

Supplementary Materials for

Attenuation of Rabies Virulence: Takeover by the Cytoplasmic Domain of Its Envelope Protein

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Supplementary Text

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Fig. S3. Comparison of the spreading of G-vir-HE, G-vir-HQ and G-att-LE rRABVs in culture.

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Supplementary Text

Neurite outgrowth

A standard functional assay of neurite outgrowth with the human neuroblastoma cell line SH-SY5Y is obtained when signaling pathways are stimulated after treatment with dibutyryl-cyclic AMP (1). During this study, we discovered that RABV has the intrinsic property to trigger neurite elongation. In uninfected conditions, neurites are small (29 to 34 μm , Fig. 3C). After infection with a virulent strain (Fig. 1D and Fig. 3C) the range of average neurites length per neuron was 97 to 110 μm , a three-fold increase compared to neuritis in uninfected cells. This outgrowth phenotype was not observed in cells infected with an attenuated strain of virus (38 to 41 μm , Fig 1D, Fig. 3C).

Deletion of the PDZ-BS attenuates growth and production of G at the membrane in human neuronal cell lines; however neurite outgrowth depends neither upon the total amount of G produced by infected cells nor on the quantity of G associated with the plasma membrane

When human neuroblastoma cells were infected synchronously at an MOI of 3, the two PDZ-BS-deleted rRABVs (G-vir-H Δ and G-att-L Δ) grew at markedly lower titres than those of the parental rRABVs strains: 16- and 166-times lower for G-vir-H Δ and G-att-L Δ , respectively (Fig. 2B). In the meantime, the amount of G detected by flow cytometry in G-vir-H Δ and G-att-L Δ cells 48 hours after infection was reduced by 33% and 56%, respectively. The other rRABVs did not exhibit an extensive defect in their growth properties (Fig. 2B). Therefore, the presence of a PDZ-BS substantially affected rRABVs production and expression of G at the membrane (Fig. 5, A and D). This is consistent with a previous observation that deletion of Cyto-G reduces viral production about six-fold (2). This could be linked to perturbed interactions of Cyto-G with protein M, a key player with G for viral budding and assembly (2, 3). Regulation of RNA transcription and replication of RABV is thought to involve G through an as-yet unknown mechanism (4). Quantitative RT-PCR analysis of the viral transcripts (mRNA) or the replicative viral genomes (gRNA) was performed after infection with rRABVs to test whether deletion of PDZ-BS affected viral transcription and replication. Overall, the viral transcription at late time points (72 hours PI) was not drastically hampered. On the contrary, viral transcription was diminished compared to that in rRABVS parental strains when the PDZ-BS was deleted in both a VIR genetic background (G-vir-H Δ , two-fold) and an ATT genetic background (G-att-L Δ , five-fold) (Fig. 2C).

In mouse models, abnormal primary transcription of rRABV also prevents proper onset of the disease (5). Both G-att-LE and G-vir-HE exhibited lower transcription and replication 8h pi compared to G-vir-HQ (respectively, 6-fold and 8-fold lower transcription and 9-fold and 22-fold lower replication, Fig. 2C). These lower transcription and replication rates at early time points, which are reminiscent of disabled primary transcription, may contribute to a change in virulence. The swap of the PDZ-BS in the G-vir background (G-vir-HE) did not drastically affect transcription and replication at later points. Conversely, the G-att-LQ was highly defective in replication and transcription at 24 hours PI (respectively 27-fold and 4.5-fold, fig. S1B). Experimental conditions, such as the test time of in vitro experiments, have been selected here to minimize these effects.

For some RABV strains, apoptosis is positively correlated with the amount of G (3). Because G-vir-HQ exhibits prosurvival properties whereas G-att-LE exhibits pro-apoptotic properties, the question was raised about any possible correlation between the survival or death phenotypes of infected cells and the amount of G. Therefore, the quantity of G was precisely

determined by Western blotting (to determine the total amount of G) or by flow cytometric analysis of cell-surface protein (to determine the amount of cytoplasmic membrane-associated G). Correlation analysis was undertaken for the neurite outgrowth phenotype at 24 hours PI for G-vir-HQ, G-att-HQ, G-vir-LE, G-att-LE, and G-vir-HE (Fig. 3D) or at 8 hours pi for G-vir-HQ and G-vir-H Δ (Fig. 3D). The calculated Pearson's correlation coefficients (*r*s) were all less than 1, indicating that, in our case, neurite outgrowth did not correlate with the amount of G. The same result was obtained when correlation analyses were undertaken with either the retraction or the apoptosis phenotypes and the amount of G. The survival or death properties are therefore linked to the sequence of *G* and not to its accumulation. In conclusion, despite clear differences in viral multiplication, neurite outgrowth depends neither upon the total amount of G nor on the quantity of G associated with the membrane.

The PDZ-BS is a key element for conferring neurites with protection against retraction

A functional assay to test the ability of differentiated neurites to resist the retraction conferred by treatment with a growth cone collapsing drug such as LPA was used to further assess the survival capacity of our RABV strains (*I*). Neurites of SH-SY5Y cells, which were differentiated with db-c-AMP, were sensitive to LPA (fig. S2A). On the contrary, neurites which had developed after infection with G-vir-HQ or G-att-HQ were not affected by LPA (fig. S2A). This was not the case when cells were infected either by G-vir-HE or G-vir-H Δ (fig. S2, B and C).

The reduced pathogenicity of G-vir-HE or G-att-LE does not result of an impaired cell-to cell spread

To further elucidate the role of PDZ-BS in RABV virulence, the intranasal route was chosen instead of the intramuscular route (as described in Fig. 3F) to infect mice because ATT strains, in contrast to VIR strains, do not cause disease in immunocompetent mice when injected intramuscularly (Fig. 1A). It could be argued that the reduced virulence of G-att-LE and G-vir-HE compared to that of G-vir-HQ resulted from impaired cell-to-cell spreading. However, when virus spread was assessed in Vero cells, the sizes of RABV-positive foci after infection with G-vir-HE or G-att-LE were not different from those observed after infection with G-vir-HQ (fig S3). Thus, in these assays, and in contrast to reports proposing that the pathogenicity of some rRABVs can be regulated by cell-to-cell spread, the reduced virulence observed for G-vir-HE cannot be related per se to a difference in in vitro spreading.

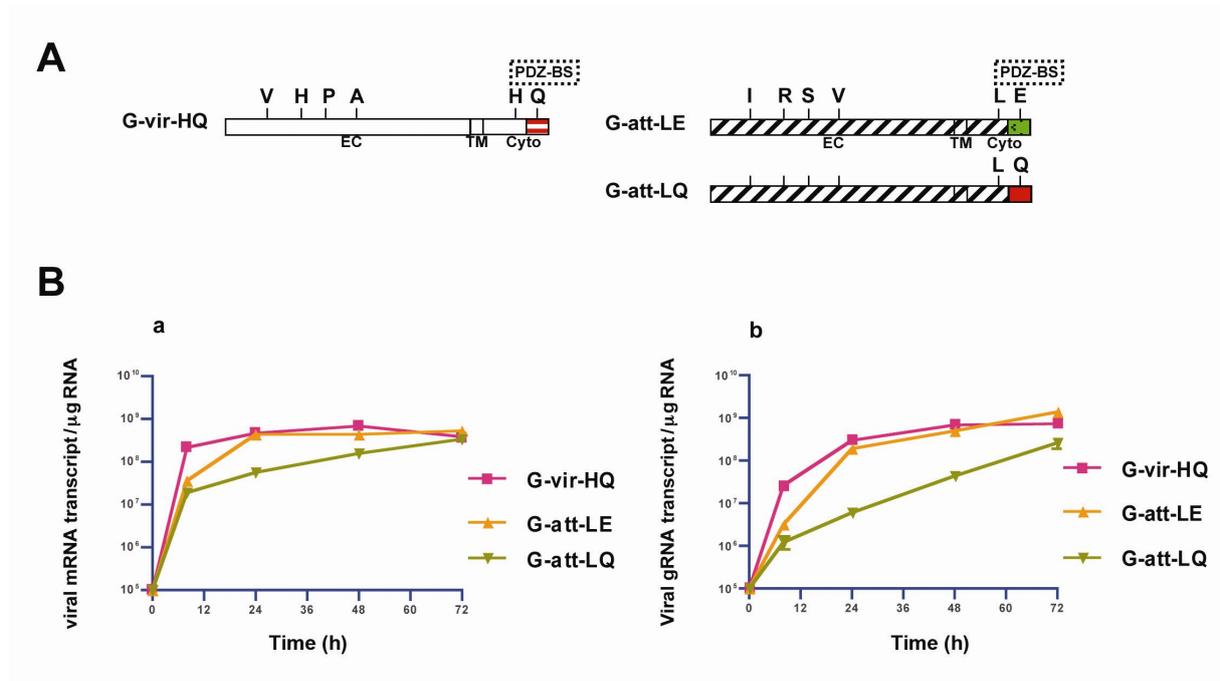


Fig. S1. G-att-LQ exhibits defective transcription and replication. **(A)** Schematic representation of the two original RABV G envelope proteins, G-vir-HQ and G-att-LE, and of the G variant, G-att-LQ, that were used in this study. **(B)** Quantification of viral transcription (mRNA) and replication (gRNA) by real-time PCR was performed in cells infected with rRABVs at an MOI of 3. Data are representative of three independent experiments. (SDs were less than 5%).

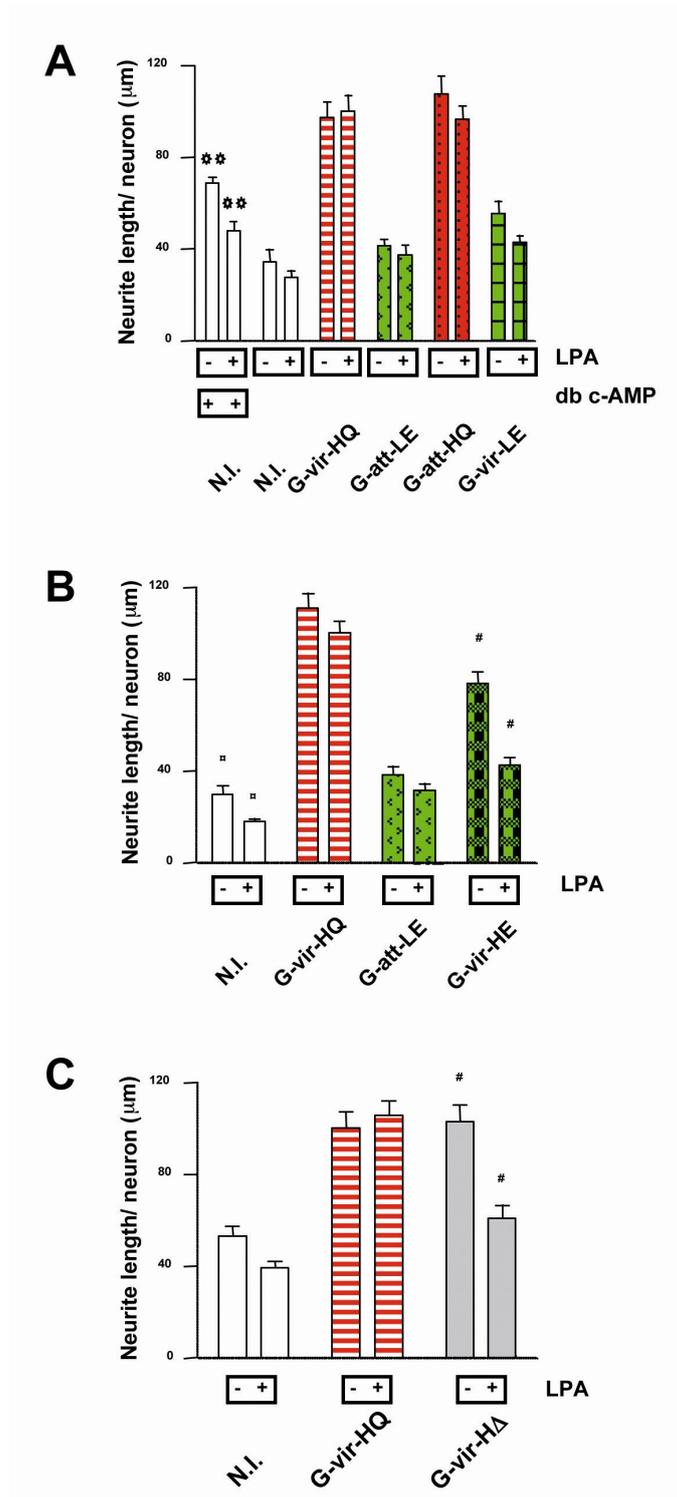


Fig. S2. The PDZ-BS is also a critical factor for neurite protection (neurosurvival). Protection of neurites from retraction was assayed after treatment with LPA in SH-SY5Y cells infected with (A) G-vir-HQ, G-att-LE, G-att-HQ, or G vir-LE, (B) G-vir-HE, or (C) G-vir-HΔ. Cells infected with the rRABVs at an MOI of 3 were treated 24 hours after infection with 10 μM LPA to monitor the resistance of neurites to retraction. **, $P < 0.002$; α, $P < 0.006$; #, $P < 0.001$, by student's t test.

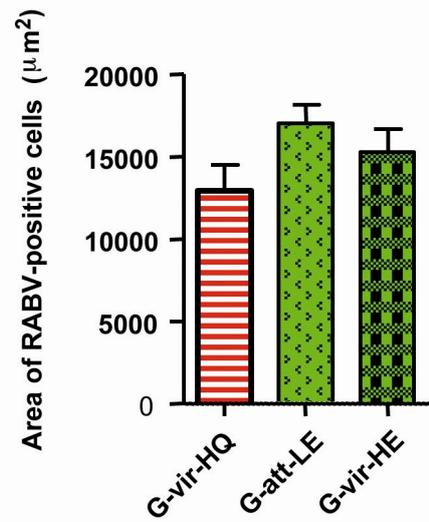


Fig. S3. Comparison of the spreading of G-vir-HE, G-vir-HQ and G-att-LE rRABVs in culture. Vero cells were infected at an MOI of 0.01 and overlaid with semisolid agar. At 48 hours PI, the agar overlay was removed and the cells were fixed with 80% acetone and incubated with a FITC-labeled anti-body against RABV nucleocapsid. Fluorescent foci were captured, and the sizes of fluorescent RABV foci were calculated with ImageJ. Each bar represents means (\pm SEM) of results for 30 foci. The analysis of variance (ANOVA) showed that the means were not statistically different ($P = 0.13$)

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