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Early events in rabies virus infection—Attachment, entry, and intracellular trafficking

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ABSTRACT

Rabies virus (RABV), an enveloped virus with a single-stranded and negative-sense RNA genome, is the type species of the *Lyssavirus* Genus within the *Rhabdoviridae* family. As the causative agent of rabies with a nearly 100% fatality, the neurotropic RABV pose a serious threat to the global public health. Though a great effort has been made toward understanding the molecular mechanism underlying virus infection cycle, there are still many aspects need to be elucidated, especially on the early events during virus replication cycle. With the application of the multiple advanced technologies, much progress has been made on these aspects. To date, multiple receptors, such as nAChR, NCAM, p75NTR, mGluR2, carbohydrates, and gangliosides, have been identified. Following initial attachment, RABV internalization occurs through clathrin-mediated endocytosis (CME) with the help of actin. After viral entry, intracellular trafficking occurs. Two retrograde trafficking models, stating that either whole virions are parceled into vesicles or only the viral capsids are transported, have been proposed. Moreover, complete enveloped virions or G-containing vesicle-associated ribonucleoproteins (RNPs) may be formed during anterograde transport, which remains poorly characterized but is important for viral budding. Combining the data elucidating the molecular mechanisms of RABV attachment, entry, and intracellular trafficking, this review provides an integrated view of the early events in the viral life cycle.

1. Introduction

Rabies virus (RABV) is a member of the genus *Lyssavirus* within the family *Rhabdoviridae*. It is the causative agent of rabies, posing a severe threat to human and animal health (Fooks et al., 2014). With a 100% fatality rate and high global death count per year, rabies is considered one of the most important zoonotic diseases. According to the report of the World Health Organization (WHO, 2017), the majority of human rabies cases occur in developing countries due to the limited medical resources and inadequate use of vaccines.

Much progress has recently been made towards clarifying the molecular mechanisms involved in the intracellular life cycle of RABV, and exploring deep into the pathogenesis. In this review, we will discuss some aspects on virus life cycle, viral trafficking including attachment, internalization, and intracellular transport, and then propose a complete model of early events in the RABV life cycle. This may be helpful for seeking novel therapeutic targets and developing clinical treatments.

1.1. The structure of RABV and viral encoded proteins

Similar to other *Rhabdovirus* enveloped virions, RABV has a distinctive bullet- or rod-shaped structure with a single nonsegmented negative-sense RNA genome (Matsumoto, 1962; Tordo and Poch, 1988). The complete genome of RABV is approximately 12 kb in length and encodes five viral proteins, namely, nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and the viral RNA polymerase (L), in the order 3′-N-P-M-G-L-5′. The linear RNA is encapsulated by N proteins to form the N-RNA complex of the nucleocapsid (N-RNA) and is believed to be the core of the genetic information (Albertini et al., 2006). The N-RNA complex, along with the P and L proteins, constitutes a helical ribonucleoprotein (RNP) (Emerson and Yu, 1975; Harmon et al., 1985). The structure of the RNP complex is critical for mRNA synthesis and replication when the viral genome is released inside the cells (Pattnaik and Wertz, 1990; Patton et al., 1984). The M protein, located beneath the viral membrane that wraps the RNP core, is closely related to the nucleocapsid and the lipid bilayer and contributes to the compact structure of virions. The G protein forms

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spikes outside the lipid envelope, recognizes cell receptors by direct interaction, and mediates cell membrane fusion with the M protein to allow the virus to enter the cells. The M and G proteins also play vital roles in the assembly and budding of infectious virus particles (Mebatsion et al., 1999; Finke and Conzelmann, 2005).

1.2. The life cycle of RABV infection

RABV is generally thought to be transmitted to hosts either by a bite or scratch at the periphery and then to migrate to the central nervous system (CNS). During this process, the virus must overcome a series of obstacles to traverse long distances, i.e., from the periphery to the CNS. Elaborate trafficking mechanisms are required and various factors are involved intracellularly. The life cycle of RABV is entirely cytoplasmic. Viral entry is initiated by the interaction between cellular receptors and the viral glycoprotein. After the virus attaches to the cell membrane, it enters the cell through the endocytic pathway. The low pH environment in early endosomes (EEs) induces conformational changes in the G protein, facilitating the fusion of the viral envelope with the endosomal membrane (Gaudin et al., 1993). Thereafter, uncoating of the viral genome occurs, releasing the viral RNP into the cytosol (Mire et al., 2010). Transcription occurs in a specialized “virus factory”, the Negri body, with the RNP as the template (Lahaye et al., 2009). The G protein is translocated into the endoplasmic reticulum (ER), while the other four viral proteins are synthesized on free ribosomes, presumably proximal to the inclusion bodies (Gaudin, 1997). After replication, the intact virions are assembled with genomic RNA and proteins in the inclusion bodies—the Negri bodies—and then released extracellularly via budding (Albertini et al., 2011).

2. Attachment to host cells dependent on receptors

Attachment is the first step in viral life cycle and is mediated by interactions of glycoprotein G with cellular receptors. The G protein is synthesized in ER and forms trimeric spikes on surface of virus particles when assembled (Gaudin, 1997). G-deficient RABV cannot be transmitted in cell cultures or infected mice, revealing the critical role of the G protein in viral infection (Etessami et al., 2000; Mebatsion et al., 1996). Several studies have been conducted to identify the host cell surface molecules that interact with the glycoprotein to mediate viral entry into the cell. These studies focused on the roles of nicotinic acetylcholine receptor (nAChR), neuronal cell adhesion molecule (NCAM), p75 neurotrophin receptor (p75NTR), metabotropic glutamate receptor subtype 2 (mGluR2), carbohydrates, and gangliosides in RABV infection.

2.1. nAChR

Lentz was the first to suggest nAChR as a receptor for RABV (Lentz et al., 1982). Among several types of nAChR, the nAChR α1 subunit, a form composed of the α1 gene product, is expressed in muscle and binds to α-bungarotoxin (α-BTX) (Fertuck and Salpeter, 1974). RABV attaches to the regions with a high density of nAChRs at the postsynaptic membrane of neuromuscular junctions (NMJs) (Burrage et al., 1985; Mcgehee and Role, 1995). A 32-residue peptide located at positions 173-204 on the nAChR α1 subunit is the main RABV G-binding site (Lentz et al., 1987). The region located at positions 190–203 on the viral G protein shares substantial identity with the receptor-binding region of neurotoxins, contributing to the direct interaction (Burrage et al., 1985; Lentz et al., 1984; Bracci et al., 1988). Soon after this discovery, the researchers provided evidences for the binding of RABV to nAChR via anti-idiotype network (Hanham et al., 1993) and virus overlay protein binding assay (Gastka et al., 1996). nAChR not only acts as a RABV receptor for muscle infection but also affects the infectivity of RABV in neuronal cells. Treatment of mouse dorsal root ganglion (DRG) cells with various nicotinic antagonists, such as dihydro-β-erythroidine, mecamylamine, d-tubocurarine, hexamethonium, α-bungarotoxin, and erabutoxin, showed that mecamylamine and d-tubocurarine reduce the percentage of infected neurons (Castellanos et al., 1997). Taken together, these observations lead to the hypothesis that nAChR serves as a RABV receptor in vitro.

Although the hypothesis that RABV attaches to the cell surface by direct interaction with nAChR has been proposed and validated through various methods, the exact role of nAChR during RABV uptake remains unclear. Interestingly, nAChR is located at the postsynaptic muscle membrane of NMJs, not at the presynaptic nerve membrane, suggesting that nAChR determines RABV infection in muscle cells. Since NMJs are the major sites of entry into neurons, the concentration of nAChRs at NMJs allows more amplification of virions in front of NMJs, which facilitates the subsequent uptake to nerve terminals (Lewis et al., 2015). The detailed role of nAChR and the possibility that other isoforms are involved in the process of RABV attachment still need further investigation.

2.2. NCAM

The second receptor discovered is NCAM. NCAM, also known as CD56, is a cell surface glycoprotein that belongs to the immunoglobulin (Ig) superfamily. NCAM is divided into three main isoforms according to the length of the cytoplasmic tail: the glycosylphosphatidylinositol (GPI)-linked NCAM 120, NCAM140, and NCAM180 (Santoni et al., 1989). The extracellular domains of these three forms contain five Ig-like domains and two fibronectin type III (FNIII) domains. NCAM isoforms can be posttranslationally modified via the addition of polysialic acid, which is associated with cell adhesion and is important in cell migration and invasion. NCAM accumulates at the cell surface of neurons and plays a crucial role in the maintenance of synaptic structure and the regulation of synaptic plasticity as well as in connecting developing neurons with the extracellular environment (Leshchyns'Ka and Sytnyk, 2015).

RABV-susceptible cell lines express NCAM on the cell surface, whereas resistant cell lines do not. Incubation with RABV decreases the expression of NCAM, while treatment with ligands of or antibodies against NCAM reduces RABV infection. In addition, RABV infectivity is neutralized after preincubation with soluble NCAM protein. Moreover, transfection with the NCAM-encoding gene induces RABV susceptibility, whereas NCAM deficiency reduces viral infection and production in primary cortical cells. These results provide evidence that NCAM serves as a receptor for RABV in vitro. Moreover, in NCAM-deficient mice, rabies mortality is delayed, and RABV brain invasion is drastically restricted, suggesting that NCAM is a receptor in vivo (Thoulouze et al., 1998).

However, RABV is considered lethal in NCAM-deficient mice (Thoulouze et al., 1998), indicating that NCAM is not essential for infection and that other receptors may also be utilized to mediate RABV entry into host cells. Until then, the identification of NCAM as a RABV receptor was widely acknowledged. Although an increasing number of studies have revealed the close association of NCAM with numerous intracellular signaling cascades, studies on the molecular mechanisms of NCAM-mediated RABV cell surface attachment are rather limited.

2.3. p75NTR

p75NTR, also called the low-affinity nerve growth factor receptor (LNGFR), belongs to the nerve growth factor receptor (NGFR) superfamily and has a transmembrane conformation. p75NTR consists of four extracellular cysteine-rich domains (CRDs) and an intracellular type II death domain (Kraemer et al., 2014). This receptor plays a crucial role in the development of the nervous system and is involved in a diverse array of cellular responses, including apoptosis, survival, neurite outgrowth, migration, and cell cycle arrest (Roux and Barker, 2002). A series of studies demonstrated that the p75NTR signaling cascade is

closely related to neurological disorders, such as Alzheimer's disease (AD), schizophrenia, major depressive disorder (MDD), posttraumatic stress disorder (PTSD), amyotrophic lateral sclerosis (ALS), and Parkinson's disease (PD). Recently, p75NTR has been regarded as a potential therapeutic target due to its diverse biological functions and close relationship with neurological disorders (Shu et al., 2015).

In 1998, Tuffereau first proposed that the neurotrophin receptor p75NTR interacts with RABV G and acts as a RABV receptor in vitro (Tuffereau et al., 1998). The N-terminal CRDs of p75NTR and viral G trimerization are responsible and important for this specific interaction with high affinity (Langevin et al., 2002; Sissoëff et al., 2005). The ability of p75NTR to interact with the G proteins of representative lyssaviruses from each genotype of rabies was subsequently investigated. The specific interaction has been observed with the G proteins of genotypes 1 (challenge virus standard or Pasteur virus strains) and 6 (European bat lyssavirus type 2) but not with those of genotypes 3 (Mokola virus), 4 (Duvenhage virus) or 5 (European bat lyssavirus type 1), indicating that p75NTR serves as a receptor for only a few lyssavirus glycoproteins (Tuffereau et al., 2001). Furthermore, RABV cannot bind or infect nearly half of the p75NTR-expressing DRG neurons that are often capsaicin-sensitive and in which the infectivity is unchanged in mice when the extracellular receptor domains of p75NTR are knocked out or RABV is mutated at the p75NTR binding site (Tuffereau et al., 2007). Accordingly, p75NTR is considered unessential for RABV infection both in vivo and in vitro. Although p75NTR has been reported to facilitate and accelerate RABV intracellular retrograde axonal transport (described later) (Gluska et al., 2014), the complete role of p75NTR in RABV attachment and transport mechanisms remains unclear.

2.4. mGluR2

Recent studies have identified a new receptor for RABV, mGluR2 (Wang et al., 2018). mGluR2, one of the eight different types of mGluRs, belongs to the group C family of G protein-coupled receptors (GPCRs) (Kammermeier et al., 2003). This structurally conserved protein has seven transmembrane domains. In addition, it is expressed abundantly in the CNS but rarely in other tissues (Ohishi et al., 1993). mGluR2 performs vital functions in the central and peripheral nervous systems because it is a receptor for a major excitatory neurotransmitter (L-glutamate). Abnormalities in mGluR2 expression can lead to multiple neurological diseases. Studies have shown that mGluR2 is a promising target for the treatment of schizophrenia, while its negative allosteric modulators have potential use as antidepressant drugs (Niswender and Conn, 2010; Foster and Conn, 2017; Jaso et al., 2017).

The crucial role of mGluR2 in RABV infection was first elucidated via a global RNAi strategy. In addition, coimmunoprecipitation (Co-IP) and immunohistofluorescence assays have revealed the direct interaction of mGluR2 with RABV G to mediate virus entry, internalization, and transport to EEs and late endosomes (LEs). Knockdown of mGluR2 drastically decreases RABV infection. Furthermore, antibodies against mGluR2 block RABV infection in vitro. When soluble protein neutralization is utilized either in vitro or in vivo, the infectivity of RABV cell-adapted strains and a street strain are neutralized. These results all suggest that mGluR2 is an additional functional receptor mediating RABV entry. However, some brain cells with little or no expression of mGluR2 can be infected by RABV. Though the detailed mechanisms remain to be addressed, this finding certainly offers a new approach to studying RABV attachment and entry (Wang et al., 2018).

In addition to these molecules that mediate RABV entry, other factors that play crucial roles in RABV attachment have been discovered (Lafon, 2005). Phospholipids, gangliosides, sialic acid, and carbohydrates are involved in RABV entry into host cells (Superti et al., 1984, 1986; Conti et al., 1986). Additionally, cellular heparan sulfate (HS) proteoglycans support the adhesion and subsequent entry of fixed RABV strains through direct interaction with the viral G protein. However, HS is expressed ubiquitously on nonpermissive as well as permissive cells, indicating that other cofactors may contribute to this HS-mediated entry pathway (Sasaki et al., 2018).

Although extensive studies have revealed that these molecules serve as RABV receptors, no specific molecule has been confirmed as an essential attachment factor for RABV infection. Moreover, it is inexplicable that cells expressing none of these revealed receptors can be susceptible. For example, African green monkey kidney cells (BS-C-1), a kind of epithelial cell, express none of the abovementioned receptors but can be infected during the final stages of diseases (Piccinotti et al., 2013), indicating that other unknown factors are involved in RABV recognition and anchoring at the cell surface. Though many efforts have been made, the mechanism of attachment and entry of RABV still requires further elucidation.

3. Internalization pathways

Attachment to specific receptors on host cells triggers the following viral internalization process. Cell surface receptor clustering is associated with the activation of downstream pathways. Viruses utilize various pinocytic mechanisms of endocytosis, among which clathrin-mediated endocytosis (CME), macropinocytosis, and caveolar/raft-dependent endocytosis (CavME) are best studied. CME is commonly utilized as the uptake mechanism for viruses such as vesicular stomatitis virus (VSV) (Johannsdottir et al., 2009; Cureton et al., 2009), dengue virus (Schaar et al., 2008; Acosta et al., 2009) and influenza A virus (IAV) (Rust et al., 2004; Chen and Zhuang, 2008). Following attachment to specific receptors, the virus promotes clustering of clathrin at the cell membrane by recruiting adaptors, such as AP2, eps15, and epsin1. Along with clathrin polymerization and cell membrane invagination, the formation of clathrin-coated vesicles (CCVs) occurs through clathrin-coated pits (CCPs) (Kaksonen and Roux, 2018). Dynamin pinches off budding vesicles at the neck and mediates their release into the cytoplasm (Bashkirov et al., 2008). Macropinocytosis is a transient, growth factor-induced, actin-dependent endocytic process and is associated with a complex signaling pathway. During this process, the plasma membrane ruffles and folds back to form a fluid-filled cavity that is closed by membrane fusion (Mercer and Helenius, 2009). Large viruses, such as poxviruses (Mercer and Helenius, 2008; Huang et al., 2008), adenoviruses (Amstutz et al., 2014; Sirena et al., 2004) and HIV1 (Maréchal et al., 2001; Liu et al., 2002), are internalized through macropinocytosis. CavME depends on cholesterol, lipid rafts, and regulatory factors such as tyrosine kinases, phosphatases, PKC, and RhoA. In CavME, as in CME, the cargo undergoes intracellular trafficking through the endosomal network after scission by dynamin (Mercer et al., 2010). Viruses in the polyomavirus family, including Simian virus 40 (SV40) (Pelkmans et al., 2002), BK virus (BKV) (Moriyama et al., 2007), and John Cunningham virus (JCV) (Achiron et al., 2016) are internalized by the caveolar/raft-dependent pathway. In addition, other endocytic pathways, such as caveolar- and clathrin-independent pathways and phagocytosis, are less well understood; thus, much work remains to be done.

3.1. Clathrin-mediated endocytosis of RABV

Rhabdoviruses, such as Australian bat lyssavirus (ABLV) (Weir et al., 2014), VSV (Sun et al., 2005), and infectious hematopoietic necrosis virus (IHNV) (Liu et al., 2011), are believed to utilize clathrin-dependent pathways for host cell entry. The internalization of RABV was first studied in chicken embryo-related (CER) cells. Ammonium chloride and chloroquine treatment inhibit the early steps of RABV infection, suggesting that viral entry is somehow obstructed. Via electron microscopy, virions have been visualized to be located in the coated pits of the plasma membrane, transferred in vesicles, and then fused with lysosomes at 30 min post infection (p.i.) (Superti et al., 1984). A similar phenomenon has been observed in cultured

hippocampal neurons, where the virus particles are adsorbed into the coated vesicles at 10 min p.i. and are located in the endosomes shortly after uptake (Lewis and Lentz, 1998). RABV has been shown to enter the cells most likely through endocytosis, but the identity of the involved molecules is not completely clear.

A recombinant VSV, in which the endogenous G protein is replaced by that of RABV, has been employed as a surrogate to examine the mechanisms of RABV endocytosis. Live cell imaging successfully captures the kinetics of CCP-mediated RABV entry into primary peripheral neurons, epithelial cells, and Vero cells (Piccinotti and Whelan, 2016; Piccinotti et al., 2013; Xu et al., 2015). The theoretical mechanism of the clathrin-mediated RABV entry pathway has been verified in pharmacological experiments. Pharmacological perturbation of dynamin by dynasore significantly inhibits viral internalization and infection. Moreover, data in Vero cells show that viral entry is inhibited by the pretreatment of cells with sucrose (an inhibitor of CME that acts by causing the dissociation of clathrin vesicles from the plasma membrane), chlorpromazine (an inhibitor of CME that acts by blocking the assembly of CCPs) and methyl-beta-cyclodextrin (MβCD) (an inhibitor of CME that acts by inhibiting CCP budding through cholesterol depletion) but is unaffected by nystatin or filipin (inhibitors of caveolar-dependent endocytosis) (Xu et al., 2015). However, surprisingly, conflicting data were obtained in Vero cells, whereas the depletion of cellular cholesterol by MβCD treatment increases RABV adsorption and infection in BHK-21 and Hep-2 cells, raising the possibility that RABV occupies other ports of entry in addition to those on cholesterol-rich microdomains and employs different mechanisms to enter nonneuronal cells (Hotta et al., 2009).

3.2. Actin-dependent internalization of RABV

Under spatiotemporal regulation of actin-binding protein activity, globular monomers of actin (G-actin) polymerize to form a linear polymer microfilament (F-actin), which is a kind of cytoskeleton (Bezanilla et al., 2015). The dynamic actin cytoskeleton is involved in many crucial cellular processes, such as morphogenesis, cell division, and vesicle trafficking (Doherty and Mcmahon, 2008). Moreover, actin dynamics are essential for membrane invagination and scission during the endocytosis process. During clathrin-mediated internalization, the force provided by the endocytic proteins is not sufficient alone, while actin recruitment offers more assistance. Polymerized actin pulls the membrane inwards when CCVs are assembled and resists the increased membrane tension created by cellular turgor pressure (Boulant et al., 2011; Kaksonen et al., 2006).

The uptake process for RABV is actin-dependent, which share an endocytic pathway with the viruses such as ABLV, VSV, and IHNV (Weir et al., 2014; Cureton et al., 2009; Liu et al., 2011). Pharmaceutical pretreatment with an actin-depolymerizing drug such as latrunculin B (Lat B) or cytochalasin D (Cyto D) does not block coated pit formation but impedes the entry of virion-containing pits by disrupting actin formation to inhibit infection (Piccinotti et al., 2013).

Thus far, the RABV endocytic pathway has been indicated to be indistinguishable from that of the rhabdovirus VSV. After virion attachment and docking on the cell plasma membrane, clathrin is recruited in the presence of its adaptor molecule AP2 and initiates cell plasma invagination. Under conditions of elevated membrane tension, actin is required to provide sufficient force to counteract membrane resistance and constrict the membrane neck of CCPs for GTP-bound dynamin-executed membrane scission. Subsequently, vesicles containing virions are separated from the plasma membrane and transported intracellularly (Fig. 1A).

The identified RABV receptors p75NTR and NCAM are internalized through the CME pathway (Taranta et al., 2003; Miñana et al., 2001), which are consistent with observations for RABV. Interestingly, a minor proportion of RABV particles are internalized in an AP2-independent manner, indicating that CME may not be the only pathway of RABV uptake (Piccinotti et al., 2013).

The endocytosis mechanism for the viruses to enter the host cells and be transported to the endosomal network is precise and complex. Some viruses can utilize more than one internalization pathway, an ability by the engagement of different receptors. Though CME has been determined in several cell lines for RABV uptake, more conclusive evidences in vivo are needed. Furthermore, whether any other pathways or molecules are involved in the endocytosis mechanism remains unclear.

4. Intracellular trafficking

RABV infects hosts at peripheral sites via a bite and then travels long distance to reach the cell soma and eventually the CNS, resulting in neurological symptoms. Since the virus entry site is not a favorable environment for the protein synthesis, RABV must transfer to and occupy the core region for further transcription and replication. Intracellular trafficking after internalization by endocytosis is thus initiated.

4.1. Endosomal trafficking of RABV

Similar to physiological ligands and membrane components, endosomal systems have also been reported to be utilized by viruses for sorting, anchoring, and targeting transport until the viruses fuse with endosomal membranes and subsequently release the viral genome into the cytosol. Viruses are initially delivered into EEs, wherein the process occurs very rapidly and efficiently. Then, mature endosomes (MEs), LEs, recycling endosomes (REs), and lysosomes successively control viral delivery. Among various molecules and factors, Rab proteins act as small GTPases and serve as central regulators of membrane transport, facilitating and assisting this process through recruiting effector proteins (Stenmark, 2009; Mercer et al., 2010).

RABV shares a similar mechanism of internalization and endosomal trafficking with other rhabdoviruses such as VSV. Studies have indicated that upon clathrin-mediated uptake of RABV, AP-2 is considered the major clathrin adaptor to contribute to the formation of CCVs and presents CCVs to EEs through interaction with Rab5 (a marker of EEs) (Semerdjieva et al., 2008). Consistent with these findings, RABV has been shown to colocalize with EE markers in nerve cells. Fusion of the endocytic vesicles with EEs occurs within 10 min after endocytosis (Albertini et al., 2012), and lysosomal transfer follows after 2 h (Lewis and Lentz, 1998; Lewis et al., 1998). Immunofluorescence results show that RABV colocalizes not only with Rab5/EEA1 (early endosomal proteins) but also with Rab7/LAMP1 (late endosomal proteins) at 24 and 48 h p.i. in neurons as well as in human (SH-SY5Y) cells. Downregulation of Rab7 and Rab5 by siRNA interference significantly reduces RABV infection, suggesting that these endosomal proteins play critical roles in RABV neuronal transport and infection (Ahmad et al., 2017).

The endogenous RABV G protein colocalizes with Rab5a (EEs) and Rab11a (REs) but not with Rab9 (LEs). However, when RABV G binds to a monoclonal specific anti-RABV G antibody, its colocalization shifts to Rab5a (EEs), Rab9a (LEs), and low pH lysosomes (Pierre et al., 2011). This relocalization initiates the degradation pathway, which is different from that of endogenous RABV G. Live cell imaging also shows that after the glycoprotein binds to p75NTR with high affinity, RABV particles hijack p75NTR to undergo clathrin-dependent endocytosis and fuse with p75NTR-positive endosomes for a faster migration route (Deinhardt et al., 2010). These observed results are consistent with previous findings that p75NTR is internalized by the clathrin-mediated pathway and transported through the endosomal pathway (Taranta et al., 2003). RABV does not strictly rely on p75NTR for internalization and can be transported without p75NTR (Gluska et al., 2014). Other identified RABV receptors, such as NCAM or other undiscovered receptors, are assumed to participate similarly in the retrograde axonal

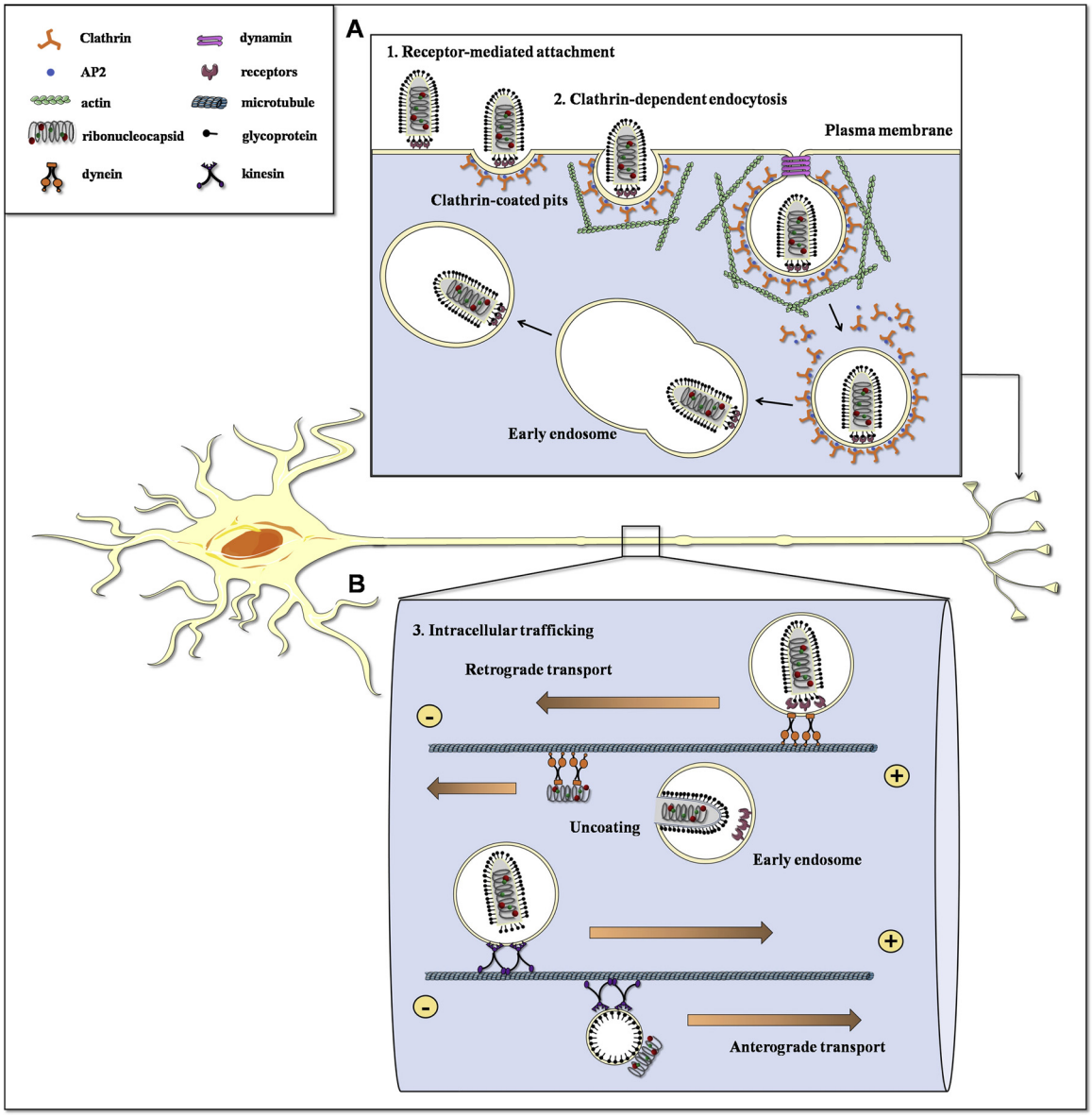

Fig. 1. Model of RABV entry and intracellular transport in neurons. A. Following the attachment to host cell mediated by the interaction between the viral glycoprotein (G) and cellular receptors (1), the RABV particles are endocytosed within endosomes coated with clathrin, which require actin to complete envelopment and dynamin to scission from the plasma membrane (2). The internalized vesicles uncoat gradually, then traffic to and fuse with the early endosomes. B. There coexists bi-directional intraneuronal transport on MTs (3). Two plausible different mechanisms were proposed in either direction, wherein dynein or kinesin motors involve. Retrograde-trafficking particles transport as the intact virions parceled inside endosomes or the free nucleocapsids (after uncoating). In the case of anterograde route, either fully-assembled postreplicative virions are transported within vesicles or G-containing vesicles hijack RNPs to move together.

transport of RABV within endosomes. In addition, retroviruses pseudotyped with fusion RABV G have been shown to enable retrograde transport and cell trafficking in Rab5-positive endosomes and Rab7 compartments (Hislop et al., 2014; Mentis et al., 2006). Together, these data support the hypothesis that the G protein of RABV not only recognizes membrane receptors but also facilitates endosomal trafficking.

The mildly acidic environment characteristic of the endosomal compartment triggers glycoprotein-mediated viral and endosomal membrane fusion, which results in the uncoating of the viral genome. Though RABV G has been demonstrated to colocalize with Rab7 as well as Rab5, fusion events are generally considered to occur when incoming viruses are passed to EEs because of the correlation of the optimal pH for RABV G-mediated fusion with the EE pH (Gaudin et al., 1993). The viral G protein spike can undergo conformational changes at low pH and assumes at least three different states, including the native (N) state, the activated (A) hydrophobic state and the fusion-inactive conformation (I). The prefusion N state is seen on the viral surface at pH 7. Upon acidification (pH 5.8–6), glycoproteins convert to the A state and interact with the target membrane when the hydrophobic domains at their top are exposed, which initiates the fusion process. Concurrently, the formation of the stalks and the initial fusion pore is facilitated. Stepwise protonation helps to enlarge the pore and complete membrane fusion. Subsequently, RABV G elongates its ectodomain and transforms to the I state (pH 6.75), which prevents fusion during the transport of the G protein in the acidic vesicles. The pH-dependent structural transition is reversible, and equilibrium between the different states of the G protein guarantees effective viral transport through the acidic compartments, ending with successful incorporation into latex particles (Gaudin et al., 1993; Gaudin, 2000, 2002; Gaudin et al., 1995).

4.2. Association of RABV with microtubules and dynein motors

The intracellular trafficking process is inevitably dependent on the cellular microtubule (MT) network and MT motors. MTs are long, polar cytoskeletal filaments formed from tubulin α/β heterodimers and play multiple roles in many essential biological processes. They are assembled in the perinuclear microtubule organizing center (MTOC) with their growing plus ends usually pointing towards the plasma membrane and axon terminals (Akhmanova and Steinmetz, 2015). MTs provide platforms for molecular motors to move cargoes throughout the cytoplasm—i.e., anterograde transport, mediated by kinesin, and retrograde transport, mediated by cytosolic dynein. Cytoplasmic dynein is a large multiprotein complex that contains heavy, intermediate, light intermediate, and light chains. Globular heads formed by two heavy chains walk along the MTs bound by the stalks, while light chains bind to cargoes, with assistance from dynactin. The cellular dynein machinery is necessary for numerous biological functions, such as organelle transport, centrosome assembly, and intracellular trafficking utilized by various viruses (Mallik et al., 2013).

Axonal transport of RABV can be inhibited upon the treatment of cells with the MT targeting drugs vincristine and colchicine (Tsiang, 1979), suggesting that the mode of RABV trafficking is dependent on the MT network. NCAM is found to tether to MTs at the synapse and mediates RABV-MT interaction (Perlson et al., 2013). The binding of the RABV P3 protein to the MT network has been quantified using confocal/live cell imaging and super-resolution direct stochastic optical reconstruction microscopy (dSTORM) (Brice et al., 2016). Moreover, the RABV phosphoprotein interacts directly with a dynein motor light chain (DLC8) through the DLC8-binding domain in its central region (residues 138–172) (Raux et al., 2000; Jacob et al., 2000; Poisson et al., 2001). Nevertheless, deletion of the DLC8-binding domain in RABV P does not abrogate the ability of incoming viruses to be ferried from a peripheral site to the CNS, while it affects the viral replication by attenuating primary transcription (Mebatsion, 2001; Tan et al., 2007). In addition, RABV L binds to DLC1 by a DLC1-binding motif at positions 1079 to 1083 to manipulate the association of MTs, achieving fast and efficient virus transport and regulating primary viral transcription (Bauer et al., 2015). From these studies, it can be speculated that RABV undergoes MT-mediated, dynein-dependent movement through direct interaction between the viral RNP and motor molecules. The fusion and uncoating events precede and allow the viral RNP's separation from the M protein wrapped outside. Whereafter, the viral RNP is released into the cytoplasm and available for the association with dynein motors. However, very little is known about the details of RABV particle uncoating.

4.3. Retrograde transport models of RABV

From the above-mentioned findings, two retrograde transport models for RABV have been proposed along the neuronal cell axon to the cell body (Fig. 1B). Both models favor further viral RNA transcription and replication. The first model postulates the whole virion is transported inside a vesicle. The alternative viewpoint is that the uncoating and fusion event occurs at some time after endocytosis, and the RNP containing viral genomic RNA is released into the cytoplasm and transported alone to the cell body.

In the first model, RABV G plays a dominant role in receptor recognition, CME, and intracellular trafficking. Previous empirical studies demonstrated that RABV G determines the transport direction and provides the driving force for long distance transport by employing retroviruses pseudotyped with RABV G to mimic the similar retrograde trafficking process of RABV (Mazarakis et al., 2001; Mentis et al., 2006.). Endocytic pits containing virions fuse with EEs, mediated by interaction between AP2 and Rab5. The whole intact virus particle is carried inside vesicles and transported along the MTs in a manner dependent on the surface G protein. Rab proteins switch on the membrane, and mediate RABV localization and delivery to EEs, LEs, and lysosomes successively through serving as central regulators of endosome trafficking. Consistent with this hypothesis, double-labeled RABV particles comprising green fluorescent RNPs and red fluorescent envelopes are transported as intact membrane-enveloped virions inside the vesicles by retrograde axonal transport (Klingen et al., 2008). Though RABV G is enclosed in the vesicle and is presumably unable to interact directly with specific motor complexes or MTs, it could still manipulate the transport direction through accompanying receptor linkers such as p75NTR or other undiscovered components.

The second model is based on the direct interaction of the dynein motor with RNPs, where RABV P and L interact with dynein light chain components, the DLC8 and DLC1, respectively. Following viral internalization and subsequent transfer to endosomal compartments, conformational changes in the G protein are induced by the low pH and enable the fusion of the viral and endosomal membranes. After the fusion, uncoating is initiated where RNP is dissociated from the M and G proteins, then released into the cytoplasm. Dynein binds to the RNP, links it to MTs, and drives retrograde trafficking, while the M and G proteins may remain associated with endosomal membranes and undergo intracellular trafficking separately from the RNP. However, RABV mutants deficient in the DLC binding site can be transported from the periphery to the CNS, implying it has the influence on viral transcription. Therefore, the model in which motor-driven transport relies on the interaction between RNP and dynein has been questioned.

4.4. Anterograde transport of RABV

In addition to the widely accepted retrograde route of RABV transport, bidirectional intracellular spread is gradually recognized. Retrograde transport assures the transmission of RABV from the periphery to the cell soma for replication and synthesis, while anterograde transport, which directs RABV towards MT plus ends, allows post replicative delivery of the newly formed virion particles for release and transmission at the presynaptic membranes. In the DRG and other peripheral neurons, anterograde axonal RABV transport is observed similarly to retrograde one, but at a relatively faster velocity (Tsiang et al., 1989; Zampieri et al., 2014; Astic et al., 1993; Coulon et al., 1989). Though insights into this process remain poor, the glycoprotein-dependent transport of RABV particles has been revealed when G gene-deleted virions fail to undergo anterograde transport. In addition to colocalizing with glycoproteins, green fluorescent protein (GFP)-labeled RNPs are cotransported with membrane-anchored mCherry fusion proteins in which the ectodomain of RABV G is replaced by the red fluorescent mCherry protein. Given the confirmed dependency on the G protein and the visualized cotransport of the RNP with G, a plausible model of anterograde trafficking is formulated (Fig. 1B), in which either complete enveloped virus particles are transported within cellular vesicles or glycoprotein-containing vesicles hijack cytoplasmic RNPs to move along MTs (Bauer et al., 2014).

As an important pathway for transport of the newly formed virus, the anterograde post replication route is as important as the retrograde route for spreading the RABV particles through peripheral neurons. Compared to the studies on retrograde transport, the studies on the mechanisms of RABV anterograde transport are far from sufficient. Differences in the velocities and kinetics of transmission in these opposite directions may be due to the involvement of different cellular motor complexes. Kinesins, members of the kinesin superfamily (KIF) of molecular motor proteins, with intracellular cargoes loaded, move along MTs towards the positive end to drive transport from the neuronal cell body to the periphery (Hirokawa and Takemura, 2005). However, the role of kinesin motors in the anterograde axonal route has not been revealed, and no kinesin binding motifs have been described in any RABV protein. However, there are reasons to believe that kinesin is closely involved in ways that are not yet clear. Moreover, the observation that in any case of anterograde transport, the expression of

the viral G glycoprotein is relied on or cotransported with vesicles is intriguing. The viral G protein likely provides a sorting signal that directs the loading of complete virions into transport vesicles or allows the recruitment of viral RNPs for transport along the MTs into axons. However, the detailed molecular mechanisms require further investigation.

To date, bidirectional intracellular transmission of RABV has been observed, and the models have been developed that either vesicles containing complete virions or RNPs directly associated with motor molecules are transported on MTs (Fig. 1B). Whether the two patterns of intracellular trafficking exist simultaneously or switch at a certain time point is still under speculation, which requires more confirmatory evidence. In addition to the identification of different motor complexes involved in retrograde and anterograde transport, the determination of other factors accounting for the differences in the kinetics of these two bidirectional translocation mechanisms requires to be investigated urgently. Overall, the precise mechanisms of RABV intracellular trafficking remain elusive, and the ways in which these factors cooperate requires further elucidation.

5. Conclusion

Though rabies has been studied for hundreds of years and the development of RABV vaccines has contributed greatly to preventing the rabies from spreading, the limited comprehension on rabies pathogenesis remains an obstacle for improving therapeutics. Attachment, entry, and trafficking of RABV are regarded as early events in the viral life cycle and vital for subsequent processes such as replication, assembly, and budding. This review focuses mainly on the significant progress achieved in understanding the intracellular steps of RABV entry and transport. Here, we summarize the identified receptors important for RABV attachment (nAchR, NCAM, p75NTR, mGluR2, carbohydrates, and gangliosides), highlight the CME pathway, and discuss the mechanisms of intracellular trafficking (Fig. 1).

Because of the efforts by the researchers, our picture on the intracellular steps of RABV transport is increasingly clarified but yet far from complete. Several problems have yet to be addressed. For example, though multiple receptors have been found, none have been confirmed as essential in vitro. Whether unknown factors exist or whether the receptors utilized differs in neurotropic or nonneurotropic cell lines during different infection stages remain to be elucidated. Considering that viral particles can be found in uncoated vesicles (Superti et al., 1984), the question that whether RABV can be internalized through pathways in addition to the generally accepted clathrin-mediated pathway is an interesting subject to explore. The mechanisms by which RABV undergoes bidirectional intracellular trafficking are highly intricate and not yet fully understood. In the retrograde route, whether the viral capsid is transported alone or as the whole virion remains under debate. In addition, whether receptor specificity can determine the intracellular route that the incoming virus follows remains largely unknown. Moreover, whether uncoating occurs immediately after internalization or whether a specific time point, such as at fusion with EEs, links the two trafficking patterns need to be determined. Notably, most current studies are performed in cell cultures with purified virus added to the medium. Further studies should be conducted in situ in tissues and live animal hosts.

In conclusion, further research is required. The interaction between viral and host cell proteins may be the key in providing a complete complement of targets with exploitation potential although little is known about this interaction. Thus, more details about the molecular regulatory mechanisms should be elucidated. With the implementation of powerful new technologies including large-scale RNAi screening, single-virus tracking, quantitative live cell imaging, advanced cryogenic electron microscopy (cryo-EM) techniques, or combinations of these approaches (Cherry, 2009; Brandenburg and Zhuang, 2007; Nketia et al., 2017; Elmlund and Elmlund, 2015), an in-depth understanding of the biological mechanism of the virus will help unravel the mystery of RABV neuropathogenesis and lead to improved antiviral therapeutics.

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