

1 **Functional investigation of conserved glutamate receptor subunits reveals a**  
2 **new mode of action of macrocyclic lactones in nematodes**

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8

9 **Abstract**

10 Glutamate-gated chloride channels receptors (GluCl) are involved in the inhibition of  
11 neurotransmission in invertebrates and represent major molecular targets for therapeutic drugs.  
12 Among these drugs, macrocyclic lactones (MLs) are widely used as anthelmintic to treat  
13 parasitic nematodes impacting both human and animal health. Despite massive use of MLs  
14 since the 80's, the exact molecular targets of these drugs are still unknown in many important  
15 parasite species. Among the GluCl subunit encoding genes, *avr-14*, *glc-2*, *glc-3* and *glc-4* are  
16 highly conserved throughout the nematode phylum. Using the *Xenopus* oocyte as an expression  
17 system, we pharmacologically characterized these GluCl subunits from the model nematode  
18 *Caenorhabditis elegans*, the human filarial nematode *Brugia malayi* and the horse parasitic  
19 nematode *Parascaris univalens*. In contrast with *C. elegans*, expression of parasitic nematode  
20 subunits as homomeric receptors was not reliable and needed glutamate application at the mM  
21 range to induce low currents at the nA range. However, the co-expression of GLC-2 and AVR-  
22 14B lead to the robust expression of ML-sensitive receptors for the three nematode species. In  
23 addition, we demonstrated that for *C. elegans* and *P. univalens*, GLC-2 co-assembled with  
24 GLC-3 to form a new GluCl subtype with distinct pharmacological properties. Whereas 1µM  
25 ivermectin, moxidectin and eprinomectin acted as agonist of the GLC-2/GLC-3 receptor from

26 *C. elegans*, they did not directly activate GLC-2/GLC-3 of *P. univalens*. In contrast, these MLs  
27 potentialized glutamate elicited currents thus representing a unique pharmacological property.  
28 Our results highlight the importance of GLC-2 as a key subunit in the composition of  
29 heteromeric channels in nematodes and demonstrate that MLs act on novel GluCl subtypes that  
30 show unusual pharmacological properties, providing new insights about MLs mode of action.

## 31 **Author summary**

32 The filarial and ascarid parasitic nematodes include some of the most pathogenic or  
33 invalidating species in humans, livestock and companion animals. Whereas the control of these  
34 worms is critically dependent on macrocyclic lactones (MLs) such as ivermectin, the mode of  
35 action of this anthelmintic class remains largely unknown in these parasites. In the model  
36 nematode *Caenorhabditis elegans*, MLs target GluCl pentameric glutamate-sensitive chloride  
37 channels (GluCl). Because MLs are potent anthelmintics on *C. elegans*, ascarid and filarial  
38 nematodes, in the present study we investigated GluCl subunits highly conserved between these  
39 distantly related worms. Using the *Xenopus* oocyte as a heterologous expression system, we  
40 identified and performed the pharmacological characterization of novel GluCl receptors  
41 from *C. elegans*, the human filarial parasite *Brugia malayi* and the horse parasite *Parascaris*  
42 *univalens*. Our results highlight heteromeric GluCls from parasites as molecular targets for a  
43 wide range of MLs. We report an original mode of action of MLs on a new GluCl subtype made  
44 of the GLC-2/GLC-3 subunit combination. This study brings new insights about the diversity  
45 of GluCl subtypes in nematodes and opens the way for rational drug screening for the  
46 identification of next generation anthelmintic compounds.

## 47 **Introduction**

48 The phylum *Nematoda* is divided into five major clades (I to V) that include free living  
49 and parasitic species impacting both human and animal health (1,2). Among these parasitic

50 nematodes, *Filarioidea* and *Ascaridoidea* belonging to clade III are considered as the most  
51 impacting on both human and animal health (3). In this study, we focus our research on two  
52 parasitic nematode species representative of human filarids and animal ascarids : *Brugia*  
53 *malayi*, a human lymphatic filarid which is the causative agent of chronic elephantiasis in the  
54 south and south-east of Asia (4) and *Parascaris spp.* which are responsible for equine  
55 ascaridiosis (5,6).

56 Without effective vaccine or alternative strategies (7), the use of anthelmintic treatments  
57 remains the standard control strategy for parasitic nematodes. Among the available anthelmintic  
58 drugs, the broad-spectrum macrocyclic lactones (MLs) are highly effective and are massively  
59 used in human and veterinary medicine (8). The MLs include avermectins (ivermectin,  
60 doramectin, eprinomectin, abamectin, selamectin, emamectin) and milbemycins (moxidectin,  
61 milbemycin-oxime, nemadectin) that are potent against both endo- and ectoparasites (9). The  
62 morbidity and socio-economic impact of human lymphatic filariasis motivated control  
63 programs led by the World Health Organization (WHO) with ivermectin (IVM) as a spearhead  
64 of eradication operations (10). For the control of *Parascaris spp.* infestations, three drug classes  
65 currently have marketing approval including benzimidazoles, pyrantel and MLs (ivermectin  
66 and moxidectin), corresponding to the most widely used family. Unfortunately, because of the  
67 intensive use of MLs, treatment failures and resistant parasites have been reported worldwide  
68 (11). Resistance to MLs is spreading fast and has currently been reported in a wide range of  
69 parasitic nematode species such as *Onchocerca volvulus* (12), *Cooperia oncophora* (13,14),  
70 *Dirofilaria immitis* (15,16), *Haemonchus contortus* (17) and *Parascaris spp.* (18–25).  
71 Resistance is considered as multifactorial as it can raise from different molecular events such  
72 as: receptor subunit mutations (26–31), decrease of the target expression level (17,32) and the  
73 efflux mechanisms involving cell membrane transporter such as P-glycoprotein (33,34).

74           The molecular targets of the MLs as well as the mechanisms involved in resistance  
75 remains unclear for most of the parasitic nematodes. In this context, a better understanding of  
76 MLs mode of action is essential for the control of resistance and the development of novel  
77 therapeutical strategies (35).

78           In the free-living model nematode *Caenorhabditis elegans*, MLs act as allosteric  
79 modulators of glutamate-gated chloride channels (GluCl<sub>s</sub>) (36). Exposure to MLs  
80 hyperpolarizes the membrane and inhibits the neurotransmission (37–39) leading to flaccid  
81 paralysis of the worms (26). GluCl<sub>s</sub> are made of five subunits combining together to form either  
82 homo- or heteromeric receptors (40,41). In order to investigate the subunit composition and the  
83 pharmacological properties of recombinant nematode GluCl<sub>s</sub>, the *Xenopus laevis* oocyte has  
84 proven to be an efficient heterologous expression system (42).

85           In *C. elegans*, six GluCl genes were identified and named *avr-14* (26,28), *avr-15*  
86 (38,43), *glc-1* (36,44), *glc-2* (36,43), *glc-3* (45) and *glc-4* (46). With the exception of GLC-4,  
87 all the subunits are able to form functional homomeric receptors when expressed in *Xenopus*  
88 *laevis* oocytes. All functional homomeric receptors were ivermectin-sensitive with the  
89 exception of Cel-GLC-2 which is not (36). However, it has been reported that the *C. elegans*  
90 GLC-2 subunit co-assemble with Cel-GLC-1 (36) or Cel-AVR-15 (43) to form two ivermectin-  
91 sensitive heteromeric GluCl<sub>s</sub> subtypes with distinct pharmacological properties.

92           In contrast with *C. elegans*, only few functional GluCl<sub>s</sub> have been characterized so far  
93 in parasitic nematodes. The AVR-14B subunit was reported to form a functional homomeric  
94 GluCl<sub>s</sub> in *H. contortus* (28,47,48), *Cooperia oncophora* (27) and *Dirofilaria immitis* (49).  
95 Interestingly, in *Cooperia oncophora* (27) and *Haemonchus contortus* (50), GLC-2 also  
96 combined with AVR-14B to form a heteromeric GluCl subtype sensitive to ivermectin.  
97 Whereas GluCl<sub>s</sub> investigations were mainly focused on clade V nematodes, the GluCl diversity

98 and the mode of action of MLs remains poorly understood in human and animal parasitic  
99 nematodes from the clade III.

100 In the present study, we describe new GluCl subtypes made of highly conserved subunits  
101 from *C. elegans*, *B. malayi* and *P. univalens* providing new insights about the pharmacology of  
102 nematode GluCl subtypes as well as the mode of action of MLs.

103

## 104 **Results**

### 105 **Four distinct GluCl subunits are conserved between *C. elegans*, *B. malayi* and *P.*** 106 ***univalens***

107 Searches for homologs of GluCl subunits encoding genes from *C. elegans* (i.e. *glc-1*,  
108 *glc-2*, *glc-3*, *glc-4*, *avr-14* and *avr-15*) (26,36,38,43,45,46) in *B. malayi* and *P. univalens*  
109 genomic/transcriptomic databases, allowed the identification of 4 independent sequences  
110 corresponding to putative homologs of *avr-14*, *glc-2*, *glc-3* and *glc-4* from both species. In  
111 contrast, no homolog could be found in *B. malayi* and *P. univalens* for *glc-1* nor *avr-15*. The  
112 corresponding full-length cDNA of *avr-14*, *glc-2*, *glc-3* and *glc-4* from the three nematode  
113 species were cloned into a transcription vector for subsequent functional analysis. All GluCls  
114 identified including AVR-14B, GLC-2, GLC-3 and GLC-4 from *C. elegans*, *B. malayi* and *P.*  
115 *univalens* present the typical characteristics of a cys-loop receptor subunit. This includes, a  
116 predicted signal peptide in the extracellular N-terminal part (with the exception of Bma-GLC-  
117 2), the first cys-loop domains specific of ligand-gated ion channels (LGIC) which is composed  
118 of two cysteines that are 13 amino acid residues apart, the second cys-loop domain found in  
119 GluCls and four transmembrane domains (TM1-4) (**Fig. S2 and Fig. S3**).

120 In comparison with *C. elegans*, the GluCl deduced amino-acid sequences of *B. malayi*  
121 and *P. univalens* respectively show an identity of 78.4% and 80.3% for AVR-14B, 64.1% and

122 67.2% for GLC-2, 66.5% and 67.8% for GLC-3 and 64.8% and 66.6% for GLC-4 (**S1 Table**).  
123 A phylogenetic analysis including GluCl sequences from *C. elegans* (Cel), *B. malayi* (Bma), *H.*  
124 *contortus* (Hco) and *P. univalens* (Pun) confirmed the orthologous relationship of the parasitic  
125 subunit sequences with their respective counterparts in *C. elegans* (**Fig. 1**). The identified  
126 sequences were then named according to the nomenclature proposed by Beech *et al* (51) and  
127 submitted to genbank under the accession numbers provided in the Material and Methods  
128 section.

129 **In contrast with *C. elegans*, GluCl subunits from *B. malayi* and *P. univalens* do not form**  
130 **robustly expressed homomeric receptors**

131 In order to test the ability of *C. elegans*, *B. malayi* and *P. univalens* GluCl subunits to form  
132 functional homomeric receptors, their respective cRNA (*avr-14b*, *glc-2*, *glc-3* and *glc-4*) were  
133 injected singly in the *Xenopus laevis* oocyte. Three to five days post injection, currents elicited  
134 by 1 mM glutamate were recorded using the two-electrode voltage-clamp technic (**Fig. 2A**).

135 Here, the *C. elegans* AVR-14B, GLC-2 and GLC-3 GluCl subunits were used as positive  
136 controls as their ability to form functional glutamate-sensitive homomeric channels has been  
137 previously reported (26,28,36,45). In our hands, expression of Cel-AVR-14B or Cel-GLC-2  
138 alone formed functional homomeric receptors with robust glutamate-elicited currents in the  $\mu\text{A}$   
139 range:  $2.1 \pm 0.4 \mu\text{A}$  (n = 16) and  $9.1 \pm 0.5 \mu\text{A}$  (n = 32) for Cel-AVR-14B and Cel-GLC-2  
140 respectively (**Fig. 2A**). Whereas Cel-GLC-3 was also able to form functional receptors, the  
141 glutamate application (1 mM) induced significantly smaller peak currents ( $285 \pm 54 \text{ nA}$ , n =  
142 50) in comparison with homomeric channels composed of Cel-AVR14B (p < 0.001) or Cel-  
143 GLC-2 (p < 0.001) (**Fig. 2A**). These results are in agreement with previous studies (26,36,45).  
144 Noteworthy, no glutamate-induced current was observed on oocytes injected with Cel-GLC-4  
145 cRNA (n = 18).

146 Unlike *C. elegans*, where three out of four GluCl subunits formed functional homomeric  
147 receptors, for *B. malayi*, only the expression of AVR-14B subunit in oocytes allowed the  
148 reliable recording of currents ( $413 \pm 132$  nA,  $n = 23$ , **Fig. 2A**), significantly smaller than those  
149 obtained with Cel-AVR-14B ( $p < 0.001$ ). Similarly, for *P. univalens*, the expression of AVR-  
150 14B subunit also allowed the formation of a homomeric receptor, with current amplitudes  
151 similar to the Bma-AVR-14B receptor ( $200 \pm 80$  nA,  $n = 9$ ;  $p = 1$ , **Fig. 2A**). Furthermore, on  
152 oocytes expressing Pun-GLC-3, 1 mM glutamate application also resulted in low currents with  
153 mean amplitudes ( $77 \pm 14$  nA,  $n = 11$ ) significantly smaller than for Cel-GLC-3 receptors ( $p <$   
154  $0.05$ , **Fig. 2A**). Finally, in our hands, neither the expression of Bma-GLC-2 ( $n = 11$ ), Pun-GLC-  
155 2 ( $n = 11$ ), Bma-GLC-3 ( $n = 14$ ), Bma-GLC-4 ( $n = 13$ ) nor Pun-GLC-4 ( $n = 12$ ) resulted in the  
156 expression of functional receptors.

157 Taken together, these results suggested that in contrast with *C. elegans*, parasitic nematode  
158 GluCl subunits are not able to form robustly expressed homomeric channels.

### 159 **GLC-2 has a critical role in heteromeric GluCl composition**

160 Because previous studies highlighted the involvement of GLC-2 in heteromeric GluCls  
161 (27,36,43,50), oocytes expressing a combination of GLC-2 with either AVR-14B, GLC-3 or  
162 GLC-4 were challenged with 1 mM glutamate (**Fig. 2B**).

163 Firstly, we investigated the /AVR-14B/GLC-2 combination for the three different  
164 species. For *C. elegans*, the co-expression of both subunit cRNAs led to the robust expression  
165 of receptors with a mean current amplitude of  $4.8 \pm 0.4$   $\mu$ A ( $n = 21$ ), significantly higher than  
166 Cel-AVR-14B alone ( $p < 0.05$ ) but smaller than Cel-GLC-2 alone ( $p < 0.05$ ). For *B. malayi*,  
167 the combination of AVR-14B and GLC-2 led to the robust expression of receptors sensitive to  
168 1 mM glutamate (**Fig. 2B**) with a current peak of  $5.6 \pm 0.5$   $\mu$ A ( $n = 31$ ), corresponding to current  
169 peaks 13-fold higher than for Bma-AVR-14B alone ( $p < 0.001$ ). Similarly, for *P. univalens*, the



170 combination of AVR-14B and GLC-2 led to the robust expression of functional receptors with  
171 1 mM glutamate-elicited currents of  $2.3 \pm 0.3 \mu\text{A}$  ( $n = 17$ , **Fig. 2B**), 11-fold higher than for  
172 Pun-AVR-14B alone ( $p < 0.001$ ).

173 These first results suggested that AVR-14B/GLC-2 were able to form heteromeric  
174 receptors potentially distinguishable from homomeric receptors.

175 Secondly, we tested the ability of the GLC-2 subunit to assemble with GLC-3.  
176 Strikingly, for *C. elegans*, we recorded strong currents elicited by 1 mM glutamate ( $3.7 \pm 0.4$   
177  $\mu\text{A}$ ,  $n = 59$ ) significantly different from homomeric receptors made of GLC-2 ( $p < 0.001$ ) or  
178 GLC-3 ( $p < 0.001$ ), thus suggesting that GLC-2/GLC-3 were able to form heteromeric  
179 receptors. Similarly, for *P. univalens*, combination of GLC-2/GLC-3 led to the robust  
180 expression of glutamate sensitive receptors with a current amplitude of  $5.3 \pm 0.6 \mu\text{A}$  ( $n = 45$ ),  
181 significantly higher than the homomeric receptor formed by Pun-GLC-3 ( $p < 0.001$ , **Fig. 2B**).  
182 Noteworthy, Pun-GLC-2/GLC-3 receptor responses were similar to those of Cel-GLC-2/GLC-  
183 3 ( $p > 0.2$ ). In contrast, for *B. malayi*, the GLC-2/GLC-3 combination failed to give rise to a  
184 functional receptor ( $n = 8$ ).

185 Thirdly, we tested the combination of GLC-2 with GLC-4 for all species. Here, only very  
186 low currents ( $490 \pm 106 \text{ nA}$ ,  $n = 14$ ) were recorded from the Cel-GLC-2/GLC-4 combination  
187 (lower than for Cel-GLC-2 alone,  $p < 0.001$ ) (**Fig. 2B**). Note that such a reduction of glutamate  
188 sensitivity has been previously reported, when GLC-2 was co-expressed with GLC-1 (36). In  
189 contrast with *C. elegans*, none of the Bma-GLC-2/GLC-4 and Pun-GLC-2/GLC-4 combination  
190 gave rise to glutamate-responsive receptor ( $n = 8$  and  $n = 11$  respectively).

191 Finally, we tested the combination of GLC-3 with GLC-4 subunit for all species. No  
192 currents were recorded with 1 mM glutamate application on oocytes injected with Bma-GLC-  
193 3/GLC-4 ( $n = 13$ ), though nA currents were recorded for Cel-GLC-3/GLC-4 and for Pun-GLC-



194 3/GLC-4. However, these currents were not different in comparison with homomeric GLC-3  
195 channels ( $p = 1$  and  $p = 0.12$  for *C. elegans* and *P. univalens* respectively (**Fig. 2B**).

196 Taken together, these results demonstrate that GLC-2 from the three different species plays  
197 a pivotal role in the formation of glutamate-sensitive heteromeric receptor including AVR-14B  
198 or GLC-3.

199 **AVR-14B/GLC-2 from *C. elegans*, *B. malayi* and *P. univalens* form GluCl subtypes**  
200 **responsive to a wide range of macrocyclic lactones**

201 As a first step, in order to distinguish the putative Cel-AVR-14B/GLC-2 heteromeric  
202 receptor from the homomeric Cel-AVR-14B and Cel-GLC-2, we challenged oocytes  
203 expressing both subunits singly or in combination and established their respective glutamate  
204 concentration-response curve. Representative currents induced by glutamate on Cel-AVR-  
205 14B/GLC-2 are shown in **Fig. 3A**. Glutamate  $EC_{50}$  values of  $112.3 \pm 13 \mu\text{M}$  ( $n = 5$ ),  $214.6 \pm$   
206  $13.1 \mu\text{M}$  ( $n = 5$ ) and  $20.5 \pm 1.3$  ( $n = 8$ ) were determined for the Cel-AVR-14B, Cel-GLC-2 and  
207 Cel-AVR-14B/GLC-2 receptor respectively (**Fig. 3B**). Cel-AVR-14B/GLC-2 channel showed  
208 a higher sensitivity for glutamate in comparison with the homomeric receptors formed by AVR-  
209 14B ( $p < 0.001$ ) or GLC-2 ( $p < 0.001$ ), with 5- and 10-fold lower  $EC_{50}$ , respectively. These  
210 results confirmed that Cel-AVR-14B/GLC-2 corresponds to a heteromeric GluCl receptor.

211 For *B. malayi*, for which GLC-2 alone did not form a functional homomeric receptor,  
212 we determined the glutamate  $EC_{50}$  values for Bma-AVR-14B and Bma-AVR-14B/GLC-2  
213 respectively. For the homomeric AVR-14B receptor glutamate  $EC_{50}$  was  $758.9 \pm 82 \mu\text{M}$  ( $n =$   
214  $5$ ), whereas for Bma-AVR-14B/GLC-2 glutamate  $EC_{50}$  was  $32.5 \pm 5.1 \mu\text{M}$  ( $n = 7$ ), (**Fig. 2**;  
215 **Fig. 3C**). Such difference of glutamate sensitivity between these two receptors provides strong  
216 evidence that AVR-14B and GLC-2 subunits can associate to form a heteromeric GluCl  
217 subtype, distinct from the homomeric receptor made of AVR-14B ( $p < 0.001$ ; **Fig. 3D**).

218 Strikingly, for the homomeric Pun-AVR-14B receptor, application of high  
219 concentrations of glutamate only induced small currents. Indeed, even with 100 mM glutamate  
220 applications ( $n = 7$ ), the currents never reached a plateau value, suggesting that the receptor was  
221 not saturable. In sharp contrast, the co-injection of AVR-14B and GLC-2 led to the robust  
222 expression of a functional receptor sensitive to a micromolar range of glutamate with an  $EC_{50}$   
223 value of  $13.6 \pm 1.6 \mu\text{M}$  ( $n = 5$ , **Fig. 3F**), thus confirming that Pun-GLC-2/AVR-14B form a  
224 heteromeric receptor (**Fig. 3E**).

225 Subsequently, in order to get new insight about the respective pharmacological  
226 properties of the heteromeric AVR-14B/GLC-2 GluCl subtypes from *C. elegans*, *B. malayi* and  
227 *P. univalens*, their sensitivity to a wide range of MLs available in the market has been  
228 investigated (i.e. abamectin, doramectin, emamectin, eprinomectin, ivermectin, moxidectin,  
229 and selamectin). Representative currents of the two most potent MLs on the AVR-14B/GLC-2  
230 receptor from each species are presented in **Fig. 4A**, **Fig. 4C** and **Fig. 4E**. All the MLs tested  
231 acted as potent agonist on these receptors, inducing their permanent activation. Receptor  
232 activation by MLs was not reversible and slower in comparison with glutamate. Interestingly,  
233 whereas emamectin was found to be among the most potent ML on Cel-AVR-14B/GLC-2 and  
234 Bma-AVR-14B/GLC-2 (with  $1 \mu\text{M}$  emamectin induced-current corresponding to  $78 \pm 7 \%$  ( $n$   
235  $= 7$ ) and  $81 \pm 6 \%$  ( $n = 7$ ) of the maximum current amplitude induced by glutamate,  
236 respectively), for Pun-AVR-14B/GLC-2 no significative difference of sensitivity was observed  
237 between the different MLs (**Fig. 4B**, **Fig. 4D** and **Fig. 4F**).

238

239 **GLC-2/GLC-3 from *C. elegans* and *P. univalens* forms a new functional GluCl subtype**  
240 **with original pharmacological properties**

241 As described for Cel-AVR-14B/GLC-2, in order to distinguish the putative Cel-GLC-  
242 2/GLC-3 heteromeric receptor from the homomeric Cel-GLC-2 and Cel-GLC-3 receptors, our  
243 objective was to determine and compare their respective glutamate EC<sub>50</sub> values (**Fig. 5A**). For  
244 Cel-GLC-2/GLC-3, glutamate EC<sub>50</sub> value was  $46.8 \pm 3.7 \mu\text{M}$  (n = 5), corresponding to a 4-fold  
245 reduction in comparison with the glutamate EC<sub>50</sub> value previously determined for Cel-GLC-2  
246 alone ( $214.6 \pm 13.1 \mu\text{M}$ , n = 5). The shift between these two EC<sub>50</sub> values clearly indicated that  
247 *C. elegans* GLC-2 and GLC-3 can combine to form a new GluCl subtype with a higher affinity  
248 for glutamate in comparison with the homomeric receptors (p < 0.001 ; **Fig. 5B**). Unfortunately,  
249 the maximum effect of glutamate application was not reached for Cel-GLC-3 (even with 30 mM  
250 glutamate), thus preventing the EC<sub>50</sub> value determination for this receptor.

251 For *P. univalens*, the GLC-2/GLC-3 glutamate EC<sub>50</sub> was  $120.2 \pm 5.7 \mu\text{M}$  (n = 21)  
252 whereas the EC<sub>50</sub> for Pun-GLC-3 was  $1482 \pm 111 \mu\text{M}$  (n = 11) (**Fig. 5C, Fig.5D**). As mentioned  
253 for *C. elegans*, this drastic shift of EC<sub>50</sub> values also confirmed that Pun-GLC-2/GLC-3 forms a  
254 novel subtype of heteromeric GluCl distinct from Pun-GLC-3 (p < 0.001).

255 In order to evaluate the involvement of GLC-2/GLC-3 as putative molecular targets for  
256 MLs, we decided to focused our attention on ivermectin, moxidectin and eprinomectin  
257 representing commonly used anthelmintic compounds (i.e. ivermectin and moxidectin used for  
258 *Parascaris spp.* treatment; eprinomectin used for clade V parasites on lactating animals).

259 The representative current traces obtained after drug applications on both Cel-GLC-  
260 2/GLC-3 and Pun-GLC-2/GLC-3 are shown in **Fig. 6A** and **Fig. 6C**. The Cel-GLC-2/GLC-3  
261 receptor was activated by ivermectin, moxidectin and eprinomectin. The maximum current  
262 amplitude was induced by ivermectin with a current amplitude corresponding to  $15 \pm 2 \%$  (n =  
263 9) of the maximum response obtained with glutamate (**Fig. 6B**) which is very similar to the  
264 ivermectin effect on Cel-AVR-14B/GLC-2 heteromeric receptor ( $18 \pm 3 \%$ , n = 6).  
265 Surprisingly, for *P. univalens*, none of the tested MLs induced a current (**Fig. 6D**). This result

266 was further confirmed using a wider panel of MLs (**Fig. S4A**). In order to investigate if the drug  
267 application time could potentially impact the ML agonist effect, 1  $\mu\text{M}$  ivermectin was perfused  
268 during 90 s on Pun-GLC-2/GLC-3. Even with this long-lasting application, ivermectin showed  
269 a weak agonist effect with a current amplitude corresponding to  $3.56 \pm 0.54 \%$  ( $n = 7$ ) of the  
270 maximum response obtained with glutamate (**Fig. S4B**).

271 Because of this unexpected lack of activity as agonists, we hypothesized that MLs could  
272 potentially act as antagonists or potentialize the effect of glutamate on Pun-GLC-2/GLC-3. In  
273 order to test the hypothesis, 100  $\mu\text{M}$  glutamate (corresponding approximately to the receptor  
274 glutamate  $\text{EC}_{50}$ ) were applied before, during and after the addition of 1  $\mu\text{M}$  ivermectin  
275 (**Fig. 7A**) or moxidectin (**Fig. 7B**) on the receptor. Strikingly, both drugs potentialized the effect  
276 of glutamate on Pun-GLC-2/GLC-3. Current amplitudes in response to 100  $\mu\text{M}$  glutamate were  
277 increased by  $45 \pm 10 \%$  ( $n = 5$ ,  $p < 0.05$ ; **Fig. 7C**) and  $93 \pm 19 \%$  ( $n = 8$ ,  $p < 0.01$ ; **Fig. 7D**)  
278 when co-applied with ivermectin or moxidectin respectively. This effect was reversible for both  
279 ivermectin ( $p < 0.05$ ) and moxidectin ( $p < 0.001$ ).

280 This is to our knowledge, the first report of a nematode GluCl potentialized by MLs at  
281 such concentration, representing a novel receptor subtype with unique pharmacological  
282 properties.

283 The pharmacological properties of the functional GluCl receptors described in the present  
284 work are summarized in S2 Table.

285

## 286 **Discussion**

287 Among the six distinct genes encoding GluCl subunits in *C. elegans*, AVR-14, GLC-2,  
288 GLC-3 and GLC-4 are highly conserved in distantly related nematode species from different

289 phylogenetic clades (52). Therefore, we reasoned that receptors including these subunits could  
290 be involved in the broad-spectrum activity of MLs on nematodes.

291 In the present study, using the xenopus oocyte as a heterologous expression system, we  
292 identified a panel of functional homomeric and heteromeric receptors made of the GLC-2,  
293 GLC-3 and AVR-14B subunits from the free-living model nematode *C. elegans* and *B. malayi*  
294 and *P. univalens*, two parasitic nematodes presenting a major impact for human and equine  
295 health respectively.

296 Among the *C. elegans* GluCl subunits, we confirmed that homomeric receptors made of  
297 AVR-14B or GLC-2 are responsive to glutamate in the  $\mu\text{M}$  range with elicited currents at the  
298  $\mu\text{A}$  range, whereas in contrast, GLC-3 homomeric receptor only respond to mM glutamate  
299 application resulting in small currents at the nA range. Strikingly, for the latter, we showed that  
300 addition to GLC-2 led to the formation of a novel heteromeric GLC-2/GLC-3 receptor,  
301 responsive to more physiologically relevant glutamate concentrations. Even though it remains  
302 highly speculative to consider that such difference could reflect their existence *in vivo*, it clearly  
303 highlights the need to further investigate heteromeric GluCl s as potential contributors to MLs  
304 sensitivity in nematodes.

305 In other clade V nematode species such as *H. contortus* (47,50) and *C. oncophora* (27)  
306 homomeric glutamate sensitive channels made of AVR-14B or GLC-2 have also been  
307 described, suggesting that such recombinant homomeric GluCl channels could also been  
308 obtained from other parasitic nematode species. However, in the present study, we showed that  
309 none of the GluCl subunits from the parasites *B. malayi* nor *P. univalens* (i.e. AVR-14B, GLC-  
310 2, GLC-3 and GLC-4) gave rise to robust functional channel when expressed in the *Xenopus*  
311 oocytes (i.e. no glutamate elicited current, or small current at the nA range elicited by mM range  
312 glutamate). These results further supported the need to explore heteromeric GluCl s in target  
313 parasitic species.

314

315 **Highly conserved nematode GluCl subunits play a pivotal role in heteromeric**  
316 **receptors composition**

317 In the present study, our hypothesis that GLC-2 could combine with other GluCls  
318 subunits was supported by previous studies reporting that GLC-2 can associate with GLC-1 or  
319 AVR-15 in *C. elegans* and with AVR-14B in *H. contortus* and *C. oncophora* (27,36,43,50). In  
320 addition, the *C. elegans* AVR-14, GLC-2, GLC-3 and GLC-4 subunits has been shown to be  
321 expressed in pharyngeal neurons, suggesting potential interactions between these subunits in  
322 the worm (53,54).

323 For *C. elegans*, *B. malayi* and *P. univalens*, the combination of AVR-14B/GLC-2 led to  
324 the robust expression of glutamate-sensitive receptors. The drastic reduction of the glutamate  
325 EC<sub>50</sub> of AVR-14B/GLC-2 in comparison with their respective homomeric receptor counterparts  
326 strongly support the association of the two distinct subunits into functional heteromeric  
327 receptors. Subsequently, we described for the first time that GLC-2 and GLC-3 can associate  
328 to form a novel glutamate-sensitive GluCl subtype in *C. elegans* and *P. univalens*, but  
329 surprisingly not in *B. malayi*. Because Bma-GLC-2 has proven to be functional in the obligate  
330 heteromeric channel including AVR-14B, we first speculated that a putatively non-functional  
331 Bma-GLC-3 could be responsible for the failure to get the predicted GLC-2/GLC-3 receptor.  
332 However, the successfully expression of a functional chimeric receptor made of Pun-GLC-2  
333 and Bma-GLC-3 (**Fig. S5**, n = 12), confirmed that Bma-GLC-3 was a functional subunit.  
334 Therefore, we could only speculate that additional subunits combining to GLC-3 alone or GLC-  
335 2/GLC-3 of *B. malayi* are required to form functional receptors. In addition, we cannot exclude  
336 that ancillary proteins might be required for the functional expression of GluCl receptor in  
337 *Xenopus* oocytes as reported for some acetylcholine-receptor subtypes (55,56).

338 In summary, our results highlight that subunit combination is critical for clade III  
339 parasites to form functional glutamate-sensitive receptor in *Xenopus* oocyte expression system  
340 but might also contribute to the diversity of GluCl subtypes in clade V nematodes.

341

### 342 **New insights about macrocyclic lactones mode of action**

343 The identification of novel functional GluCl from three distinct nematode species  
344 opened the way for detailed pharmacological characterization to decipher their relative  
345 sensitivity to different MLs. In accordance with previous studies performed on AVR-14B/GLC-  
346 2 of *H. contortus* (50) and *C. oncophora* (27), we showed that AVR-14B/GLC-2 from *C.*  
347 *elegans*, *B. malayi* and *P. univalens* were sensitive to ivermectin and moxidectin corresponding  
348 to the two MLs with marketed authorization in human health (57) and the most widely used in  
349 equine health with abamectin and doramectin (58).

350 In addition, we showed that this GluCl subtype is also sensitive to a wide range of MLs  
351 including abamectin, doramectin, emamectin, eprinomectin and selamectin. Presently, only  
352 ivermectin has marketed authorization for a wide variety of hosts and parasites. Others MLs  
353 have more specific marketed authorization such as emamectin, which is mostly used as  
354 insecticide in veterinarian aquaculture as well as in terrestrial agriculture (59). Interestingly, the  
355 present work highlighted emamectin as the most efficient agonist of the AVR-14B/GLC-2  
356 receptors in the three species in comparison with the currently used MLs against *B. malayi* and  
357 *Parascaris spp.* However, whether the high potency of emamectin on AVR-14B/GLC-2 could  
358 be correlated or not with an efficacy of this drug *in vivo* remains to be established. Importantly,  
359 this also raise the question of the relative contribution of the different nematode GluCl subtypes  
360 in MLs sensitivity. Indeed, in some case of ivermectin resistance, moxidectin remains effective  
361 to treat lambs infected with *H. contortus* (17) suggesting that both molecules could



362 preferentially activate distinct pharmacological targets in the worms. Because stable  
363 transformation remains an elusive goal for numerous parasitic nematode species (60,61), RNAi  
364 experiments could represent an attractive alternative to investigate the respective role of the  
365 distinct GluCl subtypes in MLs susceptibility (62). Recently RNAi has been successfully used  
366 in *B. malayi* to invalidate the expression of nAChR (63) and SLO-1 subunits (64). Undoubtedly,  
367 such an approach combined with phenotypic assays (65) or the recently developed *in vivo*  
368 imaging system (IVIS) optimized to study *B. malayi* on a gerbil model (66) would represent a  
369 major opportunity to investigate in more details the relative contribution of AVR-14B/GLC-2  
370 in the MLs sensitivity.

371

### 372 **Distinct pharmacology between *C. elegans* and parasites**

373 In the present study, we reported that GLC-2/GLC-3 of *C. elegans* and *P. univalens*  
374 form a new subtype of functional glutamate-sensitive receptors. However, depending on the  
375 nematode species, they presented very distinct pharmacological properties. Indeed, whereas  
376 ivermectin, moxidectin and eprinomectin act as agonist on Cel-GLC-2/GLC-3, in sharp  
377 contrast, these drugs had a reversible glutamate potentializing effect at the same concentration  
378 on the Pun-GLC-2/GLC-3 receptor. Such a pharmacological property appears to be rare in  
379 GluCl subtypes of invertebrates, opening the way for future investigations of GLC-2/GLC-3 in other  
380 parasitic species.

381

382 In conclusion, our study provides new insight about the GluCl diversity and highlights  
383 the importance of GLC-2 as a core subunit in heteromeric GluCl subtypes from *C. elegans* and the clade  
384 III nematodes *B. malayi* and *P. univalens*. This work opens the way for the systematic  
385 investigation of heteromeric GluCl subtypes in target parasitic species in order to lay a strong

386 basis for the rational use of MLs and the discovery of novel drug targets for the development  
387 of next generation anthelmintics.

388

## 389 **Material and Methods**

### 390 **A. Sample supply**

391 *C. elegans* used in this study are Bristol N2 wild-type strain worms supplied by the  
392 *Caenorhabditis* Genetics Center (CGC), St. Paul, MN, USA, which is funded by NIH Office of  
393 Research Infrastructure Programs (P40 OD010440). *B. malayi* microfilariae were supplied by  
394 NIH/NIAID Filariasis Research Reagent Resource Center, University of Georgia, Athens, GA,  
395 USA ([www.filariasiscenter.org](http://www.filariasiscenter.org)). Adults *P. univalens* were collected in faeces of naturally  
396 infested foals from UEPAO (Experimental Unit of Orfrasière Animal Physiology, INRAE  
397 Centre Val de Loire, Nouzilly, 37380, France) 50 h after a treatment with ivermectin. All  
398 samples were store at -80°C in RNA later solution (Ambion) before used.

399

### 400 **B. Karyotyping of *Parascaris* sp samples**

401 *Parascaris equorum* and *Parascaris univalens* are the two *Parascaris* species described as  
402 morphologically identical. They can be discriminated by karyotyping as *P. univalens* has one  
403 pair of chromosome while *P. equorum* has two pair (67). We confirmed the species status by  
404 karyotyping *Parascaris* eggs from foals in Nouzilly, France. Ascarid eggs were extracted from  
405 a pool of faecal samples from four foals from the UEPAO. Feces were mixed with tap water  
406 and deposited on two sieves stacking in order of size (125 µm on the top and 63 µm on the  
407 bottom, respectively). Eggs were collected and washed with a large amount of tap water on the  
408 60 µm sieve. Karyotyping was performed as described previously (68). Briefly, eggs were  
409 decorticated by three washing steps with 2% sodium hypochlorite in 16.5% sodium chloride,

410 and subsequently by six washing steps with cold tap water. Then, eggs were briefly  
411 centrifugated and split in pool of 1000-1500 eggs which were incubated for 1.5 h, 2 h or 3 h at  
412 37 °C for the first or second embryonic division to occur. Eggs were fixed with a mixture of  
413 methanol, acetic acid and chloroform (6:3:1) during 1 h, then washed twice with tap water.  
414 Approximately 500 eggs were placed between a slide and a coverslip and were crushed by  
415 pressing hard manually 1 min on the slides and then frozen in liquid nitrogen for 1 min. The  
416 coverslip was removed and let the glass air dried. Dried slides and first coverslips were mounted  
417 with new coverslips and slides respectively, using ProLong® Diamond Antifade Mountant with  
418 DAPI (Life Technologies). Six mounted slides were incubated 24 h at room temperature in the  
419 dark and examined using a fluorescent microscope (Nikon Eclipse E600). As expected, the  
420 worldwide specie *P. univalens* was identified as the only specie present in the infected foals  
421 since all eggs had a single pair of chromosomes (**Fig. S1**).

422

### 423 **C. RNA extraction and cDNA synthesis**

424 Total RNA was extracted from a pool of adults for *C. elegans* and from a pool of L4  
425 larvae for *B. malayi*. For *P. univalens*, total RNA was extracted from the head of one worm,  
426 including pharynx. Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA,  
427 USA) following the manufacturer's recommendations. cDNA synthesis was performed with  
428 0.5-5 µg of total RNA using the Maxima H minus Reverse Transcriptase kit (Thermo Scientific,  
429 Waltham, MA, USA) according to the manufacturer's recommendations.

430

### 431 **D. Identification and cloning of full-length GluCl<sub>s</sub> coding sequences from nematodes**

432 PCR amplification were performed according to the manufacturer's recommendations  
433 with the Phusion High Fidelity Polymerase (New England BioLabs, Ipswich, MA, USA) using  
434 cDNA as template. Full-length coding sequences were cloned into the transcription vector pTB-  
435 207 and RACE-PCR product were cloned into pGEM-T (Promega, Madison, WI, USA).

436 Eurofins Genomics (Luxembourg, Luxembourg) sequenced all constructs. Sequences of Cel-  
437 AVR-14B (CAA04170), Cel-GLC-2 (AAA50786), Cel-GLC-3 (CAB51708) and Cel-GLC-4  
438 (NP\_495489.2) from *C. elegans* were available on Genbank as well as Pun-AVR-14B  
439 (ABK20343) subunit coding sequence from *P. univalens*. Using the GluCl sequences of *C.*  
440 *elegans*, *Haemonchus contortus* and Pun-AVR-14B as queries, tBLASTn searches in NCBI  
441 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and WormbaseParasite  
442 (<https://parasite.wormbase.org/Tools/Blast?db=core>) allowed the identification of full-length  
443 coding sequence of GluCl sequences from *B. malayi* (Bma-GLC-2 : XM\_001893073.1 ; Bma-GLC-4 :  
444 XM\_001900205.1 ; Bma-AVR-14B : supercontig:Bmal-  
445 4.0:Bm\_v4\_Ch3\_scaffold\_001:1374074:1388079:1 ; Bma-GLC-3 : Bmal-  
446 4.0:Bm\_v4\_Ch4\_scaffold\_001:1513094:1542759:-1) and partial sequences of GluCl sequences from *P.*  
447 *univalens* (Pun-GLC-2 : NODE\_2545302 ; Pun-GLC-3 : NODE\_2129897 and  
448 NODE\_2250308 ; Pun-GLC-4 : NODE\_1817943, NODE\_2402242 and NODE\_2418647).  
449 Primers designed for RACE PCR and for amplification of the full-length coding sequences of  
450 each subunit are indicated in **S3-5 Tables**. For GluCl subunits of *P. univalens*, the  
451 corresponding 5' and 3' cDNA ends were obtained by nested RACE-PCR experiments as  
452 previously described (56). After identification of the 5' and 3' ends, two pairs of new primers  
453 per subunit were designed to amplify the full-length coding sequence of all GluCl subunits by  
454 nested PCR with the proofreading Phusion High-Fidelity DNA Polymerase (Thermo  
455 Scientific). Then, PCR products were cloned into the transcription vector pTB-207 (69) using  
456 the In-Fusion HD Cloning kit (Clontech) as described previously (70). Recombinant constructs  
457 were purified using EZNA Plasmid DNA Mini kit (Omega Bio-Tek) and sequence-checked  
458 (Eurofins Genomics). The novel complete coding sequences of Bma-AVR-14B, Bma-GLC-2,  
459 Bma-GLC-3, Bma-GLC-4, Pun-AVR-14B, Pun-GLC-2, Pun-GLC-3 and Pun-GLC-4 were  
460 deposited to Genbank. Constructs were linearized with MspI, PaeI or PacI restriction enzymes

461 (Thermofisher) depending on the construct. Linearized plasmids were used as DNA templates  
462 for cRNA synthesis using the mMessage mMachine T7 transcription kit (Ambion). cRNAs  
463 were precipitated with lithium chloride and were resuspended in a suitable volume of RNase-  
464 free water and stored at -20°C before use.

465

#### 466 **E. Sequence analysis and phylogeny**

467 Prediction of signal peptides and transmembrane domains were performed with SignalP4.0  
468 (71) and Simple Modular Architecture Research Tool (72). Deduced amino-acid sequences of  
469 GluCls from *B. malayi*, *C. elegans*, *H. contortus* and *P. univalens* were aligned using the  
470 MUSCLE algorithm and further processed with GENEDOC (IUBio). Percentage of identity  
471 between deduced amino acid of mature protein without peptide signal were obtained with EBI  
472 Global Alignment EMBOSS Needle (73). The distance trees were constructed using the  
473 SeaView software (74) with BioNJ (Poisson) parameters. Significance of internal tree branches  
474 was estimated using bootstrap resampling of the dataset 1000 times. The tree was edited using  
475 the FigTree software (<http://tree.bio.ed.ac.uk/software/figtree/>).

476 The sequences used in this study are available on GenBank under the followed accession  
477 numbers : *Brugia malayi* (Bma) : AVR-14B (MW196269), GLC-2 (MW196266), GLC-3  
478 (MW196267), GLC-4 (MW196268); *Caenorhabditis elegans* (Cel) : AVR-14A (AAC25481),  
479 AVR-14B (MW196270), AVR-15A (CAA04171), AVR-15B (CAA04170), GLC-1  
480 (AAA50785), GLC-2 (AAA50786), GLC-3 (CAB51708), GLC-4 (NP\_495489.2), UNC-49B1  
481 (AAD42383); *Haemonchus contortus* (Hco) : AVR-14A (CAA74622), AVR-14B  
482 (CAA74623), GLC-2 (CAA70929), GLC-4 (ABV68894), GLC-5 (AAD13405); *Parascaris*  
483 *univalens* (Pun): AVR-14B (MW187941), GLC-2 (MW187938), GLC-3 (MW187939), GLC-  
484 4 (MW187940) and GLC-5 (QBZ81966).

485

## 486 **F. Electrophysiological recording and data analysis in *Xenopus laevis* oocytes**

487 Defolliculated *Xenopus laevis* oocytes were purchased from Ecocyte Bioscience (Germany)  
488 and maintained in incubation solution (100 mM NaCl, 2 mM KCl, 1.8 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 1 mM  
489 MgCl<sub>2</sub>·6H<sub>2</sub>O, 5 mM HEPES, 2.5 mM C<sub>3</sub>H<sub>3</sub>NaO<sub>3</sub>, pH 7.5 supplemented with 100 U/mL  
490 penicillin and 100 µg/mL streptomycin) at 19 °C. Using the Drummond nanoject II  
491 microinjector, each oocyte was microinjected with 72 ng of cRNA when subunits were  
492 expressed singly or with 50 ng for each subunit when expressed in combination (1 :1 ratio).  
493 Three to six days after cRNA microinjection, two-electrode voltage-clamp recordings were  
494 performed with an Oocyte clamp OC-725C amplifier (Warner instrument) at a holding potential  
495 of -80 mV to assess the expression of the GluCl channels. Currents were recorded and analyzed  
496 using the pCLAMP 10.4 package (Molecular Devices).

497 Dose responses relationships for glutamate were carried out by challenging oocytes with 5-  
498 10 s applications of increasing concentration of glutamate (between 1 µM to 30 mM depending  
499 on the receptor) with 2 min washing steps with Ringer solution between each application. The  
500 peak current values were normalized to the maximum response obtained with a saturated  
501 concentration of glutamate. The concentration of agonist required to mediate 50% of the  
502 maximum response (EC<sub>50</sub>) and the Hill coefficient (nH) were determined and compared using  
503 non-linear regression on normalized data with R Studio using the *drc* package v3.0-1 (75).  
504 Results are shown as mean ± SEM.

505 Comparison of the MLs effect on a receptors were performed as described previously  
506 (27,49). Briefly, 1 mM glutamate was first perfused on the oocytes as a reference peak current  
507 (maximum response) before application of a ML at 1 µM for 5 s. Current responses were  
508 normalized to the maximum current amplitude obtained with 1 mM glutamate. For Pun-GLC-  
509 2/GLC-3, the potentializing effect of MLs was evaluated by a first application of each MLs  
510 alone for 5 s, followed by the co-application with 100 µM glutamate for 5 s. The observed

511 responses were normalized to the response induced by 100  $\mu$ M glutamate (corresponding  
512 approximately to the glutamate EC<sub>50</sub> for Pun-GLC-2/GLC-3) alone performed prior to  
513 challenging with the ML. In order to investigate the reversibility of the potentializing effect of  
514 the MLs, 100  $\mu$ M glutamate was applied after 2 min washing. Statistical analyses were  
515 performed using Wilcoxon's test with Bonferroni adjustments to compared glutamate and MLs  
516 current amplitudes.

517

## 518 **G. Chemicals**

519 Glutamate, piperazine and the macrocyclic lactones (selamectin, ivermectin, doramectin,  
520 emamectin, eprinomectin, abamectin and moxidectin) were purchased from Sigma-Aldrich.  
521 Macrocyclic lactones were first dissolved in DMSO as 10 mM and then diluted in recording  
522 solution to the required concentration with a final concentration of DMSO which not exceed  
523 1 %. Glutamate was directly prepared in recording solution.

524

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538

## 539 **Figure legends**

540 **Fig. 1: Distance tree of GluCl deduced amino-acid sequences from the nematodes *B.***  
541 ***malayi* (Bma), *C. elegans* (Cel), *H. contortus* (Hco) and *P. univalens* (Pun).**

542 The bootstrap values (% from 1000 replicates) are indicated at each node. Scale bar represents  
543 the number of substitutions per site. Accession numbers for the sequences used in this analysis  
544 are provided in the Methods sections. Sequences of AVR-14B, GLC2 and GLC-3 from *B.*  
545 *malayi*, *C. elegans* and *P. univalens* are highlighted in blue, yellow and green respectively. The  
546 GABA receptor subunit UNC-49B from *C. elegans* was used as an outgroup.

547

548 **Fig. 2: Functional expression of GluCl subunits from *C. elegans*, *B. malayi* and *P.***  
549 ***univalens* in *Xenopus laevis* oocytes.**

550 Mean current amplitude in response to 1 mM glutamate application on *Xenopus* oocytes  
551 expressing homomeric (A) or heteromeric (B) receptors for *C. elegans*, *B. malayi* and *P.*  
552 *univalens*. Boxplots represent mean +/- SEM (\*\*\*) p < 0.001). Dark red boxes show subunit  
553 combinations that led to robust expression of receptors responding to 1 mM glutamate with  
554 peak current in the  $\mu$ A range. Light red boxes correspond to combinations which respond to 1  
555 mM glutamate with small currents in the nA range. Black boxes correspond to combinations  
556 that did not respond to 1 mM glutamate application. Representative glutamate-elicited currents

557 traces are provided under each subunit combination, application times are indicated by the black  
558 bars.

559

560 **Fig. 3: Functional characterization of AVR-14B/GLC-2 from *C. elegans*, *B. malayi* and *P.***  
561 ***univalens*.**

562 **A.** Representative current traces of Cel-AVR-14B/GLC-2 expressed in *Xenopus* oocytes in  
563 response to the application of increasing concentrations of glutamate (3-1000  $\mu$ M). Glutamate  
564 application times are indicated by the black bars.

565 **B.** Glutamate concentration-response curve for Cel-AVR-14B, Cel-GLC-2 and the combination  
566 Cel-AVR-14B/GLC-2 (mean  $\pm$  SEM, n = 5-8). Current amplitudes were normalized to the  
567 maximal effect obtained with a saturating glutamate concentration (\*\*\*) p < 0.001).

568 **C.** Representative current traces of Bma-AVR-14B/GLC-2 expressed in *Xenopus* oocytes in  
569 response to the application of increasing concentrations of glutamate (3-1000  $\mu$ M). Glutamate  
570 application times are indicated by the black bars.

571 **D.** Glutamate concentration-response curve for Bma-AVR-14B and the combination Bma-  
572 AVR-14B/GLC-2 (mean  $\pm$  SEM, n = 5-7). Current amplitudes were normalized to the  
573 maximal effect obtained with a saturating glutamate concentration (\*\*\*) p < 0.001).

574 **E.** Representative current traces of Pun-AVR-14B/GLC-2 expressed in *Xenopus* oocytes in  
575 response to the application of increasing concentrations of glutamate (1-1000  $\mu$ M). Glutamate  
576 application times are indicated by the black bars.

577 **F.** Glutamate concentration-response curve for Pun-AVR-14B/GLC-2 (mean  $\pm$  SEM, n = 5).  
578 Current amplitudes were normalized to the maximal effect obtained with a saturating glutamate  
579 concentration.

580

581 **Fig. 4: Effects of marketed macrocyclic lactones on AVR-14B/GLC-2 from *C. elegans*, *B.***  
582 ***malayi* and *P. univalens*.**

583 **A.** Representative recording traces from a single oocyte injected with AVR-14B and GLC-2 of  
584 *C. elegans* induced by the two most potent activators, emamectin and eprinomectin after a first  
585 application of 1 mM glutamate. Application times are indicated by the black bars.

586 **B.** Comparison of macrocyclic lactones effects at 1  $\mu$ M after 5 s application on Cel-AVR-  
587 14B/GLC-2. All responses were normalized to the maximum responses obtained with  
588 glutamate at 1 mM (\*  $p < 0.05$ ).

589 **C.** Representative current traces from a single oocyte injected with AVR-14B and GLC-2 of *B.*  
590 *malayi* induced by the two most potent activators, emamectin and moxidectin after a first  
591 application of 1 mM glutamate. Application times are indicated by the black bars.

592 **D.** Comparison of macrocyclic lactones effects at 1  $\mu$ M after 5 s application on Bma-AVR-  
593 14B/GLC-2. All responses were normalized to the maximum responses obtained with  
594 glutamate at 1 mM (\*  $p < 0.05$ ).

595 **E.** Representative current traces from a single oocyte injected with AVR-14B and GLC-2 of *P.*  
596 *univalens* induced by the two most potent activators, emamectin and ivermectin after a first  
597 application of 1 mM glutamate. Application times are indicated by the black bars.

598A. **F.** Comparison of macrocyclic lactones effects at 1  $\mu$ M after 5 s application on Pun-AVR-  
599 14B/GLC-2. All responses were normalized to the maximum responses obtained with  
600 glutamate at 1 mM (\*  $p < 0.05$ ).

601

602 **Fig. 5: Pharmacological characterization of GLC-2/GLC-3 from *C. elegans* and *P.***  
603 ***univalens*.**

604 **A.** Representative current traces of Cel-GLC-2/GLC-3 expressed in *Xenopus* oocytes in  
605 response to an increasing concentration of glutamate (3-10000  $\mu$ M). Application times are  
606 indicated by the black bars.

607 **B.** Glutamate concentration response curve on Cel-GLC-2 and on the combination Cel-GLC-  
608 2/GLC-3 (mean  $\pm$  SEM, n = 5). Current amplitudes were normalized to the maximal effect  
609 with glutamate (\*\*\*) p < 0.001).

610 **C.** Representative current traces of Pun-GLC-2/GLC-3 expressed in *Xenopus* oocytes in  
611 response to an increasing concentration of glutamate (3-3000  $\mu$ M). Application times are  
612 indicated by the black bars.

613 **D.** Glutamate concentration response curve on Pun-GLC-3 and on the combination Pun-GLC-  
614 2/GLC-3 (mean  $\pm$  SEM, n = 11-21). Current amplitudes were normalized to the maximal  
615 effect with glutamate (\*\*\*) p < 0.001).

616

617 **Fig. 6: Pharmacological characterization of GLC-2/GLC-3 from *C. elegans* and *P.***  
618 ***univalens*.**

619 **A.** Representative current traces induced by some macrocyclic lactones (ivermectin,  
620 moxidectin and eprinomectin) on Cel-GLC-2/GLC-3. Application time are indicated by the  
621 black bars.

622 **B.** Comparison of some macrocyclic lactones effects at 1  $\mu$ M after 5 s application on Cel-  
623 GLC-2/GLC-3. All responses are normalized to the maximum responses obtained with  
624 glutamate at 1 mM (NS = Not significative)

625 **C.** Representative current traces induced by some macrocyclic lactones (ivermectin,  
626 moxidectin and eprinomectin) on Pun-GLC-2/GLC-3. Application time are indicated by the  
627 black bars.

628 **D.** Comparison of some macrocyclic lactones effects at 1  $\mu$ M after 5 s application on Pun-  
629 GLC-2/GLC-3. All responses are normalized to the maximum responses obtained with  
630 glutamate at 1 mM (NS = Not significant).

631

632 **Fig. 7: Modulation of glutamate effect by ivermectin and moxidectin on GLC-2/GLC-3**  
633 **from *P. univalens*.**

634 **A-B.** Representative current traces induced by glutamate 100  $\mu$ M followed by a co-application  
635 of ivermectin 1  $\mu$ M (**A**) or moxidectin 1  $\mu$ M (**B**) with glutamate 100  $\mu$ M. Application times are  
636 indicated by the black bars.

637 **C.** Box plot of the potentializing effect of ivermectin on Pun-GLC-2/GLC-3 normalized and  
638 compared with the response to 100  $\mu$ M glutamate (\*  $p < 0.05$ ).

639 **D.** Box plot of the potentializing effect of moxidectin on Pun-GLC-2/GLC-3 normalized and  
640 compared with the response to 100  $\mu$ M glutamate (\*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ )

641

642

643

644 **Supporting information**

645 **Fig. S1 : Karyotype of *Parascaris univalens***

646 *Parascaris* eggs were DAPI-stained during the first mitotic division. The single pair of chromosomes is  
647 representative of *P.univalens* (x400).

648

649 **Fig. S2: Amino-acid alignments of AVR-14B (A), GLC-2 (B) and GLC-3 (C) subunit sequences**  
650 **from the four nematode species *Brugia malayi* (Bma), *Caenorhabditis elegans* (Cel), *Haemonchus***  
651 ***contortus* (Hco) and *Parascaris univalens* (Pun)**

652 Predicted signal peptides in the N-terminal region are highlighted in grey. Amino acids share between  
653 the four species are highlight in blue. The four transmembrane segments (TM 1-4) and cys-loops are  
654 indicated by the black bars.

655

656 **Fig. S3: Amino-acid alignment of GLC-4 from the four nematode species *Brugia malayi* (Bma),**  
657 ***Caenorhabditis elegans* (Cel), *Haemonchus contortus* (Hco) and *Parascaris univalens* (Pun)**

658 Predicted signal peptides in the N-terminal region are highlight in grey. Amino acids share between the  
659 four species are highlight in blue. The four transmembrane segments (TM 1-4) and cys-loops are  
660 indicated by the black bars.

661

662 **Fig. S4: Pharmacological characterization of GLC-2/GLC-3 from *P. univalens***

663 **A.** Comparison of the macrocyclic lactones effects at 1  $\mu$ M after 5 s application on Pun-GLC-2/GLC-  
664 3. All responses are normalized to the maximum responses obtained with glutamate at 1 mM.

665 **B.** Representative current traces induced by ivermectin at 1  $\mu$ M during 90 s on Pun-GLC-2/GLC-3.

666 Application time is indicated by the black bar.

667

668 **Fig. S5: Representative current traces after application of glutamate 1 mM on *Xenopus* oocytes**  
669 **expressing chimera receptors made of Pun-GLC-2 and Bma-GLC-3 (n = 12)**

670 Application time are indicated by the black bars.

671

672 **S1 Table : Percentage amino acid sequence identity between GluCl subunits from *C.***  
673 ***elegans*, *B. malayi* and *P. univalens***

674

675 **S2 Table : Effect of glutamate and macrocyclic lactones on GluCl<sub>s</sub> from *C. elegans*, *B.***  
676 ***malayi* and *P. univalens* expressed in *Xenopus* oocytes.**

677 **S3 Table : List of primers used for PCR and cloning experiments of GluCl<sub>s</sub> subunits from**  
678 ***C. elegans*.**

679

680 **S4 Table : List of primers used for PCR and cloning experiments of GluCl<sub>s</sub> subunits from**  
681 ***Brugia malayi*.**

682

683 **S5 Table : List of primers used for PCR and cloning experiments of GluCl<sub>s</sub> subunits from**  
684 ***Parascaris univalens*.**

685



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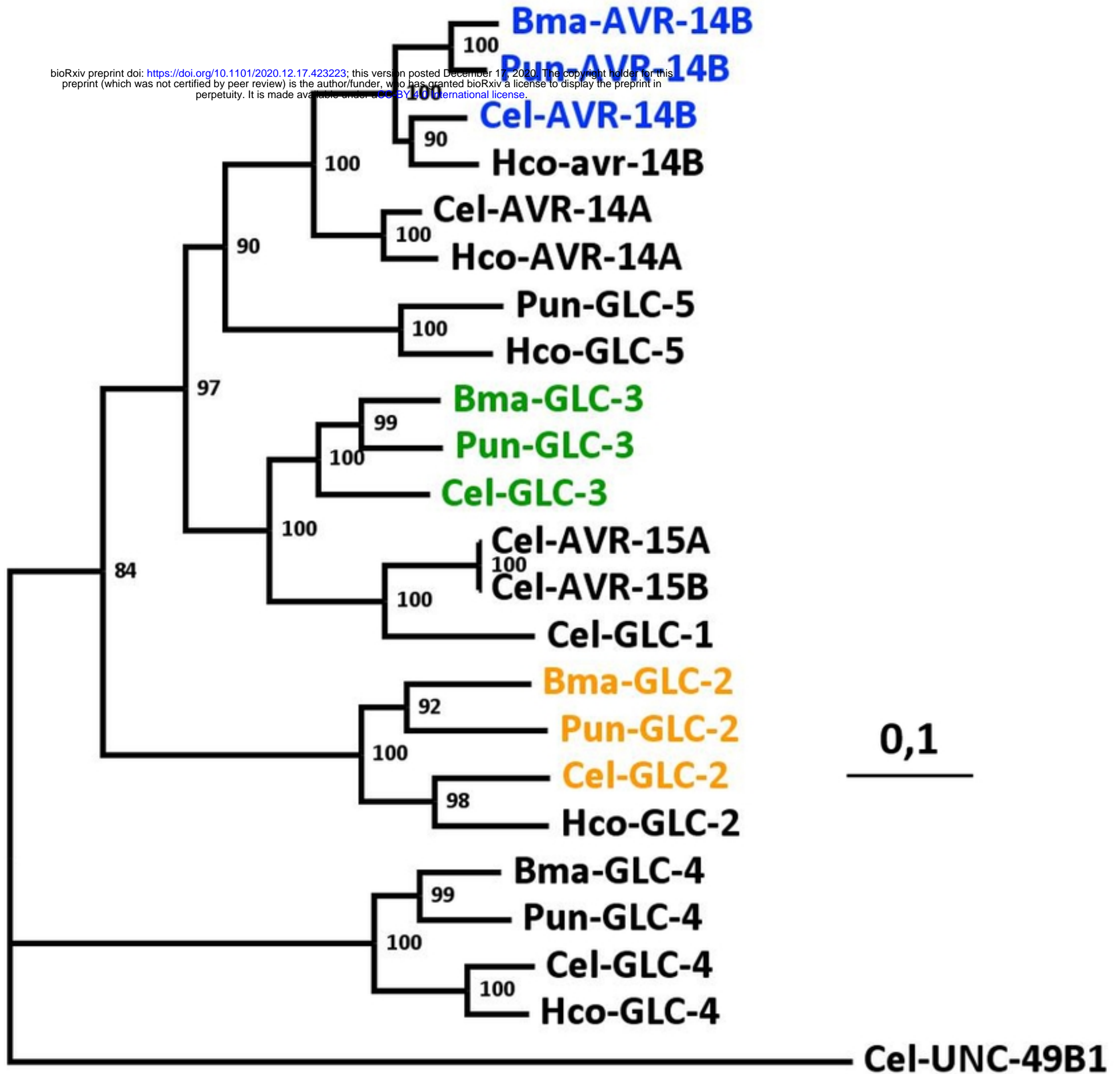


Figure 1

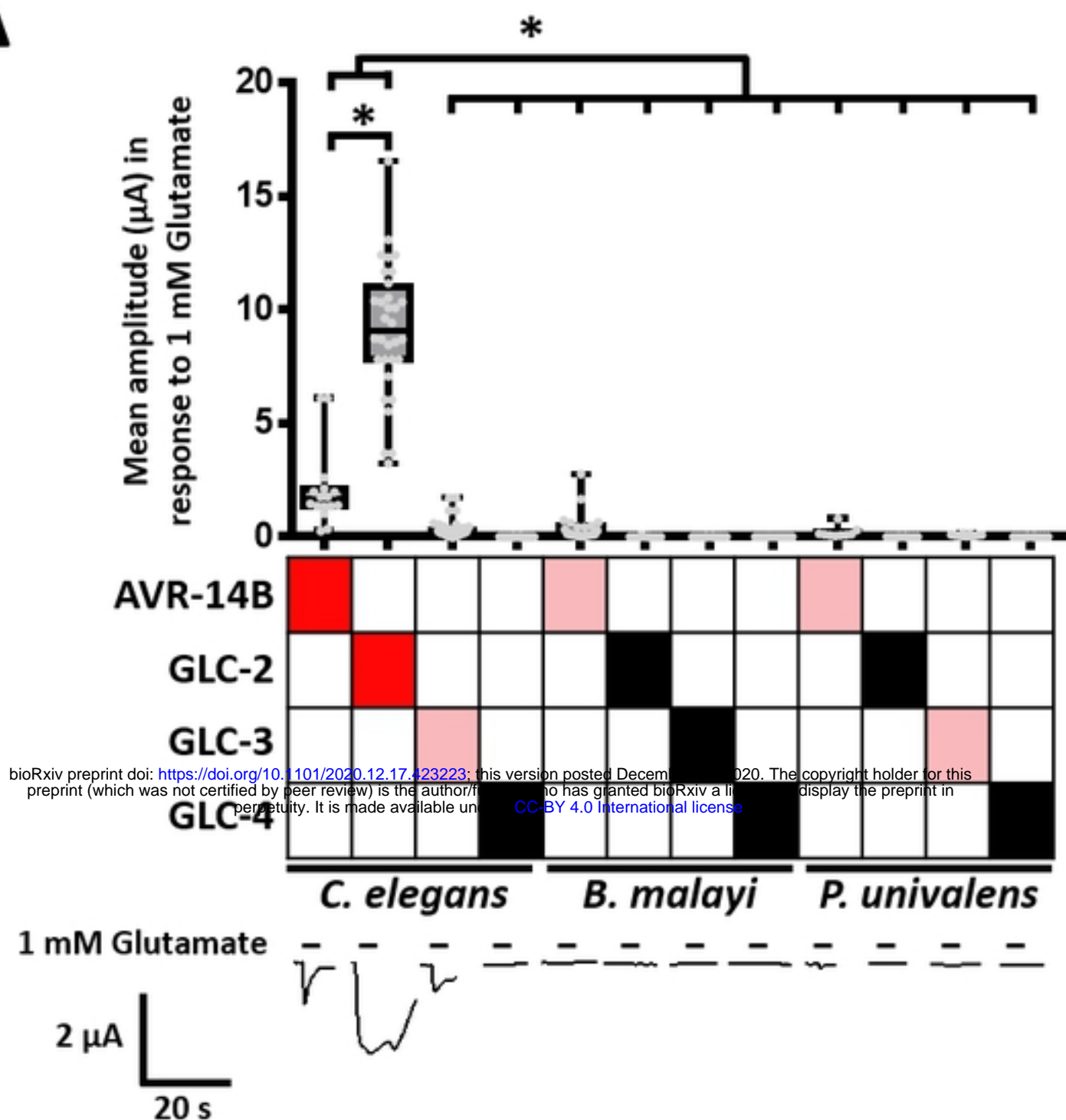
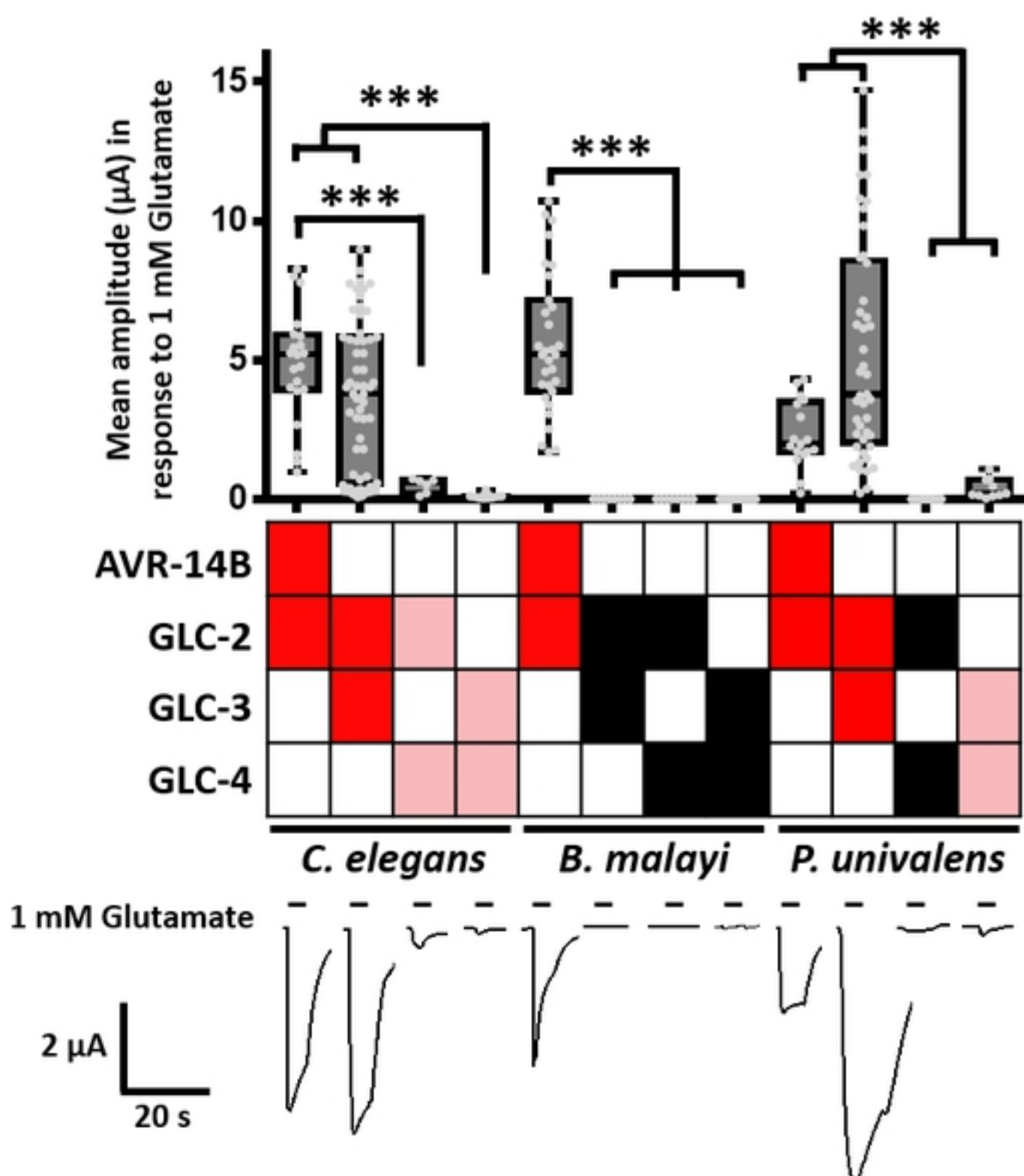
**A****B**

Figure2

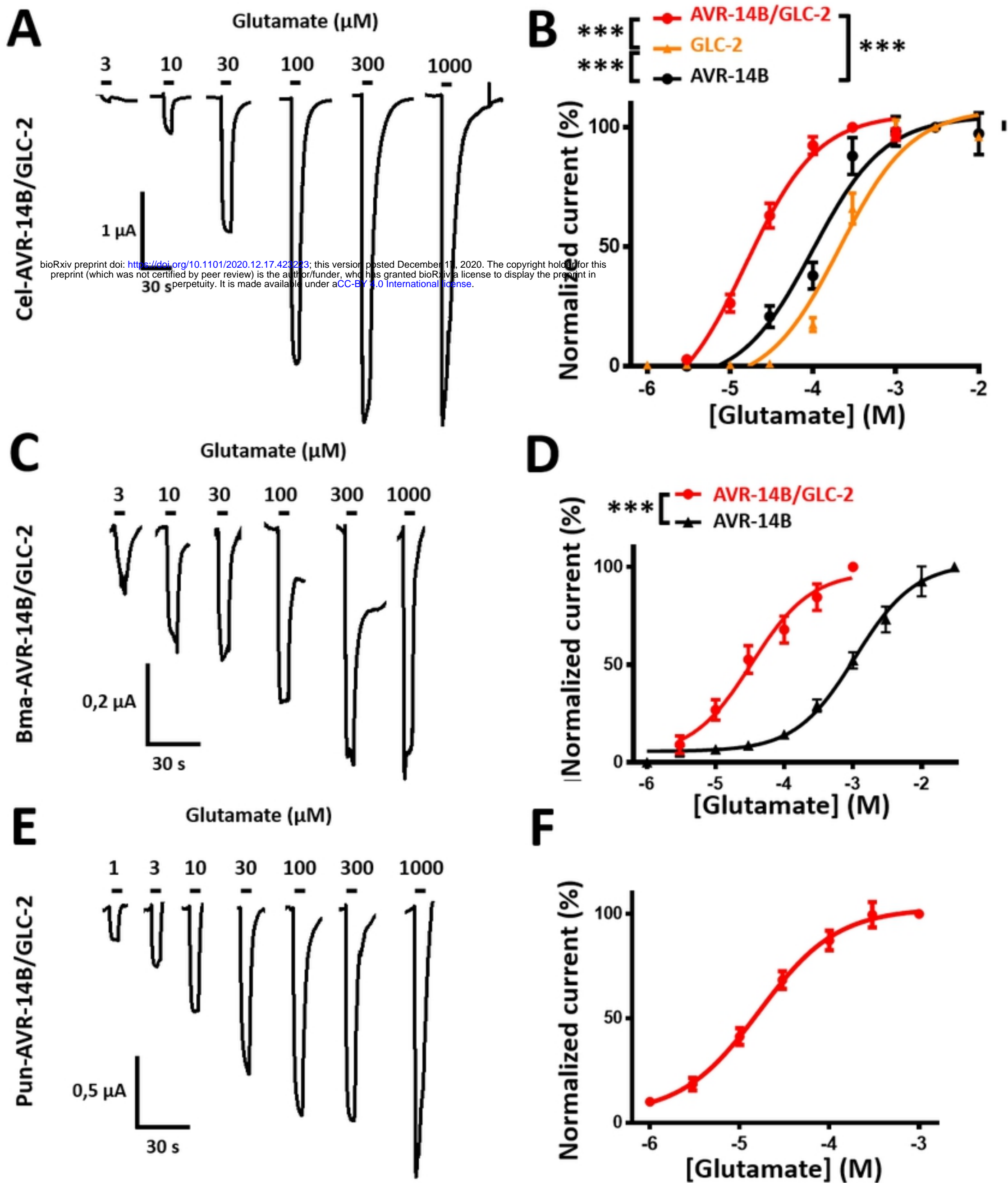


Figure3



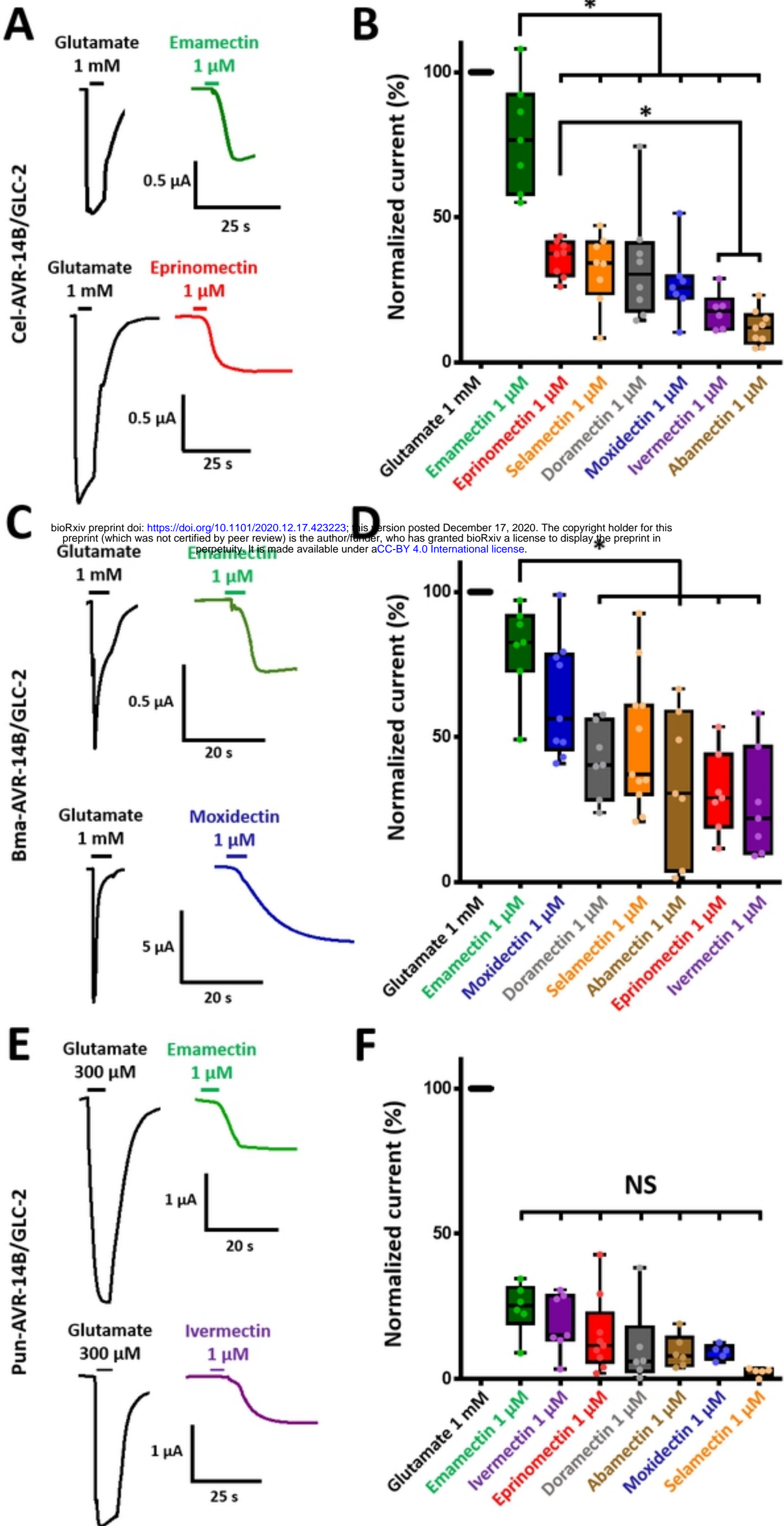


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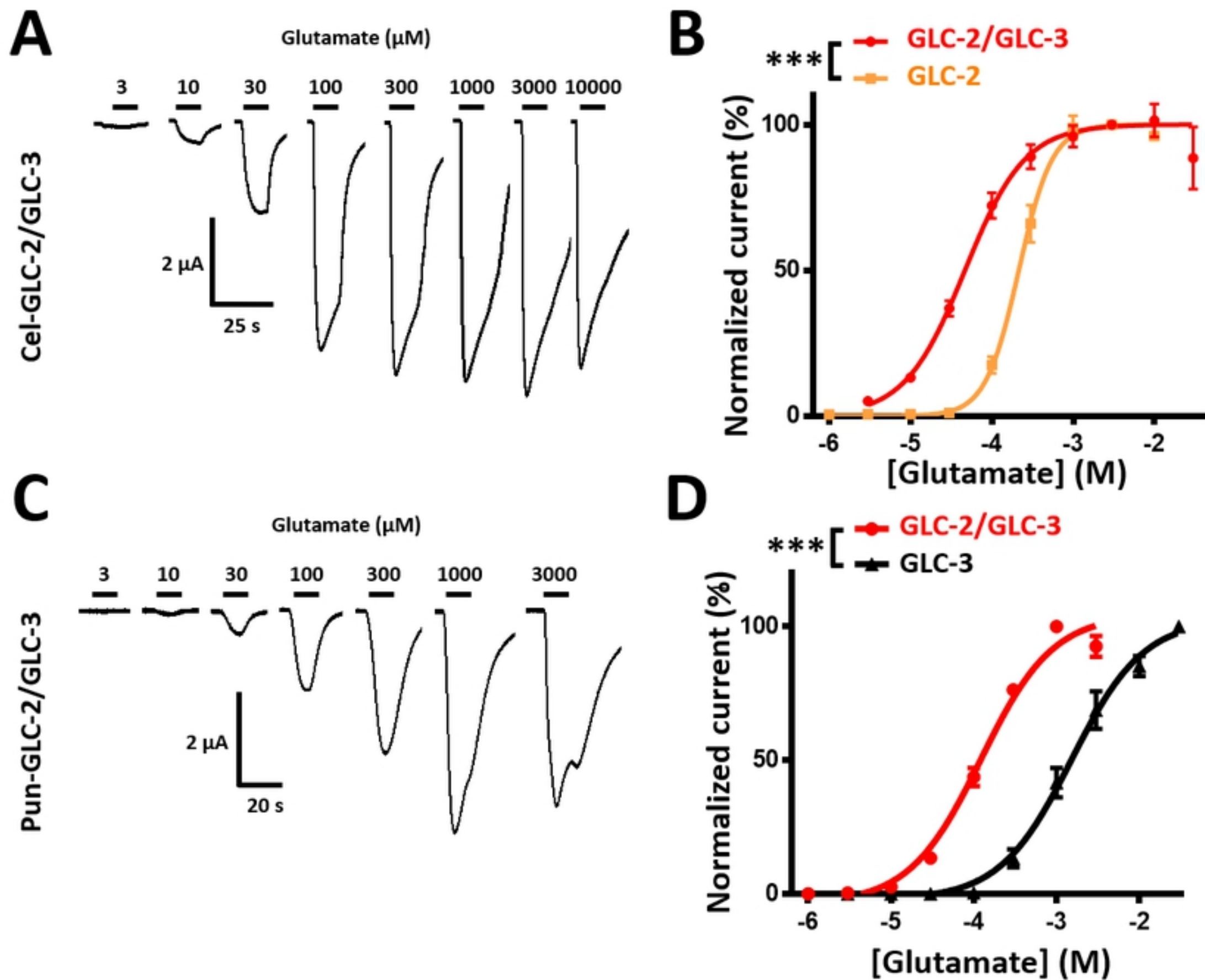


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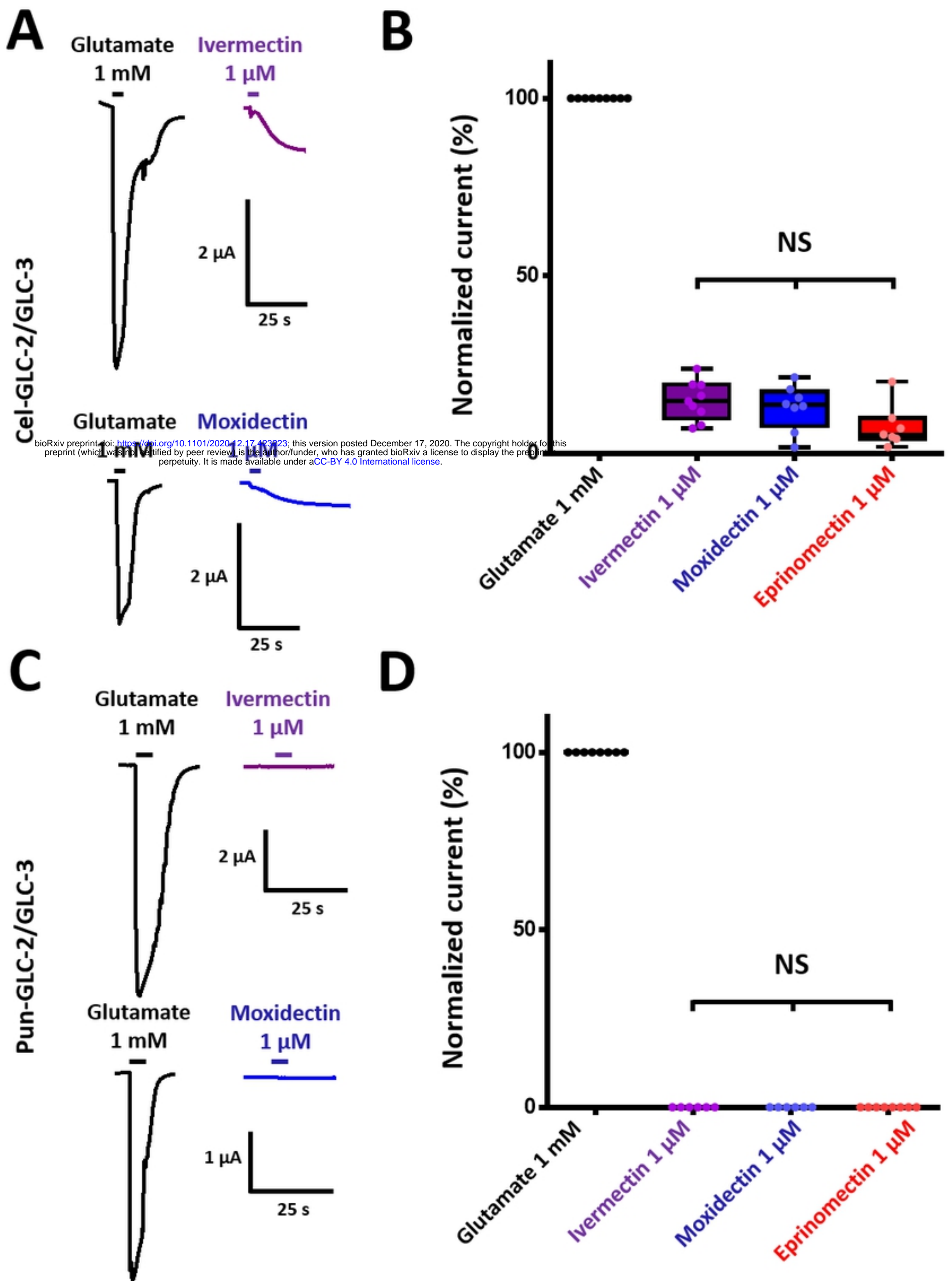


Figure6

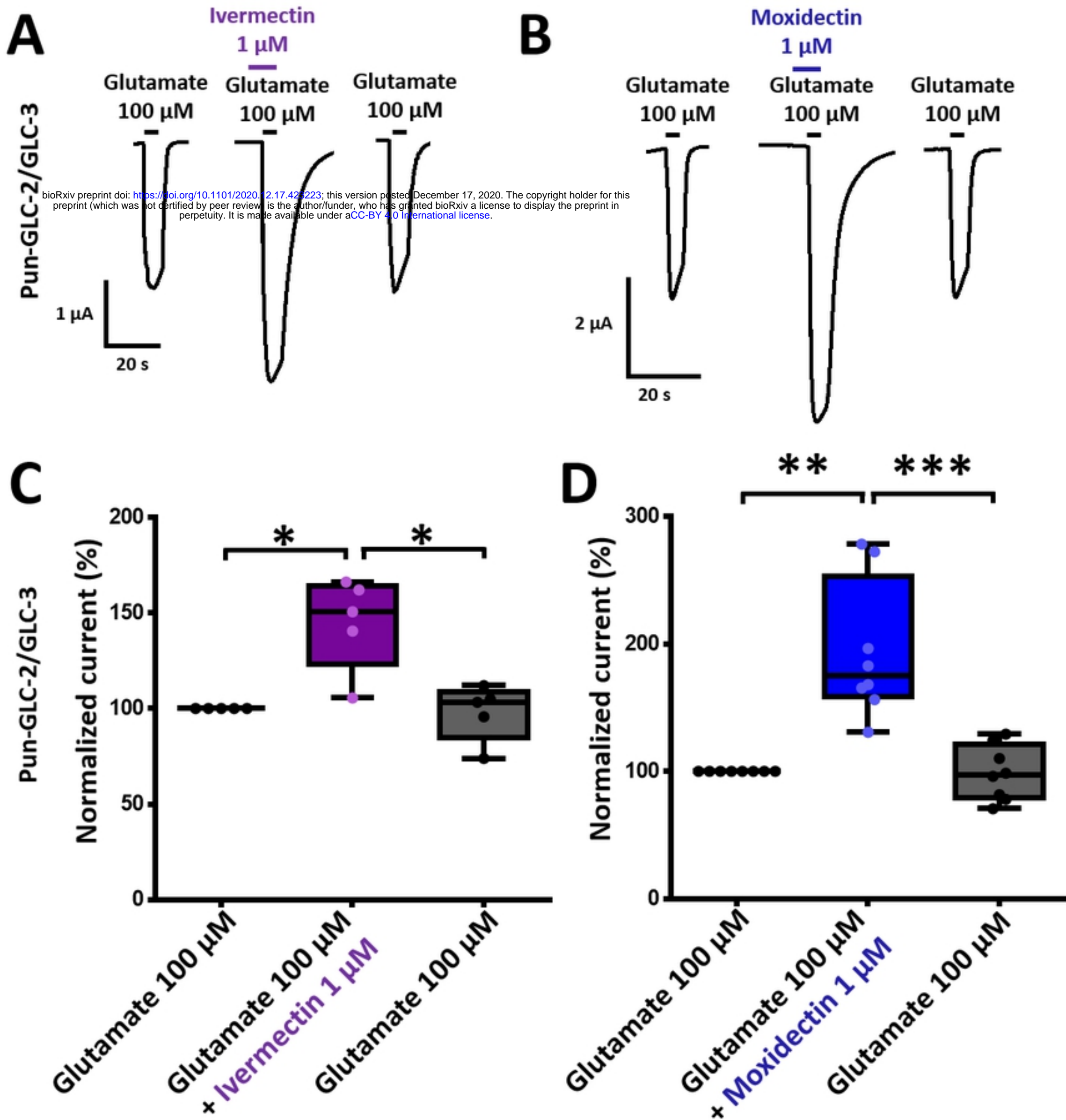


Figure 7