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# Neuronal apoptosis does not play an important role in human rabies encephalitis

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**It is generally accepted that there are not prominent features of neuronal cell death in rabies encephalitis. However, Hemachudha and coworkers recently reported widespread apoptosis in the central nervous system of several human rabies cases *(BMC Infect Dis* 5: 104, 2005). In this study we have evaluated morphological features and markers of neuronal apoptosis in postmortem brain tissue from 12 cases of human rabies who died in four different countries. Histopathological analysis, TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling) staining, and immunostaining for cleaved (activated) caspase-3 were performed on paraffin-embedded tissues from the cerebral cortex, hippocampus, and brainstem, and additional regional areas from one of the cases. We did not find morphological evidence of neuronal apoptosis or TUNEL staining in any of the cases of rabies encephalitis. Similarly, immunostained cleaved caspase-3 was not seen in neurons, but prominent staining was observed in microglial processes. We conclude that neuronal apoptosis does not play an important pathogenetic role in human rabies encephalitis.** *Journal of NeuroVirology* (2008) **14,** 368–375.

**Keywords:** encephalitis; neurovirulence; pathogenesis; rabies; virulence

## Introduction

Rabies virus is a highly neurotropic virus that causes fatal encephalomyelitis in humans and animals (Hanlon *et al,* 2007; Jackson, 2007b). Many questions about the pathogenesis of rabies remain unanswered (Jackson, 2007a). Because neurons in human rabies cases typically do not show prominent morphological features of cell degeneration or death with routine histopathological analysis (Rossiter and Jackson, 2007), this has led to the concept that there is likely neuronal dysfunction without well-defined structural changes (Fu and Jackson, 2005; Jackson, 2007a). The basis for the neuronal dysfunction has remained elusive (Fu and Jackson, 2005; Jackson, 2007a). However, there is recent evidence in a mouse model of experimental rabies that structural changes in neuronal processes may underlie what was thought to be neuronal dysfunction without recognized morphological changes (Scott *et al*, 2008).

Hemachuda and coworkers have recently reported observations suggesting that extensive neuronal apoptosis in the central nervous system may play an important role in fatal human rabies. This was based on their finding of varying degrees of immunostaining for cytochrome *c* in the cytoplasm of neurons in the brain and spinal cord and widespread TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling) staining of neurons throughout the neuraxis in all of their cases (Juntrakul *et al*, 2005). In the present study, we have evaluated brain tissue sections from three different regional areas from 12 human rabies cases for evidence of neuronal apoptosis, using

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morphological assessment of hematoxylin and eosin–stained sections and two biochemical markers for apoptosis in tissue sections: TUNEL staining and immunohistochemical staining for activated (cleaved) caspase-3, a downstream executioner of the apoptotic process.

## Results

*Rabies virus antigen*
Immunoperoxidase staining for rabies virus antigen showed that sections prepared from all 33 paraffin blocks demonstrated staining in neurons. Heavy infection was seen in cerebellar Purkinje cells (Figure 1B), hippocampal pyramidal neurons (Figure 2B) and granular neurons in the dentate gyrus (Figure 2F), amygdalar neurons (Figure 3B), pyramidal neurons of the cerebral isocortex (Figure 4B), medullary neurons (Figure 4F), and in thalamic neurons (Figure 4L).

*Histopathology*
Detailed light microscopic examination (at magnifications of 400 × to 630 ×) of the hematoxylin and eosin–stained sections was undertaken independently by three of the investigators (A.C.J., E.R., J.P.R.) that included a neuropathologist (J.P.R.) with extensive experience in the identification of apoptotic cells. This analysis revealed a complete lack of morphological features of apoptosis (e.g., perikaryal shrinkage and karyorrhectic condensation of the nuclear chromatin) in neurons, including neurons with strong staining for rabies virus antigen. On many of the sections there were prominent foci of perivascular mononuclear inflammatory cell infiltration (Figure 4I), with collections of activated microglia in the surrounding neuropil.

*Caspase-3*
As a positive-control, strong immunostaining for cleaved caspase-3 was confirmed in human pontine neurons with apoptotic morphology in a case of perinatal hypoxic-ischemic brain injury (Figure 5E) (from case 5 by Rossiter *et al* [2002]). In all of the rabies cases, neuronal perikarya were uniformly immunonegative for caspase-3, despite prominent staining for rabies virus antigen. However, on sections from 19 of the 33 (58%) blocks from 9 of the 12 (75%) cases (cases 1 to 8 and 11), there was multifocal positive staining for caspase-3. This was found to be primarily localized in activated microglia, particularly in perivascular regions (Figure 3C and 4K). In order to confirm this, adjacent sections were stained for CD68, a monocyte/macrophage marker that also stains microglia. Examination of several fields from adjacent sections showed a very similar distribution of staining for caspase-3 and

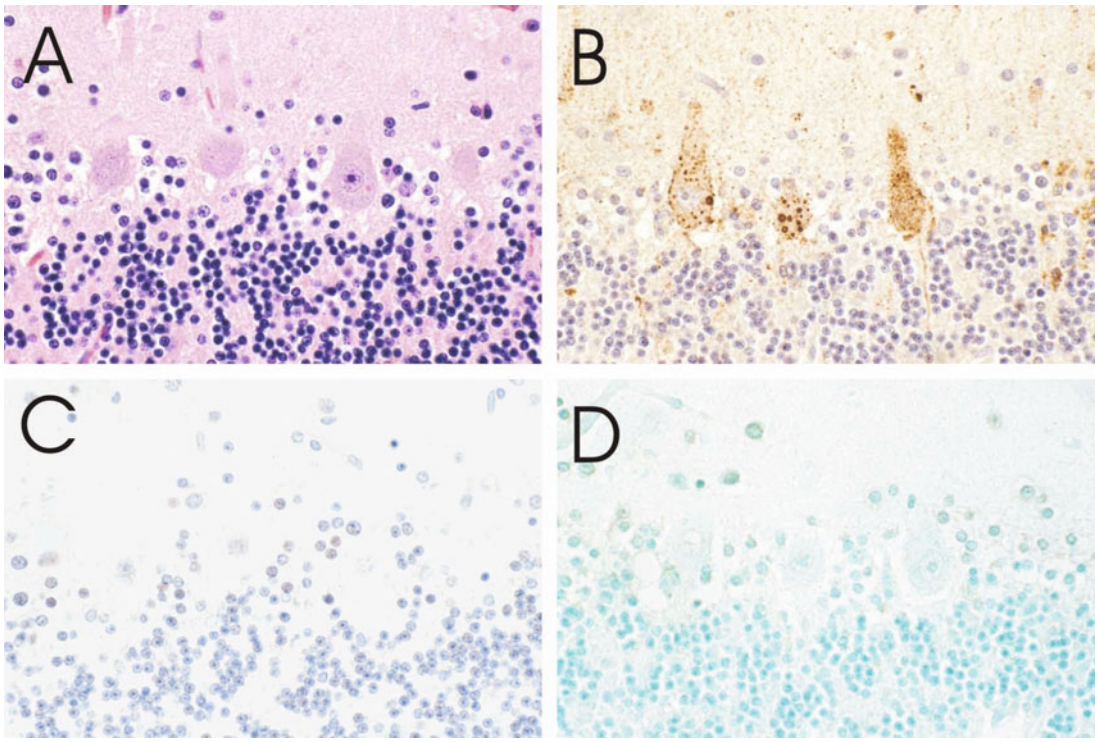


**Figure 1** Hematoxylin and eosin staining (**A**), immunostaining for rabies virus antigen (*B*) and caspase-3 (**C**), and TUNEL staining (**D**) in the cerebellum from case 2 due to a bat variant. Normal morphology (**A**) associated with strong expression of rabies virus antigen in Purkinje cells (**B**) and without expression of caspase-3 (**C**) or TUNEL staining (**D**). **A** to **D**, ×265.

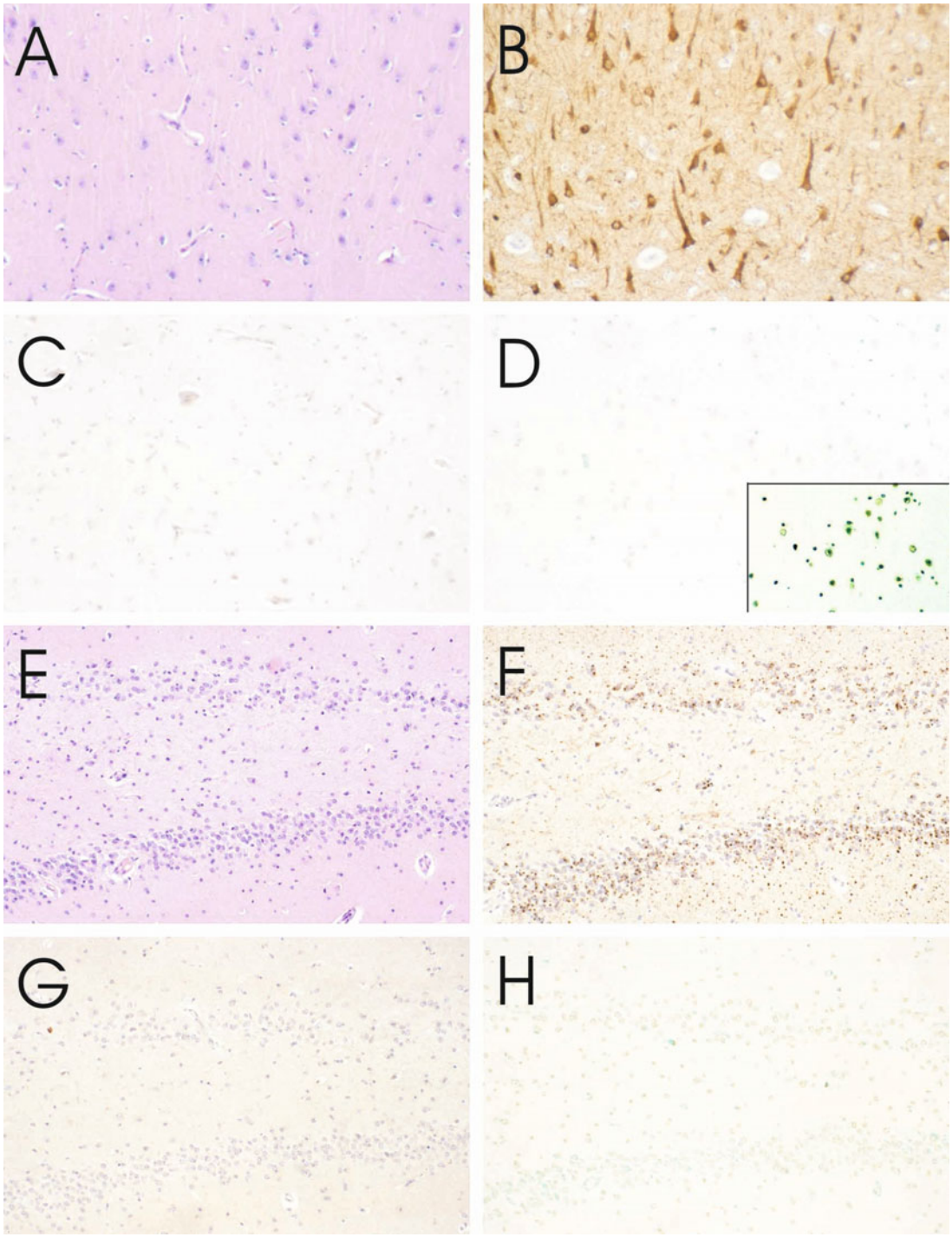


**Figure 2** Hematoxylin and eosin staining (**A, E**), immunostaining for rabies virus antigen (**B, F**) and activated caspase-3 (**C, G**), and TUNEL staining (**D, H**) in hippocampal pyramidal neurons (**A**–**D**) from case 2 and in dentate granular neurons (**E**–**H**) from case 5 and a DNase positive control for TUNEL staining on an adjacent section (**D**, *inset*). There is normal neuronal morphology (**A, E**) associated with strong expression of rabies virus antigen in neurons (**B, F**) associated with a lack of expression of caspase-3 (**C, G**) and negative TUNEL staining (**D, H**), with strong TUNEL staining on the positive control (**D**, *inset*). **A** to **H** and **D,** *inset*, ×80.

CD68 (Figure 5A to D), although CD68 more prominently stained the cell bodies of microglia (and also perivascular monocytes/macrophages) and caspase-3 more prominently stained the processes of microglia.

*TUNEL staining*

The DNase positive controls showed little TUNEL staining on all 13 tissue sections examined from all of the five cases from Thailand. Consequently, these cases were excluded from further analysis of TUNEL

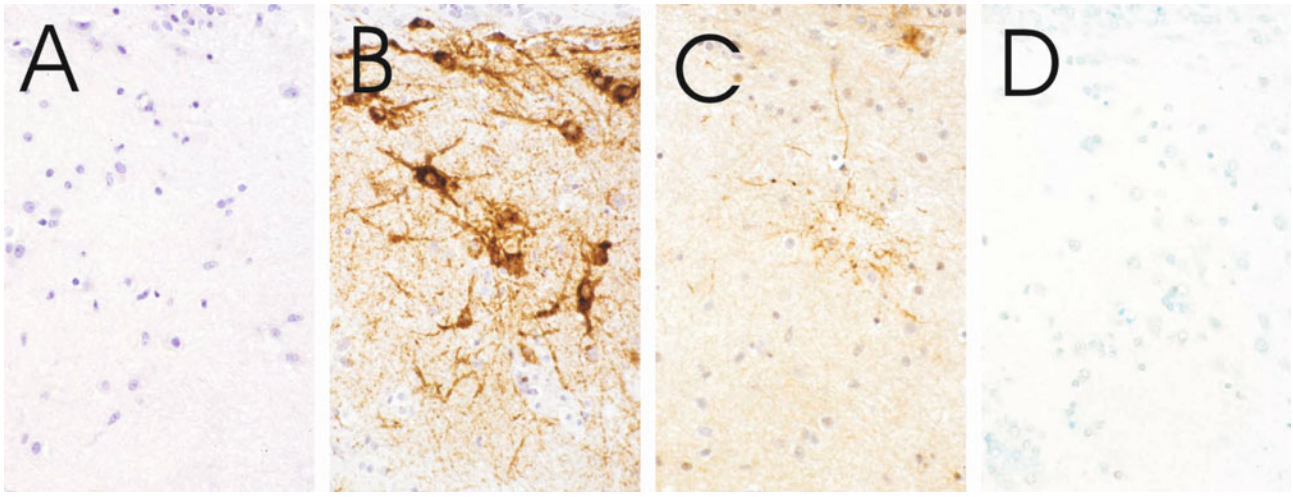


**Figure 3** Hematoxylin and eosin staining (**A**), immunostaining for rabies virus antigen (**B**) and caspase-3 (**C**), and TUNEL staining (**D**) in the amygdala of case 2. Normal neuronal morphology (A) associated with strong expression of rabies virus antigen in a group of neurons (**B**) and expression of caspase-3 in microglial processes (**C**) with negative TUNEL staining (**D**). **A** to **D**, ×170.

staining. DNase controls were positive on the 20 tissue sections from the other seven rabies cases. However, detailed microscopic examination of sections from the latter cases showed no evidence of TUNEL staining of neurons. As a positive internal control, there was TUNEL staining of scattered non-neuronal cells within the neuropil of these cases and also focally of apoptotic perivascular inflammatory cells. As a positive external control, there was positive TUNEL staining in human pontine neurons from a case (case 5) of perinatal hypoxic-ischemic brain injury (Figure 5F) reported by Rossiter *et al* (2002).

## Discussion

Most descriptions of the neuropathology of human rabies describe variable, but frequently mild, inflammatory changes and a paucity of ''degenerative-type'' changes in neurons, including morphological features of either apoptosis or necrosis (Perl and Good, 1991; Iwasaki and Tobita, 2002; Rossiter and Jackson, 2007). Neuronophagia may be present, but this is frequently not prominent or widespread. The relative lack of morphologic changes involving neurons combined with severe and fatal clinical disease has given rise to the concept that ''neuronal dysfunction'' must explain the severe disease in rabies (Fu and Jackson, 2005; Jackson, 2007a). However, the bases for this neuronal dysfunction have remained unexplained, and no consistent fundamental abnormality of neuronal function has been identified that explains the disease. Recently, structural abnormalities have been recognized involving neuronal processes of challenge virus standard CVS-infected transgenic mice that express the yellow fluorescent protein in a subpopulation of neurons (Scott *et al*, 2008). However, it is unknown whether these morphologic changes also occur in natural rabies or if they are limited to certain experimental models and are dependent on the viral strain, host, or possibly other factors.

Neuronal apoptosis was first reported in rabies in adult mice infected with CVS by the intracerebral route of inoculation, and involves multiple regional brain areas, including the cerebral cortex, hippocampus, diencephalon, and brainstem (Jackson and Rossiter, 1997). Neuronal apoptosis is even more extensive in CVS-infected young mice (Jackson and Park, 1998) and is also prominent with *in vitro* infection in CVS-infected non-neuronal cells (Jackson and Rossiter, 1997), neuroblastoma cells (Theerasurakarn and Ubol, 1998), and in primary neurons (Morimoto *et al*, 1999; Weli *et al*, 2006). Neuronal apoptosis has not been observed in neurons of street virus–infected mice with a bat rabies virus variant (Yan *et al*, 2001; Sarmento *et al*, 2005). TUNEL staining was reported in foci of brainstem and hippocampal neurons in a human patient with rabies who had the acquired immunodeficiency syndrome (Adle-Biassette *et al*, 1996), but there have not been other reports indicating apoptosis of neurons in human rabies until the recent report by Juntrakul *et al* (2005). In a series of 10 cases, they observed cytoplasmic cytochrome *c* immunoreactivity in neurons in many regions of the central nervous system (CNS) with relative sparing of the spinal cord, despite the presence of abundant rabies virus antigen within the cord. The cytoplasmic cytochrome *c* signal was interpreted as evidence of mitochondrial outer membrane permeabilization, an important feature of the mitochondrial pathway of apoptotic cell death. In addition, numerous TUNEL-labeled cells were observed throughout the neuraxis (Juntrakul *et al*, 2005). However, the TUNEL assay

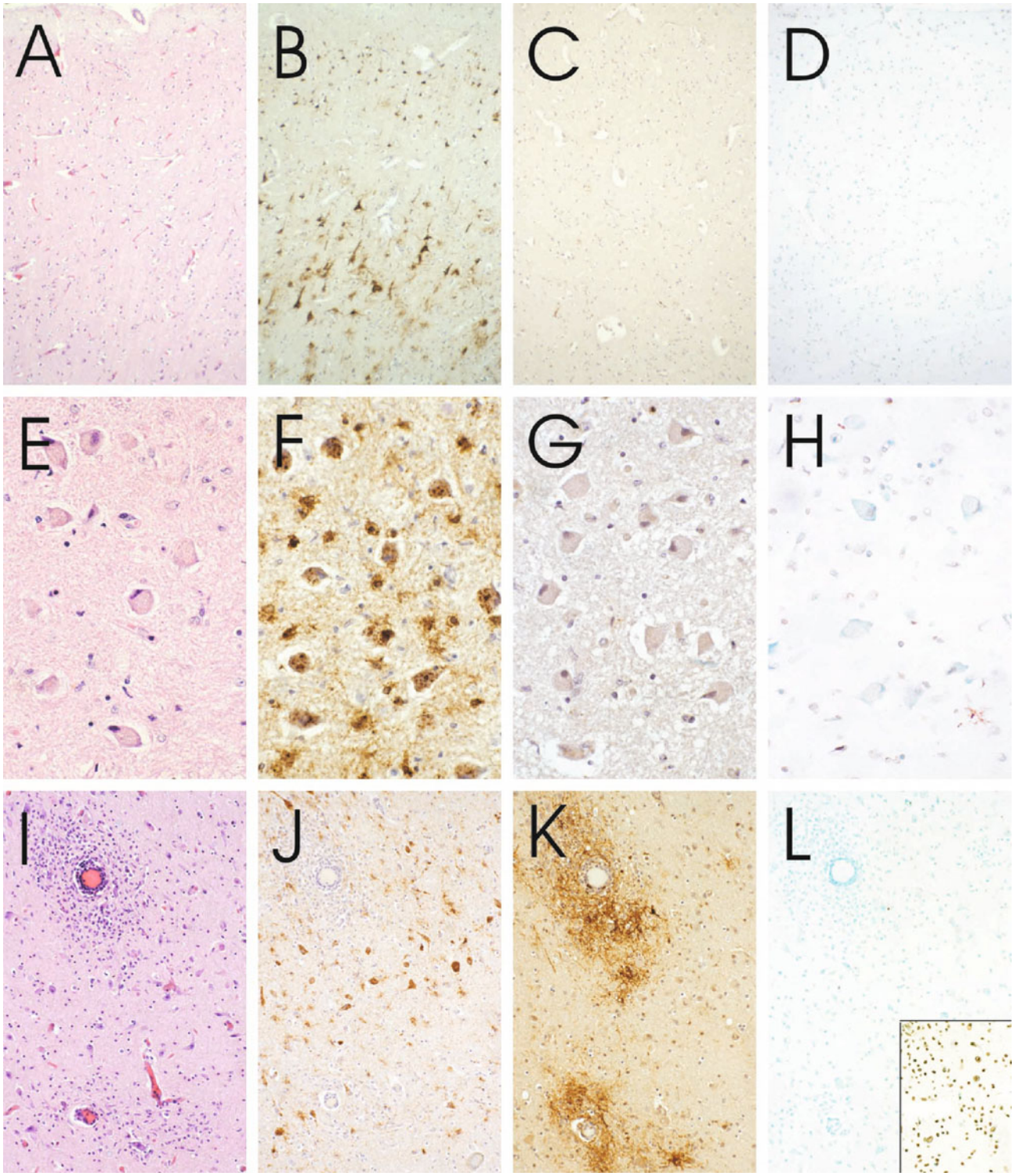


**Figure 4** Hematoxylin and eosin staining (**A, E, I**), immunostaining for rabies virus antigen (**B, F, J**) and caspase-3 (**C, G, K**), and TUNEL staining (**D, H, L**) in the cerebral cortex (**A**–**D**) and inferior olivary nucleus of the medulla (**E**–**H**) from case 1 and from the thalamus (**I**–**L**) from case 2 and a DNase positive control on an adjacent section (**L**, *inset*). These images show normal neuronal morphology in the cerebral cortex (**A**) and inferior olivary nucleus (**E**) and perivascular mononuclear infiltrates in the thalamus (**I**). There is strong expression of rabies virus antigen in neurons in the cerebral cortex (**B**), inferior olivary nucleus (**F**), and thalamus (**J**). There is lack of expression of activated caspase-3 in the cerebral cortex and inferior olivary nucleus, but strong expression in a perivascular distribution in the thalamus (**K**). TUNEL staining is negative in the cerebral cortex (**D**), medulla (**H**), and thalamus (**L**) with strong staining on the positive control (**L**, *inset*). **A** to **D**, $\times 45$; **E** to **H**, $\times 205$; and **I** to **L** and **L**, *inset,* $\times 80$.

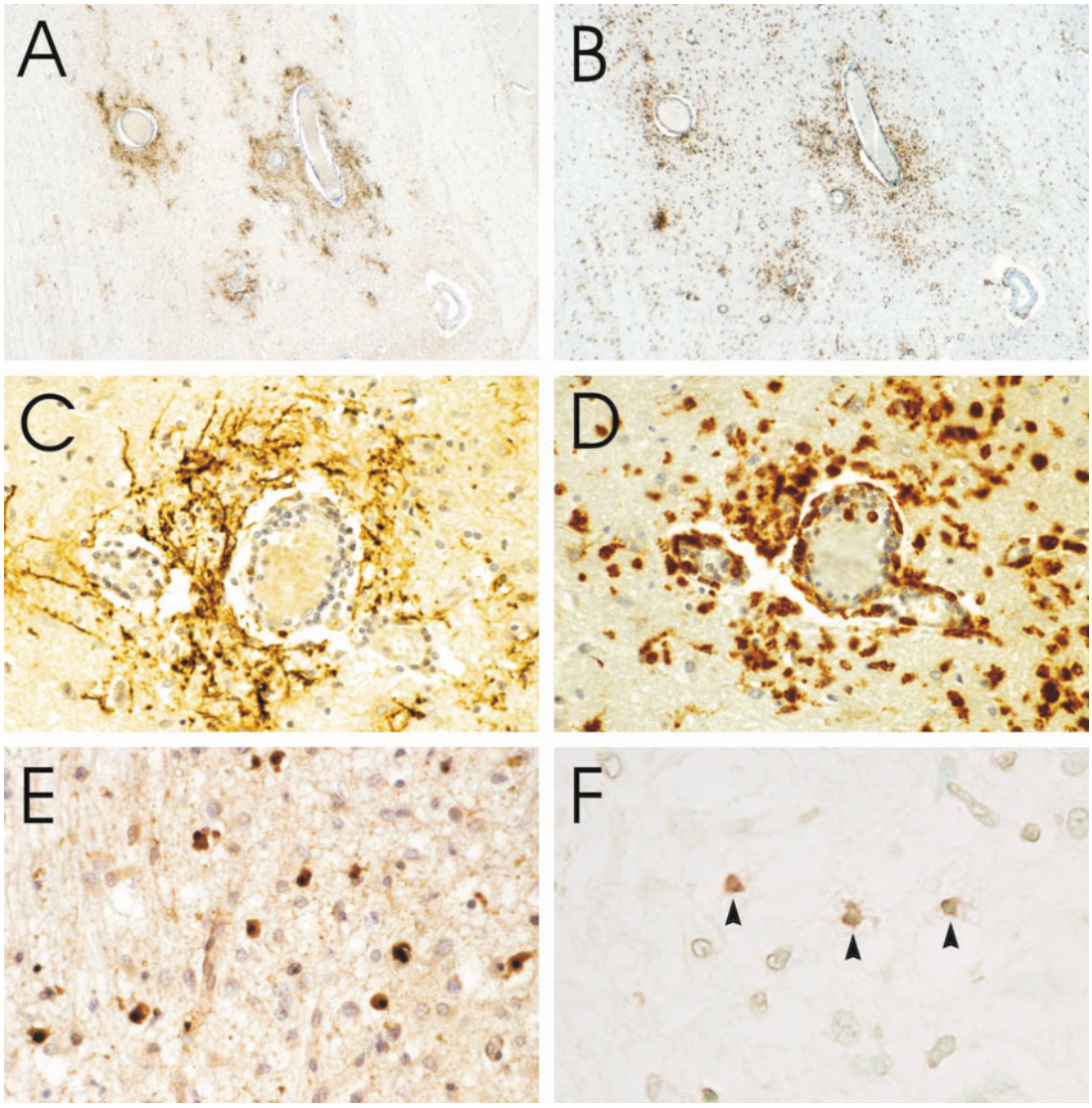


**Figure 5** Immunostaining for caspase-3 (**A** and **C**) and CD68 (**B** and **D**) on adjacent sections of the midbrain of case 5. Caspase-3 immunostaining (**E**) and TUNEL staining (**F**) in the pons of a positive-control case. There is strong perivascular staining for caspase-3 (**A** and **C**) that correlates well with the distribution of staining for CD68 (**B** and **D**). Microglial processes are more prominently stained with caspase-3 (**C**). Caspase-3 immunostaining (**E**) and TUNEL staining (**F**) are positive in apoptotic neurons (*arrowheads* in **F**) in positive-control sections of perinatal hypoxic-ischemic brain injury (from case 5 by Rossiter *et al* [2002]). **A, B**, × 30; **C, D**, × 240; **E** × 215; **F**, × 350.

has a number of limitations, including the potential for false-positive staining (Watanabe *et al*, 2002). Furthermore, apoptotic cell death cannot be reliably diagnosed in the absence of morphological features such as cell shrinkage, nuclear karyorrhexis, and formation of apoptotic bodies, and these features were not described by Juntrakul *et al* (2005).

It has been recognized that less neurovirulent strains of rabies virus are stronger inducers of apoptosis than more neurovirulent strains in primary neuron cultures (Morimoto *et al*, 1999) and also *in vivo* in young mice after peripheral inoculation (Jackson *et al*, 2006). Neuronal apoptosis probably serves as a protective mechanism by the host to reduce viral spread and dissemination in the host, and adaptive immunity is likely not important in this process (Rutherford and Jackson, 2004). In mature mice this protective mechanism plays an important role in preventing viral invasion into the nervous system. With intracerebral inoculation the normally neuroprotective mechanisms actually result in widespread neuronal apoptosis (Jackson and Rossiter, 1997; Jackson and Park, 1998).

The present study fails to demonstrate morphological features of neuronal apoptosis, TUNEL staining, or activated caspase-3 immunostaining of neurons in the brains of fatal cases of human rabies encephalitis. Hence, it is unlikely that significant

numbers of CNS neurons undergo apoptosis and result in clinical features of rabies or play an important role in the fatal outcome of the disease.

The observation of activated caspase-3 expression in microglial processes in this study was unexpected. In human immunodeficiency virus-1 encephalitis, TUNEL-stained and caspase-3–immunoreactive microglia were observed, but there were associated TUNEL-stained and caspase-3–positive neurons, which made it difficult to determine whether the microglia were actually undergoing apoptosis or phagocytosing caspase-3 and fragmented nuclear DNA (James *et al*, 1999). Phagocytosis of material of neuronal origin was not likely to be important in microglial expression of caspase-3 in the rabies cases in the present study, so the significance of this observation is unclear, especially because the great majority of these cells was TUNEL negative. However, the absence of such staining in microglia in sections from 3 of the 12 cases (25%) indicates that is unlikely to be an essential feature of rabies pathogenesis.

In summary, based on the present findings we conclude that neuronal apoptosis is not prominent in the brains of humans with fatal rabies encephalitis and it is unlikely that this process plays an important role in the clinical disease or fatal outcome. The finding of caspase-3 expression in microglial processes requires further study.

## Materials and methods

*Preparation of tissue sections*

Tissue sections (5 to 6 μm) were prepared from archived formalin-fixed paraffin-embedded blocks of the cerebral cortex ($n = 10$), hippocampus ($n = 7$), and brainstem ($n = 13$) from 12 postmortem cases of human rabies from four countries (Table 1). In addition, tissue sections from the cerebellum, thalamus, and amygdala were evaluated from case 2 (total of 33 blocks evaluated).

*Immunostaining*

Immunoperoxidase staining was performed on brain sections for rabies virus antigen using mouse anti-rabies virus nucleocapsid monoclonal antibody (5DF12; obtained from A. I. Wandeler, Centre of Expertise for Rabies, Canadian Food Inspection Agency, Nepean, Ontario, Canada) at a 1:160 dilution (Jackson *et al*, 2007) and for cleaved (activated) caspase-3 using a polyclonal rabbit antibody (Cell Signaling Technology, Danvers, MA; catalog no. 96611) at a 1:200 dilution using a standard avidin-biotin complex ABC technique, as previously described (Rasalingam *et al*, 2005). Using the same methodology, we previously demonstrated specific caspase-3 immunostaining in many apoptotic brain neurons in an experimental model of rabies in mice (Rasalingam *et al*, 2005). Immunoperoxidase staining for the monocyte/macrophage marker CD68 (mouse monoclonal M0814, clone KP1, DAKO, at a 1:100 dilution) was also performed on selected serial sections of brain that showed positive staining for caspase-3.

*TUNEL staining*

Paraffin-embedded tissue sections were deparaffinized and rehydrated, and then heated in a microwave for 1 min at high power and 9 min at medium power in citrate buffer (pH 3). Sections were successively treated with 15 μg/ml proteinase K (Sigma), 3% $H_2O_2$, 1 × blocking solution (Boehringer Mannheim), TdT buffer (5 × buffer is 1 M sodium cacodylate and 150 mM Tris, pH 6.6 with bovine serum albumin), TdT enzyme solution (20 μl 5 × TdT buffer, 4 μl 25 mM $CoCl_2$, 0.24 μl biotin-16-dUTP [Roche], 75.72 μl $dH_2O$, and 0.04 μl TdT enzyme [Roche]), 300 mM sodium chloride, 30 mM sodium citrate (pH 7.2), avidin-biotinylated horseradish peroxidase complex, and 3,3-diaminobenzidine tetrachloride with 0.01% $H_2O_2$. Slides were counterstained with methyl green. Pretreatment with 1 μg/ml DNase was used as a positive control.

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

**Table 1** Human rabies cases

| Case no. | Country of death | Age of patient | Variant | Reference |
|---|---|---|---|---|
| 1 | United States (NY) | 54 | dog | (Van Fossan *et al,* 2000) |
| 2 | United States (CA) | 49 | bat | (Van Fossan *et al,* 2000) |
| 3 | United States (VA) | 25 | raccoon | (Silverstein *et al,* 2003) |
| 4 | Canada (QC) | 9 | bat | (Turgeon *et al,* 2000) |
| 5 | Mexico | 8 | dog | (Jackson *et al,* 2001) |
| 6 | Mexico | 4 | dog | Unpublished |
| 7 | Mexico | 8 | dog | (Jackson and Lopez-Corella, 1996) |
| 8 | Thailand | 9 | dog | (Juntrakul *et al,* 2005) |
| 9 | Thailand | 15 | dog | (Juntrakul *et al,* 2005) |
| 10 | Thailand | 81 | dog | (Juntrakul *et al,* 2005) |
| 11 | Thailand | 55 | dog | (Juntrakul *et al,* 2005) |
| 12 | Thailand | 61 | dog | (Juntrakul *et al,* 2005) |

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