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## Apoptotic cell death in experimental rabies in suckling mice

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**Abstract** A fatal encephalomyelitis developed after intracerebral inoculation of 6-day-old ICR mice with the challenge virus standard (CVS) strain of fixed rabies virus. The brains of CVS-infected mice showed widespread morphologic changes of apoptosis, which were particularly prominent in pyramidal neurons of the hippocampus and in the cerebral cortex. Evidence of oligonucleosomal DNA fragmentation was sought in situ using the TUNEL method. TUNEL staining was observed in many neurons, and rabies virus antigen was usually demonstrated with immunoperoxidase staining in similar regions. Neurons in the dentate gyrus of the hippocampus demonstrated expression of viral antigen, apoptotic changes, and positive TUNEL staining. This region normally demonstrates little infection in CVS-infected adult mice. Double labeling of neurons with TUNEL and viral antigen indicated that infected neurons actually underwent apoptosis. Increased immunoreactivity against the Bax protein was demonstrated compared to uninfected mice. Purkinje cells expressed viral antigen, but did not show significant morphologic changes of apoptosis or TUNEL staining. In contrast, neurons in the external granular layer of the cerebellum did not express viral antigen, but demonstrated greater morphologic changes of apoptosis and positive TUNEL staining than uninfected controls. Apoptotic cell death likely plays an important role in the pathogenesis of rabies virus infection in suckling mice. There was evidence of more apoptosis in the brains of suckling mice than in those of adult mice and this finding explains the greater neurovirulence of rabies virus in younger mice. Rabies virus likely induces apoptosis in vivo by both direct and indirect mechanisms.

**Key words** Apoptosis · Encephalitis · Rabies · Pathogenesis

### Introduction

Apoptosis has recently been recognized to be a common pathologic mechanism by which viruses cause injury to their host cells [15, 18]. We recently reported the occurrence of apoptotic cell death in the brains of adult mice experimentally infected intracerebrally with the challenge virus standard (CVS) strain of rabies virus [7]. Age-dependent susceptibility and virulence are common features of viral infections [12, 17]. Suckling mice are more susceptible than adult mice to intracerebrally inoculated rabies virus and, for this reason, suckling mice are used for inoculation of tissues from suspected animals in a mouse inoculation test [9]. Although previous studies have examined the role of apoptosis in experimental infections of newborn mice due to neurotropic viruses, including Sindbis virus [10], La Crosse virus [14], and reovirus [13], the topographic distribution of apoptosis in the brain was only assessed in reovirus infection [13]. In the present work we have examined CVS infection of suckling mice to determine the role and distribution of apoptotic cell death in rabies virus infection during an important period of postnatal development of the brain.

### Materials and methods

Virus, animals, and inoculations

The CVS-11 strain of fixed rabies virus, which was obtained from Dr. William H. Wunner (The Wistar Institute, Philadelphia, Pa.), was grown in BHK-21 cells and plaque purified. Six-day-old ICR mice (Charles River Canada, St.-Constant, Quebec) were used. A viral dose of 840 plaque-forming units of CVS was administered intracerebrally in 0.02 ml phosphate-buffered saline (PBS) with 2% fetal bovine serum (FBS). Uninfected control mice were inoculated with PBS with 2% FBS.

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### Preparation of tissue sections

Mice were anesthetized with methoxyflurane and perfused with buffered 4% paraformaldehyde. Brains of three to ten infected and control mice were removed daily from 2 to 4 days after inoculation and immersion-fixed in the same fixative for 18 h at 4°C. Tissue sections (6 µm) were prepared in the coronal plane after dehydration and embedding in paraffin. Tissues for histologic examination were stained with cresyl violet.

### Immunoperoxidase staining

Sections were stained for rabies virus antigen by the avidin-biotin-peroxidase complex (ABC) method using polyclonal rabbit anti-nucleocapsid protein serum as primary antibody as previously described [8]. Immunostaining for the Bax protein was performed by the same method using polyclonal rabbit anti-Bax protein (Santa Cruz Biotechnology, Santa Cruz, Calif.) as primary antibody. No enzymatic digestion was performed prior to application of the primary antibody (diluted 1:500). Tissues from uninfected mice were used as controls, and normal rabbit serum was substituted for the primary antibody on tissues as another control.

### DNA nick end labeling of tissue sections (TUNEL staining)

DNA fragmentation was assessed in situ in sections of infected and control animals using the terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) method as previously described [6]. Selected slides were double-labeled with the TUNEL method followed by immunostaining for rabies virus antigen without counterstaining.

## Results

Mice infected intracerebrally with CVS developed signs of rabies, including growth retardation, ataxia, and paralysis, and the mice became moribund 4 days after inoculation. At 2–3 days after inoculation there was a widespread distribution of rabies virus antigen in neurons throughout the brain and staining for viral antigen was maximal on day 4 (Table 1). The cerebral isocortex and pyriform cortex were heavily infected. There was prominent infection of pyramidal neurons of the hippocampus and less marked infection of the dentate gyrus predominantly affecting neurons in the outer layer (Fig. 1A, B). The neostriatum showed much less prominent infection. Viral antigen was not observed in the periventricular germinal layer (Fig. 2A). The diencephalon and brain stem demonstrated widespread viral antigen. Antigen was also noted in trigeminal ganglia (Fig. 2B), indicating centrifugal spread of the virus. Viral antigen was prominent in Purkinje cells and in deep cerebellar nuclei (Fig. 1C). Multifocal infection was noted in the internal granular layer of the cerebellum, but viral antigen was not observed in the external granular layer (Fig. 1C).

TUNEL staining was prominent in neurons in the cerebral cortex (Fig. 2C), diencephalon, and brain stem. In the hippocampus there was marked TUNEL staining in pyramidal neurons (Fig. 1D). TUNEL staining in neurons in the dentate gyrus was not confined to the outer layer (Fig. 1E), which showed viral antigen (Fig. 1B) (see above). Variable numbers of cells demonstrated TUNEL staining

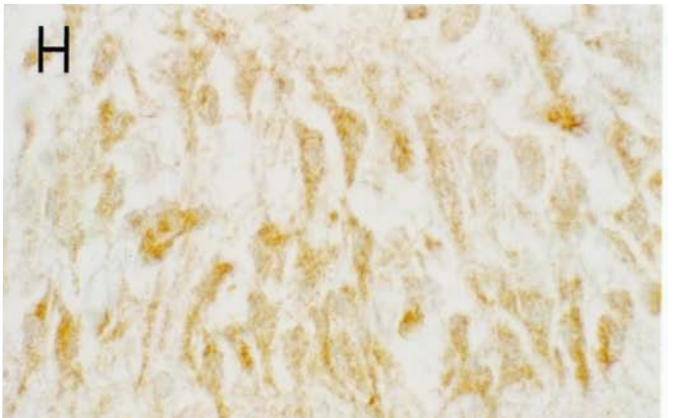
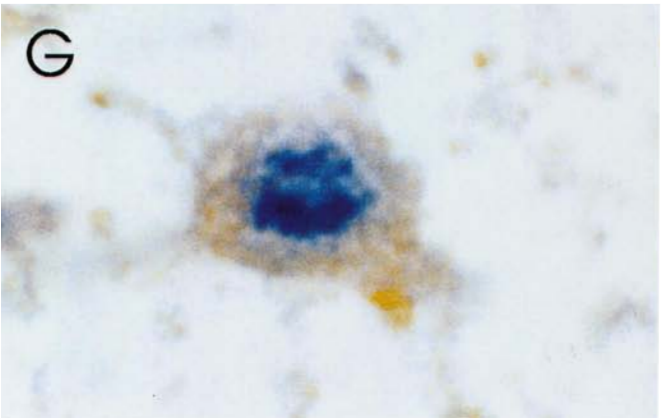
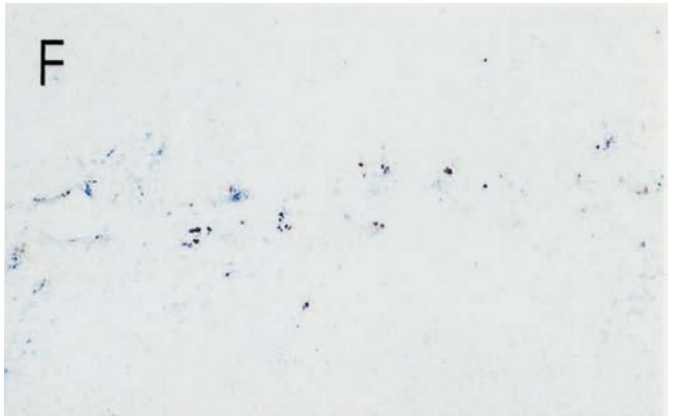
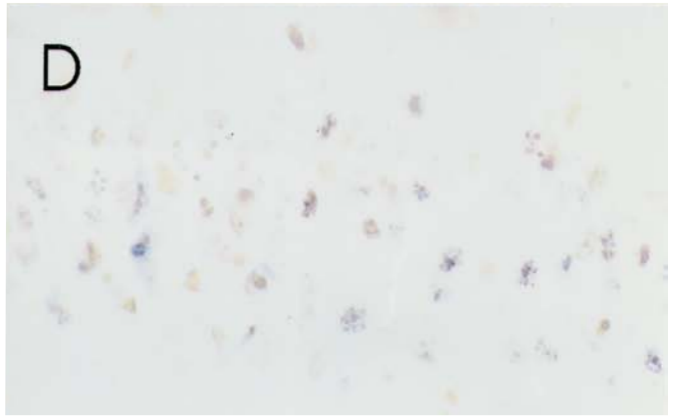
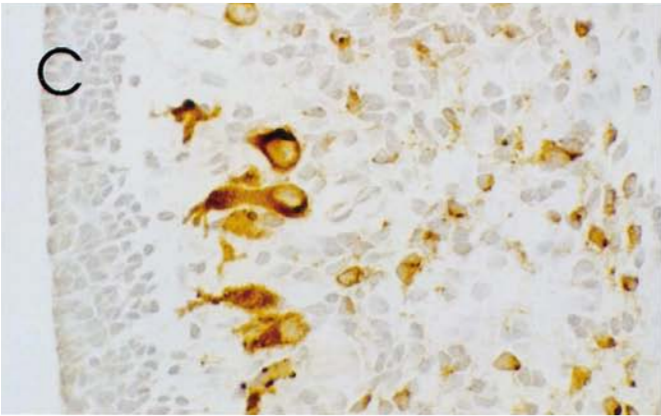
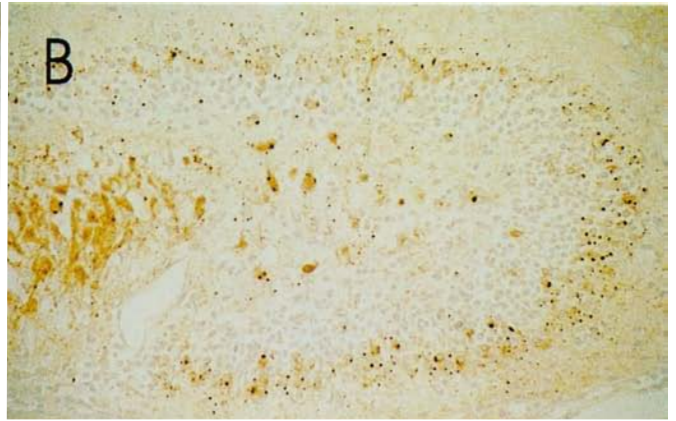
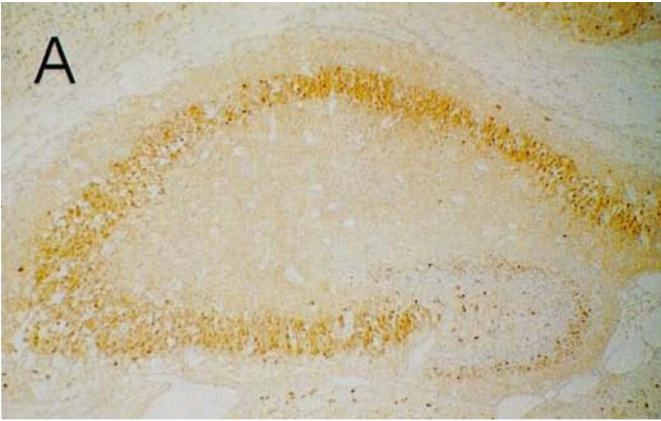
**Table 1** Rabies virus antigen distribution and morphologic changes of apoptosis in regional areas of the brain (semiquantitative evaluation: + weak staining/mild changes, ++ moderate staining/changes, +++ strong staining/severe changes, ++++ very strong staining/very severe changes)

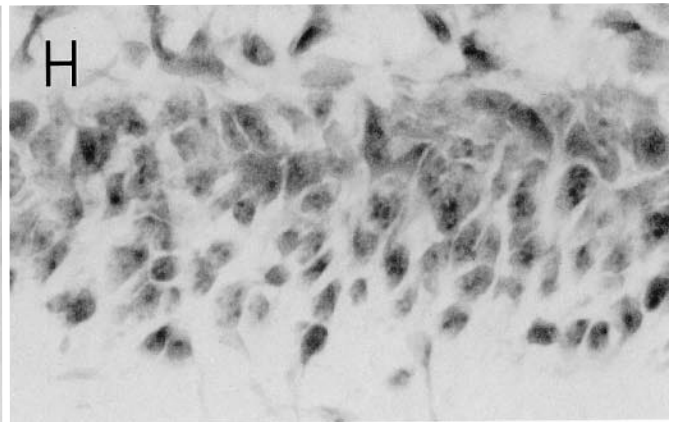
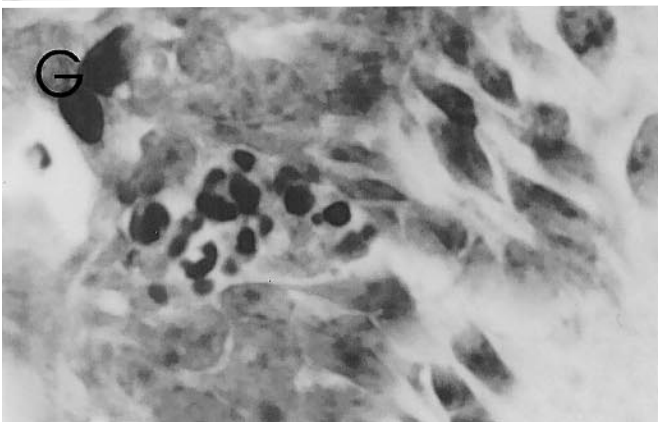
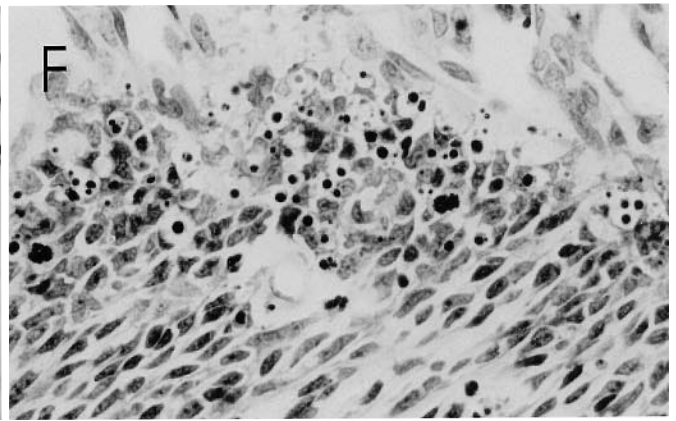
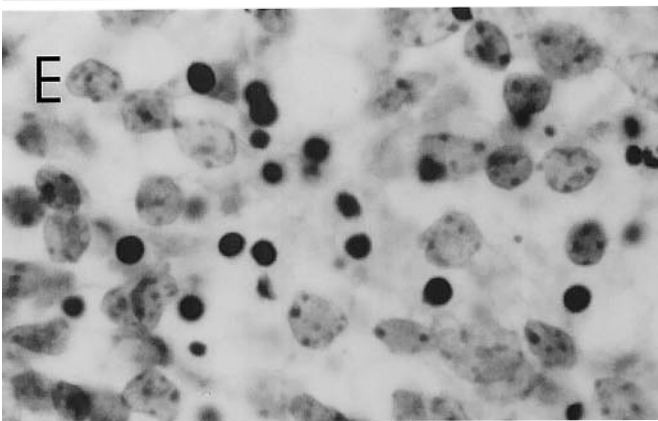
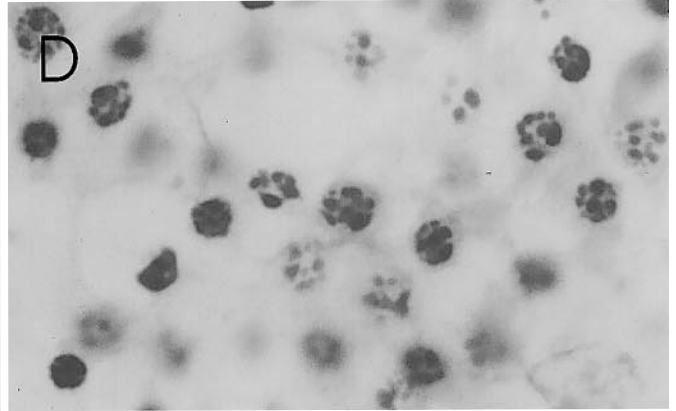
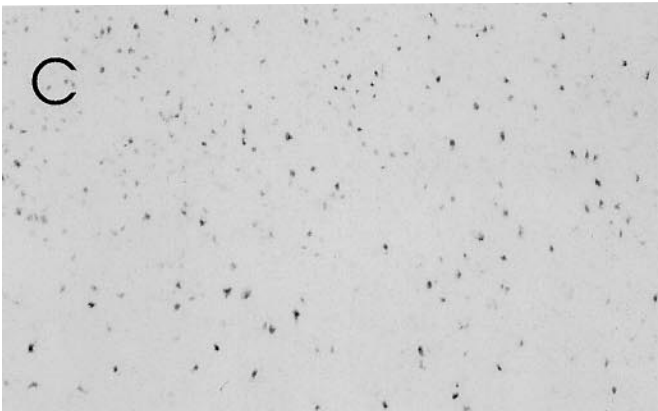
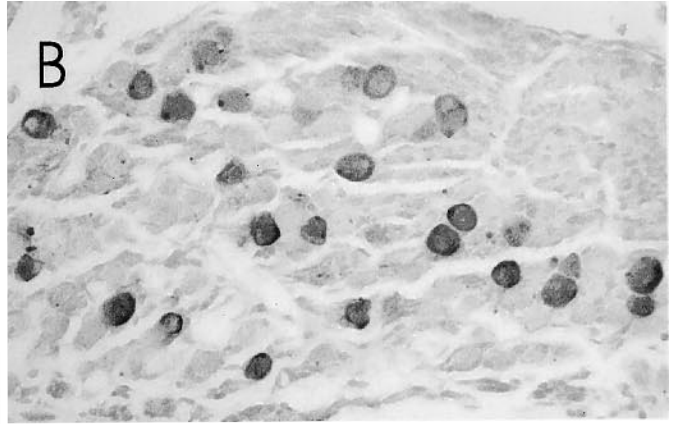
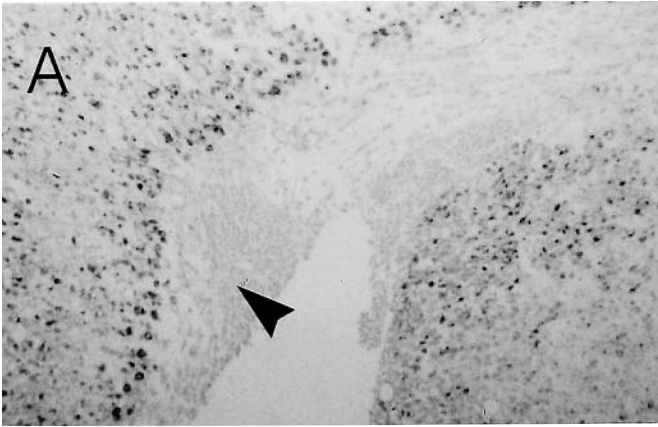
Brain region	Day 2	Day 3	Day 4
<b>Rabies virus antigen</b>			
Cerebral cortex	++	++++	++++
Hippocampus	+++	++++	++++
Diencephalon	+-++	++++-++++	++++
Cerebellum	0-+	+++	++++
Brain stem	+-++ (n = 2)	++++ (n = 2)	++++ (n = 8)
<b>Morphologic changes of apoptosis</b>			
Cerebral cortex	0	++	++++-++++
Hippocampus	0	++	++++
Diencephalon	0	+	++
Cerebellum	0	+	++
Brain stem	0 (n = 4)	++ (n = 4)	++ (n = 9)

in the external granular layer of the cerebellum (Fig. 1F) and there was staining of scattered cells in the internal granular layer. TUNEL staining was not observed in Purkinje cells.

Morphologic changes of apoptosis were found in neurons with a widespread distribution in the brain. The changes were first observed on day 3 and they were maximal on day 4 (Table 1). Multiple condensations of nuclear chromatin were seen in association with cytoplasmic shrinkage. The most marked changes were found in cortical neurons and in pyramidal neurons of the hippocampus (Fig. 2D). Less marked changes were also noted in the diencephalon and brain stem. The neostriatum was relatively uninvolved, but there was much less viral antigen in neurons in this region. Neurons demonstrated large condensations of chromatin that involved the entire nucleus in the dentate gyrus of the hippocampus, and these findings were present in all layers of the dentate gyrus (Fig. 2E). In the external gran-

**Fig. 1** A Immunoperoxidase staining showing rabies virus antigen in pyramidal neurons and neurons in the dentate gyrus of the hippocampus 4 days after intracerebral inoculation of CVS. B Viral antigen in neurons in the outer layer of the dentate gyrus of the hippocampus. C Viral antigen in Purkinje cells and in neurons in the internal granular layer of the cerebellum. Note that antigen is not present in the external granular layer. D TUNEL staining in multiple pyramidal neurons in the CA1 region of the hippocampus of a CVS-infected mouse. E TUNEL staining in neurons in the dentate gyrus of the hippocampus of an infected mouse. F TUNEL staining in the external granular layer of the cerebellum in a CVS-infected mouse. G Double labeling of a neuron in the cerebral cortex showing strong TUNEL staining in the nucleus and mild staining for viral antigen in the perikaryon, indicating that infected neurons actually undergo apoptosis. H Immunostaining for the Bax protein in pyramidal neurons in the CA4 region of the hippocampus. (CVS challenge virus standard, TUNEL terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling). A × 20; B, E, F × 65; C × 160; D × 120; G × 225; H × 140





ular layer of the cerebellum there were cells with multiple condensations of nuclear chromatin and others with chromatin condensations involving the entire nucleus (Fig. 2F, G). The changes in the external granular layer involved many more cells than in uninfected control mice of the same age, which demonstrated a few apoptotic cells in the external granular layer (Fig. 2H). Occasional cells with apoptotic morphology were also observed in the internal granular layer. Double labeling of neurons in multiple regions by TUNEL and immunoperoxidase staining for rabies virus antigen demonstrated that infected neurons actually underwent apoptosis (Fig. 1G).

Immunostaining for the Bax protein was observed in uninfected mice, especially involving the cerebral cortex, hippocampus, and Purkinje cells. Infected mice demonstrated increased immunostaining for the Bax protein, particularly in the cerebral cortex and in pyramidal neurons of the hippocampus (Fig. 1H).

## Discussion

Young animals are often highly susceptible to viral infections and some viruses (e.g., Sindbis virus) that cause fatal disease in newborn mice cause no disease or only mild disease in older mice [3]. The age-dependent neurovirulence of alphavirus (Sindbis) infection in mice was recently shown to be related to an increased resistance to virus-induced apoptosis with neuronal maturation [3, 4]. Suckling mice infected with CVS by intracerebral inoculation developed progressive neurologic disease more rapidly than adult mice, and infection in suckling mice was associated with infection of many neurons throughout the brain. There were widespread and marked apoptotic changes in neurons assessed by both morphologic and biochemical criteria. More neurons underwent apoptotic cell death in suckling mice than in adult mice, and additional neuronal cell types were infected. Infection was demonstrated in neurons in the dentate gyrus of the hippocampus of suckling mice, and these neurons are only occasionally infected in adult mice [5]. In addition, these neurons showed morphologic changes of apoptosis and

positive TUNEL staining. Hence, a neuronal cell type that had a low susceptibility to rabies virus infection in adults was susceptible to infection in suckling mice and there was induction of apoptosis.

Similar to CVS infection of adult mice [7], infection of Purkinje cells was demonstrated with immunostaining, but there was no morphologic evidence of apoptosis or positive TUNEL staining in these neurons. This suggests that there may be induction of an inhibitor of apoptosis, possibly a protein of the Bcl-2 family, although *bcl-2* mRNA was not detected in rat Purkinje cells using *in situ* hybridization [2]. In a porcine hypoxia-ischemia model, Purkinje cells were observed to die by necrosis but never by apoptosis, while cerebellar granule cells were frequently apoptotic, but never necrotic [20]. Hence, Purkinje cells may be generally more resistant to apoptotic cell death than other neuronal cell types.

Double labeling of neurons for rabies virus antigen and TUNEL staining demonstrated that infected neurons actually underwent apoptosis in the suckling mouse model, which is similar to the situation in adult mice [7]. CVS infection of cultured rat prostatic adenocarcinoma (AT3) cells resulted in apoptosis [7], indicating that immune mechanisms are clearly not essential for rabies virus-induced apoptosis. However, immune and other indirect mechanisms may also be important in the induction of apoptosis. In the dentate gyrus of the hippocampus, rabies virus antigen was found predominantly in neurons in the outer layer, but morphologic changes and TUNEL staining of cells were not restricted to this distribution in the dentate gyrus, suggesting that indirect mechanisms may be important in the induction of apoptosis in this location.

Apoptosis, assessed by morphologic changes and positive TUNEL staining, was more extensive in the external granular layer of the cerebellum of CVS-infected mice than in uninfected control mice. During normal brain development, it has been recognized that nuclear DNA fragmentation occurs in both the external and internal granular layers of the cerebellum between postnatal days 5 and 9, which is an important time in the histogenesis of the cerebellum [19]. Since the number of viable granule cells that are generated and migrate to the internal granular layer must be regulated to match the number of Purkinje cells, Wood et al. [19] have speculated that this naturally occurring apoptotic cell death may tailor the granule cell population to match the Purkinje cell number prior to synaptogenesis. Consistent with this hypothesis, neonatal Borna disease virus infection of rats causes persistent infection of Purkinje cells, but not granule cells, and this is associated with loss of granule cells during cerebellar development [1]. Purkinje cells likely play an important role in the normal replication, maturation, and migration of granule cells during development [16] and we speculate that non-cytopathic viral infection of Purkinje cells may impair this normal function of these neurons, resulting in increased apoptosis in the external granular layer.

The Bax protein is a Bcl-2 family protein with proapoptotic activity [11]. Compared with uninfected control mice, increased immunostaining for the Bax protein was

◀ **Fig. 2** **A** Rabies virus antigen in the cerebral cortex (*left*) of a CVS-infected mouse. Note the absence of antigen in the periventricular germinal layer (*arrowhead*). **B** Viral antigen in multiple neurons in a trigeminal ganglion, indicating centrifugal spread of the virus. **C** TUNEL staining in multiple neurons in the cerebral cortex of an infected mouse. **D** Pyramidal neurons in the CA1 region of the hippocampus of a CVS-infected mouse showing multiple nuclear condensations of chromatin, which are typical features of apoptosis. **E** Multiple neurons in the dentate gyrus of the hippocampus showing chromatin condensations involving entire nuclei. **F** The external granular layer of the cerebellum of a CVS-infected mouse showing many cells with condensations of nuclear chromatin. **G** Nuclear chromatin condensations in multiple cells in the external granular layer of the cerebellum in an infected mouse. **H** External granular layer of the cerebellum of an uninfected mouse of the same age showing the absence of typical apoptotic morphology. **D–H** Cresyl violet staining. **A**  $\times 15$ ; **B**  $\times 90$ ; **C**  $\times 20$ ; **D**, **E**  $\times 340$ ; **F**  $\times 120$ ; **G**  $\times 285$ ; **H**  $\times 140$

observed in regions of the brain that demonstrated prominent apoptosis, including the cerebral cortex and pyramidal neurons of the hippocampus. We believe that the Bax protein and possibly other Bcl-2 family proteins may be important inducers of rabies virus-induced apoptosis in neurons in this model of experimental rabies.

The high neurovirulence of rabies virus in this suckling mouse model was associated with more widespread infection of neurons in the brain, including involvement of a greater number of neurons and neuronal cell types, and greater induction of apoptosis than in adult mice. Infection of all neuronal cell types is not associated with an equal propensity to undergo apoptotic cell death. It is unclear whether more efficient viral replication resulting in a greater viral load is important in the induction of apoptosis. Infected neurons undergo apoptosis and evidence from the present study suggests that indirect mechanisms also play a role in producing apoptosis in uninfected neurons. A greater understanding of the mechanisms responsible for the induction of apoptosis in rabies and other viral infections will be useful in the development of new therapeutic strategies for the management of these infections.

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## References

- Bautista JR, Rubin SA, Moran TH, Schwartz GJ, Carbone KM (1995) Developmental injury to the cerebellum following perinatal Borna disease virus infection. *Dev Brain Res* 90: 45–53
- Castren E, Ohga Y, Berzaghi MP, Tzimagiorgis G, Thoenen H, Lindholm D (1994) Bcl-2 messenger RNA is localized in neurons of the developing and adult rat brain. *Neuroscience* 61: 165–177
- Griffin DE, Levine B, Tyor WR, Tucker PC, Hardwick JM (1994) Age-dependent susceptibility to fatal encephalitis: alphavirus infection of neurons. *Arch Virol [Suppl]* 9: 31–39
- Griffin DE, Levine B, Ubol S, Hardwick JM (1994) The effects of alphavirus infection on neurons. *Ann Neurol* 35: S23–S27
- Jackson AC, Reimer DL (1989) Pathogenesis of experimental rabies in mice: an immunohistochemical study. *Acta Neuropathol* 78: 159–165
- Jackson AC, Rossiter JP (1997) Apoptotic cell death is an important cause of neuronal injury in experimental Venezuelan equine encephalitis virus infection of mice. *Acta Neuropathol* 93: 349–353
- Jackson AC, Rossiter JP (1997) Apoptosis plays an important role in experimental rabies virus infection. *J Virol* 71: 5603–5607
- Jackson AC, Wunner WH (1991) Detection of rabies virus genomic RNA and mRNA in mouse and human brains by using in situ hybridization. *J Virol* 65: 2839–2844
- Koprowski H (1996) The mouse inoculation test. In: Meslin FX, Kaplan MM, Koprowski H (eds) *Laboratory techniques in rabies*, 4th edn. World Health Organization, Geneva, pp 80–87
- Lewis J, Wesselingh SL, Griffin DE, Hardwick JM (1996) Alphavirus-induced apoptosis in mouse brains correlates with neurovirulence. *J Virol* 70: 1828–1835
- Merry DE, Korsmeyer SJ (1997) Bcl-2 gene family in the nervous system. *Annu Rev Neurosci* 20: 245–267
- Mims CA (1987) Host and microbial factors influencing susceptibility. In: *The pathogenesis of infectious disease*, 3rd edn. Academic Press, London, pp 270–295
- Oberhaus SM, Smith RL, Clayton GH, Dermody TS, Tyler KL (1997) Reovirus infection and tissue injury in the mouse central nervous system are associated with apoptosis. *J Virol* 71: 2100–2106
- Pekosz A, Phillips J, Pleasure D, Merry D, Gonzalez-Scarano F (1996) Induction of apoptosis by La Crosse virus infection and role of neuronal differentiation and human bcl-2 expression in its prevention. *J Virol* 70: 5329–5335
- Razvi ES, Welsh RM (1995) Apoptosis in viral infections. *Adv Virus Res* 45: 1–60
- Smeyne RJ, Chu T, Lewin A, Bian F, Crisman SS, Kunsch C, Lira SA, Oberdick J (1995) Local control of granule cell generation by cerebellar Purkinje cells. *Mol Cell Neurosci* 6: 230–251
- Smith AL, Barthold SW (1997) Methods in viral pathogenesis. In: Nathanson N, Ahmed R, Gonzalez-Scarano F, Griffin DE, Holmes K, Murphy FA, Robinson HL (eds) *Viral pathogenesis*. Lippincott-Raven, Philadelphia, pp 483–506
- Teodoro JG, Branton PE (1997) Regulation of apoptosis by viral gene products. *J Virol* 71: 1739–1746
- Wood KA, Dipasquale B, Youle RJ (1993) In situ labeling of granule cells for apoptosis-associated DNA fragmentation reveals different mechanisms of cell loss in developing cerebellum. *Neuron* 11: 621–632
- Yue X, Mehmet H, Penrice J, Cooper C, Cady E, Wyatt JS, Reynolds EOR, Edwards AD, Squier MV (1997) Apoptosis and necrosis in the newborn piglet brain following transient cerebral hypoxia-ischaemia. *Neuropathol Appl Neurobiol* 23: 16–25