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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 subunits¹⁻³, and can form oligomers⁴. Auxiliary subunits can also associate with
52 such complexes and affect their overall structure, function, and localization⁵. 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-consuming⁶. They often rely on tissue treatments,
58 membrane fraction preparation, solubilization, or mass spectrometry analysis^{6,7} 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)^{8,9}. It is
61 then difficult to rule out the possibility of proteins not interacting directly¹⁰. Often, these
62 techniques requires covalent labeling¹⁰, or recombinant fusion proteins.

63 An attractive approach to examine protein complexes is the use of time-resolved FRET
64 (TR-FRET)^{4,11} (**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 acceptor¹², 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 antibodies¹¹, antibody
70 fragments¹³ or even small ligands⁴ 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 tissues⁴.

73 The use of small antibody fragments, such as camelid single-domain antibodies, called
74 V_{HH} or nanobodies (15 kDa, ~2.5 nm)¹⁴, can result in a high spatial resolution of the protein
75 complexes detected by TR-FRET, higher than with conventional antibodies (~15 nm)^{11, 13}.
76 Moreover nanobodies can recognize conformational epitopes, allowing the detection of
77 specific conformations of membrane proteins, such as the active¹⁵⁻¹⁷ or resting state^{18, 19}.
78 Nanobodies that potentiate or activate metabotropic glutamate (mGlu) receptors were recently
79 reported^{15, 17}.

80 mGlu receptors are G protein-coupled receptors activated by the neurotransmitter
81 glutamate, which tune the activity of many synapses²⁰. 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 activity²¹⁻²³. This is well-illustrated by the latest structures of full-length mGlu dimers in
86 their active and inactive states^{24, 25}. 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 receptors²⁶. Since then, specific pharmacological properties of these heterodimers have
93 been reported, providing indirect evidence of their existence in the brain²⁷⁻²⁹. 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 ^{26, 30}, the mGlu2-4
107 heterodimer is the most investigated ²⁷⁻²⁹, 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 ¹⁵. 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 ¹⁵ that is located
117 at an interface of the two ECDs of the mGlu2 homodimer in its active form exclusively ²⁴, 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 ¹⁵

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)^{31, 32}.
135 No signal was detected from *Grm2* knockout (*Grm2*^{-/-}; 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^{33, 34} (Fig. 2d). The potencies also correlated well
142 with those measured by the accumulation of inositol phosphate-1 (IP₁) (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^{33, 35}. 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 EC₈₀.

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_{C1} and mGlu2_{C2} subunits, as previously

170 described ^{26, 36} (**Extended Data Fig. 5d**). In these constructs, the C-terminal of the mGlu2
171 subunits was replaced by that of the modified GABA_{B1} (C1) or GABA_{B2} (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 ²⁶. However, when using a similar controlled mGlu2-4 heterodimer
174 made of mGlu2_{C1} and mGlu4_{C2} 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 ²⁶ as well
178 as the mGlu2 homodimer ^{26, 37, 38} 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*^{-/-}; 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_B 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^{26, 37}.

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 cerebellum³¹, 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 data³¹
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 lines²⁷, as well as in the PFC, striatum and hippocampus²⁷⁻²⁹ as suggested by
230 electrophysiological, pharmacological and biochemical data. Although convincing, the results
231 provide indirect evidence of the endogenous mGlu2-4 heterodimer²⁷⁻²⁹. 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_{C1} and mGlu4_{C2} (**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**)^{26, 27}. 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^{31, 39, 40} (**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^{17, 27}, 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^{17, 27, 34}, 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 IP₁ 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 ⁴¹. 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 ⁴. 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 ⁴².
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 ⁵. 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 signal⁴³. 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 ^{27-29, 44}. First, it analyzes the heterodimer entity directly and not its downstream

344 signaling that could result from cross-talk between signaling pathways⁴⁵. Second, our
345 biosensors are good reporters of the conformational change of the receptor during activation,
346 as other sensors of the mGlu receptors^{27, 33, 34}. 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^{35, 46}. 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 synapses²⁸, and at
361 corticostriatal synapses²⁹ 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^{26, 30} 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 ²⁸, 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 ⁴⁷.

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 ⁴⁸ whereas it has no effect on the mGlu4 homodimer ²⁹. In addition, mGlu7
377 heterodimers could also contribute to the enigmatic function of the mGlu7 subunit due to its
378 very low glutamate potency ⁴⁹, and the effect of mGlu7 negative allosteric modulators with a
379 context-dependent activity ⁵⁰. 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 ^{13, 16, 18}.

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388

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421 **Author contributions**

422 J.M. developed the FRET-based detectors and the FRET-based conformational change sensors,
423 and performed the sensors experiments, IP₁ 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 IP₁ 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 μ M) or antagonist LY341495 (10 μ 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 μ 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 IP₁
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 (pEC₅₀) determined with the indicated agonists on the conformational biosensor
472 (*d*) and IP₁ 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_{C1} and
499 mGlu4_{C2} (*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_{C1} and mGlu4_{C2} (*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_{C1} and mGlu4_{C2} 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_{C1} and mGlu4_{C2} (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 μ M) or L-AP4 (100 μ M) and/or the antagonist LY341495 (10 μ 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_{C1} and
533 mGlu4_{C2} (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 μ M) and
537 LY341495 (10 μ 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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672

673

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 mice⁵¹ were kindly provided by Dr. Gonzalez Maeso (Virginia Commonwealth University
680 School of Medicine, Richmond, VA, USA), while knock-out mGlu₄ mice⁵² 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% CO₂. HEK-293F cells were cultured in suspension in SMM
693 293-TI animal-free and serum-free at 37°C and 5% CO₂ 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 previously²⁶. 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
700³⁶. The sequence coding for C1 and C2 contains the coiled-coil region of the C terminus of
701 GABA_{B1a} or GABA_{B2}, 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 α_{q9} , were previously described²⁶.

704 To construct the cDNA of the Fc-DN42 and Fc-GFP nanobodies⁵³, 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 IgG₁. 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_HH library after all the procedures
713 including the llama immunization, library construction, and selection of nanobodies targeting
714 mGlu4 were performed as described previously ¹⁷.

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 2×10^7 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 ¹⁵. 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 0.6×10^6 mL⁻¹ with 180 mL fresh medium in a 2 L culture bottle at
731 37°C and 5% CO₂, shaken at 200 rpm. Of the cells, $1-1.5 \times 10^6$ mL⁻¹ 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% CO₂ 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 ⁻¹) 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⁻¹ cm⁻¹ for Lumi4-Tb, and OD at
752 650 nm and ε = 225,000 M⁻¹ cm⁻¹ for d2), while that of nanobodies was determined by the OD
753 at 280 nm (OD₂₈₀). The conjugated concentration was calculated as $OD_{280} - (OD_{fluo}/RZ_{max})/\epsilon$
754 nanobody, with $RZ_{max} = OD_{fluo}/OD_{280}$. 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 μ s delay and 450 μ 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 ¹⁵.

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% CO₂ 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 CaCl₂, 0.5 mM MgCl₂, 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 2×10^4 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 μ M quisqualic acid for mGlu1;
775 5 or 100 μ M L-AP4 for mGlu4, 6, 7, and 8; 100 nM LY379268 for mGlu2 and 3), or the
776 antagonist (10 μ M LY341495) were applied to labeled cells in adherent cell experiments, with
777 a total volume of 60 μ 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 μ 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% CO₂; 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 μ s delay and 450 μ 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*^{-/-} 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/>)⁵⁴. 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 μ L of Versene solution (Thermo
803 Fisher) for 10 min at 20°C, and the cells were dissociated by pipetting. Then, 200 μ 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 μ 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 μ 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% CO₂. 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 μ L per well. For TR-FRET measurements in cell suspensions, 5 μ 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 2×10^4 cells/well. All reagents were added to reach
819 a total volume of 20 μ L per well. For TR-FRET measurements in dissociated brain cells, 10
820 μ 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 μ 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 ¹⁵.

824 **BCA assay for protein quantification**

825 The dissociated cells (30 μ 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% CO₂ 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_{q19} 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 (IP₁) 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 previously²⁷, 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 MgCl₂, 1.15
854 mM CaCl₂, 10 mM D-glucose, 10 mM HEPES, pH 7.3), supplemented with 1% BSA. Images
855 were acquired with a homebuilt TR-FRET microscope⁴³, following a previously described
856 protocol²⁷.

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 μ 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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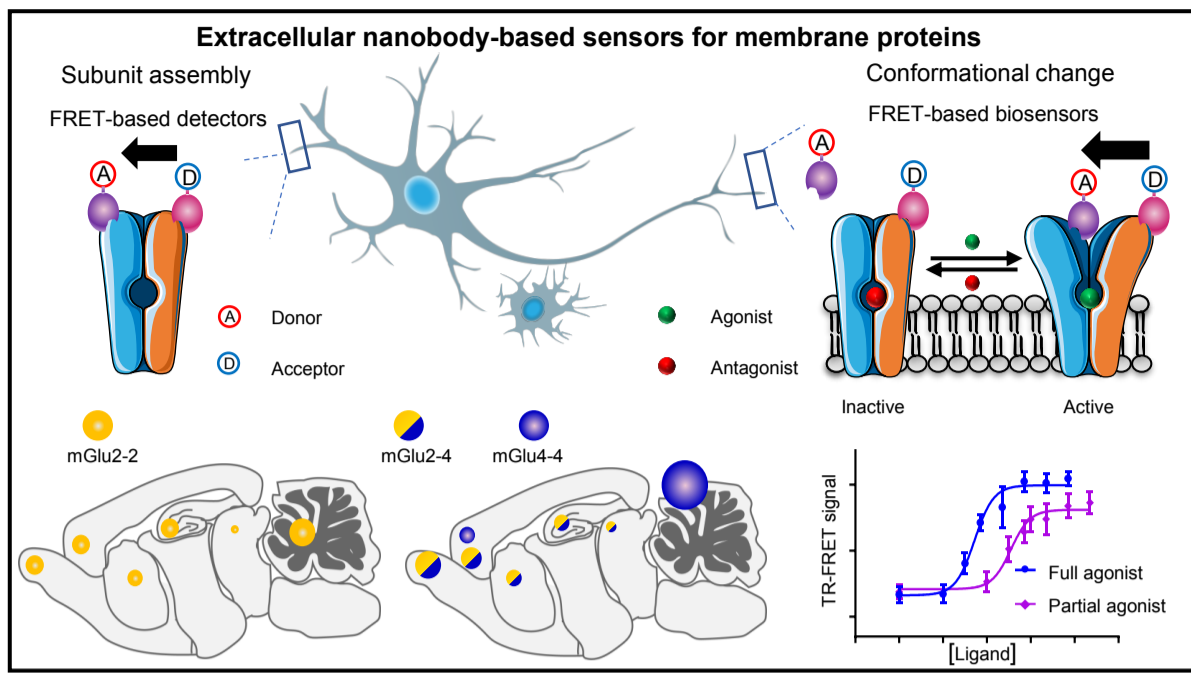
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926

927

928

Graphical Abstract



Main figures

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

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Figure 1.

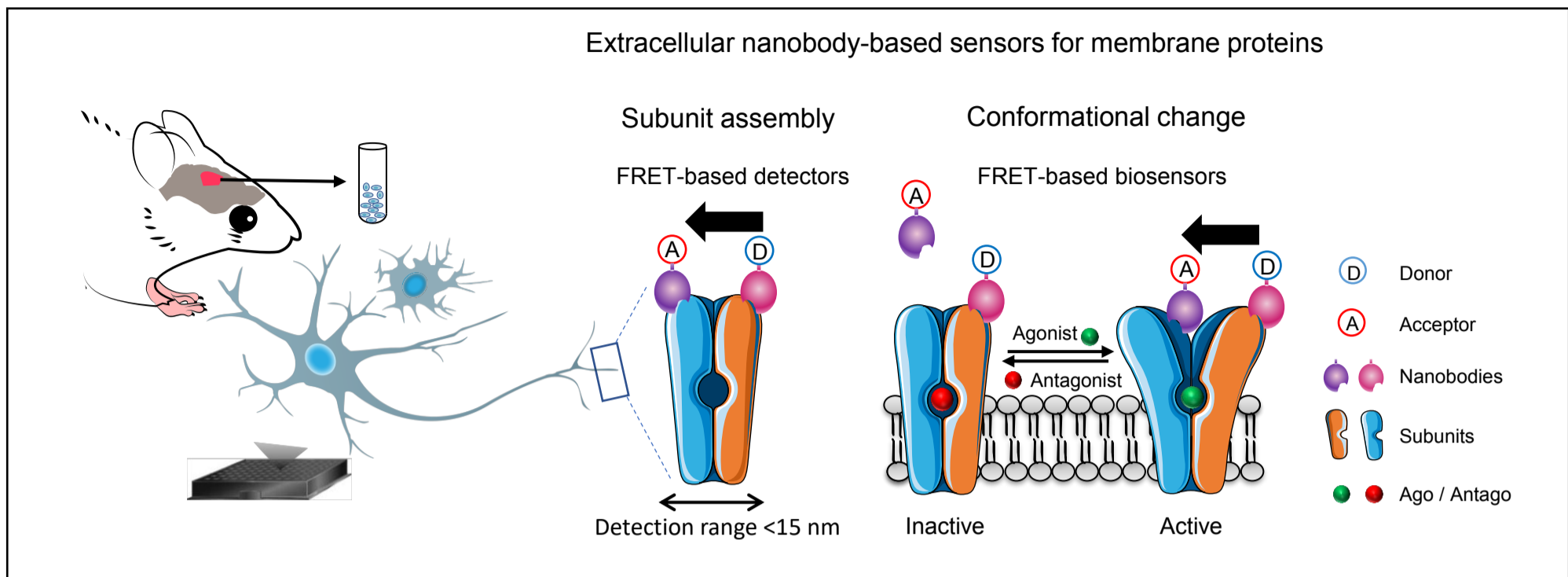


Figure 2. A nanobody-based biosensor to detect the expression and activation of the mGlu2 receptor both on transfected cells and cell dissociated brain tissues.

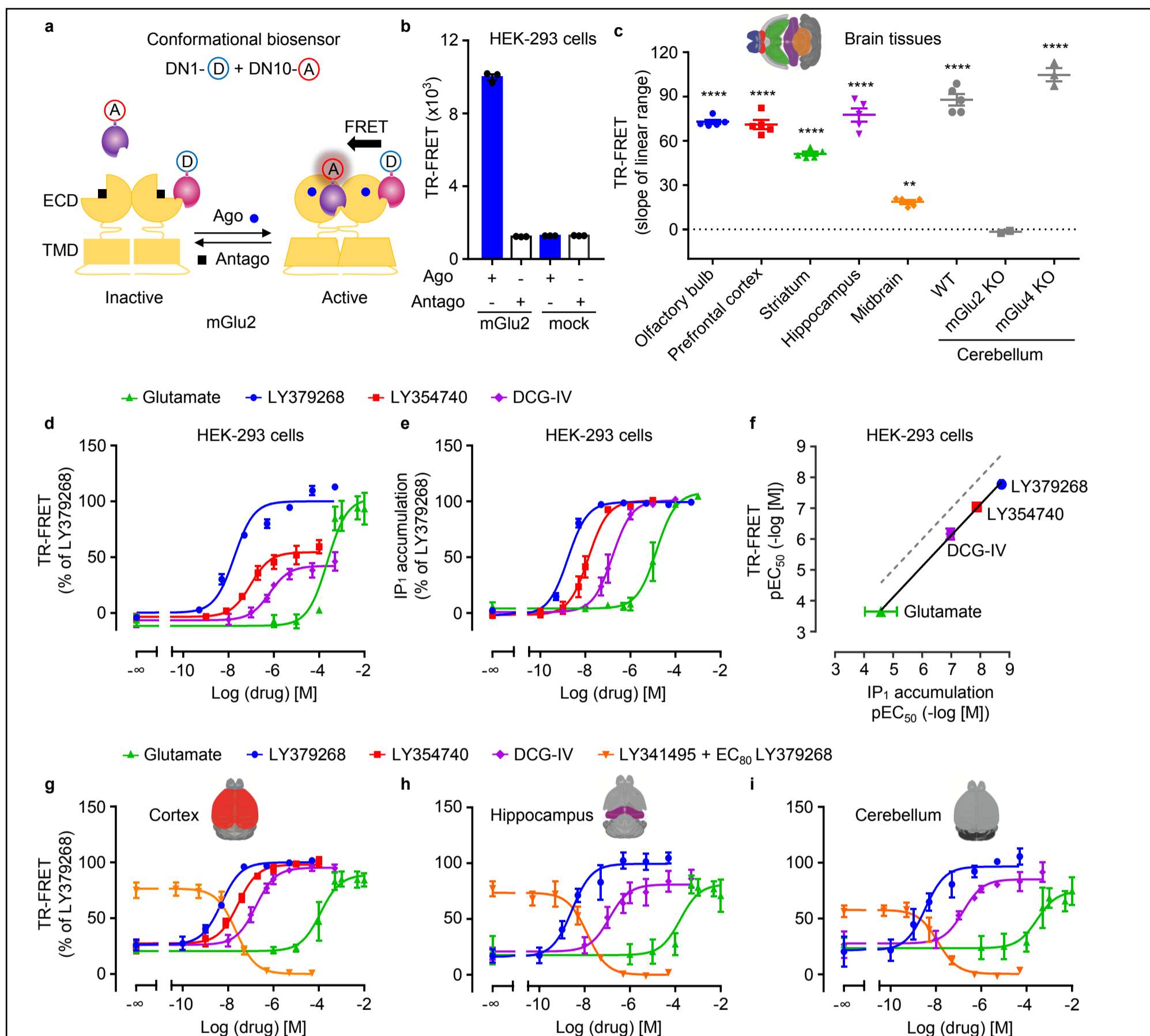


Figure 3. Relative quantification of endogenous mGlu2 and mGlu4 homodimers by FRET.

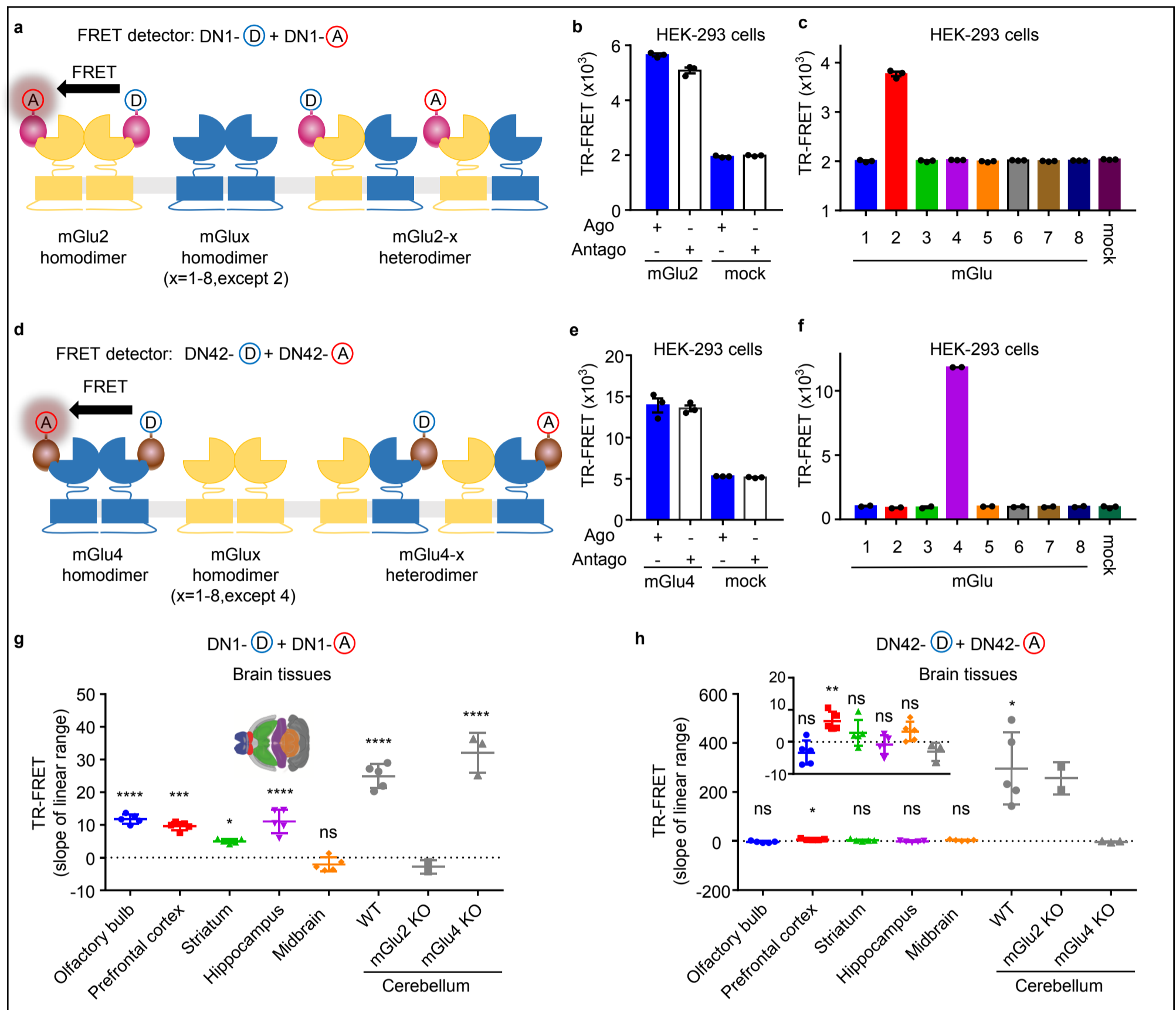
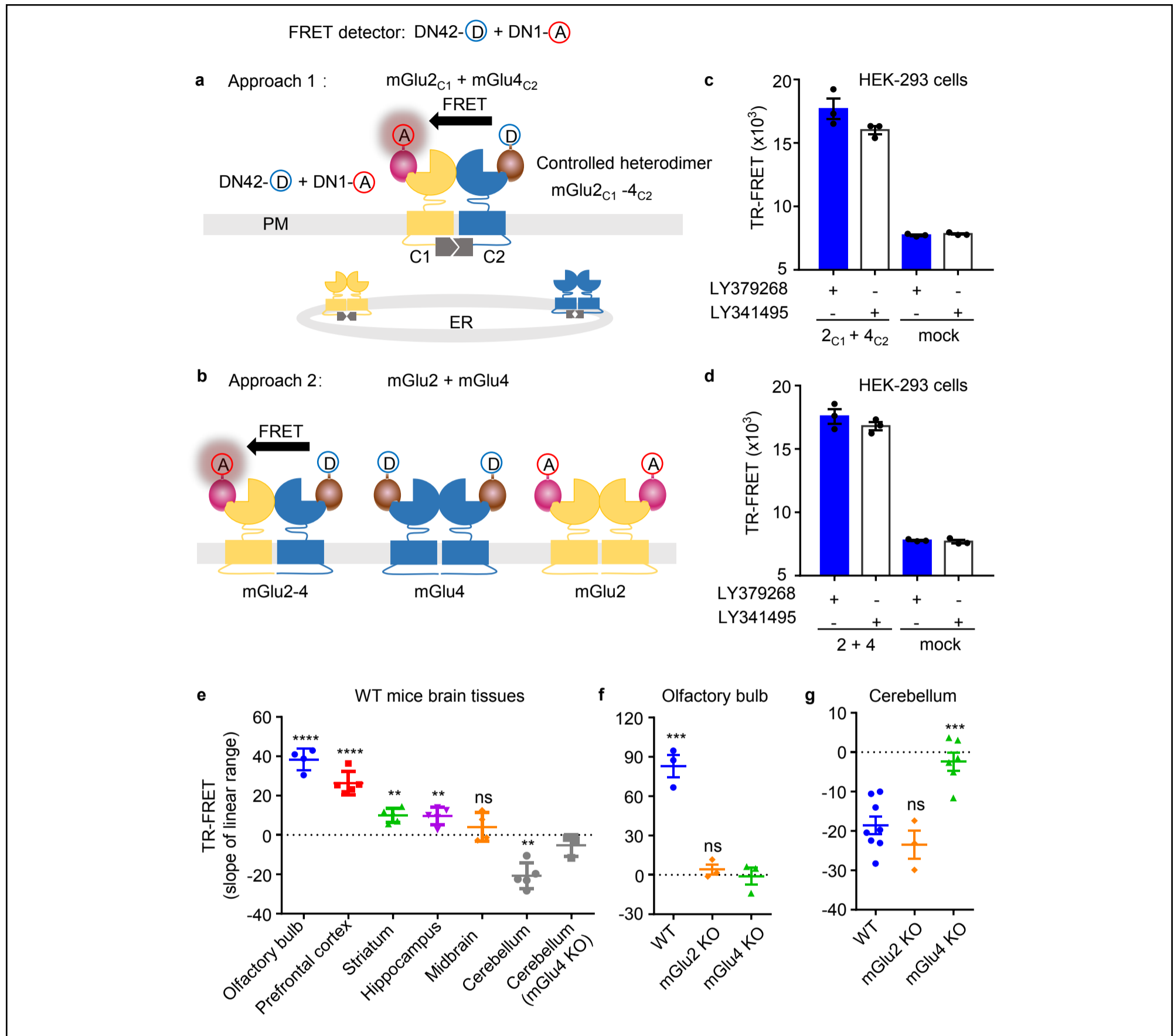
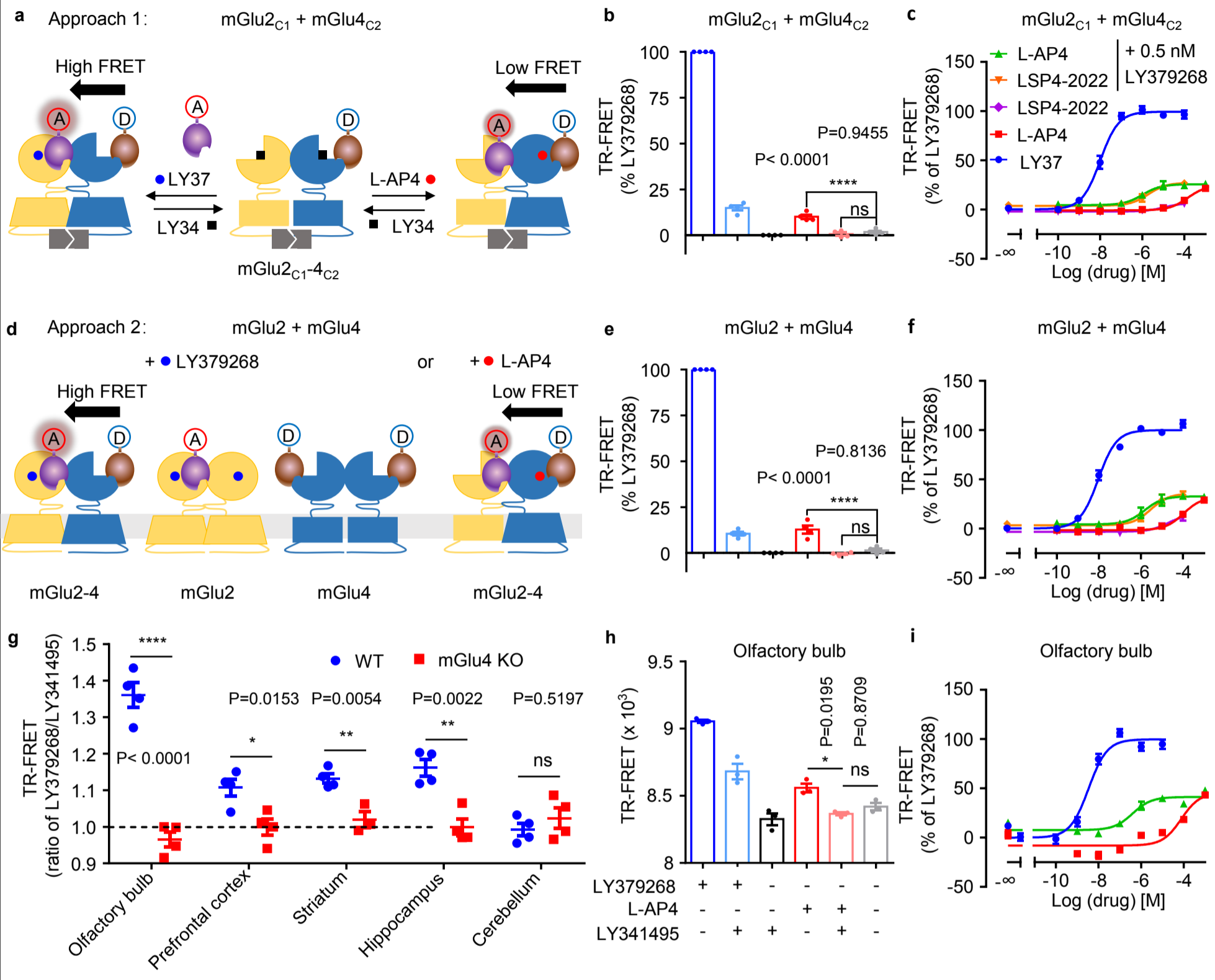


Figure 4. Relative quantification of the mGlu2-4 heterodimer by FRET.



Conformational biosensor: DN42-D + DN10-A



Conformational biosensor: DN42-D + DN10-A

