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# G gene-deficient single-round rabies viruses for neuronal circuit analysis

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ABSTRACT

Rhabdoviruses like the neurotropic rabies virus are fully amenable to pseudotyping with homologous and heterologous membrane proteins, which is being harnessed for the study of viral envelope proteins, viral retargeting, or immunization purposes. Particularly, pseudotyped delta G rabies viruses are emerging as safe and superb tools for mapping direct synaptic connections and analyzing neuronal circuits in the central and peripheral nervous system, which is a fundamental pillar of modern neuroscience. Such retrograde rabies mono-transsynaptic tracers in combination with optogenetics and modern in vivo imaging methods are opening entirely new avenues of investigation in neuroscience and help in answering major outstanding questions of connectivity and function of the nervous system. Here, we provide a brief overview on the biology and life cycle of rabies virus with emphasis on neuronal infection via axon ends, transport, and transsynaptic transmission of the virus. Pseudotyping of single-round, G-deleted virus with foreign glycoproteins allows to determine tropism and entry route, resulting in either retro- or anterograde labeling of neurons. Pseudotyping in vitro also allows specific targeting of cells that serve as starter cells for transsynaptic tracing, and pseudotyping in situ for a single (mono-transsynaptic) step of transmission to presynaptic neurons. We describe principle and experimental variations for defining "starter" cells for mono-transsynaptic tracing with ΔG rabies virus and outline open questions and limitations of the approach.



## 1. Rabies virus: one of a kind

### 1.1. Virus and disease

Rabies is among the longest known and feared infectious diseases for humans and animals (Steele, 1991). The causative agent is the highly neurotropic rabies virus (RABV), the prototype of the *Lyssavirus* genus in the *Rhabdoviridae* family (Fu, 2005). The disease is characterized by an invariably fatal encephalomyelitis once the infection has reached the brain. RABV has a broad animal reservoir, including terrestrial animals and bats, with dogs being mainly responsible for the more than 55,000 human deaths per year (Knobel et al., 2005). Other members of the *Lyssavirus* genus ("rabies-related viruses") are almost completely restricted to bat reservoirs, but can sporadically cause rabies-like encephalitis and death in terrestrial animals and humans (Luis et al., 2013). The ten-thousands of annual deaths of mostly children are undue from a medical point of view as rabies can be easily prevented, even post exposure. Highly effective, inactivated cell culture vaccines are available in all industrialized countries. In developing countries, however, access to the vaccines for pre- or post-exposure prophylaxis is often limited to metropolitan areas, and/or by the inability of people to purchase the vaccines, making rabies largely a disease of poverty (Warrell and Warrell, 2015).

Although great progress has been made in the past decades in the fields of molecular biology and epidemiology of RABV, rabies disease and pathogenesis is still enigmatic. Composed of highly immunogenic proteins (Gomme et al., 2011), the virus is prone to quickly enter the nervous system in which it is partially protected against immune responses. Rabies virus has developed a variety of traits to provide for the necessary time to reach and replicate in the CNS. These include effective axonal transport mechanisms, tools to dampen innate and adaptive immune response and to prevent premature neuronal damage, as well as ways to interfere with migration of immune cells through the blood-brain barrier (Chai et al., 2015; Conzelmann, 2015; Lafon, 2011). Typically, the dramatic clinical outcome of RABV infection is not reflected by overt pathological changes in neurons, as is the case in other virus infections of the CNS. A somewhat greater affinity to the limbic system

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rather than the neocortex seems to correlate with aberrant behavior and aggressiveness, and to compel the host to transmit the virus to others (for reviews see Dietzschold et al., 2008; Jackson, 2013). Therefore, covert neurotropism, or "stealth" (Schnell et al., 2010) and modulation of brain function rather than demolition seems to be the basis of successful maintenance of RABV transmission cycles.

RABV is considered the only member of the *Rhabdoviridae* family with eminent clinical relevance. While it is a prototypical member of the family in terms of genome and virus structure as well as gene expression mechanisms it is completely atypical in terms of biology. RABV and the other members of the *Lyssavirus* genus have adapted to direct transmission between their hosts, while other mammalian rhabdoviruses are transmitted in nature by insect vectors. The latter include important livestock pathogens such as vesicular stomatitis virus (VSV) causing an acute illness in animals typified by skin lesions, which is usually resolved without further sequelae. Infections of humans by VSV are rare, and healthy individuals are asymptomatic or exhibit mild flu-like symptoms. However, the recent isolation of VSV-like Chandipura virus or of the novel Bas-Congo virus from patients suffering from encephalitis or hemorrhagic fever, respectively, indicate a pathogenic potential of rhabdoviruses (Basak et al., 2007; Grard et al., 2012). The contrasting hit-and-run and stealth strategies of VSV and RABV, respectively, are illustrated already in tissue culture. VSV infection is rapid, causes extensive cytopathic effects (CPE) and within a day yields very high infectious supernatant titers of more than $10^{10}$ $mL^{-1}$ plaque-forming units (pfu), while RABV infection is slow and non-cytopathic and reaches modest infectious titers of up to $10^{8}$ $mL^{-1}$ focus-forming units (ffu) after 3–4 days.

RABV has entirely adapted to growth in the nervous system and polarized neurons. It is therefore important to keep in mind that most studies on RABV cell biology, biochemistry, and reverse genetics have been performed in non-polarized cell culture systems, including baby hamster kidney (BHK) cell derived lines like BSR, human HEK 293, or mouse neuroblastoma cell lines like N2A. The results of these studies do not necessarily reflect the natural behavior of wt RABV or "street" isolates from animals. In fact, most RABV street isolates are very poor in infecting the above tissue cultures and require several passages for adaptation. The changes required to yield so called "fixed" RABV, or RABV "strains", are poorly defined and may affect receptor usage, replication, and intracellular transport and budding (Dietzschold et al., 2008). Importantly however, tissue culture adapted strains have largely preserved many key RABV features, like transsynaptic transmission between neurons (see below for details).

### 1.2. Rabies virions

The *Rhabdoviridae* family [*rhabdos*; Greek: rod] together with the *Paramyxoviridae*, *Filoviridae*, and *Bornaviridae* families belongs to the order of *Mononegavirales*, which are enveloped viruses with a single non-segmented negative sense RNA, or non-segmented negative-strand RNA viruses (NNSV). Negative-strand RNA viruses are distinguished from all other viruses and living organisms by using a permanent nucleoprotein (N)-RNA complex as a template for transcription and replication, rather than naked RNA. RABV and VSV have the smallest rhabdovirus genomes and comprise 5 genes encoding structural proteins, which are components of the virus particle: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and a large polymerase subunit protein (L). The virions as observed in electron microscopy or cryo-electron-tomography are characterized by the eponymous rod- or bullet-shaped morphology with one flat and one conical end, a diameter of about 75 nm, and a length of in average 180 nm (Fig. 1) (Ge et al., 2010; Guichard et al., 2011; Matsumoto, 1962). In the virion, the N-RNA complex comprising 12 kb RNA and approximately 1300 N protomers is present in a highly condensed superhelical form and associated with approximately 650 copies of P protein dimers, which bind between two adjacent N molecules, and 50 molecules of the large RNA polymerase subunit L. The superhelix is surrounded by an envelope comprising a continuous inner M protein mesh, a lipid membrane, and trimers of the transmembrane glycoprotein G. The overall shape of the virion is imposed by the M protein layer, which determines the number of N subunits per turn, the diameter of the trunk, and the pitch of the RNP superhelix (Green et al., 2006). As described for VSV, the conical end is a consequence of N-RNA helix self-assembly and comprises the 5′-end of the viral RNP genome (Albertini et al., 2006; Desfosses et al., 2013; Green et al., 2006; Luo et al., 2007).

Rhabdovirus particles contain a single RNP, in contrast to pleomorphic NNSV like paramyxoviruses which may contain multiple copies of RNPs (Rager et al., 2002). Due to the helical nature of the virion, however, strict RNA packaging restrictions do obviously not apply. In fact, natural miniature versions of rhabdovirus particles with standard diameter but shorter helical trunks are long known and contain defective interfering (DI) RNAs (Huang and Baltimore, 1970; Wiktor et al., 1977). As confirmed by analyses of genetically engineered RABV, deletions or insertions of extra sequences in recombinant RABV seem to be faithfully reflected by the length of virus particles (Fig. 1B).

### 1.3. RABV genomes and gene expression

Once released into the cytoplasm of a cell and upon dissociation of the M corset, the RNP takes a relaxed structure in which it can serve as a template for RNA synthesis by the polymerase which is brought into the cell with the RNP (Baltimore et al., 1970). RNA synthesis includes sequential transcription of subgenomic monocistronic mRNAs and replication of full-length RNPs, following a canonical NNSV "stop-start" model of gene expression (Fig. 2).

In the N-RNA template complex, the RNA is completely buried in a cavity between the two globular domains of an N subunit, and adjacent subunits are further linked by N- and C-terminal protein extensions. This rigid structure allows access of the polymerase to the RNA only at the RNP 3′-end, and suggests that the template RNA must be temporarily released from the N cavity – in a zipper-like fashion – to allow the viral polymerase to read the template (Albertini et al., 2011, 2008). This important "chaperoning" function is assigned to the P protein, which is an essential co-factor of the L polymerase and mediates L binding to the RNP (Leyrat et al., 2011).

The genome of RABV is 12 kb in length and comprises the five genes in the strictly conserved order 3′-N-P-M-G-L-5′ (Fig. 2A). The genome RNA is flanked by short terminal regulatory sequences, known as 3′-leader- and 5′-trailer regions, respectively. The 11 3′- and 5′-terminal nucleotides of leader and trailer regions are conserved and complementary, such that the 3′-ends of genome and antigenome RNA are identical (3′-UGCGAAUUGUU. . .5′) (Tordo et al., 1988).

The 3′-terminal leader region acts as the "genome promoter" (GP) and initially directs the transcription of monocistronic capped, and polyadenylated mRNAs by L/P complexes (Fig. 2A). Due to the 3′-end-restricted access of the polymerase, transcription of the genes is obligatory sequential (Abraham and Banerjee, 1976). Transcription initiation and 5′-capping is directed by conserved start-sequences (UUGUAACAYYNCT) upstream of an ORF and transcription termination, polyadenylation, and release of the mature mRNA by downstream stop signals (TGAAAAAAA). Without leaving the template RNP, the polymerase can re-initiate at the near-by downstream start-signal. The sequences in-between the signals are known as intergenic sequences, which in RABV genomes comprise 2–5 nucleotides between the N, P, M, and G genes, and up to 24 nucleotides upstream of the L gene. Due to eventual