoptosis, because both TUNEL and caspase-3 activation were inhibited by ΔRR, but not ΔPK. 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 ΔRR 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 ΔRR with NMDA corrected the rotational behavior induced by apomorphine test by 72.8% as compared to NMDA alone. ΔPK 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.

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Fig 1.
HSV-2 but not ΔRR induces an inflammatory response in the striatum. Mice were injected with 2500 pfu of either HSV-2 or ΔRR in the striata. At day 5 post-injection, striatal-containing tissue sections were stained with antibodies to microglial proteins, TNFα, F4/80 or antibody to CD8+ T cells (a). Duplicate sections of tissues in (a) were stained with VP5 and the neuronal specific antibody βIII tubulin (b). Yellow stain in merged panel indicates VP5 and βIII tubulin co-localization.
Fig 2.
ΔRR reduces the excitotoxic lesion volume after intrastriatal injection. Mice were intrastriatally injected with PBS (n=5), NMDA (n=10), NMDA+ΔRR (n=5) or NMDA+ΔPK (n=5). After four hours, mice previously given NMDA were injected with ΔRR (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 ± s.e.m. is graphed. (b). **p<0.01, *p<0.05 for lesion volume compared to NMDA.
Fig 3.
ΔRR and ΔPK distribution is similar after intrastriatal injection but only ΔRR increases survival. Mice were injected with PBS, NMDA, NMDA+ΔRR or NMDA+ΔPK (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 ± 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.
ΔRR decreases the number of TUNEL+ striatal cells after NMDA injection. Mice were intrastriatally injected with PBS, NMDA NMDA+ΔRR or NMDA+ΔPK. 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 ± s.e.m. are graphed. **p<0.01.

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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 ± 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 intrastriatally 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.
ΔRR decreases apoptosis through ERK activation. Mice were intrastriatally injected with PBS or ΔRR. 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 ± s.e.m. are graphed. ***p<0.001.
Fig 8.
MEK inhibition prevents ΔRR neuroprotection. Mice were intrastriatally injected with NMDA +ΔRR (n=5) or with U0126 (U, n = 5) 30 min prior to NMDA+ΔRR 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 ± s.e.m. are graphed. **p<0.01.
Fig 9.
ΔRR reduces NMDA lesion related behavior. Mice intrastriatally 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

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ipsilateral (Right)</td>
<td>PBS</td>
<td>NMDA</td>
<td>NMDA+ΔRR</td>
<td>NMDA+ΔPK</td>
</tr>
<tr>
<td>Contralateral (Left)</td>
<td>PBS</td>
<td>PBS</td>
<td>PBS</td>
<td>PBS</td>
</tr>
</tbody>
</table>

¹ In all cases, injection volume was 0.5 μl. The concentration of NMDA was 40.5 n mole/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.  

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
<th>Group E</th>
<th>Group F</th>
<th>Group G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ipsilateral (Right)</td>
<td>PBS</td>
<td>NMDA</td>
<td>NMDA +ΔRR</td>
<td>NMDA</td>
<td>NMDA</td>
<td>NMDA</td>
<td>ΔRR</td>
</tr>
<tr>
<td>Contralateral (Left)</td>
<td>PBS</td>
<td>PBS</td>
<td>PBS</td>
<td>PBS</td>
<td>NMDA</td>
<td>NMDA</td>
<td>PBS</td>
</tr>
</tbody>
</table>

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.