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Mini-Review—The Rabies Virus

Rabies virus receptors

Monique Lafon

Unité de Neuroimmunologie Virale, Institut Pasteur, Paris, France

There is convincing *in vitro* evidence that the muscular form of the nicotinic acetylcholine receptor (nAChR), the neuronal cell adhesion molecule (NCAM), and the p75 neurotrophin receptor (p75NTR) bind rabies virus and/or facilitate rabies virus entry into cells. Other components of the cell membrane, such as gangliosides, may also participate in the entry of rabies virus. However, little is known of the role of these molecules *in vivo*. This review proposes a speculative model that accounts for the role of these different molecules in entry and trafficking of rabies virus into the nervous system. *Journal of NeuroVirology* (2005) **11,** 82–87.

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Introduction

Rabies virus (RABV) is a well-adapted pathogen of the mammalian nervous system (NS), where it mostly infects neurons. This virus is typically transmitted by animal bites, although some cases of aerosol contamination have been described. Virus particles from the saliva of infected animals or progeny virus particles produced by muscle infection enter the NS via a sensory nerve through nerve spindles or via the neuromuscular junctions (NMJs) where motor axons bifurcate in invaginations of the muscle surface. RABV particles then travel along the spinal cord to the brain, before spreading to the salivary glands. Virions are excreted in the saliva and are transmitted to other hosts by bites.

There is considerable evidence showing that RABV enters motor nerves through NMJs (Burrage *et al*, 1985; Lentz *et al*, 1982; Lewis *et al*, 2000; Watson *et al*, 1981). Virus antigens have been detected in sensory spindles, stretch receptors, and proprioceptors, showing that RABV can also travel along sensory nerves (Murphy, 1977). It is not yet known whether the route of entry affects RABV pathogenicity or the pattern of NS infectivity. It has only been shown that after peripheral inoculation of monkey or rats with RABV, the motoneurons of the ventral horns are infected before the dorsal root ganglion cells (Kelly and Strick, 2000; Tang *et al*, 1999). Electron microscopy suggested that in the RABV-infected central nervous system (CNS), RABV transport occurs primarily at synaptic junctions (Charlton *et al*, 1997; Iwasaki and Clark, 1975). In cultures of rat hippocampal neurons and in cocultures of rat myotubes and nerves, RABV was found in endosomal vesicles and in synaptic vesicles (synapsin 1–positive vesicles) shortly after uptake (Lewis *et al*, 2000). RABV is transported to anatomically connected sites exclusively by the retrograde pathway (Gillet *et al*, 1986; Kelly and Strick, 2000). RABV transport is blocked by colchicine, which causes microtubules depolymerization (Ceccaldi *et al*, 1989).

After crossing the NMJs, RABV is seen both in neutral vesicles and in some acidic vesicles, which may trigger the fusion of the virus envelope and the release of nucleocapsids into the axoplasm (Lewis *et al*, 2000; Lewis and Lentz, 1998). This raises questions about the form in which RABV travels along the nerve endings to the soma of the neurons where replication can occur: intact virus trapped in vesicles or nucleocapsid entities linked to microtubules?

The viral envelope is made of host lipids and two proteins, G and M. RABVG is a 505–amino acid, type I membrane glycoprotein with three potential *N*-glycosylation sites. It adopts a trimeric form in the endoplasmic reticulum. The G trimer is responsible for the attachment of RABV to target cells. RABVG enables the virus to be transported to the CNS via the retrograde pathway. This was demonstrated by comparing the neuroinvasiveness of lentivirus vectors pseudotyping envelope proteins from different viruses. RABVG conferred the equine infection anemia lentivirus the ability to be transported into the

Address correspondence to Monique Lafon, Unité de Neuroimmunologie Virale, Département de Neuroscience, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris cedex 15, France. E-mail: mlafon@pasteur.fr

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CNS via the retrograde pathway after peripheral muscle injection (Mazarakis *et al*, 2001). Conversely, unlike G-expressing viruses, G-deficient RABV are not transmitted transsynaptically after being stereotaxically inoculated into the rat striatum (Etessami *et al*, 2000). In addition, immunohistochemistry using an anti-idiotypic monoclonal antibody (mAb) mimicking the RABVG showed that the RABVG recognizes molecules localized at the synaptic junctions. This mAb labeled structures resembling the NMJs and synaptic junctions in the NS of mouse and monkey (Hanham *et al*, 1993). These data strongly suggest that RABVG is the key element in the virus entry and propagation, consistent with the observations that the nature of RABVG is a key element of RABV neuroinvasiveness and pathogenicity (Ito *et al*, 2001; Morimoto *et al*, 1998, 1999, 2000; Yan *et al*, 2002).

Several different experimental approaches have been used to identify membrane molecules that can bind RABVG and that facilitate virus entry *in vitro*. The roles of nicotinic acetylcholine receptor (nAChR), neuronal cell adhesion molecule (NCAM), p75 neurotrophin receptor (p75NTR), and gangliosides in RABV infection are reviewed below.

nAChR

nAChR is a pentameric ligand-gated ion channel that mediates and modulates interneuronal communication in the CNS and in the peripheral NS. Several types of nAChR are present in the NS. Only one form, containing the α1 gene product, is expressed in muscle. The nAChR α1 subunit binds α-bungarotoxin (α-BTX). Most (99%) of the nAChr molecules produced by adult muscle accumulate at the postsynaptic membrane of NMJs. Autoradioradiography using ^{125}I-α-BTX showed that nAChR molecules are located at the tops of the junctional folds (Fertuck and Salpeter, 1974). The first observation suggesting that the entry of RABV is mediated by nAChR was made by Lentz *et al* (1982), who showed that RABV bound to mouse diaphragms at the location of nAChR. An anti-idiotypic mAb mimicking RABVG binds to AChR and to structures resembling NMJs and synaptic junctions with a pattern similar to the anti-AChR mAb (Hanham *et al*, 1993). The αl subunit of the muscular nAChR interacts with RABVG (Bracci *et al*, 1988). The main RABVG-binding site on the αx subunit of nAChR is the fragment between positions 173 and 204 (Lentz *et al*, 1987). These data demonstrate that the RABVG binds to the muscular nAChR present at the NMJs. The abilities of α-BTX, *d*-turbocurarine, and a mAb directed against the α subunit of AChR monomers to inhibit partially infection of cultured myotubes and the binding of RABV to purified AChR and dorsal root ganglia cells led to the hypothesis that nAChR acts as a RABV receptor *in vitro* (Burrage *et al*, 1985; Lentz *et al*, 1982, 1986; Gastka *et al*, 1996; Castellanos *et al*, 1997).

Although *in vitro* experiments have clearly shown that muscular nAChR binds RABVG, the role played by AChR in RABV infection is still not completely understood. It is puzzling that in the NMJs, nAChR are located at the postsynaptic muscle membrane and not at the presynaptic nerve membrane. This shows that nAChR is a receptor for muscle infection. According to this hypothesis, nAChR may allow the amplification of the virus inoculum before entry into the NS. Alternatively, nAChR may concentrate virus particles in front of the NMJ, improving the probability of virus particles being taken up by the nerve terminal (Lentz *et al*, 1982; Lewis *et al*, 2000). This is consistent with nAChR molecules being located on the top of junctional folds in areas where nerves and muscles are in close contact. The involvement of other isoforms of nAChR for further synaptic passage in the CNS has not yet been investigated. Given the susceptibility of the interaction between RABV and nAChR to α-BTX treatment, α7 nAChR, which is also bound by α-BTX (Pugh *et al*, 1995) and expressed at synaptic junctions in the brain, may also be involved in the synaptic passage of RABV in the CNS. However, there is currently no experimental evidence supporting such an involvement.

NCAM

The neural cell adhesion molecule, NCAM, is a cell adhesion glycoprotein from the immunoglobulin superfamily. Three major splicing isoforms are expressed on the surface of cells: a glycosyl phosphatidylinositol (GPI)-linked NCAM 120, and two transmembrane forms, the NCAM140 and NCAM180, which have cytoplasmic tails of different lengths. They all contain the same ectodomain composed of five immunoglobulin (Ig)-like and two fibronectin-like domains. All three forms can be posttranslationally modified by the addition of polysialic acid. NCAM is concentrated in synaptic regions and at the NMJs. In the presynaptic membrane of NMJs, NCAM is present in the area occupied by the nerve terminal and its Schawnn cell cap. It also accumulates at the postsynaptic membrane deep within the junctional folds of the NMJs (Covault and Sanes, 1986). NCAM is involved in the mobilization and cycling of synaptic vesicles at the NMJs. NCAM180, the cytoplasmic tail of which interacts with spectrin, plays a role in synaptogenesis by mediating the transport and accumulation of synaptic organelles and protein at the sites of synapse formation (Sytnyk *et al*, 2002). In addition to its adhesion properties, NCAM is a signaling receptor for glial-derived neurotrophic factor, GDNF (Paratcha *et al*, 2003).

The observation that laboratory cell lines susceptible to RABV infection express NCAM on their surface whereas resistant cells do not suggested that NCAM plays a role in RABV entry. Incubation with RABV decreases NCAM expression on the surface of

cells. Pretreatment of cells with the natural ligand of NCAM or antibodies directed against NCAM significantly reduces RABV infection. Preincubation of infectious viruses with soluble NCAM protein neutralizes RABV infectivity. Moreover, expression of NCAM140 or NCAM180 in a resistant cell line makes it susceptible to RABV. In contrast, RABV infection is drastically reduced in primary cortical cell cultures prepared from NCAM-deficient mice (Cremer *et al*, 1994). These data demonstrate that NCAM is a receptor for RABV *in vitro* (Thoulouze *et al*, 1998).

The *in vivo* role of NCAM as a receptor was assayed in NCAM-deficient mice (Cremer *et al*, 1994). After injection into the masseter, RABV invaded the brain of NCAM-deficient mice much less efficiently than that of wild-type mice and progression was drastically impaired in all regions except the cerebellum and brain stem in which the pattern of infection was similar to in wild-type mice. In addition, NCAM-deficient mice injected with RABV die 4 days after wild-type mice (Thoulouze *et al*, 1998). Thus, NCAM plays a major role in RABV neuroinvasiveness. Synapses and NMJs are normal in mice lacking NCAM. Even the number of synapses is not modified (Dityatev *et al*, 2000). However, in NCAM knockout mice, the NMJs are smaller, and the strength of synapses and the ability of junctions to maintain transmitter output following repetitive stimulation are diminished (Polo-Parada *et al*, 2001; Rafuse *et al*, 2000). Thus, it cannot be completely excluded that the reduced size of the NMJs and the functional anomalies in the recycling of synaptic vesicles slow early RABV trafficking in NCAM-deficient mice.

The role of the NCAM that accumulates at the postsynaptic membrane within the junctional folds of the NMJs during RABV infection remains to be investigated. The location of NCAM at the presynaptic membrane makes it a serious candidate for the passage of NMJs and synapses by RABV.

p75NTR

The neurotrophin receptor, p75NTR, also named the low-affinity receptor for the nerve growth factor (NGF), is a type I transmembrane protein from the tumor necrosis factor receptor family. Its ectodomain consists of four cysteine-rich domains (CRDs). p75NTR binds to neurotrophins, including NGF, at the junction of the second and third CRDs (Rodriguez-Tebar *et al*, 1992). Wheat germ agglutinin binds to the *N*-glycosylation site of CRD1. The p75NTR is a ligand for the amyloid β peptide that accumulates in Alzheimer's disease and for the scrapie prion protein PrPsc. It also binds to ganglioside GT1b. p75NTR promotes survival and differentiation of neurons. It also plays a role in cell death, synaptic transmission, and axonal elongation (Dechant and Barde, 2002).

The p75NTR is mainly expressed during development. In adults, it is expressed after axonal injury or in pathological situations such as epilepsy and neurodegeneration. Nevertheless, the NGF receptor has been detected on the axons and cell bodies of some neuron populations in the adult rat CNS (Pioro and Cuello, 1990a, 1990b; Dougherty and Milner, 1999). p75NTR is not expressed in spinal cord motoneurons in motor nerve endplates or at terminal intramuscular axon branches (Sheard *et al*, 2002; Copray *et al*, 2003). In the spinal cord of adult rats, it is essentially seen in the dorsal horn and rarely in the ventral horn (Pioro and Cuello, 1990b).

Analysis of a cDNA library of a mouse neuroblastoma cell line showed that p75NTR is a ligand for the soluble form of RABVG (Tuffereau *et al*, 1998b). This association was confirmed by immunoprecipitation of RABV-infected cells with anti-RABVG antibodies, as p75NTR coimmunoprecipitates with RABVG (Tuffereau *et al*, 1998b). CRD1 of p75NTR has been shown to have a high affinity (K_d 30 to 35 pM) for trimeric RABVG (Langevin *et al*, 2002). These data clearly show that p75NTR is a ligand for RABVG. A reverse binding assay in insect cells revealed a specific interaction between p75NTR and representatives of genotypes 1 (PV and CVS) and 6 (European bat lyssavirus type 2) but not with representatives of genoytpes 3 (Mokola virus), 4 (Duvenhage), or 5 (European bat lyssavirus type 1) (Tuffereau *et al*, 2001). The restriction of p75NTR to certain RABV strains was also observed with a RABV strain isolated from a rabid fox (Tuffereau *et al*, 1998a). In this experiment, transfection of fibroblasts with p75NTR conferred susceptibility to the fox virus isolate, leading to the conclusion that p75NTR is a receptor for certain RABV strains. In contrast, and despite the specific interaction of p75NTR with CVS (Tuffereau *et al*, 2001), the expression of p75NTR did not increase the ability of CVS to infect the fibroblast cell line, suggesting that the ability of p75NTR to bind RABVG and the role of p75NTR in RABV entry are distinct functions.

There is currently no *in vivo* evidence that p75NTR is a receptor; the first attempt to address this point used mice knockout for the $p75NTR^{exon\ III}$ (Lee *et al*, 1992), which still express the first CRD that binds RABVG with high affinity. This possibly explains why RABV infection develops equally well in p75NTR knockout and normal mice (Jackson and Park, 1999). Studies of RABV infection in mice lacking CRD1 of p75NTR (knockout for $p75NTR^{exon\ IV}$) and in normal mice with RABV mutants deficient in p75NTR binding (Langevin and Tuffereau, 2002) are now required to analyze the role of p75NTR in rabies infection. Nevertheless, it will be difficult to study RABV infection in complete p75NTR knockout mice as they display severe NS abnormalities, reflecting the importance of neurotrophins in the development of peripheral sympathetic and sensory neurons and in the development of certain neuron populations in the CNS (von Schack *et al*, 2001).

The absence of p75NTR at the NMJ suggests that p75NTR is not involved in the passage of RABV across the NMJ. In contrast, the fact that p75NTR is mainly found in the dorsal horn of the spinal cord implies that it might participate in the trafficking of RABV by a sensory route which is not the main entry of RABV. When expressed, p75NTR is mostly found at presynaptic locations, at least in the rat dendate gyrus (Dougherty and Milner, 1999). This suggests that p75NTR may participate in the retrograde transport of RABV in the CNS. NGF is transported via the p75NTR into endocytic compartments, possibly caveolae, without being degraded (Tsui-Pierchala *et al*, 2002). It has been proposed that p75NTR plays a role not only in the internalization and trafficking of neurotrophins, but also in the internalization and trafficking of proteins that bind p75NTR such as wheat germ, β-amyloid, and prion proteins as well as tetanus toxoid indirectly through its binding to the ganglioside GT1b (Butowt and von Bartheld, 2003). Similarly, it is possible that the binding of RABV to p75NTR may allow retrograde axonal trafficking of RABV. Thus, binding of RABV to p75NTR may permit RABV to follow caveolae transcytosis, allowing the retrograde transport of RABV particles along the axons to the soma of the neurons, where the virus can replicate without being degraded. However, there is still no experimental proof that RABV uses p75NTR-decorated vesicles for its transport. Moreover, the numerous discrepancies between the sites where the NGF receptor is expressed and the areas of the brain infected by RABV suggest that p75NTR is not the only candidate for the axonal transport of RABV or that the role of p75NTR in RABV transport is controlled locally by unidentified factors.

Other components

Other components of the cell surface might be involved in RABV infection. A virus overlay protein binding assay showed that RABV associates with four proteins of between 66 and 200 kDa (Broughan and Wunner, 1995). These proteins have not yet been characterized. Based on their molecular weights, it is possible that some of them correspond to the NCAM140 and NCAM180 isoforms. Phospholipids, glycolipids, sialic acid, and carbohydrates have been proposed to play a role in RABV entry into fibroblasts (Superti *et al*, 1984; Wunner *et al*, 1984). The susceptibility of fibroblasts previously desialylated by neuraminidase treatment to RABV can be restored by feeding the fibroblasts with the gangliosides GT1b and GQ1b (Superti *et al*, 1986). These data suggest that highly sialylated gangliosides, which are important constituents of the presynaptic membrane, participate in RABV infection. Nevertheless, there is currently no evidence that RABVG directly binds gangliosides. Polysialylated gangliosides, the main constituents of lipid rafts, may be indirectly involved in the ability of RABV to cross membranes. As NCAM is associated with lipid rafts in brain membranes (Delling *et al*, 2002), it is tempting to propose that gangliosides concentrate RABV receptors into "lipid raft" microdomains, thereby allowing the simultaneous binding of G proteins and improving the membrane fusion process. This synergy between NACM and gangliosides of "lipid rafts" remains to be investigated.

Proposed scheme of RABV entry and trafficking

The present data do not allow to identify the molecules that bind and concentrate the virus at the membrane and those that internalize the virus. The mechanism of crossing the NMJ is completely unknown. Nevertheless, the localization of the nAChR, NCAM, and p75NTR in the NS allows us to propose the following scheme of RABV entry and trafficking.

Saliva from rabid animals contains RABV particles that are transferred by bites to the vicinity of NMJs and sensory terminations. At the NMJs, free RABV particles bind to nAChR located on the top of junctional folds, in areas where nerves and muscles are in close contact. This concentrates virus particles in front of the NMJs and improves the probability of RABV being taken up by the nerve terminal. RABV particles bind to NCAM present at the presynaptic membrane. The presence of gangliosides in this membrane concentrates NCAM into "lipid raft" microdomains, thereby allowing the simultaneous binding of G proteins and improving the membrane fusion process or allowing the detachment of RABV from nAChR. After crossing the NMJ, RABV is internalized by neutral and acidic vesicles, which may trigger the fusion of the virus envelope and the release of nucleocapsids. Alternatively, intact RABV remain in vesicles and travel along the nerve endings to the soma of the neurons where replication can occur. Possibly in sensory endings or at subsequent steps of the RABV journey, but probably not at the NMJs where p75NTR is not detected, the binding of RABV to p75NTR may enable RABV to follow caveolae transcytosis, allowing the retrograde transport of RABV particles along the axons.

Further investigations are required to test this proposed model. Fluorescent tracers, recombinant viruses carrying green fluorescent protein (GFP), and new microscopy techniques will be valuable tools for these investigations.

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