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Reovirus-induced neuronal apoptosis is mediated by caspase 3 and is associated with the activation of death receptors

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Reovirus infection of the central nervous system (CNS) is an important experimental system for understanding the pathogenesis of neurotropic viral infection. Infection of neonatal mice with T3 reoviruses causes lethal encephalitis in which injury results from virus-induced apoptosis. We now show that this apoptosis *in vivo* is associated with activation of caspase 3, and use neuroblastoma and primary neuronal cultures to identify the cellular pathways involved. Reovirus-induced apoptosis in neuronal cultures is initiated by activation of the tumor necrosis factor (TNF) receptor superfamily death receptors and is inhibited by treatment with soluble death receptors (DRs). The DR-associated initiator caspase, caspase 8, is activated following infection, this activation is inhibited by a cell-permeable peptide inhibitor (IETD-CHO). In contrast to our previous findings in non-neuronal cell lines, reovirus-induced neuronal apoptosis is not accompanied by significant release of cytochrome *c* from the mitochondria or with caspase 9 activation following infection. This suggests that in neuronal cells, unlike their non-neuronal counterparts, the mitochondria-mediated apoptotic pathway associated with cytochrome *c* release and caspase 9 activation does not play a significant role in augmenting reovirus-induced apoptosis. Consistent with these results, peptide caspase inhibitors show a hierarchy of efficacy in inhibiting reovirus-induced apoptosis, with inhibitors of caspase 3 > caspase 8 >>> caspase 9. These studies provide a comprehensive profile of the pattern of virus-induced apoptotic pathway activation in neuronal culture. *Journal of NeuroVirology* (2002) **8,** 365–380.

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Viruses cause disease by injuring or killing discrete populations of cells in host organs. Cell loss or damage is particularly detrimental in tissues with limited ability to regenerate, such as the mature central nervous system (CNS). In cell culture, many neurotropic viruses have the capacity to kill target cells, including neurons, by inducing apoptosis. A small group of viruses, including human immunodeficiency virus (HIV) (Petito *et al*, 1999), Dengue (Despres *et al*, 1996), Sindbis (Nava *et al*, 1998; Jan and Griffin, 1999), Theiler's (Jelachich and Lipton, 2001), rabies (Jackson and Rossiter, 1997a), and reovirus (Oberhaus *et al*, 1997), have been shown to induce apoptosis in experimental or natural models

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of encephalitis *in vivo*. Despite the importance of apoptosis as a mechanism of virus-induced cell death, little is known about the nature of apoptotic signaling pathways activated during neurotropic viral infection. Identifying mechanisms of virus-induced apoptosis is critical not only to understanding the pathogenesis of CNS viral infections but may also be relevant to neurodegenerative and other neurological diseases in which apoptosis contributes to neuronal loss (Allsopp and Fazakerley, 2000; Honig and Rosenberg, 2000; Mattson, 2000; Raghupathi *et al*, 2000; Yuan and Yankner, 2000).

Apoptosis is a distinct form of cell death in which affected cells undergo characteristic morphological and biochemical changes, including cytoplasmic shrinkage, condensation and fragmentation of nuclear chromatin, membrane alterations, and changes in gene and protein expression (Reed, 2000; Hengartner, 2000). Most forms of apoptosis are associated with sequential activation of cysteine-aspartyl proteases (caspases) by extracellular and/or intracellular stimuli, ultimately causing cleavage of cellular substrates, including laminins, poly-ADP-ribose polymerase (PARP), and DNA (Nunez *et al*, 1998; Earnshaw *et al*, 1999). Caspase activation has been implicated in neuronal apoptosis induced by diverse stimuli, including potassium deprivation (D'Mello *et al*, 2000), N-methyl-D-aspartate (NMDA) excitotoxicity (Ma *et al*, 1998; Budd and Lipton, 1999), and ischemic insults (Chien *et al*, 2000; Rentsch *et al*, 2001; Velier *et al*, 1999).

Experimental infection with mammalian reoviruses has provided a classic model for studying the pathogenesis of viral infection *in vivo* and virus-cell interactions *in vitro* (Tyler, 2001). Reoviruses induce apoptosis in a variety of cells in culture, including fibroblasts, kidney cells, cardiac myocytes, and cells derived from a variety of human cancers. Our laboratory has shown that reovirus-induced apoptosis of undifferentiated epithelial and cancer cells is initiated by death receptor (DR) activation and involves sequential activation of caspase cascades, starting with the DR-associated initiator caspase, caspase 8 (Clarke *et al*, 2000, 2001). Caspase 8 activation leads to the cleavage of the Bcl-2 family protein Bid, which translocates to the mitochondrion where it facilitates release of apoptosis-inducing factors including cytochrome *c* (Kominsky, 2002). Once in the cytoplasm, cytochrome *c* forms part of the apoptosome complex that leads to the activation of caspase 9 (Li *et al*, 1997). Caspases 8 and 9 contribute to the activation of effector caspases, notably caspase 3, which act on cellular substrates, resulting in the morphological hallmarks of apoptosis.

Although reovirus can induce apoptosis in neurons *in vivo* (Oberhaus *et al*, 1997), studies of the cellular pathways involved in reovirus-induced apoptosis have been limited to non-neuronal cell lines. We now show that reovirus-induced apoptosis in neurons *in vivo* involves activation of the effector caspase, caspase 3, and use both neuroblastoma-derived cell lines and primary cultures of terminally differentiated neurons to investigate cellular pathways leading to this activation.

Results

Reovirus-induced apoptosis in the neonatal mouse brain is associated with caspase 3 activation

We have previously shown that type 3 reovirus strain Dearing (T3D)-induced encephalitis in neonatal mice is associated with apoptosis in the CNS (Oberhaus *et al*, 1997). To better understand the mechanisms by which this occurs, we looked for activation of caspase 3, a key effector caspase that is activated in many forms of apoptosis. Activated caspase 3 was detected by immunohistochemistry in mouse brain tissue at day 7 post infection and correlated with distribution of viral antigen and Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP Nick-End Labeling (TUNEL) staining on serial tissue sections. We found that activated caspase 3 staining colocalized to regions of mouse brain also containing viral antigen and apoptotic cells (TUNEL positive), including the cingulate cortex, hippocampus, and thalamus (Figure 1). Having shown that reovirus-induced neuronal apoptosis involved caspase activation *in vivo*, we next used neuronal cultures to investigate the cellular pathways involved *in vitro*.

Reovirus induces apoptosis in mouse neuroblastoma-derived cells and mouse primary cortical cultures

Major morphological features of apoptosis include modification of the plasma membrane, compaction and margination of nuclear chromatin, and oligonucleosomal DNA fragmentation. We looked for evidence of these apoptotic features in reovirus-infected mouse neuroblastoma-derived cell line NB41a3 (NB4) and in primary mouse cortical cultures derived from embryonic (E20) mice (MCC). Translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane is an early morphological feature of apoptosis that can be detected by annexin V staining (Martin *et al*, 1995). We detected PS translocation (annexin V labeling) in T3D-infected NB4 as early as 6 h following infection. By 10 h post infection, there was a significant ($P < .001$) increase in annexin V labeling in T3D-infected cells (53% ± 3%) as compared to mock (15% ± 1%) (Figure 2**A**). Propidium iodide (PI), which can only enter cells with compromised plasma membranes, was used as a second label in the annexin V assay, staining late apoptotic cells (also annexin V positive) and necrotic cells (only PI positive). Cells were treated with 30% ethanol for 1 h prior to the annexin V assay to generate necrotic cells as a negative control. In contrast to virus-infected cells, these necrotic (only PI-positive) cells did not show annexin V labeling (5% ± 1%).

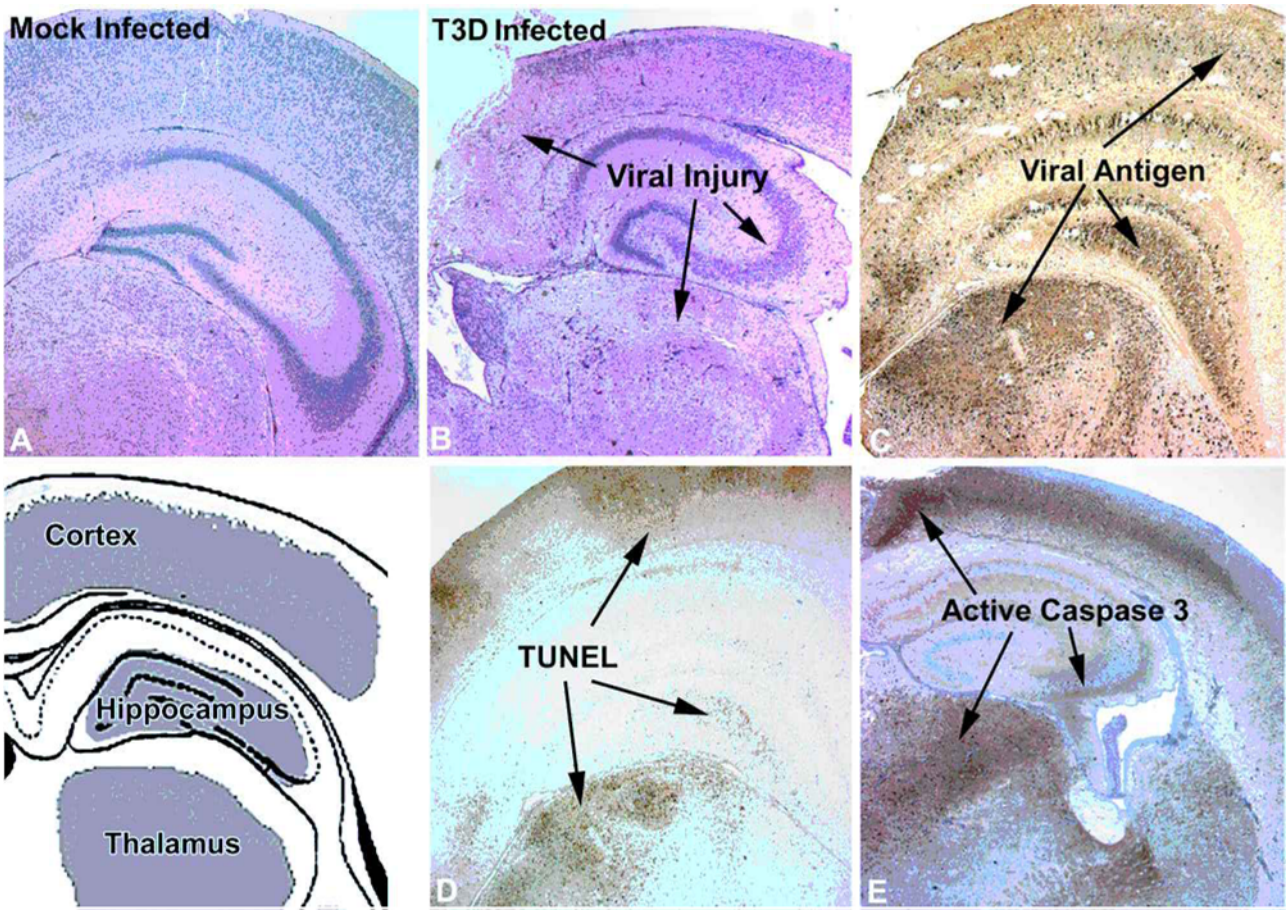

Figure 1 Coronal sections of neonatal mouse brain 7 days after intracranial inoculation of 10,000 PFU of strain T3D reovirus or mock inoculation (eight mice per treatment group). Hematoxylin and eosin–stained tissue reveals marked destruction of brain tissue in the T3D infected brain (**B**) as compared to the uninfected brain (**A**). Using immunohistochemistry, serial sections of T3D-infected brain tissue were stained for viral antigen (**C**), TUNEL/apoptosis marker (**D**), and active caspase 3 (**E**). Staining for viral antigen, TUNEL, and active caspase 3 was undetectable in the mock-infected brains (data not shown).

These results were confirmed by finding changes in nuclear structure characteristic of apoptosis, including chromatin condensation and margination in cells stained by the fluorescent nuclear dye, Hoechst 33342 (Figure 2**B**). Proapoptotic signal transduction leads to activation of a caspase-activated endonuclease that cleaves DNA at internucleosomal points (located at ~180-bp intervals) and exposes 3′ OH ends normally bound by nuclear proteins. These two features of apoptosis were detected by DNA ladder assay and TUNEL (Gavrieli *et al*, 1992), respectively. Apoptotic DNA fragmentation detected by DNA ladder assay was present in T3D-infected NB4 and MCC (Figure 2**C**). There was significantly greater levels of free 3′ OH ends detected by TUNEL in T3D-infected NB4 and MCC as compared to mock infected cells at 48 h post infection (Figure 2**D**). As an additional control, we also infected the neuronal cultures with type 1 reovirus strain Lang (T1L), a reovirus strain that induces significantly less apoptosis than T3D in non-neuronal cell cultures (Tyler *et al*, 1995). The percentage of TUNEL-positive cells in T1L-infected neuronal cultures was similar to that in mock-infected cultures (see Figure 2**D**).

Reovirus-induced neuronal apoptosis requires viral binding at host cell receptors

In order to insure that apoptosis seen in reovirus-infected neuronal cultures occurred as a direct consequence of viral binding, we tested whether a monoclonal antibody (9BG5, hereafter "anti-T3D") specific for the T3D σ1 viral attachment protein would inhibit virus-induced apoptosis. Anti-T3D has been shown to protect neonatal mice from T3D-induced neuronal injury (Virgin *et al*, 1988; Tyler *et al*, 1989), and inhibits apoptosis in non-neuronal cultures *in vitro* (Tyler *et al*, 1995; Oberhaus *et al*, 1997). T3D inoculum was preincubated (1 h) with either anti-T3D or an isotype-matched control antibody specific for the T1L σ1 protein (5C6) (Virgin *et al*, 1988; Tyler *et al*, 1989) prior to infection of neuronal cultures. Nuclear morphology assays and TUNEL were performed at 48 h post infection to

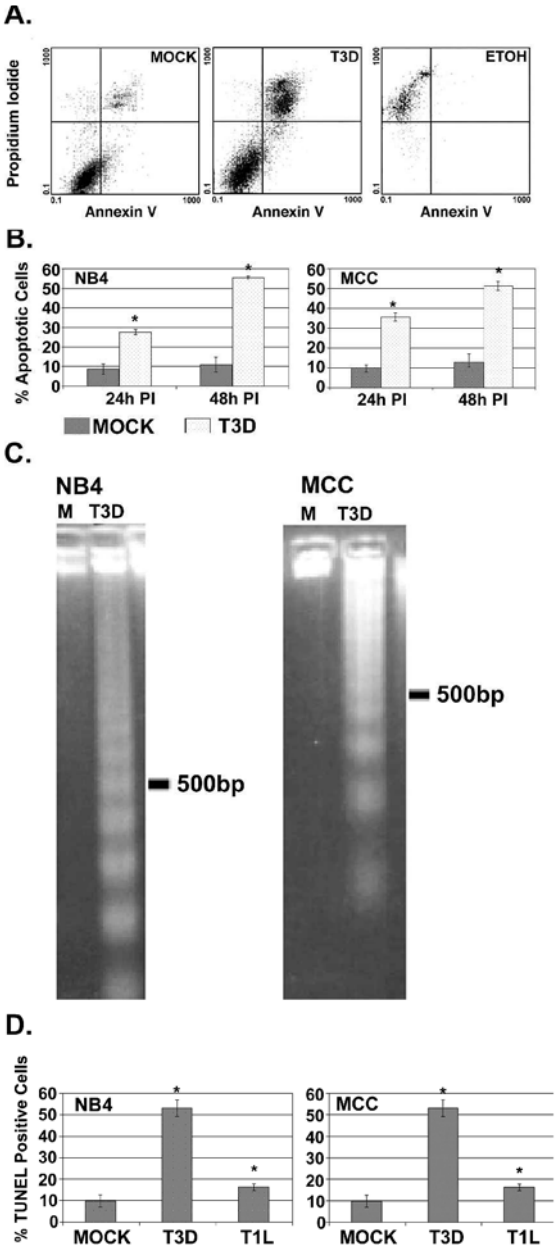

Figure 2 (**A**) Scatter plots representing flow cytometric analysis of mock (M)-infected, T3D-infected, and ethanol (ETOH)-treated mouse NB41a3 neuroblastoma cells (NB4), stained with annexin V–FITC and propidium iodide at 10 h post infection generated following flow cytometry. Annexin V–FITC fluorescence intensity is on the *x* axis (FL1 channel) and propidium iodide fluorescence is on the *y* axis (FL3 channel). (**B**) Percent apoptosis in M- and T3D-infected NB4 and mouse primary cortical cultures (MCC) at 24 and 48 h post infection. Data are presented as mean ± standard error of three to four experiments with 300 cells counted per condition per experiment, $^{*}P < .001$ for M versus T3D at both 24 and 48 h post infection by Tukey-Kramer multiple-comparison test. PI = post infection. (**C**) By 48 h PI, T3D infection–induced oligonucleosomal DNA fragmentation ("ladders") in NB4 and MCC. (**D**) The percentage of TUNEL-positive NB4 and MCC following M infection, T3D infection, and T1L infection. Data are presented as mean ± standard error of six experiments with 300 cells counted per condition per experiment, $^{*}P < .001$ for M versus T3D and T3D versus T1L by Tukey-Kramer multiple-comparison test.

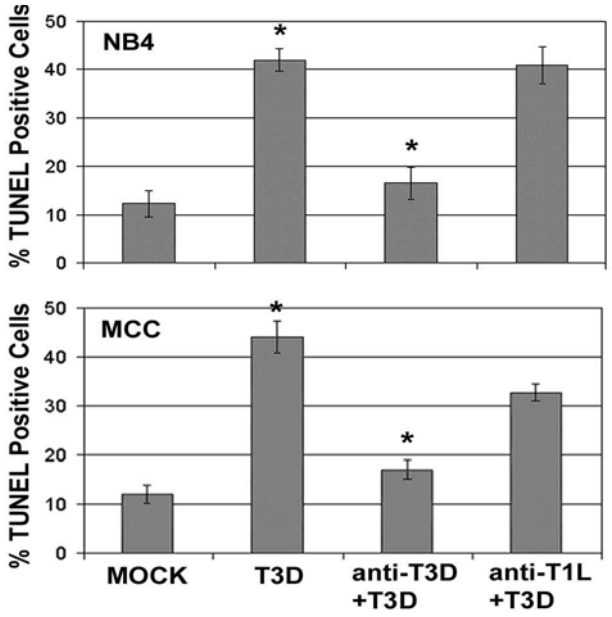

Figure 3 Effects of incubating T3D with monoclonal antibody (anti-T3D) specific for the T3D viral attachment protein or with an isotype-matched control antibody (anti-T1L) specific for the T1L viral attachment protein prior to infection of mouse neuroblastoma cells (NB4) and mouse primary cortical cultures (MCC). These are compared with T3D- and mock-infected NB4 and MCC. Data are percentage TUNEL-positive cells in each treatment. Data are presented as mean ± standard error of three experiments with 300 cells counted per condition per experiment, $^{*}P < .001$ for anti-T3D + T3D versus anti-T1L + T3D and anti-T3D + T3D versus T3D by Tukey-Kramer multiple-comparison test.

determine percentage of apoptotic cells present in each sample. Treatment of T3D with anti-T3D but not with control anti-T1L significantly inhibited reovirus-induced apoptosis in both types of neuronal culture (Figure 3).

Reovirus induces both direct and bystander apoptosis

Reovirus encephalitis is associated with both direct and "bystander" apoptosis as indicated by many cells in T3D-infected mouse cortex dual labeled for reovirus antigen and apoptosis and fewer cells positive for apoptosis marker alone (Oberhaus *et al*, 1997) (Figure 4**A**). In order to determine if both these mechanisms of apoptosis also occurred in neuronal cultures, we performed dual labeling by immunocytochemistry and TUNEL to determine whether cells infected with T3D were also undergoing apoptosis. We found that although the great majority of cells were undergoing apoptosis, there was a subset of apoptotic cells that were uninfected (antigen negative) but located in proximity to virus-infected cells. Thus, although most apoptotic cells in reovirus-infected neuronal cultures were virus-infected, some cells appeared to be undergoing "bystander" apoptosis (Table 1; Figure 4**B**, **C**).

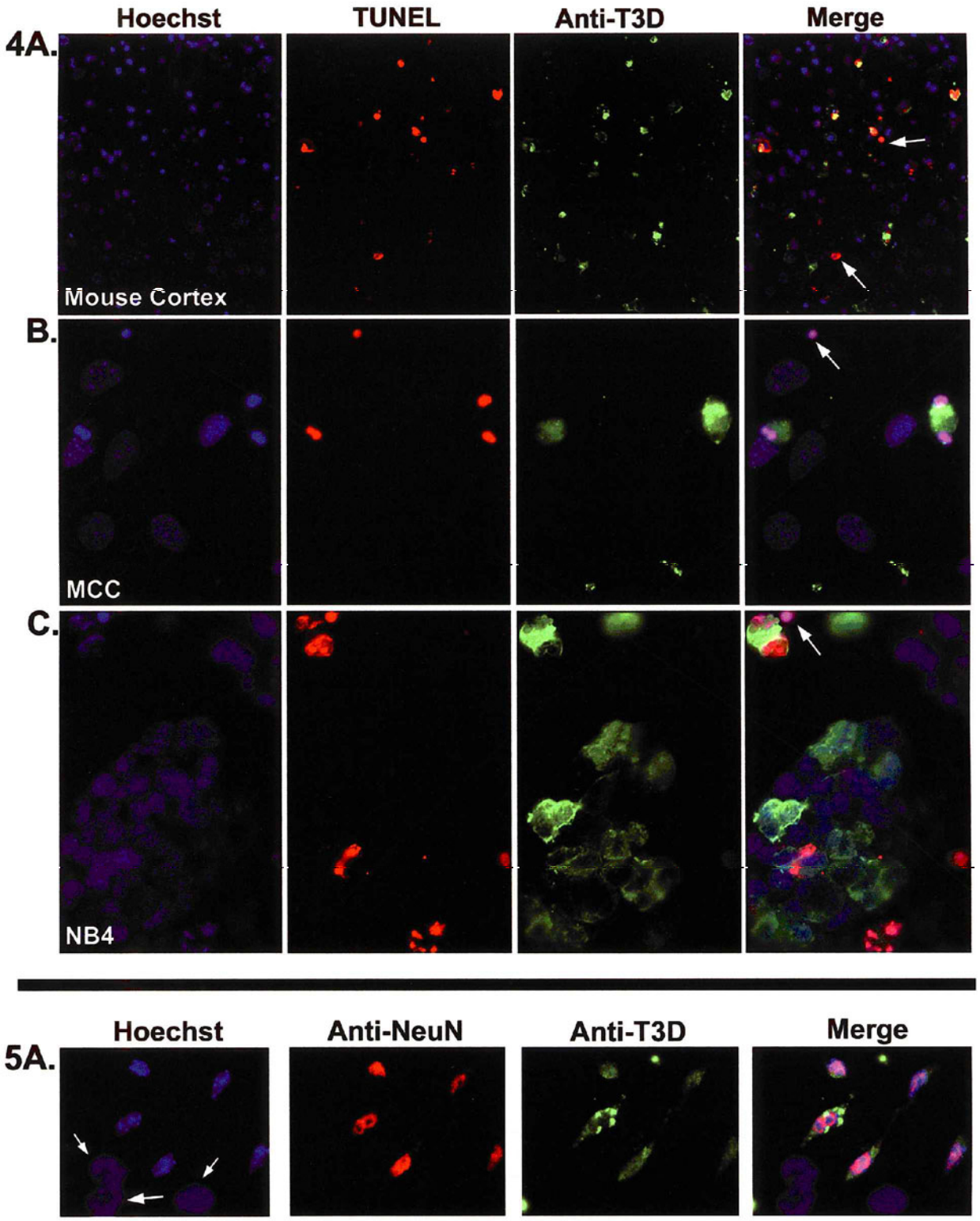

Figure 4–5A

Figure 4 (**A**) A coronal section of T3D-infected neonatal mouse cortex was triple labeled with Hoechst 33342 (nuclear dye), TUNEL-Cy3 (apoptosis marker), and immunohistochemistry for viral antigen–FITC (anti-T3D) to determine whether cells positive for viral antigen were also undergoing apoptosis. Colocalization of viral antigen and apoptosis was also detected in (**B**) mouse primary cortical cultures (MCC) and (**C**) mouse neuroblastoma cells (NB4) by triple labeling with Hoechst 33342, TUNEL-Cy3, and anti-T3D–FITC. Apoptotic cells that are not T3D-infected may be undergoing "bystander" apoptosis (indicated by white arrows). **Figure 5**(**A**) T3D infected mouse primary cortical cultures (MCC) were triple labeled with Hoechst 33342, immunocytochemistry for neuron nuclear protein-Cy3 (anti-NeuN), and immunocytochemistry for viral antigen-FITC (anti-T3D) to determine if the T3D infected cells were neurons. The primary cortical cultures are heterogeneous with approximately 70% neurons and 30% glia. Neurons (Cy3-red) are positive for NeuN and T3D (FITC-green). The large nuclei (Hoechst-blue) of glia are neither NeuN positive nor T3D positive (indicated by white arrows).

Table 1 Percentage distribution of apoptotic (TUNEL +) and infected (antigen +) cells in neuronal cultures

	Antigen + *TUNEL+*	*Antigen +* *TUNEL −*	*Antigen −* *TUNEL +*	*Antigen −* *TUNEL −*
NB4*				
24 h PI**	7% (± 2%)	52% (± 2%)	11% (± 1%)	31% (± 3%)
48 h PI	33% (± 2%)	38% (± 3%)	13% (± 2%)	16% (± 3%)
MCC***				
24 h PI	6% (± 1%)	36% (± 4%)	11% (± 2%)	47% (± 4%)
48 h PI	38% (± 2%)	26% (± 3%)	12% (± 3%)	25% (± 3%)

*NB4 = NB41a3; mouse neuroblastoma cell line.
**PI = post infection.
***MCC = mouse primary cortical cultures.

T3 reovirus grows in mouse neuroblastoma-derived cell lines and mouse primary cortical cultures and infects neurons in mixed primary cortical cultures

To determine if T3D infects neurons *in vitro* as seen *in vivo*, we used dual lable immunocytocchemistry to detect neurons (anti-neuron nuclear protein; NeuN) and reovirus infected cells (anti-T3D) in mixed (70% neurons, 30% glia) primary cortical cultures. Reovirus antigen was only detectable in cells that were also expressing neuron nuclear protein, indicating that following infection of mouse cortical cultures *in vitro*, T3D infection is restricted to neurons (Figure 5**A**). Although we have previously shown that reovirus-induced apoptosis in non-neuronal cells does not require viral replication, and that differences in the capacity of reovirus strains to induce apoptosis is not correlated with their replication efficiency in target cells (Rodgers *et al*, 1997; Tyler *et al*, 1995), we wished to determine whether this was true in neuronal cultures. We assessed viral yield by plaque assay following infection with T3D in NB4 and MCC. T3D grows in both types of neuronal culture. T1L grows to a significantly lower titer in NB4 as compared to T3D infected cells and does not grow in MCC (Figure 5**B**).

Reovirus-induced apoptosis in neuronal cultures is mediated by caspase 3 and caspase inhibition protects neurons from apoptosis

Many proapoptotic signaling pathways involve activation of caspase cascades that originate with pathway-specific initiator caspases (e.g., caspases 8 and 9) and converge on common downstream effector caspases (e.g., caspase 3). Having shown that caspase 3 was activated in the brains of T3D-infected neonatal mice, we wished to determine whether it was also activated in T3D-infected neuronal cultures. We first looked for caspase 3 activation in NB4 using a fluorogenic caspase substrate assay. Cell lysate was mixed with DEVD-AFC, a fluorogenic substrate that binds active caspase 3, allowing its

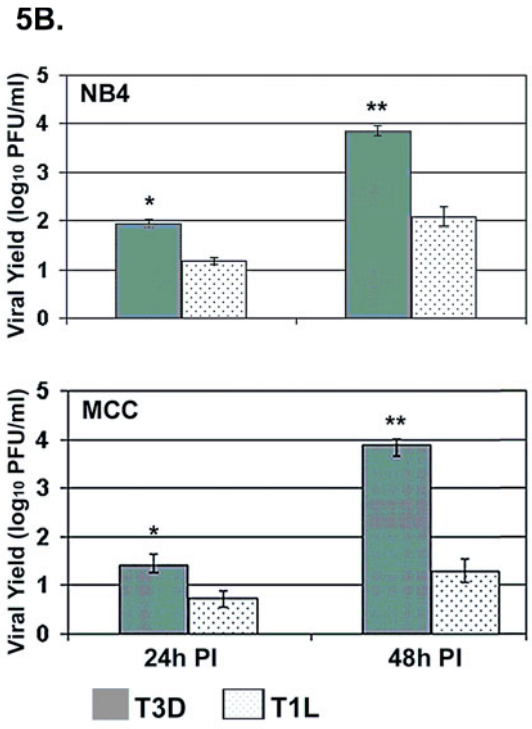

Figure 5 (**B**) One step growth curves were performed for both T3D and T1L in mouse neuroblastoma cells (NB4) and mouse primary cortical cultures (MCC). The data are represented as mean viral yield (log10 PFU/ml) ± standard error obtained at the designated timepoint after an initial exposure to viral inoculum at time 0 ($^{*}P < 0.01$ and $^{**}P < 0.001$ by Tukey-Kramer multiple comparisons test).

detection by fluorometry. We detected significantly greater levels of active caspase 3 in T3D-infected NB4 as compared with mock-infected cells by 24 h post infection (Figure 6**A**). We next performed immunocytochemistry to detect the active caspase 3 fragment in T3D-infected NB4 and MCC at 24 h post infection. In both types of neuronal culture, T3D infection significantly increased the number of cells positive for active caspase 3 as compared to mock-infected cells (Figure 6**B**). Lastly, we wished to understand whether inhibition of caspase 3 activity could protect neuronal cultures from T3D-induced apoptosis. NB4 and MCC were treated with the cell-permeable caspase 3 inhibitor, DEVD-FMK, for 1 h prior to infection with T3D and throughout the infection. At 48 h post infection, TUNEL or apoptotic morphology assay was performed. We found that caspase 3 inhibition protected NB4 and MCC from T3D-induced neuronal apoptosis (Figure 6**C**).

Reovirus-induced apoptosis in neuronal cultures is associated with activation of caspase 8 and death receptors of the TNF receptor superfamily

Having established that reovirus-induced apoptosis was associated with activation of the downstream effector caspase, caspase 3, we next wished to determine which upstream caspases were involved in initiating this process. Having previously shown that caspase 8 activation is an early event in reovirus-induced apoptosis in non-neuronal cells, we looked for the presence of caspase 8 activation that preceded caspase 3 activation in NB4 (Clarke *et al*, 2001). Using a cell-free fluorogenic substrate assay for detecting active caspase 8, we found that caspase 8 was active in T3D-infected NB4 by 20 h post infection (Figure 7**A**). We also performed Western blot analysis to detect antibody of caspase 8 in whole cell lysates from infected NB4. This showed the presence of caspase 8 cleavage products, indicative of caspase 8 activation at ~20 h post infection (Figure 7**B**). Furthermore, we used immunocytochemistry to detect active caspase 8 in T3D-infected MCC at 20 h post infection (Figure 7**B**).

Having shown that caspase 8 was activated in reovirus-infected neuronal cultures, we wished to determine whether inhibition of this activity could protect neuronal cultures from reovirus-induced apoptosis. We pretreated NB4 and MCC with the cell-permeable caspase 8 inhibitor IETD-CHO (IETD) or the caspase 9 inhibitor Z-LEHD-FMK (LEHD) 1 h prior to and during T3D infection, then assayed apoptosis by TUNEL staining at 48 h post infection. In T3D-infected NB4 and MCC (MCC data not shown), inhibition of caspase 8 activity, but not inhibition of caspase 9 activity, significantly decreased the percentage of TUNEL-positive NB4 (Figure 7**D**). We next assayed cells treated with IETD to determine whether inhibition of caspase 8 activation was associated with inhibition of effector caspase activation. IETD did not

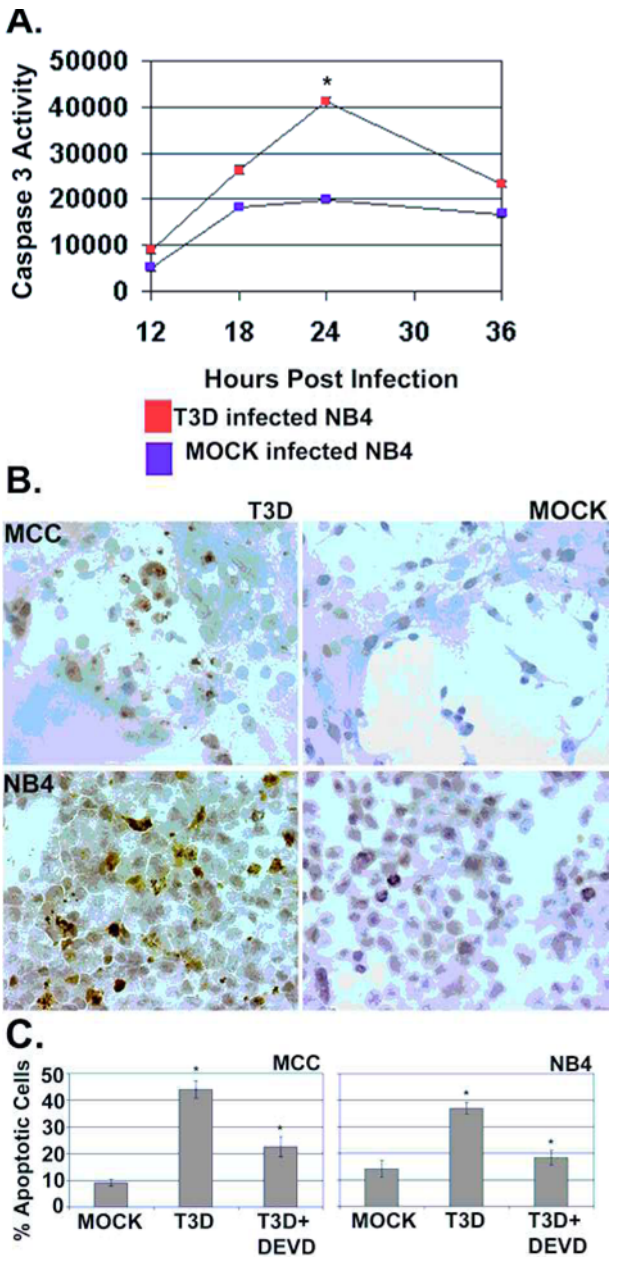

Figure 6 (**A**) Caspase 3 activity is increased in T3D-infected mouse neuroblastoma cells (NB4) as compared to mock infected cells at 24 h post infection. Data are raw fluorescent values obtained at 405 nm excitation and 500 nm emission. Data are mean raw fluorescence ± SEM ($^{*}P < .01$ by Tukey-Kramer multiple-comparison test). (**B**) Immunocytochemistry was performed on mouse primary cortical cultures (MCC) and NB4 at 24 h post infection (PI) to detect the presence of active caspase 3 (anti–active caspase 3). Active caspase 3 is detectable in T3D-infected neuronal cultures but not in mock infected neuronal cultures. Cells were counterstained with Blue Counterstain. (**C**) The effect of inhibition by specific cell-permeable peptide inhibitor of caspase 3, DEVD-CHO (DEVD) on T3D-induced apoptosis in NB4 and MCC. Data are percentage of TUNEL-positive cells in NB4 or percentage apoptotic cells in MCC cells in each treatment. Data are presented as mean ± standard error of six experiments with 300 cells counted per condition per experiment, $^{*}P < .001$ for M versus T3D and T3D versus T3D-DEVD by Tukey-Kramer multiple-comparison test.

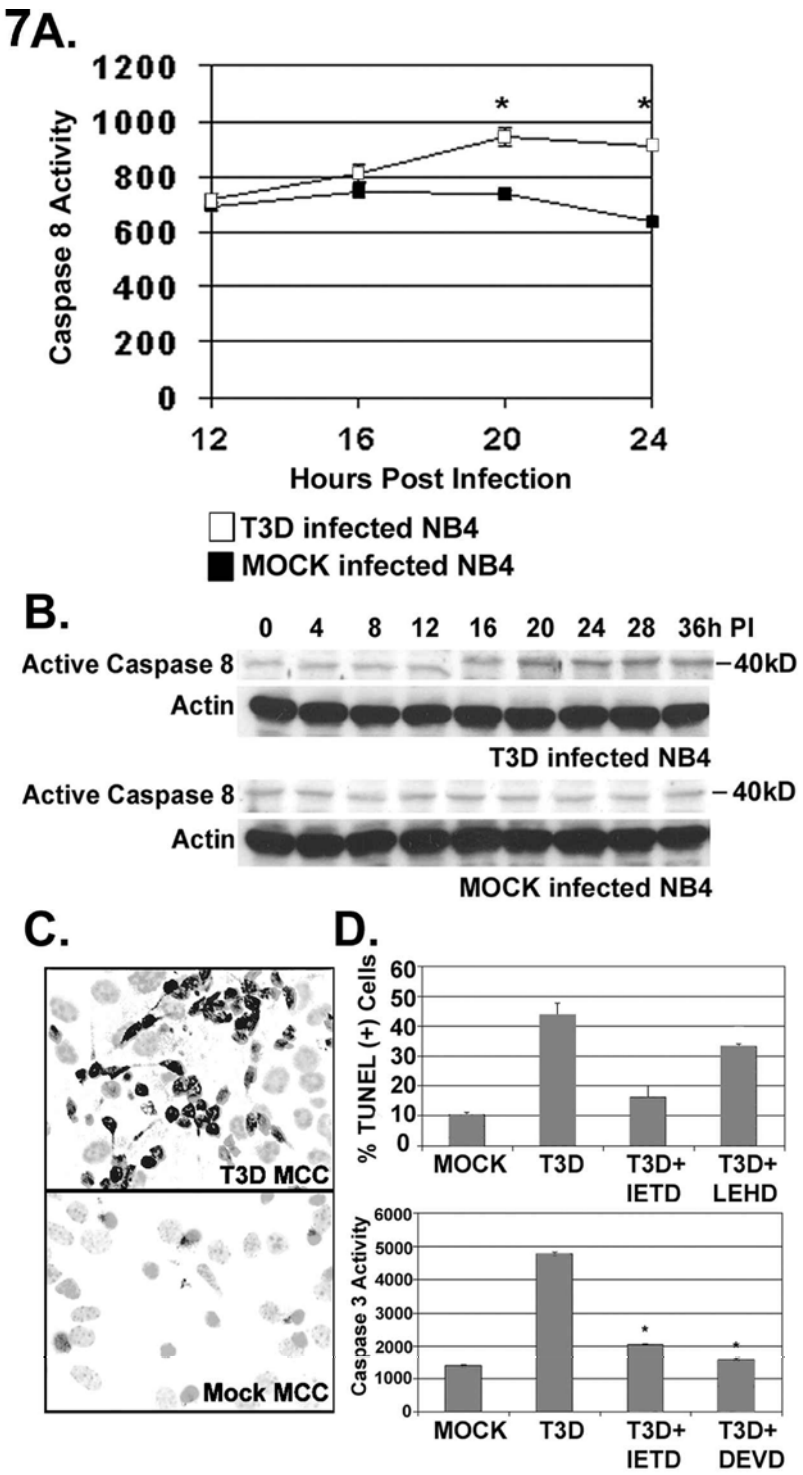

Figure 7 (**A**) Caspase 8 activity is increased in T3D-infected mouse neuroblastoma cells (NB4) as compared to mock-infected cells at 20 and 24 h post infection. Data are raw fluorescent values obtained at 405 nm excitation and 500 nm emission. Data are mean fluorescence ± SEM ($^{*}P < .01$ by Tukey-Kramer multiple-comparison test). (**B**) Active caspase 8 (40-kDa cleavage fragment of pro-caspase 8) is increased in T3D-infected NB4 as compared to mock infected NB4 by 20 h post infection (PI) as detected by Western blot analysis. Protein loading was normalized by Western blot detection of actin levels. (**C**) Active caspase 8 is detectable in T3D-infected mouse primary cortical cultures (MCC) at 20 h PI by immunocytochemistry. (**D**) The effect of inhibition by specific cell-permeable peptide inhibitors of caspase 8, IETD-CHO (IETD) and caspase 9, Z-LEHD-FMK (LEHD), on T3D-induced apoptosis in NB4. Data are percentage of TUNEL-positive cells in each treatment at 48 h PI. Data are presented as mean ± standard error of six experiments with 300 cells counted per condition per experiment, $^{*}P < .001$ T3D versus T3D + IETD by Tukey-Kramer multiple-comparison test. At 24 h PI, caspase 3 activity was decreased in T3D-infected NB4 treated with either the caspase 8 inhibitor, IETD-CHO (IETD), or the caspase 3 inhibitor, DEVD-CHO (DEVD), as compared to untreated T3D-infected NB4. Data are raw fluorescent values obtained at 405 nm excitation and 500 nm emission. Data are mean raw fluorescence ± SEM ($^{*}P < .01$ for T3D versus T3D + IETD, T3D versus DEVD by Tukey-Kramer multiple-comparison test).

directly inhibit caspase 3 activity at the concentrations tested (data not shown). Caspase 3 activity was measured in reovirus-infected IETD-treated cultures by fluorogenic substrate assay. We found that caspase 3 activity was significantly decreased by treatment of T3D-infected NB4 with IETD, suggesting that in T3D-induced neuronal apoptosis, caspase 3 activity was at least in part mediated by active caspase 8 (Figure 7**D**).

Caspase 8 is a DR-associated initiator caspase. The presence of active caspase 8 in T3D-infected neuronal cultures, and the inhibition of reovirus-induced apoptosis by caspase 8 inhibitor, suggested that reovirus-induced neuronal apoptosis was mediated by activation of DRs. Tumor necrosis factor (TNF) receptor superfamily DRs are transmembrane proteins activated by death-inducing ligands, including TNFα, TNF-related apoptosis-inducing ligand (TRAIL), and Fas ligand (FasL) (Ashkenazi and Dixit, 1998). To test whether reovirus activates DR signaling in neuronal cultures, we treated T3D-infected NB4 and MCC with several different soluble recombinant death receptors (Fc:DR5/TRAIL-R2, Fc:TNFR-1, Fc:CD95/FASR), and assayed apoptosis by TUNEL staining at 48 h post infection. In T3D-infected NB4, we found that treatment with either Fc:DR5/TRAIL-R2 or Fc:TNFR-1 significantly decreased the percentage of TUNEL-positive cells. Treatment of T3D-infected NB4 with Fc:CD95/FASR had no effect on T3D-induced apoptosis (Table 2). In T3D-infected MCC, we found that treatment with either Fc:TNFR-1 or Fc:CD95/FASR significantly decreased the percentage of TUNEL-positive cells. Treatment of T3D-infected MCC with Fc:DR5/TRAIL-R2 had no effect on T3D-induced apoptosis (see Table 2). These results indicate that DRs play a key role in reovirus-induced neuronal apoptosis.

Mitochondria-associated proapoptotic signaling mediated by cytochrome c *and caspase 9 is not a major factor in reovirus-induced apoptosis in neuronal cultures*

We have recently shown that reovirus-induced DR-initiated apoptosis of HEK293 cells is amplified by mitochondria-associated apoptotic signaling, as indicated by robust release of cytochrome *c* from the mitochondria and activation of caspase 9 (Kominsky, 2002). We wanted to understand whether there was similar involvement of the mitochondria in reovirus-induced apoptosis in neuronal cultures. Using Western blot analysis of cell lysates from T3D-infected NB4 separated into cytoplasmic and membrane/mitochondrial fractions, we did not find evidence of significant release of cytochrome *c* in the cytoplasm (Figure 8**A**). Cytosolic cytochrome *c* participates in the apoptosome-mediated activation of caspase 9 (Li *et al*, 1997). Small amounts of active caspase 9 were detectable by Western blot in reovirus-infected NB4, but only at late times (28 to 36 h) post infection, well after the peak of caspase 3 activation (Figure 8**B**). In T3D-infected MCC, there was no increase in cytosolic cytochrome *c* and active caspase 9 was not detected (data not shown). In contrast to the effects seen with caspase 3 inhibitor (see Figure 6**C**), treatment of T3D-infected NB4 with caspase 9 inhibitor (Z-LEHD-FMK) did not inhibit T3D-induced apoptosis (see Figure 7**D**). Taken together, these data suggest that mitochondria-mediated apoptosis associated with release of cytochrome *c* from the mitochondria and activation of caspase 9 does not significantly contribute to reovirus-induced neuronal apoptosis.

Table 2 Percent inhibition of T3D-induced apoptosis in neuronal cultures by soluble death receptors

Fc:Receptor	*Fc:DR5*	*Fc:TNFR*	*Fc:FASR*
NB4*	23% (± 4%)	22% (± 4%)	1% (± 5%)
	$P < .001$	$P < .01$	NS**
MCC***	4% (± 4%)	25% (± 6%)	49% (± 6%)
	NS	$P < .05$	$P < .001$

*NB4 = NB41a3; mouse neuroblastoma cell line.
**NS = not significant.
***MCC = mouse primary cortical cultures.

Discussion

In this paper, we show that T3 reovirus-induced neuronal apoptosis in the neonatal mouse CNS involves activation of caspase 3, a key effector downstream of many proapoptotic signaling molecules. Active caspase 3 staining was found in brain regions with reovirus-induced tissue injury and colocalized with reovirus antigen and TUNEL (apoptosis marker). To further elucidate the signal transduction mechanisms by which reovirus induces apoptosis in neurons, we investigated reovirus-induced neuronal apoptosis *in vitro* using neuron-derived cell lines and primary cultures of terminally differentiated neurons.

T3D kills and also grows in mouse neuroblastoma-derived cell line NB41a3 (NB4) and mouse primary cortical cultures derived from embryonic (E20) mice (MCC). Using a variety of different assays, we show that T3D-induced neuronal cell death results from apoptosis as demonstrated by changes in the location of plasma membrane PS as detected by annexin V staining, nuclear condensation and/or margination as shown by nuclear dye assays, and apoptosis-specific DNA fragmentation as seen using TUNEL and DNA laddering. We also found that caspase 3 is activated in reovirus-infected neuronal cultures, paralleling results seen in the CNS following infection *in vivo*.

Having shown that caspase 3 was activated in reovirus-infected neuronal cultures, we wished to determine the upstream events in this process. We found that caspase 8, a DR-associated initiator caspase, is activated in both NB4 and MCC cultures as early as 18 h following T3D infection.

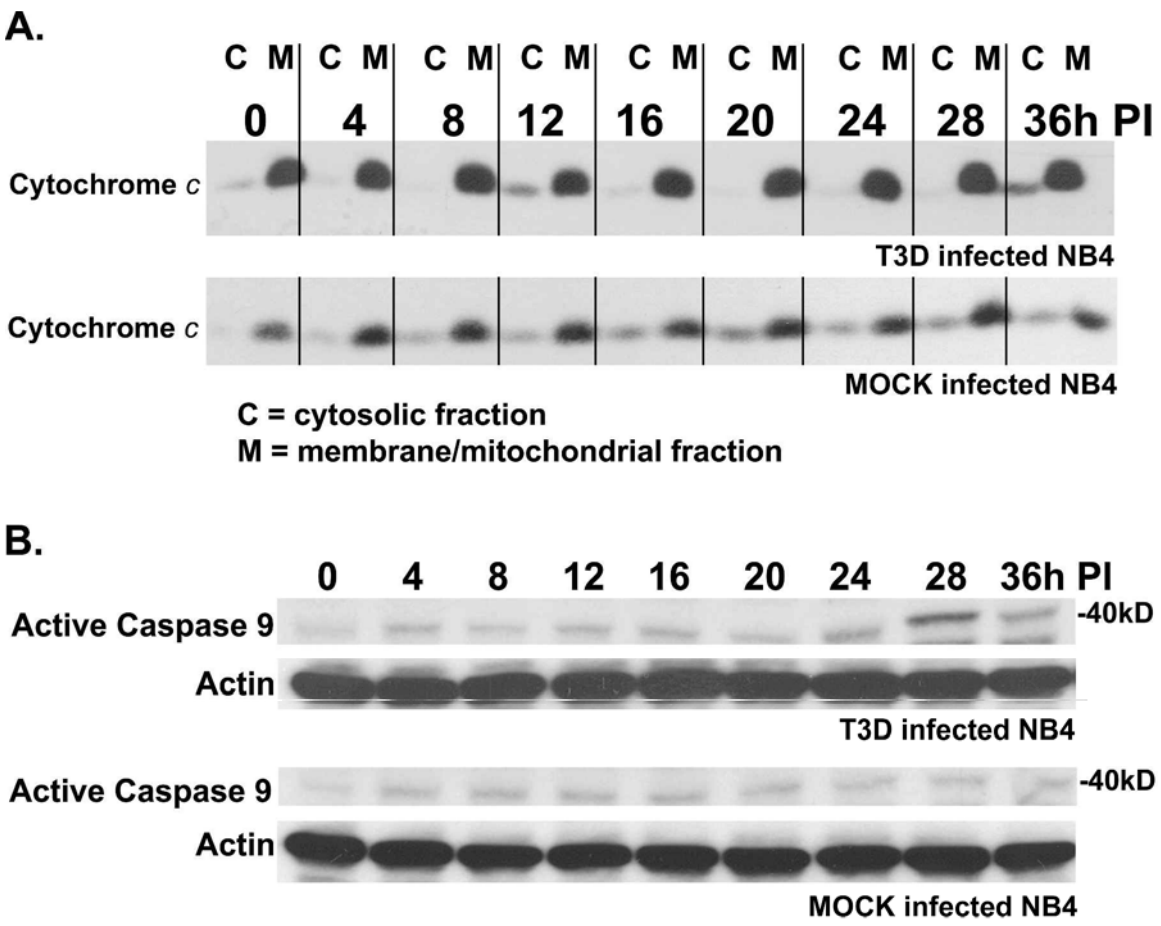

Figure 8 (**A**) Significant levels of cytosolic cytochrome *c* are not detected in T3D-infected mouse neuroblastoma cells (NB4) nor in mock-infected cells by Western blot analysis. (**B**) There is no difference in levels of active caspase 9 (40-kDa cleavage fragment of pro-caspase 9) between T3D-infected and mock-infected NB4 at time points preceeding caspase 3 activation (approximately 24 h post infection) as detected by Western blot analysis. Protein loading was normalized by Western blot detection of actin levels. PI = post infection.

Caspase 8 is activated by stimulation of the TNF receptor superfamily DRs following binding of death-inducing ligands such as TNFα, TRAIL, and FasL (Ashkenazi and Dixit, 1998). Caspase 8 activation has been implicated in apoptosis induced by viruses including HIV (Patel *et al*, 2000), Epstein-Barr virus (Tepper and Seldin, 1999), and Sendai virus (Bitzer *et al*, 1999). Recent studies from our laboratory indicate that in epithelial cell lines, and in certain human breast and lung cancer cell lines, reovirus stimulates TRAIL release from infected cells, activating DR4/DR5 (TRAILR1/TRAILR2) signaling pathways, leading to caspase 8–dependent apoptosis (Clarke *et al*, 2000, 2001). Reovirus-induced release of death ligands such as TRAIL may be a possible mechanism of induction of "bystander" apoptosis, which has been seen in the CNS following reovirus infection (Oberhaus *et al*, 1997), and also occurs in T3D-infected neuronal cultures (see Figure 4).

The expression of DRs and their role in apoptosis have been extensively studied in both epithelial and cells in the immune system (Dorr *et al*, 2002; Pettersen, 2000; Gupta, 2000; Strater and Moller, 2000), but less is understood about the role of DRs in neuronal apoptosis. Similarly, the role of DRs in virus-induced apoptosis remains poorly understood. CNS-specific changes in the expression or activity of CD95/FASR and TNF receptors and/or their corresponding death-inducing ligands have been implicated in neuronal apoptosis *in vitro* (McGuire *et al*, 2001), as well as in a variety of models of both viral and nonviral apoptosis *in vivo* (Tan *et al*, 2001; Rosenbaum *et al*, 2000; Sporer *et al*, 2000; Jelachich and Lipton, 2001). We now show that inhibition of reovirus-induced apoptosis in neuronal cultures is achieved using Fc-coupled soluble forms of TNF receptor superfamily DRs, specifically Fc:DR4/DR5 (TRAIL-R1/TRAIL-R2), Fc:FASR (CD95), or Fc:TNFR1. These results differ from those

in non-neuronal cells in which only Fc:DR4/DR5 significantly inhibited reovirus-induced apoptosis (Clarke *et al*, 2000), and suggest that the pathways of DR activation induced by a common stimulus, in this case viral infection, may differ between different populations of cells in specific target organs.

Sequential activation of caspases occurs in most forms of apoptosis, including during reovirus-induced apoptosis. Therefore, it is conceivable that if caspase activation could be inhibited early or at various stages in the apoptotic signal cascade, then dying cells may be able to recover and survive. Caspase inhibitors have been used successfully to protect neurons from apoptosis both *in vivo* and *in vitro* following several types of apoptotic stimuli (Kondratyev and Gale, 2000; Ma *et al*, 1998; Hara *et al*, 1997; Ray *et al*, 2000; Jiang *et al*, 2001; Jan *et al*, 2000). Here we demonstrate that cell-permeable peptide caspase inhibitors show a hierarchy of efficacy in their capacity to inhibit reovirus-induced neuronal apoptosis, with caspase 3 and caspase 8 inhibitors being significantly more than caspase 9 inhibitor (see Figure 7**D**). We have previously shown that inhibition of apoptosis can limit reovirus-induced myocardial injury *in vivo* (DeBiasi *et al*, 1999, 2001). It will be important to see whether caspase inhibition, which effectively inhibits reovirus-induced neuronal death *in vitro*, can also prevent reovirus-induced CNS injury *in vivo*.

Activation of the mitochondrial apoptotic pathway plays an integral role in augmenting DR-initiated signaling in reovirus-induced apoptosis of HEK293 cells. In these cells, apoptosis is associated with Bid cleavage, robust release of cytochrome *c* into the cytoplasm, and strong caspase 9 activation (Kominsky, 2002). In contrast to these findings, we did not detect increased levels of cytochrome *c* in the cytoplasm of reovirus-infected neurons, nor was there significant activation of the mitochondrion-associated initiator caspase, caspase 9. These results suggest that, in contrast to reovirus infected non-neuronal cells, mitochondria-associated apoptotic signaling mediated by cytochrome *c* and caspase 9 does not significantly contribute to reovirus-induced neuronal apoptosis.

These studies provide a profile of the activation of apoptotic signaling pathways in neuronal cells following viral infection, and indicate that these pathways may differ in important features from those in non-neuronal cells. It is increasingly evident that apoptosis is a factor in acute and chronic neurological diseases, including stroke, epilepsy, traumatic brain injury, and neurodegenerative diseases. Many neurotropic viral infections, including those caused by HIV (Patel *et al*, 2000; Ohagen *et al*, 1999; Kaul *et al*, 2001; Gray *et al*, 2000), La Crosse virus (Pekosz *et al*, 1996), Sindbis virus (Nava *et al*, 1998; Jan *et al*, 2000; Lewis *et al*, 1996), Dengue virus (Despres *et al*, 1996), Venezuelan equine encephalitis virus (Jackson and Rossiter, 1997b; Jackson *et al*, 1991), Rabies virus (Jackson and Rossiter, 1997a), herpes simplex virus (Thompson and Sawtell, 2000), and poliovirus (Lopez-Guerrero *et al*, 2000; Girard *et al*, 1999), are associated with apoptosis, suggesting that this form of cell death is likely to be a common feature of many CNS infections. This suggests that strategies designed to inhibit apoptosis may provide a novel approach for the treatment of virus-induced CNS diseases.

Materials and methods

Tissue culture

Neuroblastoma-derived cell line The mouse neuroblastoma-derived cell line, NB41a3 (ATCC CCL-147) was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in minimal essential media supplemented with 2 mM glutamine, 50 U/ml of penicillin and streptomycin (P/S), 1 mM nonessential amino acids, and 10% heat-inactivated fetal bovine serum (FBS; GibcoBRL, Gaithersburg, MD). Cells were plated at densities of 2×10^5 cells per milliliter in tissue culture–treated 6-well, 12-well, or 96-well plates (Costar, Acton, MA), or polystyrene 8-well chamber slides (Lab-Tek II, Nunc/Nalgene International, Fisher Scientific, Pittsburgh, PA) and maintained at 37°C in 5% CO_2.

Primary cultures Primary cortical cultures were prepared from embryonic day 20 (E20) Swiss-Webster mice. Pregnant mice were euthanized by isofluorane inhalation followed by cervical dislocation. Fetuses were removed from the uterus, separated, and immediately submerged in ice cold sterile Hanks buffer (without calcium chloride, magnesium chloride, magnesium sulfate, and phenol red; GibcoBRL). Fetuses were decapitated with sterile scissors, and brains were removed and immediately submerged in fresh ice-cold sterile Hanks buffer. The frontal cortex was dissected, the meninges were removed, and the cortical tissue was washed three times in fresh ice-cold Hanks buffer, then dissociated with a 1-ml pipette tip. The percentage of viable cells was quantified by trypan blue staining using a hemocytometer.

Cells were plated at densities of 10^5 cells per milliliter in poly-D-lysine (PDL)-coated 6-well polystyrene plates or 8-well glass chamber slides (BioCoat, BD Biosciences, San Jose, CA) and maintained in Neurobasal media supplemented with 2 mM Glutamax, 50 U/ml P/S, 100 μM glucose, and 10% heat-inactivated FBS (GibcoBRL) at 37°C in 5% CO_2. At 24 h following plating, the serum-containing medium was replaced by serum-free medium containing the nonserum nutritional supplement B27 (Gibco BRL) to limit non-neuronal cell proliferation. Half the medium was replaced every 3 to 4 days, and cells were allowed to mature for 10 to 14 days before use in experiments.

Viral infections

Cells For most apoptosis and signal transduction assays, subconfluent cell monolayers were infected with plaque-purified second passage laboratory stocks of T3D or T1L at a MOI of 100 PFU per cell. This multiplicity of infection (MOI) was selected to generate a synchronized infection of all susceptible cells in the culture. For infections, the cell culture medium was aspirated and replaced with viral stock diluted in a minimal volume of cold gelatin saline, then incubated at 37°C for 1 h, with rocking every 15 min. Following the 1-h infection, fresh medium was added to the cells, which continued to be maintained at 37°C with 5% CO_2.

Mice Postnatal day 2 mice were infected with either T3D (10^5 PFU) or mock-infected with an equivalent volume of diluent medium via intracerebral (IC) injection. Injections were made using a 29-gauge needle in a 10-μl volume. Animals were sacrificed 7 days after infection.

Histology

For histopathological and immunohistochemical staining, eight whole mouse brains per treatment group (e.g., mock-infected, T3D-infected) were fixed by immersion in 10% buffered formalin for 24 to 30 h at room temperature (RT), then cut in half along the mid-coronal line for sectioning. Fixed tissues were transferred to 70% ethanol, paraffin-embedded, and sectioned at 4 μm thickness. For each animal, a coronal section that showed cingulate gyrus, hippocampus, and thalamus was stained with hematoxylin and eosin for studies of the extent of virus-induced pathology. Paraffin-embedded sections were baked at 57°C for 5 min to enhance antigen retrieval, then deparaffinized by immersion in mixed xylenes, followed by rehydration in a series of descending ethanol concentrations followed by phosphate-buffered saline.

Viral growth assays

Viral growth in neuronal cell cultures was assayed by determining viral titer at 0, 24, or 48 h following reovirus infection at a MOI of 10. This MOI resulted in more distinct one-step growth curves but essentially similar peak titers to that seen with MOI 100 (data not shown). At the indicated times, infected neuronal cells were lysed via three freeze (−70°C)–thaw (37°C) cycles, followed by manual disruption with a 1-ml pipette tip. Viral titer in cell lysates was determined by plaque assay on monolayers of L929 mouse fibroblasts, as previously described (Tyler *et al*, 1985).

Apoptosis assays

Flow cytometric analysis For the annexin V–PI assay (Flow-TACS, Trevigen, Gaithersburg, MD), 6 wells per treatment group of 5×10^5 cells were gently harvested with a 1:1 mixture of trypsin-versene (0.25%, GibcoBRL) and Accumax (Innovative Cell Technologies, San Diego, CA), then resuspended and washed in PBS. Once in suspension, cells were incubated with 1.5 μg annexin V–fluorescein isothiocyanate (FITC) in 100 μl binding buffer for 15 min in the dark at RT. PI (0.25 μg in 400 μl binding buffer) was added, then cells were analyzed by flow cytometry (Coulter Epics; Beckman Coulter, Fullerton, CA). Annexin V–FITC was measured by the FL1 channel (*x*-axis), and PI was measured by the FL3 channel (*y*-axis).

Nuclear morphology assays Apoptotic cells were identified by evaluating nuclear morphology at various times following reovirus infection by staining fixed cells with the fluorescent nuclear DNA intercalating dye, Hoechst 33342 (Molecular Probes, Eugene, OR). Apoptotic nuclei were identified by the presence of condensed and/or marginized chromatin. Cells were grown and infected in chamber slides, fixed at 24 or 48 h following infection with 3.7% formaldehyde/PBS for 20 min at RT, then incubated with Hoechst 33342 (1 μg/ml PBS) in the dark for 15 min at RT. The slides were mounted with an antifade mounting media (4 mg phenylenediamine in 1 ml PBS and 3 ml glycerol or Anti-Fade Kit from Molecular Probes). Apoptotic cells were quantified and imaged by fluorescence microscopy at 200× magnification (Zeiss Axioplan 2 Digital Microscope with Cooke SensiCam 12 bit Camera). For each condition, percentage of apoptotic cells was determined by counting 300 hundred cells in at least three individual samples.

Apoptotic DNA ladder assays We evaluated the fragmentation patterns of DNA isolated from control and reovirus-infected cells using the method described by Gong *et al* (1994). Briefly, 1×10^6 neuronal cells were harvested 48 h after infection with 0.25% trypsin/versene (GibcoBRL) for 5 min at RT, then resuspended in 1 ml Hanks buffer, fixed with 10 ml cold 70% ethanol, and placed at −20°C overnight. The next day, the ethanol was removed, DNA was extracted with a sodium phosphate-citrate buffer (92 parts 0.2 M Na_2HPO_4, 8 parts 0.1 M citric acid, pH 7.8), vacuum-dried, then incubated with 0.25% Nonidet-P 40, RNase A (3 μg) and proteinase K (3 μg) at 37°C for 30 min. Extracted DNA was separated by electrophoresis in a 2% TBE (25 mM tris-borate, 0.5 mM EDTA)/agarose gel containing 10 μg ethidium bromide at 22 V for 18 h. DNA ladder pattern was visualized by ultraviolet (UV) illumination. A 100-base pair (bp) DNA molecular weight marker with high intensity bands at 600 bp, 1.5 kilobases (kb), and 2.0 kb (GibcoBRL) was used to estimate molecular weight of DNA fragments.

TUNEL A biotin/streptavidin-based TUNEL kit optimized for neuronal tissues and cells was used

(NeuroTACS II; Trevigen, Gaithersburg, MD). At 48 h following reovirus infection, cells grown in chamber slides were fixed with 3.7% formaldehyde/PBS for 10 min, post-fixed in methanol for 20 min, then permeabilized in Neuropore (Trevigen) for 30 min at RT under hydrophobic coverslips. For each condition, percentage of TUNEL-positive cells was determined by counting 300 hundred cells in at least three individual samples.

Brain tissue sections underwent antigen retrieval, deparaffinization, and rehydration, then were permeabilized with Neuropore for 30 min at 37°C under hydrophobic coverslips. The remainder of the TUNEL assay for both neuronal cells and brain tissue sections was performed in accordance with the manufacturer's guidelines.

Inhibitor assays Nuclear morphology assays and TUNEL were used to determine whether treatment of T3D-infected neuronal cultures with anti-reovirus sigma 1 antibodies (anti-T3D sigma 1, 9BG5; anti-T1L sigma 1, 5C6) from (Virgin *et al*, 1988), cell-permeable caspase inhibitors, or soluble recombinant DRs could block T3D-induced apoptosis. Caspase inhibitors used include DEVD-CHO, IETD-CHO, and Z-LEHD-FMK (Calbiochem, San Diego, CA). Neuronal cultures were incubated at 37°C with 25 μM caspase inhibitor for 1 h prior to infection and throughout infection. The cytopathic effect induced by vehicle (DMSO) alone was minimal (data not shown). Soluble DRs used include Fc:DR5 (TRAILR2), Fc:CD95(FAS), and Fc:TNFR1 (Alexis Corporation, San Diego, CA). Neuronal cultures were incubated at 37°C with 1 to 5 μg/ml soluble receptor for 1 h prior to infection and throughout infection. For assays using Fc:receptors, neuronal cultures were infected by T3D at MOI 50.

Caspase 3 and 8 activation assays Caspase 3 or caspase 8 activity in reovirus-infected and control neuroblastoma cells was detected via ApoAlert Caspase 3 and 8 Activity Fluorometric Assays per manufacturer's guidelines (Clontech, Palo Alto, CA). Samples were transferred to 96-well enzyme-linked immunosorbent assay (ELISA) plates for detection of fluorescent activity with a fluoremeter (Cytofluor Series 4000; PerSeptive Biosystems) set at 400 nm excitation filter and 505 nm emission filter.

Immunocytochemistry and immunohistochemistry

Single antibody In preparation for immunoassays, neuronal cell cultures were grown and infected in 8-well chamber slides, fixed at various times following virus infection with 3.7% formaldehyde/PBS for 1 h at RT, and permeabilized with Neuropore for 30 min at RT. Brain tissue underwent deparaffinization, rehydration, and permeabilization in Neuropore for 30 min at RT. Samples were washed in Tris-buffered saline (TBS; 140 mM NaCl, 20 mM Tris, pH 7.6), nonspecific binding was blocked with 5% normal goat serum in TBS (NGS; Vector Laboratories, Burlingame, CA), then samples were incubated (overnight at 4°C) with primary antibody (1:50 to 1:100) diluted in 3% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) in TBS/0.1% Tween (TBST). Samples were next washed in TBST and incubated in blocking buffer with the appropriate secondary antibody conjugated to horseradish peroxidase (HRP) (Amersham, Piscataway, NJ) or FITC (Jackson Immunoresearch Laboratories, West Groue, PA) for 1 h at 37°C. Samples were washed with TBST and either exposed to diaminobenzidine (DAB) for HRP-conjugated secondary antibody then permanently mounted, or immediately mounted with antifade medium if the secondary antibody was conjugated to FITC. Mounted samples were stored as described for TUNEL. Primary antibodies used for immunocytochemistry include rabbit polyclonal anti–active caspase 3, mouse monoclonal anti–active caspase 8, and rabbit polyclonal anti–active caspase 9 (Cell Signaling Technology, Beverly, MA), rabbit polyclonal anti–active caspase 8, mouse monoclonal anti–cytochrome *c* (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-NeuN (Chemicon, Temecula, CA), and rabbit polyclonal reovirus antisera (Tyler *et al*, 1985).

Double antibody labeling The protocol described for fluorescent single antibody labeling was used with the addition of another incubation (usually 2 h at 37°C) for the second primary antibody following the incubation and washes for the first primary antibody. The secondary antibodies were conjugated to different fluorophores with differing spectra, such as FITC and Cy3. The secondary antibodies were mixed together, diluted in 3% BSA/TBST, and incubated for 1 h at 37°C. Samples were then washed in TBST, incubated with Hoechst 33342 (100 ng/ml)/PBS for 10 min at RT in the dark as counterstain, mounted with antifade medium, and stored in the dark at −20°C until imaging.

Double label (antibody + TUNEL) For both neuronal cells and brain tissue sections, binding of the primary antibody for the immunoassay was performed prior to TUNEL staining by diluting the primary antibody (1:100) in Neuropore and incubating for 1 h at 37°C or overnight at 4°C under hydrophobic coverslips. The TdT reaction was performed as described for TUNEL. A mixture of strep-Cy3 (Jackson Immunoresearch Laboratories) to detect TUNEL and secondary antibody (1:100) conjugated to FITC to detect the primary antibody. Samples were washed and mounted as described for double-antibody labeling.

Western blots

Cell lysates Whole-cell lysates, mitochondrial/membrane lysates, and cytosolic/mitochondria-free lysates were prepared from neuronal cell cultures

by growing and infecting the cells in 6-well plates. At various times following viral infection, cells were harvested by incubation with 0.25% trypsin/versene (GibcoBRL), washed in PBS, and resuspended in the appropriate lysis buffer. Two wells of a 6-well plate were used for whole cell lysates. The cell pellet was resuspended in 150 μl of whole cell lysis buffer (1% Nonidet P40, 0.15 M NaCl, 5 mM EDTA, 0.01 M Tris pH 8.0, 1 M PMSF, 0.02 mg/ml leupeptin, 0.02 mg/ml trypsin inhibitor), briefly sonicated with a microtip probe, then mixed with 150 μl of Laemmli buffer (4% sodium dodecyl sulfate, 20% glycerol, 10% beta-mercaptoethanol, 0.004% bromophenol blue, 0.125 M Tris-HCl, pH 6.8). The remaining 4 wells of the 6-well plate was used for preparation of both mitochondrial/membrane lysates and cytosolic/mitochondria-free lysates. The cell pellet was resuspended in 300 μl of mitochrondria-free extraction lysis buffer (220 mM mannitol, 68 mM sucrose, 50 mM PIPES-KOH, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT; protease inhibitor cocktail, Boehringer Mannheim, Indianapolis, ID), incubated on ice for 30 min allowing mitochondria to swell, homogenized in a 2-ml glass dounce-homogenizer with 40 strokes, then centrifuged at 14,000 $\times$ *g* for 15 min at 4°C to remove mitochondria and cell membranes. The supernatant was transferred to a fresh microfuge tube and 300 μl of Laemmli buffer was added. The mitochondria/membrane pellet was resuspended in 150 μl whole cell lysis buffer, briefly sonicated with a microtip probe, and mixed with 150 μl Laemmli buffer. All lysates were stored at −20°C until use.

Gels and immunoblots Lysates were boiled for 7 min and electrophoresed (Hoefer Pharmacia Biotech, San Francisco, CA) in 10% or 15% glycine/polyacrylamide gels or 10% or 15% Tricine/polyacrylamide gels (for small proteins and peptides) at constant voltage of 60 V through the stacking gel and 150 V through the resolving gel. Proteins were electroblotted onto Hybond-C nitrocellulose membranes (Amersham) and immunoblotting was performed as described (Poggioli *et al*, 2000). Primary antibodies used for immunoblots include anti-actin (Calbiochem), anti–caspase 9 (Cell Signaling Technology, Beverly, MA), and anti–cytochrome *c* and anti–caspase 8 (BD-Pharmingen, La Jolla, CA).

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