

# Comparative pathogenesis of the SAD-L16 strain of rabies virus and a mutant modifying the dynein light chain binding site of the rabies virus phosphoprotein in young mice

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## Abstract

Recent reports have suggested that rabies virus phosphoprotein (P) interaction with dynein minus-end-directed microtubule motor proteins may be of fundamental importance in the axonal transport of rabies virus. A deletion of 11 amino acids was introduced into recombinant rabies virus SAD-L16 (L16) that modified the dynein light chain (LC8) binding site of the rabies virus P, producing mutant L- $\Delta$ P11. This mutant is a useful tool for determining the role of P–LC8 interaction in viral spread and pathogenesis. Seven-day-old ICR mice were inoculated into a hindlimb thigh muscle with L16 or L- $\Delta$ P11. Histopathological and immunohistochemical analyses of their brains were performed at serial time points in order to determine the pattern of viral spread. L16 spread to the brain and caused a severe encephalitis with apoptotic neuronal changes. L- $\Delta$ P11 infected specific brain areas (brainstem and hippocampus) 1–2 days later than L16 and involved a smaller number of neurons in some brain regions. However, the neuronal apoptotic changes produced by both viruses were similar in most brain regions. Following peripheral inoculation, deletions modifying the LC8 binding site had an effect on delaying viral spread, but did not significantly alter the pattern of rabies virus encephalitis. The precise role of the rabies virus P–dynein interaction in the axonal transport of rabies virus, particularly the importance of this interaction during natural infection, merits further study.

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## 1. Introduction

Rabies virus is a highly neurotropic virus that produces fatal encephalomyelitis in humans and animals (Jackson, 2002b; Jackson and Wunner, 2002; Niezgodna et al., 2002). After peripheral inoculation rabies virus spreads through peripheral nerves to the central nervous system (CNS) by retrograde fast axonal transport (Tsiang, 1979; Gillet et al., 1986; Jackson, 2002a). It has been recognized that a variety of toxins and pathogens disseminate in the nervous system by hijacking cellular transport machinery (Mueller et

al., 2001; Von Bartheld, 2004). Cytoplasmic dynein serves an important role in retrograde axonal transport (Vallee et al., 2004). The cytoplasmic dynein light chain (LC8) has recently been shown to interact with the rabies virus P using laser confocal microscopy and immunoprecipitation (Jacob et al., 2000; Raux et al., 2000). Analyses of a series of deletions mapped the LC8-binding domain to residues 138–172 of the P (Raux et al., 2000). LC8 is an important component of both cytoplasmic dynein and myosin V, which are important in microtubule minus-end-directed organelle transport and in actin-based vesicle transport, respectively. Consequently, it has been proposed that the rabies virus P–LC8 interaction might play an important role in the axonal transport of rabies virus along microtubules in neurons (Jacob et al., 2000; Raux et al., 2000). However, since rabies virus is internalized by receptor-mediated endocytosis (Superti et al., 1984),

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viral uncoating would need to occur shortly after viral entry for rabies virus P–LC8 interaction to take place for initiation of retrograde axonal transport from, for example, axon terminals unless there is an unknown mechanism by which an intact endosome containing rabies virus could attach to the dynein motor protein and be transported along the axon (Schnell et al., 2005). Experimental evidence is lacking that viral uncoating actually occurs at this site, and the actual role of rabies virus P–LC8 interaction in axonal transport remains speculative.

In an effort to develop a new live attenuated rabies vaccine with impaired neuronal transport, Mebatsion (2001) generated recombinant rabies viruses with deletions encompassing a conserved LC8-interacting motif ((K/R)XTQT) in the LC8 binding site (Lo et al., 2001). The deletions blocked incorporation of LC8 into mature virions and completely abolished the P–LC8 interaction. Intriguingly, mortality data at the end of a 21 day observation period indicated that SAD-L16 (L16) and the deletion mutants derived from it were equally virulent in 1-week-old mice after intramuscular inoculation, indicating that LC8 is dispensable for the spread of a pathogenic rabies virus from a peripheral site to the CNS in young mice. However, this mortality data may not reflect differences in virus transport. In the present study, we compare the time course of viral spread and neuropathological changes of a mutant with a deletion of 11 amino acids of the LC8 binding site, L- $\Delta$ P11, with parent virus L16 after intramuscular inoculation in 7-day-old mice in order to further assess the biological importance of the LC8 binding site in experimental rabies pathogenesis.

## 2. Materials and methods

### 2.1. Viruses

*Rabies virus* belongs to the *Lyssavirus* genus in the *Rhabdoviridae* family in the *Mononegavirales* order. The generation of recombinant rabies viruses L16 and L- $\Delta$ P11 was previously described (Mebatsion, 2001).

### 2.2. Animals and inoculations

Seven-day-old ICR mice (Charles River Canada, St. Constant, Quebec) were used. Mice were inoculated intramuscularly into the right hindlimb thigh muscle with 20  $\mu$ L containing 1000 focus-forming units of either L16 or L- $\Delta$ P11 diluted in PBS with 4% fetal bovine serum. Uninfected control mice were inoculated with only the diluent.

### 2.3. Preparation of tissue sections

Two to three mice were killed at daily intervals. Mice were anesthetized with methoxyflurane and perfused with buffered 4% paraformaldehyde. Brains were removed and immersion-fixed in the same fixative for 24 h at 4 °C. Coronal brain tissue

sections (6  $\mu$ m) were prepared after dehydration and embedding in paraffin, and were stained with cresyl violet for light microscopic examination.

### 2.4. Immunoperoxidase staining for rabies virus antigen

Sections were stained for rabies virus antigen by the avidin-biotin-peroxidase complex method using monoclonal mouse anti-rabies virus IgG 5DF12 primary antibody (obtained from Alexander I. Wandeler, Centre of Expertise for Rabies, Canadian Food Inspection Agency, Nepean, Ontario) as previously described (Jackson et al., 1999). Brain sections from uninfected mice were used as controls. In brief, tissue sections were deparaffinized, hydrated, and were successively reacted with 5% normal rabbit serum, monoclonal mouse anti-rabies virus nucleocapsid protein IgG 5DF12 diluted 1:160, biotinylated rabbit anti-mouse IgG secondary antibody, diluted 1:100 (Vector Laboratories, Burlingame, CA), 1% hydrogen peroxide in methanol, avidin-biotinylated horseradish peroxidase complex (Vector Laboratories), 3, 3'-diaminobenzidine tetrachloride (Polysciences, Warrington, PA) with 0.01% hydrogen peroxide, 0.5% cupric sulfate in 0.15 M sodium chloride. The sections were counterstained with hematoxylin.

## 3. Results

### 3.1. Clinical data

L16-infected mice developed hindlimb paralysis on day 4 post-inoculation (p.i.), by which time there was evidence of growth retardation. Over the next 3 days there was progression to quadriplegia and all surviving mice were moribund by day 8 p.i.

L- $\Delta$ P11-infected mice demonstrated hindlimb paralysis 1 day later than L16 (on day 5 p.i.) and also had growth retardation. Over the next 3–6 days the mice progressed to quadriplegia or a moribund state. Some mice survived up to 3 days longer than the L16-infected mice.

### 3.2. Rabies virus antigen distribution and histopathology

#### 3.2.1. Virus distribution

In L16 infection rabies virus antigen was first detected in brainstem neurons at 4 days p.i. By day 6 p.i. L16 had spread to involve the thalamus, deep cerebellar nuclei (Fig. 1A), Purkinje cells in the cerebellar cortex, cerebral cortex, and pyramidal neurons of the hippocampus. The infection in the cerebral cortex was usually prominent before significant involvement of hippocampal pyramidal neurons (Fig. 2A). Many hippocampal pyramidal neurons were infected on day 7 or 8 (Fig. 2C). Antigen was not observed in the dentate gyrus of the hippocampal formation or in the internal or external granular cell layers of the cerebellum.

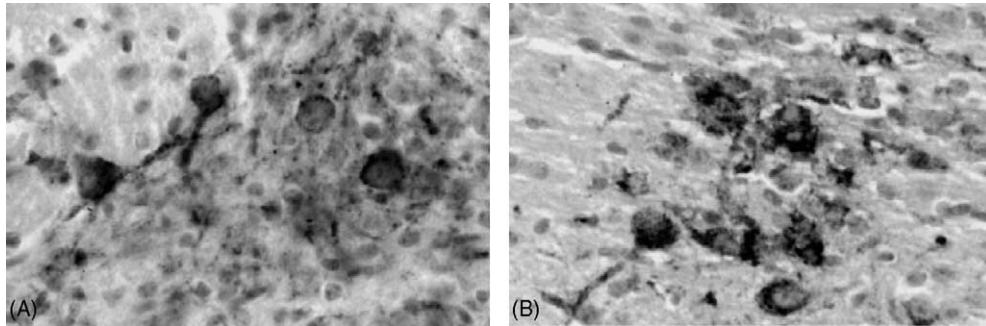


Fig. 1. Immunoperoxidase staining for rabies virus antigen in deep cerebellar nuclei 6 days after inoculation of a thigh muscle with L16 (A) and L- $\Delta$ P11 (B). Rabies virus antigen was present in the cytoplasm and neuronal processes of multiple neurons in both viral infections. Immunoperoxidase-hematoxylin; (A and B) 480 $\times$ .

In L- $\Delta$ P11 infection viral antigen was first detected 5 days p.i. in brainstem neurons. By day 6 p.i., L- $\Delta$ P11 had spread to the thalamus, deep cerebellar nuclei (Fig. 1B), Purkinje cells, and cerebral cortex (Fig. 2B). Mild infection of the hippocampal pyramidal neurons developed on day 8 p.i. (Fig. 2D), and progressively increased until day 10. L- $\Delta$ P11 did not infect neurons in the dentate gyrus or in the external granular cell layer of the cerebellum, and there were occasional foci of infected neurons in the internal granular cell layer.

Viral spread occurred less rapidly for L- $\Delta$ P11 than L16 to the brainstem (day 5 versus day 4) and hippocampal pyramidal neurons (day 8 versus day 6). Otherwise, infected neurons were observed in at least one mouse at the same time points for

L16 and L- $\Delta$ P11 in the other major brain regions (Table 1). Neurons containing rabies virus antigen were counted at daily time points, in order to compare the burden of infection in major brain regions in the L16 and L- $\Delta$ P11 infections (Fig. 3). The numbers of infected neurons were similar in the brainstem after day 4 p.i. (data not shown) and in the thalamus (Fig. 3A). In the cerebellum there was a greater number of infected Purkinje cells in L16 infection only at day 6 p.i., but not at either day 7 or 8 (data not shown). There were more L16 infected neurons at day 8 in both the cerebral cortex (Fig. 3B) and hippocampal pyramidal neurons ( $p < 0.05$ ). In summary, L- $\Delta$ P11 spread less rapidly than L16 and infected a smaller number of neurons in specific brain regions at a limited number of time points.

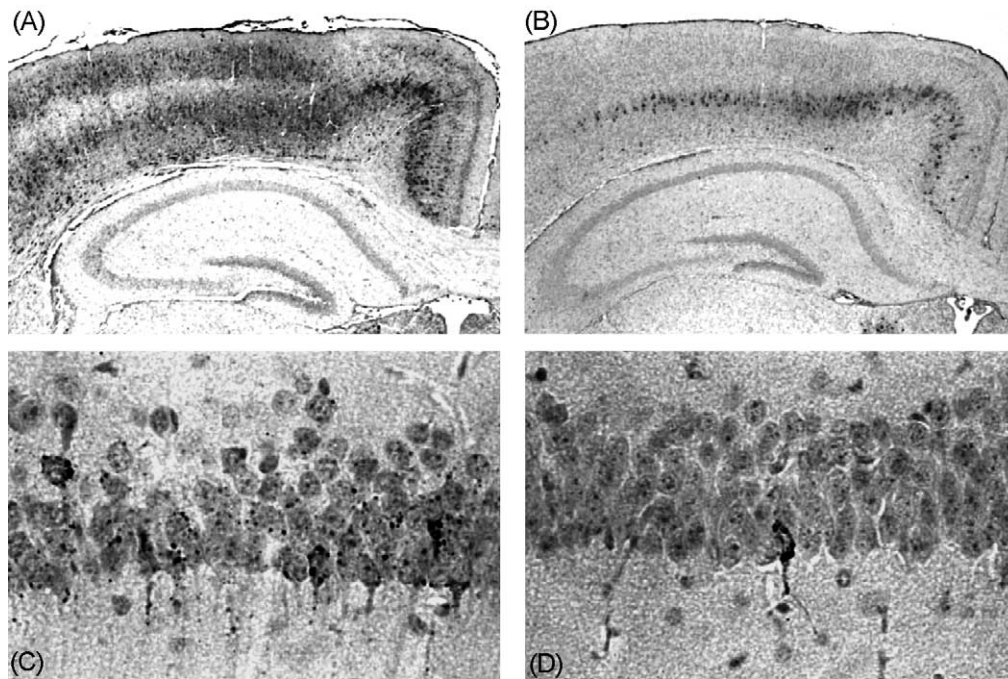


Fig. 2. Immunoperoxidase staining for rabies virus antigen in the hippocampus and overlying cerebral cortex (A and B) 6 days p.i. and in pyramidal neurons of the hippocampus (CA1 region) (C and D) 8 days p.i. with L16 (A and C) and L- $\Delta$ P11 (B and D). Rabies virus antigen was present in many cortical neurons and a few hippocampal neurons in L16 infection (A) and in a smaller number of cortical neurons without hippocampal involvement in L- $\Delta$ P11 infection (B). (C) Numerous pyramidal neurons contained rabies virus antigen in the cytoplasm and processes. (D) Single infected pyramidal neuron. Immunoperoxidase-hematoxylin; (A and B) 25 $\times$  (C and D) 370 $\times$ .

Table 1  
Viral spread to major brain regions

Brain region	Initial detection of viral antigen (days p.i.)	
	L16	L- $\Delta$ P11
Brainstem	4	5
Thalamus	6	6
Cerebellum—Purkinje cells	6	6
Cerebellum—internal granular cell layer	ND	ND
Cerebral cortex	6	6
Hippocampus—pyramidal neurons	6	8
Hippocampus—dentate gyrus	ND	ND

ND: viral antigen not detected.

### 3.2.2. Histopathology

L16 virus-induced cytological features of apoptosis, including karyorrhectic condensation of nuclear chromatin and cytoplasmic shrinkage, that were first observed in neurons in the brainstem tegmentum at 5 days p.i. above levels observed in uninfected control mice (related to developmental cell death). On day 6 p.i. neuronal apoptosis was noted in the thalamus, deep cerebellar nuclei, internal granular cell layer of the cerebellum, and cerebral cortex (Fig. 4A), and apoptotic changes in brainstem neurons were quite severe by this time (Fig. 4C). Few cytological abnormalities were seen in

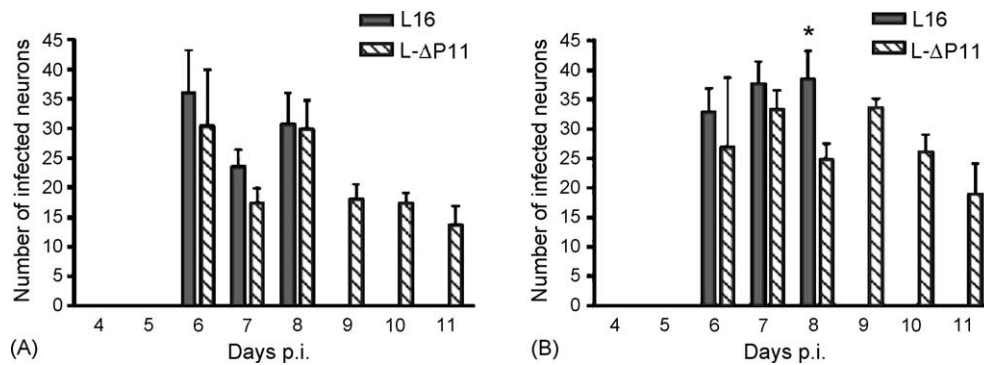


Fig. 3. Counts of the numbers of infected neurons after L16 (solid) and L- $\Delta$ P11 (hatched) infections in the thalamus (A) and cerebral cortex (B). Immunoperoxidase stained sections for rabies virus antigen were examined and the numbers of infected neurons were counted in three different fields using a high power (40 $\times$ ) objective in areas with the most marked staining. L16 and L- $\Delta$ P11 infections were compared at each time point using the Student's *t*-test (\*) represents statistical significance at a confidence level of 95% and the error bars represent the standard error of the mean. There were no survivors in L16 infection after day 8.

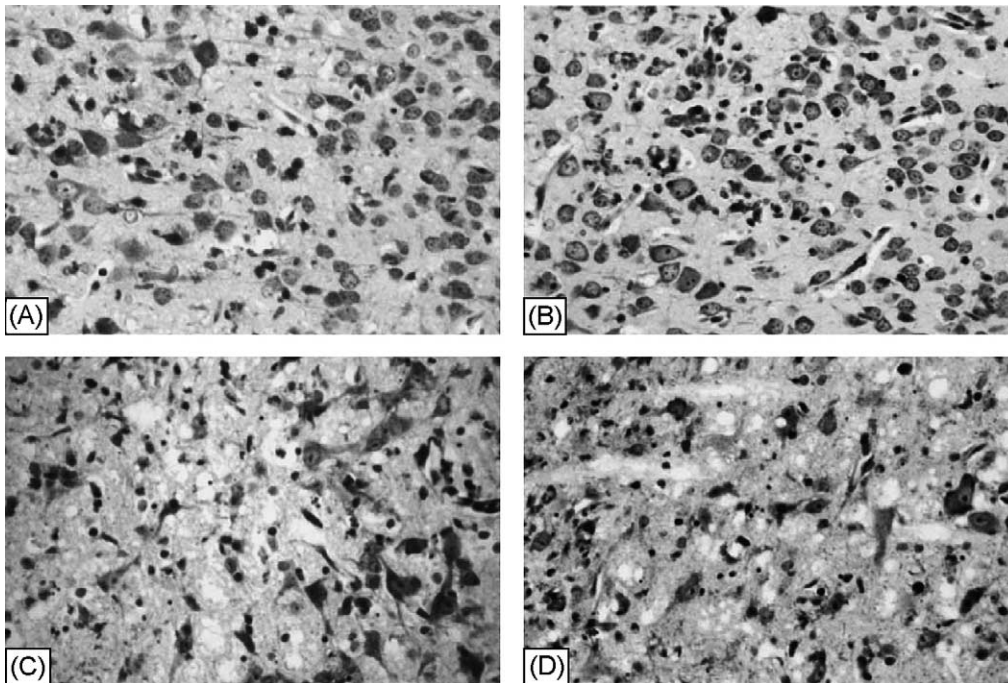


Fig. 4. Histology of cerebral cortex (A and B) and tegmentum of medulla (C and D) 8 days p.i. with L16 (A and C) and L- $\Delta$ P11 (B and D). In both locations there were changes of neuronal apoptosis with condensations of nuclear chromatin and cytoplasmic shrinkage in both infections. There was also evidence of neuronal loss and vacuolation in the tegmentum of the medulla in both infections (C and D). Cresyl violet; A–D 200 $\times$ .

cerebellar Purkinje cells, external granular cell layer of the cerebellum, hippocampal pyramidal neurons or dentate granular neurons. Around 4 days p.i. monocuclear inflammatory cell infiltrates appeared in the leptomeninges, perivascular regions, and progressively increased in severity at later time points.

After inoculation of L- $\Delta$ P11, neuronal apoptosis was first observed in brainstem neurons at 5 days p.i. Apoptotic changes were present in the thalamus on day 5 p.i. and in deep cerebellar nuclei, internal granular layer of the cerebellum, and cerebral cortex on day 6 p.i. There was marked apoptosis of brainstem neurons by 6 days p.i. Few changes of neuronal apoptosis were observed in other regions. Mononuclear inflammatory infiltration was present in the leptomeninges, perivascular regions, and brain parenchyma by day 4 p.i. and subsequently increased in severity. In summary, the neuropathological findings were similar in L16 and L- $\Delta$ P11 infections.

#### 4. Discussion

There is strong experimental evidence of rabies virus P interaction with the LC8 dynein light chain (Jacob et al., 2000; Raux et al., 2000). However, the functional importance of this interaction in rabies pathogenesis and in natural rabies is unclear. Pathogenicity of L- $\Delta$ P11 after intramuscular inoculation in 1-week-old mice was unexpected (Mebatsion, 2001). This observation has raised doubt that P-LC8 interaction is of fundamental importance for the spread of rabies virus in young mice. Although suckling mice are very permissive hosts for rabies virus infection, there is no evidence that there are important differences in the mechanisms of viral spread via axonal transport in very young versus older animals. However, adult mice would not be a suitable host for studying the pathogenesis of the L16 strain or derived mutants after peripheral routes of inoculation, because they are attenuated viruses that are poorly neuroinvasive in older animals. In addition, the L16 strain, which is actually a recombinant clone of the attenuated SAD-B19 vaccine strain (Schnell et al., 1994), is highly attenuated and only neuroinvasive in young mice. It is possible that more virulent rabies virus strains, including street rabies viruses, employ additional mechanisms for their neuroinvasiveness that may not be evident in this model.

In previous experiments, survival at the end of a 21-day observation period of 1-week-old mice after peripheral inoculation was used to compare the pathogenicity of L16 and mutant derivatives (Mebatsion, 2001). The experiments described here were designed to systematically compare the spread and neuropathological changes in L16 and L- $\Delta$ P11 infections at an early time in the course of infection. After intramuscular inoculation in 7-day-old mice we have found that viral spread of deletion mutant L- $\Delta$ P11 to major brain regions was delayed to the brainstem by 1 day and to hippocampal pyramidal neurons by 2 days. Hippocampal pyra-

midal neurons become infected relatively late compared to other regions after a peripheral route of inoculation with the challenge virus standard (CVS)-11 strain of fixed rabies virus (Jackson and Reimer, 1989). Consequently, it was not unexpected that the greatest effect was observed on spread to this brain region. L- $\Delta$ P11 infected fewer neurons in only some brain regions at some time points, indicating that efficiency of viral spread between neurons was not markedly reduced.

There are multiple factors that influence rabies virus neurovirulence (Jackson, 1994). The site and dose of inoculation as well as age of the animal are among the factors that considerably influence the course of rabies virus pathogenicity. Previous experiments, involving more attenuated strains possessing deletions at the LC8 binding site and replacement of the arginine at position 333 (R333) of the rabies virus glycoprotein, showed as much as 30-fold attenuation in pathogenicity when the mutants were administered by intramuscular route, but no difference from the parent virus when inoculated directly into the brain. In addition, greater differences in pathogenicity were observed at low viral doses than at high doses, indicating that the effect of blocking rabies virus P-LC8 interaction is more evident after peripheral inoculation of low virus doses (Mebatsion, 2001). Likewise, the 1–2 days delay in virus spread to certain brain areas and the longer survival time of L- $\Delta$ P11 inoculated mice might become more pronounced at lower dose ranges. Considering that very few infectious rabies virus particles may gain access to the body of a victim during natural infection, the role of P-LC8 interaction might be to secure an efficient transport of the few infectious particles along peripheral nerves, so that they ultimately gain access to the brain. Further studies are needed to uncover the precise role of the P-LC8 interaction in the axonal transport of rabies virus. Of course, the mild reduction in the rate of viral spread to specific brain regions and the limited effects on the number of infected neurons may not have been due to a specific effect on viral transport mechanisms in neurons, since the mutation could also have effects prior to uptake into neurons, on in vivo viral replication, or on trans-synaptic neuronal spread.

Both L16 and deletion mutant L- $\Delta$ P11 caused severe and fatal neurological disease in infected mice after intramuscular inoculation. Neuronal apoptosis is prominent after intracerebral inoculation of CVS in both adult (Jackson and Rossiter, 1997) and suckling mice (Jackson and Park, 1998). However, peripheral inoculation of CVS in adult mice (Jackson, 2003) and bats (Reid and Jackson, 2001) is associated with relatively little evidence of neuronal apoptosis, although the clinical neurological illness is severe and fatal. We have found that L16 (and also L- $\Delta$ P11) inoculated intracerebrally in 7-day-old mice produces marked neuronal apoptosis in widespread brain regions (Rasalingam et al., 2003; Rasalingam et al., 2005). In this study, we have shown that L16 and deletion mutant L- $\Delta$ P11 induce neuronal apoptosis after peripheral inoculation, although the number of affected neurons and distribution is much less than after the intracerebral route of inoculation. The distribution of the neuronal apoptosis was

most prominent in the brainstem tegmentum after intramuscular inoculation, and it may have played an important role in producing the neurological signs of disease and the fatal outcome. No major differences were observed in the severity of the neuropathological changes between L16 and L- $\Delta$ P11 infections, although there were differences in the time course.

It can be concluded that the deletion mutation in the rabies virus P affecting the LC8 binding site did not produce biologically important impairment of viral transport in the nervous system under these experimental conditions. The rabies virus glycoprotein (G) may actually play a more important role in axonal transport of rabies virus than the phosphoprotein (P). Mazarakis et al. (2001) showed that a lentivirus vector (equine infectious anemia virus) pseudotyped with the rabies virus G, but not with the vesicular stomatitis virus glycoprotein, enabled retrograde axonal transport to the spinal cord after inoculation into rat gastrocnemius muscle. The p75 neurotrophin receptor (NTR) is a ligand for the rabies virus G and has been postulated to serve as a rabies virus receptor (Tuffereau et al., 1998). The p75NTR has important functions in the retrograde axonal transport of neurotrophins and also a variety of other proteins (Butowt and Von Bartheld, 2003). The binding of rabies virus to the p75NTR has recently been postulated to be relevant for the retrograde axonal transport of rabies virus (Lafon, 2005). Clearly, the functional role of the rabies virus P–LC8 dynein interaction, and particularly the importance of this interaction during natural infection, merits further study.

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