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Caspase-3 activation is required for reovirus-induced encephalitis *in vivo*

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Reovirus infection of neonatal mice provides a classic experimental system for understanding the molecular pathogenesis of central nervous system (CNS) viral infection. CNS tissue injury, caused by many human neurotropic viruses, including herpes viruses and West Nile virus, is associated with caspase-dependent apoptotic neuronal cell death. We have previously shown that reovirus-induced CNS tissue injury results from apoptosis and is associated with activation of both death-receptor and mitochondrial apoptotic pathways culminating in the activation of the downstream effector caspase, caspase-3. In order to directly investigate the role of caspase-3 in virus-induced neuronal death and CNS tissue injury during encephalitis, we have compared the pathogenesis of reovirus CNS infection in mice lacking the caspase-3 gene (*caspase-3 (−/−)*) to syngeneic wild-type mice. Prior studies of antiapoptotic treatments for reovirus-infected mice have indicated that protection from reovirus-induced neuronal injury can occur without altering the viral titer in the brains of infected mice. We now show that reovirus infection of *caspase-3 (−/−)* mice was associated with dramatic reduction in severity of CNS tissue injury, decreased viral antigen and titer in the brain, and enhanced survival of infected mice. Following intracerebral inoculation, the authors also show that virus spread from the brain to the eyes in reovirus-infected *caspase-3 (−/−)* mice, indicating that viral spread was intact in these mice. Examination of brains of long-term survivors of reovirus infection among *caspase-3 (−/−)* mice showed that these mice eventually clear their CNS viral infection, and do not manifest residual or delayed CNS tissue injury. *Journal of NeuroVirology* (2010) **16,** 306–317.

Keywords: apoptosis; caspase; encephalitis; neuron; reovirus

Introduction

Viral infections of the central nervous system (CNS) cause significant worldwide morbidity and mortality. Therapy is limited to selected viruses and when available is of suboptimal efficacy. For example, herpes simplex virus (HSV) is the most common cause of sporadic viral encephalitis in the United States; yet, despite treatment with acyclovir, the mortality rate is 15% and only 14% of the survivors return to normal function (Raschilas *et al,* 2002). West Nile virus (WNV) is the most common cause of epidemic viral encephalitis in the United States (Sejvar *et al,* 2003) and Japanese encephalitis virus (JEV), a closely related flavivirus, is the most important cause of acute viral encephalitis in the world; yet, neither disease has effective therapies of proven benefit. JEV alone causes approximately 50,000 infections and 15,000 deaths per year, with only 41% of survivors returning to normal neurologic function after infection (Ooi *et al,* 2008; Solomon *et al,* 2000). The development of more effective therapies for these and other forms of viral encephalitis has

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been hampered by a lack of understanding of the exact signaling pathways involved in virus-induced neuronal cell death.

WNV, HSV, and other viral infections of the CNS cause neuronal death by activating caspase-3 (DeBiasi *et al,* 2002; Griffin, 2005; Michaelis *et al,* 2007; Samuel *et al,* 2007). Studies of human brain tissue from patients infected with HSV and cytomegalovirus (CMV) show that apoptosis plays an important role in mediating neuronal death and CNS tissue injury (DeBiasi *et al,* 2002). *In vitro* studies of viral pathogenesis have shown that caspase-3–dependent neuronal cell death is important in multiple models of neurotropic viral infection, including infections caused by WNV, JEV, and reovirus (Clarke *et al,* 2009; Kleinschmidt *et al,* 2007; Richardson-Burns *et al,* 2002; Tsao *et al,* 2008). *In vitro* studies have also shown that inhibition of neuronal cell death following viral infection can occur independently of any parallel effect on viral replication, as both pharmacological and genetically mediated inhibition of caspases can mediate neuroprotection in the absence of significant effects on viral replication (Kleinschmidt *et al,* 2007; Richardson-Burns *et al,* 2002; Samuel *et al,* 2007). However, no studies to date have evaluated the long-term effects of caspase-3 inhibition on viral replication and spread in the infected host.

Several studies have evaluated the role of apoptosis inhibition as a potential therapeutic strategy for viral encephalitis. In murine models of viral encephalitis, studies have shown moderate improvement in survival using nonspecific inhibitors of apoptosis such as minocycline (Michaelis *et al,* 2007; Richardson-Burns and Tyler, 2005) and specific pathway inhibitors related to apoptotic signaling such as c-Jun N-terminal kinase (JNK) inhibitors (Beckham *et al,* 2007). These studies show that a small percentage of mice survive; yet, viral replication in the CNS is not affected by inhibitors of apoptosis in these studies. However, ongoing replication of virus in the CNS of mice treated with apoptosis inhibitors is unlikely to be compatible with prolonged disease-free survival, and it remains critical to fully understand the effect of inhibition of the primary executioner caspase (caspase-3) on viral replication, tissue injury, and survival.

In order to fully understand the role of caspase activation in models of viral encephalitis, other executioner caspases must be evaluated as well. Prior studies of Fas-induced apoptosis in mammalian cells have shown that alternative caspases can be activated in the absence of caspase-3 (Zheng *et al,* 2000). It is also well established that caspase-3 is only one of a group of executioner caspases (Slee *et al,* 1999b). For example, caspase-6 and -7 are important executioner caspases that play a primary role in caspase execution instead of or along with caspase-3 (Eguchi *et al,* 2009; Walsh *et al,* 2008). Additionally, caspase-9 is an initiator caspase that can be activated by the mitochondrial signaling pathway of apoptosis and may be involved in activation of caspase-3, -6, and -7 (Slee *et al,* 1999b). Indeed, activation of the mitochondrial signaling pathway and caspase-9 is associated with reovirus-induced apoptosis (Richardson-Burns *et al,* 2002). Therefore, it is critical to understand the effect of caspase-3 inhibition on other initiator caspases (Slee *et al,* 1999a).

We used the reovirus model of viral encephalitis to further evaluate the role of caspase-3 activation in viral induced tissue injury and viral replication *in vivo.* Reovirus infection in the CNS is a classical model of viral encephalitis in which neonatal mice are injected with serotype 3 reovirus by intracranial injection, resulting in characteristic encephalitis with injury, caspase-3 activation, and viral antigen colocalizing in areas including the cingulate cortex, CA2–3 region of the hippocampus, and the thalamus (Richardson-Burns *et al,* 2002; Richardson-Burns and Tyler, 2004). We utilized this experimental model to show, for the first time, that caspase-3 activation is critical for reovirus-induced viral injury in the CNS as well as maintenance of a productive viral infection over time in the brains of infected mice. We also show that other effector caspases do not play a significant role in reovirus-induced apoptosis in the CNS of *caspase-3 (−/−)* mice and that viral spread occurs in reovirus-infected *caspase-3 (−/−)* mice.

Results

Caspase-3 is required for reovirus-induced injury in the CNS

We first determined whether caspase-3 was required for reovirus-induced CNS tissue injury. Two-day-old mice born to *caspase-3 (−/+)* breeder pairs were infected with 10^3 plaque-forming units (PFU) of reovirus serotype 3 Dearing (T3D) by intracranial (i.c.) injection. Intracranial inoculation of phosphate-buffered saline (PBS) was used as a vehicle control. At day 8 post infection, pups were sacrificed and brains were formalin-fixed and paraffin-embedded. Sections were stained with hematoxylin and eosin (H&E; Figure 1), then analyzed using a previously validated rater-blinded scoring system to quantitatively evaluate the extent and severity of tissue injury. T3D-infected *caspase-3 (−/−)* mice had markedly reduced CNS injury compared to T3D-infected *caspase-3 (+/−)* and *caspase-3 (+/+)* controls (Table 1).

Viral antigen expression and viral titers are decreased in the brains of caspase-3 *(−/−) mice*

Having shown that tissue injury was reduced in reovirus-infected *caspase-3 (−/−)* mice, we determined whether caspase-3 gene deletion had an effect on viral replication within the CNS. Mice were infected as described above and sacrificed for analysis at day 8 post infection. Virus-infected cells

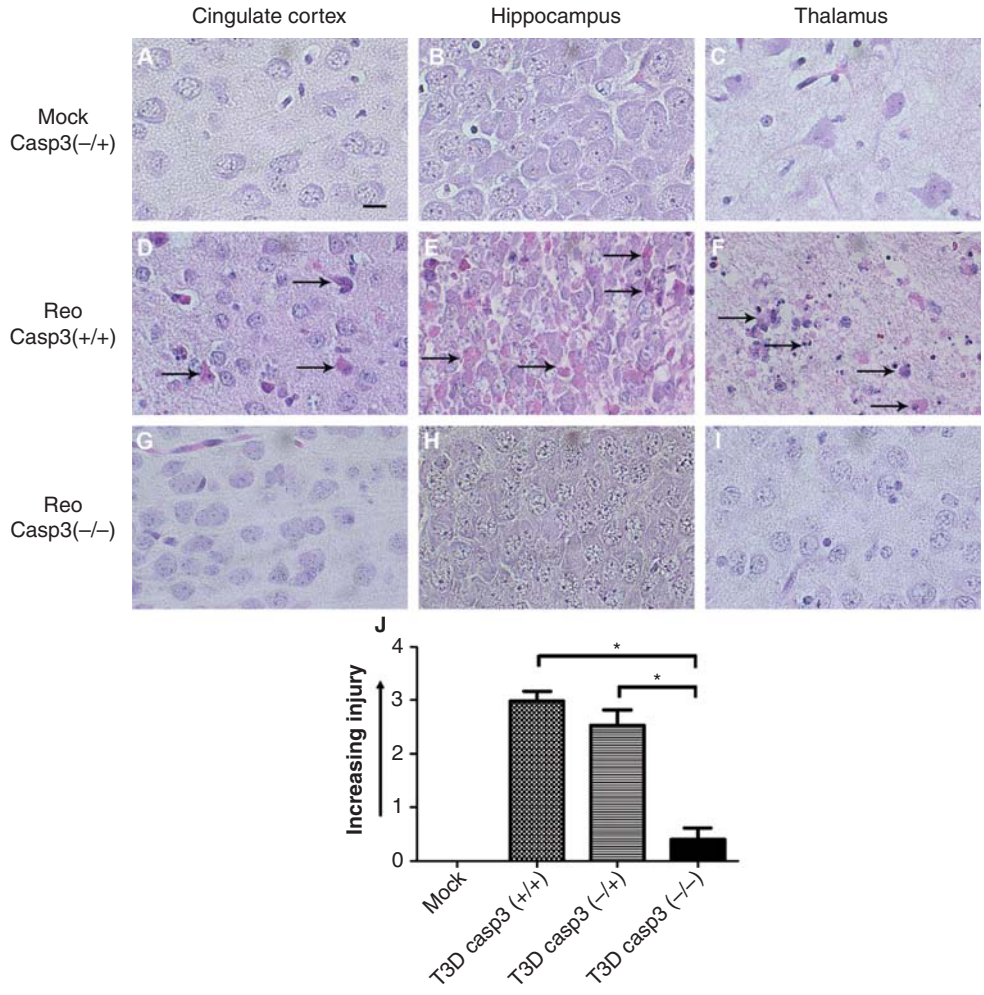

Figure 1 Caspase-3 is required for reovirus-induced injury in the CNS. Two-day-old pups from *caspase3 (−/+)* breeder pairs were infected with T3D (10^3 PFU, i.c.) or mock infected, sacrificed at day 8 post infection, formalin-fixed, and paraffin-embedded. Compared to (**A–C**) mock-infected *caspase3 (−/+)* mice (n = 5), H&E-stained histological brain sections of (**D–F**) T3D-infected *caspase3 (+/+)* mice exhibit increased tissue injury characterized by acidophilic staining as well as cellular/nuclear condensation and fragmentation (*black arrows*). (**G–I**) T3D-infected *caspase3 (−/−)* mice exhibit little injury and resemble mock-infected animals. (**J**) Rater-blinded histological scoring (0 = no injury to 4 = severe, confluent areas of injury) revealed significantly decreased injury scores in T3D-infected *caspase3 (−/−)* mice (n = 10) compared to T3D-infected *caspase3 (−/+)* (n = 9) and *caspase3 (+/+)* mice (n = 20). Mice from six different litters were used. Images are 400× original magnification. *P < .0001.

were identified using a monoclonal antibody that detected the T3D σ3 major viral outer capsid protein (fluorescein isothiocyanate [FITC]/green secondary antibody) (Figure 2). T3D-infected *caspase-3 (−/−)* mice had only rare T3D antigen–positive cells (Figure 2J–L). By comparison, both T3D-infected *caspase-3 (+/+)* and *caspase3 (−/+)* mice had abundant viral antigen associated with robust caspase-3 activation (cy3/red secondary antibody) (Figure 2D–I).

Table 1 Brain injury histology scores following reovirus infection

Mouse genotype	Mean histology score	SEM	Number of mice (n)
Caspase-3 (−/−)	0.40	0.66	10
Caspase-3 (+/−)	2.53	0.88	9
Caspase-3 (+/+)	2.98	0.81	20

Note. P < .0001 for caspase-3 (−/−) versus caspase-3 (+/−) or (+/+); P = ns for caspase-3 (−/+) versus caspase-3 (+/+).

In order to quantitatively evaluate the reduction in number of infected cells in the brains of T3D-infected *caspase-3 (−/−)* mice, we performed quantitative cell counts of infected cells in defined brain regions. Because CNS tissue injury (Figure 1) was similar between caspase-3 wild-type and heterozygote mice, histological sections of caspase-3 wild-type and knockout mice were rater-blinded and numbers of reovirus σ3 antigen–positive cells were

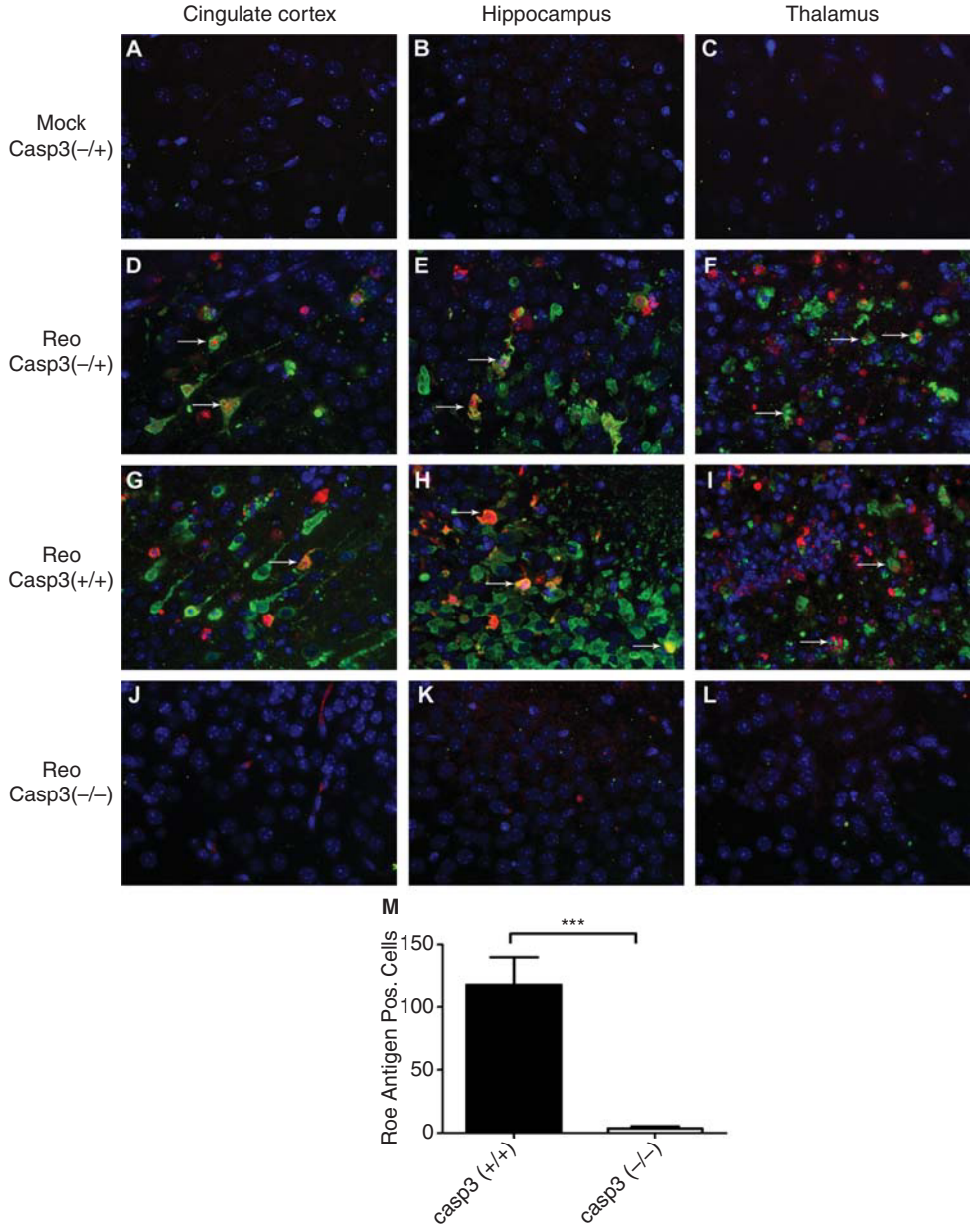

Figure 2 Caspase-3 (−/−) mice exhibit decreased viral antigen in the brain. Two-day-old pups from *caspase3 (−/+)* breeder pairs were infected with T3D (10^3 PFU, i.c.) or mock infected, sacrificed at day 8 post infection, formalin-fixed, and paraffin-embedded. Immunohistochemistry sections labeled with primary antibodies to cleaved caspase-3 (cy3/red conjugated polyclonal anti-rabbit IgG) and anti-σ3 reovirus antigen (FITC/green-conjugated monoclonal anti-mouse IgG) revealed extensive reovirus antigen and caspase3 activation in T3D-infected (**D–F**) *caspase3 (−/+)* mice and (**G–I**) *caspase3 (+/+)* mice compared to (**A–C**) mock-infected *caspase3 (−/+)* mice. (**J–L**) T3D-infected *caspase3 (−/−)* mice exhibited reduced reovirus antigen and caspase-3 activation compared to wild-type controls. (**M**) The total number of reovirus antigen–positive cells in the cingulate cortex, hippocampus, and thalamus per 400× field were significantly decreased in T3D-infected *caspase3 (−/−)* mice compared to littermate controls. White arrows indicate colocalization of cleaved caspase-3 and reovirus antigen. ***P = .0002.

determined by taking the average number of infected cells from three high-power fields in each of three anatomic regions that are typically affected during reovirus encephalitis (the cingulate cortex, the CA3 region of the hippocampus, and the thalamus). The sum of the number of infected cells in these three regions was used as an index of the extent of viral CNS infection. There was a 97% reduction (31-fold) in the number of infected cells in T3D-infected *caspase-3 (−/−)* mice compared to T3D-infected *caspase-3 (+/+)* controls (Figure 2M, Table 2).

Table 2 Viral antigen cell counts in reovirus-infected brains

Mouse genotype	Mean number viral antigen (+) cells per HPF	SEM	Number of mice (*n*)
Caspase-3 (−/−)	3.8	1.4	15
Caspase-3 (+/+)	117	22.8	16

$P = .0002$; HPF, high power field (400× magnification).

To further quantify the reduction in viral growth in brains with the caspase-3 gene deletion, we determined the viral titer from whole brains of infected mice. Two-day-old mice from litters of *caspase-3 (−/+)* breeder pairs were infected with T3D (10^3 PFU, i.c.). At days 4, 6, and 8 post infection, mice were sacrificed and brains were processed for viral titer analysis. There was no significant difference in titers between groups at day 4 or 6 post infection. However, by day 8 post infection viral titers were 99% lower (81-fold) in *caspase-3 (−/−)* mice ($4.3 \times 10^7 \pm 3.8 \times 10^7$) compared to littermate wild-type and heterozygote infected controls ($3.5 \times 10^9 \pm 1 \times 10^9$) ($P = .004$; Figure 3). Using a one-way analysis of variance (ANOVA) with a Tukey's multiple comparison test, brain viral titers did not significantly increase in *caspase-3 (−/−)* mice from day 6 to day 8 (mean difference -5×10^6 PFU/ml); however, viral titers significantly increased in wild-type mice from day 6 to day 8 post infection (mean difference $+2.8 \times 10^9$ PFU/ml; $P < .05$).

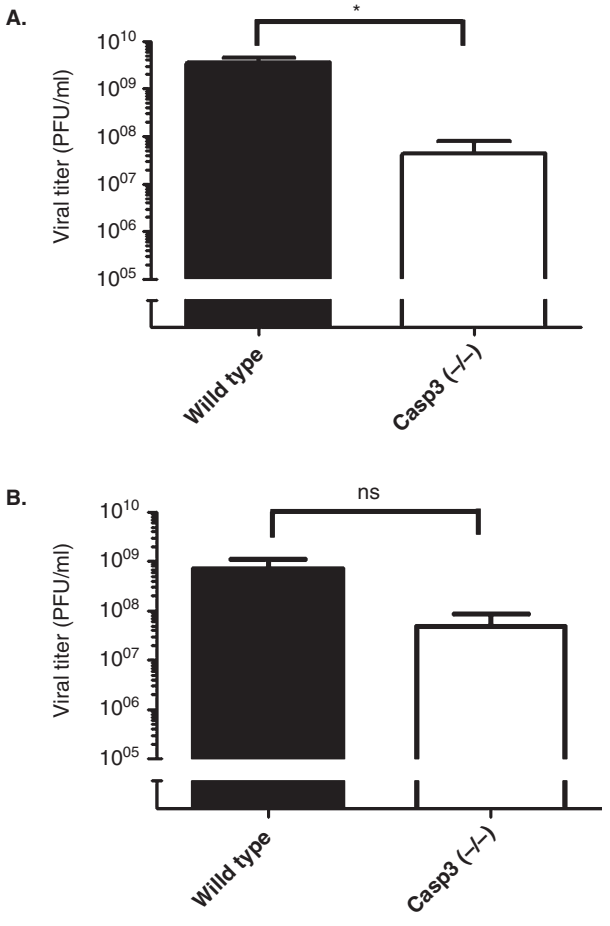

Figure 3 Caspase-3 activation is important for efficient viral infection *in vivo.* Two-day-old pups from *caspase3 (−/+)* breeder pairs were infected with T3D (10^3 PFU, i.c.) or mock infected with PBS, sacrificed at specified days post infection, and processed for whole-brain viral titer assay. (**A**) At day 8 post infection, T3D-infected *caspase-3 (−/−)* mice ($n = 8$) exhibit a 100-fold decrease in viral titer compared to T3D-infected *caspase3 (+/+)* mice and *caspase3 (−/+)* mice ($n = 14$; $^*P = .004$). (**B**) At day 6 post infection, viral titers did not significantly differ when comparing T3D-infected *caspase3 (−/−)* mice ($n = 7$) to *caspase3 (+/+)* mice and *caspase3 (−/+)* mice ($n = 11$; $P = .14$, ns). Mice from six different litters were used.

Caspase-3 activation is not required for viral spread in CNS

Our earlier results show that caspase-3 activation is required for efficient long-term infection in the CNS. Viral replication in the brains of *caspase-3 (−/−)* mice is not significantly different from wild-type mice at day 6 post infection; however, after day 6, viral titers do not significantly change in reovirus-infected *caspase-3 (−/−)* mice. Therefore, we determined whether reduced viral titer in the brains of *caspase-3 (−/−)* mice could reflect diminished efficiency of viral spread in the CNS. We have previously shown that following intracranial infection, reovirus spreads from the brain to the neural cells (retinal ganglion and bipolar cells) of the retina (Tyler *et al,* 1985). In order to determine the role of caspase-3 in viral spread in the CNS, we evaluated viral titers in the eyes of reovirus-infected mice. Two-day-old pups from *caspase-3 (−/+)* breeder pairs were infected with T3D (10^3 PFU, i.c.) as above. At day 8 post infection, mice were euthanized and brains and eyes were harvested for viral titer. Eyes from each mouse were homogenized in pairs in 150 μl of cold PBS and processed for viral titer assay. At day 8 post infection, reovirus was able to spread from the brain to the eyes in *caspase-3 (−/−)* mice but, as in the brain, viral titers in the eyes were significantly lower in T3D-infected *caspase-3 (−/−)* mice ($n = 5$; $3.5 \times 10^4 \pm 3.1 \times 10^4$) compared to T3D-infected caspase-3 wild-type mice ($n = 9$; $2.7 \times 10^7 \pm 7.7 \times 10^6$; $P = .008$; Figure 4).

Alternative executioner caspases are not activated in the brains of reovirus-infected caspase-3 *(−/−) mice*

Caspase-6 and -7 are alternative executioner caspases in other models of apoptosis, and caspase-9 is associated with activation of alternative executioner caspases (Inoue *et al,* 2009; Slee *et al,* 1999b). Given that T3D-infected *caspase-3 (−/−)* mice exhibited measurable but decreased viral titers and decreased viral spread in the CNS, we determined whether

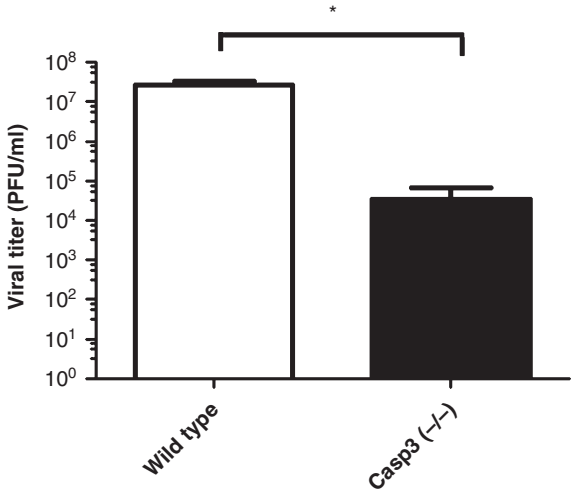

Figure 4 Activation of caspase3 contributes to viral spread *in vivo*. Brain tissue used for viral titer assays was collected in conjunction with paired eye samples for viral titer assay as a measure of viral spread from the brain to another part of the CNS, the eye. At day 8 post infection, viral titers from the eyes of T3D-infected *caspase3 (−/−)* mice (n = 5) were significantly reduced compared to litter mate controls (n = 9; *P = .008).

deletion of caspase-3 resulted in compensatory up-regulation and activation of other effector caspases. Two-day-old pups from *caspase-3 (−/+)* breeder pairs were infected with T3D (10^3 PFU, i.c.) and sacrificed at day 8 post infection. We first wanted to confirm that reovirus-induced caspase-3 activation was absent in *caspase-3 (−/−)* mice. As expected, caspase-3 was not activated in the brains of reovirus-infected *caspase-3 (−/−)* mice (Figure 5A). Immunoblots of whole-brain lysates revealed a significant decrease in cleaved caspase-7 (Asp198; Cell Signaling) in T3D-infected *caspase-3 (−/−)* mice compared to T3D-infected caspase-3 wild-type mice (P = .0007; Figure 5B and C). Additional immunoblots for caspase-9 revealed that deletion of caspase-3 had no significant effect on the expression of caspase-9 cleavage products (37-, 39-kDa) following reovirus-infection in the brain (Figure 5D and E). Immunoblots for cleaved caspase-6 (Asp162; Cell Signaling) in the same treatment groups revealed no evidence of cleavage products in any of the infected animals (data not shown). Thus, the deletion of the key effector caspase, caspase-3, is not associated with compensatory up-regulation or activation of other effector or initiator caspases. However, caspase-7 activation is dependent on caspase-3 activation in the CNS of reovirus-infected mice.

Caspase-3 gene deletion in mice prolongs survival following lethal viral challenge

Our data indicated that caspase-3 was important for viral injury and maintenance of viral infection in the CNS. Having shown that caspase-3 deletion resulted in decreased CNS tissue injury and poor infection efficiency, we determined whether these effects resulted in enhanced survival of infected mice. Two-day-old pups from breeder pairs consisting of a *caspase-3 (−/−)* male and a *caspase-3 (−/+)* female were infected with T3D (10^2 PFU, i.c.). Mice were sacrificed when moribund, tail snips obtained for genotyping, and organs harvested for histology and viral titer assays. Genotypes were matched to mice post mortem so that all decisions to sacrifice moribund mice were made without knowledge of their genotype. T3D-infected *caspase-3 (−/−)* mice had markedly enhanced survival compared to their *caspase-3 (−/+)* littermates. Eight of nine (89%) of the T3D-infected *caspase-3 (−/−)* mice survived as compared to 1/12 (8%) of T3D-infected *caspase-3 (−/+)* mice (P = .0008; Figure 6).

Infected caspase-3 knockout mice clear viral infection from the CNS

Next, we determined the status of viral replication in T3D-infected *caspase-3 (−/−)* mice that survived long term. Previous studies with minocycline suggested that some neuroprotective treatments might simply delay rather than prevent virus-induced CNS tissue injury (Richardson-Burns and Tyler, 2005), suggesting the possibility that inhibiting apoptosis might simply allow viral injury to occur through alternate mechanisms (e.g., caspase-independent apoptosis or necrosis). However, T3D-infected *caspase-3 (−/−)* mice surviving long term (30 days post infection [p.i.]) exhibited little or no CNS tissue injury (Figure 7D–F) compared to reovirus-infected caspase-3 heterozygotes (Figure 7A–C), indicating that deletion of caspase-3 did not simply delay virus-induced CNS tissue injury but in fact prevented it. Consecutive sections were immunostained using a polyclonal antibody to T3D antigens as above. Reovirus antigen was abundant in moribund (days 9 to 11 p.i.), T3D-infected *caspase-3 (−/+)* mice (Figure 7G–I); however, there was no detectable reovirus antigen in T3D-infected *caspase-3 (−/−)* mice at day 20 or later post infection (Figure 7J–L). We also quantified viral titer from brains and eyes of T3D-infected *caspase-3 (−/−)* mice that survived long term (>20 days). Five (n = 6) brains harvested from T3D-infected *caspase-3 (−/−)* mice at day 30 post infection exhibited undetectable viral titers (data not shown). Seven (n = 9) of the T3D-infected *caspase-3 (−/−)* mice exhibited no detectable viral titer in the eyes; yet, all of the T3D-infected *caspase-3 (−/+)* mice had detectable viral titer in the eyes when moribund ($9.98 \times 10^7 \pm 3.31 \times 10^7$; $P < .0001$; Figure 7M). Interestingly, two of the nine infected *caspase-3 (−/−)* mice had detectable viral titers in the eye even at 30 days p.i. (10^3 PFU/eye). To our knowledge, this is the first time that infectious virus has been shown to persist this long in the CNS of immunocompetent reovirus-infected mice.

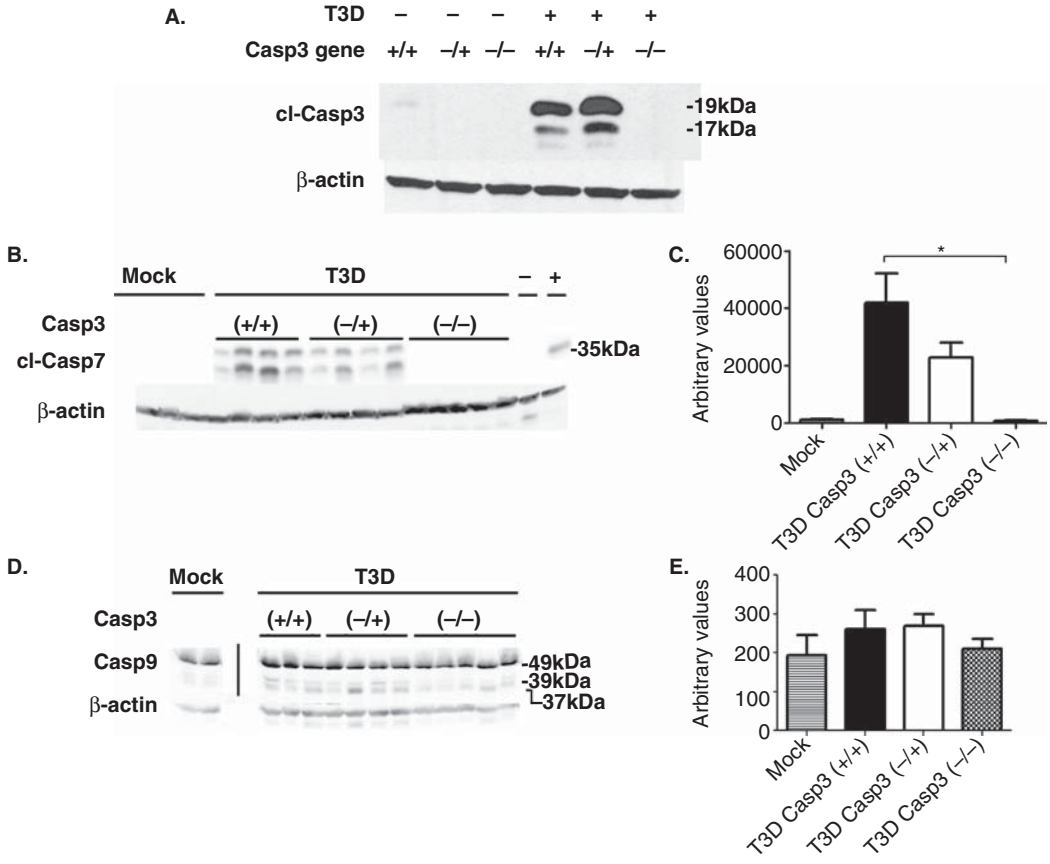

Figure 5 Alternative executioner caspases are not activated in the brains of reovirus-infected *caspase-3 (−/−)* mice. Two-day-old pups from *caspase-3 (−/+)* breeder pairs were infected with T3D (10^3 PFU, i.c.) or mock infected, sacrificed at specified days post infection, and whole-brain lysates prepared for Western blot analysis. Whole-brain lysates were resolved on 10% polyacrilamide gel, transferred to PVDF membrane, and labeled with primary antibodies to cleaved caspase-3, cleaved caspase-7 (cl-casp7) and caspase-9. (**A**) Western blot analysis of whole-brain lysates show that caspase-3 cleavage occurs in T3D-infected *caspase-3 (+/+)* mice and *caspase-3 (−/+)* mice but is eliminated in *caspase-3 (−/−)* mice. (**B**) Western blot analysis of whole-brain lysates from T3D-infected *caspase-3 (−/−)* mice show no evidence of caspase-7 cleavage (35kDa) compared to littermate controls. +, positive control; −, negative control. (C) T3D-infected *caspase-3 (−/−)* mice exhibit a significant reduction in band density of caspase-7 cleavage product compared to littermate controls ($n = 4$ per group; $^*P = .0007$). (**D**) There is no significant change in caspase-9 cleavage products (39- and 37-kDa) in *caspase-3 (−/−)* mice compared to littermate controls. (**E**) Densitometry values of caspase-9 cleavage products ($n = 4$ per group).

Discussion

We examined the role of caspase-3 activation in reovirus-induced acute encephalitis *in vivo.* We show that caspase-3 is not only activated but actually required for reovirus-induced tissue injury and disease in the CNS of infected mice and that deletion of caspase-3 results in near-complete resistance to reovirus-induced encephalitis, as shown by the prolonged survival of reovirus-infected *caspase-3 (−/−)* mice.

Following viral infection of the brain, *caspase-3 (−/−)* mice exhibited decreased CNS tissue injury that was associated with a dramatic decrease in the amount of detectable viral antigen compared to wild-type and heterozygote caspase-3 mice. In reovirus-infected wild-type mice and caspase-3 heterozygotes, activated caspase-3 colocalized with reovirus antigen within cells of the CNS of infected mice. We also observed caspase-3–positive cells in the absence of reovirus antigen. These observations are consistent with previous data indicating that reovirus infection and caspase-3 activation occur primarily in neurons; however, some degree of bystander apoptosis likely occurs in neighboring cells as well (Beckham *et al,* 2007; Richardson-Burns *et al,* 2002; Richardson-Burns and Tyler, 2004, 2005). Cells that were reovirus antigen positive in the absence of caspase-3 activation likely represent a subset of neurons that have not expressed significant levels of cleaved caspase-3, as previous studies indicate that neuronal viral infection precedes terminal expression of activated caspase-3 (Richardson-Burns *et al,* 2002).

The effects of viral-induced caspase-3 on CNS tissue injury appear to be due, in large part, to the requirement of caspase-3 for efficient long-term viral infection in the CNS. In the absence of caspase-3, virus is able to replicate to near normal levels early

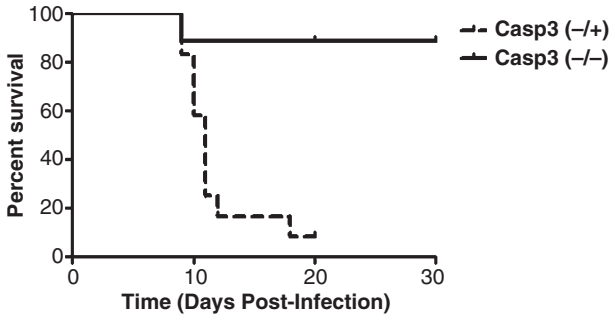

Figure 6 Caspase-3 gene deletion in mice prolongs survival following lethal viral challenge in the CNS. Two-day-old pups from three litters of breeder pairs using *caspase-3 (−/+)* females and *caspase-3 (−/−)* males were infected with T3D (10^2 PFU, i.c.) and sacrificed when moribund. Decisions to sacrifice animals due to clinical symptoms of encephalitis were blinded to the genotype of the mice. T3D-infected *caspase-3 (−/−)* mice (n = 9) exhibit prolonged survival compared to T3D-infected littermate controls (n = 12; P = .0008). One T3D-infected *caspase-3 (−/−)* mouse was sacrificed at day 9 post infection due to illness, all other T3D-infected *caspase-3 (−/−)* mice lived to the endpoint analysis. An endpoint analysis of 20 days was used for the first experiment and an endpoint analysis of 30 days was used for replicate litters 2 and 3.

in infection. However, mean viral titers in reovirus-infected *caspase-3 (−/−)* mice showed no significant change between day 6 post infection (mean viral titer 4.78×10^7 PFU/ml) and day 8 post infection (mean viral titer 4.3×10^7 PFU/ml). This implied that caspase-3 is required for efficient reovirus replication within neurons or is required for efficient spread of reovirus to other neurons. In order to understand the mechanism for these changes *in vivo,* we evaluated viral spread by quantifying viral titers in a distal portion of the CNS, the eye. Virus is able to spread to the eye in caspase-3 knockout mice and is able to persist in a small subset of surviving mice. Further studies evaluating the role of caspase-3 activation on viral replication are complicated by limited techniques to model the *in vivo* time course of infection in an *in vitro* model. Further improvements of *in vitro* models of CNS infection will allow improved understanding of the complex interactions between caspase-3 activation and reovirus replication.

The exact mechanism of caspase-3–dependent reovirus infection efficiency is not known. Viral-induced apoptosis may increase the amount of virus released from initially infected cells that is available for uptake, and/or infection, and subsequent trans-neuronal transport via neighboring cells. In this model apoptosis is required for efficient release of virus from infected cells, and this release in turn allows for spread to neighboring neurons. Alternatively, it is possible that caspase-3 is inhibiting the efficiency of viral spread through an alternate mechanism that is independent of its effects on apoptosis. Caspase-3 is known to interact with postsynaptic densities in Alzheimer's patients resulting in degradation of the synaptic terminal during disease progression (Louneva *et al,* 2008), and synaptic transmission is modulated by the presence of the BCL-2 family protein, BCL-xL (Jonas *et al,* 2003). Moreover, electrophysiological measures in hippocampal slice neurons show that high synaptic localization of caspase-3 results in neuroplastic processes in synapses (Kudryashova *et al,* 2009). These studies suggest the possibility that inhibition of caspase-3 could also inhibit the efficiency of viral spread through effects on synaptic terminals or synaptic transmission that may be independent of its role in apoptotic signaling pathways. Further studies are needed to understand the interactions of host-cell proapoptotic proteins, viral particles, and the synaptic terminal.

Because caspase-3 is the main executioner caspase associated with reovirus-induced apoptosis in the CNS (Richardson-Burns *et al,* 2002), the murine caspase-3 knockout model of infection offered an opportunity to evaluate other executioner caspases that may be involved in reovirus-induced apoptosis *in vivo.* Previous studies of neuronal cell death and degeneration in the CNS have shown that caspase-6 and caspase-7 are important executioner caspases (Gafni *et al,* 2009; Graham *et al,* 2006). Our studies show no evidence of caspase-6 activation in the brains of reovirus-infected mice despite robust activation in positive controls (data not shown). On the other hand, caspase-7 is activated in the brains of wild-type and caspase-3 heterozygote reovirus-infected mice, and caspase-3 deletion results in a significant decrease in reovirus-induced caspase-7 activation. These studies show that reovirus infection in the CNS of mice activates caspase-7 and activation is caspase-3 dependent. The specific role of activated caspase-7 in reovirus-infected neurons is not known and is the subject of ongoing evaluation. Prior studies have also shown that caspase-9 is activated late in reovirus-infected primary neuronal cultures (Richardson-Burns *et al,* 2002). Our studies show no significant change in reovirus-induced casapase-9 cleavage between treatment groups. However, there is a trend toward increased caspase-9 cleavage in reovirus-infected wild-type and heterozygote caspase-3 mice compared to mock-infected mice and a trend towards decreased caspase-9 cleavage in the brains of reovirus-infected caspase-3 knockout mice. These results largely support prior studies showing that caspase-9 activation is not likely to play an important role in reovirus-induced apoptosis in the CNS.

We also evaluated the role of caspase-3 activation on survival in acute viral encephalitis. Based on our studies of caspase-3 activation, tissue injury, viral antigen, and viral titer, there was no evidence of an intermediate phenotype in reovirus-infected heterozygote caspase-3 mice. Therefore, we evaluated survival between mixed litters of caspase-3 homozygote

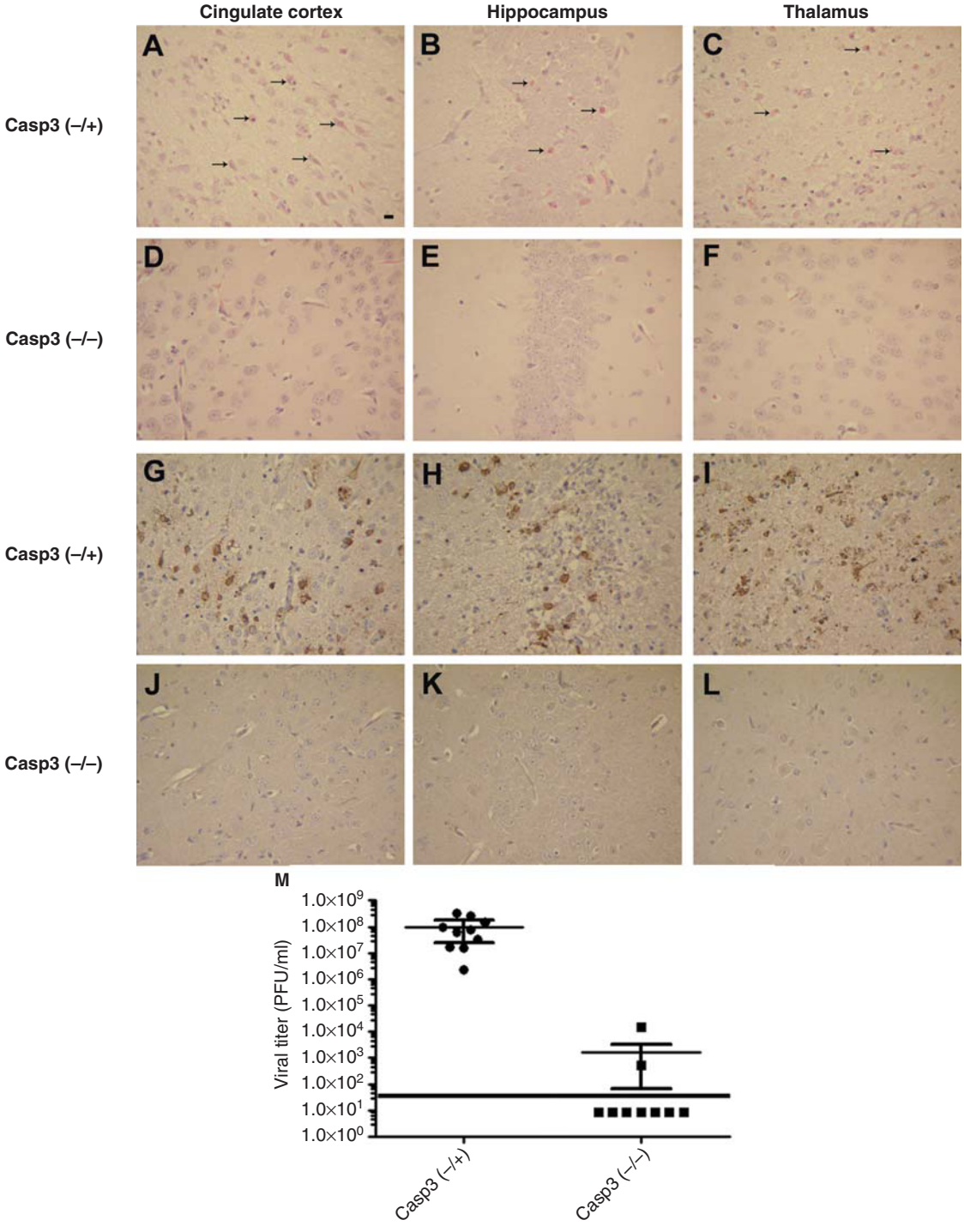

Figure 7 Reovirus-infected *caspase-3 (−/−)* mice eliminate viral infection in the CNS. Two-day-old pups from three litters of breeder pairs of *caspase-3 (−/+)* females and *caspase-3 (−/−)* males were infected with T3D (10^2 PFU, i.c.) and sacrificed when moribund. Brains were formalin fixed for histological analysis and pairs of eyes were processed for viral titer. (**A–C**) T3D-infected *caspase-3 (−/+)* mice were sacrificed when moribund at a mean of 11 days post infection and H&E staining shows evidence of injury in the cingulate cortex, hippocampus, and thalamus. Arrows indicate injured cells. (**D–F**) T3D-infected *caspase-3 (−/−)* littermates exhibited little evidence of injury up to 30 days post infection. Immunohistochemistry analysis using a polyclonal antibody to T3D antigens revealed extensive antigen was present in (**G–I**) T3D-infected *caspase-3 (−/+)* littermates that were sacrificed at a mean of 11 days post infection. (**J–L**) There was no evidence of reovirus antigen–positive cells in T3D-infected *caspase-3 (−/−)* mice at day 30 post infection. (**M**) Viral titer of eye pairs revealed that seven of nine T3D-infected *caspase-3 (−/−)* mice had cleared virus from the CNS at endpoint analysis with titers less than the limit of detection (50 PFU/ml; *horizontal black line*); however, T3D-infected *caspase-3 (−/+)* mice exhibited mean eye titers of 9.98×10^7 PFU/ml at a mean of 11 days post infection ($P < .0001$). Images shown at 400× original magnification.

and heterozygote mice, sacrificed the mice for evaluation, and genotyped the mice after they were sacrificed. *Caspase-3 (−/−)* mice infected with 10^2 PFU (~10× LD_{50}) exhibited significant survival benefit compared to heterozygote littermate controls. In reovirus-infected *caspase-3 (−/−)* mice that survive an otherwise lethal viral infection, there is no evidence of histological injury and the virus is cleared from the CNS in the majority of mice. Mice show near complete protection from lethal viral encephalitis after selective knockout of a host cell gene, caspase-3. Future studies aimed at discovering highly potent, selective inhibitors of caspase-3 that can cross the blood-brain barrier may offer significant benefit as a therapeutic agent for viral encephalitis.

Although the majority of reovirus-infected *caspase-3 (−/−)* mice that survived infection cleared virus from the CNS, two of nine *caspase-3 (−/−)* mice maintained detectable viral titers in the eye at 30 days post infection. Prior studies with reovirus isolates from persistently infected L-cell cultures have shown that viral persistence is linked to mutations in the S1 gene, which encodes the σ1 viral attachment protein, and the S4 gene, which encodes the σ3 viral outercapsid protein (Baer and Dermody, 1997; Wetzel *et al,* 1997). Previous studies have shown that reovirus isolates from persistently infected L-cell cultures (PI viruses) can persist in the CNS of mice for up to 25 days post inoculation but were eventually cleared (Morrison *et al,* 1993). This is the first demonstration of persistence of wild-type reovirus infection in the CNS of immunocompetent mice, and although this phenomenon was unusual and did not seem to be associated with tissue injury, it does raise the possibility that anti-apoptotic treatments may enhance the possibility of chronic or persistent infections if unaccompanied by specific antiviral therapy.

Materials and methods

Cell lines and virus stocks

L929 mouse fibroblasts (ATCC CL1) were used for viral titer assays and were maintained in 2× 199 medium supplemented with 10% heat-inactivated fetal bovine serum and 4 mM L-glutamine. Reovirus serotype 3 Dearing (T3D) is a laboratory stock that was plaque purified and passaged twice in L929 cells to generate working stocks (Tyler, 1998).

In vivo experiments

Caspase-3 gene deficient mice were a kind gift from Richard Flavell (Yale University) and were backcrossed on C57B6 background (Kuida *et al,* 1996). Two-day-old pups were inoculated by intracerebral injection (i.c.) with 10^3 PFU or 10^2 PFU of reovirus (T3D) in a 10-μl volume of phosphate-buffered saline as described previously (Beckham *et al,* 2007, 2009; Richardson-Burns *et al,* 2002). All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and performed in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited animal facility.

Western blotting

Whole-brain lysates were prepared as previously described (Goody *et al,* 2005). Lysates were loaded onto 10% or 15% polyacrylamide/tricine gel (Hoefer Pharmacia Biotech, San Francisco, CA), separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to 0.22μm polyvinylidene fluoride (PVDF) membrane (Millipore). Immunoblotting was performed as described previously (Poggioli *et al,* 2000). Membranes were probed with antibodies to cleaved (Asp 175) caspase-3 (Cell Signaling; 1:1000), cleaved (Asp198) caspase-7 (Cell Signaling: 1:1000), cleaved (Asp162) caspase-6 (Cell Signaling; 1:1000), caspase-9 (Cell Signaling; 1:1000), and β-actin (Jackson, 1:5,000). Secondary antibodies used for Western blots included mouse and rabbit specific horseradish peroxidase–conjugated anti-immunoglobulin G (IgG) (Calbiochem; 1:5000). Following antibody incubations, membranes were developed using enhanced chemiluminescence (ECL) reagent (Pierce). Western blots were imaged and densitometry values obtained using the Alpha Innotec FlourChemQ imaging system. All densitometry values were normalized to the corresponding β-actin band density to correct for protein loading.

Histological studies

Brain tissue was formalin-fixed, paraffin-embedded, and cut into 5 μm thick coronal sections. Histology sections were stained with hematoxylin and eosin (H&E) and scored for degree of tissue injury, by an observer blinded to the experimental condition, based on a previously published neuropathological injury scoring system (Thoresen *et al,* 1996). Consecutive sections were deparaffinized in xylene and rehydrated in sequential ethanol washes. Tissue was subjected to antigen retrieval (Vector Labs, Antigen unmasking solution), permeabilization, and blocking (10% normal goat serum in PBS plus 0.3% Triton), followed by incubation in primary antibodies: monoclonal reovirus σ3-reovirus antibody (4F2; 1:100), polyclonal reovirus antibody, and cleaved (Asp 175) caspase-3 (Cell Signaling; 1:100). Sections were incubated in secondary antibodies (Cy3-conjugated AffiniPure goat anti-rabbit IgG (Jackson ImmunoResearch; 1:300) and Alexaflour 488–conjugated goat anti-mouse IgG (Invitrogen; 1:200) for experiments using fluorescent antibodies. Nuclei were stained (1 μg/ml; Hoechst 33342; Invitrogen) and were mounted (VectorShield; Vector Laboratories) prior to visualization of immunofluorescence using a Zeiss Axioplan 2 digital

deconvolution microscope with a Cooke Sensicam 12-bit camera. Positive staining cells were counted by evaluating three to five contiguous high-power fields (400×) in specific areas of the brain (cingulate cortex, CA2 and CA3 regions of the hippocampus, and the lateral nucleus of the thalamus) by a rater blinded to the experimental condition. Immunohistochemistry experiments using biotinylated goat anti-rabbit IgG (Invitrogen; 1:100) as a secondary antibody were incubated with ABC streptavidin reagent (Vector Laboratories) followed by staining with diaminobenzadine (Invitrogen) and counterstaining with hematoxylin (Dako).

Viral titer assays

L929 mouse fibroblasts were used for viral titer assays and were maintained in 2× 199 medium (10% heat-inactivated fetal bovine serum [FBS] and 4 mM L-glutamine). Both whole brain and eyes from mice were harvested for viral titer assays. Whole brain and paired eyes were homogenized fresh in cold PBS (1 ml and 150 μl, respectively). Homogenates underwent three freeze-thaw cycles followed by sonication as previously described (Beckham *et al,* 2007). Serial dilutions of the homogenate were prepared in gel saline and viral titer determined by plaque assays as previously described (Debiasi *et al,* 1999).

Statistical analysis

All statistical analyses were performed using Prism Software (GraphPad Software, San Diego, CA). Statistical comparisons were made using a two-tailed, unpaired *t* test with Welch correction, one-way ANOVA with Tukey's posttest analysis, and the Mann-Whitney *U* test for nonparametric comparison of viral titer values that included values below the limits of detection for the assay. Log-rank test was used for analysis of survival curves.

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

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