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Mini-Review—The Rabies Virus

Neuronal dysfunction and death in rabies virus infection

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Because morphologic changes in natural rabies are usually relatively mild, it is thought that the severe clinical disease with a fatal outcome must be due to neuronal dysfunction of rabies virus–infected neurons. The precise bases of this functional impairment are unknown, and current knowledge on electrophysiological alterations, effects on ion channels and neurotransmission, and neurotoxicity are reviewed. Rabies virus may induce neuronal death, possibly through apoptotic mechanisms. Neuronal apoptosis has been observed *in vitro* and also *in vivo* under particular experimental conditions. The relevance of neuronal apoptosis in these situations to natural rabies has not yet been fully elucidated. *Journal of NeuroVirology* (2005) **11,** 101–106.

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Introduction

Rabies is an ancient disease, but only over the last few decades have we begun to gain a real understanding of the pathogenesis of the disease. This has been largely based on experimental studies in animal models of rabies virus (RV) infection. However, our understanding of the disease remains incomplete even though there have been particularly marked advances in rabies prevention. Despite the dramatic and severe clinical neurological signs in rabies, the neuropathological findings under natural conditions are relatively mild and degenerative neuronal changes are not prominent (Iwasaki and Tobita, 2002). These observations suggest that neuronal dysfunction, rather than neuronal death, is likely responsible for the clinical disease and fatal outcome in rabies under natural conditions. However, under particular experimental conditions neuronal death may be prominent. We will review what is currently known about the consequences of RV infection of neurons, including neuronal dysfunction and death.

Electrophysiological alterations

RV infection may have important effects on the electrophysiological properties of neurons. Electroencephalographic (EEG) recordings of mice infected with the challenge virus standard (CVS) of fixed RV showed that the initial changes were alterations of sleep stages, including the disappearance of rapid eye movement (REM) sleep and the development of pseudoperiodic facial myoclonus (Gourmelon *et al*, 1986). As the disease progressed, there was generalized EEG slowing (at 2 to 4 cycles per second), and terminally there was an extinction of hippocampal slow activity with flattening of cortical activity. Brain electrical activity terminated about 30 min before cardiac arrest, indicating that cerebral death in experimental rabies occurs prior to failure of vegetative functions. Street virus–infected mice showed progressive disappearance of all sleep stages with a concomitant increase in the duration of waking stages, and these changes occurred before the development of clinical signs of rabies (Gourmelon *et al*, 1991). There was an absence of other EEG abnormalities in street virus–infected mice that lasted through the preagonal phase of the disease. Again, brain electrical activity ceased about 30 min before cardiac arrest. These studies highlight the differences between infection with fixed and street viruses, but provide few insights into the mechanisms involved.

Effects on ion channels

Dysfunction of ion channels has been shown in RV–infected cultured mouse neuroblastoma NA cells with the whole-cell patch clamp technique

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(Iwata *et al*, 1999). Infection with the RC-HL strain reduced the functional expression of voltage-dependent sodium channels and inward rectifier potassium channels, and there was a decreased resting membrane potential reflecting membrane depolarization. There was no change in the expression of delayed rectifier potassium channels, indicating that nonselective dysfunction of ion channels had not occurred. The reduction in sodium channels and inward rectifier potassium channels could prevent infected neurons from firing action potentials and generating synaptic potentials, resulting in functional impairment.

Infection of NG108-15 cells with the RC-HL strain *in vitro* was not found to alter the functional expression of voltage-dependent calcium ion channels (Iwata *et al*, 2000). NG108-15 cells express both α_2-adrenoreceptors and muscarinic receptors. Induced voltage-dependent calcium ion channel current inhibition with noradrenaline (α_2-adrenoreceptors) was significantly decreased in RV infection, whereas carbachol (muscarinic receptors) inhibition remained unchanged. Because α_2-adrenoreceptor–mediated inhibition of voltage-dependent calcium ion current serves as a brake mechanism to keep neurons from releasing their neurotransmitters beyond physiological requirements, the impaired modulation by α_2-adrenoreceptors could possibly contribute to clinical features of rabies, including hyperexcitability and aggressive behavior (Iwata *et al*, 2000). Evaluation of the effects of RV infection on ion channels merits further study.

Effects on cellular RNA and protein synthesis

It is not clear what causes the functional alterations in RV–infected neurons. It has been shown that RV infection does not inhibit host gene synthesis in cell culture (Ermine and Flamand, 1977; Madore and England, 1977; Tuffereau and Martinet-Edelist, 1985). Baer (1990) also suggested that RV replication *in vivo* probably does not shut off cellular DNA, RNA, or protein synthesis due to the paucity and late onset of cytopathic effects in virus-infected brain. However, Gosztonyi (1994) proposed that cytopathic neuronal changes may indicate a virus-induced inhibition of host cell synthetic processes. Fu *et al* (1993) reported that although extensive RV transcription and replication coincided with induction of host immediate-early gene expression, there was also suppression of the expression of neuronal specific genes such as preproenkephalin. At later stages of infection, mRNA transcripts for preproenkephalin were no longer detectable by *in situ* hybridization and immunohistochemical studies also indicated the depletion of enkephalin in the brain. Other host gene products such as 5-hydroxytryptamine receptor (Ceccaldi *et al*, 1993) and neuronal constitutive nitric oxide synthase (Akaike *et al*, 1995) were also found to be significantly decreased in the later stages of RV infection. Even the expression of a housekeeping gene such as glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was drastically reduced in the later stages of the disease process (Fu *et al*, 1993). Recently, Prosniak *et al* (2001) reported that infection of mice with CVS-N2c resulted in down-regulation of about 90% of genes in the normal brain at more than fourfold lower levels by using subtraction hybridization. These studies indicate that RV infection suppresses host gene synthesis, which leads to neuronal dysfunction, particularly in late stages of infection.

Effects on neurotransmission

A variety of experimental studies in RV infection have investigated possible abnormalities in neurotransmission involving acetylcholine. Tsiang (1982) initially reported reduction in specific binding to muscarinic acetylcholine receptors in CVS-infected rat brains, which was most marked in the hippocampus, by using a 3H-labeled antagonist, quinuclidinyl benzylate (QNB). When cholinergic neurotransmission was examined in CVS-infected and uninfected control mice, the enzymatic activities of choline acetyltransferase and acetylcholinesterase, which are required for the synthesis and degradation of acetylcholine, respectively, were similar in the cerebral cortex and hippocampus of moribund CVS-infected and control mice (Jackson, 1993). In this model, binding to muscarinic acetylcholine receptors, which was assessed with ^{3}H-labeled QNB using Scatchard plots, was not significantly different in the cerebral cortex or hippocampus of CVS-infected and uninfected control mice. These findings cast doubt on the importance of RV binding to muscarinic acetylcholine receptors in the brain. Mildly reduced specific binding of ^{3}H-labeled QNB in the hippocampus and brainstem was reported in naturally infected rabid dogs in Thailand compared with uninfected control dogs (Dumrongphol *et al*, 1996). K_d values were increased, indicating a decrease in receptor affinity, and B_{max} values, reflecting receptor content, were unchanged in rabid dogs. Curiously, increased K_d values were found to be similar in the hippocampus whether or not RV antigen was detectable at that site. These findings argue against alteration of muscarinic receptor binding as a specific consequence of RV infection of neurons. They suggest an unknown indirect mechanism for altered receptor affinity that is probably not related to the viral load.

Defective neurotransmission involving neurotransmitters other than acetylcholine could be important in the pathogenesis of rabies, and both serotonin and γ-amino-*n*-butyric acid (GABA) have been studied. Ligand binding to serotonin (5-HT) receptor subtypes was studied in the brains of CVS-infected rats

(Ceccaldi *et al*, 1993). Binding to 5-HT_1 receptor sites using [^{3}H]5-HT was showed a marked decrease in maximum binding (B_{max}) in the cerebral cortex 5 days after inoculation of CVS into the masseter muscles. Evaluation with ligands specific for receptor subtypes suggested that RV infection specifically affects 5-HT_{1D}–like receptors in the cerebral cortex. Furthermore, the reduced binding was demonstrated before RV antigen was detected in the cerebral cortex. Hence, the effect of RV on receptor binding is unlikely due to direct effects of viral replication in cortical neurons. There are important serotonergic projections from the dorsal raphe nuclei in the brainstem to the cerebral cortex, which can lead to early infection of the midbrain raphe nuclei in experimental rabies in skunks (Smart and Charlton, 1992). It is possible that the reduced binding to 5-HT_{1D}–like receptors is an indirect effect of the infection at noncortical sites by unknown mechanisms or that it is part of a physiological response to the stress produced by the infection. In support of impaired serotonergic neurotransmission in rabies, mildly decreased potassium-evoked release of [^{3}H]5-HT synaptosomes from the cerebral cortex of CVS-infected rats was found compared with controls (Bouzamondo *et al*, 1993). Hence, there is evidence of both impaired release and binding of serotonin, which might play roles in producing the neuronal dysfunction in rabies.

Impairments of both release and uptake of GABA have been found in CVS-infected primary rat cortical neuronal cultures (Ladogana *et al*, 1994). A 45% reduction of [^{3}H]GABA uptake was found 3 days after infection, which coincided with the time of peak viral growth in the cultures. Kinetic analysis revealed major reductions in V_{max}, indicating a decrease in the number of fully active GABA transport sites. There were no significant changes in K_m in infected cultures in comparison to controls, reflecting the affinity of the GABA transport system. Potassium- and veratridine-induced [^{3}H]GABA release were increased in infected cultures by 98% and 35%, respectively, compared to controls. The importance of these abnormalities in both the uptake and release of GABA on rabies pathogenesis *in vivo* has yet to be determined.

The dynamics of neurotransmission essentially involves four steps, namely, synthesis, storage, release and interaction with postsynaptic receptors, and intracellular events (Muller and Nistico, 1988). Measurement of one, some, or all of the aspects of neurotransmission has been used as an indicator of neuronal activity. To this end, the release of neurotransmitters in rat hippocampus after inoculation with RV CVS-24 by the intranasal route was determined by push-pull techniques (MohanKumar *et al*, 1999) in one of our laboratories (ZFF). Perfusates were collected at days 0, 1, 3, 5, and 7 after inoculation and neurotransmitter concentrations were determined in the perfusates using high-performance liquid chromatography with electrochemical detection (HPLC-EC) (MohanKumar *et al*, 1999). The concentrations of the following neurotransmitters were analyzed: norepinephrine (NE), dopamine (DA), dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA). As shown in Figure 1, neurotransmitter release was increased at day 1, reached a peak at day 3, and then declined by day 5 after inoculation. By day 7 after infection, the level of neurotransmitter release was either at or below the level prior to infection. We interpret these findings as evidence that neurotransmitter release was stimulated by the virus in an early stage of the infection. Previous studies using the same route of infection (Fu *et al*, 1993) indicate that RV is still in the process of spreading to the brain by day 3 after intranasal inoculation when the neurotransmitter release reached the peak. By day 5, and particularly by day 7 after inoculation, animals developed clinical rabies when neurotransmitter release was at or below the level prior to infection. It is possible that neurons are no longer capable of releasing neurotransmitters at the synaptic junctions and this may be the basis of clinical signs, including paralysis.

Neurotoxicity

Koprowski *et al* (1993) have hypothesized that nitric oxide neurotoxicity may mediate neuronal dysfunction in rabies, and induction of inducible nitric oxide synthase (iNOS) mRNA was observed in mice experimentally infected with street RV. iNOS mRNA was detected using reverse transcriptase–polymerase chain reaction (RT-PCR) amplification in the brains of three of six paralyzed mice 9 to 14 days after inoculation of RV in the masseter muscle. iNOS mRNA expression was rapidly induced in the brains of the rabid mice. The onset of clinical signs in RV–infected rats and the clinical progression of disease correlated with increasing quantities of nitric oxide in the brain to levels up to 30-fold more than in controls, which was determined using spin trapping of nitric oxide and electron paramagnetic resonance spectroscopy (Hooper *et al*, 1995). iNOS was detected by immunostaining in CVS-infected rats in many cells throughout the brain near blood vessels, which were identified as microglia and macrophages (Van Dam *et al*, 1995). CVS-24–infected rats developed a reduction in neuronal nitric oxide synthase (nNOS) activity with reductions in nNOS mRNA and nNOS immunoreactivity and an increase in iNOS activity in the brain in a time-dependent manner (Akaike *et al*, 1995). Choline acetyltransferase activity in the brain remained unchanged, indicating that the decrease in nNOS activity did not reflect generalized neuronal loss. The nitric oxide produced by macrophages may be neurotoxic because its reaction with superoxide anion O_2^- leads to the formation of peroxynitrate, which is a reactive oxidizing agent capable of causing tissue damage (Akaike *et al*, 1995). Ubol *et al*

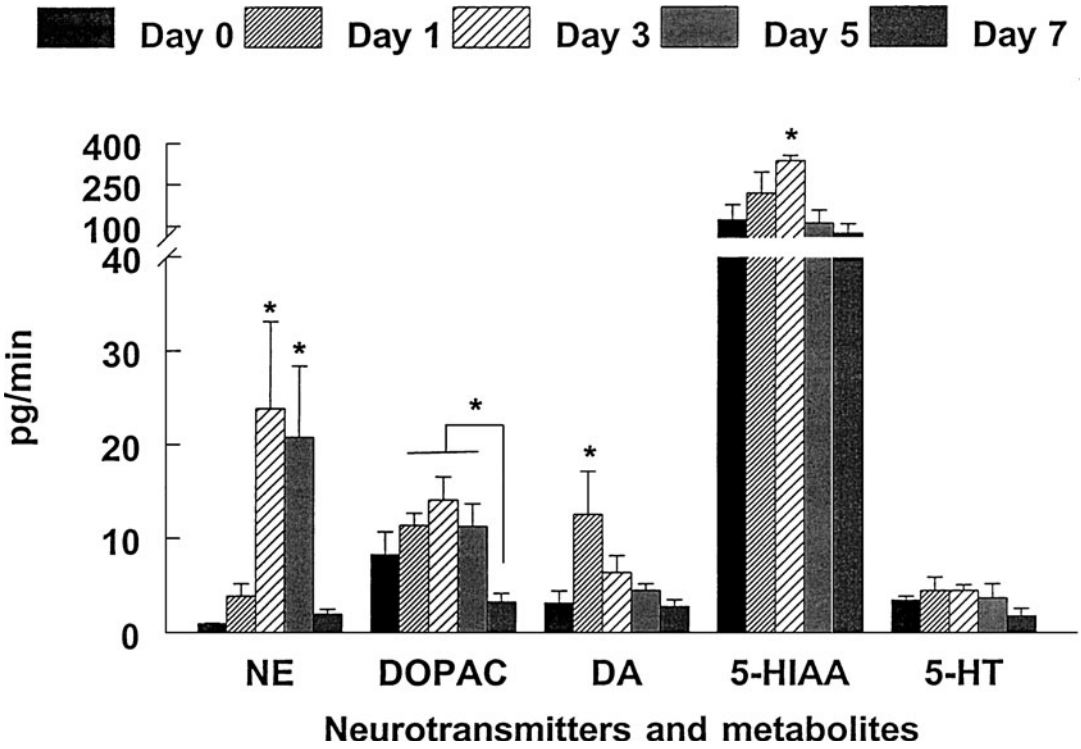

Figure 1 Neurotransmitter release at the synaptic junctions in the hippocampus. Push-pull cannulae were inserted into rat hippocampus 1 week before intranasal infection with rabies virus CVS-24. Perfusates were collected from the rats ($n = 5$) and the concentrations of the above neurotransmitters were determined by HPLC-EC. *Statistical significance at $P < .05$.

(2001) found that mice treated with the iNOS inhibitor aminoguanidine (AG) delayed the death of CVS-11–infected mice by 1.0 to 1.6 days (depending on the dose). A delay in rabies virus replication was observed in the AG-treated mice. The role of nitric oxide in rabies pathogenesis clearly needs further study.

Neuronal death

Neurotropic viruses may cause cell death by either apoptosis or necrosis (Griffin and Hardwick, 1999; Allsopp and Fazakerley, 2000; Fazakerley and Allsopp, 2001). Apoptosis depends on synthesis of macromolecules and requires energy, whereas, in contrast, necrosis is associated with energy failure. Each of these forms of cell death is associated with typical morphologic features. CVS has been observed to induce apoptotic cell death in rat prostatic adenocarcinoma cells (Jackson and Rossiter, 1997), mouse and human lymphocytes (Thoulouze *et al*, 1997), mouse neuroblastoma cells (Theerasurakarn and Ubol, 1998), and in mouse embryonic hippocampal neurons (Morimoto *et al*, 1999). Morimoto and coworkers have observed that variants that are more neurovirulent in adult mice produce less apoptosis over a period of 72 h in primary hippocampal neurons than produced by less neurovirulent variants (Morimoto *et al*, 1999). Guigoni and Coulon (2002) observed that primary cultures of CVS-infected purified rat spinal motoneurons did not show major evidence of apoptosis over a period of 7 days, whereas infected purified hippocampal neurons showed apoptosis in over 90% of neurons within 3 days, indicating that different neuronal cell types respond differently to RV infection. Recently it has been reported that CVS and PV strains induce only limited apoptosis whereas two vaccine strains, ERA and SN-10, but induce strong apoptosis in human neuroblastoma SK-N-SH cell line and in lymphoblastoid Jurkat cells (Baloul and Lafon, 2003; Lay *et al*, 2003; Prehaud *et al*, 2003; Thoulouze *et al*, 1997, 2003). Thus the induction of apoptosis is via both virus-dependent and cell-dependent mechanisms. Furthermore, RV-induced apoptosis is activated in caspase-dependent and caspase-independent fashions and can be blocked by Bcl-2 overexpression (Thoulouze *et al*, 2003).

In animal models, prominent apoptotic death of neurons has been observed in the brains of mice of various ages inoculated intracerebrally with the CVS strain of fixed RV (Jackson and Rossiter, 1997; Jackson and Park, 1998) and immunosuppression of adult mice did not reduce the apoptotic process (Theerasurakarn and Ubol, 1998). However, apoptosis in infected cultured cells, including embryonic cells, does not closely correspond to what is observed in infected animals. Peripherally inoculated

animals with CVS strains do not show the prominent apoptosis that is observed in neurons after intracerebral inoculation (Reid and Jackson, 2001). Conflicting results have been reported by different investigators with respect to the occurrence of neuronal apoptosis after intracerebral inoculation of different street (wild-type) RV variants in mice (Ubol and Kasisith, 2000; Yan *et al*, 2001). Furthermore, it has recently been reported that different apoptotic mechanisms are induced by different virus strains. In CVS-infected mice, CD3 T cells were the main contributor to the pool of apoptotic cells whereas in PV-infected animals, infected neurons were an important fraction of the deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive neurons (Baloul and Lafon, 2003).

In RV infection there may be complex mechanisms involved in cell death or survival of neurons both *in vitro* and in animal models using different viral strains and routes of inoculation. Both *in vitro* and *in vivo* observations demonstrate that apoptosis may be a protective rather than a pathogenic mechanism in RV infections because less pathogenic viruses induced more apoptosis than more pathogenic viruses in both *in vitro* and *in vivo* using peripheral routes of inoculation (Morimoto *et al*, 1999; Prehaud *et al*, 2003; Yan *et al*, 2001). This is not surprising because neuronal cell death is not prominent in natural rabies (Iwasaki and Tobita, 2002). Thus preservation of the neuronal network by inhibition of apoptosis and limitation of the inflammation and the destruction of T cells that invade the central nervous system (CNS) in response to the infection is crucial for RV neuroinvasion and for transmission of RV to another animal (Baloul and Lafon, 2003). Hopefully, a greater understanding of the mechanisms involved in neuronal apoptosis in experimental models may provide insights into the pathogenesis of neuronal dysfunction that occurs in natural rabies.

Conclusions

Although we have made progress in understanding the bases for neuronal dysfunction and neuronal death in RV infection, there are still many more questions than answers. No fundamental abnormality has yet been identified for the neuronal dysfunction in rabies. We are hopeful that identification of this functional impairment may lead to novel therapies for rabies and perhaps also be relevant to other viral infections of the nervous system and other diseases. Although at the present time rabies can be effectively prevented, all therapeutic approaches to date have been very disappointing. Further basic research on rabies pathogenesis is essential if we are going to make progress in truly conquering this ancient disease.

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