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Short Communication

Expression of Toll-like receptor 3 in the human cerebellar cortex in rabies, herpes simplex encephalitis, and other neurological diseases

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There is recent *in vitro* evidence that human neurons express the innate immune response receptor, Toll-like receptor-3 (TLR-3), and that expression is enhanced in viral infections. The authors examined the immunohistochemical expression of TLR-3 in the cerebellar cortex of postmortem human brains. Purkinje cells were found to express TLR-3 in all cases of rabies (4 of 4) and herpes simplex encephalitis (2 of 2) as well as in cases of amyotrophic lateral sclerosis (1 of 2), stroke (1 of 2), and Alzheimer's disease (3 of 3). In cases of viral infection, direct viral infection was not necessary for enhanced neuronal TLR-3 expression, suggesting that soluble factors likely play an important role in inducing TLR-3 expression. In addition to neurons, occasional Bergmann glia expressed TLR-3 in some cases. This study has provided evidence that human brain neurons can express TLR-3 *in vivo* and suggests that neurons may play an important role in initiating an inflammatory reaction in a variety of neurological diseases. *Journal of NeuroVirology* (2006) **12,** 229–234.

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The innate immune response is an early line of defense against microbes that precedes the adaptive immune response. It is characterized by the production of inflammatory cytokines and chemokines, complement activation, and attraction of macrophages, neutrophils, and natural killer (NK) cells into infected tissues. In the case of a viral infection, cells sense the infection by detecting viral proteins (TenOever *et al*, 2004) and/or nucleic acids, and, in particular, double-strand RNA (dsRNA), which is a by-product of the replicative cycle of many viruses (Jacobs and Langland, 1996; Karpala *et al*, 2005). Viral proteins and dsRNA are recognized through receptors, including the evolutionarily conserved Toll-like receptors (TLRs) (Finberg and Kurt-Jones, 2004; Takeda and Akira, 2004). Of the 10 TLRs identified in humans, TLR-3 has been shown to respond to dsRNA (Alexopoulou *et al*, 2001; Finberg and Kurt-Jones, 2004). The signaling pathway triggered by TLR-3 leads to interferon (IFN)-regulatory factor 3 (IRF3) phosphorylation and nuclear factor (NF)-κB activation (Sato *et al*, 2003; Sen and Sarkar, 2005). This induces inflammatory cytokines (tumor necrosis factor [TNF]-α, interleukin [IL]-6, and IL-1α) and chemokines (CCL-5 and CXCL-10) and also activation of the IFN-ß promoter for IFN expression. INF responses are important not only for their antiviral activity, but also because of the links between innate and adaptive immunity (Le Bon and Tough,

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2002). TLR-3 expression is up-regulated by IFN type 1 (Miettinen *et al*, 2001; Siren *et al*, 2005), which is produced in viral infection and in neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease (Yamada *et al*, 1994).

To date glia have been identified as the main producers of TLR (Farina *et al*, 2005; Jack *et al*, 2005) and initiators of inflammation in the central nervous system (CNS). In response to pathogens, glial cells dynamically and differentially regulate TLR gene activation (McKimmie and Fazakerley, 2005). We have recently reported TLR-3 expression in human neurons *in vitro* (Prehaud *et al*, 2005). NT2-N cells are pure populations of terminally differentiated human postmitotic cells with many properties similar to human CNS neurons (Pleasure *et al*, 1992). We have established that cultured NT2-N cells express TLR-3 and mount an innate immune response with the production of IFN-β, chemokines, and inflammatory cytokines in response to rabies virus infection, IFN-β, or dsRNA [poly(I:C)] (Prehaud *et al*, 2005). Hence, there is evidence that human neurons, even in the absence of astrocytes, oligodendrocytes, and microglia, have the machinery to sense and initiate innate immune responses and to react to IFN-ß. We have now investigated the neuronal expression of TLR-3 in postmortem brain tissues in cases of infection with neurotropic viruses (rabies and herpes simplex encephalitis) as well as a variety of other neurological disorders and control case material.

Tissue sections (5 to 6 μm) were prepared from archived formalin-fixed paraffin-embedded blocks of cerebellar cortices from four postmortem cases of human rabies and two cases of herpes simplex encephalitis, which were obtained from sources as indicated in Table 1. Sections were also prepared

Table 1 Neurological diagnosis, age, and source of postmortem brain tissues

Neurological diagnosis	Age of patient	Country of origin	Reference
Rabies	43	Canada	Picard, 1984; Webster *et al*, 1985
Rabies	13	Mexico	Lopez-Corella and Jackson, 1996
Rabies	8	Mexico	Jackson *et al*, 2001
Rabies	81	Thailand	
Herpes simplex encephalitis	14	Canada	Jackson *et al*, 2002
Herpes simplex encephalitis	94	Canada	
Cardiac arrest	77	Canada	
Cardiac arrest	55	Canada	
Amyotrophic lateral sclerosis	64	Canada	
Amyotrophic lateral sclerosis	75	Canada	
Stroke	46	Canada	
Stroke	75	Canada	
Alzheimer's disease	87	Canada	
Alzheimer's disease	88	Canada	
Alzheimer's disease	81	Canada	

from two cases of sudden death due to cardiac arrest, two cases of amyotrophic lateral sclerosis, two cases of stroke (cerebral infarction) in another vascular territory, and three cases of Alzheimer's disease (Table 1). Tissue sections from rabies cases were deparaffinized, hydrated, and were successively reacted with 1% hydrogen peroxide in methanol (30 min), mouse monoclonal anti-rabies virus nucleocapsid protein immunoglobulin G (IgG) (obtained from Dr. Reto Zanoni, Institute of Veterinary Virology, Bern, Switzerland) diluted 1:40 in 2% normal goat serum in phosphate-buffered saline (PBS) (15 h), DakoCytomation EnVision + System horseradish peroxidase (HRP)-labeled polymer anti-mouse (K4000) (DakoCytomation, Glostrup, Denmark) (40 min), 3,3′-diaminobenzidine tetrachloride (Sigma FAST DAB; Sigma-Aldrich, St. Louis, MO, USA), and the slides were lightly counterstained with Mayer's hematoxylin and examined using a Leica DM 5000B microscope equipped with a DC 300FX camera. Images were processed with Leica FW 4000 software. Tissues from cases without rabies were used in order to exclude nonspecific staining.

For TLR-3 immunoperoxidase staining tissue sections were deparaffinized, hydrated, and then heated in a microwave for 1 min at high power, followed by 9 min at medium power in 0.01 M sodium citrate buffer (pH 6.0) for antigen unmasking. Sections were successively reacted with 5% normal goat serum (20 min), 1% hydrogen peroxide in methanol (30 min), polyclonal rabbit anti-TLR3 (H-125) (sc-10740; Santa Cruz Biotechnology, Santa Cruz, CA, USA), which has previously validated specificity in transfected cultured cells (Guillot *et al*, 2005), diluted 1: 100 in PBS (15 h), DakoCytomation EnVision + System HRP-labeled polymer anti-rabbit (K4002) (40 min), 3,3′-diaminobenzidine tetrachloride (Sigma FAST DAB), and the slides were lightly counterstained with Mayer's hematoxylin and examined by light microscopy (see above). An irrelevant primary antibody, *Helicobacter pylori* Ab-1 (Lab Vision Corporation, Fremont, CA, USA) was substituted on a section from every case with TLR-3 expression at the same protein concentration (2.0 μg/ml) in order to exclude nonspecific staining. Each of the slides was masked and slides stained for TLR-3 and the irrelevant primary antibody were graded on a scale of 0 (no staining) to 10 (marked staining intensity) in Purkinje cells. Enhanced staining was noted when the TLR-3–stained slides were at least three grades higher than irrelevant primary antibody-stained slides.

Immunohistochemical staining of TLR-3 in cardiac arrest cases

Staining was absent (Figure 1A) or showed only very low levels of signal in the perikarya of Purkinje cells and was absent in other cell types in the two cardiac arrest cases.
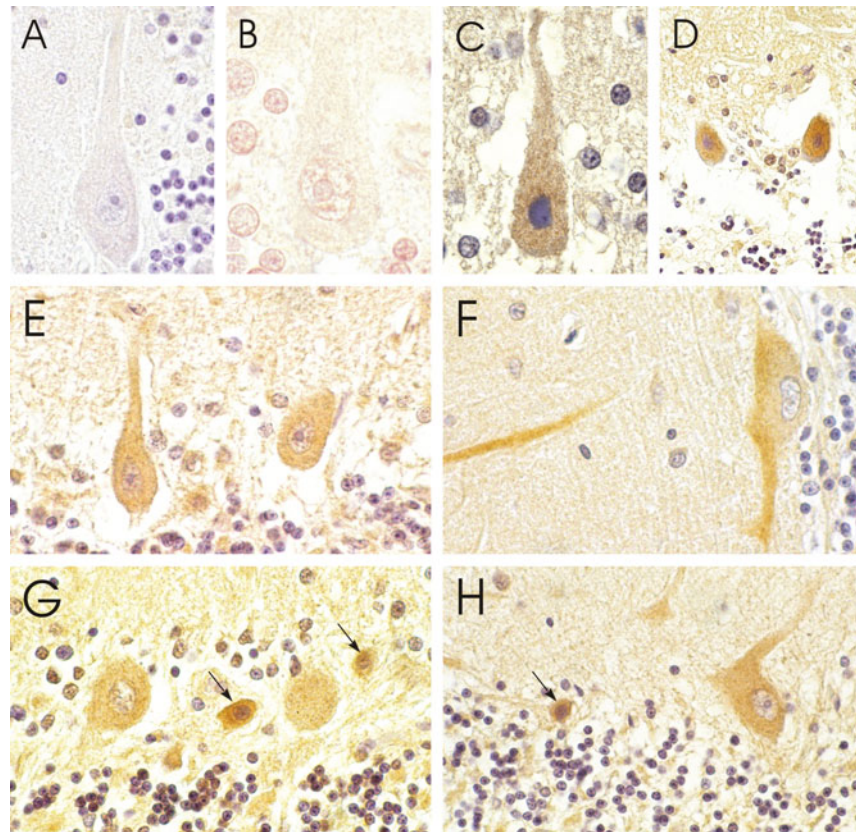
Figure 1 A cardiac arrest case showed no staining of a Purkinje cell for TLR-3 (**A**). An irrelevant primary antibody showed no staining of a Purkinje cell from a rabies case (**B**). Purkinje cells show moderate staining for TLR-3 in perikarya and proximal dendrites in a rabies case (**C, D**). Another rabies case (same case as in **B**) shows TLR-3 expression in Purkinje cells (**E**). A case of herpes simplex encephalitis shows moderate TLR-3 staining in the perikaryon of a Purkinje cell and also in a dendritic process (left of field) in the molecular layer (**F**). In stroke (**G**) and Alzheimer's disease (**H**) cases there is TLR-3 expression in Purkinje cells and Bergmann glia (*arrows*) (**G**). Immunoperoxidase-hematoxylin; **A**, ×140; **B**, ×530; **C**, ×260; **D**, ×300; **E**, ×275, **F**, ×200; **G**, ×205; **H**, ×220.
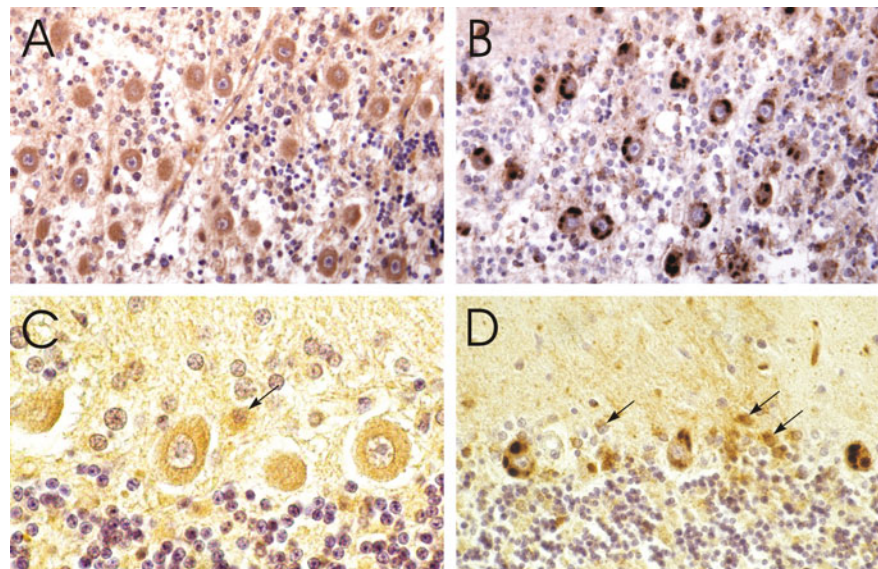

Figure 2 A field of Purkinje cells expresses TLR-3 (**A**) and another section shows expression of rabies virus antigen in a nearby field (**B**), providing indirect evidence that infected Purkinje cells can express TLR-3. Purkinje cells and a Bergmann glial cell (*arrow*) stain for TLR-3 in a rabies case (**C**). Rabies virus antigen is also, observed in Purkinje cells and Bergmann glia (*arrows*) (**D**). Immunoperoxidase-hematoxylin; **A**, **B**, ×85; **C**, ×240; **D**, ×125.

Immunohistochemical staining of TLR-3 in rabies encephalitis

Moderately intense staining for TLR-3 was observed in the perikarya and proximal dendrites of the majority of the Purkinje cells (Figures 1C–E and 2A and C) in two of the rabies cases, with mild staining for TLR-3 in the other two rabies cases. There was some variability in the intensity of staining in Purkinje cells within each cerebellum. Some TLR-3–immunoreactive Purkinje cells showed cytoplasmic shrinkage and shrunken hyperchromatic nuclei, suggestive of ischemic neuronal injury (Figure 1C and D). Two of the four rabies cases (with moderate staining of Purkinje cells) also showed staining for TLR-3 in a minority of Bergmann glia (Figure 2C). There was low background staining with the irrelevant antibody with a mean grade of 1.3 of 10 and a range of 0 to 3 of 10 (Figure 1B).

Immunohistochemical staining of rabies virus antigen in rabies encephalitis

There was prominent rabies virus antigen staining in the great majority of Purkinje cells in three of the four cases (Figure 2B and D). In two of these three cases, rabies virus was also observed in many Bergmann glia (Figure 2D). Both of these cases showed TLR-3 staining in Bergmann glia (see above). One case, in which rabies virus antigen was limited to rare Purkinje cells in more superficial sections of the paraffin block showed no rabies virus antigen in adjacent sections immediately before and after a section showing moderate TLR-3 staining of many Purkinje cells.

Immunohistochemical staining of TLR-3 in herpes simplex encephalitis

Staining was observed in Purkinje cells in both cases, which was typically mild and occasionally moderate in intensity (Figure 1F). One of the two cases also showed mild staining of occasional Bergmann glia.

Immunohistochemical staining of TLR-3 in amyotrophic lateral sclerosis, stroke, and Alzheimer's disease cases

One of the two cases of amyotrophic lateral sclerosis showed mild staining of Purkinje cells, without staining in other cell types. Mild to moderate staining for TLR-3 was present in many Purkinje cells and occasional Bergmann glia in one of the two stroke cases (Figure 1G) and in all three cases with Alzheimer's disease (Figure 1H). Each of the three cases of Alzheimer's disease also showed mild to moderate expression of TLR-3 in occasional Bergmann glia (Figure 1H).

In this study of human cerebellar cortex from 15 postmortem cases, we provide evidence for the first time that human brain neurons and glial cells can express TLR-3 in neuropathological conditions. The finding of low intensity immunohistochemical staining for TLR-3 in rare Purkinje cells of the cardiac arrest cases is consistent with a low level of basal expression in the normal brain. Before this investigation the mouse brain was reported to express TLR transcripts, including TLR-3, and TLR-3 expression was markedly enhanced in mice infected with rabies virus or with Semliki Forest virus (McKimmie *et al*, 2005; Wang *et al*, 2005), but the nature of the cells expressing TLR-3 was not described. After a first *in vitro* report indicating that mouse fetal neurons can express TLR-2 (Kurt-Jones *et al*, 2004), only one report has indicated that human neurons express TLR-3 *in vitro* both as transcripts and protein (Prehaud *et al*, 2005). Thus the present work fully establishes that human brain neurons can express TLR-3 *in vivo* in pathological conditions such as rabies and herpes simplex encephalitis and also in neurological diseases with an inflammatory component, including stroke and Alzheimer's disease. TLR-3 is a type I integral membrane protein located in intracellular endosomal membranes (Matsumoto *et al*, 2003). We showed here that TLR-3 expression was located in the cytoplasm of human neurons, which is consistent with the previous observations in cultivated neurons (Prehaud *et al*, 2005) and dendritic cells (Matsumoto *et al*, 2003) or transfected cells (Sen and Sarkar, 2005). In addition to Purkinje cells, we have observed that some Bergmann glia expressed TLR-3 in the same pathological conditions. This is consistent with the previous *in vitro* finding that fetal astrocytes express TLR-3 (Farina *et al*, 2005; Jack *et al*, 2005) and the observation of enhanced glial expression of TLR-3 in multiple sclerosis white matter lesions (Bsibsi *et al*, 2002).

In human viral encephalitides the expression of TLR-3 was increased in Purkinje cells in the brains of cases with rabies and herpes simplex encephalitis. Enhanced TLR-3 expression was observed in Purkinje cells in locations where virtually all of the cells expressed rabies virus antigen (Figure 2), providing strong indirect evidence that infected neurons can express TLR-3. This observation is supported by our previous *in vitro* studies in which rabies virus-infected NT2-N cells were shown to express TLR-3 (Prehaud *et al*, 2005). However, uninfected Purkinje cells in rabies also exhibited moderate expression of TLR-3, strongly implicating indirect mechanisms rather than a requirement for direct viral infection of neurons to stimulate the expression of TLR-3. This observation was facilitated by the study of a rabies case with TLR-3 expression in Purkinje cells in which an unusually low proportion of Purkinje cells were infected (LopezCorella and Jackson, 1996) and of two cases of herpes simplex encephalitis, in which the cerebellum is not directly involved in the viral

infection. Purkinje cells showed increased expression of TLR-3 in herpes simplex encephalitis, albeit less marked than in rabies. In rabies virus infection there is a primary INF response in which INF-β is released, and a secondary response follows by the secreted INF-β through INF-α/β receptors and from activation of transcription of genes containing INF-stimulated response elements in their promoters, including TLR-3 (Miettinen *et al*, 2001). Furthermore, the findings strongly implicate that one or more soluble factors are likely responsible for stimulating TLR-3 expression in uninfected neurons. These factors may be produced by infected cells in other brain areas. INF-β is one important candidate for stimulating TLR-3 expression in rabies encephalitis. This possibility is supported by the observation of McKimmie *et al.* (2005) that TLR-3 transcript expression in the mouse brain required an intact INF-α/β response in Semliki Forest virus infection. However, INF is likely not the only modulating factor involved with TLR-3 expression by Purkinje cells in the herpes simplex encephalitis cases. Differing innate immune responses developed *in vitro* to rabies virus and herpes simplex virus type 1 (HSV-1) infections, in which rabies virus infection resulted in production of INF-β and TNF-α, whereas HSV-1 infection did not (Prehaud *et al*, 2005).

What could be the function of TLR3 in the brain? As we have shown in cultures of human postmitotic neurons (Prehaud *et al*, 2005), TLR-3 could play a role as an immune molecule, sensing viral infection and initiating inflammatory, chemoattractive, and antiviral responses. Enhanced expression of TLR-3 by infected neurons in rabies could potentially play a role in promoting neuronal survival by effectively limiting the viral burden. Alternatively, the inflammatory response may contribute to neuronal damage. However, neuronal death is not prominent in natural rabies despite widespread infection of neurons in the CNS (Jackson, 2002), and rabies encephalitis usually shows relatively modest inflammatory changes, considering the typically high burden of neuronal infection (Iwasaki and Tobita, 2002). This suggests that a subtle balance and control of innate immune responses exist in the rabies virus infected brain (Conzelmann, 2005; Wang *et al*, 2005). How TLR-3 signaling is modulated during viral encephalitides and, in particular, in nonfatal viral encephalitides, is a point that deserves future attention. In contrast, inflammation is an important pathological characteristic of amyotrophic lateral sclerosis, stroke, and Alzheimer's disease (McGeer and McGeer, 2002a, 2002b, 2004). TLR-3 expression in Purkinje cells and also in glial cells in some of the cases with amyotrophic lateral sclerosis, stroke, and Alzheimer's disease could be consistent with the inflammatory component in these diseases.

TLR-3 may also have functions other than in immunosurveillance, which could be relevant at a late time point with expression of TLR-3 in infected or inflamed neurons. Neurons express classical major histocompatibility complex (MHC) class I (Boulanger and Shatz, 2004) subunits of the MHC receptor such as CD3 ζ chain (Corriveau *et al*, 1998) and TCR β chain (Huh *et al*, 2000). They also express nonclassical MHC class Ib molecules, including H-2M (Loconto *et al*, 2003; Ishii *et al*, 2003), Qa-1 (Huh *et al*, 2000), and HLA-G (Maier *et al*, 1999; Lafon *et al*, 2005). A role for some of these "immune molecules" has been assigned in the development and organization of neuronal networks, in synaptic organization, and also in pheromone perception (Boulanger and Shatz, 2004). This opens new avenues for the role of TLR-3 in the brain. Further studies are needed to establish the precise role of TLR-3 in neuron physiology and its modulation in diverse brain diseases.

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