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

3

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25 **Abstract**

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

37

38

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

40 PDZ

41

42 **1. Introduction**

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

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

65 Amongst the five RABV proteins, the glycoprotein (G-protein), an integral trans-membrane
66 protein, has been identified as playing a critical role in the fate of viral properties [3, 5-12].

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

88 In this study we designed such an *in vitro* test, and challenged this test by assaying whether *in*
89 *vitro* virulence features of three representative South African RABV isolates (a canid, a
90 mongoose and a spill over corresponding to a dog infected by a mongoose RABV biotype) and
91 whose Cyto-Gs were distinct by a few mutations correlate with the virulence of the strains as

92 previously determined in an experimental mouse model of rabies (Seo, W et al. companion
 93 paper). Despite the limited number of strains used for the correlate, this pilot study gave
 94 promising results suggesting that virulence, at least in the case of RABV infection, might be
 95 forecast by performing *in vitro* tests.

96

97 **2. Material and Methods**

98 *2.1 Cells*

99 Human embryonic kidney cells HEK 293-T were grown at 37 °C with 5% CO₂ in Dulbecco's
 100 Modified Eagle Medium with Glutamax-1 supplemented with 10% fetal calf serum (FCS),
 101 penicillin (10,000 IU/ml) and streptomycin (10 mg/ml). Neuroscreen-1 (Cellomics), derived
 102 from rat pheochromocytoma were grown in RPMI 1640 supplemented with 10% horse serum,
 103 5% FCS, 200 mM glutamine, penicillin (10,000 IU/ml) and streptomycin (10 mg/ml).

104

105 *2.2 Construction and Recovery of Recombinant lentivirus*

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

Canid	Canid 1	5'TGGGAATCATAACAAGAGTGGGGGTGAGACCAGACTGTGAGGCCAAG3'
	Canid 2	3'ACCCTTAGTATGTTCTCACCCCCACTCTGGTCTGACACTCCGGTTC5'
Mongoose	Mong 1	5'TGGGAATCATAACAAGAATGGGGAGGAGACCAGAATGTGAGGCCAAG3'
	Mong 2	3'ACCCTTAGTATGTTCTTACCCCTCTCTGGTCTTACACTCCGGTTC5'
Spill over	Spill over 1	5'TGGGAATCATAACAAGAATGGGGGTGAGACCAGAATGTGAGGCCAAG3'
	Spill over 2	3'ACCCTTAGTATGTTCTTACCCCCACTCTGGTCTTACACTCCGGTTC5'
ERA	ERA 1	5'TGGGAATCACACAAGAGTGGGGGTGAGACCAGACTGTGAGGCCAAG3'

ERA 2 3'ACCCTTAGTGTGTTCTCACCCCCACTCTGGTCTGACACTCCGGTTC5'

111

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

126

127 *2.3 Western Blotting and Immunocytochemistry*

128 Lentivirus and non-infected cells were lysed with RIPA buffer (Sigma) supplemented with
129 anti-protease and anti-phosphatase cocktails (Roche Life Science) and stored at -20 °C. Cell
130 lysates were electrophoresed on a 4-20% gradient polyacrylamide gel (Pierce Biotechnology)
131 and blotted on PVDF membranes (Hybond-P, GE HealthCare). The membranes were incubated
132 with a customized polyclonal rabbit antibody directed against the Cyto-G (Proteogenix)
133 followed by an incubation with a secondary antibody coupled to horseradish peroxidase
134 (Jackson Immuno Research). Signals were revealed with SuperSignal[®] West Femto Substrate
135 (Pierce Biotechnology), acquired and analyzed with chemiluminescence imaging system

136 (G:Box, Syngene).

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

142

143 *2.4 Neurite Outgrowth Assay*

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

150

151 *2.5 Statistical Analysis*

152 Student's *t-test* ($P < 0.05$ was considered significant) was undertaken using GraphPad Prism
153 version 6.0 program.

154

155 **3. Results**

156 *3.1 Construction and expression of Cyto-Gs*

157 The commitment of RABV-infected neurons toward death (i.e. ERA-NIV) or survival (i.e.
158 CVS-NIV) is controlled by the Cyto-G and the PBM located at the COOH terminus of the Cyto-
159 G protein in particular [14]. We wanted here to assay whether Cyto-G expressed alone, in the
160 absence of any other RABV elements, retains the neurosurvival features. We chose to construct

161 a Cyto-G of the CVS-NIV (44 amino acids long) and 4 chimeric Cyto-Gs replacing the 12
162 original amino acid residues of CVS-NIV Cyto-G by the 12 amino acids of the COOH terminal
163 of ERA-NIV Cyto-G or those of the three South African RABV (Canid, Mongoose and Spill
164 over) (Fig. 1A). The reason to engraft 12 COOH amino acids and not the 4 COOH amino acids,
165 which is the most common length of a PBM, is motivated by the results of nuclear magnetic
166 resonance (NMR) and X-rays studies indicating that up to 12 amino acids of RABV Cyto-G
167 could be involved in the formation of the PBM/PDZ complexes [15, 16]. The Cyto-G constructs
168 included the transmembrane domain (TM) of the G-protein and G,K amino acids of ectodomain
169 proximal to the TM as well as the entire signal peptide 19 amino acid long (Fig. 1A) to allow
170 sorting and trafficking of the Cyto-Gs from the endoplasmic reticulum. A negative control,
171 consisting in the G-CVS-NIV deleted of the last 4 amino acids, G-CVS-NIV Δ , was also
172 constructed. Lentiviruses were chosen as a mean to express the Cyto-Gs in Neuroscreen-1 cells.
173 The mRNA transcription levels of lentivirus constructs were evaluated by relative RT-qPCR
174 (Fig. 1B) and ERA-NIV and Spill over constructs exhibited the lowest value, however, the
175 mRNA expression of CVS-NIV showed the highest value. Interestingly, the G-canid and G-
176 mongoose constructs displayed similar mRNA transcriptional levels. These data suggested that
177 point mutations in the nucleotide sequence may slightly modify the intrinsic stability of the
178 mRNA transcripts. Nevertheless, when the Cyto-G protein expression was assayed by
179 immunoblotting (Fig. 1C) and immunocytochemistry (Fig. 1D), all chimeric constructs
180 exhibited high levels of protein expression. To note, in contrast to CVS-NIV infected cells, in
181 which the full length G-protein is localized both in the cytoplasm and at the cytoplasmic
182 membrane (data not shown), or in cells infected by a lentiviral vector expressing the entire G-
183 protein only [16], the Cyto-Gs were mainly localized in the cytoplasm of the cells. This may
184 suggest that RABV G-protein trafficking was modified by the absence of the ectodomain (but
185 two amino acids) of the RABV G-protein.

186

187 *3.2 The Cyto-G of CVS-NIV expressed out of a RABV context is sufficient to trigger neurite*
188 *outgrowth.*

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

196 Neuroscreen-1 cells were either non-infected or infected with the recombinant lentiviruses G-
197 CVS-NIV, G-ERA-NIV, G-CVS-NIV- Δ and the mean neurite length was measured 72-hour-
198 post infection (Fig. 2 and table 2). The CVS-NIV construct triggered significantly higher neurite
199 outgrowth compared to non-infected group and to G-CVS-NIV- Δ or ERA-NIV constructs
200 (mean length of 420 μm for CVS-NIV versus 200 μm for the other conditions: non-infected or
201 G-CVS-NIV- Δ or ERA-NIV), indicating that the increased neurite outgrowth triggered by
202 CVS-NIV Cyto-G could also be observed after Cyto-Gs has been delivered in rat Neuroscreen-
203 1 cells by a lentiviral vector. The lentiviral vector Cyto-G CVS-NIV- Δ triggered a mean neurite
204 outgrowth similar to those observed in non-infected cells (left panel in Fig. 2C and table 2).
205 This observation rules out the possibilities that the lentiviral vector has an effect on neurite
206 outgrowth besides the specific contribution of the Cyto-G-PBM.

207 These data established that Cyto-G of CVS-NIV has an intrinsic property to trigger neurite
208 outgrowth even in the absence of G ectodomain or any other viral components. In this test, the
209 property of Cyto-G of ERA-NIV or of CVS-NIV- Δ (to not stimulate neurite outgrowth) is also
210 maintained. Thus, the *in vitro* test of neurite outgrowth using lentivirus for the ERA or CVS

211 Cyto-G delivery reproduce the *in vitro* neurosurvival and attenuation features triggered by the
212 complete viruses.

213

214 *3.3 Comparison of neurite outgrowth induced by the three South African chimeric constructs in* 215 *a recombinant lentivirus system*

216 Using these experimental conditions, the capacity of the three South African chimeric Cyto-
217 Gs to trigger neurite outgrowth was compared to the positive (CVS-NIV) and negative (ERA-
218 NIV) Cyto-G constructs. The G-canid chimeric construct showed the highest level of neurite
219 outgrowth, in the same range as those triggered by the positive control CVS-NIV, whereas G-
220 spill over and G-mongoose Cyto-G constructs triggered relatively lower levels of neurite
221 outgrowth, but significantly higher than those induced by chimeric G-ERA-NIV (Fig. 2 and
222 Table 2 for statistical analysis). G-spill over and G-mongoose triggered similar levels of neurite
223 outgrowth. The difference between the efficiency of G-spill over and G-mongoose constructs
224 compared to G-canid construct was not due to a reduced G-protein expression because no
225 significant differences in the level of expression of G constructs were observed after lentiviral
226 vector delivery (Fig. 2D, left plot: 4.99, 5.04 and 5.20 x 10⁷AU for Canid, Mongoose and Spill-
227 over G constructs expression respectively). Moreover, as shown in Fig. 2D, when the level of
228 expression of G constructs (G-CVS-NIV, G-CVS-NIV Δ , G-ERA-NIV and G-canid chimera, G-
229 mongoose chimera, G-spill over-chimera) was plotted with the neurite outgrowth scores, it
230 appeared there was no obvious correlation between the expression of G construct and neurite
231 outgrowth values. For example (Fig. 2D, left panel), the neurite outgrowth score of the G-
232 mongoose chimera construct was lower compared to those of G-canid chimera, despite a similar
233 G expression, it is the same result for G-CVS-NIV (highest neurite score, lowest level of G
234 expression) and G-CVS-NIV Δ (Lowest neurite score, highest level of G expression) as shown
235 on Fig 2D, right panel. This confirm previous observations establishing that RABV virulence

236 is not correlated to the G-protein expression level, but in fact to the genetic nature of the
237 delivered G-protein [14, 22].

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

244

245 **4. Discussion**

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

255 Viruses are masters for manipulating the proliferation or death of the infected cells of a host,
256 mainly by interfering with crucial endogenous interactions. RABV pathogenicity relies on its
257 potential to keep the infected neurons alive, thereby allowing efficient viral transmission from
258 one neuron to the next order neuron, from the site of infection up to the brain stem and finally
259 to be secreted by the salivary glands [13]. It has been shown that virulent laboratory RABV
260 strains such as CVS-NIV trigger survival of the infected neurons [3]. In contrast, the attenuated

261 laboratory RABV strain, ERA-NIV, induces neuronal death. The neurosurvival phenotype is
262 characterized by an increase of the neurite length of human neuroblastoma cells and by the
263 activation of the neurosurvival Pi3k-Akt signaling pathway [14]. By using isogenic
264 recombinant RABVs, we previously demonstrated these features are controlled by the
265 cytoplasmic domain of the G protein, and in particular by the C-terminus PBM.

266 Here, we showed that the expression of the last 12 C-terminus of G-CVS-NIV in
267 Neuroscreen-1 cells assumes the neurosurvival properties of CVS-NIV, whereas the expression
268 of the last 12 COOH terminus of G-ERA-NIV does not have such an effect. This indicates that
269 the neurosurvival properties of CVS-NIV and ERA-NIV could be described *in vitro* without the
270 need of manipulating RABV isolates, by simply studying the characteristics of the C-terminus
271 of one of the RABV proteins.

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

282 It was previously established by comparing in a recombinant RABV system the role of G-
283 protein of two laboratory strains CVS-NIV and ERA-NIV that the control of neurosurvival or
284 death relies on the nature of the PBM. In particular, a mutation resulting in a single amino acid
285 change [glutamine (Q) to glutamic acid (E) at -3] in the PBM was sufficient to switch the fate

286 of the infected cells and the neurite outgrowth suggesting that expression of ETRL_{COOH} in this
287 genetic context (Fig. 1A) was a marker of attenuation [14]. However, ETRL_{COOH} alone might
288 not be a signature of attenuation whatever the genetic context is. Indeed, the virulent canid
289 strain stimulates neurite outgrowth while Cyto-G terminates by ETRL. It is likely that another
290 mutation such as the replacement of the histidine (H) of CVS by Tyrosine (Y) at position -8
291 modifies the pattern. In that case, the combination of Y at -8 with -ETRL_{COOH} should trigger a
292 virulent property. This is strongly supported by the observation that this sequence combination
293 (H at -8 and ETRL_{COOH}) is expressed by the G-protein of several other virulent strains including
294 the CVS-AJ506997 (GenBank Acc. AJ506997.1). The possibility that Y at -8 contributes to the
295 interaction of Cyto-Gs with the PDZ of cellular partners is strongly supported by structural
296 studies resolving the complex formed by the PBM of RABV Cyto-Gs and the PDZ of the
297 cellular partner [16].

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

303 The reasons why the replacement of H by Y changes the phenotype driven by the PBM
304 terminated by ETRL are still unclear. A change in the nature of the cellular partners is unlikely
305 because the 12 COOH termini Cyto-Gs of the three South African strains have highly similar
306 patterns of reactivity, when assayed in a PDZ array including 220 distinct human PDZs (Renaud
307 Vincentelli, Nicolas Wolff and Gilles Travé, personal communication). It has been shown in
308 other models, that phosphorylation of residues in the PBM can modulate PBM/PDZ interactions
309 [23, 24]. Thus, we may hypothesize that the swap H/Y at -8 modifies the phosphorylation status
310 of the Cyto-Gs. Such a hypothesis deserves complete studies determining at first whether Cyto-

311 Gs terminated by ETRL_{COOH} and expressing Y at -8 instead of H have distinct patterns of
312 phosphorylation and second, whether phosphorylation status of the PBM modulates the pattern
313 of interaction with cellular partners.

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

317 In an attempt to replace animal use in post-licensing safety tests for live viral vaccines, this
318 pilot study using RABV as a model, proposes that virulence of a RABV strain might be
319 predicted by simply assaying *in vitro* the capacity of the COOH terminus of its G-protein to
320 trigger neurite outgrowth. We showed that it is feasible to predict virulence of a RABV strain
321 by using appropriate *in vitro* tests. To note, none of the RABV vaccine for human belongs to
322 the category of live viral vaccines, all of RABV vaccines for human use contain inactivated
323 viral particles. Thus, the test we set up has no ambition to be substituted to safety tests currently
324 in use to check for inactivation or potency of rabies vaccines. We hope such a study may pave
325 the way to propose new tests replacing the injection of animals in the post-licensing safety tests
326 currently requested for several live viral vaccines for human use.

327

328 **Conflicts of interest**

329 All authors confirm that there are no conflicts of interest.

330

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333

334

335

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- 404

405 *Legends*406 **Fig. 1. Construction and characterization of lentivectors expressing RABV Cyto-G**

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

423

424 **Fig. 2 Neurite outgrowth.** Cultures of Neuroscreen cultivated for 72 hours after lentivirus

425 transduction. Cells were fixed and stained with crystal violet solution to visualize neurite
 426 processes. **A)** Representative fields of cultures. **B)** Neurite outgrowth drawing (ImageJ 1.44p,
 427 Neuron J plug-in) of representative N.I (non-infected) or 72h-lentivirus transduced
 428 Neuroscreen-1 cells. **C)** Comparison of mean neurite outgrowth (μ m) triggered in N.I or cells
 429 expressing either: G-CVS NIV and G-CVS NIV Δ (left panel) or N.I., G CVS-NIV, G-canid,

430 G-mongoose, G-spill-over or G-ERA-NIV chimera (right panel). The data are representative
431 of duplicate independent experiments. Neurites were counted in 55 fields for each condition
432 (with at least one neurite per field). See table 2 for Statistical significance. **D)** Absence of
433 correlation between G constructs and neurite outgrowth experimental values. The level of
434 expression of the G constructs and the mean neurite length obtained are not correlated each
435 other, whatever the nature of the chimera construct (the left and right panels represent
436 independent experiments).