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## Nanobody-based sensors reveal a high proportion of mGlu heterodimers in the brain

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► **To cite this version:**

Jiyong Meng, Chanjuan Xu, Pierre-André Lafon, Salomé Roux, Michaël Mathieu, et al.. Nanobody-based sensors reveal a high proportion of mGlu heterodimers in the brain. *Nature Chemical Biology*, 2022, 18, pp.894-903. 10.1038/s41589-022-01050-2 . hal-03699580

**HAL Id: hal-03699580**

**<https://hal.science/hal-03699580>**

Submitted on 4 Aug 2022

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1 **Nanobody-based sensors reveal a high proportion of mGlu heterodimers in the**  
2 **brain**

3

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28

29 **Keywords:**

30 **G protein-coupled receptor | metabotropic glutamate receptor | nanobody | time-resolved**

31 **FRET | heterodimer | neurotransmitter receptor | neuroreceptor | membrane receptor |**

32 **olfactory bulb**

33

34 Abstract

35 **Membrane proteins, including ion channels, receptors and transporters are often**  
36 **composed of multiple subunits and can form large complexes. Their specific composition**  
37 **in native tissues is difficult to determine and remains largely unknown. In this study, we**  
38 **developed a method for determining the subunit composition of endogenous cell surface**  
39 **protein complexes from isolated native tissues. Our method relies on nanobody-based**  
40 **sensors, which enable the proximity detection between subunits in time-resolved FRET**  
41 **measurements. Additionally, given the conformation-specific nanobodies, the activation**  
42 **of these complexes can be recorded in native brain tissue. Applied to the metabotropic**  
43 **glutamate receptors in different brain regions, this approach revealed the clear existence**  
44 **of functional mGlu2-4 heterodimers in addition to mGlu2 and mGlu4 homodimers.**  
45 **Strikingly, the mGlu4 subunits appear to be mainly heterodimers in the brain. Overall,**  
46 **these versatile biosensors can determine the presence and activity of endogenous**  
47 **membrane proteins in native tissues with high fidelity and convenience.**

48

49 **Main Text**

50 Many membrane proteins, including receptors, ion channels, and transporters, are composed  
51 of multiple subunits<sup>1-3</sup>, and can form oligomers<sup>4</sup>. Auxiliary subunits can also associate with  
52 such complexes and affect their overall structure, function, and localization<sup>5</sup>. A major  
53 challenge is to investigate these membrane complexes in native conditions without disrupting  
54 their environment, since their interactions with lipids and associated proteins could be critical  
55 for their assembly and function.

56 Only a limited number of methods enable the validation of native membrane protein  
57 complexes, and are often difficult and time-consuming<sup>6</sup>. They often rely on tissue treatments,  
58 membrane fraction preparation, solubilization, or mass spectrometry analysis<sup>6,7</sup> and chemical  
59 cross-linking is sometimes required. Good alternatives are proximity interaction assays that  
60 rely on optical detection, but several of these lack spatial resolution (around 40 nm)<sup>8,9</sup>. It is  
61 then difficult to rule out the possibility of proteins not interacting directly<sup>10</sup>. Often, these  
62 techniques requires covalent labeling<sup>10</sup>, or recombinant fusion proteins.

63 An attractive approach to examine protein complexes is the use of time-resolved FRET  
64 (TR-FRET)<sup>4,11</sup> (**Fig. 1**). It relies on resonance energy transfer between two fluorophores, with  
65 a distance limit of 15 nm between the long-lasting emission donor and acceptor<sup>12</sup>, compatible  
66 with the size of multi-subunit proteins. A major advantage is the long lifetime of the donor,  
67 which enables a delay between its excitation and the measurement of the emission of the  
68 sensitized acceptor, strongly reducing the background fluorescence from biological systems.  
69 Fluorophores compatible with TR-FRET can be easily attached to antibodies<sup>11</sup>, antibody  
70 fragments<sup>13</sup> or even small ligands<sup>4</sup> specific to the protein complex under analysis, allowing  
71 the analysis of native protein complexes. TR-FRET is thus a highly sensitive technique that is  
72 compatible with the detection of endogenous and low-expressed proteins in native tissues<sup>4</sup>.

73           The use of small antibody fragments, such as camelid single-domain antibodies, called  
74 V<sub>HH</sub> or nanobodies (15 kDa, ~2.5 nm)<sup>14</sup>, can result in a high spatial resolution of the protein  
75 complexes detected by TR-FRET, higher than with conventional antibodies (~15 nm)<sup>11, 13</sup>.  
76 Moreover nanobodies can recognize conformational epitopes, allowing the detection of  
77 specific conformations of membrane proteins, such as the active<sup>15-17</sup> or resting state<sup>18, 19</sup>.  
78 Nanobodies that potentiate or activate metabotropic glutamate (mGlu) receptors were recently  
79 reported<sup>15, 17</sup>.

80           mGlu receptors are G protein-coupled receptors activated by the neurotransmitter  
81 glutamate, which tune the activity of many synapses<sup>20</sup>. They are therapeutic targets for several  
82 drugs under development for neurological and psychiatric diseases. Eight genes encoding  
83 mGluRs have been identified that generate receptors mGlu1 to 8. These are mandatory  
84 homodimers with both subunits linked by a disulfide bond, a quaternary structure essential for  
85 their activity<sup>21-23</sup>. This is well-illustrated by the latest structures of full-length mGlu dimers in  
86 their active and inactive states<sup>24, 25</sup>. These structural studies confirmed a specific symmetrical  
87 conformation of the large extracellular glutamate binding domains (ECD), with both domains  
88 closed and in a specific active orientation, while the dimer of 7TM domains is asymmetric,  
89 with only one capable of G protein activation.

90           Ten years ago, we reported that these mGlu subunits could also assemble into  
91 heterodimers with specific combinations, revealing the possible existence of 16 additional  
92 mGlu receptors<sup>26</sup>. Since then, specific pharmacological properties of these heterodimers have  
93 been reported, providing indirect evidence of their existence in the brain<sup>27-29</sup>. However, such  
94 data could also be explained by functional cross-talk between colocalized homodimeric  
95 receptors. In addition, these studies did not reveal the proportion of such heterodimers in the  
96 brain compared to homodimers.

97 In the present study, using specific nanobodies for both mGlu2 and mGlu4 subunits,  
98 we were able to reveal the existence of mGlu2-4 heterodimers, in addition to mGlu2 and mGlu4  
99 homodimers, in various brain regions. We also confirm their specific pharmacological  
100 properties and reveal that mGlu2-4 is a major type of receptor containing the mGlu4 subunit in  
101 the brain outside the cerebellum. These data demonstrate the effectiveness of our approach in  
102 deciphering the subunit composition of membrane protein complexes in their native  
103 environment and in providing relative quantification of endogenous membrane receptor species  
104 in native tissues.

## 105 **Results**

106 Among the possible 16 mGlu heterodimers observed in recombinant cells <sup>26, 30</sup>, the mGlu2-4  
107 heterodimer is the most investigated <sup>27-29</sup>, but its existence and abundance in the brain remain  
108 unclear. To clarify this issue in different brain areas, we have developed two kinds of  
109 nanobody-based TR-FRET sensors (**Fig. 1**): 1) conformational sensors, or “biosensors,” that  
110 reveal the activation of these receptors upon agonist binding; and 2) the “detectors” that enable  
111 the relative quantification of both mGlu homo- and heterodimers.

### 112 **mGlu2 FRET-based conformational biosensor**

113 We first developed a “biosensor” for the mGlu2 homodimer, by taking advantage of a pair of  
114 specific and high affinity nanobodies for the mGlu2 receptor, DN10 and DN1 <sup>15</sup>. DN10  
115 specifically recognizes the receptor dimer in its active state, whereas DN1 is not sensitive to  
116 the conformational state (**Fig. 2a**). The DN10 epitope overlaps that of DN13 <sup>15</sup> that is located  
117 at an interface of the two ECDs of the mGlu2 homodimer in its active form exclusively <sup>24</sup>, the  
118 nanobody contacting both subunits. In contrast to DN13, DN10 can also bind to the active  
119 mGlu2-4 heterodimer, as shown below, likely because the mGlu4 part of the epitope is  
120 compatible with DN10 binding. In contrast, the DN1 epitope remains unknown <sup>15</sup>

121 (Supplementary Table 1 and Table 2). When DN1 and DN10 were covalently labeled with  
122 donor Lumi4-Tb and acceptor d2, respectively, a FRET signal was measured in cells expressing  
123 mGlu2 in the presence of the mGlu2/3 agonist LY379268, but not with the antagonist  
124 LY341495 (Fig. 2b). No signal was measured when DN10-d2 was absent (Extended Data Fig.  
125 1a), and the FRET signal followed a saturation curve with the increase of DN10-d2 and a fixed  
126 concentration of DN1-Tb (Extended Data Fig. 1b). The signal was specific to mGlu2, as no  
127 signal was measured with other mGluRs (Extended Data Fig. 1c). Finally, DN10-Tb and  
128 DN1-d2 can also detect endogenous mGlu2 in rat hippocampal neurons by TR-FRET  
129 microscopy (Extended Data Fig. 2).

130 This pair of nanobodies could also be used with dissociated cells from different mouse  
131 brain regions (Extended Data Fig. 3a,b). The more cells, the higher the FRET signal with the  
132 nanobodies in the presence of agonists (Extended Data Fig. 3c). The slopes, representative of  
133 the FRET signal per amount of brain cells, revealed a high signal in the cerebellum and other  
134 regions (Fig. 2c), consistent with DN1-d2 staining of brain slices (Extended Data Fig. 4)<sup>31, 32</sup>.  
135 No signal was detected from *Grm2* knockout (*Grm2*<sup>-/-</sup>; called mGlu2 KO in this study) mice  
136 (Fig. 2c, Extended Data Fig. 4). Altogether, these data validate the use of this pair of  
137 nanobodies in the detection of endogenous active mGlu2 receptors.

138 This conformational biosensor is a sensitive tool to report the rearrangement of the  
139 mGlu2 ECD upon agonist activation. In transfected cells, the TR-FRET signal generated using  
140 increasing concentrations of various full and partial agonists revealed potencies and efficacies  
141 consistent with SNAP-tag FRET based assay<sup>33, 34</sup> (Fig. 2d). The potencies also correlated well  
142 with those measured by the accumulation of inositol phosphate-1 (IP<sub>1</sub>) (Fig. 2e,f). Such a good  
143 correlation between the potencies of partial and full agonists observed in both assays was not  
144 expected, as the amplification resulting from receptor reserve is expected to increase more the  
145 potencies of full agonists than those of partial agonists. The good correlation may be due to the

146 fact that the efficacy of partial agonists is closer to a full efficacy in the presence of the G  
147 protein bound to the active receptor<sup>33,35</sup>. Taken together, these data show that this nanobody-  
148 based biosensor constitutes a new generation of untagged mGlu conformational sensors.

149 This biosensor can also reveal the activation of endogenous mGlu2 receptors in  
150 dissociated cells from various brain regions. The agonist-induced change in FRET observed in  
151 the cortex, hippocampus, and cerebellum was similar to that found with transfected cells (**Fig.**  
152 **2g-i**). The antagonist LY341495 was also found to inhibit both the basal signal likely generated  
153 by ambient glutamate in the assay, and the agonist at it EC<sub>80</sub>.

154 Altogether, our data demonstrate that this optical nanobody-based biosensor can be  
155 used to reveal the activation of endogenous mGlu2 receptors in native dissociated tissues. It  
156 represents an innovative assay for throughput screening of the efficacy of drugs on native  
157 mGlu2Rs in brain tissue.

### 158 **Quantification of the mGlu2 homodimer**

159 We next aimed at quantifying of mGlu2 homodimers using DN1-Tb and DN1-d2 as TR-FRET  
160 pair, first on the surface of transfected cells (**Fig. 3a**). As expected, this “detector” signal is  
161 independent of the conformation of the homodimer, whether bound to an agonist or an  
162 antagonist (**Fig. 3b**). The concentrations of the nanobodies were optimized to have a FRET  
163 signal proportional to the quantity of mGlu2 over a wide range of receptor amounts (**Extended**  
164 **Data Fig. 5a-c**). This “detector” was also specific for the mGlu2 among all mGlu homodimers  
165 (**Fig. 3c**). We also believe that the FRET signal was mostly due to the mGlu2 homodimer, and  
166 not higher order oligomers. First, the FRET signal between the DN1 nanobodies was  
167 proportional to the number of SNAP-mGlu2 subunits on the cell surface over a wide range of  
168 receptor amounts (**Extended Data Fig. 5c**). Second, this linearity was also observed with the  
169 “controlled” mGlu2 homodimer formed by the mGlu2<sub>C1</sub> and mGlu2<sub>C2</sub> subunits, as previously

170 described <sup>26, 36</sup> (**Extended Data Fig. 5d**). In these constructs, the C-terminal of the mGlu2  
171 subunits was replaced by that of the modified GABA<sub>B1</sub> (C1) or GABA<sub>B2</sub> (C2) subunits,  
172 respectively, preventing any of these to reach the cell surface alone. Indeed, only C1-C2 dimers  
173 can reach the cell surface <sup>26</sup>. However, when using a similar controlled mGlu2-4 heterodimer  
174 made of mGlu2<sub>C1</sub> and mGlu4<sub>C2</sub> subunits, no FRET signal with the DN1 “detector” was  
175 measured (**Extended Data Fig. 5d**). Under these conditions, the mGlu2-4 heterodimer is  
176 present at the cell surface in the absence of both mGlu2 and mGlu4 homodimers. This is  
177 consistent with our previous demonstration that the controlled mGlu2-4 heterodimer <sup>26</sup> as well  
178 as the mGlu2 homodimer <sup>26, 37, 38</sup> do not have the tendency to form oligomers in transfected  
179 cells. Third, the highest FRET signal measured for equal concentrations of donor (DN1-Tb)  
180 and acceptor (DN1-d2) was also consistent with the presence of strict mGlu2 homodimers  
181 (**Extended Data Fig. 5b**).

182 By comparing the mGlu2 homodimer "detector" signal in different brain areas (**Fig. 3g**  
183 and **Extended Data Fig. 5e**), mGlu2 was found more abundant in the cerebellum (**Fig. 3g**). As  
184 a control, no signal was observed in the cerebellum of mGlu2 KO mice. However, this  
185 "detector" appears less sensitive than the "biosensor", but this was expected for two main  
186 reasons. First, only half of the mGlu2 homodimers can be labeled with two FRET compatible  
187 DN1 nanobodies (DN1-Tb and DN1-d2). In contrast, each mGlu2 subunit will be labeled with  
188 two FRET compatible nanobodies in the biosensor assay, DN1-Tb and DN10-d2, such that  
189 each mGlu2 homodimers carry two pairs of FRET compatible nanobodies. Second, this  
190 biosensor assay detects any active form of the mGlu2 subunits, whether in a homo or  
191 heterodimer, in contrast to the "detector" that reveals mGlu2 homodimers only.

## 192 **Quantification of the mGlu4 homodimer**

193 To quantify the mGlu4 homodimer, we isolated and characterized the nanobody DN42, highly  
194 specific for mGlu4 (**Extended Data Fig. 6a,b**). For this study, we used the Fc-DN42 dimeric

195 construct (80 kDa) as the DN42 monomer has a low affinity after labeling with a fluorophore  
196 (~100 nM) that is not compatible with its use with native tissues. Interestingly, Fc-DN42 has a  
197 sub-nanomolar affinity for the mGlu4 ECD, and a similar affinity for the inactive and active  
198 conformations (**Supplementary Table 1 and Table 2; Extended Data Fig. 6c**). We verified  
199 by immunofluorescence that Fc-DN42 was able to specifically detect mGlu4 subunits in brain  
200 slices of wild-type mice but not in *Grm4* knockout (*Grm4*<sup>-/-</sup>; called mGlu4 KO in this study)  
201 mice (**Extended Data Fig. 6d**).

202 We used Fc-DN42, from now on referred to as DN42, as a “detector” to quantify mGlu4  
203 homodimers, similarly to what was done with DN1 for the mGlu2 homodimers. With optimized  
204 concentrations of DN42 (**Extended Data Fig. 7a,b**), a strong FRET signal was measured  
205 between DN42-Tb and DN42-d2 in mGlu4 transfected cells specifically (**Fig. 3d-f**),  
206 independent from the state of the receptor. As observed with mGlu2, the FRET signal appeared  
207 mostly owing to the mGlu4 homodimer, and not to higher order oligomers. First, the signal  
208 was proportional to the number of SNAP-mGlu4 subunits on the cell surface over a wide range  
209 of receptor amounts (**Extended Data Fig. 7c**). Second, this linearity was also observed with a  
210 controlled mGlu4 homodimer, where the C-terminal of mGlu4 subunits was replaced by that  
211 of the GABA<sub>B</sub> receptor (**Extended Data Fig. 7d**). Lastly, the low FRET signal between mGlu4  
212 subunits measured with the controlled mGlu2-4 heterodimer (**Extended Data Fig. 7d**) may be  
213 due to random proximity between these heterodimers, although one cannot exclude a very few  
214 mGlu2-4 oligomers. This is also consistent with several reports showing the low probability of  
215 mGlu dimers to oligomerize under basal condition<sup>26, 37</sup>.

216 In native brain tissues, this pair of labeled DN42 nanobodies revealed a detectable  
217 signal mainly in the cerebellum of the wild-type and mGlu2 KO mice, but not in mGlu4 KO  
218 mice (**Fig. 3h** and **Extended Data Fig. 7e**). Significant signal could also be observed in the  
219 prefrontal cortex (PFC) (**Figure 3h, inset**), but the signals measured in other brain areas were

220 not significant indicating that our assay is not sensitive enough to detect mGlu4 homodimers  
221 in these areas, if any. This agrees with the strong expression of mGlu4 in the cerebellum<sup>31</sup>, as  
222 shown also by DN42-stained brain slides, especially in the molecular layer (**Extended Data**  
223 **Fig. 6d**). Altogether, our results show a very low expression of the mGlu4 homodimer in most  
224 brain regions except for the cerebellum, not really consistent with the immunostaining data<sup>31</sup>  
225 (**Extended Data Fig. 6d**), suggesting that mGlu4 subunits could be associated with other mGlu  
226 subunits outside the cerebellum.

### 227 **An mGlu2-4 heteromer FRET-based detector**

228 Recent studies argue in favor of the existence of endogenous mGlu2-4 receptors in neuronal  
229 cell lines<sup>27</sup>, as well as in the PFC, striatum and hippocampus<sup>27-29</sup> as suggested by  
230 electrophysiological, pharmacological and biochemical data. Although convincing, the results  
231 provide indirect evidence of the endogenous mGlu2-4 heterodimer<sup>27-29</sup>. More direct evidence  
232 for this heterodimer could come from a proximity assay based on FRET between the mGlu2  
233 and mGlu4 subunits in tissues, owing to the nanobodies described above.

234 Thus, we used DN1 and DN42 nanobodies to detect mGlu2-4 heterodimers first on  
235 transfected cells. Using the optimized concentrations of DN1 and DN42 (**Supplementary**  
236 **Table 1, Extended Data Fig. 8a,b**), a strong FRET signal was measured, proportional to the  
237 amount of controlled mGlu2-4 heterodimers on the cell surface (**Extended Data Fig. 8c**). We  
238 also showed that the FRET signal could not result from mGlu homodimers (**Extended Data**  
239 **Fig. 8d**). Because mGlu2-4 is likely co-expressed with mGlu2 and mGlu4 homodimers in  
240 native tissues, we used two methods to detect mGlu2-4 heterodimers in transfected cells. First,  
241 we used cells co-expressing mGlu2<sub>C1</sub> and mGlu4<sub>C2</sub> (**Extended Data Fig. 5d and 7d**) such that  
242 only heterodimers could reach the surface (**Fig. 4a**). Second, the wild-type mGlu subunits were  
243 co-transfected to obtain a mix of mGlu2 and mGlu4 homodimers together with the mGlu2-4  
244 heterodimer on the cell surface (**Fig. 4b**)<sup>26, 27</sup>. In both cases, a strong FRET signal was

245 measured whether the receptors were activated with the mGlu2 agonist LY379268 or  
246 antagonized with LY341495 (**Fig. 4c,d**).

247 Applied to isolated brain cells, this nanobody FRET pair generated a strong signal in  
248 the olfactory bulb (OB), PFC, striatum and hippocampus (**Fig. 4e** and **Extended Data Fig. 8e**)  
249 where mGlu2 (**Fig. 3g**) but not mGlu4 (**Fig. 3h**) homodimers have been previously detected.  
250 As a control, no signal was measured in the OB from mGlu2 KO or mGlu4 KO mice (**Fig. 4f**  
251 and **Extended Data Fig. 8g**). In agreement with these results, co-immunoprecipitation  
252 experiments with the OB using DN42 revealed the presence of endogenous mGlu2 in the same  
253 complexes as mGlu4 (**Extended Data Fig. 8f**). Notably, the cerebellum did not produce a  
254 specific signal between DN1 and DN42 (**Fig. 4e, g**), consistent with mGlu2 and mGlu4  
255 subunits being expressed in different cell types<sup>31, 39, 40</sup> (**Extended Data Fig. 4** and **Extended**  
256 **Data Fig. 6d**). Surprisingly, the slope for the cerebellum was even negative (**Fig. 3e, g** and  
257 **Extended Data Fig. 8e,g**), most probably due to the relatively high amount of mGlu4  
258 homodimers in this region that is sufficient to titrate the DN42 present at 1.6 nM in the assay,  
259 then resulting in a slight but significant decrease in FRET. In agreement with this hypothesis,  
260 the slope was no longer negative for samples from mGlu4 KO mice, whereas it remained  
261 negative in samples from mGlu2 KO mice (**Extended Data Fig. 8e, g**).

#### 262 **mGlu2-4 heterodimer FRET-based biosensor**

263 Although the above data provide strong evidence for the presence of mGlu2-4 heterodimers in  
264 various brain regions, one cannot exclude that the signal come from the proximity between  
265 mGlu2 and mGlu4 homodimers. To bring further evidence of the existence of the endogenous  
266 mGlu2-4 heterodimer, we developed a nanobody-based FRET conformational sensor for this  
267 heterodimer using DN10 and DN42, as DN10 can bind to the mGlu2-4 heterodimer in the  
268 active state only (**Extended Data Fig. 9a,b; Supplementary Table 1 and Table 2**). Of note,  
269 L-AP4, a partial agonist of the mGlu2-4 heterodimer<sup>17, 27</sup>, remains partial in promoting DN10

270 binding (**Extended Data Fig. 9a,b**), as monitored by the FRET between the nanobody and the  
271 SNAP-mGlu4 subunits. Interestingly, DN10 binding to mGlu2-4 in the presence of the mGlu4  
272 agonist L-AP4 (**Extended Data Fig. 9a,c**) was strongly potentiated by the mGlu2 agonist  
273 LY379268 (**Extended Data Fig. 9c,e**), whereas L-AP4 did not induce binding of DN10 to the  
274 control mGlu2 homodimer (**Extended Data Fig. 9d**).

275 By combining DN42 that specifically binds to the mGlu4 subunit, and DN10, which  
276 binds to the active form of mGlu2-4, we could detect mGlu2-4 activation by FRET (**Fig. 5a-f**,  
277 **Extended Data Fig. 9f**), a signal that could not result from either homodimers (**Extended Data**  
278 **Fig. 9g**). Similar data were obtained in cells expressing the controlled mGlu2-4 (**Fig. 5a-c**) and  
279 in cells expressing both mGlu2 and mGlu4 subunits (**Fig. 5d-f**). Under the latter conditions,  
280 both homodimers were present on the cell surface along with the heterodimer (**Fig. 5d**),  
281 demonstrating that they do not interfere with the specific signal generated by the active  
282 heterodimer. Under these conditions, the agonist LY379268 generated a large signal that is  
283 largely inhibited by the antagonist LY341495 while L-AP4 generated a smaller signal (**Fig. 5b**,  
284 **d,e**). Moreover, as previously reported<sup>17, 27, 34</sup>, a strong positive cooperativity was observed  
285 between the mGlu2 and the mGlu4 agonists on the heterodimer, illustrated here with the large  
286 increase in L-AP4 potency by a low concentration of LY379268 (**Fig. 5c,f** and **Extended Data**  
287 **Fig. 10d**). These data are also consistent with the IP<sub>1</sub> production data obtained in cells  
288 expressing controlled mGlu2-4, or both mGlu2 and mGlu4 (**Extended Data Fig. 10a-c**).  
289 Altogether, these results show that DN10 and DN42 can be used to detect the active form of  
290 the mGlu2-4 heterodimer.

291 This mGlu2-4 biosensor also detected the activation of endogenous mGlu2-4  
292 heterodimer in dissociated brain cells as revealed by the synergy between the mGlu2 and  
293 mGlu4 agonists. The activation of the endogenous mGlu2-4 was revealed by the large FRET  
294 signal induced by LY379268 in all regions where the mGlu2-4 heterodimer was detected – i.e.

295 the OB, PFC, striatum and hippocampus-, but not in the cerebellum (**Fig. 5g**). As expected, the  
296 LY379268 effect disappeared in the mGlu4 KO brain samples. In addition, in the OB (**Fig. 5h**),  
297 L-AP4 potency was increased by a low concentration of LY379268 (**Fig. 5i** and **Extended**  
298 **Data Fig. 10e**), as observed in transfected cells. Finally, a nice correlation in agonist potencies  
299 measured in transfected cells and in brain samples was observed (**Extended Data Fig. 10f** and  
300 **Extended Data Table 3**). Altogether these results bring further direct evidence for the  
301 existence of the mGlu2-4 heterodimer in different brain regions.

### 302 **mGlu2-4 heterodimer brain distribution**

303 Intriguingly, we detected higher signals using the mGlu2-4 “detector” (**Fig. 4e**), than the  
304 mGlu4 homodimer “detector” (**Fig. 3h**) in most brain areas outside the cerebellum. Such a  
305 difference in signal intensity was not due to a difference in FRET efficacy between the  
306 nanobodies per dimeric combination. This is best illustrated by the perfect correlation between  
307 the FRET obtained with either the mGlu4 specific homodimer DN42 “detector,” or the mGlu2-  
308 4 specific heterodimer DN1-DN42 “detector,” relative to the cell surface expression of the  
309 SNAP or CLIP subunits (**Fig. 6a**). These data indicate that there are more mGlu2-4  
310 heterodimers than mGlu4 homodimers in most regions outside the cerebellum (**Fig. 6b**).

### 311 **Discussion**

312 Our study describes an innovative and general method for the quantification and analysis of  
313 endogenous multi-subunit membrane proteins using nanobody-based optical sensors. Using  
314 this method, we provide direct evidence for the existence of mGlu2-4 heterodimers in different  
315 brain areas. Surprisingly, our results revealed that most mGlu4 subunits are likely associated  
316 with another subunit, such as mGlu2, in most brain regions outside the cerebellum.

317 Our method combines the high spatial resolution of the TR-FRET technology (< 15  
318 nm) with the small size of single-domain nanobodies (~ 2.5 nm) to detect low amounts of

319 endogenous subunits in native tissues. Obtaining such information in native membranes is  
320 essential, as lipid composition and ions likely play an important role in stabilizing protein  
321 complexes <sup>41</sup>. No chemical fixation or biochemical treatments of the biological sample are  
322 required, in contrast to other analyses, thus preventing the conformational changes of the  
323 complex. In addition, our results prove that nanobodies have great potential as TR-FRET  
324 probes, which help to solve the shortcomings of small molecules in terms of specificity, which  
325 limits their use in TR-FRET experiments <sup>4</sup>. Nanobodies have hydrophilic properties, in contrast  
326 to small molecules that can be hydrophobic, and help overcome the limitations of classical  
327 antibodies in recognizing specific protein conformations. Nanobodies are small antibodies (ten  
328 times smaller than IgGs), easy to engineer, and display good and rapid tissue penetrance <sup>42</sup>.  
329 They often recognize conformational and cryptic epitopes, not accessible to classical  
330 antibodies. Our method can be applied to any cell surface proteins, including ligand-gated ion  
331 channels or transporters <sup>5</sup>. In addition, our method is versatile as the fluorophores can be  
332 covalently attached to small ligands, antibody fragments or common antibodies. Finally, our  
333 method does not require a high level of expertise or expensive equipment. It only entails  
334 working in microplates with standard biochemical protocols and a standard commercial TR-  
335 FRET reader. However, the TR-FRET approach may not be appropriate for the detection of  
336 heterodimers using microscopy in brain slices, due to the low quantum yield of the donor and  
337 the need for a special equipment for the time delay between the excitation and the measurement  
338 of the emission signal<sup>43</sup>. It can however be used on cultured neurons (**Extended Data Figure**  
339 **2**). However, the use of fluorophores compatible with conventional FRET microscopy may  
340 allow the detection of dimers in neuronal sub-compartments using microscopy in cultured  
341 neurons with a better precision.

342 Our approach has two major advantages in investigating the endogenous mGlu  
343 heterodimers <sup>27-29, 44</sup>. First, it analyzes the heterodimer entity directly and not its downstream

344 signaling that could result from cross-talk between signaling pathways<sup>45</sup>. Second, our  
345 biosensors are good reporters of the conformational change of the receptor during activation,  
346 as other sensors of the mGlu receptors<sup>27, 33, 34</sup>. However, whether this approach can be used to  
347 detect mGluR activation in real time remains to be tested. For this, the use of fluorophores  
348 compatible with conventional FRET measurement will be necessary. It will also be essential  
349 to take into consideration the ON rate of binding of the nanobody that recognizes the active  
350 form of the receptor, as this may be much slower than the ON rate of mGluR activation that  
351 occurs in the sub-millisecond time scale<sup>35, 46</sup>. It is clear that this second point will generate  
352 limitations for such analysis. Finally, the pharmacological signature of our new sensors using  
353 orthosteric ligands could be defined in transfected cells, and could then be observed in native  
354 brain samples.

355         Our study reveals an intriguing distribution of mGlu4 homodimers, mainly found in the  
356 cerebellum, where they do not form detectable heterodimers with mGlu2 as expected as these  
357 two subunits are expressed in different types of neurons in the cerebellum. These data appear  
358 as an excellent control for our assay. The absence of significant detection of mGlu4 homodimer  
359 in most brain regions does not exclude that some homodimers may be present. Indeed, mGlu4  
360 homodimers were proposed at hippocampal-mPFC and amygdala-mPFC synapses<sup>28</sup>, and at  
361 corticostriatal synapses<sup>29</sup> suggesting that our approach is not sensitive enough to detect those  
362 homodimers. However, this conclusion was based on the use of mGlu4 PAMs inactive at  
363 mGlu2-4 such as *N*-phenyl-7-(hydroxyimino)cyclopropa[b] chromen-1a-carboxamide  
364 (PHCCC). Since the effect of these compounds on mGlu4-3, 4-7 or 4-8 heterodimers<sup>26, 30</sup> is not  
365 known, further pharmacological studies on these heterodimers will be necessary to clarify this  
366 issue. Whatever, our data clearly show that in many brain regions, there are more mGlu2-4  
367 than mGlu4-4, as a larger signal could be detected with the mGlu2-4 detector, despite a very  
368 similar FRET efficacy per dimer. Interestingly, an astonishing distribution of the mGlu2-4

369 heterodimer was observed, with a high expression in the OB and PFC, in agreement with the  
370 demonstration of mGlu2-4 at thalamo-mPFC synapses <sup>28</sup>, where they would coexist with  
371 mGlu2 homodimers, without excluding low amounts of mGlu4 homodimers. This is consistent  
372 with the link between the mGlu2 subunit and psychiatric diseases involving the PFC <sup>47</sup>.

373 Future studies on the existence of other mGlu heterodimers are crucial for assessing the  
374 physiological role of the mGlu receptors in the brain, potential new drug targets. Indeed, mGlu4  
375 heterodimers could explain the effect of an mGlu4 allosteric modulator acting in the basal  
376 ganglia <sup>48</sup> whereas it has no effect on the mGlu4 homodimer <sup>29</sup>. In addition, mGlu7  
377 heterodimers could also contribute to the enigmatic function of the mGlu7 subunit due to its  
378 very low glutamate potency <sup>49</sup>, and the effect of mGlu7 negative allosteric modulators with a  
379 context-dependent activity <sup>50</sup>. Further studies are necessary to clarify these issues, as well as  
380 the functional role and therapeutic potential of these mGlu heterodimers, a step that will require  
381 the development of specific ligands for these receptor species.

382 In conclusion, we have reported a general and versatile approach compatible with the  
383 quantification and functional analysis of membrane proteins from endogenous native tissues,  
384 without disrupting the membrane environment, but the availability of specific ligands is a major  
385 limitation. However, the number of antibodies targeting these proteins, including nanobodies,  
386 is rapidly expanding, even those selective of a conformational state <sup>13, 16, 18</sup>.

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388

### 389 **Acknowledgments**

390 We wish to thank Damien Nevoltris and Damien Meyer (IRCM, Marseille, France) for the  
391 screening of the nanobodies, and preparing the Fc-DN42 construct, respectively. We thank the  
392 Arpège platform of the Institut de Génomique Fonctionnelle for providing facilities and

393 technical support, the imaging facility Montpellier Ressources Imagerie (MRI), member of the  
394 national infrastructure France-BioImaging supported by the French National Research Agency  
395 (ANR-10-INBS-04, Investments for the Future), the animal facility RAM-iExplore from  
396 BioCampus (Montpellier, France) and Perkin Elmer Cisbio for providing reagents. We thank  
397 Cyril Goudet and Thierry Durroux (IGF, Montpellier, France) for providing the mGlu4 KO  
398 mice and for the assistance in the TR-FRET microscopy, respectively. J.M. and C.X. were  
399 supported by Sino-French Cai Yuanpei program (grant number 201604490217 and  
400 201304490188, respectively). P. R. and J.-P. P. were supported by the Centre National de la  
401 Recherche Scientifique (CNRS, PICS n°07030, PRC n°1403), the Institut National de la Santé  
402 et de la Recherche Médicale (INSERM, IRP BrainSignal), the Fondation pour la Recherche  
403 Médicale (FRM) (Equipe DEQ20170336747), the Eidos collaborative laboratory with Perkin  
404 Elmer Cisbio, the Franco-Chinese Joint Scientific and Technological Commission (CoMix)  
405 from the French Embassy in China and the LabEx MAbImprove (Grant NR-10-LABX-5301).  
406 P.R. and J.-P. P. were supported by the ANR (Grant ANR-15-CE18-0020-01, ANR-20-CE18-  
407 0011-01 and ANR-20-CE44-0006-02). J. L. was supported by the Program of Bioland  
408 Laboratory (Guangzhou Regenerative Medicine and Health Guangdong Laboratory; grant  
409 number 2010A080813001), the Ministry of Science and Technology of China (grant number  
410 2018YFA0507003), the National Natural Science Foundation of China (NSFC) (grant numbers  
411 81720108031, 31721002, and 81872945), the Program for Introducing Talents of Discipline to  
412 the Universities of the Ministry of Education (grant number B08029), Key Program of Natural  
413 Science Foundation of Hubei Province (grant number 2019ACA128) and Wuhan  
414 (2019020701011481). P.C. were supported by the FUI of the French government (FUI,  
415 Cell2Lead project). J.G.M. was supported by the National Institutes of Health grants  
416 R01MH084894 and R01MH111940. Mouse picture in Figure 1 and Extended Data Fig. 3a was  
417 modified from Servier Medical Art (<http://smart.servier.com/>), licensed under a Creative

418 Common Attribution 3.0 Generic License (<https://creativecommons.org/licenses/by/3.0/>).  
419 Mouse brain in Extended Data Fig. 3a was from Figdraw (<https://www.figdraw.com/>). Pictures  
420 of the microplate in Figure 1 and Extended Data Fig. 3a were from Perkin Elmer Cisbio.

#### 421 **Author contributions**

422 J.M. developed the FRET-based detectors and the FRET-based conformational change sensors,  
423 and performed the sensors experiments, IP<sub>1</sub> assays, co-immunoprecipitation and  
424 immunoblotting. C.X. proposed the idea and set up the protocol for the nanobody-based sensors  
425 for the detection of the expression and activation of mGlu2 in brain dissociated cells, and  
426 performed the DN1-DN10 sensor experiments in HEK-293 cells and brain dissociated cells  
427 and IP<sub>1</sub> assays. P.-A. performed the tissue immunofluorescence. S.R. designed and performed  
428 experiments with the FRET-based detectors and conformational change sensors. M.M.  
429 performed TR-FRET microscopy imaging, nanobody production, purification and labeling.  
430 R.Z. performed nanobody production, purification, labeling and co-immunoprecipitation. P.S.  
431 and E.B. developed the FRET-based conformational change biosensor for the mGlu2  
432 homodimer in HEK-293 cells. J.A.J.B. and J.L.M. provided brain samples and trained J.M. for  
433 brain samples preparation. J.G.-M. prepared brain samples for the wild-type and mGlu2 KO  
434 mice. P.C. screened for the mGlu2 and mGlu4 nanobodies, and prepared the Fc versions. J.L.,  
435 J.-P.P. and P.R. conceived experiments, supervised the work and wrote the manuscript.

#### 436 **Competing interests**

437 P.R. and J.-P.P. are funded by Perkin Elmer CisBio through the collaborative laboratory Eidos.  
438 The remaining authors declare no competing interests.

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441

442 **Figure legends**

443 **Fig. 1 | Nanobody-based sensors to detect the expression and activation of endogenous**  
444 **membrane proteins.** Schemes illustrating the design of two kinds of extracellular nanobody-  
445 based sensors either to detect membrane protein subunit assembly or their conformational  
446 change upon activation. These sensors are compatible with the detection of endogenous  
447 membrane proteins in dissociated cells from different mouse brain regions.

448 **Fig. 2 | A nanobody-based biosensor to detect the expression and activation of the mGlu2**  
449 **receptor in both transfected cells and dissociated brain tissues.** (a) Schematic  
450 representation of the TR-FRET-based mGlu2 receptor conformational sensor in presence of  
451 the agonist or antagonist to stabilize the active and inactive states, respectively. This sensor  
452 was made of 7.5 nM donor nanobody DN1-Tb is in pink (Lumi4-Tb shown as a circled “D”) and  
453 15 nM acceptor nanobody DN10-d2 in purple (d2 shown as a circled “A”). (b) TR-FRET  
454 signal measured in mGlu2 transiently transfected HEK-293 cells or in mock cells in presence  
455 of the biosensor with agonist LY379268 (1  $\mu$ M) or antagonist LY341495 (10  $\mu$ M). Data are  
456 represented as mean  $\pm$  SEM of triplicate measurements in three independent experiments. (c)  
457 Analysis of the relative expression of mGlu2 receptor in brain tissues in the presence of  
458 LY379268 (1  $\mu$ M). TR-FRET signal indicated the slope values of the relative linear  
459 quantification experiments. Each dot represents a TR-FRET experiment performed on the  
460 indicated brain tissue of one mouse (n=5 for all samples of wild-type mice, n=2 for cerebellum  
461 of mGlu2 KO mice group and n=3 for cerebellum of mGlu4 KO mice group). Data are mean  
462  $\pm$  SEM and analyzed using one-way ANOVA followed by Dunnett’s *post-hoc* test (compared  
463 with the cerebellum of mGlu2 KO group), with \*\*\*\* $P < 0.0001$  for all, except for midbrain  
464 (\*\* $P = 0.0044$ ). (d and g-i) Dose-dependent effects of the ligands on the TR-FRET signal of  
465 the biosensor measured in HEK-293 cells transiently co-transfected with mGlu2 and the high  
466 affinity glutamate transporter EAAC1 (d) or in dissociated cells from the cortex (g),

467 hippocampus (*h*), or cerebellum (*i*). **(e)** Dose-dependent effects of the indicated ligands on IP<sub>1</sub>  
468 accumulation measured on HEK-293 cells co-transfected with mGlu2, EAAC1, and the  
469 chimeric G protein Gqi9. In *d-e* and *g-i*, data are mean ± SEM of three independent experiments  
470 performed in triplicate and normalized to the response of LY379268. **(f)** Correlation between  
471 the potencies (pEC<sub>50</sub>) determined with the indicated agonists on the conformational biosensor  
472 (*d*) and IP<sub>1</sub> assay (*e*). Data are mean ± SEM of three independent experiments.

473 **Fig. 3 | Relative quantification of endogenous mGlu2 and mGlu4 homodimers by FRET.**

474 **(a and d)** Schematic representation of mGlu2 (*a*) and mGlu4 (*d*) homodimer that can be  
475 detected by FRET-based “detectors”. mGlu2 homodimer “detector” (*a*) was made of donor and  
476 acceptor labeled nanobodies (DN1-Tb and DN1-d2 both at 25 nM). mGlu4 homodimer  
477 “detector” (*d*) was made of DN42-Tb and DN42-d2 (both at 1.6 nM). **(b and e)** TR-FRET  
478 signal measured in HEK-293 cells transfected with the mGlu2 (*b*) or mGlu4 receptors (*e*), or  
479 in mock cells with the indicated “detectors” and the agonist LY379268 (1 μM) (*b*) or L-AP4  
480 (100 μM) (*e*) and the antagonist LY341495 (10 μM). **(c and f)** TR-FRET signal measured in  
481 HEK-293 cells transfected with the indicated mGlu receptors or in mock cells with LY341495  
482 (10 μM). In *b*, *e* and *c*, *f*, data are mean ± SEM of triplicate measurements from one  
483 representative experiment in three independent experiments. **(g and h)** Relative expression of  
484 the mGlu2 (*g*) and mGlu4 (*h*) homodimers in the indicated brain tissues by their respective  
485 “detectors”. TR-FRET signal indicated the slope values of the relative linear quantification  
486 experiments. Each dot represents a TR-FRET measurement from one mouse (n=5 for all  
487 sample of wild-type mice, n=2 for cerebellum of mGlu2 KO mice group and n=3 for  
488 cerebellum of mGlu4 KO mice group). Data are mean ± SEM and analyzed using a one-way  
489 ANOVA followed by a Dunnett’s *post-hoc* test (control group is the cerebellum of mGlu2 KO  
490 group (*g*), with \*\*\*\**P* < 0.0001 for all, except for striatum (\**P* = 0.0164) and midbrain (ns  
491 *P* = 0.9996) or mGlu4 KO group (*insert h*), with ns *P* > 0.05 for olfactory bulb (> 0.9999),

492 striatum (0.0896), hippocampus (0.8253) and midbrain (0.0615) and prefrontal cortex (\*\* $P$  =  
493 0.0031), or a Welch's ANOVA test followed by a Dunnett's T3 *post-hoc* test (compared with  
494 the cerebellum of mGlu4 KO group) (*h*), with ns  $P > 0.05$  for olfactory bulb ( $> 0.9999$ ),  
495 striatum (0.2251), hippocampus (0.8436) and midbrain (0.1304) and \* $P < 0.05$  for prefrontal  
496 cortex (0.0425) and the cerebellum of the wild-type group (0.0435).

497 **Fig. 4 | Relative quantification of the mGlu2-4 heterodimer by FRET.** (a, b) Schematic  
498 representation of the FRET-based “detector” for the mGlu2-4 heterodimer with mGlu2<sub>C1</sub> and  
499 mGlu4<sub>C2</sub> (*a*) or wild-type mGlu2 and mGlu4 are co-expressed (*b*). This “detector” is made of  
500 DN42-Tb (1.6 nM) and DN1-d2 (25 nM). (c, d) TR-FRET signal measured in HEK-293 cells  
501 transiently transfected with mGlu2<sub>C1</sub> and mGlu4<sub>C2</sub> (*c*), or co-transfected with both wild-type  
502 mGlu2 and mGlu4 (*d*) or on mock cells in presence of the “detector” with LY379268 (1 μM)  
503 or LY341495 (10 μM). Data are mean ± SEM of triplicate measurements from one  
504 representative experiment in three independent experiments. (e–g) Relative expression of the  
505 mGlu2-4 heterodimer in the indicated brain tissues by the “detector”. TR-FRET signal  
506 indicated the slope values of the relative linear quantification experiments. Each dot represents  
507 a TR-FRET experiment performed on the indicated brain tissue of one mouse. For *e*, n=5 for  
508 all sample of wild-type mice except for the olfactory bulb (n=4), and cerebellum of mGlu4 KO  
509 mice group (n=3). For *f*, n=3 for the olfactory bulb of wild-type, mGlu2 KO and mGlu4 KO.  
510 For *g*, cerebellum of wild-type mice (n=8), mGlu2 KO (n=3) and mGlu4 KO mice (n=6). Data  
511 are mean ± SEM and analyzed using a one-way ANOVA followed by a Dunnett's *post-hoc*  
512 test, compared with the cerebellum of mGlu4 KO group (*e*), with \*\*\*\* $P < 0.0001$  for olfactory  
513 bulb and prefrontal cortex, \*\* $P < 0.01$  for striatum (0.0054), hippocampus (0.0062),  
514 cerebellum (0.0045) and ns  $P > 0.05$  for midbrain (0.1533); compared with the olfactory bulb  
515 of mGlu4 KO group (*f*), with \*\*\* $P < 0.001$  for wild-type group (0.0002) and ns  $P > 0.05$  for

516 mGlu2 KO group (0.8100); or compared with the cerebellum of the wild-type group (g), with  
517 ns  $P > 0.05$  for mGlu2 KO group (0.4120), \*\*\* $P < 0.001$  for mGlu4 KO group (0.0005).

518 **Fig. 5 | A conformational biosensor detects the activation of the endogenous mGlu2-4**  
519 **heterodimer. (a and d)** Schematic representation of the conformational biosensor for the  
520 mGlu2-4 heterodimer when the mGlu2<sub>C1</sub> and mGlu4<sub>C2</sub> constructs are used to have only the  
521 heterodimer at the surface (a) or when wild-type mGlu2 and mGlu4 are co-expressed (d) in the  
522 presence of the indicated mGlu2 and mGlu4 agonists and antagonist. This biosensor is made  
523 of DN42-Tb (1.6 nM) and DN10-d2 (25 nM). **(b, e and h)** TR-FRET signal measured in HEK-  
524 293 cells co-transfected with mGlu2<sub>C1</sub> and mGlu4<sub>C2</sub> (b), or with wild-type mGlu2 and mGlu4  
525 (e) or the dissociated cells from the olfactory bulb (h) with the biosensor and the agonists  
526 LY379268 (1  $\mu$ M) or L-AP4 (100  $\mu$ M) and/or the antagonist LY341495 (10  $\mu$ M). Data are  
527 analyzed using a One-way ANOVA followed by a Tukey's *post-hoc* test, with \*\*\*\* $P \leq 0.0001$   
528 and ns  $P > 0.05$ . For b and e, data are mean  $\pm$  SEM of four independent experiments performed  
529 in triplicate and normalized to the response of LY341495 (0%) and LY379268 (100%). For h,  
530 data are mean  $\pm$  SEM of triplicate measurements from one representative experiment in three  
531 independent experiments. **(c, f and i)** Dose-dependent effects of the indicated ligands on the  
532 TR-FRET signal of the biosensor measured in HEK-293 cells co-transfected with mGlu2<sub>C1</sub> and  
533 mGlu4<sub>C2</sub> (c) or with both wild-type mGlu2 and mGlu4 (f), or in the dissociated cells from the  
534 olfactory bulb (i). Data are mean  $\pm$  SEM of *n* independent experiments performed in duplicate  
535 (c and f, *n*=3) or in triplicate (i, *n*=5) and normalized to the response of LY379268. **(g)** TR-  
536 FRET signal of the biosensor where the ratio in the presence of LY379268 (1  $\mu$ M) and  
537 LY341495 (10  $\mu$ M) is measured. Each dot represents one measurement on the indicated brain  
538 tissue of WT or mGlu4 KO mouse (*n*=4, except the striatum group of mGlu4 KO (*n*=3)). Data  
539 are analyzed using two-tailed Student's *t* test, with \*\*\*\* $P \leq 0.0001$ , \*\* $P \leq 0.01$ , \* $P \leq 0.05$ ,  
540 and ns  $P > 0.05$ , and the exact *P* values have been indicated.

541

542 **Fig. 6 | mGlu2-4 heterodimers are predominant over the mGlu4 homodimers outside the**  
543 **cerebellum. (a)** Relative quantification of mGlu4-4 homodimers and mGlu2-4 heterodimers  
544 based on the TR-FRET signal between the two subunits in the dimer using either the SNAP-  
545 or CLIP-tag fluorescent substrates or the “detector” nanobodies. Data are mean  $\pm$  SEM of  
546 triplicate measurements from one out of three experiments. **(b)** Relative amounts of the mGlu4  
547 homodimer compared with the mGlu2-4 heterodimer, as indicated by the respective circle size,  
548 in the adult mouse brain according to the quantification with their respective “detectors”. The  
549 relative amounts of the mGlu2 homodimer determined with its “detector” are indicated in a  
550 separate brain since the sensitivity of this detector is different.

551

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## 674 **Materials and Methods**

### 675 **Animal ethics**

676 This project followed the Animal Welfare Body guidelines and was approved by the internal  
677 Ethics Committee of the Institut de Génomique Fonctionnelle (Montpellier, France). Wild-type  
678 mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France), knock-out mGlu2  
679 mice<sup>51</sup> were kindly provided by Dr. Gonzalez Maeso (Virginia Commonwealth University  
680 School of Medicine, Richmond, VA, USA), while knock-out mGlu4 mice<sup>52</sup> were available at  
681 the Institut de Génomique Fonctionnelle (Montpellier, France). Animals were housed under a  
682 12 h light/dark cycle at 23 ± 2°C with a relative humidity of 53 ± 10%. Mice had access to  
683 water and food *ad libitum*.

### 684 **Reagents, cell lines, and plasmids**

685 DCG-IV, LY341495, LY379268, and LY354740 were purchased from Tocris Bioscience  
686 (Bristol, UK). Glutamate was purchased from Sigma-Aldrich (Saint Louis, MO, USA). LSP4-  
687 2022 was provided by Dr. F. Acher (Paris, France). Anti-c-Myc-d2, anti-6His-d2, SNAP-  
688 Lumi4-Tb, Lumi4-Tb-NHS, d2-NHS, and Tag-lite® buffer were kindly supplied by Perkin  
689 Elmer Cisbio (Codolet, France).

690 HEK-293 cells (ATCC, CRL-1573, lot: 3449904) were cultured in DMEM (Thermo Fischer  
691 Scientific, Courtaboeuf, France) supplemented with 10% (v/v) fetal bovine serum (FBS,  
692 Sigma-Aldrich) at 37°C and 5% CO<sub>2</sub>. HEK-293F cells were cultured in suspension in SMM  
693 293-TI animal-free and serum-free at 37°C and 5% CO<sub>2</sub> with shaking at 200 rpm to produce  
694 nanobodies Fc-DN42 or Fc-GFP. The absence of mycoplasma was routinely checked using the  
695 MycoAlert Mycoplasma Detection Kit (Lonza, Amboise, France), in accordance with the  
696 manufacturer's protocol.

697 The pRK5 plasmids encoding wild-type rat mGlu1-8, with the HA- and SNAP-tags next to the  
698 human mGlu5 signal peptide, have been described previously<sup>26</sup>. For the quality control system,  
699 we used the mGlu2/4 C1 or C2 plasmids tagged with HA or Flag, with or without SNAP tags  
700<sup>36</sup>. The sequence coding for C1 and C2 contains the coiled-coil region of the C terminus of  
701 GABA<sub>B1a</sub> or GABA<sub>B2</sub>, and the endoplasmic reticulum retention signal KKTN, which allows to  
702 control the trafficking of the constructs to the cell surface. The other plasmids, namely the  
703 glutamate transporter EAAC1 and the chimeric G protein G $\alpha_{q9}$ , were previously described<sup>26</sup>.

704 To construct the cDNA of the Fc-DN42 and Fc-GFP nanobodies<sup>53</sup>, the monomeric DN42 and  
705 anti-GFP sequences were subcloned into pcDNA3.1 (+). The secreted signal peptide of human  
706 interleukin (IL-2) was introduced before the N-terminal of the nanobody, allowing the secretion  
707 of the protein into the culture medium. The C-terminus of the nanobody was fused with the  
708 sequence encoding the Fc region of human immunoglobulin IgG<sub>1</sub>. The 6xHis tagged sequence

709 was fused at the C-terminus for the purification and separation of nanobodies by affinity  
710 chromatography with metal chelation.

#### 711 **Library construction and DN42 selection**

712 DN42 targeting the mGlu4 subunit was selected from the V<sub>H</sub>H library after all the procedures  
713 including the llama immunization, library construction, and selection of nanobodies targeting  
714 mGlu4 were performed as described previously <sup>17</sup>.

715 Bacteria were then infected with the KM13 helper phage, and phage-containing pellets were  
716 purified by two selection rounds on rat mGlu4 receptor transfected into  $2 \times 10^7$  HEK-293T  
717 cells. Each round was preceded by a depletion step on cells that were not transfected, and  
718 positive selection was performed in the presence of an excess of anti-HEK-293 cells. *E. coli*  
719 TG1 bacteria were infected with eluted phages and used for the sequencing and production of  
720 the nanobody.

#### 721 **Production and purification of nanobodies**

722 For the nanobodies DN1 and DN10, production and purification were performed as previously  
723 described <sup>15</sup>. Briefly, a *E. coli* BL21DE3 colony transformed with the pHEN phagemid carrying  
724 cDNA of the nanobody of interest was grown in LB medium. The bacteria were cultured in  
725 large scale at 37°C and the expression of the nanobody was induced by 1 mM IPTG at 28°C.  
726 Bacteria were then collected after centrifugation and treated with different buffers to extract  
727 the periplasmic proteins. Nanobody in the periplasmic space was collected from the supernatant  
728 after a centrifugation at 4°C.

729 For large-scale production of the Fc-DN42 and Fc-GFP nanobodies, HEK-293F cells were  
730 cultured at a density of  $0.6 \times 10^6$  mL<sup>-1</sup> with 180 mL fresh medium in a 2 L culture bottle at  
731 37°C and 5% CO<sub>2</sub>, shaken at 200 rpm. Of the cells,  $1-1.5 \times 10^6$  mL<sup>-1</sup> were transfected with a  
732 mixture of Fc-DN42 or Fc-GFP plasmids (225 µg in 12 mL OMEM) and PEI (675 µg in 12

733 mL OMEM). Cells were cultured for 4–7 days at 37°C and 5% CO<sub>2</sub> with 200 rpm shaking. The  
734 supernatant was collected after a 10-min centrifugation at 2,000 × g and 4°C.

735 The His-tagged nanobodies from both bacteria and HEK-293F cells were then purified from  
736 the supernatant using Ni-NTA purification (Qiagen, Hilden, Germany) in accordance with the  
737 manufacturer's instructions. Finally, the nanobodies were purified by size-exclusion  
738 chromatography on a Superdex 200 10/300 column for Fc-DN42 and Fc-GFP, and Superdex  
739 75 10/300 column for DN1 and DN10 (GE Healthcare, UK) in phosphate buffer saline (PBS,  
740 pH = 7.4).

#### 741 **Nanobody labeling**

742 Nanobodies were dialyzed overnight at 4°C and incubated (250 µg of nanobodies per 2 mg mL  
743 <sup>-1</sup>) at 20°C with d2-NHS (Perkin Elmer Cisbio) in 0.1 M carbonate buffer (pH = 9) and Lumi4-  
744 Tb-NHS in 50 mM phosphate buffer (pH = 8) at a molar ratio of 6 or 12 for 45 or 30 min,  
745 respectively. The nanobodies were then purified using a gel filtration column (NAP-5, GE  
746 Healthcare) in 100 mM phosphate buffer (pH = 7). The final molar ratio, namely the number  
747 of fluorophores per nanobodies, was calculated as the fluorophore concentration/conjugated  
748 nanobody concentration, and the conditions were set up for a ratio between 2 and 3 (for DN1  
749 and DN10), 2 and 4 (for Fc-DN42 labeled with d2), or 5 and 8 (for Fc-DN42 labeled with  
750 Lumi4-Tb). The concentration of fluorophores in the labeled fraction was calculated as the  
751 OD/ε for each fluorophore (OD at 340 nm and ε = 26,000 M<sup>-1</sup> cm<sup>-1</sup> for Lumi4-Tb, and OD at  
752 650 nm and ε = 225,000 M<sup>-1</sup> cm<sup>-1</sup> for d2), while that of nanobodies was determined by the OD  
753 at 280 nm (OD<sub>280</sub>). The conjugated concentration was calculated as OD<sub>280</sub> - (OD<sub>fluo</sub>/RZ<sub>max</sub>)/ε  
754 nanobody, with RZ<sub>max</sub> = OD<sub>fluo</sub>/OD<sub>280</sub>. After purification, labeled nanobodies were  
755 supplemented with 0.1% bovine serum albumin (BSA) and stored at -20°C.

#### 756 **TR-FRET binding measurements**

757 The FRET signal was determined by measuring the sensitized d2 acceptor emission (Em 665  
758 nm) and Lumi4-Tb donor emission (Em 620 nm) using a 50  $\mu$ s delay and 450  $\mu$ s integration  
759 upon excitation at 337 nm. All data were obtained using PHERAstar FS (BMG LabTech,  
760 Ortenberg, Germany). The TR-FRET ratio was calculated as  $\text{Em } 665 \text{ nm} / \text{Em } 620 \text{ nm} \times 10^4$  as  
761 previously described <sup>15</sup>.

762 HEK-293 cells were co-transfected with rat SNAP-tagged mGlu and EAAC1 (unless otherwise  
763 indicated) using Lipofectamine in a 100 mm cell culture dish in accordance with the  
764 manufacturer's instructions. Twenty-four hours after transfection, cells were plated in  
765 polyornithine-coated, white 96-well plates (Greiner Bio-One) at  $10^5$  cells/well and cultured  
766 overnight at 37°C and 5% CO<sub>2</sub> for adherent cell experiments. Cells were labeled with 100 nM  
767 SNAP-Lumi4-Tb in DMEM-GlutaMAX (Thermo Fischer Scientific) for 2 h at 37°C, and then  
768 washed three times with Krebs buffer (10 mM HEPES pH 7.4, 146 mM NaCl, 4.2 mM KCl, 1  
769 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 5.6 mM glucose, and 0.1% BSA). For suspension experiments,  
770 cells were frozen at -80°C with 10% DMSO and FBS, and then washed three times with Krebs  
771 buffer before use. Five microliters of cells were plated in a white, small volume 384-well plate  
772 (Greiner Bio-One) at  $2 \times 10^4$  cells/well.

773 To determine the selectivity of Fc-DN42 for the mGlu1-8 receptors, 100 nM of nanobody and  
774 200 nM of anti-His antibody labeled with d2, and the agonists (1  $\mu$ M quisqualic acid for mGlu1;  
775 5 or 100  $\mu$ M L-AP4 for mGlu4, 6, 7, and 8; 100 nM LY379268 for mGlu2 and 3), or the  
776 antagonist (10  $\mu$ M LY341495) were applied to labeled cells in adherent cell experiments, with  
777 a total volume of 60  $\mu$ L per well. After an overnight incubation at 25°C, the TR-FRET signal  
778 between Lumi4-Tb and d2 was determined.

779 To determine the affinity of the c-Myc-tagged nanobodies (DN1 and DN10) and His-tagged  
780 nanobody Fc-DN42, 200 nM of anti-c-Myc and 100 nM of anti-His antibodies, both labeled

781 with d2, were used. The different reagents were applied to labeled cells in adherent or  
782 suspension experiments, with a total volume of 20  $\mu$ L per well incubated overnight at 25°C.  
783 The TR-FRET signal between Lumi4-Tb and d2 was determined.

#### 784 **SNAP subunit quantification**

785 Transfected HEK-293 cells were plated in polyornithine-coated, white 96-well plates (Greiner  
786 Bio-One) at  $10^5$  cells/well and cultured overnight at 37°C and 5% CO<sub>2</sub>; the cells were then  
787 labeled with 100 nM SNAP-Lumi4-Tb in DMEM-GlutaMAX for 2 h at 37 °C, followed by  
788 three washes with Krebs buffer. The signal was determined by measuring the emission intensity  
789 of Lumi4-Tb at 620 nm with a 50  $\mu$ s delay and 450  $\mu$ s integration upon excitation at 337 nm.  
790 All data were obtained using a PHERAstar FS (BMG LabTech, Ortenberg, Germany).

#### 791 **Tissue cell sample preparation**

792 To obtain dissociated cells from brain tissue, 6–8 weeks-old C57BL/6 mice (including wild-  
793 type or *Grm4*<sup>-/-</sup> male and female) were euthanized; the whole brain was dissected in cold PBS  
794 (pH = 7.4) to obtain the regions of interest, according to the Allen Mouse Brain Atlas  
795 (<https://mouse.brain-map.org/>)<sup>54</sup>. Tissues were quickly cut into small pieces using a scalpel,  
796 collected into a 1.5 mL cryogenic tube (Thermo Fisher Scientific Nalgene system 100TM,  
797 Boston, MA, USA) in 1 mL of cold cryopreservation medium (DMEM GlutaMAX  
798 supplemented with 10% FBS and 10% DMSO), frozen at –80°C in a freezing box, and stored  
799 at –80°C if not used immediately. When tissues were needed, the samples were rapidly thawed  
800 in a water bath at 37°C. The cryopreservation medium was replaced with a precooled medium  
801 (DMEM-GlutaMAX supplemented with 10% FBS), and the tissues were washed with cold  
802 PBS. Frozen tissues or fresh tissues were digested with 400  $\mu$ L of Versene solution (Thermo  
803 Fisher) for 10 min at 20°C, and the cells were dissociated by pipetting. Then, 200  $\mu$ L of DMEM  
804 supplemented with 10% FBS was added. After 5–8 min of incubation, the supernatant was

805 transferred into a new 2 mL tube. The remaining precipitate was resuspended in 300  $\mu$ L DMEM  
806 GlutaMAX supplemented with 10% FBS. After 3 min of incubation, the supernatants were  
807 transferred and combined with the supernatant. This step was repeated two more times to obtain  
808 the largest number of dissociated cells. Finally, the total dissociated cells were centrifuged at  
809 3,000 x g for 5 min, and the cell pellet was resuspended in 400  $\mu$ L of cold PBS.

#### 810 **TR-FRET measurement with the nanobody-based sensors**

811 For TR-FRET measurements in attached cells, transfected HEK-293 cells were plated in  
812 polyornithine-coated, white 96-well plates (Greiner Bio-One) at  $10^5$  cells/well and cultured  
813 overnight at 37°C and 5% CO<sub>2</sub>. Cells were starved in DMEM-GlutaMAX for 2 h at 37°C, and  
814 then washed once with Krebs buffer to reduce ambient glutamate. Donor and acceptor  
815 nanobodies and ligands were prepared in Krebs buffer and added to the plate to reach a total  
816 volume of 60  $\mu$ L per well. For TR-FRET measurements in cell suspensions, 5  $\mu$ L of HEK-293  
817 cells alone or transfected with mGlu receptor encoding plasmids were added to a low-volume  
818 384-well microplate (Greiner Bio-One) at  $2 \times 10^4$  cells/well. All reagents were added to reach  
819 a total volume of 20  $\mu$ L per well. For TR-FRET measurements in dissociated brain cells, 10  
820  $\mu$ L of cells in PBS, corresponding to different amounts of total proteins, were added to a half-  
821 area 96-well microplate (Greiner Bio-One); all reagents were added to reach a total volume of  
822 40  $\mu$ L. In all cell preparations, the plates were incubated at 25°C for 4 h or overnight, then the  
823 TR-FRET signal was measured as previously described <sup>15</sup>.

#### 824 **BCA assay for protein quantification**

825 The dissociated cells (30  $\mu$ L) in PBS were diluted twice with PBS containing 2% Triton X-100  
826 and incubated at 20°C for 1 h. The total protein quantity was measured in triplicate using a  
827 bicinchoninic acid kit (BCA1, Sigma-Aldrich) in accordance with the manufacturer's  
828 instructions using Infinite F500® microplate reader (Tecan, Crailsheim, Germany).

## 829 **Relative quantification of mGlu4-4 homodimer and mGlu2-4 heterodimer by TR-FRET**

830 Twenty-four hours after transfection, cells (100,000 cells/well in a 96-well microplate) were  
831 labeled in Tris-Krebs buffer for 1 h at 37°C and 5% CO<sub>2</sub> with the “detector” nanobodies (1.6  
832 nM Fc-DN42-Tb and 1.6 nM Fc-DN42-d2 for the SNAP-tagged mGlu4 homodimer; 1.6 nM  
833 Fc-DN42-Tb and 25 nM DN1-d2 for the co-expressed wild-type CLIP-tagged mGlu2 and  
834 SNAP-tagged mGlu4 to obtain the mGlu2-4 heterodimer) or the SNAP/CLIP substrates (100  
835 nM SNAP-Lumi4-Tb and 100 nM SNAP-d2 for the SNAP-tagged mGlu4 homodimer; 100 nM  
836 CLIP-Lumi4-Tb and 100 nM SNAP-d2 for the co-expressed wild-type CLIP-tagged mGlu2  
837 and SNAP-tagged mGlu4). After labeling, the cells were washed thrice with Tris-Krebs buffer,  
838 and the signal was recorded with 100 µL of Tris-Krebs buffer per well. The TR-FRET signal  
839 was measured using a PHERAstar FS, by the emission of d2 at 665 nm with a 50 µs delay and  
840 a 450 µs integration time after excitation at 337 nm.

## 841 **Measurements of inositol phosphate**

842 HEK-293 cells were transiently co-transfected with the mGlu receptors, EAAC1, and the  
843 chimeric G<sub>q19</sub> protein using Lipofectamine 2000. Sixteen hours after transfection, the cells were  
844 incubated with the indicated ligands and 10 mM LiCl for 30 min. The accumulated inositol-  
845 phosphate-1 (IP<sub>1</sub>) was quantified using a PHERAstar FS and the IP One HTRF assay kit (Perkin  
846 Elmer Cisbio, Codolet, France) in accordance with the manufacturer’s instructions.

## 847 **Neuronal culture and TR-FRET microscopy imaging**

848 The primary hippocampal neurons were cultured following the procedures described  
849 previously<sup>27</sup>, and after 17 days of culture, neurons were imaged. Neurons were labeled with  
850 120 nM DN1-d2 and 80 nM DN10-Tb in the presence or absence of ligands (150 nM  
851 LY379268 or 1 µM LY341495) for 2 h at 37°C. After labeling by nanobodies, the neurons

852 were washed four times before imaging to remove the unbound nanobodies. Labeling and  
853 washing were performed in imaging buffer (127 mM NaCl, 2.8 mM KCl, 1.1 mM MgCl<sub>2</sub>, 1.15  
854 mM CaCl<sub>2</sub>, 10 mM D-glucose, 10 mM HEPES, pH 7.3), supplemented with 1% BSA. Images  
855 were acquired with a homebuilt TR-FRET microscope<sup>43</sup>, following a previously described  
856 protocol<sup>27</sup>.

### 857 **Brain collection and fixation**

858 Mice were euthanized with 140 mg/kg sodium pentobarbital (Euthasol Vet®, Dômes Pharma  
859 Vétérinaire TVM, Lempdes, France) followed by cardiac perfusion with PBS. Brains were  
860 extracted and incubated overnight at 4°C in a 4% paraformaldehyde solution (Euromedex,  
861 Souffelweyersheim, France), cryoprotected for 4 days at 4°C with a 30% sucrose solution,  
862 included in an optimal cutting temperature compound (Tissue-Tek® O.C.T., Sakura Finetek,  
863 Villeneuve d'Ascq, France), and quickly frozen in ethanol cooled on dry ice. Brains were stored  
864 at -80°C until use. Frozen brains were mounted on a cryostat (Leica Biosystems, Nanterre,  
865 France) and 16 µm sagittal sections were obtained. Sections were mounted on Superfrost Plus  
866 glass slides (Microm France, Francheville, France) and kept at -20°C until use.

### 867 **Tissue immunofluorescence**

868 Fifteen to twenty sections per mouse (n = 2–3 per genotype) were used. Sections were rinsed  
869 in PBS and incubated for 1 h at 20°C with a blocking solution (3% BSA and 0.1% Triton X-  
870 100 in PBS). Sections were then incubated with the appropriate nanobody: 200 nM of DN1-d2  
871 (overnight at 4 °C) or 300 nM of Fc-DN42-d2 (1.5 h at 20°C). Sections were first washed in  
872 PBS, then in distilled water, and mounted in Fluoroshield® mounting medium with DAPI  
873 (Sigma-Aldrich, Saint-Quentin-Fallavier, France). All images were taken with a slide scanner  
874 Axio scan Z1 microscope (Carl Zeiss Microscopy, Jena, Germany) by performing full-section  
875 mosaics at 20× magnification.

## 876 **Co-immunoprecipitation and immunoblotting**

877 The mice were decapitated, brains were rapidly removed, and the OB was separated. Samples  
878 from 4–5 mice were combined and homogenized in a tissue grinder with 10  $\mu$ L lysis buffer (25  
879 mM HEPES, 150 mM NaCl, 1.0% LMNG, protease inhibitor cocktail, pH 8.0) per mg of tissue  
880 and incubated for 1 h at 4 °C. The lysate was then centrifuged at 12,000  $\times$  g for 45 min at 4 °C,  
881 and the supernatant was collected. The lysate was precleared with protein A beads (Millipore  
882 16-125, Sigma-Aldrich (Shanghai, China) for 2 h at 4°C. Fc-DN42 or Fc-GFP nanobodies were  
883 bound to protein A beads by rotating at 4°C for 3 h. The precleared lysate was then added to  
884 the antibody-bound protein A beads and incubated overnight at 4°C. The beads were washed  
885 four times with wash buffer (25 mM HEPES, 150 mM NaCl, 0.01% LMNG, protease inhibitor  
886 cocktail, pH 8.0), and proteins were eluted with unheated SDS sample buffer. Samples were  
887 subjected to SDS–PAGE using 10% polyacrylamide gels and then transferred to nitrocellulose  
888 membranes (GE Healthcare). After the transfer, membranes were blocked in TBST (25 mM  
889 Tris, 150 mM NaCl, and 0.1% Tween 20) containing 5% BSA at 20°C for 1 h. mGluR2 (Abcam,  
890 ab15672, Shanghai, China) and mGluR4 antibodies (Abcam, ab184302, Shanghai, China) were  
891 diluted 1:1,500 in blocking buffer and incubated with the membranes at 4°C overnight.  
892 Membranes were washed four times with TBST and then incubated in HRP-conjugated goat  
893 anti-mouse IgG secondary antibody (Cell Signaling Technology (CST),7076S, Massachusetts,  
894 USA) or incubated in HRP-conjugated goat anti-rabbit IgG secondary antibody (CST, 7074S)  
895 diluted 1:10,000 in TBST containing 5% nonfat milk for 1 h at 20°C. Membranes were then  
896 washed four times with TBST, and an enhanced chemiluminescent assay (Thermo Scientific)  
897 was performed to detect immunoreactive proteins. The membrane was scanned using an imager.

## 898 **Statistical analysis**

899 All data are presented as mean  $\pm$  SEM and were initially analyzed using GraphPad Prism  
900 (version 9.1.2 for Windows, GraphPad software, San Diego, California, USA) using Shapiro-  
901 Wilk's normality test. Normally distributed data sets ( $P > 0.05$ ) were analyzed using parametric  
902 tests, a two-tailed Student's t test or a one-way analysis of variance (ANOVA) followed by a  
903 Dunnett's or Tukey's *post-hoc* analysis, or a two-way ANOVA followed by a Tukey's *post-*  
904 *hoc* test, depending on the experiments analyzed. For data analyzed with the one-way ANOVA  
905 and having a significant Brown-Forsythe test ( $P < 0.05$ , meaning there is unequal variance  
906 between the different groups), data sets were analyzed using Welch's ANOVA test followed  
907 by Dunnett's T3 *post-hoc* test (recommended for  $n < 50$  /group). For all statistical analyses, a  
908 probability of 0.05 has been defined as a significant difference. The exact P values were  
909 indicated in figures or in figure legends.

#### 910 **Data availability**

911 All raw data of the main and extended Figures and Supplementary information are all included  
912 in the Data Source files. Materials and protocols are available on request.

913

#### 914 **References**

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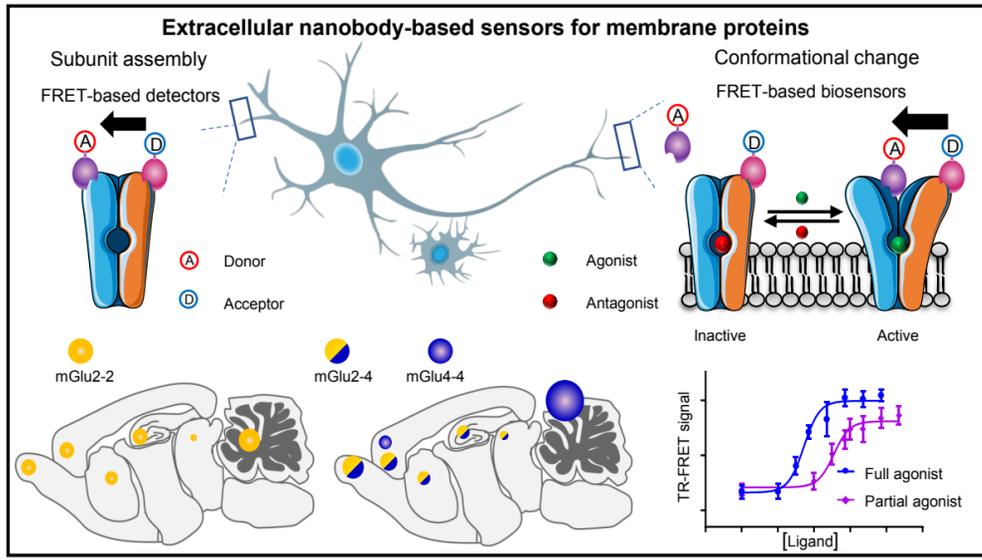
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## Graphical Abstract



## Main figures

### Optical biosensors of native membrane protein complexes reveal high proportion of mGlu heterodimers in the brain

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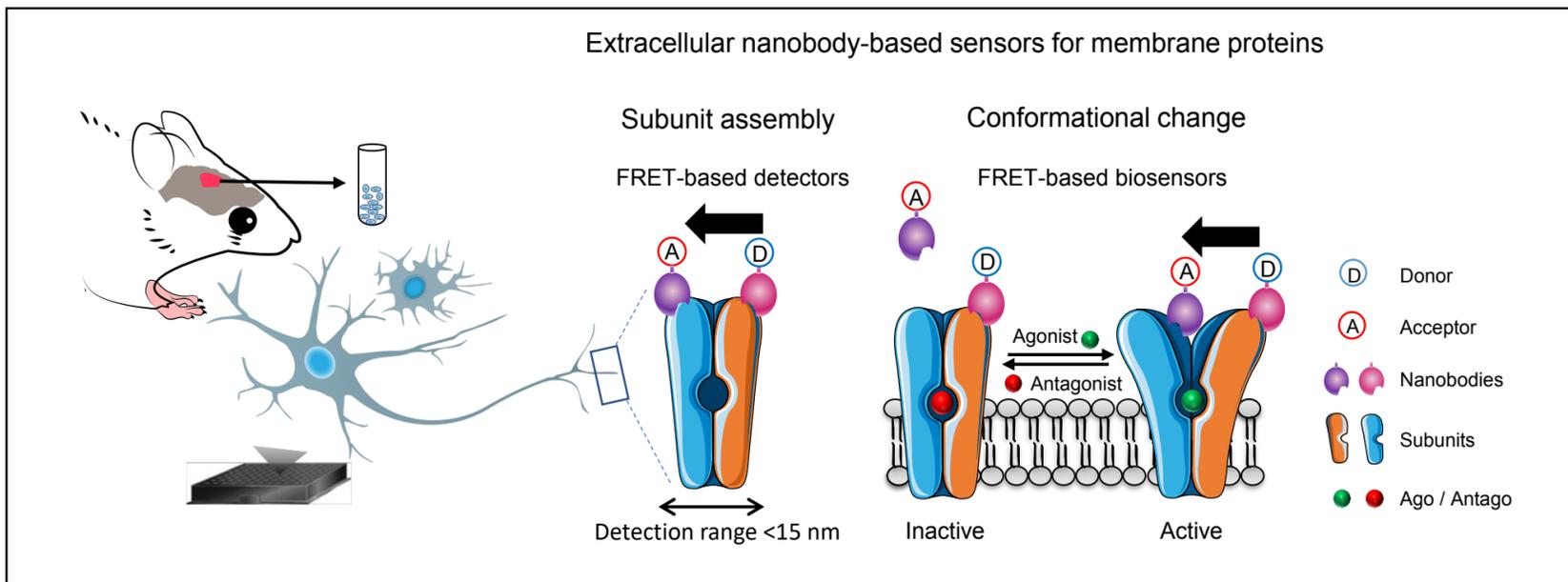
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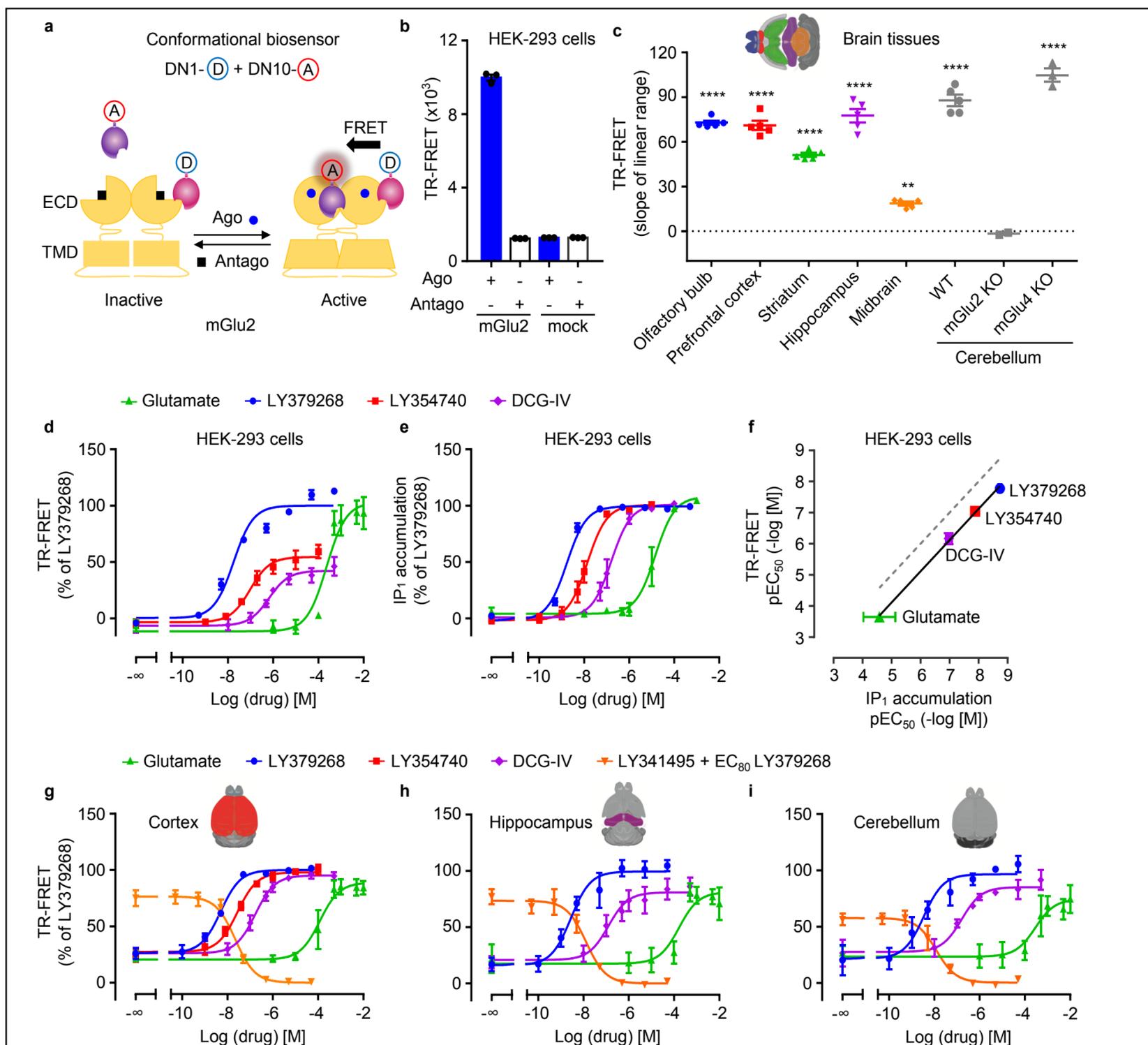
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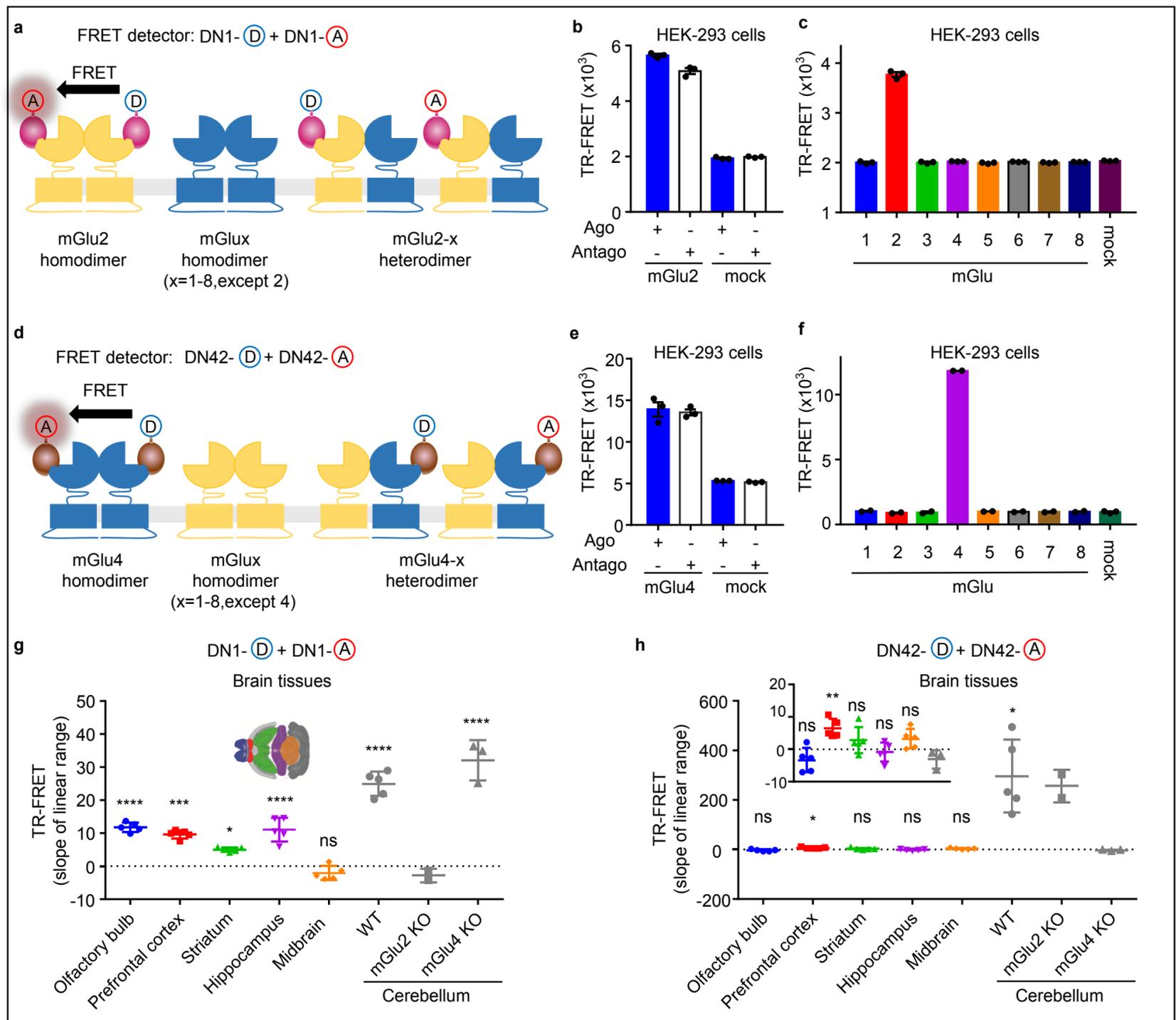
Figure 1.



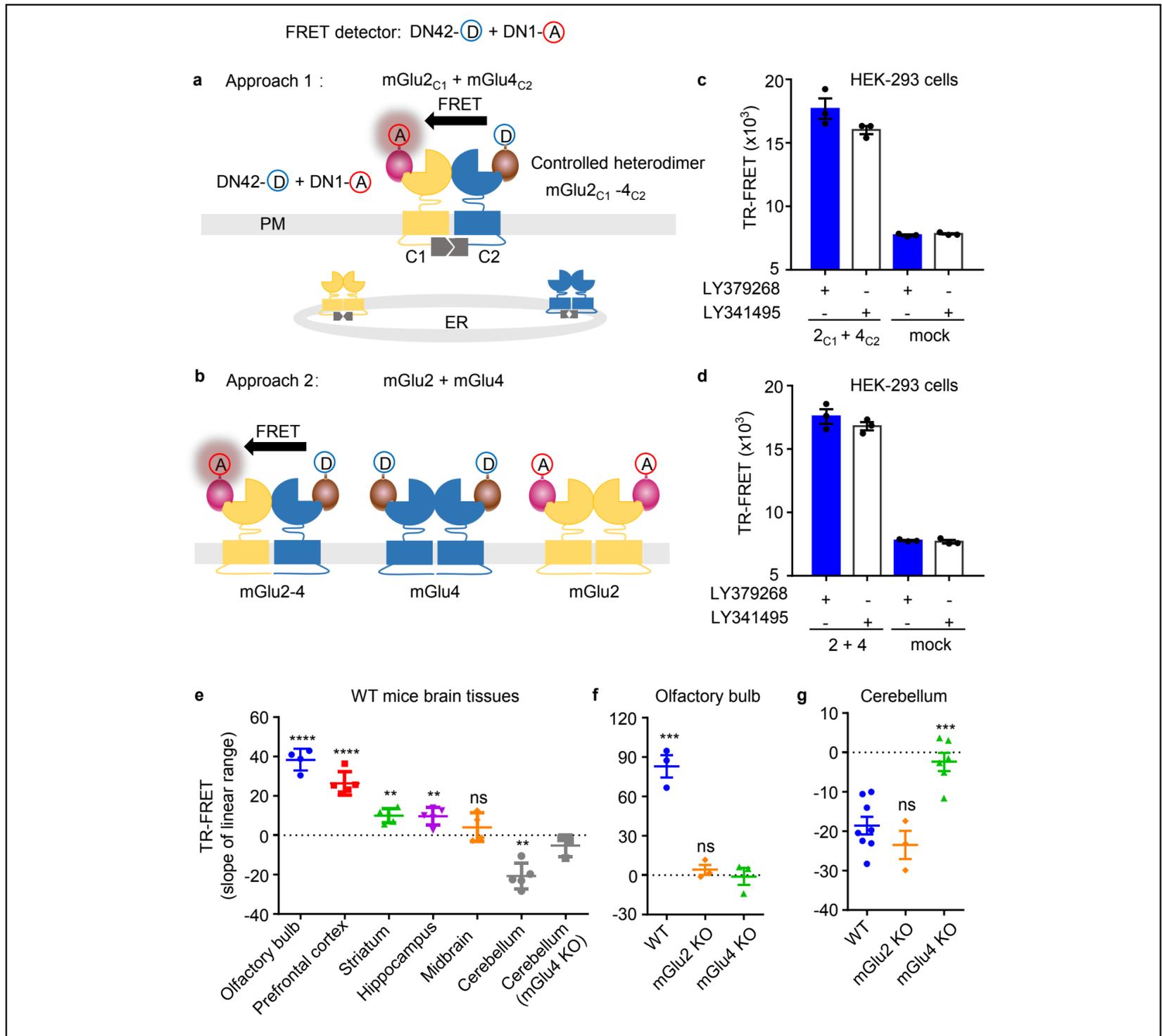
**Figure 2. A nanobody-based biosensor to detect the expression and activation of the mGlu2 receptor both on transfected cells and cell dissociated brain tissues.**



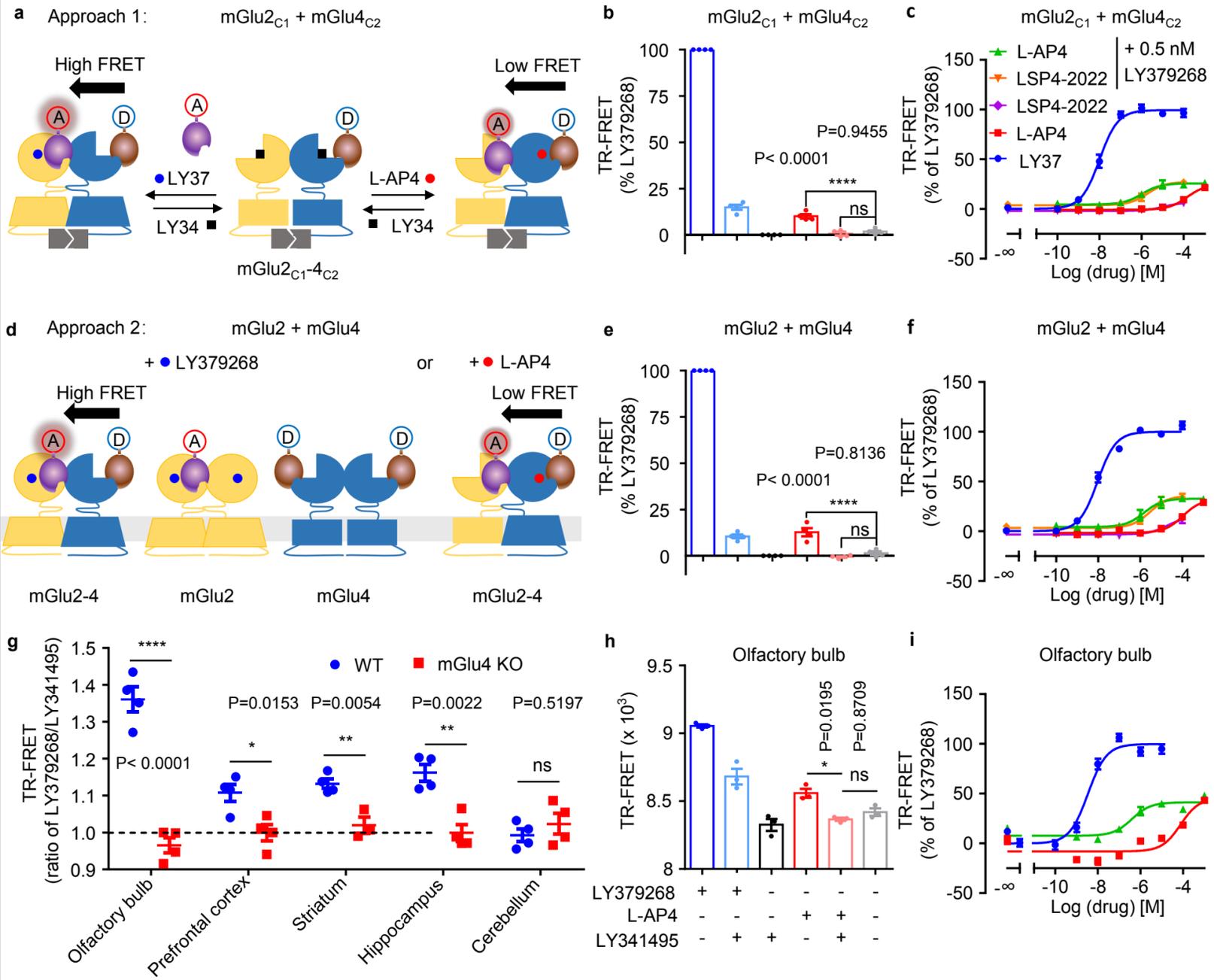
**Figure 3. Relative quantification of endogenous mGlu2 and mGlu4 homodimers by FRET.**



**Figure 4. Relative quantification of the mGlu2-4 heterodimer by FRET.**



Conformational biosensor: DN42-D + DN10-A



Conformational biosensor: DN42-D + DN10-A

