Everything You Always Wanted to Know About Rabies Virus (But Were Afraid to Ask)

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Abstract

The cultural impact of rabies, the fatal neurological disease caused by infection with rabies virus, registers throughout recorded history. Although rabies has been the subject of large-scale public health interventions, chiefly through vaccination efforts, the disease continues to take the lives of about 40,000–70,000 people per year, roughly 40% of whom are children. Most of these deaths occur in resource-poor countries, where lack of infrastructure prevents timely reporting and postexposure prophylaxis and the ubiquity of domestic and wild animal hosts makes eradication unlikely. Moreover, although the disease is rarer than other human infections such as influenza, the prognosis following a bite from a rabid animal is poor: There is currently no effective treatment that will save the life of a symptomatic rabies patient. This review focuses on the major unanswered research questions related to rabies virus pathogenesis, especially those connecting the disease progression of rabies with the complex dysfunction caused by the virus in infected cells. The recent applications of cutting-edge research strategies to this question are described in detail.

RABIES VIRUS TAXONOMY, STRUCTURE, AND LIFE CYCLE WITHIN THE INFECTED CELL

Rabies is registered throughout recorded history (1–3) and continues to kill an estimated 40,000–70,000 people yearly, with a high percentage of those being children (4, 5). The causative agent of rabies, rabies virus (RABV), is a negative-stranded RNA virus of the genus *Lyssavirus* (Greek: *Lyssa*, the goddess of rage or madness) (2). RABV is a member of the Rhabdoviridae family (Greek: *rhabdos*, rod), named for the characteristic rod- or bullet-shaped rhabdovirus virion observed by electron microscopy (6). Rhabdovirus virions, like other negative-stranded RNA viruses, are composed of a highly stable and organized complex of genomic RNA and nucleoprotein, contained in a lipid envelope derived from the host cell membrane (7).

RABV is the most prominent member of the *Lyssavirus* genus, with a global distribution and a long history of study. However, there are several other lyssaviruses that can also cause fatal rabies-like disease. Fourteen rabies-related lyssaviruses are currently known, including Mokola virus, European bat lyssavirus 1 and 2, and Australian bat lyssavirus (2). These lyssaviruses tend to be geographically restricted, and they cause a minority of human rabies fatalities but may emerge more prominently as humans encroach on new areas and habitats in which they are endemic. Most of our current knowledge of these viruses has come from ecological studies of bats, but recently, several teams have used experimental virology techniques to learn more about lyssavirus diversity (8–11).

All rhabdoviruses encode five structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and an RNA-directed RNA polymerase (L) (**Figure 1*a***) (7). N encapsulates the RNA genome, forming a tightly wound N-RNA complex known as a ribonucleoprotein (RNP) (12, 13). The RNP is condensed, along with L and P, into a helical nucleocapsid (NC) (12). P is a noncatalytic cofactor for the polymerase L. M surrounds the NC, forming a bridge between the NC and the viral envelope (14, 15). G, which is trimeric and interacts at its cytoplasmic side with M, is the only protein exposed on the surface of the rhabdovirus envelope (16) and is the sole ligand for the cellular receptor. Many rhabdoviruses express additional proteins of diverse function; the RABV genome consists only of these five (17). The contributions that the individual RABV proteins make to RABV pathogenesis go far beyond their structural functions and are discussed throughout this review. For instance, RABV-P has functions other than serving as polymerase cofactor, such as the disruption of host interferon (IFN)-mediated antiviral defense (18).

A generalized life cycle is observed across the rhabdovirus family (**Figure 1*b***) (7). The virus G protein interacts with host cellular receptors, triggering endocytosis of the virion (19, 20). The lower pH of the endosome catalyzes a G-mediated fusion between the viral envelope and the endosomal membrane, releasing the NC into the cytoplasm (20). The functional viral polymerase, which is a complex of L and P, uses the released RNP as a template for repetitive rounds of transcription (21). The expression levels of rhabdovirus mRNAs, and therefore the proteins they encode, are maximal at the 3′ end of the genome and become sequentially less abundant toward the 5′ end of the genome (**Figure 1*c***). Thus, N is most abundant, followed by P, M, G, and L. This decrement is due to disassociation of the polymerase from the RNP during transcription when a termination signal is reached, requiring the polymerase to reengage to initiate transcription of the downstream gene (22, 23). Viral replication starts once a certain threshold of viral N has been produced, and for RABV, this is thought to be regulated by M levels (24, 25). For replication, the viral polymerase switches to a more processive mode, producing a full-length, positive-sense RNA antigenome (25, 26). This intermediate then serves as the template for the production of full-length negative-sense genomes. Finally, the rhabdovirus budding mechanism begins with the

insertion of G into the host cell membrane (27, 28). The M in the nascent NC interacts with the cytoplasmic tail of G, triggering budding of the virus from the host cell membrane (15, 28).

Despite close similarities in morphology and life cycle, the various members of the rhabdovirus family have a wide array of species host range and in vivo phenotypes. For instance, the best-studied rhabdovirus besides RABV, vesicular stomatitis virus (VSV), is a pathogen of cattle that does not cause severe human disease. RABV's narrow cellular tropism, absence of cytopathic effect, and long incubation time can be contrasted with VSV's broad cellular tropism, cytotoxic life cycle, and fast replication (5, 29, 30). RABV has tropism for mammals; other rhabdoviruses infect fish, insects, and plants (17).

THE JOURNEY OF RABIES VIRUS INFECTION THROUGH THE HOST: OVERVIEW

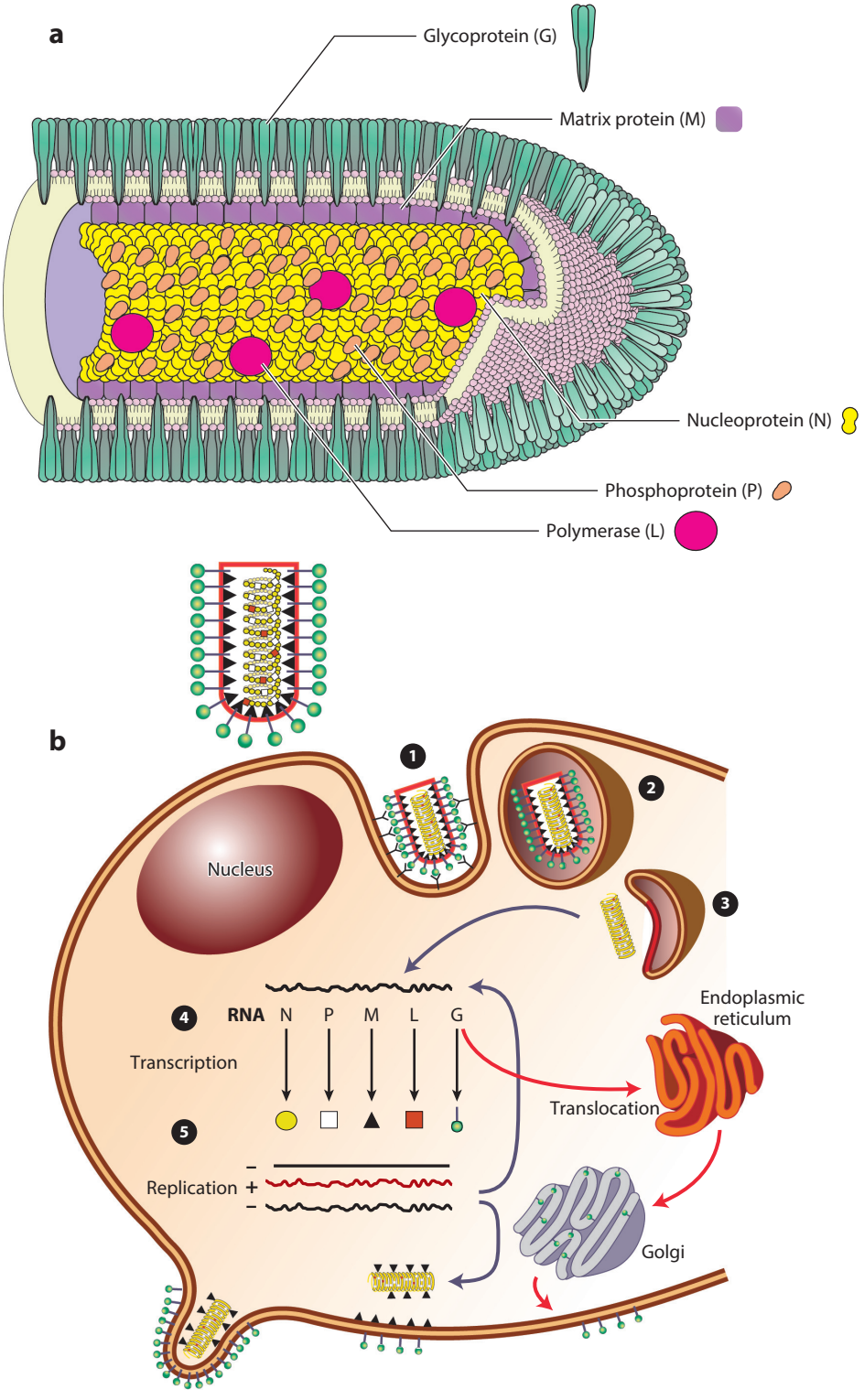
Most human RABV exposures are a result of animal bites or scratches, which expose muscle tissue to animal saliva containing RABV particles (**Figure 2**) (31). One of the known receptors for RABV, the nicotinic acetylcholine receptor (nAchR), is restricted to muscle cells, which is evidence for primary infection of muscle cells followed by transmission to neurons (**Figure 3**) (31). In the current model of RABV neuroinvasion, the RABV particles enter the central nervous system (CNS) by budding from muscle cells into the synaptic cleft of neuromuscular junctions (NMJs), the specialized synapses between efferent nerve terminals and muscle fibers. It is thought that RABV entry into primary motor neurons is followed by retrograde axonal transport, replication and assembly in the neuronal cell body, and then transport to and budding from another synapse to start a new round of infection and resultant neuron-to-neuron spread. In this model, the process of transsynaptic spread continues until RABV is widely distributed in the CNS (**Figure 2**). This causes behavioral changes that support the spread of the virus to a new host. Simultaneously, RABV undergoes a centrifugal spread from the CNS into several extraneural organs, including the skin, hair follicles, heart, adrenal glands, tongue, and salivary glands (32–35).

Viral CNS invasion is not unique to RABV, but several characteristics set RABV apart from other neuroinvasive viruses. During early stages of infection, RABV penetrates and is transported exclusively by primary motor neurons, rather than sensory and autonomic neurons as is the case for neuroinvasive alphaherpesviruses (32, 36). Once in the CNS, RABV displays (*a*) strict preference for neurons rather than other CNS cells such as microglia and astrocytes, (*b*) exclusive transsynaptic spread of virus without release at other parts of the neuron, and (*c*) lack of histopathological evidence of damage to infected tissue (32). Although these are reliably observed features of RABV infections, the ways in which RABV biology determines these properties are not well understood.

The lack of apparent damage to neurons is especially compelling to current researchers. How does RABV infection kill the host without inducing widespread neuronal death? Unraveling this question at the molecular level is a major challenge in the field of RABV virology and is perhaps key to understanding rhabdoviral pathogenesis, designing improved treatments, and gaining insights into neuronal biology. The genetic, morphological, and potentially inflammatory changes in infected cells are the subject of much ongoing study and are discussed at the end of this review.

EXPERIMENTAL USE OF RABIES VIRUS

When addressing RABV pathogenicity experimentally, it is important to first consider the diversity of RABV strains available to researchers. Different strains may produce conflicting results, even


a
Glycoprotein (G)
Matrix protein (M)
Nucleoprotein (N)
Phosphoprotein (P)
Polymerase (L)
b
Nucleus
1
2
3
4
RNA
N
P
M
L
G
Transcription
5
Replication
+
–
Endoplasmic reticulum
Translocation
Golgi

in the same system. RABV strains can be categorized a number of ways, including by passage history [laboratory-adapted (fixed virus) or wild-type (street virus) isolate], animal source, and pathogenicity (none, conditional, or high). These are discussed briefly below.

Laboratory-adapted strains of RABV are those viruses that have a long passage history in tissue culture or animals. These have the advantage of well-defined incubation periods and a predictable clinical course in experimental models. However, fixed strains may have lost properties of their wild-type ancestors, such as local replication in muscle prior to CNS invasion. Fixed RABV is not necessarily apathogenic; for example, the pathogenic CVS (challenge virus standard) strain is commonly used for postvaccination challenge studies, in which a pathogenic virus is required (37).

The animal source, such as bat-associated or dog-associated, of a wild-type virus may also be predictive of its phenotype. For instance, it has been suggested that bat-associated strains can

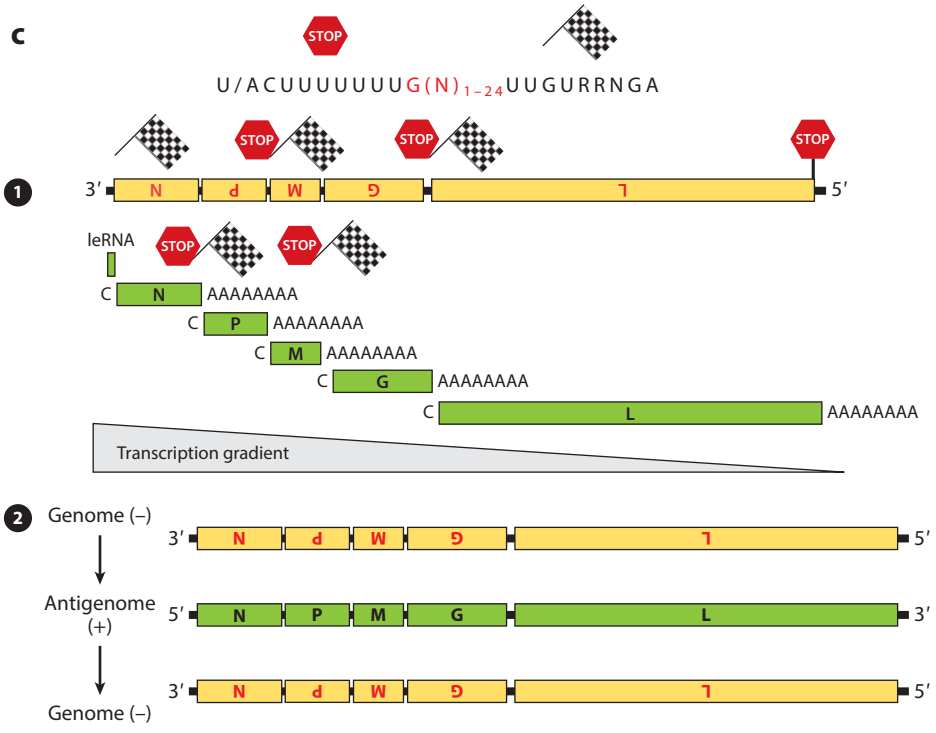


Figure 1

(*a*) A rabies virus (RABV) particle, composed of the host cell–derived membrane and the five viral proteins. (*b*) RABV life cycle. An RABV particle attaches to a host cell receptor (step ❶) and is engulfed by the host cell membrane (step ❷). After pH-mediated fusion of the virion membrane with the endosomal membrane (step ❸), the capsid is released, the individual genes transcribed (step ❹), and the genome replicated (step ❺). (*c*) The RABV transcription and replication strategy. The negative-sense genomic RNA (*yellow*) is the template for the L-P polymerase complex. (❶) During transcription, five 5′end-capped (C) and polyadenylated (AAAAAAAA) mRNAs (*green*) encode the viral proteins. The polymerase complex disassociates from the template at each termination signal (STOP). The polymerase does not always reengage successfully, leading to a negative transcription gradient from 3′ to 5′. (❷) During replication, the negative-sense genome is transcribed into a positive-sense antigenomic RNA intermediate (*green*) by a more processive form of the viral polymerase. The antigenome is then transcribed back into a negative-sense RNA to complete replication. Figure modified with permission from Reference 5.

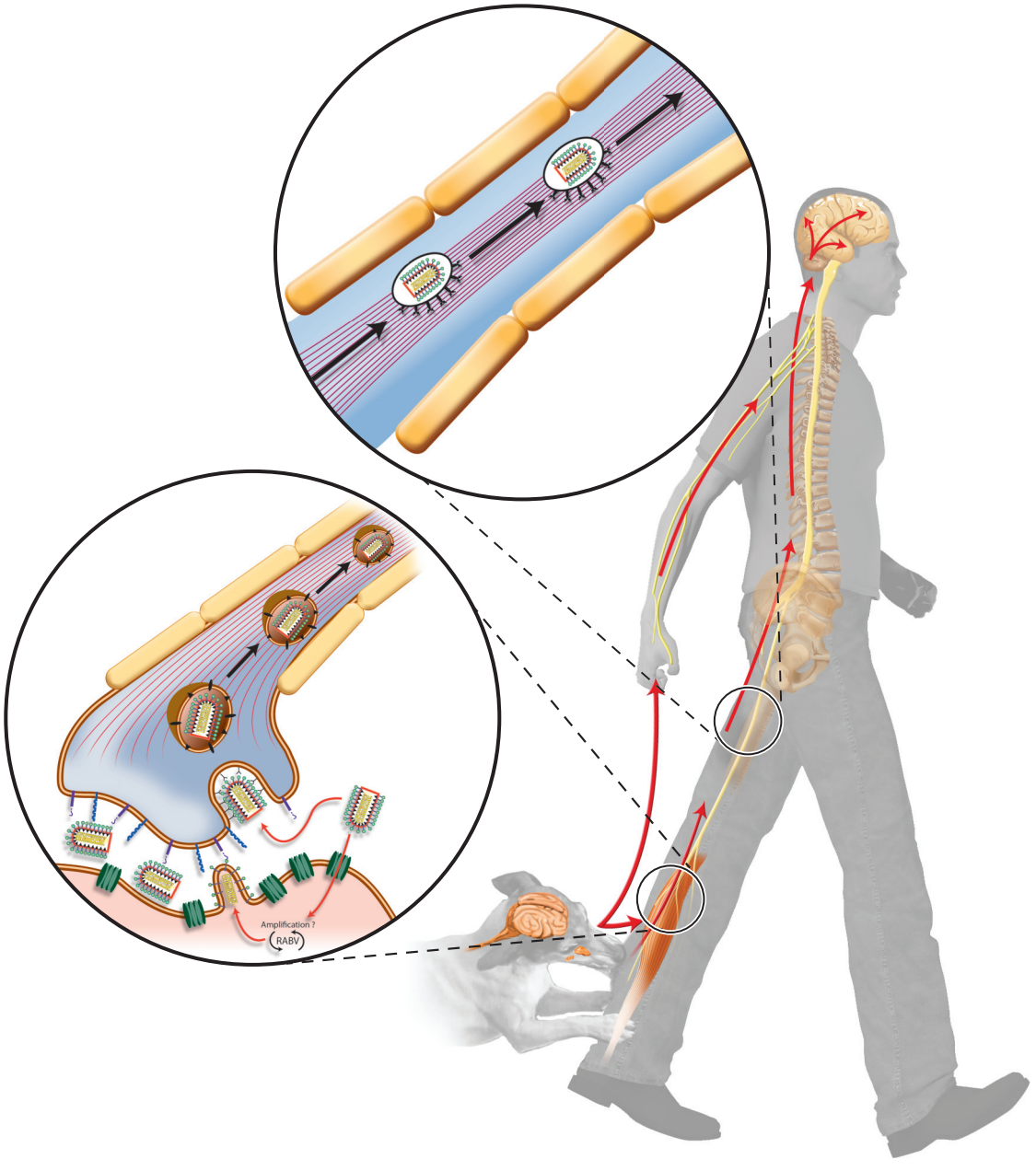


Figure 2

The path of rabies virus (RABV) infection through the host. Most natural RABV infections start with exposure of muscle tissue to RABV particles by an animal bite or scratch. The infection spreads to the peripheral nervous system through neuromuscular junctions (*bottom inset*). Virus particles travel as an enveloped vesicle using dynein-mediated retrograde axonal transport pathways (*top inset*), spreading transsynaptically from postsynaptic to presynaptic neurons until widespread infection of the central nervous system is achieved.

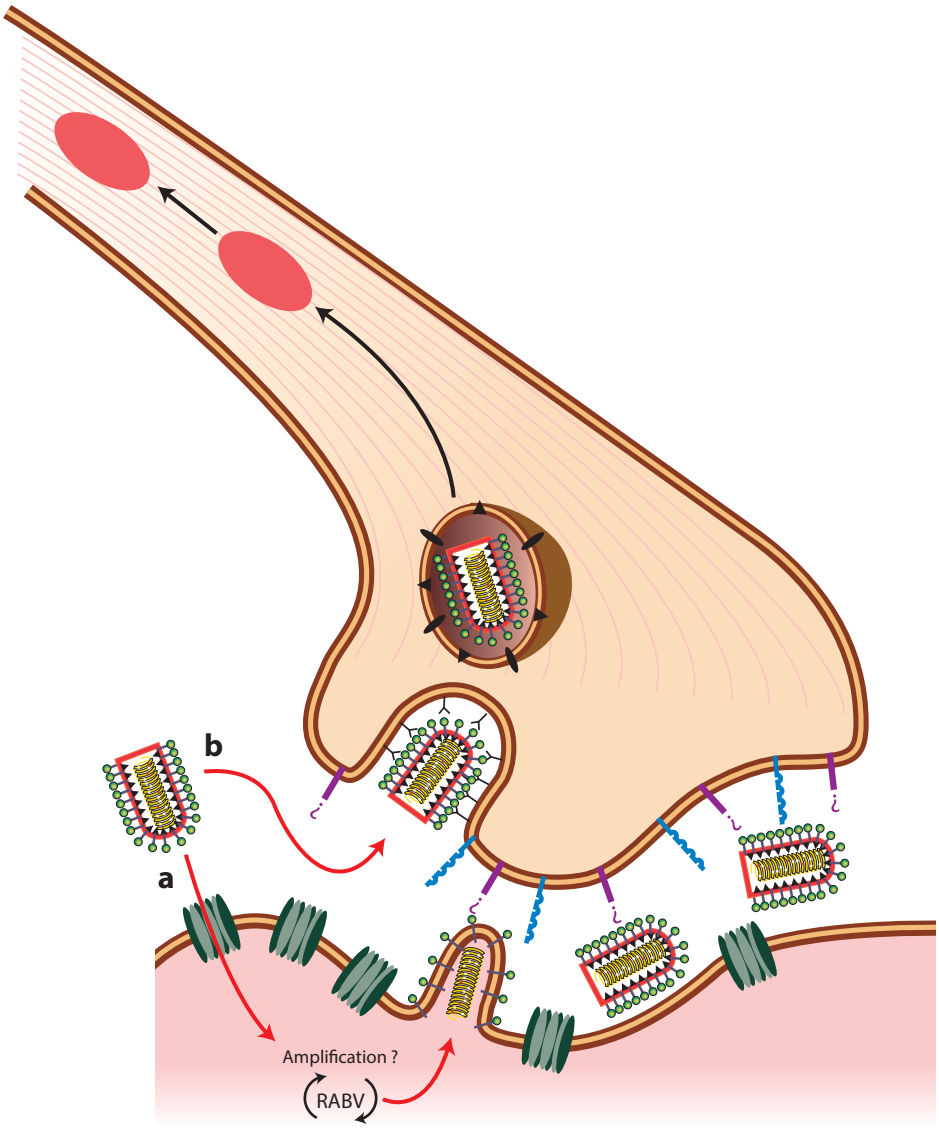


Figure 3

The rabies virus (RABV) neuroinvasive strategy. Two possible mechanisms are depicted, both of which have been observed experimentally. (*a*) The RABV glycoprotein (RABV-G) interacts with the nicotinic acetylcholine receptor (nAchR; *green*), mediating entry into muscle cells. This leads to local replication of the virus before budding into the synaptic cleft of the neuromuscular junction. (*b*) Alternatively, RABV-G interacts with one of several cellular receptors on the neuron, such as NCAM (*purple*) or p75NTR (*blue*). This leads to direct entry into the neuron without prior local replication in muscle.

initiate CNS invasion following relatively minor bite wounds, whereas dog-associated strains require deep penetration into muscle to do the same (38–40). This difference in infectivity may reflect an increased ability of bat-associated viruses to infect and replicate in epithelial or subcutaneous tissue, but the mechanism underlying this observation is not well understood (40).

Finally, the availability of pathogenic and apathogenic (attenuated) substrains of similar viruses allows investigators to study the contribution made by individual RABV genetic elements to pathogenicity. This has been a productive line of research in the years since molecular cloning of RABV became possible (41). In these studies, researchers generated chimeric RABV strains by exchanging genes or parts of genes between more or less pathogenic counterparts and characterized the differences in pathogenicity (5, 41). Several of these studies are discussed throughout this review.

Another important distinction among rabies research strategies is whether peripheral or CNS inoculation sites are used. Direct infection of the CNS is usually accomplished by intranasal or intracranial inoculation. These strategies conceptually separate RABV CNS replication and spread from the peripheral neuroinvasive mechanism necessary for most natural infections. For the most part, however, neuroinvasion, neurotropism, and neuropathogenicity of RABV are correlated in vivo (38). Models of natural RABV infections usually use intramuscular inoculation of laboratory animals.

NEUROINVASION: PENETRATION OF THE CENTRAL NERVOUS SYSTEM

From a peripheral site of exposure, neurotropic pathogens such as RABV must enter the CNS to spread and cause disease. However, the mammalian CNS has several anatomical and biochemical barriers that separate it from the rest of the body. Among the most widely studied natural defense barrier is the blood-brain barrier (BBB), the highly selective permeability barrier of the neurovascular epithelium, made up of tight junctions between the epithelial cells. In addition to the BBB, the CNS's lack of lymphatic drainage, low MHC expression, and elevated levels of immunosuppressive molecules collectively contribute to a state of immunological isolation relative to the rest of the body (42). This concept, once referred to as immune privilege, has been revised, as immune responses can and do occur within the CNS and thus, technically, the brain is not privileged. Rather, the details of the nature of the immune response—from the signals that induce it to the processes that govern the antiviral response in the CNS—are fundamentally distinct from those that have been defined in peripheral organs.

There are several mechanisms by which a virus can overcome barriers that limit neuroinvasion. Retrograde transport along axons from the periphery, the primary mechanism of RABV neuroinvasion, is also a route of infection for alphaherpesviruses and possibly others (43–45); it allows viral infections to bypass the BBB, which would limit hematogenous entry. A related mechanism of viral neuroinvasion is through olfactory receptors, which are the only CNS cells directly exposed to the exterior environment and are therefore likely gateways to CNS infections (46). Intranasal infections are widely used in research, but aside from the possibility of airborne virus in bat-infested caves, sufficiently dense aerosols of neuroinvasive viruses are not encountered in nature (46). Finally, the most common mechanism for viral CNS entry is a hematogenous route, through which viral particles penetrate the BBB by infecting the neurovascular epithelium or a CNS-infiltrating lymphocyte or monocyte. CNS complications of flavivirus or paramyxovirus infections are thought to occur primarily through this route (42, 46). Although hematogenous spread of RABV from the periphery to the brain has been demonstrated in the laboratory for silver-haired bat RABV (SHBRV), it is not thought to be a major contributor to natural RABV infections and was not observed for a dog-derived RABV (47).

In natural infections, RABV neuroinvasion takes place at the NMJ. The strongest early evidence for this came when a protein-protein interaction between RABV and the nAchR was discovered, making the nAchR the first known RABV cellular receptor (CNS-restricted receptors would be

discovered later) (48). The nAchR is a widely disseminated receptor in the peripheral nervous system, located on the postsynaptic membrane of NMJs (5). The discovery of the nAchR-RABV interaction was soon solidified by immunofluorescence and electron microscopic evidence, which showed fixed RABV localizing to NMJs in vivo (49). An nAchR binding site on RABV-G was subsequently identified, providing further evidence for a RABV-G-mediated entry mechanism (50).

Curiously, the nAchR is located at postsynaptic muscle membranes and not presynaptic nerve membranes as initially suspected. This suggests that RABV has conserved a strategy of local replication in the muscle prior to neuroinvasion (31). Concentrating virus at an NMJ, or exposing additional NMJs to viral particles, may increase the likelihood of CNS uptake (51). This may also explain the long incubation periods of natural RABV infections, during which a latent or low-replication stage of RABV may occur in muscle cells. Such a hypothesis is supported by experiments in which street-virus RABV replication occurred in vivo despite denervation of the inoculated muscle (52, 53). However, entry of RABV from the periphery into the CNS without prior local replication has also been observed in laboratory animals inoculated with fixed RABV (54, 55).

The relative contributions to human rabies made by latent or low-replication RABV in muscle and by direct neuronal infection are not currently known. This is an important ongoing question in the field, as it may influence the efficacy of RABV-targeted interventions. For example, a drug that does not penetrate the CNS may be effective if RABV replicates locally in muscle before entering a neuron but will fail if RABV enters the CNS directly after exposure.

It is thought that RABV penetrates the nervous system exclusively through primary motor neurons, and not through sensory or autonomic neurons (32). The field of RABV transneuronal tracing, a method by which RABV spread is closely monitored, has provided much of the evidence for this hypothesis. For example, fixed RABV was used to map muscles controlling eye movement in guinea pigs (36), the facial and bulbospongiosus muscles of rats (56, 57), and the motor innervation of primate hand muscle (58). RABV was found to label only motor neurons in these studies. This hypothesis is complicated by conflicting results elsewhere, however. After intramuscularly inoculating mice with a fixed strain of RABV, another group found simultaneous infection of sensory and motor neurons at early time points postinfection (54). Another study, in which fixed RABV was inoculated into the mouse footpad, found RABV antigen in dorsal root ganglion (DRG) sensory neurons (59), albeit at a time point (72 hours postinfection) that could indicate reciprocal spread of RABV between the motor and sensory routes rather than primary infection of sensory neurons (54).

The expression pattern of the peripheral RABV receptor, the nAchR, may clarify this issue. The nAchR is present on postsynaptic motor endplates and does not play a role in transmitting signals from sensory neurons (32). Therefore, it follows that RABV particles may become concentrated at NMJs, rather than sensory endings, and first penetrate the nervous system through a primary motor neuron (5, 32). It should be noted that these circumstances are relevant only for the first stages of RABV infection. At later stages of infection, the distinction between motor and sensory pathways becomes irrelevant as the virus spreads in the CNS centrifugally toward its end organs and is readily detected in both motor and sensory neurons (32).

RABIES VIRUS AXONAL TRANSPORT

Even before humankind understood what viruses were and how they caused disease, RABV was suspected to be an agent that traveled through nervous tissue (1). Early studies of axonal transport used the drug colchicine as an inhibitor of axoplasmic flow, which we now know occurs by inhibiting

microtubule polymerization. The inhibition of RABV propagation through a colchicine-treated rat sciatic nerve provided early evidence for RABV retrograde axonal transport (60, 61).

In recent years, studies of RABV-G and RABV-P structure and host cell interactions have guided research in RABV axonal transport mechanisms. RABV-G was found to be sufficient to confer retrograde axonal spread to a pseudotyped lentivirus vector, as well as to a VSV deleted of its own G and transcomplemented with RABV-G (30, 62). This is strong evidence that RABV-G plays a role in retrograde axonal transport. A screen for lyssavirus P–interacting proteins revealed an interaction between RABV-P and the cytoplasmic dynein light chain (LC8), a protein involved in minus-end-directed microtubule transport (63, 64). This evidence suggests a mechanism for RABV retrograde transport involving a dynein–RABV-P interaction (dynein is a family of motor proteins that transport cargo in a retrograde manner). However, deletion of RABV-P's LC8-binding site did not abrogate transport of RABV from the peripheral site of inoculation to the brain. This was taken as evidence for other microtubule-virus interactions that have yet to be described (65, 66).

RABV axonal transport has been directly observed with live cell in vitro imaging. RABV containing a fluorescently labeled RABV-G was observed traveling in neuronal processes completely enveloped in endosomal vesicles, confirming the repurposing of intracellular transport mechanisms by one or more viral proteins (67). Another study using compartmentalized rat DRGs showed efficient anterograde transport of RABV subsequent to retrograde transport and viral replication in the cell soma (68). This complicates the concept that RABV is transported in only a retrograde fashion, a belief that has been taken for granted by researchers employing the virus as a transneuronal tracer (32). However, the most likely contribution of anterograde transport is in late-stage infections, when multiorgan deposition of RABV is observed (34). The consequences of anterograde transport for early events in the RABV life cycle, such as neuroinvasion or spread through the CNS, remain controversial.

RABIES VIRUS TRANSSYNAPTIC SPREAD

When infecting a host organism, viruses are confronted with a complex mosaic of cell types, only some of which may be susceptible to infection, as well as intrinsic and innate host defenses (46). One way to look at RABV tropism is to consider the advantage a virus derives from accessing a densely connected network of related cells such as neurons. Furthermore, as a nonrenewable cell population, neurons have evolved mechanisms (such as the BBB) to protect themselves from cytotoxic immune effectors such as $CD8^+$ T cells, which may otherwise recognize and destroy a virus-infected cell (42).

As discussed above, the unique features of RABV once inside the CNS are exclusive infection of neurons, transsynaptic spread, and lack of apparent damage to infected tissue (32). Studies of these phenomena have yielded diverse insights into RABV–host cell interactions. The cellular tropism of RABV, for example, is thought to be determined by the virus's ability to bind to at least two cell surface targets specific to neurons, the neuronal cell adhesion molecule (NCAM) and p75NTR (recall that the other cellular receptor, the nAchR, is present only on postsynaptic membranes and therefore most likely plays a role in entry into muscle cells rather than neurons) (48, 69, 70). Because deletion of the genes encoding these viral receptors does not entirely abrogate RABV infection in vivo, it is likely that RABV uses these receptors in combination with other molecules such as carbohydrates, gangliosides, and lipids (71) or that other receptors exist to facilitate entry. In the face of this incomplete picture, another way to approach the study of tropism is to study the viral ligand of the cellular receptor—RABV-G.

ROLE OF THE RABIES VIRUS GLYCOPROTEIN IN VIRAL TROPISM AND PATHOGENICITY

Shortly after RABV-G was characterized as a RABV surface protein, anti-RABV-G monoclonal antibodies were observed to protect laboratory animals from RABV challenge (72). This finding had substantive implications for contemporary vaccine design, but it also offered an indirect means of examining the puzzle of RABV pathogenicity. By chemically mutagenizing the virus and screening for RABV that escaped neutralization by these antibodies, researchers generated a panel of fixed RABV-G mutants with reduced virulence (73, 74). These pathogenic and apathogenic counterpart strains have continued to enable investigations into RABV-G as a molecular determinant of pathogenicity. One mutant discovered in this manner, which substitutes the arginine at position 333 with glutamate or isoleucine, has become a well-studied model of RABV-G-mediated RABV attenuation (54, 75, 76). The G-333 mutant displays stunted spreading, infecting primary motor neurons following intramuscular inoculation but becoming blocked after the first cycle of infection (54).

These first-generation RABV-G mutants are all tissue culture–adapted virus strains, which raises the concern that they do not recapitulate natural infections (77). The recovery of infectious RABV from cDNA clones has allowed researchers to make more precise changes to the viral genome, and to manipulate wild-type isolates without extensive passage in tissue culture. For instance, one study exchanged the RABV-G of a fixed, nonpathogenic strain of RABV (SN-10) either with the RABV-G of a bat-associated street virus (SHBRV) or with that of two different fixed but pathogenic strains (CVS-N2c and CVS-B2c) (77). This resulted in significant, but incomplete, restoration of the pathogenic phenotype after intramuscular inoculation of mice for each of the chimeric viruses. Similar results were observed for the substitution of SN-10's RABV-G and RABV-M with SHBRV's, and for several other combinations (78, 79). Together, these results suggest that RABV pathogenicity is partially, but not exclusively, determined by RABV-G.

RABIES VIRUS IMMUNE EVASION

RABV, like all pathogens, has coevolved with the sophisticated immune systems of its hosts. As discussed above, the tropism of RABV for an immunologically isolated tissue is itself an immune evasion strategy. RABV strains that replicate at lower levels in vivo and that cause less overt tissue damage are often more pathogenic (80–82). Therefore, it seems that RABV is evading the adaptive immune system by staying under the radar. Some mechanisms of this adaptive immune evasion have been studied, such as a possible manipulation of BBB permeability and the RABV-induced apoptosis of CNS-infiltrating lymphocytes during infection.

RABV also needs to escape innate immunity, which is highly conserved in all mammalian cells and might be especially important in neurons. For these reasons, RABV directly disrupts inter- and intracellular signaling pathways involved in host cell defense. The best known and understood of these is the disruption of IFN signaling by RABV-P, a mechanism of innate immune evasion.

Rabies Virus and the Interferon Response

Most mammalian cells can detect viral infections through pattern-recognition receptors, which stimulate production of type I IFN (includes IFN-α and IFN-β) (18, 83, 84). IFNs act in an autocrine and paracrine manner to induce expression of IFN-stimulated genes, which have diverse antiviral functions (84, 85). The importance of the IFN system in the context of RABV infection was demonstrated by the increased susceptibility to RABV of (*a*) mice given IFN-blocking

immunoglobulin and (*b*) type I IFN receptor–knockout mice (*Ifnar*$^{-/-}$) (86, 87). The fact that *Ifnar*$^{-/-}$ mice have elevated or faster morbidity after both peripheral and central inoculations (86, 88) suggests that IFNs act as an anti-RABV defense mechanism at the levels of both initial neuroinvasion and intra-CNS spread.

Researchers have elucidated two distinct mechanisms of IFN inhibition by RABV, both mediated by RABV-P: (*a*) inhibition of initial IFN induction and (*b*) downstream induction of IFN-stimulated genes. Both these mechanisms involve the binding by RABV-P of cytoplasmic signal transducers and nuclear transcription factors, requiring both cytoplasmic and nuclear forms of the protein. This is accomplished by the use of internal start codons. Full-length RABV-P has a nuclear import and a nuclear export sequence, but a truncated form is also produced that lacks the nuclear export sequence and therefore is trafficked to and remains in the nucleus (18, 89).

The first of the RABV-P-mediated IFN-inhibitory mechanisms to be discovered was the nuclear binding of IFN regulatory factor 3 (IRF3) (18, 90). IRFs are transcription factors that activate IFN production in response to upstream detection of viral nucleic acids; RABV-P prevents IRF activation, blocking the IFN response (18). The first evidence of this was the observation that strains of RABV engineered to express low levels of RABV-P induced higher levels of IFN production (91). A follow-up study showed that truncated RABV-P also induces high levels of IFNs but that IFN inhibition can be restored by complementing with the full-length version, and linked this effect in vitro to a possible IRF3–RABV-P protein-protein interaction (90). This interaction was later confirmed and mapped to specific sites on RABV-P (92).

In addition to counteracting transcription of IFNs, RABV has a separate mechanism to prevent cells from responding to IFNs. RABV-P sequesters the transcription factor STAT1/2 (a heterodimer) in the cytoplasm, preventing it from reaching its nuclear IFN-stimulated gene target promoters (93). The interaction between RABV-P and STATs was mapped to sites on RABV-P that were distinct from the IRF interaction sites (92). Additionally, the nuclear form of RABV-P, responsible for IRF binding, also binds STAT1 in the nucleus, implicating a separate, nuclear inhibition mechanism for STAT1 (94). This mechanism is conserved across several lyssavirus species (10).

Predictably, RABV-P is a determinant of RABV pathogenicity. This has been studied using chimeric viruses, similar to the exchanges of RABV-G between pathogenic and nonpathogenic strains described above. In one study, a fixed RABV that killed laboratory animals after intracranial injection was attenuated by repeated passage in cell culture (95). When the RABV-P of the pathogenic parent strain was inserted into its nonpathogenic derivative, pathogenicity was restored. The attenuated strain was found to be specifically impaired for blocking STAT nuclear translocation, directly linking STAT inhibition to pathogenicity. A follow-up study recapitulated these data after intramuscular infection, showing that STAT inhibition by RABV is also important in the neuroinvasion step of RABV infection (96).

Importance of Central Nervous System–Infiltrating Lymphocytes in Controlling Rabies Virus

The migration of lymphocytes into the CNS has been implicated in the control of several neurotropic pathogens, RABV included (80). Although the means by which T or B cells clear a RABV infection in the CNS are still not known, the importance of these migratory cells is becoming evident. For instance, peripheral inoculation of nude mice, which lack functional T lymphocytes, with a normally apathogenic RABV results in a fatal outcome (97). In contrast, the presence or absence of functional lymphocytes does not change the outcome of a pathogenic

RABV peripheral inoculation (98). One explanation for this result is that apathogenic RABV activates T cells in the periphery prior to neuroinvasion, preventing the virus from entering the CNS in a T cell–dependent manner. However, differences in the immune response, in terms of neutralizing antibody titers, are not necessarily observed between peripherally administered strains of different pathogenicity (80, 99). Rather, it is also possible that RABV gene products actively block the entry or survival of lymphocytes in the CNS.

Entry of immune effectors may be blocked at the level of the BBB. A difference in pathogenicity between two RABV strains may be explained by the difference in BBB permeability to lymphocyte chemoattractants (100). By maintaining a higher level of BBB integrity, wild-type RABV may block lymphocyte diapedesis across the neurovascular epithelium and thereby prevent lymphocytes from accessing RABV-infected neurons (100, 101). In a comparison of several RABV strains, researchers found significant lymphocyte CNS infiltration and increased BBB permeability in animals infected with attenuated RABV, but not in animals infected with any pathogenic RABV strains (101). Additionally, increasing BBB permeability during wild-type RABV infection increased survival (102). However, it is still not known whether this means that pathogenic RABV strains have an active mechanism for maintaining BBB integrity. It is also possible that the attenuated RABV has gained an immunostimulatory property that triggers a danger signal, resulting in immune cell infiltration and viral clearance.

There is also evidence that RABV-infected cells are stimulated to kill CNS-infiltrating lymphocytes. When comparing peripherally administered pathogenic and attenuated fixed RABV strains (CVS and Pasteur virus, respectively), one group observed that although both viruses triggered $CD4^+$ and $CD8^+$ T cell CNS infiltration, migratory T cells were lost over the course of pathogenic RABV infection but continued to accumulate in the transient infection (103). This loss corresponded to an increase in T cell apoptosis, indicating that lymphocytes were able to invade the BBB but not survive in the CNS. Deletion of the cell surface molecule FasL, a major trigger of T cell apoptosis in the normal immune system, abrogated this effect and increased survival after pathogenic fixed virus challenge (103). A similar reduction of RABV virulence was observed in mice lacking B7-H1, another natural inhibitor of T cell responses (104).

NEUROPATHOGENICITY AND SPREAD OF RABIES VIRUS

Despite much research into RABV biology, the mechanism by which RABV infection causes fatal disease is still not known. As noted above, symptomatic rabies patients die with only mild histological lesions in the brain, and without significant loss of neurons (105). The immune evasion strategies outlined above explain a part of this situation: RABV seems to avoid inflammatory or cytolytic host defense and may lose its ability to cause disease if apoptosis, local inflammation, or lymphocyte diapedesis is restored (80–82, 106). Therefore, RABV's "invisibility" can be regarded as a successful adaptation. Neuronal dysfunction induced by wild-type RABV may not depend on any known immune response (i.e., viral proteins alone may be responsible for it).

Researchers approach the study of this neuronal dysfunction in a number of ways, mainly morphological, metabolic, and molecular genetic analysis. These lines of research most directly address the central mystery of RABV pathogenesis: the gross behavioral changes and death that occur in human clinical rabies cases.

Morphological, Metabolic, and Genetic Analysis of Infected Cells

For many years, the major observation that distinguished RABV-infected and normal cells was the appearance of cytoplasmic inclusions called Negri bodies, named for the researcher who described

them in 1903. Negri bodies are now known to be centers of RABV translation and replication, and they contain at least one cellular protein, Hsp70, with a positive role in viral production (107, 108). However, despite their clear role in the RABV life cycle, the known properties of Negri bodies do not explain the aberrant phenotype of RABV-infected neurons.

More details have emerged with advances in labeling and microscopy. In one study, degeneration of axons or dendrites—indicated by obvious disorganization or disappearance during microscopy and loss of microtubule- and neurofilament-specific immunostaining—was observed in animals peripherally inoculated with a pathogenic fixed virus but in animals with an attenuated fixed virus (109). Consistent with findings in other studies, this degeneration occurred without evidence of inflammation or apoptosis. When another team used fluorescently labeled recombinant RABV in a similar system, infected neurons displayed bead-like swellings in neuronal processes, especially axons (110).

These morphological changes in infected cells have been linked to metabolic changes. One report recapitulated the axonal swelling phenotype in mouse DRGs cultured ex vivo that were infected with pathogenic fixed RABV (111). In addition to RABV antigen, these swellings were found to contain aggregations of mitochondria and markers of oxidative stress, characteristics of sensory neurons in the context of diabetic neuropathy (111, 112). A follow-up study gathered evidence that RABV-infected cells, including DRGs, have increased generation of potentially toxic reactive oxygen species during normal cellular metabolism (113). The loss of neuronal structural integrity suggested by these studies is especially interesting. Because one of the defining features of the CNS is its connectedness, a pathogen that physically disrupts axons or dendrites could cause major damage to the CNS without killing any cells, for example, by affecting neuronal networks.

The tools of modern genetic analysis have recently been brought to bear on this question as well. Our laboratory's recent study used a virally expressed Cre recombinase–based reporter system to permanently mark RABV-infected neurons in vivo (114). To the existing techniques of viral antigen staining and fluorescently labeled virus, this reporter system adds a noninvasive and permanent way of marking infected cells. Transgenic mice are used that express a fluorescent TdTomato protein in all cells. This gene is flanked by *loxP* sites such that in the presence of a functional Cre recombinase, the intervening sequence is removed, the expression of TdTomato is lost, and the expression of a downstream green fluorescent protein gene is induced. This shift reflects a somatic cellular change that is indicative of infection and would remain even if the virus were eventually cleared. The Cre-based reporter system is a promising method for studying RABV spread to various brain regions. In this approach, neurons that were once infected with RABV but had cleared the virus can be separated from uninfected neurons using fluorescence-activated cell sorting, and the transcriptome changes between these two cell populations can be analyzed by microarray. The application of this technique has yielded a large data set that researchers can use to probe the molecular genetics of RABV neuronal dysfunction.

Late Stages of Infection: Behavioral Changes, Centrifugal Spread of Rabies Virus, and Death

Human cases of rabies present across a spectrum of clinical forms, roughly divided into furious or paralytic rabies (1). Briefly, furious rabies refers to classical rabies symptoms, including severe agitation and hydrophobia (1). During the course of several days, this phase of excitement subsides into worsening paralysis and impaired consciousness, and finally coma. Death occurs by cardiac arrest, circulatory insufficiency, or respiratory failure. In paralytic rabies, ascending paralysis is the principal presenting feature, followed by a similar progression into coma. Both phenotypes may be observed in laboratory animals following infection, sometimes unpredictably with the same

strain of virus and method of administration (B.M. Davis, unpublished observation). The same may be true of natural RABV infections: In one report, furious rabies and paralytic rabies were observed in two human patients after exposure to the same rabid dog (115).

In the past, the behavioral changes observed following RABV infection have been ascribed to the observation that RABV preferentially infects the structures associated with the limbic system, responsible for aspects of emotion and motivation (46). However, there is no evidence that RABV is more able to infect the neurons of one brain region or another (P. Strick, personal communication). Rather, it is more likely that this observation is due to the closer synaptic connectivity of these regions with the common sites of RABV entry. In either case, rabies-associated aggression seems to make transmission of RABV more likely, especially given the synchronous shedding of the virus into saliva.

In the late stages of infection, the spread of RABV to salivary glands occurs alongside the observed centrifugal propagation of RABV to diverse end organs, such as skin, hair follicles, and muscle fibers (32, 35). In order to reach the salivary glands from the CNS, RABV needs to travel in an anterograde fashion through autonomic sensory ganglia—an inversion of the unidirectional (retrograde) propagation of RABV observed early in infection (32). RABV enters the saliva by budding from the apical plasma membranes of mucous cells, an apparent loss of strict neurotropism (116). These changes highlight the sharp differences between RABV phenotypes early and late in infection.

Rabies presents with some variation across species (117). Small mammals such as bats may have a milder, more prolonged disease progression or may even recover (118, 119). Nonfatal or abortive human rabies cases are more controversial. In case reports of patients surviving clinical rabies, partial resistance from previous exposure plays an ambiguous role alongside intensive care (120). Naturally acquired resistance to RABV was suggested by a study of two communities in rural Peru, where unvaccinated but seropositive individuals were observed (121). For the most part, however, knowledge of nonfatal RABV exposures or disease has not changed the grim prognosis for clinical rabies patients. Outcomes in human rabies patients seem to be similar among individuals infected by large or by small mammals, despite any reported differences in the infectiousness of RABV strains associated with these animals (1, 122). This suggests that (*a*) RABV causes similar damage at the cellular level in all mammals, but presents differently due to differences in CNS architecture, and/or (*b*) RABV can initiate distinct diseases depending on the host organism. Neuronal dysfunction may be a consequence of disruption or deregulation of particular neuronal networks or pathways. A better understanding of both RABV cellular pathogenesis and synaptic connectivity is required before these concepts can be meaningfully related to each other.

OUTLOOK AND FUTURE DIRECTIONS

The mystery of RABV neuropathogenesis is still being unraveled despite many years of research. However, the advancements made during the past few years hold great promise for the field. The structure and life cycle of RABV are now well understood, an important step in enabling efforts to develop interventions that block replication or budding of the virus. Moreover, the ability to experimentally manipulate the RABV genome by reverse genetics allows researchers to link pathogenicity to specific sites in the viral genome. These genetic determinants of pathogenicity are themselves becoming better understood, given progress in fields such as RABV neuroinvasive mechanisms, intracellular targeting, and immune evasion. Furthermore, advances in in vitro imaging have recently allowed researchers to observe RABV traveling through host cells at high resolution. The same may be said about RABV spread through different brain regions, which is becoming

easier to visualize. Finally, the powerful toolbox of modern high-throughput genetics platforms, such as microarrays, has just begun to be applied to the field of RABV–host cell interactions.

It is hoped that the next generation of RABV virologists will integrate these advancements with the central observations about the unique disease. For this to occur, researchers will have to somehow connect these observations with the ways RABV uses neurons not just as a substrate—as most other neurotropic viruses seem to do—but as a platform for manipulation of target cells.

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