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# Blue eye disease porcine rubulavirus (PoRv) infects pig neurons and glial cells using sialo-glycoprotein as receptor

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**Abstract**

Pig neural cells express glycoproteins with sialylated N-linked oligosaccharide chains (SNOC) which are used by the porcine rubulavirus (PoRv) as receptors. Pig neuronal or glial cell cultures were employed to investigate (a) whether PoRv infects such cells using a molecule expressing SNOC, and (b) the role of viral envelope glycoproteins in establishing the infection. Enriched neuronal or glial cell cultures were exposed to PoRv and infection was detected immunocytochemically. Neuronal cultures prepared from neonatal pigs were treated enzymatically to eliminate sialic acid or N-linked oligosaccharide chains. Primary neural cultures were exposed to anti-HN or anti-F preincubated with PoRv to study the role of the viral glycoproteins. In enriched cultures, PoRv infected neurons and glial cells, and sialic acid expressed in N-linked oligosaccharide chains appeared to play a central role in infection. It was concluded that HN and F viral glycoproteins are required to infect neurons and glial cells.


*Keywords:* Rubulavirus; Neuronal culture; Viral infection; Glycosidase; Cell receptor

## 1. Introduction

Porcine rubulavirus (PoRv), also known as porcine rubulavirus La Piedad Michoacan, or PRLPM (Moreno-López et al., 1986; Sundqvist et al., 1990, 1992; Berg et al., 1991, 1992; Hernández-Jáuregui et al., 2004), is the aetiological agent of a disease causing fatal encephalomyelitis in neonatal pigs (Stephano-Hornedo and Gay, 1982; Stephano-Hornedo and Guy-Gutiérrez, 1986).

PoRv belongs to the Rubulavirus genera of the Paramyxovirus family; it is pleomorphic and its lipid envelope exhibits two embedded glycoproteins, a haemagglutinin–neuraminidase protein (HN, 66 kDa), and a fusion protein (F, 59 kDa) (Moreno-López et al., 1986). Rubulavirus envelope glycoproteins are responsible for virus attachment and fusion. We found in ex vivo experiments that antibodies against haemagglutinin (HN) viral glycoprotein effectively inhibited attachment, but antibodies against F viral protein did not (Mendoza-Magaña et al., 2001). However, it is unknown whether in experiments using living cells in vitro the F protein also participates in the viral infection.

The PoRv cell receptor was partially identified as a surface molecule containing sialic acid bound to lactose, which could be associated to a specific protein molecule in order to function as a true receptor in cell surface (Reyes-Leyva et al., 1993). In ex vivo studies, we have found that PoRv binds to a 116 kDa pig neuronal membrane glycoprotein, containing sialic acid in N-linked oligosaccharide chains (Mendoza-Magaña et al., 2001). However, it is not known whether PoRv infects neurons and/or glial cells using an N-linked glycoprotein.

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The present study analysed whether PoRv infects pig neurons and glial cells in enriched primary neuronal cell culture, the role of sialic acid and lactose in N-linked oligosaccharides in cell binding and infection, and whether HN or F viral glycoproteins participate in infecting neural cells.

## 2. Materials and methods

### 2.1. Animals

All procedures were performed according to protocols approved by the Bioethics Committee of the Physiology Department and by National Council of Science and Technology (CONACYT).

Newborn pigs were obtained from the University of Guadalajara Livestock Centre and were known to be free of PoRv viral infection. The animals were transported immediately after birth.

### 2.2. Virus harvest

PoRv was obtained from an experimentally infected pig brain. Infected tissue was prepared in a 10% homogenate diluted with phosphate buffered saline (PBS), centrifuged at 1600*g* for 10 min and then filtered through a 0.22 μm nitrocellulose membrane. The virus suspension was centrifuged at 40,000*g* for 2 h, the pellet resuspended in one-tenth of the original volume and virus was titrated by haemagglutination. Then, PK-15 (pig kidney) monolayers were infected with 256 haemagglutinating units/100 μL. For infecting neuronal cell cultures, the virus suspension was adjusted to a concentration of 30 μg of viral protein/mL corresponding to $10^{4.5}$ SFU/mL.

### 2.3. Mixed neuron and glial cell culture

Newborn pig neuronal cells were obtained as follows: pigs were anaesthetised with sodium pentobarbital (35 mg/kg). Each animal was then decapitated and the head placed in 70% cold ethanol for 3 min. The brain was aseptically extracted and placed in ice cold phosphate buffer with antibiotics (gentamycin 150 μg/mL, or penicillin 1000 U/mL and streptomycin 100 μg/mL). Meningeal membranes were removed using fine forceps.

The temporal brain cortex, hippocampus and cerebellum were dissected and approximately 1 g samples of neuronal tissue were cut into small pieces and enzymatically dissociated with 0.25% trypsin and 0.6% bovine serum albumin in calcium-free Hank's solution at 37 °C for 5 min (Barnes et al., 1994). An ulterior mechanical dissociation was performed using fire polished Pasteur glass pipettes. Trypsin solution was decanted and enzyme activity was stopped by addition of fetal calf serum (FCS). Thereafter, cortex and hippocampal neuronal cells were suspended in D-MEM F-12 supplemented with glucose 25 mM, L-glutamine 4 mM, HEPES 50 mM, gentamycin 150 μg/mL and 20% FCS.

Cerebellar cells were grown in D-MEM F-12 medium supplemented with glucose 25 mM, L-glutamine 4 mM, HEPES 50 mM, potassium chloride 5 mM and gentamycin 150 μg/mL and 20% horse serum (HS). Cell viability was determined using a trypan blue exclusion method (Freshney, 1991). Cells were plated in polylysine treated 24 well plastic plates at cell density of $3 \times 10^5$ cells per well. Fresh medium was added every three days; morphological differentiation was accomplished by day 3 and experiments were carried out on day 10.

### 2.4. Enriched neuron cell culture

To obtain an enriched neuron cell culture, cytosine-1B–D arabinofranoside (C-Ara, 10 μM) diluted in D-MEM was added to 1-day old neuronal cell cultures and incubated for 72 h. Later, medium containing C-Ara was eliminated, replaced with fresh media with 20% FCS or HS as previously indicated, and incubated until differentiation was accomplished. This enrichment procedure yielded a culture purity approaching 90% in most cases as determined by immunocytochemistry detecting immunoreactivity to MAP-2, a microtubule associated protein specific of neurons. (The most common contaminant cell is the astrocyte type II, but this was excluded by immunocytochemistry.)

### 2.5. Enriched glial cell culture

Glial cell culture started as mixed neuron and glial cell culture plating $6 \times 10^5$ cells per well and incubated for 2 h. Thereafter, media was eliminated collecting non-adherent cells. Early adherent cells, mainly glial cells were maintained for 10 days. Fresh media was added as previously indicated. This allowed us to obtain glial cell enrichment close to 85%, as determined by immunocytochemistry detecting immunoreactivity to glial fibrillar acidic protein (GFAP) as a glial cell marker.

### 2.6. PoRv infection of neurons and glial cells

Neurons and glial cell cultures were exposed to 0.5 mL PoRv suspension for 30 min at 37 °C. Virus excess was eliminated, cells were washed with sterile PBS and incubated with fresh media for 24 h. Afterwards, cells were fixed with 4% paraformaldehyde in PBS 0.1 M pH 7.3 and the immunocytochemical study was performed to establish whether PoRv infected neurons or glial cells.

### 2.7. Immunocytochemistry

A mouse polyclonal antiserum against PoRv was immunoadsorbed using a nervous tissue homogenate prepared from newborn mice and processed as follows: first, 50% brain homogenate was prepared in 0.1 M of NaCl and filtered; acetone was added and the suspension was chilled to −20 °C for 1 h. Then, the homogenate was centrifuged at