Investigation of rabies virus glycoprotein carboxyl terminus as an *in vitro* predictive tool of neurovirulence. A 3R approach

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Abstract

In the field of live viral vaccines production, there is an unmet need for *in vitro* tests complying a 3R approach (Refine, Replace and Reduce the use of animal experimentation) to replace the post-licensing safety tests currently assayed in animals. Here, we performed a pilot study evaluating whether virulence of rabies virus, RABV, can be forecast by an *in vitro* test of neurite outgrowth. The rationale to use neurite outgrowth as a read-out for this test is based on the salient property of the cytoplasmic domain of the G-protein (Cyto-G) of virulent RABV strains - not of attenuated RABV strains - to stimulate neurite outgrowth *in vitro*. We observed that neurite elongation triggered by the Cyto-Gs encoded by different RABV field isolates correlate with the distinct virulence scores obtained in a mouse model of experimental rabies. Our results cast the idea that it could be feasible to predict RABV virulence by testing the *in vitro* property of a RABV strain to promote neurite outgrowth without the use of animal experimentation.

***Key words*:** 3R; post-licensing safety test; live viral vaccine; rabies virus; neurite outgrowth; PDZ

1. Introduction

Vaccines are invaluable tools to prevent diseases and to increase the live expectancy of humans. Some vaccines are prepared with live non-virulent microbes. Their safe use requires that they are devoid of mutants which have acquired virulent features back. Before release for human immunization, batches of live viral vaccines such as those of Yellow Fever, Measles or Polioviruses, have to be tested for the absence of neurovirulence. At the moment, this post-licensing safety testing is performed in animals. Ethical considerations questioning the use of animals, monkeys in particular, in research strongly advocate for alternative methods. The 3R approach (Reduce, Refine, Replace the use of animals) is now clearly a priority for the world health authorities and a real challenge for scientists to stimulate their ingenuity on alternative *in vitro* tests that do not require animal experimentation [1]. A cellular test, which could amplify and make detectable phenotypic trait linked to neurovirulence is highly desirable. Nevertheless, because neurovirulence is a polygenic trait often not completely elucidated, the design of relevant *in vitro* test may not be an easy task.

Here, we performed a pilot study to bring a proof of concept to validate the relevance of such an approach. To this purpose, the neurotropic rabies virus (RABV) was chosen as a model since some mechanisms controlling RABV pathogenicity start to be unravelled. The virulent RABV strain CVS, referred as CVS-NIV, which invades the nervous system of the mouse and on being injected in the hind limb causes a fatal encephalitis [2], whereas the non-neurotropic strain ERA-NIV does not [3, 4] and triggers striking distinct features (attenuation, apoptosis and a robust antibody induction). Surprisingly enough for such a lethal neurotropic virus, CVS-NIV promotes neuron survival and triggers neurite elongation (neurite outgrowth), whereas ERA-NIV does not share these properties and instead induces the death of the infected neurons.

Amongst the five RABV proteins, the glycoprotein (G-protein), an integral trans-membrane

protein, has been identified as playing a critical role in the fate of viral properties [3, 5-12]. These features are controlled not only by the ectodomain of the G-protein, but also by the cytoplasmic domain, Cyto-G which was found to control the fate of the infected cell (cellular death or survival) through the interaction with some cellular partners [13, 14]. Commitment of RABV-infected neurons towards cellular survival or death is under the control of the carboxyl terminus (C-terminus) of Cyto-G forming a PDZ Binding Motif (PBM), which interacts with the PDZ domain (Post synaptic density protein, Drosophila disc large tumor suppressor, and Zonula occludens-1 protein domain) of a select group of cellular partners [14-17]. PDZ domains are protein-protein interacting domains and play a central role in cell signaling by favoring spatial contact between enzymes and their substrates, and more generally by assembling and/or regulating protein networks [18, 19]. Remarkably, in a model of strictly isogenic recombinant RABVs the introduction of a single point mutation in the C-terminus of Cyto-G (COOH Cyto-G) of the CVS-NIV switched neurosurvival to neuronal death. This single change is sufficient to induce the loss of virulence markers (neurite outgrowth, increase in AKT phosphorylation and protection against apoptosis) and the acquisition of markers of attenuation (increase in the number of cellular partners of the Cyto-G, induction of apoptosis). This change also modifies pathogenicity in a mouse model of RABV encephalitis [14]. With this background, it can be expected that the expression of the Cyto-G CVS-NIV or ERA-NIV alone and in the absence of any other RABV components may be sufficient to reproduce the *in vitro* neurosurvival and attenuation features of RABVs. The delivery of Cyto-Gs could be obtained for example by infecting the cells with recombinant lentiviral vectors. If this was the case, it could be possible to predict virulence of a RABV strain by performing an *in vitro* test of neurite outgrowth.

In this study we designed such an *in vitro* test, and challenged this test by assaying whether *in vitro* virulence features of three representative South African RABV isolates (a canid, a

mongoose and a spill over corresponding to a dog infected by a mongoose RABV biotype) and whose Cyto-Gs were distinct by a few mutations correlate with the virulence of the strains as previously determined in an experimental mouse model of rabies (Seo, W et al. companion paper). Despite the limited number of strains used for the correlate, this pilot study gave promising results suggesting that virulence, at least in the case of RABV infection, might be forecast by performing *in vitro* tests.

2. Material and Methods

2.1 Cells

Human embryonic kidney cells HEK 293-T were grown at 37 °C with 5% CO_2 in Dulbecco's Modified Eagle Medium with Glutamax-1 supplemented with 10% fetal calf serum (FCS), penicillin (10,000 IU/ml) and streptomycin (10 mg/ml). Neuroscreen-1 (Cellomics), derived from rat pheochromocytoma were grown in RPMI 1640 supplemented with 10% horse serum, 5% FCS, 200 mM glutamine, penicillin (10,000 IU/ml) and streptomycin (10 mg/ml).

2.2 Construction and Recovery of Recombinant lentivirus

Chimeric G-protein constructs were generated by using the plasmid with the deleted ectodomain that resulted in delta EC. The mutant clones were generated by amino acid substitutions using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) with mutagenic primers (Eurogentec Co). The primer sets for site-directed mutagenesis of the 3 constructs are shown below.

Canid	Canid 1	5'TGGGAATCATACAAGAGTGGGGGTGAGACCAGACTGTGAGGCCAAG3'
	Canid 2	3'ACCCTTAGTATGTTCTCACCCCCACTCTGGTCTGACACTCCGGTTC5'
Mongoose	Mong 1	5'TGGGAATCATACAAGAATGGGGAGGAGACCAGAATGTGAGGCCAAG3'
	Mong 2	3'ACCCTTAGTATGTTCTTACCCCTCCTCTGGTCTTACACTCCGGTTC5'
Spill over	Spill over 1	5'TGGGAATCATACAAGAATGGGGGTGAGACCAGAATGTGAGGCCAAG3'
	Spill over 2	3'ACCCTTAGTATGTTCTTACCCCCACTCTGGTCTTACACTCCGGTTC5'
ERA	ERA 1	5'TGGGAATCACACAAGAGTGGGGGTGAGACCAGACTGTGAGGCCAAG3'
	ERA 2	3'ACCCTTAGTGTGTTCTCACCCCCACTCTGGTCTGACACTCCGGTTC5'

Then, the chimeric transgenes were cloned in the lentivirus vector by using the pLenti6.3/V5-TOPO® TA Cloning® Kits (Invitrogen). A total number of 5×10^6 HEK 293-T cells were grown overnight to 80% confluence in 10 cm diameter Cell$^+$ dishes (Sarstedt). The transfection was conducted using the calcium phosphate method. HIV vectors were prepared as previously described by co-transfecting HEK 293-T cells with a four-plasmid system including the above plasmids carrying the genes of interest, the pMDLg/pRRE, the pRSV-Rev packaging plasmids and the VSV-G envelope protein expression plasmid (pMDG) [20]. HIV Gag protein p24 measured in 48 hours supernatants by the enzyme-linked immunosorbent assay (HIV p24 ELISA, Perkin Elmer). The infectivity of each stock was monitored on Neuroscreen-1 cells before use. A dose of 33 ng of p24 was needed to infect 5×10^4 Neuroscreen-1 cells. One hundred percent of infection was achieved 48-hour-post infection in these conditions. This dose was determined by using an eGFP (enhanced green fluorescent protein) expressing lentivirus included in each experiment to check the infectivity. Real-time relative qPCR (RT-qPCR) was further conducted and the level of 18S rRNA expression was used to as a house keeping gene.

2.3 Western Blotting and Immunocytochemistry

Lentivirus and non-infected cells were lysed with RIPA buffer (Sigma) supplemented with

anti-protease and anti-phosphatase cocktails (Roche Life Science) and stored at -20 °C. Cell lysates were electrophoresed on a 4-20% gradient polyacrylamide gel (Pierce Biotechnology) and blotted on PVDF membranes (Hybond-P, GE HealthCare). The membranes were incubated with a customized polyclonal rabbit antibody directed against the Cyto-G (Proteogenix) followed by an incubation with a secondary antibody coupled to horseradish peroxidase (Jackson Immuno Research). Signals were revealed with SuperSignal® West Femto Substrate (Pierce Biotechnology), acquired and analyzed with chemiluminescence imaging system (G:Box, Syngene).

For immunocytochemistry Neuroscreen-1 cells were infected for 48 hours with recombinant lentiviruses (20 ng of p24/well) on coverslips in a 24-well plate. Fixed cells were incubated with anti Cyto-G polyclonal customized rabbit antibodies (Proteogenix) followed by goat-anti rabbit labeled with Alexa 488 (Molecular Probe). Nuclei were stained with Hoescht 333420 and analyzed with a Leica confocal microscope Zeiss LSM 510.

2.4 Neurite Outgrowth Assay

A total number of 5×10^4 Neuroscreen-1 cells were seeded in 24 well poly-D-lysine-coated tissue culture dishes (Cell Bind, Corning). After 6-hour-incubation with 200 ng of neuronal growth factor (NGF), recombinant lentiviral stock containing 60 ng of p24 was added and cells were further cultivated for 72 hours. Cells were fixed and stained with crystal violet solution to visualize neurite processes. Cell images were captured (3 field/well, 10 wells/group) with a phase contrast Leica microscope and analyzed with ImageJ 1.44p, Neuron J plug-in.

2.5 Statistical Analysis

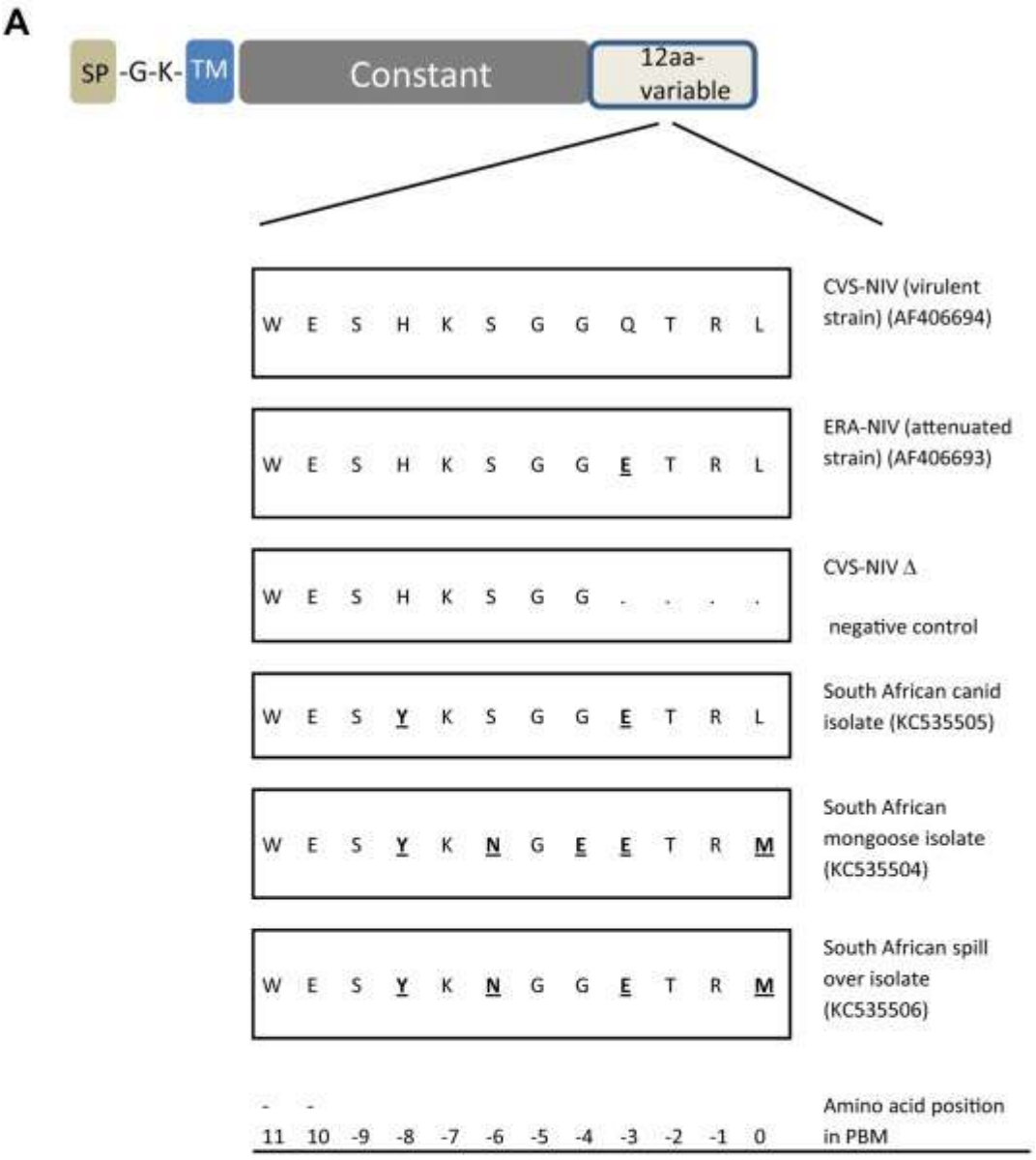
Student's *t-test* ($P<0.05$ was considered significant) was undertaken using GraphPad Prism

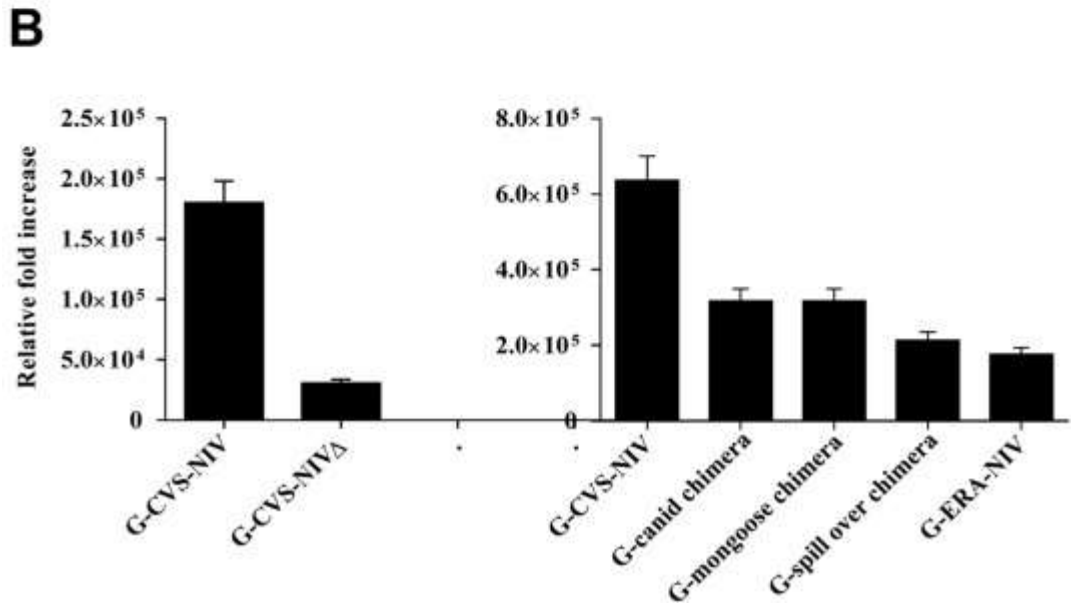
version 6.0 program.

3. Results

3.1 Construction and expression of Cyto-Gs

The commitment of RABV-infected neurons toward death (i.e. ERA-NIV) or survival (i.e. CVS-NIV) is controlled by the Cyto-G and the PBM located at the COOH terminus of the Cyto-G protein in particular [14]. We wanted here to assay whether Cyto-G expressed alone, in the absence of any other RABV elements, retains the neurosurvival features. We chose to construct a Cyto-G of the CVS-NIV (44 amino acids long) and 4 chimeric Cyto-Gs replacing the 12 original amino acid residues of CVS-NIV Cyto-G by the 12 amino acids of the COOH terminal of ERA-NIV Cyto-G or those of the three South African RABV (Canid, Mongoose and Spill over) (Fig. 1A). The reason to engraft 12 COOH amino acids and not the 4 COOH amino acids, which is the most common length of a PBM, is motivated by the results of nuclear magnetic resonance (NMR) and X-rays studies indicating that up to 12 amino acids of RABV Cyto-G could be involved in the formation of the PBM/PDZ complexes [15, 16]. The Cyto-G constructs included the transmembrane domain (TM) of the G-protein and G,K amino acids of ectodomain proximal to the TM as well as the entire signal peptide 19 amino acid long (Fig. 1A) to allow sorting and trafficking of the Cyto-Gs from the endoplasmic reticulum. A negative control, consisting in the G-CVS-NIV deleted of the last 4 amino acids, G-CVS-NIVΔ, was also constructed. Lentiviruses were chosen as a mean to express the Cyto-Gs in Neuroscreen-1 cells. The mRNA transcription levels of lentivirus constructs were evaluated by relative RT-qPCR (Fig. 1B) and ERA-NIV and Spill over constructs exhibited the lowest value, however, the mRNA expression of CVS-NIV showed the highest value. Interestingly, the G-canid and G-mongoose constructs displayed similar mRNA transcriptional levels. These data suggested that point mutations in the nucleotide sequence may slightly modify the


A
SP
-G-K-
TM
Constant
12aa-variable
W E S H K S G G Q T R L
CVS-NIV (virulent strain) (AF406694)
W E S H K S G G E T R L
ERA-NIV (attenuated strain) (AF406693)
W E S H K S G G . . . .
CVS-NIV Δ
negative control
W E S Y K S G G E T R L
South African canid isolate (KC535505)
W E S Y K N G E E T R M
South African mongoose isolate (KC535504)
W E S Y K N G G E T R M
South African spill over isolate (KC535506)
11 10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0
Amino acid position in PBM


B
Relative fold increase
2.5×10⁵
2.0×10⁵
1.5×10⁵
1.0×10⁵
5.0×10⁴
0
8.0×10⁵
6.0×10⁵
4.0×10⁵
2.0×10⁵
0
G-CVS-NIV
G-CVS-NIVΔ
G-CVS-NIV
G-canid chimera
G-mongoose chimera
G-spill over chimera
G-ERA-NIV

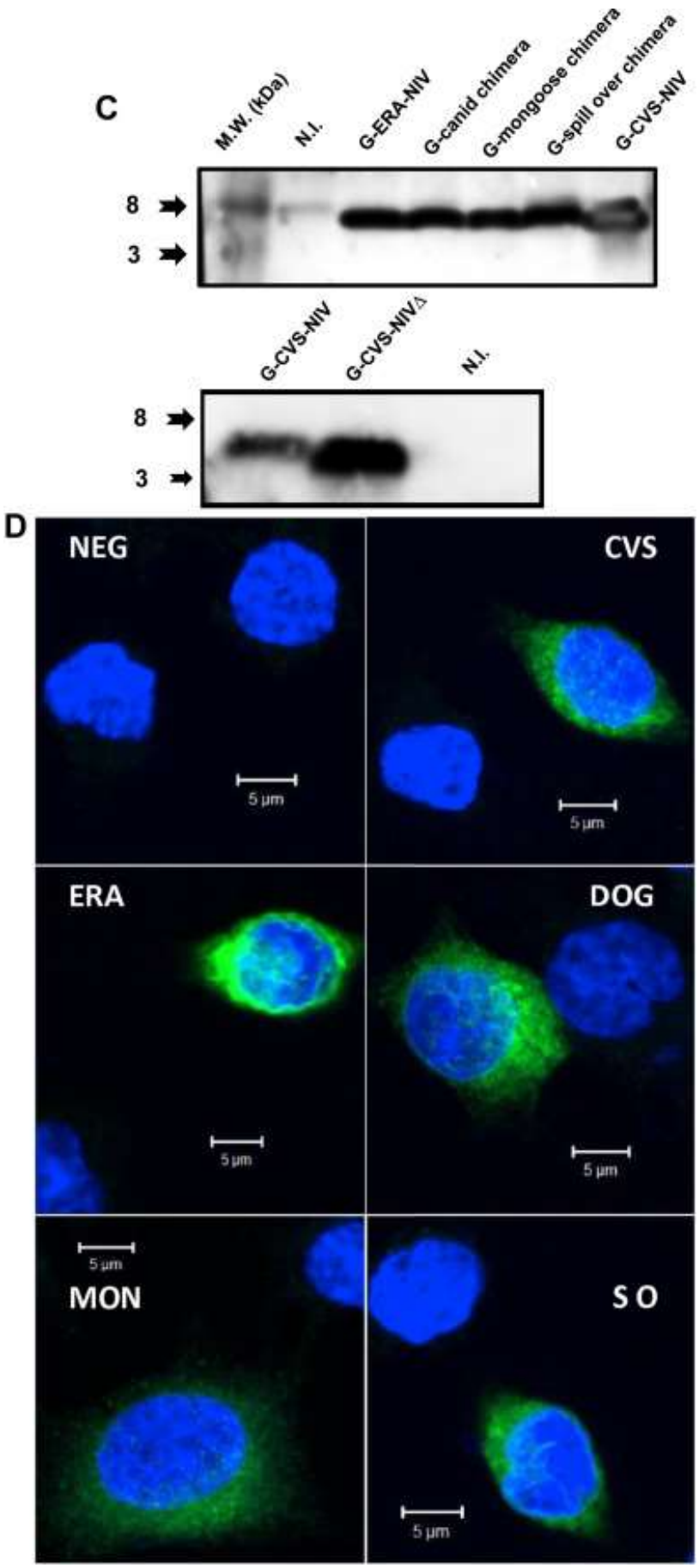

C
M.W. (kDa)
N.I.
G-ERA-NIV
G-canid chimera
G-mongoose chimera
G-spill over chimera
G-CVS-NIV
8
3
G-CVS-NIV
G-CVS-NIVΔ
N.I.
8
3
D
NEG
CVS
ERA
DOG
MON
S O
5 µm

Fig. 1. Construction and characterization of lentivectors expressing RABV Cyto-G constructs. **A)** Construction of chimera Cyto-Gs and sequences of the 12 COOH amino acids of the G protein of CVS-NIV, ERA-NIV and the three South Africa RABV strains (Canid, Mongoose or Spill-over, the latter resulting from the infection of a dog by a mongoose RABV strain). Chimera Cyto-Gs (44aa) are formed by the Signal peptide, SP, G and K amino acids from the ectodomain, transmembrane domain, TM, (19 aa), the Constant domain (32aa) from the CVS-NIV Cyto-G and a variable insert (12aa) corresponding to the 12 COOH terminus aa of Cyto-Gs of the different RABV strains distinct by 5 mutations (bold). **B)** Relative abundance of G mRNA transcription levels in a recombinant lentivirus system obtained by relative RT-qPCR (The left and right panels represent independent experiments). See Table 1 for statistical significance. **C)** Expression of the chimera Cyto-Gs (7.4 kDa) in Neuroscreen-1 cells after a 48 h-infection with lentivirus expressing either G-ERA-NIV, G-canid chimera, G-mongoose chimera, G-spill-over chimera or G-CVS-NIV (N.I. is for non-infected). M = molecular weight marker. **D)** Expression of Cyto-G constructs in 48 h transduced (CVS, ERA, DOG, MON = Mongoose or SO = Spill Over) or non-treated (NEG). Neuroscreen-1 cells was monitored by confocal microscopy using RABV specific Cyto-G polyclonal antibody (green). The nucleus is in blue after Hoechst 33342 staining. Scale bars: 10 μm.

Table 1 Statistical analysis of the lentivirus mRNA transcription. 'Statistical significance' (*P* value < 0.05) is denoted as (yes) and 'not statistical significance' is shown as (no) (*P* value > or = 0.05) by a Student's *t test*.

Pairwise comparison of constructs			*P* value	Statistical significance
G-ERA	**:**	**G-canid**	**0.0080**	**yes**
G-ERA	**:**	**G-mongoose**	**0.0080**	**yes**
G-ERA	**:**	**G-spill over**	**0.2228**	**no**
G-ERA	**:**	**G-CVS-NIV**	**0.0004**	**yes**
G-canid	**:**	**G-mongoose**	**0.9962**	**no**
G-canid	**:**	**G-spill over**	**0.0351**	**yes**
G-canid	**:**	**G-CVS-NIV**	**0.0042**	**yes**
G-mongoose	**:**	**G-spill over**	**0.0349**	**yes**
G-mongoose	**:**	**G-CVS-NIV**	**0.0042**	**yes**
G-spill over	**:**	**G-CVS-NIV**	**0.0007**	**yes**

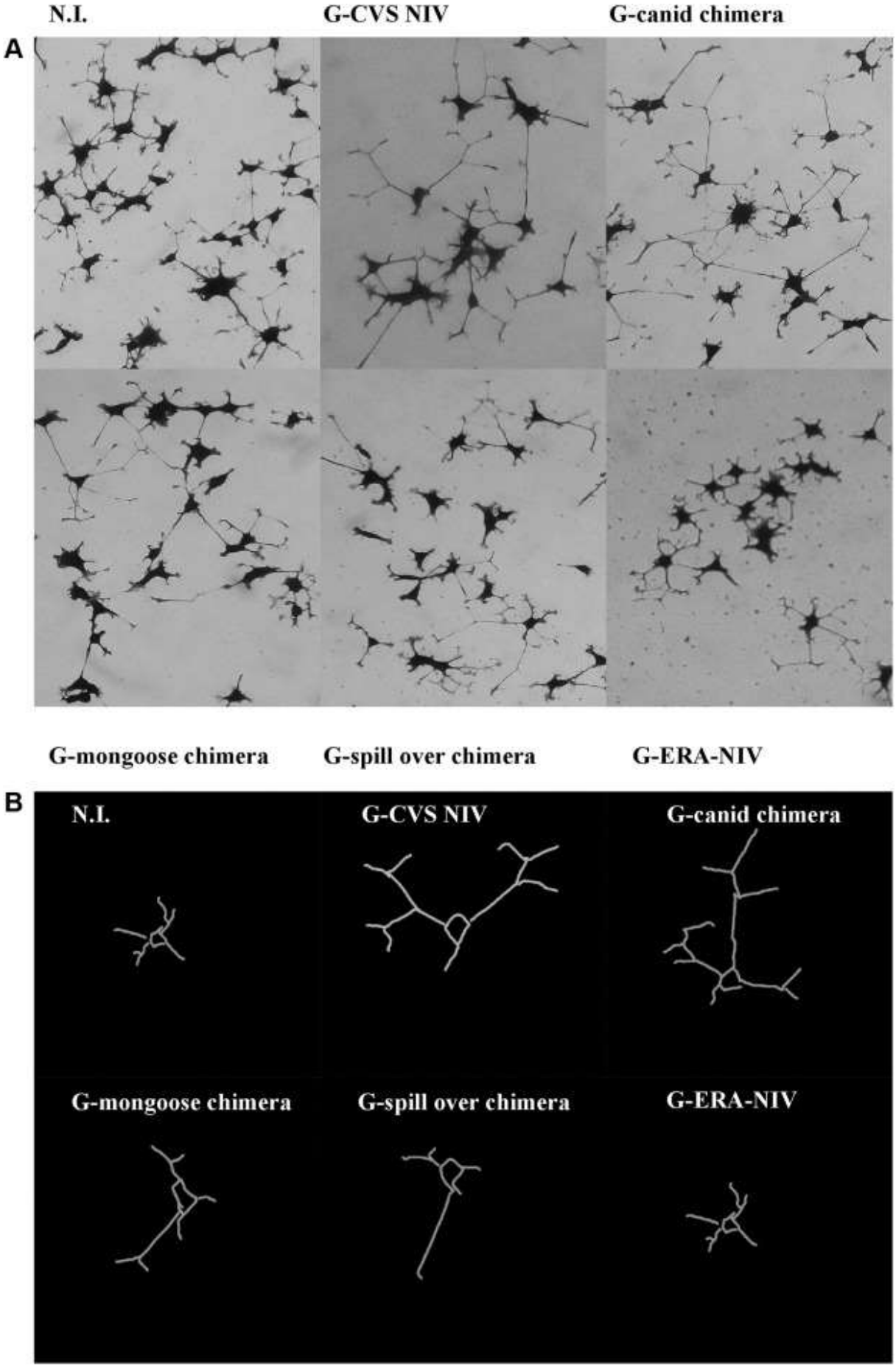
intrinsic stability of the mRNA transcripts. Nevertheless, when the Cyto-G protein expression was assayed by immunoblotting (Fig. 1C) and immunocytochemistry (Fig. 1D), all chimeric constructs exhibited high levels of protein expression. To note, in contrast to CVS-NIV

infected cells, in which the full length G-protein is localized both in the cytoplasm and at the cytoplasmic membrane (data not shown), or in cells infected by a lentiviral vector expressing the entire G-protein only [16], the Cyto-Gs were mainly localized in the cytoplasm of the cells. This may suggest that RABV G-protein trafficking was modified by the absence of the ectodomain (but two amino acids) of the RABV G-protein.

3.2 The Cyto-G of CVS-NIV expressed out of a RABV context is sufficient to trigger neurite outgrowth.

The capacity of Cyto-Gs to trigger neurite outgrowth in a PBM-dependent manner has been previously assayed in human neuroblastoma SH-SY5Y cells infected with isogenic recombinant RABVs [14]. Here, we tested whether the neurite outgrowth triggered by CVS-NIV Cyto-G could also be observed after the Cyto-Gs have been delivered in the cells by a lentiviral vector. For this assay the human neuroblastoma SH-SY5Y cell line was replaced by rat Neuroscreen-1, which compared to human neuroblastoma, are less prone to cellular aggregation, allowing for an easier evaluation of neurite outgrowth in individual cells [21].

Neuroscreen-1 cells were either non-infected or infected with the recombinant lentiviruses G-CVS-NIV, G-ERA-NIV, G-CVS-NIV-Δ and the mean neurite length was measured 72-hour-post infection (Fig. 2 and table 2). The CVS-NIV construct triggered significantly higher neurite outgrowth compared to non-infected group and to G-CVS-NIV-Δ or ERA-NIV constructs (mean length of 420 µm for CVS-NIV versus 200 µm for the other conditions: non-infected or G-CVS-NIV-Δ or ERA-NIV), indicating that the increased neurite outgrowth triggered by CVS-NIV Cyto-G could also be observed after Cyto-Gs has been delivered in rat Neuroscreen-1 cells by a lentiviral vector. The lentiviral vector Cyto-G CVS-NIV-Δ triggered a mean neurite outgrowth similar to those observed in non-infected cells (left panel in Fig. 2C


A
N.I.
G-CVS NIV
G-canid chimera
G-mongoose chimera
G-spill over chimera
G-ERA-NIV
B
N.I.
G-CVS NIV
G-canid chimera
G-mongoose chimera
G-spill over chimera
G-ERA-NIV

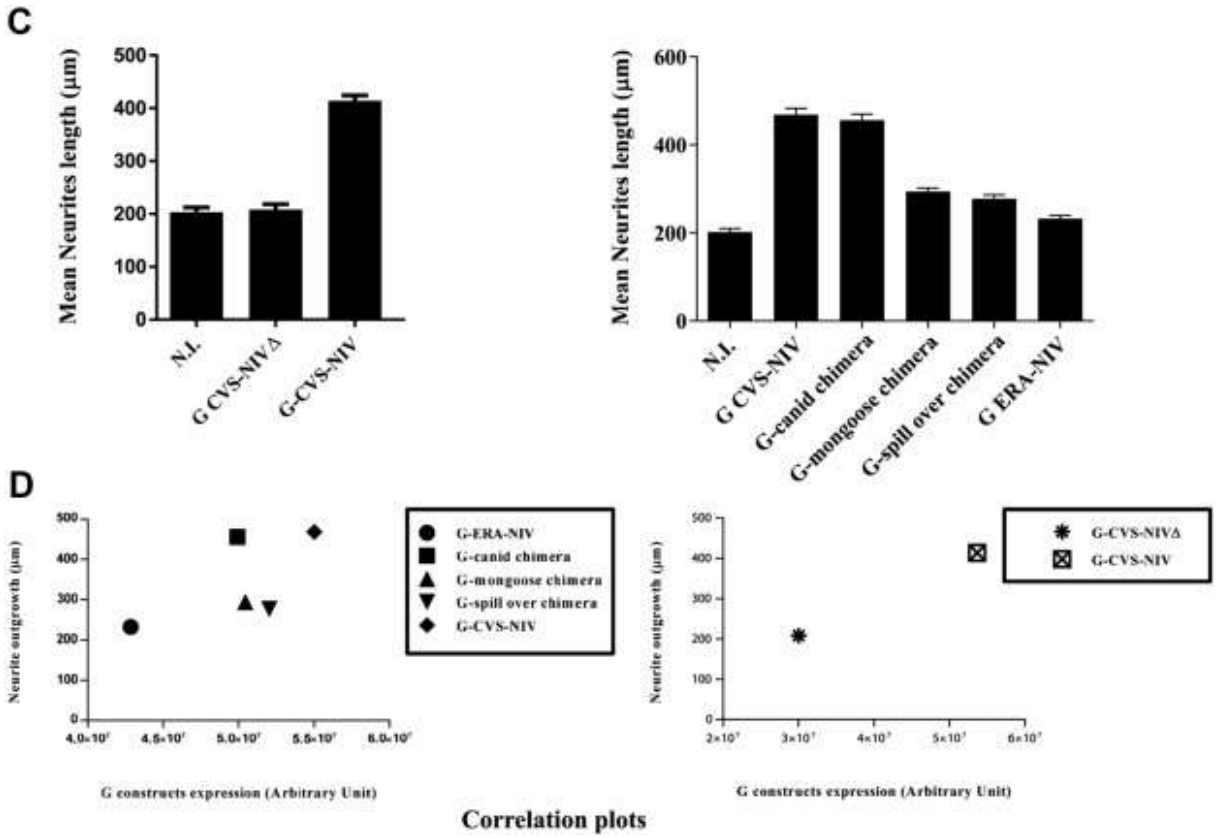


Fig. 2. Neurite outgrowth. Cultures of Neuroscreen cultivated for 72 h after lentivirus transduction. Cells were fixed and stained with crystal violet solution to visualize neurite processes. **A)** Representative fields of cultures. **B**) Neurite outgrowth drawing (ImageJ 1.44p, Neuron J plug-in) of representative N.I. (non-infected) or 72 h-lentivirus transduced Neuroscreen-1 cells. **C)** Comparison of mean neurite outgrowth (µm) triggered in N.I. or cells expressing either: G-CVS NIV and G-CVS NIV Δ (left panel) or N.I., G CVS-NIV, G-canid, G-mongoose, G-spill-over or G-ERA-NIV chimera (right panel). The data are representative of duplicate independent experiments. Neurites were counted in 55 fields for each condition (with at least one neurite per field). See Table 2 for Statistical significance. **D)** Absence of correlation between G constructs and neurite outgrowth experimental values. The level of expression of the G constructs and the mean neurite length obtained are not correlated each other, whatever the nature of the chimera construct (the left and right panels represent independent experiments).

Table 2 Statistical analysis of the neurite outgrowth. 'Statistical significance' (*P* value < 0.0001) is denoted as (yes) and 'not statistical significance' is shown as (no) (*P* value > or = 0.0001) by a Student's *t test*.

Pairwise comparison of constructs			*P* value	Statistical significance
G-ERA	**:**	**N.I**	**0.0057**	**no**
G-ERA	**:**	**G-canid**	**<0.0001**	**yes**
G-ERA	**:**	**G-mongoose**	**<0.0001**	**yes**
G-ERA	**:**	**G-spill over**	**0.0002**	**no**
G-ERA	**:**	**G-CVS-NIV**	**<0.0001**	**yes**
G-canid	**:**	**N.I**	**<0.0001**	**yes**
G-canid	**:**	**G-mongoose**	**<0.0001**	**yes**
G-canid	**:**	**G-spill over**	**<0.0001**	**yes**
G-canid	**:**	**G-CVS-NIV**	**0.5389**	**no**
G-mongoose	**:**	**N.I**	**<0.0001**	**yes**
G-mongoose	**:**	**G-spill over**	**0.1702**	**no**
G-mongoose	**:**	**G-CVS-NIV**	**<0.0001**	**yes**
G-spill over	**:**	**N.I**	**<0.0001**	**yes**
G-spill over	**:**	**G-CVS-NIV**	**<0.0001**	**yes**
G-CVS-NIV	**:**	**N.I**	**<0.0001**	**yes**
G-CVS-NIVΔ	**:**	**N.I**	**0.7169**	**no**

and table 2). This observation rules out the possibilities that the lentiviral vector has an effect on neurite outgrowth besides the specific contribution of the Cyto-G-PBM.

These data established that Cyto-G of CVS-NIV has an intrinsic property to trigger neurite outgrowth even in the absence of G ectodomain or any other viral components. In this test, the property of Cyto-G of ERA-NIV or of CVS-NIV-Δ (to not stimulate neurite outgrowth) is also maintained. Thus, the *in vitro* test of neurite outgrowth using lentivirus for the ERA or CVS Cyto-G delivery reproduce the *in vitro* neurosurvival and attenuation features triggered by the complete viruses.

3.3 Comparison of neurite outgrowth induced by the three South African chimeric constructs in a recombinant lentivirus system

Using these experimental conditions, the capacity of the three South African chimeric Cyto-Gs to trigger neurite outgrowth was compared to the positive (CVS-NIV) and negative (ERA-NIV) Cyto-G constructs. The G-canid chimeric construct showed the highest level of neurite outgrowth, in the same range as those triggered by the positive control CVS-NIV,

whereas G-spill over and G-mongoose Cyto-G constructs triggered relatively lower levels of neurite outgrowth, but significantly higher than those induced by chimeric G-ERA-NIV (Fig. 2 and Table 2 for statistical analysis). G-spill over and G-mongoose triggered similar levels of neurite outgrowth. The difference between the efficiency of G-spill over and G-mongoose constructs compared to G-canid construct was not due to a reduced G-protein expression because no significant differences in the level of expression of G constructs were observed after lentiviral vector delivery (Fig. 2D, left plot: 4.99, 5.04 and 5.20 x 10^7AU for Canid, Mongoose and Spill-over G constructs expression respectively). Moreover, as shown in Fig. 2D, when the level of expression of G constructs (G-CVS-NIV, G-CVS-NIVΔ, G-ERA-NIV and G-canid chimera, G-mongoose chimera, G-spill over-chimera) was plotted with the neurite outgrowth scores, it appeared there was no obvious correlation between the expression of G construct an neurite outgrowth values. For example (Fig. 2D, left panel), the neurite outgrowth score of the G-mongoose chimera construct was lower compared to those of G-canid chimera, despite a similar G expression, it is the same result for G-CVS-NIV (highest neurite score, lowest level of G expression) and G-CVS-NIVΔ (Lowest neurite score, highest level of G expression) as shown on Fig 2D, right panel. This confirm previous observations establishing that RABV virulence is not correlated to the G-protein expression level, but in fact to the genetic nature of the delivered G-protein [14, 22].

To conclude, in an attempt to predict relative virulence of the three South African RABVs, the in cellular assay of neurite outgrowth triggered by the C-terminus of the Cyto-Gs allowed us to propose that the G-canid construct exhibited virulent traits of CVS-NIV constructs, whereas both the spill over and the mongoose constructs showed less virulent traits. These *in vitro* findings correlate with the virulence of the three RABV strains as previously established in an experimental mouse model of rabies (Seo W et al, companion paper).

4. Discussion

In the vaccine field, there is an unmet need for a post licensing safety test capable to predict neurovirulence of live viral vaccine samples without the use of in vivo animal models. Safety tests for live vaccine (for example Yellow fever vaccines) require that vaccine samples are injected in monkeys whose brains are collected and checked for the absence of anatomo-pathology signs. The rationale of such a test is that neurotoxicity signs will reveal the presence of neurotoxic mutants which have appeared in the process of production and can cause vaccine-associated neuropathology. The growth of a pathogenic viral population within the nervous system may result into the acquisition and then fixation of few genomic mutations that confer neurovirulence.

Viruses are masters for manipulating the proliferation or death of the infected cells of a host, mainly by interfering with crucial endogenous interactions. RABV pathogenicity relies on its potential to keep the infected neurons alive, thereby allowing efficient viral transmission from one neuron to the next order neuron, from the site of infection up to the brain stem and finally to be secreted by the salivary glands [13]. It has been shown that virulent laboratory RABV strains such as CVS-NIV trigger survival of the infected neurons [3]. In contrast, the attenuated laboratory RABV strain, ERA-NIV, induces neuronal death. The neurosurvival phenotype is characterized by an increase of the neurite length of human neuroblastoma cells and by the activation of the neurosurvival Pi3k-Akt signaling pathway [14]. By using isogenic recombinant RABVs, we previously demonstrated these features are controlled by the cytoplasmic domain of the G protein, and in particular by the C-terminus PBM.

Here, we showed that the expression of the last 12 C-terminus of G-CVS-NIV in Neuroscreen-1 cells assumes the neurosurvival properties of CVS-NIV, whereas the expression of the last 12 COOH terminus of G-ERA-NIV does not have such an effect. This indicates that the neurosurvival properties of CVS-NIV and ERA-NIV could be described *in*

vitro without the need of manipulating RABV isolates, by simply studying the characteristics of the C-terminus of one of the RABV proteins.

Then we challenged the test by assaying the C-terminus properties of three South African RABV strains. We found that the C-terminus of the canid RABV isolate G-protein exhibited a survival phenotype (neurite outgrowth), whereas the C-terminus of mongoose or spill over G-protein did not. In this frame, it can be expected that canid strains are more virulent than spill over and mongoose strains. This forecast fits with the *in vivo* data since virulence of the RABV strains was in this order of magnitude: Canid and CVS virulence superior to those of mongoose and spill over RABV strains (Seo et al., companion paper). These results indicated that the virulence of these three wildlife RABV strains can be predicted by monitoring neurite outgrowth *in vitro* and again without the need of manipulating RABV isolates and infecting animals.

It was previously established by comparing in a recombinant RABV system the role of G-protein of two laboratory strains CVS-NIV and ERA-NIV that the control of neurosurvival or death relies on the nature of the PBM. In particular, a mutation resulting in a single amino acid change [glutamine (Q) to glutamic acid (E) at -3] in the PBM was sufficient to switch the fate of the infected cells and the neurite outgrowth suggesting that expression of $ETRL_{COOH}$ in this genetic context (Fig. 1A) was a marker of attenuation [14]. However, $ETRL_{COOH}$ alone might not be a signature of attenuation whatever the genetic context is. Indeed, the virulent canid strain stimulates neurite outgrowth while Cyto-G terminates by ETRL. It is likely that another mutation such as the replacement of the histidine (H) of CVS by Tyrosine (Y) at position -8 modifies the pattern. In that case, the combination of Y at -8 with $-ETRL_{COOH}$ should trigger a virulent property. This is strongly supported by the observation that this sequence combination (H at -8 and $ETRL_{COOH}$) is expressed by the G-protein of several other virulent strains including the CVS-AJ506997 (GenBank Acc. AJ506997.1). The possibility

that Y at -8 contributes to the interaction of Cyto-Gs with the PDZ of cellular partners is strongly supported by structural studies resolving the complex formed by the PBM of RABV Cyto-Gs and the PDZ of the cellular partner [16].

A PBM classically corresponds to a stretch of 4 amino acids, allowing the insertion of the peptide into the groove formed by two beta sheets of the PDZ. Nevertheless, in the case of RABV Cyto-G structural studies indicated that more than 4 amino acids of the C-terminus of Cyto-Gs contribute to the formation of the PBM-PDZ complex and that the surface of interaction recruited the 12 amino acids COOH end [16].

The reasons why the replacement of H by Y changes the phenotype driven by the PBM terminated by ETRL are still unclear. A change in the nature of the cellular partners is unlikely because the 12 COOH termini Cyto-Gs of the three South African strains have highly similar patterns of reactivity, when assayed in a PDZ array including 220 distinct human PDZs (Renaud Vincentelli, Nicolas Wolff and Gilles Travé, personal communication). It has been shown in other models, that phosphorylation of residues in the PBM can modulate PBM/PDZ interactions [23, 24]. Thus, we may hypothesize that the swap H/Y at -8 modifies the phosphorylation status of the Cyto-Gs. Such a hypothesis deserves complete studies determining at first whether Cyto-Gs terminated by ETRL $_{COOH}$ and expressing Y at -8 instead of H have distinct patterns of phosphorylation and second, whether phosphorylation status of the PBM modulates the pattern of interaction with cellular partners.

The main conclusion of this discrepancy is that it is not sufficient to look at the primary amino acid sequence of Cyto-Gs to forecast virulence of a RABV strain, emphasizing that other tests including *in vitro* tests are mandatory to answer this question.

In an attempt to replace animal use in post-licensing safety tests for live viral vaccines, this pilot study using RABV as a model, proposes that virulence of a RABV strain might be predicted by simply assaying *in vitro* the capacity of the COOH terminus of its G-protein to

trigger neurite outgrowth. We showed that it is feasible to predict virulence of a RABV strain by using appropriate *in vitro* tests. To note, none of the RABV vaccine for human belongs to the category of live viral vaccines, all of RABV vaccines for human use contain inactivated viral particles. Thus, the test we set up has no ambition to be substituted to safety tests currently in use to check for inactivation or potency of rabies vaccines. We hope such a study may pave the way to propose new tests replacing the injection of animals in the post-licensing safety tests currently requested for several live viral vaccines for human use.

Conflicts of interest

All authors confirm that there are no conflicts of interest.

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References

[1] Iabs. IABs scientific workshop on neurovirulence tests for live virus vaccines, January 31-February 1, 2005, Geneva. Biologicals : journal of the International Association of Biological Standardization 2006;34:233-6.

[2] Camelo S, Lafage M, Galelli A, Lafon M. Selective role for the p55 Kd TNF-alpha receptor in immune unresponsiveness induced by an acute viral encephalitis. Journal of neuroimmunology 2001;113:95-108.

[3] Prehaud C, Lay S, Dietzschold B, Lafon M. Glycoprotein of nonpathogenic rabies viruses is a key determinant of human cell apoptosis. Journal of virology 2003;77:10537-47.

[4] Thoulouze MI, Lafage M, Montano-Hirose JA, Lafon M. Rabies virus infects mouse and human lymphocytes and induces apoptosis. Journal of virology 1997;71:7372-80.

[5] Dietzschold B, Wunner WH, Wiktor TJ, Lopes AD, Lafon M, Smith CL, et al. Characterization of

an antigenic determinant of the glycoprotein that correlates with pathogenicity of rabies virus. Proceedings of the National Academy of Sciences of the United States of America 1983;80:70-4.

[6] Faber M, Faber ML, Papaneri A, Bette M, Weihe E, Dietzschold B, et al. A single amino acid change in rabies virus glycoprotein increases virus spread and enhances virus pathogenicity. Journal of virology 2005;79:14141-8.

[7] Faber M, Pulmanausahakul R, Nagao K, Prosniak M, Rice AB, Koprowski H, et al. Identification of viral genomic elements responsible for rabies virus neuroinvasiveness. Proceedings of the National Academy of Sciences of the United States of America 2004;101:16328-32.

[8] Ito N, Takayama M, Yamada K, Sugiyama M, Minamoto N. Rescue of rabies virus from cloned cDNA and identification of the pathogenicity-related gene: glycoprotein gene is associated with virulence for adult mice. Journal of virology 2001;75:9121-8.

[9] Ito Y, Ito N, Saito S, Masatani T, Nakagawa K, Atoji Y, et al. Amino acid substitutions at positions 242, 255 and 268 in rabies virus glycoprotein affect spread of viral infection. Microbiol Immunol 2010;54:89-97.

[10] Lafay F, Coulon P, Astic L, Saucier D, Riche D, Holley A, et al. Spread of the CVS strain of rabies virus and of the avirulent mutant AvO1 along the olfactory pathways of the mouse after intranasal inoculation. Virology 1991;183:320-30.

[11] Morimoto K, Hooper DC, Spitsin S, Koprowski H, Dietzschold B. Pathogenicity of different rabies virus variants inversely correlates with apoptosis and rabies virus glycoprotein expression in infected primary neuron cultures. Journal of virology 1999;73:510-8.

[12] Prehaud C, Coulon P, LaFay F, Thiers C, Flamand A. Antigenic site II of the rabies virus glycoprotein: structure and role in viral virulence. Journal of virology 1988;62:1-7.

[13] Lafon M. Evasive strategies in rabies virus infection. Advances in virus research 2011;79:33-53.

[14] Prehaud C, Wolff N, Terrien E, Lafage M, Megret F, Babault N, et al. Attenuation of rabies virulence: takeover by the cytoplasmic domain of its envelope protein. Science signaling 2010;3:ra5.

[15] Babault N, Cordier F, Lafage M, Cockburn J, Haouz A, Prehaud C, et al. Peptides targeting the PDZ domain of PTPN4 are efficient inducers of glioblastoma cell death. Structure 2011;19:1518-24.

[16] Terrien E, Chaffotte A, Lafage M, Khan Z, Prehaud C, Cordier F, et al. Interference with the

PTEN-MAST2 interaction by a viral protein leads to cellular relocalization of PTEN. Science signaling 2012;5:ra58.

[17] Terrien E, Simenel C, Prehaud C, Buc H, Delepierre M, Lafon M, et al. 1H, 13C and 15N resonance assignments of the PDZ of microtubule-associated serine/threonine kinase 205 (MAST205) in complex with the C-terminal motif from the rabies virus glycoprotein. Biomolecular NMR assignments 2009;3:45-8.

[18] Ivarsson Y. Plasticity of PDZ domains in ligand recognition and signaling. FEBS letters 2012;586:2638-47.

[19] Javier RT, Rice AP. Emerging Theme: Cellular PDZ Proteins as Common Targets of Pathogenic Viruses. Journal of virology 2011;85:11544-56.

[20] Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat Biotechnol 1997;15:871-5.

[21] Radio NM, Breier JM, Shafer TJ, Mundy WR. Assessment of chemical effects on neurite outgrowth in PC12 cells using high content screening. Toxicol Sci 2008;105:106-18.

[22] Wirblich C, Schnell MJ. Rabies virus (RV) glycoprotein expression levels are not critical for pathogenicity of RV. Journal of virology 2011;85:697-704.

[23] Delury CP, Marsh EK, James CD, Boon SS, Banks L, Knight GL, et al. The Role of Protein Kinase A Regulation of the E6 PDZ-Binding Domain during the Differentiation-Dependent Life Cycle of Human Papillomavirus Type 18. Journal of virology 2013;87:9463-72.

[24] Lee HJ, Zheng JJ. PDZ domains and their binding partners: structure, specificity, and modification. Cell Commun Signal 2010;8:8.