J. Neurovirol. (2016) 22:8–13
DOI 10.1007/s13365-015-0351-1

MINI-REVIEW

Diabolical effects of rabies encephalitis

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Received: 4 December 2014 / Revised: 24 April 2015 / Accepted: 30 April 2015 / Published online: 21 May 2015

Abstract Rabies is an acute encephalomyelitis in humans and animals caused by rabies virus (RABV) infection. Because the neuropathological changes are very mild in rabies, it has been assumed that neuronal dysfunction likely explains the severe clinical disease. Recently, degenerative changes have been observed in neuronal processes (dendrites and axons) in experimental rabies. In vitro studies have shown evidence of oxidative stress that is caused by mitochondrial dysfunction. Recent work has shown that the RABV phosphoprotein (P) interacts with mitochondrial Complex I leading to overproduction of reactive oxygen species, which results in injury to axons. Amino acids at positions 139 to 172 of the P are critical in this process. Rabies vectors frequently show behavioral changes. Aggressive behavior with biting is important for transmission of the virus to new hosts at a time when virus is secreted in the saliva. Aggression is associated with low serotonergic activity in the brain. Charlton and coworkers performed studies in experimentally infected striped skunks with skunk rabies virus and observed aggressive behavioral responses. Heavy accumulation of RABV antigen was found in the midbrain raphe nuclei, indicating that impaired serotonin neurotransmission from the brainstem may account for the aggressive behavior. We now have an improved understanding of how RABV causes neuronal injury and how the infection results in behavioral changes that promote viral transmission to new hosts.

Keywords Aggressive behavior · Mitochondrial dysfunction · Oxidative stress · Pathogenesis · Rabies · Serotonin · Viral encephalitis

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Rabies is an acute viral infection of the central nervous system (CNS) of humans and animals caused by rabies virus infection (Hanlon 2013; Jackson 2013). The infection is usually transmitted in the saliva by biting animals. Two important fundamental questions in rabies pathogenesis are (1) What is the basis for neuronal dysfunction/injury in rabies? and (2) How can the behavioral changes in rabies vectors be explained? This review will address these basic questions with our current knowledge.

Bases for neuronal injury in rabies

Although neurons are prominently infected in rabies, the bases for the neurological disease have not yet been clearly defined because widespread neuronal injury or death has not been recognized (Fu and Jackson 2005; Jackson and Fu 2013). Recently, degeneration of neuronal processes has been recognized in experimental mouse models of rabies (Li et al. 2005; Scott et al. 2008). We comprehensively evaluated infection in adult transgenic mice expressing the yellow fluorescent protein (YFP; H clone) using hindlimb footpad inoculation of the challenge virus standard-11 strain of fixed rabies virus (CVS) (Scott et al. 2008). In these mice, YFP expression is driven in a subpopulation of neurons, and there are strong fluorescent signals in dendrites, axons, and presynaptic nerve terminals (Feng et al. 2000). Conventional histopathology showed mild inflammatory changes without significant degenerative neuronal changes. At late clinical time points, with the development of severe clinical neurological disease, fluorescence microscopy showed marked structural abnormalities, including beading and/or swelling in dendrites and axons of neurons (Fig. 1a, b). The morphological changes sufficiently explain the severe clinical disease with a fatal outcome.

The morphologic changes in neuronal processes in rabies virus (RABV) infection have a striking similarity to the neurodegenerative changes that occur in diabetic sensory and autonomic neuropathy, in which a key feature is the presence of axonal swellings that are composed of accumulations of mitochondria and cytoskeletal proteins (e.g., neurofilaments) (Lauria et al. 2003; Schmidt et al. 1997). Diabetes-induced

oxidative stress in sensory neurons and peripheral nerves is associated with increased production of reactive oxygen species (ROS) (Nishikawa et al. 2000; Russell et al. 2002), lipid peroxidation (Obrosova et al. 2002), and protein nitrosylation (Obrosova et al. 2005).

We have shown that RABV infection in cultured dorsal root ganglion (DRG) sensory neurons induces oxidative stress (Jackson et al. 2010). We evaluated CVS- and mock-infected cultures of DRG neurons derived from adult mice for neuron-specific β-tubulin and observed axonal swellings in CVS neurons (Fig. 1c, d), and performed immunostaining for RABV antigen and for amino acid adducts of 4-hydroxy-2-nonenal (4-HNE), which is a marker of lipid peroxidation and, hence, oxidative stress (Jackson et al. 2010). Neuronal viability (by trypan blue exclusion), terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, and axonal

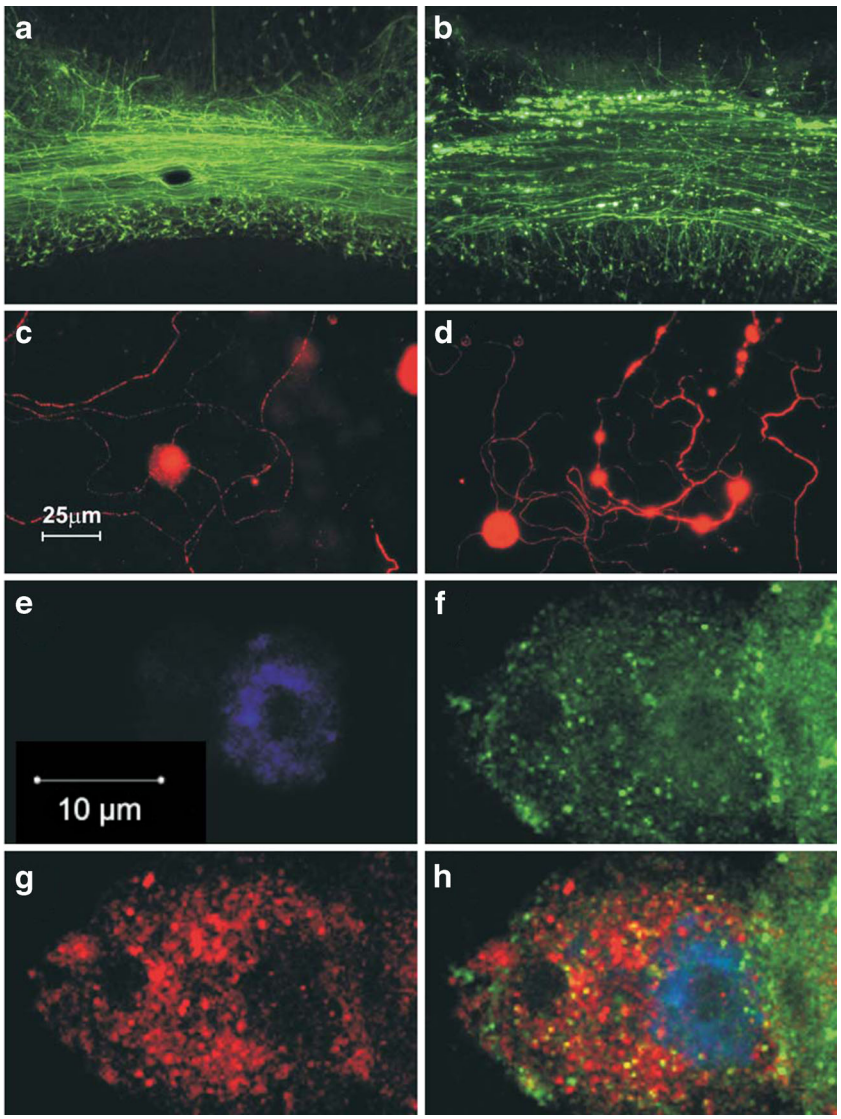

Fig. 1 Morphology of the cerebellar mossy fibers of mock-infected (**a**) and moribund CVS-infected YFP mice (**b**). Mossy fiber axons in the cerebellar commissure of moribund mice show severe beading (**b**), whereas no abnormalities were observed in mock-infected mice (**a**). CVS infection causes formation of axonal swellings in DRG cultures. Fluorescence microscopy showing CVS-infected DRG neurons at 24 h (**c**) and at 72 h (**d**) postinfection (p.i.). Staining for β-tubulin III (**c** and **d**) shows two neuronal cell bodies at 24 h p.i. (**c**) and one (*large spherical body*) at 72 h p.i. (**d**). Definite axonal swellings are not yet present at 24 h p.i. (**c**), but multiple axonal swellings are well established at 72 h p.i. (**d**). Co-localization of the RABV P and mitochondrial markers in infected cells. CVS-infected Mouse neuroblastoma cells at 72 h p.i. were stained for nuclei with DAPI (**e**) and immunostained for RABV P with anti-P monoclonal antibody M1034 (*green*) (**f**) and for mitochondrial marker VDAC with an anti-VDAC antibody (*red*) (**g**). The merged image shows co-localization of RABV P staining in the mitochondria at higher (*yellow*) and lower (*orange*) levels (**h**). **a**, **b** ×140. Adapted with permission from Scott et al. (2008), Copyright ©American Society for Microbiology, [Journal of Virology, 82, 513–21, doi:10.1128/JVI.01677-07] (**a**, **b**), Jackson et al. (2010), Copyright © American Society for Microbiology, [Journal of Virology, 84, 4697–705, doi:10.1128/JVI.02654-09] (**c**, **d**), and Kammouni et al. (2015), Copyright © Springer [Journal of Neurovirology (In press), doi:10.1007/s13365-015-0320-8)] (**e**–**h**)

growth were also assessed in the cultures. CVS infected up to about 50 % of cultured DRG neurons, similar to the findings of other investigators. Neuronal viability and TUNEL staining were similar in CVS- and mock-infected DRG neurons. There were significantly more 4-HNE-labelled puncta at 2 and 3 days postinfection in CVS-infected cultures than in mock infection. Axonal outgrowth was reduced at these time points in CVS infection vs. mock-infected cultures.

The mitochondria are considered the main source of ROS, and they play a critical role in the induction of oxidative stress. ROS are mainly generated in the electron transport chain (ETC), primarily at Complex I and Complex III (Murphy 2009). Since we found evidence of oxidative stress in cultured DRG neurons, we comprehensively evaluated CVS and mock infection in a variety of neuronal and non-neuronal cell types, including neurons with neurites (DRG neurons and PC12 cells), neurons without neurites (mouse neuroblastoma cells [MNA]), and non-neuronal cells (BHK cells) for abnormalities of mitochondrial function (Alandijany et al. 2013). No changes were found in Krebs cycle enzyme activities (e.g., citrate synthase) in CVS versus mock infection. The lack of change in citrate synthase activity indicates that there was no major effect of the viral infection on mitochondrial mass or intactness and suggests that the effects on mitochondrial respiratory activity were the result of direct interference with respiratory chain components. We found increased Complex I activity in all CVS-infected cell types that correlated with the susceptibility of the cells to CVS infection, suggesting that the increase in activity may be a direct viral effect. In contrast, Complex II–III activities were similar in CVS- and mock-infected cells. Increased Complex IV activity was found in CVS-infected cells versus mock-infected cells, but this increase did not correlate with the susceptibility of the cells to CVS infection, suggesting that the increase in activity was unlikely due to a direct viral effect. A mitochondrial respiration assay was performed in which the rate of oxygen consumption was measured under different states (rate of proton leak, coupled respiration, maximal uncoupled respiration, and Complex IV respiration) (Alandijany et al. 2013). In CVS infection, coupled respiration and the rate of proton leak were maintained, indicating a tight mitochondrial coupling. Possibly as a result of enhanced complex activity and efficient coupling, a high mitochondrial membrane potential was generated. CVS infection altered the cellular redox state as indicated by a high NADH/NAD+ ratio and reduced the intracellular ATP level. CVS infection was associated with a higher rate of succinate-driven hydrogen peroxide production than in mock infection, suggesting that the increased activity of Complex I led to generation of ROS at Complex I and other sites.

Hence, we postulate that the fundamental abnormality in RABV infection is likely increased activity of Complex I resulting in ROS generation at Complex I and other sites of the electron transport chain. This is a novel finding because most Complex I disorders (e.g., genetic or toxic disorders) are associated with reduced Complex I activity (Fato et al. 2008; Irwin et al. 2013).

We transfected the human embryonic cell line HEK-293T (HEK) with plasmids expressing RABV proteins and only the plasmid expressing the RABV phosphoprotein (P) increased Complex I activity and ROS levels, whereas none of the other rabies virus proteins increased Complex I activity or ROS levels (Kammouni et al. 2015). We found co-localization of immunofluorescent staining for the RABV P and the voltage-dependent anion channel (VDAC) as a mitochondrial marker (Fig. 1e–h). The mechanism by which P enters the mitochondria is uncertain. We found that Western immunoblots of purified mitochondrial extracts contain RABV P and that the RABV P was also detected in Complex I immunoprecipitates of purified mitochondrial extracts, providing strong supporting evidence that there is a physical interaction between the RABV P and Complex I resulting in the increase in activity of Complex I. We evaluated Complex I activity and ROS levels of cells transfected with a variety of plasmids containing deletions of the RABV P. Western blotting confirmed comparable expression of the proteins after transfection of the plasmids. The data strongly implicate the 34-amino acid region of the P from positions 139 to 172 as a key region for interaction with Complex I resulting in its increased activity and an increase in ROS levels.

Our animal model studies provide evidence that CVS infection selectively injures neuronal processes without causing neuronal death. This injury results in few morphologic changes of neurons on routine histopathological studies in adult mice inoculated peripherally with CVS and is similar to the situation in natural rabies. Our studies of CVS infection in DRG neurons indicate evidence of oxidative stress resulting in axonal swellings and degeneration and impaired axonal growth. Studies of mitochondrial function indicate increased activity of mitochondrial respiratory chain Complex I, which correlates with the susceptibility of the cell type to RABV infection, a high mitochondrial membrane potential, increased NADH/NAD+ ratio, reduced ATP content, and normal coupled respiration and rate of proton leak (Alandijany et al. 2013). This work strongly suggests that increased Complex I activity explains ROS overproduction, which was supported by Amplex Red assays for hydrogen peroxide production. We now have strong evidence that the RABV P physically interacts with Complex I and increases its activity resulting in increased ROS levels (Kammouni et al. 2015).

Behavioral changes in rabies vectors

Altered behavior of infected animal vectors of rabies, particularly aggressive behavior, is an important component of rabies pathogenesis because this leads to transmission of rabies virus in the saliva to new hosts by biting (Jackson and Fu 2013). The biological bases for the behavioral changes in rabies are poorly understood. This is largely due to the fact that

rabies pathogenesis has only rarely been studied in natural hosts under experimental conditions similar to what occurs naturally. In contrast, dead-end hosts such as rodents and humans may show behavioral changes, but these are only rarely the typical aggressive behavioral changes observed in natural hosts.

Bases for aggressive behavior in rabies

Richard Johnson related the clinical features of rabies in vectors to probable neuroanatomical localization in the limbic system. "The … localization to limbic system with relative sparing of neocortex provides a fascinating clinicopathologic correlate with the alertness, loss of natural timidity, aberrant sexual behavior and aggressiveness that may occur in clinical rabies. No other virus is so diabolically adapted … that it can drive the host in a fury to transmit the virus to another host animal" (Johnson 1971). However, Johnson did not actually experimentally demonstrate localized limbic system involvement in rabies; he performed his studies in a mouse model of rabies using laboratory-adapted CVS. Hence, Johnson's classical quotation is actually based more on his insightful speculation than on an objective evaluation of experimental studies. In humans, probably the best examples of disorders with localized limbic system anatomical involvement are herpes simplex encephalitis of adult human patients (Whitley 2014) and autoimmune limbic encephalitis (Derry et al. 2011). However, both of these conditions have very different clinical features than rabies, which may include behavioral changes that are not usually aggressive in nature.

Aggressive behavior is associated with lesions in many locations in the brain, including the posterior olfactory bulbs, the ventromedial nucleus of the hypothalamus, and the septal area (Isaacson 1989). Offensive aggression, which is often impulsive and unprovoked, has been associated low serotonergic activity and also increased testosterone levels in human and animal studies (Kalin 1999). Hence, the neuroanatomical basis of aggressive behavior is complex and presents a considerable challenge for investigation of the underlying mechanisms in rabies.

There are very few good experimental models of rabies that closely resemble natural disease and include aggressive behavioral changes in the host. Natural experimental models of rabies performed in dogs, skunks, and bats using the associated street (wild-type) rabies virus variant pose many challenges for the researcher. Of course, aggressive animals are very difficult to handle, and animal care protocols are needed that require fairly advanced disease. Incubation periods may be fairly long (lasting weeks to months), and many animals inoculated with virus may not develop disease at all. Hence, these studies are labor-intensive, time-consuming, and expensive and do not quickly answer hypothesis-based research questions and consequently are often not competitive for grant funding agencies with peer review.

The best-studied natural model is the striped skunk model of experimental rabies used by Kenneth Charlton and coworkers from Canada. In a study, Charlton et al. (1984)

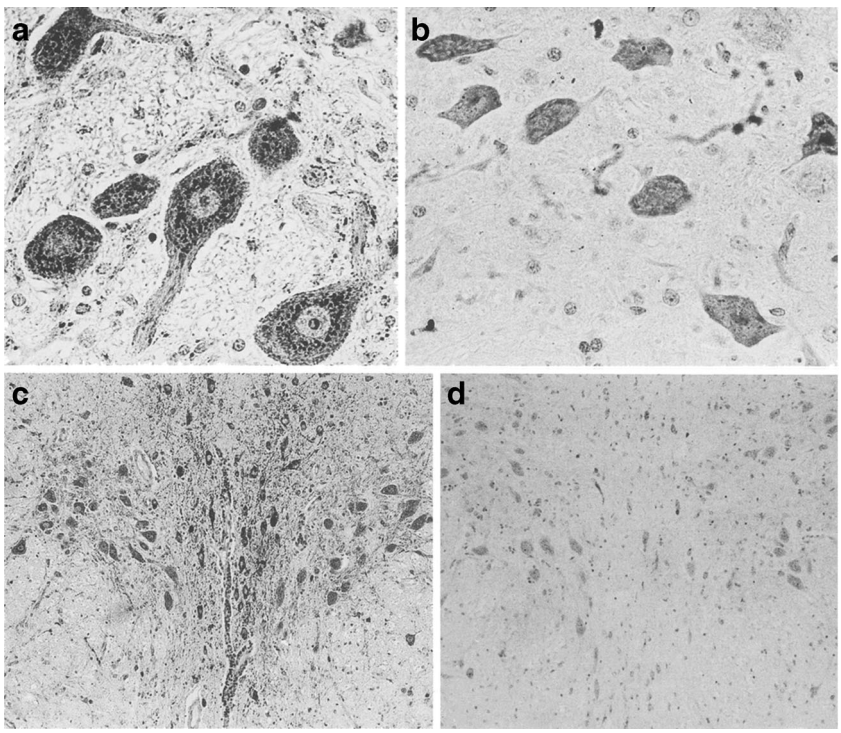

Fig. 2 Immunoperoxidase-hematoxylin-stained sections of the red nucleus (**a** and **b**) and the midbrain raphe (**c** and **d**) at the level of the oculomotor nucleus. Street virus is detectable in large amounts in the neurons of the red nucleus in (**a**) and the midbrain raphe in (**c**), whereas CVS (**b** and **d**) is detectable only in small amounts in these areas. Reproduced with permission from Smart and Charlton (1992), Copyright © Springer-Verlag [Acta Neuropathologica 84, 501–8]

immunosuppressed experimentally infected skunks with cyclophosphamide (25 mg/kg intraperitoneally on the first day and then 12.5 mg/kg on days 5, 9, 13, 17, 21, 26, and 30) and observed similar levels of aggressive behavior in both immunosuppressed and control skunks. The immunosuppressed skunks exhibited relatively few inflammatory cells in the brain, providing evidence that aggressive behavioral changes were likely not induced by the associated inflammatory reaction in infected skunks.

In the best study to date, Smart and Charlton (1992) performed studies evaluating the bases of aggressive behavior in rabies in experimentally infected skunks. In addition to infecting skunks with skunk rabies virus, they also studied infection using CVS, which has been serially passaged in animal brains and in cell culture. CVS infection is not associated with long incubation periods, and CVS has lost neuroinvasiveness after peripheral inoculation and does not result in aggressive behavior in host species. Smart and Charlton (1992) observed that skunks inoculated with skunk virus predominantly exhibited furious rabies with biting attacks. They evaluated the CNS for the presence or absence of rabies virus antigen in nuclei in order to assess the bases for the behavioral changes in rabies. They assumed that nuclei containing greater amounts of viral antigen were more likely to exhibit dysfunction. Eight skunks were infected with skunk virus by the intramuscular route into the abductor digiti quinti muscle of the hindfoot and another eight skunks were infected intranasally via drops into the nose. All developed clinical rabies except two skunks infected by the intranasal route. Of the 14 skunks with clinical rabies, 12 (86 %) demonstrated hyperresponsiveness to stimuli (noise and touch) and 12 (86 %) made an immediate attack with stick presentation. In contrast, skunks infected with CVS demonstrated limb paresis and only two of eight skunks (25 %) demonstrated a mild response on stick presentation characterized by "investigation and tentative chewing."

The brains of the infected skunks were subjected to immunohistochemical staining for detection of rabies virus antigen. The skunk virus-infected brainstems demonstrated much larger quantities of rabies antigen in the red nucleus and in the midbrain raphe nuclei than in CVS infection (Fig. 2). In contrast, in CVS infection, there were larger quantities of detectable rabies virus antigen in the pyramidal cell layers of the parietal cerebral cortex and in the Purkinje cell layer of the cerebellum than in skunk virus infection. The finding of more abundant rabies virus antigen in the midbrain raphe nuclei in skunk virus infection than in CVS infection may explain the observation of biting behavior of the skunk virus-infected skunks because the midbrain raphe nuclei produce the neurotransmitter serotonin. Serotonin is known to be involved in controlling various types of aggressive behavior (Popova 2008), and Yamamoto and Ueki (1977) reported that midbrain raphe lesions induce aggressive behavior. Hence, there is evidence that street rabies virus infects the midbrain raphe nuclei, presumably leading to depletion of serotonin that involves ascending pathways in the brain and results in aggressive behaviors. The evaluation of other brain regions that play an important role in the neuroanatomical substrate of aggressive behavior (Isaacson 1989) showed mild accumulation of rabies virus antigen in septal nuclei in both skunk virus and CVS infections, whereas marked accumulations in the hypothalamus were observed in street virus infection versus moderate accumulations in CVS infection (Smart and Charlton 1992). Hence, a greater burden of infection in hypothalamic neurons in street virus infection may have also played a role in producing aggressive behavior of the street virus-infected skunks. Of course, much more experimental work is needed in different natural animal models of rabies in order to confirm the observations that infection involving the midbrain raphe nuclei and also possibly the hypothalamus is the basis for aggressive behavior of rabies vectors. Clearly, understanding that biological bases for behavioral changes in rabies is an under-researched area because of a number of obstacles. More research is needed in good experimental animal models of natural rabies in order to gain a better understanding of the pathogenesis of this ancient disease.

Funding statement The studies on neuronal injury in rabies were supported by Canadian Institutes of Health Research / Manitoba Regional Partnership Program with the Manitoba Health Research Council (to Alan C. Jackson and Paul Fernyhough at the University of Manitoba).

Conflict of interest The author declares that he has no competing interests.

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