The cell biology of rabies virus: using stealth to reach the brain

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Abstract | Rabies virus, the prototypical neurotropic virus, causes one of the most lethal zoonotic diseases. According to official estimates, over 55,000 people die of the disease annually, but this is probably a severe underestimation. A combination of virulence factors enables the virus to enter neurons at peripheral sites and travel through the spinal cord to the brain of the infected host, where it often induces aggression that facilitates the transfer of the virus to a new host. This Review summarizes the current knowledge of the replication cycle of rabies virus and virus–host cell interactions, both of which are fundamental elements in our quest to understand the life cycle of rabies virus and the pathogenesis of rabies.

Rabies is a devastating and important, albeit neglected, infectious disease that has been feared by mankind for more than 4,000 years[1]. Although rabies was not recognized to be the result of an infection when it was first described, it was well known that a bite from a 'mad dog' would most likely result in the death of the bitten individual, and laws regulated the consequences for the dog's owner[2]. In 1802, George Zinke[3] showed that rabies could be transmitted from the saliva of a rabid dog to a healthy dog and cause disease. Pierre-Victor Galtier[4] first demonstrated the transfer of rabies from one animal species to another, and his studies were continued and extended by Louis Pasteur[2], who established that the virus is harboured in the brain. In 1885, Pasteur developed the first rabies virus vaccine from the spinal cord of rabbits with rabies[5]. This meant that rabies, which is almost always fatal after the onset of disease, was no longer an automatic 'death sentence' for those exposed[6,7].

Despite the great progress that has been made in the development of rabies virus vaccines and the control of rabies (for a review, see REF. 8) (BOX 1, FIG. 1), the annual number of deaths caused by rabies virus worldwide is estimated to be between 40,000 and 70,000 (see WHO Fact Sheet No. 99). However, a recent study in Tanzania indicated that the number of deaths caused by rabies may be up to 100 times higher than officially reported[9], and it has been suggested that only 3% of human cases of rabies virus are recorded by central health authorities[1]. As 40% of those infected are children, the years of life lost by this infection makes rabies the seventh most important global infectious disease[10]. Therefore, new approaches for therapy and cheaper, more effective vaccines are urgently needed. A better understanding of the biology of the virus might also help to develop rabies virus as a tool for neuronal labelling and neurotracer studies[11–14] and will provide insight that will facilitate the development of treatments for other diseases of the central nervous system (CNS).

In the past, the study of rabies virus was hampered by the lack of genetic manipulation; therefore, research studies were based on the use of different rabies virus strains, which were often genetically diverse. Although these studies enhanced our understanding of the virus, the reverse genetics technique established in 1994 to directly manipulate the rabies virus genome has opened new research areas for rabies virus molecular biology and pathology[15]. The ability to introduce precise mutations and to exchange genes from vaccine and pathogenic strains has allowed more detailed analysis of the virus–host cell interaction. In this Review, we describe our current knowledge of the cell biology of rabies virus, including the steps of the viral life cycle and virus–host cell interactions.

Genomic organization and replication

Rabies virus belongs to the genus lyssavirus (from the Greek word for frenzy) in the *Rhabdoviridae* family. Rabies virus is divided into two phylogroups with a total of 11 genotypes[16,17]. Genotype 1, which contains the so-called classical rabies virus (see BOX 2 for definitions) is the most prevalent and is responsible for most animal and human infections and deaths[16].

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doi:10.1038/nrmicro2260
Published online
30 November 2009

Box 1 | **Rabies symptoms and treatment**

Clinical manifestation of rabies in humans has two forms[7]: the furious (classical) form (80% of infections), and the numb (non-classical or paralytic) form (20% of infections)[126]. The furious form of rabies "is characterized by hydrophobia: terror and excitation with spasm of inspiratory muscles, larynx and pharynx precipitated by attempts to drink" (REF. 7), and episodes of hallucinations and excitement are common. Animals often present with extreme aggression and randomly attack objects, other animals or humans[127]. These behavioural changes occur simultaneously with the shedding of large amounts of rabies virus in the saliva, which facilitates the spread of the virus to a new host. The numb form of rabies is characterized by weakness and flaccid paralysis, which sometimes causes misdiagnosis at the onset of this clinical form of rabies[126]. In both cases, survival after the onset of symptoms is rarely more than 7 days[7].

Human rabies virus infections are controlled by limiting the infection in animals and by post-exposure prophylaxis (PEP) of humans after contact with a potentially infected animal. Current PEP in the United States for previously non-immunized individuals consists of one dose with rabies immunoglobulin in conjunction with five doses of immunization with inactivated rabies virus vaccines[128]. This regimen is effective, but its usefulness in developing countries, where rabies is endemic, is hampered by the high cost and lack of compliance. The WHO has approved alternative vaccine regimens in developing countries to reduce the cost of vaccination. Approaches that do not rely on the use of inactivated vaccines, including DNA vaccines[129–134] and viral vectors[135–140], are also being investigated. Although pre-exposure vaccination has been historically reserved for populations at risk of infection, such as veterinarians and laboratory workers, efforts are underway to promote and determine the efficacy, safety and cost for a broader application of rabies virus vaccines in pre-exposure settings, especially children in high risk areas[141–143].

Rabies virus is the prototypical neurotropic virus and has a small, negative-stranded RNA genome of about 12 kb, which encodes five proteins[18,19] (FIGS 2,3). Similarly to all negative-stranded RNA viruses, the RNA genome is tightly encapsidated into the nucleoprotein. Only the encapsidated RNA, termed the ribonucleoprotein (RNP), is a functional template for transcription and replication. The viral capsid also contains the viral polymerase complex (PC), which consists of the polymerase (known as L, for large protein) and the phosphoprotein, the non-catalytic subunit of the polymerase complex. The capsid is surrounded by the host cell-derived membrane that interacts with two viral proteins, the matrix protein and the glycoprotein. On a structural level, the matrix protein functions as a bridge between the rabies virus RNP and the virion membrane. The matrix protein interacts with and condenses the RNP, yielding the typical helical form of the RNP found within the virions. It also interacts with the cytoplasmic domain of the glycoprotein, which forms a trimer[20], and has a role not only in virus entry and membrane fusion, but also in virus release[21].

The life cycle of rabies virus

As for most viruses, the rabies virus life cycle can be divided into three phases (FIGS 3,4). The first phase includes binding to the host cell receptor (or receptors), entry into the host cell by endocytosis followed by fusion of the viral and endosomal membrane and release of the viral genome into the cytoplasm (known as uncoating). Although a recent study using bat-associated rabies virus has shown that the virus spreads in the blood and invades the CNS at the neurovascular junction of the hypothalamus[22], classical wild-type rabies virus most likely enters a motor neuron at the neuromuscular junction. In either case, rabies virus particles are subsequently transported in a retrograde direction through the axon of the infected neuron[11]. Once the virus particles reach the cell body of the neuron, the second phase commences, which involves the production of the virion components (transcription, replication and protein synthesis). The last phase of the life cycle comprises the assembly of the viral components, their transport to the site of budding and the release of mature rabies virus particles, which can then start a new round of infection. Every step of this process is highly regulated and only partly understood (FIG. 3).

The rabies virus receptor — still a mystery? The first step in the rabies virus life cycle is the attachment to the host cell. This step requires the rabies virus glycoprotein. Indeed, rabies viruses with a deletion in the gene encoding the glycoprotein that are *trans*-complemented with the glycoprotein infect cells efficiently but cannot spread from the infected cell *in vivo*[23] or *in vitro*[24], indicating that the glycoprotein is required for the spread of the virus. However, it is less clear which host cell molecule (or molecules) interacts with the rabies virus glycoprotein and mediates its entry into cells. Nicotinic acetylcholine receptor (nAchR) was the first identified potential receptor for rabies virus[25]. Because nAchRs are located at the postsynaptic muscle membrane and not at the presynaptic nerve membrane[26], it is unlikely that this receptor is used for the initial entry into motor neurons. Instead, it is possible that nAchR enriches rabies virus at the neuromuscular junction to enable more efficient infection of the connected motor neurons[26] (FIG. 4). Moreover, as rabies virus might initially replicate in striated muscle cells, nAchRs could be used to infect muscle cells[27,28].

Other potential receptors for rabies virus include neuronal cell adhesion molecule (NCAM; also known as NCAM1)[29] and low-affinity nerve growth factor receptor (p75NTR; also known as BEX3 and NGFR)[30]. It has been shown that virus-resistant cell lines are permissive to rabies virus infection after NCAM expression and that NCAM-specific antibodies and NCAM ligand treatment reduce rabies virus infection[29]. However, mice in which the gene that encodes NCAM was deleted were still susceptible to infection with the virus, although the disease was delayed. This indicates that although the receptor might not be essential for infection, it has a role in the infection process[29]. The function of p75NTR in rabies virus entry is less clear. Similarly to NCAM, expression of p75NTR enables non-permissive cells to be infected with a rabies virus field isolate, but there is no difference in disease progression between p75NTR-deficient and wild-type mice[31]. Moreover, only 25% of the primary dorsal root ganglions that express p75NTR were infected by rabies virus *ex vivo*, indicating that p75NTR might only function in combination with another cell surface molecule[31]. Carbohydrates[32,33], gangliosides[32] and lipids[34] have also been suggested to be rabies virus entry receptors, but their functions are not well established.

Trans-complemented
Complemented by expression of missing components in the host cell.

Postsynaptic
Positioned after the synapse.

Presynaptic
Positioned before the synapse.

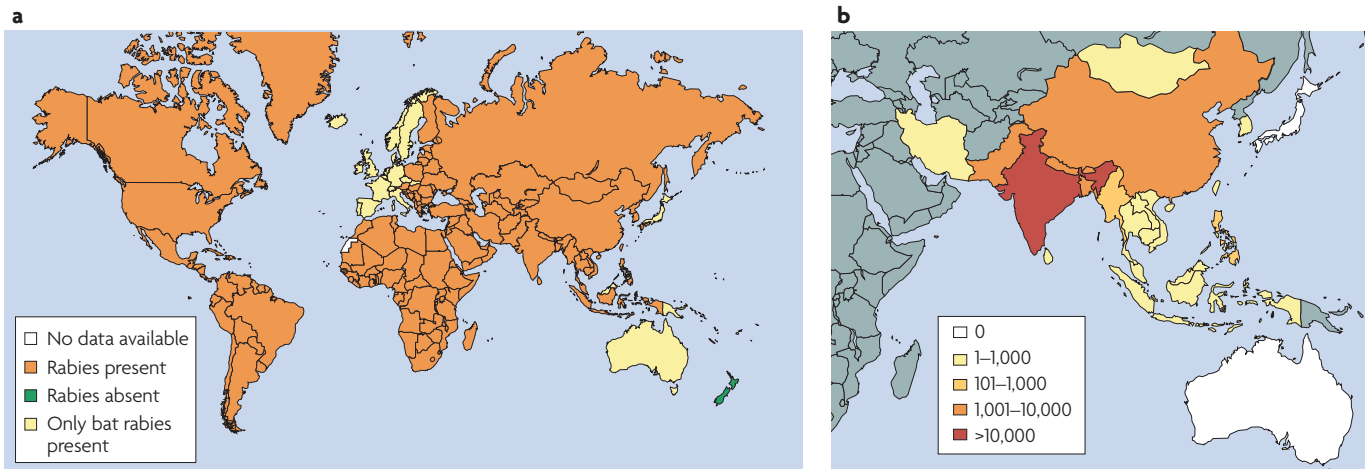

Figure 1 | **Rabies worldwide. a** | The worldwide distribution of rabies virus. The distribution of classical or terrestrial rabies virus (genotype 1 (GT1)) in 2007 is shown in orange. The distribution of rabies virus and other members of the lyssavirus genus is shown in yellow. The only known lyssavirus-free country is New Zealand (green). **b** | Number of human deaths from rabies in 2004. The map indicates the areas in South-East Asia most affected by human deaths caused by rabies for which reliable data exist.

None of the multiple potential receptors identified seems to be essential *in vitro*. The finding that a single point mutation in the rabies virus glycoprotein can render rabies virus non-neurotropic and reduce its uptake speed[35] can only be explained by the use of two different receptors. Differences in speed of uptake and spread in tissue culture of bat-derived rabies virus and a rabies virus vaccine strain also suggest that different receptors may be used[36]. It is possible that certain receptors require an additional molecule (a co-receptor), as suggested for p75NTR[31]. Distinguishing the receptor mechanism is even more complicated for *in vivo* systems because it is unclear whether the receptor (or receptors) used for the initial infection is the same as the one used for viral spread[35]. Obviously, more research is needed to understand the function of rabies virus receptors for viral entry, spread and pathogenicity.

Internalization. Rabies virus particles can be found in clathrin-coated pits, in uncoated vesicles and, at later time points after infection, in lysosomes[32]. The exact mechanism by which rabies virus is internalized into these compartments remains unclear. Fusion of the virus with these vesicles requires a change in the pH inside the vesicle. This has been shown by ammonium chloride- and chloroquine-induced inhibition of membrane fusion and release of the rabies virus capsid[32] and by the loss of pH dependence when the virus contains a foreign glycoprotein, such as HIV-1 gp160, instead of the rabies virus glycoprotein[37,38]. Thus, the rabies virus glycoprotein is solely responsible for both attachment and pH-dependent membrane fusion. The crystal structure of the glycoprotein of a related rhabdovirus, vesicular stomatitis virus (VSV), revealed that this protein combines structural features of both class I and class II fusion proteins[39], and a similar structure has been suggested for the rabies virus glycoprotein[40]. A major difference of these rhabdoviral glycoproteins from those of other viruses fusing at low pH levels is that the inhibition of fusion is reversible[40]. It remains unclear whether uncoating in primary neurons occurs immediately after internalization or only after transport through the axon in the cell body; this is discussed in more detail below.

Intracellular rabies virus transport. Because the entry site of rabies virus in axons does not provide the biochemical environment required for protein synthesis[41], rabies virus needs to reach the neuronal cell body for replication and transcription. Two different mechanisms have been proposed for the transport of rabies virus through the axon to the cell body: transport of either the rabies virus capsid alone or transport of the whole virion (FIG. 4). The transport of the rabies virus capsid would require uncoating after entry and a specific interaction of the RNP with the cellular transport machinery. Two research groups[42,43] identified an interaction between rabies virus phosphoprotein and the dynein light chain 8 (LC8), which led them to propose that the virus uses the cytoplasmic dynein motor complex for intracellular transport. However, deletion of the LC8-binding site in the phosphoprotein does not affect viral transport from a peripheral site to the CNS[44,45], and LC8 directly or indirectly affects primary transcription of rabies virus but not its transport[45].

A recent study used a capsid- and envelope-labelled rabies virus and showed that the whole virion is transported to the cell body[46] in an endosomal vesicle (FIG. 4), although the exact mechanism by which this occurs remains unclear. Transport depends on the rabies virus glycoprotein, as retroviral vectors *trans*-complemented with rabies virus glycoprotein are transported in a similar manner as rabies virus[47–50]. However, as the rabies virus glycoprotein is inside the vesicle, it should not be able to interact directly with a specific transporter complex. Based on these observations, it is speculated (but not shown experimentally) that entry into the vesicle,

Box 2 | **Definitions of rabies virus strains**

- Rabies virus street virus: an isolate from a naturally infected animal, for example a dog or fox.
- Fixed rabies virus: a rabies virus that has been passaged in tissue culture or animals. Fixed rabies virus can be pathogenic or non-pathogenic. The term fixed indicates only that the incubation period and virulence has been stabilized.
- Pathogenic rabies virus: a strain that typically causes rabies after peripheral inoculation.
- Attenuated rabies virus: a strain with a greatly reduced ability to cause rabies after inoculation into an animal.
- Neurotropic rabies virus: a strain that preferentially infects primary neurons or neuronal cell lines.
- Non-neurotropic rabies virus: a strain that infects neuronal cells at a level less than or equal to other cell types.

which is mediated by the rabies virus glycoprotein, determines the direction and provides the driving force of rabies virus transport. This indicates that the nature of the vesicle formed dictates the transport method that follows. However, this theory is at odds with the finding that different receptors are used by different rabies virus strains, unless entry using different receptors results in internalization into similar vesicles.

Replication, transcription and protein expression. Rabies virus gene expression and genome replication is highly regulated and differs in some respect from that of other rhabdoviruses, including VSV. Although it seems that VSV aims to produce as much virus in the least amount of time, rabies virus endeavours to preserve infected cells for two reasons. First, induction of cytotoxicity in the infected neuron would probably prevent neuronal transport. Second, the production of high levels of viral protein, especially glycoprotein, would induce strong humoral immune responses[51], which would probably neutralize the virus before it could complete its life cycle. Therefore, rabies virus has evolved regulatory mechanisms that produce viral components at the optimum amounts necessary for efficient viral production but low enough not to be recognized by the immune system or to interfere with the vital functions of its host cells. Most studies analysing the interactions of rhabdoviral proteins with cellular proteins for replication and transcription have been carried out using VSV and have been reviewed previously[52]. Although there are still limited data on cellular cofactors of the rabies virus replicase and transcriptase compared with what is known about the VSV proteins[52], it is not obvious that the same interactions exist for rabies virus. The finding that the dynein LC8 serves as an important factor for primary transcription of rabies virus is an example for differences between the two viruses[45].

Recent research strongly indicates that replication and transcription of rabies virus occurs in Negri bodies, which are cytoplasmic inclusion bodies that are typically found in rabies virus-infected neurons[53] and have been used as diagnostic markers for rabies virus infection. Negri bodies were shown to contain ubiquitylated proteins and heat shock protein 70 (HSP70); they are not aggresomes containing misfolded proteins but instead are functional structures for viral replication[53]. Interestingly, the formation of Negri bodies depends on Toll-like receptor 3 (TLR3)[54], which is involved in the spatial arrangement of sites of rabies virus replication.

After the release of the RNP into the host cell cytosol, the condensed and helical form of the RNP needs to switch to a relaxed stage to be synthetically active. Direct translation of viral messenger RNA cannot occur because the viral genome is negative sense RNA. In addition, the encapsidation of the genome and anti-genome by nucleoprotein[18,19,55,56] would interfere with this process. Crystal structure analysis using ring complexes of 11 nucleoprotein molecules and single-stranded RNA revealed that the nucleoprotein consists of two main domains, which contact nine nucleotides of RNA. Polymerization between the nucleoprotein protomers allows the formation of the RNP[57]. This structure nicely explains the protection of the RNA and the stability of the RNP, but it does not reveal the molecular and structural basis of the preferential encapsidation of viral RNA compared with host cell RNA (see below).

The PC, which is found in the rabies virus capsid, synthesizes the initial viral RNAs. Transcription begins at the 3′ end of the genomic RNA and results in a short uncapped and unpolyadenylated leader RNA (leRNA) of 55–58 bp. This is followed by sequential synthesis of 5′ end-capped and polyadenylated mRNAs that encode the viral proteins[19,56]. The most widely accepted model for transcription of rhabdoviruses is the so-called stop–start model, in which the PC stops transcription at a conserved signal sequence, ignores the intergenic region (IGR) of 2–24 nucleotides and restarts at the transcription start signal sequence[58] (FIG. 3). Successful reinitiation of transcription at each gene junction does not always occur, so transcription is attenuated from the 3′ to the 5′ end of the viral genome[59]. As such, the gene order partly determines the level of the respective viral mRNAs. The number of nucleotides in the IGR also regulates the transcription levels of the viral mRNAs[59] (FIG. 3). Unlike VSV, in which the IGRs always consist of 2 nucleotides[60], the IGRs in rabies virus are 2, 5, 5 and 24–29 nucleotides long[18,19]. Switching the IGRs in the rabies virus genome alters the mRNA and protein levels of the downstream genes[59], indicating that the length of the IGRs has an important role in regulating mRNA expression levels[61].

The leRNA also regulates the transcription of rabies virus genes[62]. The nucleoprotein binds to the leRNA about 10-fold stronger than to other rabies virus mRNAs; therefore, it has been proposed that the leRNA contains the encapsidation signal for the nucleoprotein[63]. It is speculated that when sufficient nucleoprotein is available, encapsidation of the leRNA results in the synthesis of an encapsidated full-length anti-genome rather than the production of individual viral mRNAs, switching from transcription to replication[64]. This hypothesis is supported by findings that rabies virus tolerates a large range of non-rabies virus RNA sequences in its

Aggresome
An inclusion body in which proteins accumulate when the cell's degradation machinery is not functioning properly or is overwhelmed.

Toll-like receptor
A receptor that recognizes viral, bacterial or fungal material and signals to induce an immune response.

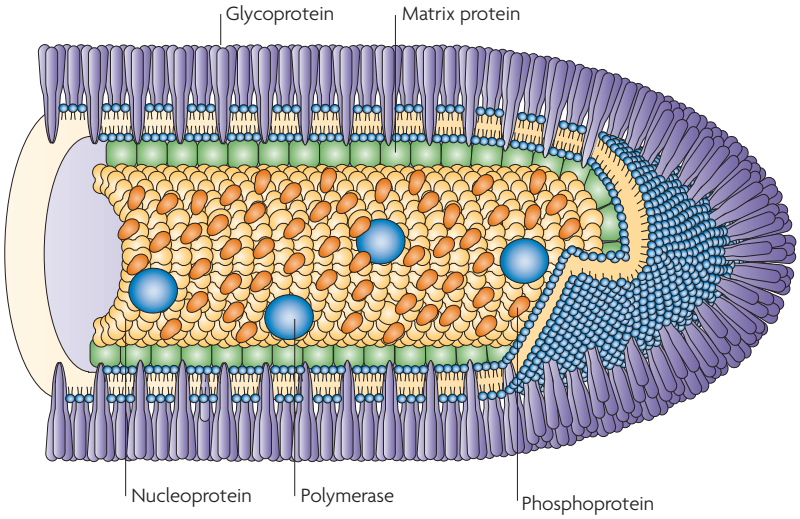

Figure 2 | **The rabies virus virion.** The negative-stranded RNA genome is tightly encapsidated into the nucleoprotein (yellow) and is termed the ribonucleoprotein (RNP). Two other viral proteins are associated with the RNP, the viral polymerase (blue) and the phosphoprotein (orange), which make up the internal core or capsid. The capsid is engulfed by the host cell-derived membrane, which is associated with two additional viral proteins: the matrix protein (green), which functions as a bridge between the capsid and the virion membrane, and the single transmembrane glycoprotein, which is organized as a trimer (purple).

genome[65], which are unlikely to contain rabies virus-specific encapsidation sequences. However, when nucleoprotein is expressed alone, it binds equally to any RNA[64], and non-specific RNA encapsidation is greatly reduced only when the nucleoprotein is expressed together with the phosphoprotein. This indicates that the phosphoprotein functions as a chaperone for the nucleoprotein to reduce non-specific RNA binding[66]. Furthermore, the phosphoprotein has been shown to prevent nucleoprotein phosporylation[64] by casein kinase II[67]. Taken together, these findings resulted in the following model. The nucleoprotein–phosphoprotein complex interacts specifically with viral RNA. During this interaction the nucleoprotein undergoes a conformational change, which enables its phosphorylation[64,68]. Therefore, the amount of the nucleoprotein–phosphoprotein complex is one factor responsible for increasing viral replication and decreasing transcription[63]. However, the removal of the phosphorylation site on rabies virus nucleoprotein impairs both viral replication and transcription[69], but this is probably an indication that two processes are linked.

The rabies virus matrix protein has an important role in regulating transcription and replication. A high concentration of VSV matrix protein inhibits RNA synthesis *in vitro*[70], and it has been proposed that the association of RNP templates and VSV matrix protein results in the condensation of the RNP, which then would not be functional for transcription and replication[71]. Rabies virus matrix protein might also be involved in the condensation of the RNP, but this has not been directly addressed. Evidence does suggest that, similarly to other rhabdoviral matrix proteins, the rabies virus matrix protein is involved in regulating transcription and replication. Heterologous expression of matrix protein increases transcription but not replication of rabies virus, and attenuation of matrix protein expression results in rabies virus with a high transcription rate[72]. In contrast to these findings, replacement of the rabies virus matrix protein of a vaccine strain with that of a bat-derived rabies virus increased both replication and transcription in a similar manner[36]. Rabies virus matrix protein was also identified as an important factor in the translation of mRNAs. One study showed that matrix protein inhibits the translation of mRNAs with classical 5′ end untranslated repeats but not of mRNAs that contain an internal ribosome entry site[73] through a specific interaction with the eukaryotic translation initiation factor 3 subunit H (EIF3H). Because rabies virus-derived mRNAs are undistinguishable from host cell mRNAs, high concentration of rabies virus matrix protein should affect not only viral transcription but also translation of viral proteins. However, this could also interfere with the overall strategy of rabies virus to conserve the function of the infected cell, as host cell protein synthesis would also be affected.

Assembly and budding — the final steps. The last steps in the life cycle of rabies virus are assembly of the viral components and release of virions (known as budding). For this process, the inner core of the virions (the capsid or RNP) must be engulfed by the host cell membrane. Budding of rabies virus takes place at the plasma membrane, but it is unknown how the capsid is transported to the site of budding. In contrast to some other enveloped viruses such as HIV-1, in which the capsid protein provides all the functions required for virion release (for a review, see REF. 74), both the rabies virus matrix protein and glycoprotein play an important part in budding. Although there is no absolute requirement for the glycoprotein, rabies virus is released 30-fold less efficiently in its absence[24]. Deletion of the cytoplasmic domain of the rabies virus glycoprotein led to a six-fold decrease in the release of virions and revealed a specific interaction between rabies virus glycoprotein and matrix protein[21]. Unlike the glycoprotein of VSV, the cytoplasmic domain of rabies virus glycoprotein is required for the incorporation of foreign glycoproteins into the virion[38,75–77]. In the absence of rabies virus matrix protein, viral titres were reduced by as much as 500,000-fold, although viral protein expression was not greatly affected[21].

Recently, the structures of the matrix proteins of VSV and Lagos bat virus (a rabies virus-related lyssavirus) have been solved[78]. These proteins share a similar overall structure and the ability to form non-covalent linear polymers even though they do not have sequence similarity. Self-association is mediated by binding of a proline-rich motif in the mostly disordered amino-terminal region to a pocket in the main globular domain of an adjacent matrix protein. Interestingly, the structure of these proteins reveals that the proline-rich self-interaction motifs differ significantly on a molecular level and notably overlap with known interaction motifs of host proteins in both rabies virus and VSV matrix proteins[78]. This important finding indicates that virus–host cell

Internal ribosome entry site
RNA sequence that allows ribosomes to bind to an mRNA at a position other than the 5′ end.

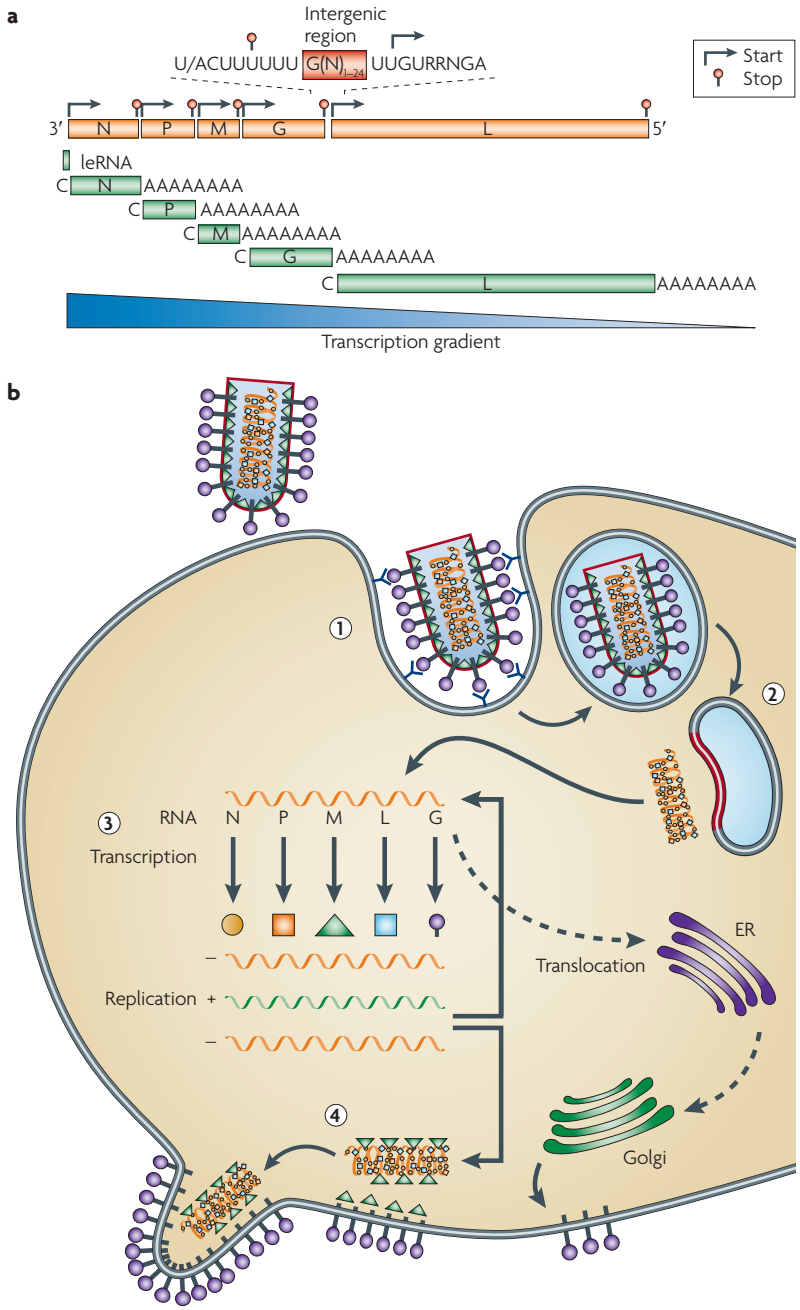

Figure 3 | **Aspects of the rabies virus life cycle. a** | Transcription of the rabies virus genome. The encapsidated negative-stranded RNA (orange) serves as a template for transcription by the polymerase complex. Transcription starts with a short uncapped leader RNA (leRNA) from the 3′ end of the genomic RNA. This is followed by the transcription of 5′ end-capped (C) and polyadenylated (A) mRNAs, which encode the viral proteins (green). The polymerase complex stops at signal sequence (U/ACUUUUUU), ignores the intergenic region of 2–24 nucleotides and restarts transcription at the transcription start signal sequence (UUGURRNGA). Successful reinitiation of transcription at each gene junction does not always occur, therefore transcription is attenuated from the 3′ to 5′ end[59] (this is illustrated by the transcription gradient). **b** | A simplified rabies virus life cycle in an infected cell can be divided into three different phases. The first phase includes binding and entry into the host cell by endocytosis (step 1), followed by fusion of the viral membrane and endosome membrane to release the viral genome (uncoating; step 2). In the second phase, virion components are produced (transcription, replication and protein synthesis; step 3). The last phase of the life cycle is the assembly of the viral components and budding and release of the rabies virus virions (step 4), which can start a new round of infection. ER, endoplasmic reticulum.

interaction could be different for these related rhabdoviruses. It also suggests that self-association and interaction with host cell proteins are regulated.

Most enveloped viruses contain late domains, so-called because mutations in these domains arrest virus morphogenesis at a late stage, when virions are mostly formed but fail to bud from the host cell membrane[79]. Late domains mediate efficient budding of virions by using host cell proteins that normally are involved in vacuolar protein sorting (VPS) pathways (for a review, see REF. 69). The rabies virus matrix protein contains at least three motifs that are similar to known late domains: ASAP (consensus sequence in other viruses is PTAP), PPEY (consensus sequence in other viruses is PPxY, in which x denotes any amino acid) and the overlapping YVPL (consensus sequence in other viruses is YxxL, in which x denotes any amino acid), forming the motif PPEYVPL[80]. All three motifs are located within the N-terminal region of the matrix protein; this region is mostly disordered apart from the PPxY, which overlaps a self-association motif, as described above. Site-directed mutagenesis confirmed the importance of the first proline in the PPEY motif and the leucine in the YVPL motif for virus production, whereas the potential role of the ASAP motif in virus budding has not been addressed yet. There is also evidence that the ubiquitin–proteasome system is important for virus budding, and *in vitro* binding studies indicate that the rhabdovirus PPxY motif can bind ubiquitin ligases[81], as has been reported for similar motifs in other enveloped viruses.

It remains to be shown to what extent rhabdoviruses use the VPS machinery, as VPS4A, an essential component of this pathway, does not seem to be involved in rhabdovirus budding[82]. Although no functional interactions with components of the VPS pathway have been shown *in vivo*, the PPxY motif is probably a hotspot for interactions with host cell proteins based on information obtained from the matrix protein crystal structure[78] and a promising target for further studies aimed at identifying such proteins. Further studies will also need to clarify whether rhabdovirus budding involves a new pathway that is distinct from the classical VPS pathway, as has been suggested for other negative-stranded RNA viruses.

Host cell response to rabies virus infection

Evading the innate immune response. The innate immune response represents the first defence of the host cell against viral infection and involves the secretion of type I interferons (IFNs; including IFNα and IFNβ) following the activation of pattern recognition receptors, such as TLRs and RNA helicases[83]. Many viruses have therefore developed mechanisms to escape these responses[83] (FIG. 5).

It is well established that rabies virus induces innate immune responses both *in vitro* and *in vivo*[84–88]. Retinoic acid-inducible gene I (RIG-I; also known as DDX58) has been shown to be a potent activator of type I IFNs during rabies virus infection[89]. RIG-I-mediated type I IFN production requires the presence of a 5′ triphosphate-ended RNA[89], but not viral replication. However, in contrast to VSV, the role of TLRs for the induction of type I IFNs

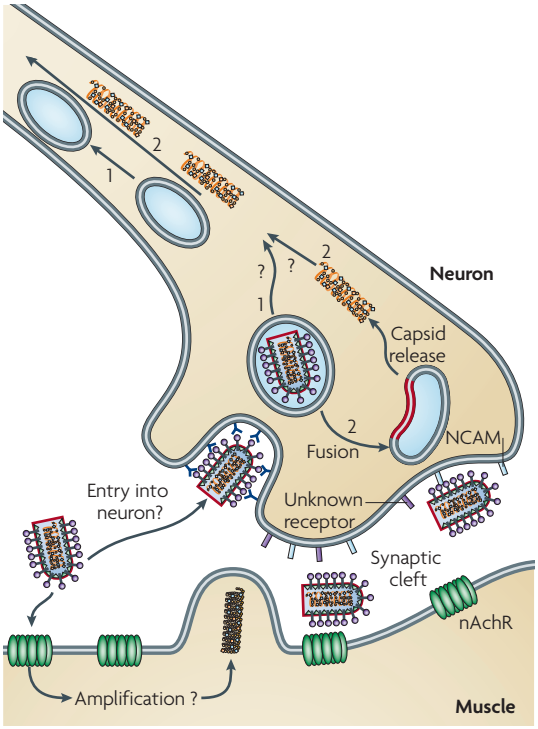

Figure 4 | **Rabies virus entry into neurons and intra-neuronal transport.** The nicotinic acetylcholine receptor (nAchR) is located at the postsynaptic muscle membrane. It has been suggested that nAchR enriches rabies virus at the neuromuscular junction or synaptic cleft, enabling more efficient infection of the connected motor neurons. Other research suggests that initial rabies virus replication is in muscle cells, indicating that nAchRs might be used to infect muscle cells. In both cases rabies virus enters the neurons using neural cell adhesion molecule (NCAM) or another, unknown receptor. Two different mechanisms have been proposed for the transport of rabies virus through the axon to the cell body: the transport of either the rabies virus capsid or the whole rabies virus virion within the vesicle. The evidence favours the transport of intact virions.

following rabies virus infection is less clear. One study showed that TLR3 is upregulated in rabies encephalitis[90], and the same laboratory recently found that TLR3 has an important function in the formation of Negri bodies[54]. However, a role for TLR3 in the induction of pro-inflammatory responses, as is the case for TLR4, TLR7 and TLR9, still needs to be shown.

Expression of IFNβ greatly reduces rabies virus pathogenesis and viral replication but not its immunogenicity[91]. Therefore, it might be expected that rabies virus suppresses such host defence mechanisms. Indeed, the rabies virus phosphoprotein interferes with the type I IFN responses by preventing the phosphorylation of IFN regulatory factor 3 (IRF3) by TANK-binding kinase 1 (REF. 61) (FIG. 5). The expression level of rabies virus phosphoprotein is crucial for the prevention of type I IFN responses, and recombinant rabies virus expressing low levels of phosphoprotein fails to suppress the production of IFNβ[61].

Furthermore, the rabies virus phosphoprotein can inhibit type I and type II IFN signal transduction pathways[62], which indicates that the inhibition of IFN synthesis by the phosphoprotein is probably not 100% efficient. Additional research indicates that rabies virus phosphoprotein does not induce the phosphorylation or the degradation of signal transducer and activator of transcription 1 (STAT1; a transcription factor that is activated by IFN signalling), but instead it interacts with and retains STAT1 in the cytoplasm[92]. Moreover, another study showed that only tyrosine-phosphorylated STAT1 and STAT2 can be bound by rabies virus phosphoprotein. This indicates that phosphoprotein binds STAT1 and STAT2 only following the activation of the IFN response, which leads to the phosphorylation of STAT1 and STAT2 by Janus kinases (FIG. 5). Binding of rabies virus phosphoprotein to STAT1 and STAT2 requires 10 carboxy-terminal amino acids in rabies virus phosphoprotein[93].

Of note, rabies virus phosphoprotein is expressed not only as full-length protein but also in four N-terminal truncated forms (phosphoprotein 2–phosphoprotein 5) that are synthesized from internal start codons[94]. All phosphoproteins contain a nuclear import sequence[94], but phosphoprotein 3, phosphoprotein 4 and phosphoprotein 5 lack the nuclear export signal found in the N-terminus and are retained in the nucleus. As a result, the rabies virus phosphoprotein can retain STAT1 in the cytoplasm and thereby prevent IFN-stimulated growth factor 3 (ISGF3) from activating the IFN-stimulated response element (ISRE) in the nucleus[95]. Interaction of rabies virus phosphoprotein in the nucleus with the homodimer of STAT1 also interferes with the activation of γ-activated sequence (GAS)[95] (FIG. 5). Recently, it has been shown that rabies virus phosphoprotein 3 can also facilitate the interaction of STAT1 and microtubules, which prevents STAT1 nuclear import. This is another illustration of the ways in which viral proteins can affect IFN signalling on multiple levels[96].

Last, there is evidence that the phosphoprotein retains the IFN-induced promyelocytic leukaemia (PML) protein in the cytoplasm[97]. This protein is found in PML nuclear bodies, which have many possible functions in nuclear trafficking, viral defence mechanisms and apoptosis[98]. How PML nuclear bodies function in viral infection is still not understood, but the binding and retention of PML by rabies virus phosphoprotein indicates that PML may have an antiviral function[97,99].

Rabies virus and apoptosis. Many neurotropic viruses use apoptosis as a mechanism of neuropathogenicity[100]. The induction of apoptosis by rabies virus has been a controversial topic, but increasing evidence indicates that pathogenic rabies virus strains do not induce apoptosis[101]. It was initially thought that apoptosis had an important role in rabies virus infection[102,103]; for example, induction of apoptosis has been detected in cultured rat prostatic adenocarcinoma cells after infection with a CVS strain of rabies virus[102]. However, rabies virus strains that are propagated by passage in animals or tissue culture

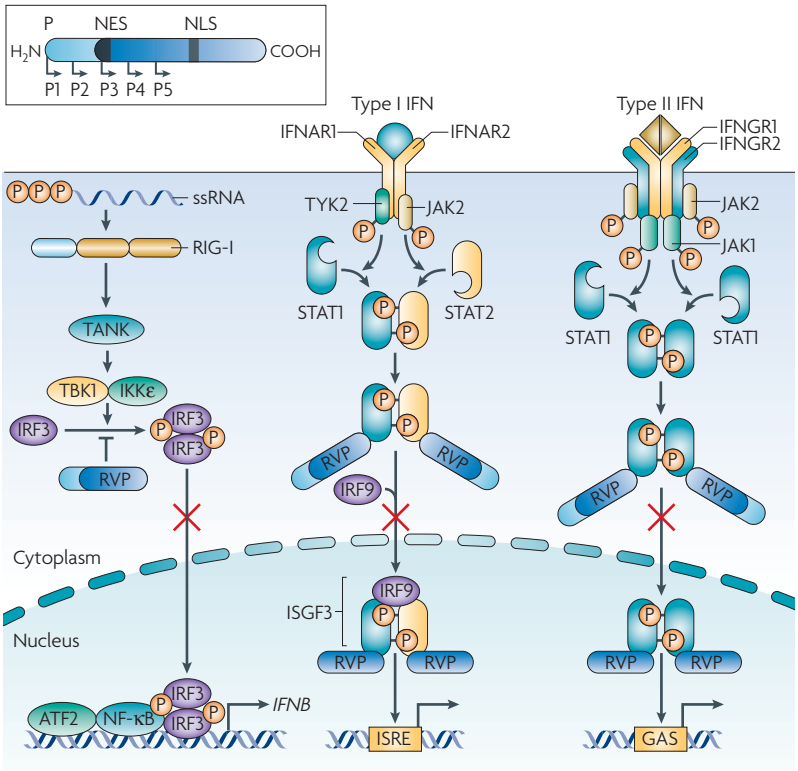

Figure 5 | **Rabies virus inhibition of the innate immune response.** Rabies virus phosphoprotein (RVP) antagonizes interferon (IFN) responses by blocking the host cell response to pathogen-associated molecular patterns and type I and II IFN signalling. For example, the 5′ triphosphate-ended RNA of rabies virus is detected by retinoic acid-inducible gene I (RIG-I), which signals through TRAF-family-member-associated NF-kB activator (TANK) and the TANK-binding kinase 1 (TBK1)– IκB kinase ε (IKKε) complex to induce the phosphorylation of IFN regulatory factor 3 (IRF3). Phosphorylated IRF3 dimerizes and is transported to the nucleus to induce the transcription of *IFNB* in conjunction with activating transcription factor 2 (ATF2) and nuclear factor-κB (NF-κB). In addition, some studies show IFNβ induction following signalling by Toll-like receptor 3 (TLR3; not shown); the function of other TLRs still needs to be elucidated. RVP inhibits the induction of IFNβ by preventing the phosphorylation of IRF3, thus retaining IRF3 in the cytosol. RVP can also inhibit type I (IFNα and IFNβ) and type II (IFNγ) IFN signal transduction pathways. Following the triggering of type I and type II IFN receptors (IFNRs), signal transducer and activator of transcription 1 (STAT1) is phosphorylated by Janus kinase (JAK). RVP binds to phosphorylated STAT1, preventing its translocation to the nucleus and the subsequent antiviral transcriptional response. In the nucleus, shorter versions of RVP bind to STAT1 and STAT2 heterodimers complexed with IRF9 (forming the IFN-stimulated growth factor 3 (ISFG3) complex) and STAT1 homodimers. This prevents transcriptional activation of the IFN-stimulated response element (ISRE) and γ-activated sequence (GAS) and type I and type II IFN-dependent immune response. Different versions of RVP are produced depending on the transcriptional start site used (see inset). Phosphoprotein 3 (P3), P4 and P5 lack the nuclear export signal (NES) and accumulate in the nucleus. TYK2, tyrosine kinase 2; NLS, nuclear localization signal.

do not always maintain a pathogenic phenotype[104]. The highly attenuated rabies virus strain ERA, but not the pathogenic CVS strain, induces apoptosis in mouse and human lymphocytes[105]. Similarly, the more attenuated isolate CVS-B2C, but not the pathogenic isolate CVS-N2c, induces apoptosis in primary mouse neurons[106]. Furthermore, there is evidence that apoptosis does not have a role in human rabies[10,107]. These findings highlight that attenuated rabies viruses (for example, vaccine strains) display a more pro-apoptotic phenotype compared with pathogenic rabies virus strains. This is supported by proteomic profiling showing that an attenuated rabies virus strain mostly upregulated the production of pro-apoptotic proteins, whereas a pathogenic strain affected the expression of proteins involved in ion homeostasis, docking and fusion of synaptic vesicles[108]. One exception to this rule is the finding that only infection by the pathogenic rabies virus strain CVS results in the apoptosis of T cells owing to early upregulation of CD95 ligand (also known as FASL) by infected neurons[85]. When detected, induction of apoptosis during rabies virus infection can occur by both caspase-dependent and caspase-independent pathways[109–111], and overexpression of the anti-apoptotic protein B cell lymphoma 2 prevented apoptosis in AT3 and Jurkat cells after rabies virus infection[102,110]. In addition, expression of a pro-apoptotic protein, such as cytochrome *c*, can reduce rabies virus pathogenicity while increasing its immunogenicity[112]. Interestingly, it was shown that high levels of rabies virus glycoprotein seem to be a key factor in the induction of apoptosis[51,113], which could explain why apoptosis is mostly detected during infection with highly attenuated rabies virus strains, which express much higher levels of viral proteins than pathogenic strains[114]. It is interesting to note that there is now clear evidence that rabies virus can actively promote neuronal survival that depends on an interaction with cellular partners recruited by the PDZ-binding site of the glycoprotein cytoplasmic domain, and silencing of certain host cell phosphatases abrogates rabies virus-mediated apoptosis[115].

In summary, most evidence indicates that pathogenic rabies virus does not induce apoptosis and that the induction of apoptosis actually reduces rabies virus pathogenesis. Induction of apoptosis depends largely on the expression levels and the amino acid sequence of the rabies virus glycoprotein[113]. Whether this is the consequence of differences in protein stability or a lower propensity to misfold and induce endoplasmic reticulum stress or due to other factors has yet to be determined. It also remains to be shown whether pathogenic strains limit apoptosis solely by reducing viral replication and transcription and changing the amino acid sequence of their glycoprotein or whether they use an additional direct mechanism to prevent apoptosis.

Inducible nitric oxide synthase. Studies have examined the mRNA levels of inducible nitric oxide synthase (iNOS) in rat brains after infection with different neurotropic viruses, including rabies virus. In contrast to herpes simplex virus 1 and Borna disease virus, iNOS mRNA was only detected in some animals infected with rabies virus[116]. Moreover, the detection of only minimal inflammation in all rabies virus-infected animals provides evidence that iNOS induction is not likely to be responsible for the observed neuropathology of rabies virus. Therefore, it is of interest to determine whether the induction of iNOS can decrease rabies virus pathogenicity. In support of this

hypothesis, Phares *et al.*[117,118] showed that the changes in the permeability of the brain–blood barrier, a well-known effect of iNOS, are indeed beneficial for resisting rabies virus infection and can promote viral clearance. This is in contrast to the findings of Ubol *et al.*[119], who showed that inhibition of iNOS production delays death of mice after rabies virus infection. However, the two studies used different rabies virus strains (namely a highly pathogenic and attenuated strain by Phares *et al.* and fixed strain by Ubol *et al.*) and inoculation routes[119,120]. Most results indicate that iNOS induction is essential for permeabilizing the brain–blood barrier and allowing entry of the necessary effector cells to clear the virus. It is worth noting that iNOS induction is rarely detected following infection with pathogenic rabies viruses but is detected following infection with attenuated ones[87,117,118,120–122].

Summary and outlook

Rabies virus has a complex life cycle, and we have only just begun to appreciate its interactions with host cells on a molecular level. Great progress has been made in understanding the functions of the viral proteins in viral replication, transcription, assembly and budding owing to the ability to directly manipulate the viral genome. Studies investigating the interaction with and use of host cell proteins by rabies virus are at an early stage but are progressing rapidly. The detailed study of the interaction of rabies virus phosphoprotein with host cell proteins that are involved in the innate immune response is a good example for such progress. Only when these basic processes of virus–host cell interaction are further understood can new therapeutic targets be identified.

Another advancement in rabies virus research could be a more restricted use of viral strains. Although it is justified and necessary to use different primary rabies virus isolates to test, for example, the efficiency of rabies virus vaccines, the use of such strains to study the biology of rabies virus needs to be revisited. In the case of rabies virus, the choice of a particular viral strain is important and should be dictated by the study's hypothesis. For example, an attenuated strain of rabies virus can be an excellent model system to study apoptosis in general but it might not be a good model system to study rabies virus transport or pathogenesis. Three different infectious clones of rabies virus vaccine strains are currently available, and two of them are almost identical[15,123,124]. In addition, infectious rabies virus cDNA was generated for two highly pathogenic rabies virus strains, silver-haired bat variant of rabies virus[125] and a fixed pathogenic rabies virus (CVS-N2c; C.W., unpublished observations). The use of such viruses and targeted mutations in their genomes might help to better compare the findings of different studies.

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Acknowledgements

We thank Gene Tan for assistance in designing figure 2 and Paul Schiffmacher for assistance in designing figures 3–5. This work was supported in part by a Center for Neuroanatomy and Neurotropic Viruses grant to Peter Strick, Center for Neuroscience, University of Pittsburgh (USPHS-NCRR P40RR028604, subcontract to M.J.S.) and internal funds of Thomas Jefferson University, Medical College, Jefferson Vaccine Center, Philadelphia, USA.

Competing financial interests

The authors declare competing financial interests: see web version for details.

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WHO Fact Sheet No. 99: http://www.paho.org/common/Display.asp?Lang=E&RecID=10209
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