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

Jiyong Meng1,2,3,8, Chanjuan Xu1,3,8, Pierre-André Lafon1, Salomé Roux2, Michaël Mathieu2, Rui Zhou1, Pauline Scholler2, Emilie Blanc2, Jérôme A. J. Becker4,5, Julie Le Merrer4,5, Javier González-Maeso6, Patrick Chames7, Jianfeng Liu1,3✉, Jean-Philippe Pin2✉ and Philippe Rondard2,2✉

Membrane proteins, including ion channels, receptors and transporters, are often composed of multiple subunits and can form large complexes. Their specific composition in native tissues is difficult to determine and remains largely unknown. In this study, we developed a method for determining the subunit composition of endogenous cell surface protein complexes from isolated native tissues. Our method relies on nanobody-based sensors, which enable proximity detection between subunits in time-resolved Förster resonance energy transfer (FRET) measurements. Additionally, given conformation-specific nanobodies, the activation of these complexes can be recorded in native brain tissue. Applied to the metabotropic glutamate receptors in different brain regions, this approach revealed the clear existence of functional metabotropic glutamate (mGlu)2-mGlu4 heterodimers in addition to mGlu2 and mGlu4 homodimers. Strikingly, the mGlu4 subunits appear to be mainly heterodimers in the brain. Overall, these versatile biosensors can determine the presence and activity of endogenous membrane proteins in native tissues with high fidelity and convenience.

Many membrane proteins, including receptors, ion channels and transporters, are composed of multiple subunits1–3 and can form oligomers4. Auxiliary subunits can also associate with such complexes and affect their overall structure, function and localization5. A major challenge is to investigate these membrane complexes in native conditions without disrupting their environment, because their interactions with lipids and associated proteins could be critical for their assembly and function. Only a limited number of methods enable the validation of native membrane protein complexes and are often difficult and time consuming5. They often rely on tissue treatments, membrane fraction preparation, solubilization or mass spectrometry analysis6,7, and chemical cross-linking is sometimes required. Good alternatives are proximity interaction assays that rely on optical detection, but several of these lack spatial resolution (around 40 nm)8–10. It is then difficult to rule out the possibility of proteins not interacting directly10. Often, these techniques require covalent labeling11 or recombinant fusion proteins.

An attractive approach to examine protein complexes is the use of time-resolved FRET (TR-FRET)11,12 (Fig. 1). It relies on resonance energy transfer between two fluorophores, with a distance limit of 15 nm between the long-lasting emission donor and acceptor13, compatible with the size of multi-subunit proteins. A major advantage is the long lifetime of the donor, which enables a delay between its excitation and the measurement of the emission of the sensitized acceptor, strongly reducing the background fluorescence from biological systems. Fluorophores compatible with TR-FRET can be easily attached to antibodies14, antibody fragments15 or even small ligands specific to the protein complex under analysis, allowing the analysis of native protein complexes. TR-FRET is thus a highly sensitive technique that is compatible with the detection of endogenous and low-expressed proteins in native tissues.

The use of small antibody fragments, such as camelid single-domain antibodies, called VHH or nanobodies (15 kDa, ~2.5 nm)16, can result in high spatial resolution of protein complexes detected by TR-FRET, higher than that with conventional antibodies (~15 nm)11,13. Moreover, nanobodies can recognize conformational epitopes, allowing the detection of specific conformations of membrane proteins, such as the active17–19 or resting state20,21. Nanobodies that potentiate or activate mGlu receptors were recently reported16,17.

mGlu receptors are G protein-coupled receptors activated by the neurotransmitter glutamate that tune the activity of many synapses22. They are therapeutic targets for several drugs under development for neurological and psychiatric diseases. Eight genes encoding mGlu receptors have been identified that generate receptors mGlu1–mGlu8. These are mandatory homodimers with both subunits linked by a disulfide bond, a quaternary structure essential for their activity21–23. This is well illustrated by the latest structures of full-length mGlu dimers in their active and inactive states24,25. These structural studies confirmed a specific symmetrical conformation of the large extracellular glutamate-binding domains (ECDs), with both domains closed and in a specific active orientation, while the dimer of seven transmembrane (7TM) domains is asymmetric, with only one capable of G protein activation.

1Cellular Signaling Laboratory, International Research Center for Sensory Biology and Technology of MOST, Key Laboratory of Molecular Biophysics of MOE and College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, China. 2Institut de Génomique Fonctionnelle, Université de Montpellier, CNRS, INSERM, Montpellier, France. 3Bioland Laboratory, Guangzhou Regenerative Medicine and Health Guangdong Laboratory, Guangzhou, China. 4Physiologie de la Reproduction et des Comportements, INRAE UMR0085, CNRS UMR7247, IFCE, Université de Tours, INSERM, Nouzilly, France. 5UMR1253, IBAB, Université de Tours, INSERM, CNRS, Tours, France. 6Department of Physiology and Biophysics, Virginia Commonwealth University School of Medicine, Richmond, VA, USA. 7Institut Paoli-Calmettes, Aix Marseille University, CNRS, INSERM, CRCM, Marseille, France. 8These authors contributed equally: Jiyong Meng, Chanjuan Xu. ✉E-mail: jfliu@mail.hust.edu.cn; jean-philippe.pin@igf.cnrs.fr; philippe.rondard@igf.cnrs.fr

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

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

Results

Among the possible 16 mGlu heterodimers observed in recombinant cells, the mGlu2–mGlu4 heterodimer is the most investigated, but its existence and abundance in the brain remain unclear. To clarify this issue in different brain areas, we have developed two kinds of nanobody-based TR-FRET sensors (Fig. 1): (1) conformational sensors or ‘biosensors’ that reveal the activation of these receptors upon agonist binding and (2) the ‘detectors’ that enable the relative quantification of both mGlu homodimers and heterodimers.

An mGlu2 FRET-based conformational biosensor. We first developed a ‘biosensor’ for the mGlu2 homodimer by taking advantage of a pair of specific and high-affinity nanobodies for the mGlu2 receptor, DN10 and DN1 (ref. 15). DN10 specifically recognizes the receptor dimer in its active state, whereas DN1 is not sensitive to the conformational state (Fig. 2a). The DN10 epitope overlaps that of DN13 (ref. 15), the nanobody contacting both subunits, which is located at an interface of the two ECDs of the mGlu2 homodimer in its active form exclusively17. In contrast to DN13, DN10 can also bind to the active mGlu2–mGlu4 heterodimer, as shown below, likely because the mGlu4 part of the epitope is compatible with DN10 binding. By contrast, the DN1 epitope remains unknown (Supplementary Tables 1 and 2). When DN1 and DN10 were covalently labeled with donor Lumi4-Tb and acceptor d2, respectively, a FRET signal was measured in cells expressing mGlu2 in the presence of the mGlu2 and mGlu3 agonist LY379268 but not with the antagonist LY341495 (Fig. 2b). No signal was measured when DN10–d2 was absent (Extended Data Fig. 1a), and the FRET signal followed a saturation curve with the increase of DN10–d2 and a fixed concentration of DN1–Tb (Extended Data Fig. 1b). The signal was specific to mGlu2, as no signal was measured with other mGlu receptors (Extended Data Fig. 1c). Finally, DN10–Tb and DN1–d2 can also detect endogenous mGlu2 in rat hippocampal neurons by TR-FRET microscopy (Extended Data Fig. 2).

This pair of nanobodies could also be used with dissociated cells from different mouse brain regions (Extended Data Fig. 3a,b). The more cells, the higher the FRET signal with the nanobodies in the presence of agonists (Extended Data Fig. 3c). The slopes, representative of the FRET signal per amount of brain cells, revealed a high signal in the cerebellum and other regions (Fig. 2c), consistent with DN1–d2 staining of brain slices (Extended Data Fig. 4). No signal was detected from Gm2-knockout (Gm2−/−; called mGlu2-KO mice in this study) mice (Fig. 2c and Extended Data Fig. 4). Altogether, these data validate the use of this pair of nanobodies in the detection of endogenous active mGlu2 receptors.

This conformational biosensor is a sensitive tool to report the rearrangement of the mGlu2 ECD upon agonist activation. In transfected cells, the TR-FRET signal generated using increasing concentrations of various full and partial agonists revealed potencies and efficacies consistent with results from a SNAP-tag FRET-based assay (Extended Data Fig. 4d). The potencies also correlated well with those measured by the accumulation of inositol phosphate-1 (IP1) (Fig. 2e,f). Such a good correlation between the potencies of partial and full agonists observed in both assays was not expected, as the amplification resulting from receptor reserve is expected to increase the potencies of full agonists more than those of partial agonists. The good correlation may be due to the fact that the efficacy of partial agonists is closer to a full efficacy in the presence of the G protein bound to the active receptor. In sum, these data show that this nanobody-based biosensor constitutes a new generation of untagged mGlu conformational sensors.

This biosensor can also reveal the activation of endogenous mGlu2 receptors in dissociated cells from various brain regions. The agonist-induced change in FRET observed in the cortex, hippocampus and cerebellum was similar to that found with transfected cells (Fig. 2g–i). The antagonist LY341495 was also found to inhibit both the basal signal likely generated by ambient glutamate in the assay and the response evoked by the concentration of agonist required to give 80% of a maximum response (the EC50).

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

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

**Quantification of the mGlu2 homodimer.** We next aimed to quantify mGlu2 homodimers using DN1–Tb and DN1–d2 as a TR-FRET pair, first on the surface of transfected cells (Fig. 3a). As expected, this ‘detector’ signal was independent of the conformation of the homodimer, whether bound to an agonist or an antagonist (Fig. 3b). The concentrations of the nanobodies were optimized to have a TR-FRET signal proportional to the quantity of mGlu2 over a wide range of receptor amounts (Extended Data Fig. 5a–c). This ‘detector’ pair was also specific for mGlu2 among all mGlu homodimers (Fig. 3c). We also believe that the FRET signal was mostly due to the mGlu2 pair, first on the surface of transfected cells (Fig. 3a). As expected, this ‘detector’ signal was independent of the conformation of the homodimer, whether bound to an agonist or an antagonist (Fig. 3b). The concentrations of the nanobodies were optimized to have a FRET signal proportional to the quantity of mGlu2 over a wide range of receptor amounts (Extended Data Fig. 5a–c). This ‘detector’ pair was also specific for mGlu2 among all mGlu homodimers (Fig. 3c). We also believe that the FRET signal was mostly due to the mGlu2 pair, first on the surface of transfected cells (Fig. 3a). As expected, this ‘detector’ signal was independent of the conformation of the homodimer, whether bound to an agonist or an antagonist (Fig. 3b). The concentrations of the nanobodies were optimized to have a FRET signal proportional to the quantity of mGlu2 over a wide range of receptor amounts (Extended Data Fig. 5a–c). This ‘detector’ pair was also specific for mGlu2 among all mGlu homodimers (Fig. 3c).
Fig. 3 | Relative quantification of endogenous mGlu2 and mGlu4 homodimers by FRET. a, d, Schematic representation of mGlu2 (a) and mGlu4 (d) homodimers that can be detected by FRET-based ‘detectors’. The mGlu2 homodimer ‘detector’ (a) was made of donor- and acceptor-labeled nanobodies (DN1–Tb and DN1–d2, both at 25 nM). The mGlu4 homodimer ‘detector’ (d) was made of DN42–Tb and DN42–d2 (both at 1.6 nM). b, e, TR-FRET signal measured in HEK293 cells transfected to express the mGlu2 (b) or mGlu4 (e) receptors or in mock cells with the indicated ‘detectors’ and the agonist LY379268 (1 μM) (b) or L-AP4 (100 μM) (e) and the antagonist LY341495 (10 μM). c, f, TR-FRET signal measured in HEK293 cells transfected to express the indicated mGlu receptors or in mock cells with LY341495 (10 μM). In b, c, e, f, data are mean ± s.e.m. of triplicate measurements from one representative experiment of three independent experiments. g, h, Relative expression of the mGlu2 (g) and mGlu4 (h) homodimers in the indicated brain tissues as shown by their respective ‘detectors’. The TR-FRET signal indicates the slope values of the relative linear quantification experiments. Each dot represents a TR-FRET measurement from one mouse (n = 5 for all samples of wild-type mice, n = 2 for the cerebellum of the mGlu2-KO mouse group and n = 3 for the cerebellum of the mGlu4-KO mouse group). Data are mean ± s.e.m. and were analyzed using one-way ANOVA followed by Dunnett’s post hoc test (the control group is the cerebellum of the mGlu2-KO group (g), with ****P < 0.0001 for all, except for the striatum (***P = 0.0164) and the midbrain (not significant (n.s.), P = 0.9996) or the mGlu4-KO group (insert, h), with n.s., P > 0.05 for the olfactory bulb (>0.9999), the striatum (0.0896), the hippocampus (0.8253) and midbrain (0.0615) and the PFC (**P = 0.0031) or Welch’s ANOVA test followed by Dunnett’s T3 post hoc test (compared with the cerebellum of the mGlu4-KO group) (h), with n.s., P > 0.05 for the olfactory bulb (>0.9999), the striatum (0.2251), the hippocampus (0.8436) and the midbrain (0.1304) and *P < 0.05 for the PFC (0.0425) and the cerebellum of the wild-type group (0.0435).

homodimer and not to higher-order oligomers. First, the FRET signal between the DN1 nanobodies was proportional to the number of SNAP–mGlu2 subunits on the cell surface over a wide range of receptor amounts (Extended Data Fig. 5c). Second, this linearity was also observed with the ‘controlled’ mGlu2 homodimer formed by the mGlu2C1 and mGlu2C2 subunits as previously described26,28 (Extended Data Fig. 5d). In these constructs, the C terminus of the mGlu2 subunits was replaced by that of the modified γ-aminobutyric acid (GABA)B1 (C1) or GABAB1 (C2) subunits, respectively, preventing any of these from reaching the cell surface alone. Indeed, only C1–C2 dimers can reach the cell surface26. However, when using a similar controlled mGlu2–mGlu4 heterodimer made of mGlu2C1 and mGlu4C2 subunits, no FRET signal with the DN1 ‘detector’ was measured (Extended Data Fig. 5d). Under these conditions, the mGlu2–mGlu4 heterodimer is present at the cell surface in the absence of both mGlu2 and mGlu4 homodimers. This is consistent...
with our previous demonstration that the controlled mGlu2–mGlu4 heterodimer26 as well as the mGlu2 homodimer26,37,38 do not have the tendency to form oligomers in transfected cells. Third, the highest FRET signal measured for equal concentrations of donor (DN1–Tb) and acceptor (DN1–d2) was also consistent with the presence of strict mGlu2 homodimers (Extended Data Fig. 5b).

By comparing the mGlu2 homodimer ‘detector’ signal in different brain areas (Fig. 3g and Extended Data Fig. 5e), mGlu2 was found to be more abundant in the cerebellum (Fig. 3g). As a control, no signal was observed in the cerebellum of mGlu2-KO mice. However, this ‘detector’ appeared less sensitive than the ‘biosensor’, but this was expected for two main reasons. First, only half of the mGlu2 homodimers can be labeled with two FRET-compatible DN1 nanobodies (DN1–Tb and DN1–d2). By contrast, each mGlu2 subunit will be labeled with two FRET-compatible nanobodies in the biosensor assay, DN1–Tb and DN10–d2, such that each mGlu2 homodimer carries two pairs of FRET-compatible nanobodies. Second, this biosensor assay detects any active form of the mGlu2 subunits, whether in a homodimer or heterodimer, in contrast to the ‘detector’, which reveals mGlu2 homodimers only.

Quantification of the mGlu4 homodimer. To quantify the mGlu4 homodimer, we isolated and characterized the nanobody DN42, highly specific for mGlu4 (Extended Data Fig. 6a,b). For this study, we used the Fc–DN42 dimeric construct (80 kDa), as the DN42 monomer has low affinity after labeling with a fluorophore (~100 nM), which is not compatible with its use in native tissues. Interestingly, Fc–DN42 has subnanomolar affinity for the mGlu4 ECD and a similar affinity for the inactive and active conformations (Supplementary Tables 1 and 2 and Extended Data Fig. 6c). We verified by immunofluorescence that Fc–DN42 was able to specifically detect mGlu4 subunits in brain slices of wild-type mice but not in Grm4-knockout (Grm4<sup>-/-</sup>; called mGlu4-KO mice in this study) mice (Extended Data Fig. 6d).

We used Fc–DN42, from now on referred to as DN42, as a ‘detector’ to quantify mGlu4 homodimers, similar to what was done with DN1 for the mGlu2 homodimers. With optimized concentrations of DN42 (Extended Data Fig. 7a,b), a strong FRET signal was measured between DN42–Tb and DN42–d2 specifically in cells transfected to express mGlu4 (Fig. 3d–f), independent of the state of the receptor. As observed with mGlu2, the FRET signal appeared mostly owing to the mGlu4 homodimer and not to higher-order oligomers. First, the signal was proportional to the number of SNAP–mGlu4 subunits on the cell surface (Extended Data Fig. 8c). We also showed that the FRET signal could not result from mGlu homodimers (Extended Data Fig. 9a,b and Supplementary Tables 1 and 2). Of note, L-AP4, a partial agonist of the mGlu2–mGlu4 heterodimer<sup>17,27</sup>, remained partial in promoting DN10 binding (Extended Data Fig. 9a,b), as

An mGlu2–mGlu4 heteromer FRET-based detector. Recent studies argue in favor of the existence of endogenous mGlu2–mGlu4 receptors in neuronal cell lines<sup>26</sup> as well as in the PFC, striatum and hippocampus<sup>27,28</sup>, as suggested by electrophysiological, pharmacological and biochemical data. Although convincing, the results provide indirect evidence of the endogenous mGlu2–mGlu4 heterodimer<sup>26,27</sup>. More direct evidence for this heterodimer could come from a proximity assay based on FRET between the mGlu2 and mGlu4 subunits in tissues, owing to the nanobodies described above.

Thus, we used DN1 and DN42 nanobodies to detect mGlu2–mGlu4 heterodimers first on transfected cells. Using the optimized concentrations of DN1 and DN42 (Supplementary Table 1 and Extended Data Fig. 8a,b), a strong FRET signal was measured, proportional to the amount of controlled mGlu2–mGlu4 heterodimers on the cell surface (Extended Data Fig. 8c). We also showed that the FRET signal could not result from mGlu homodimers (Extended Data Fig. 8d). Because mGlu2–mGlu4 is likely coexpressed with mGlu2 and mGlu4 homodimers in native tissues, we used two methods to detect mGlu2–mGlu4 heterodimers in transfected cells. First, we used cells coexpressing mGlu<sub>2,4</sub> and mGlu<sub>4,4</sub> (Extended Data Figs. 5a and 7d), such that only heterodimers could reach the surface (Fig. 4a). Second, cells were cotransfected to express the wild-type mGlu4 subunits to obtain a mix of mGlu2 and mGlu4 homodimers together with the mGlu2–mGlu4 heterodimer on the cell surface (Fig. 4b,4c). In both cases, a strong FRET signal was measured whether the receptors were activated with the mGlu2 agonist LY379268 or antagonized with LY341495 (Fig. 4c,d).

Applied to isolated brain cells, this nanobody–FRET pair generated a strong signal in the olfactory bulb, the PFC, the striatum and the hippocampus (Fig. 4e and Extended Data Fig. 8e), where mGlu2 (Fig. 3g) but not mGlu4 (Fig. 3h) homodimers have been previously detected. As a control, no signal was measured in the olfactory bulb from mGlu2-KO or mGlu4-KO mice (Fig. 4f and Extended Data Fig. 8g). In agreement with these results, co-immunoprecipitation experiments with the olfactory bulb using DN42 revealed the presence of endogenous mGlu2 in the same complexes as mGlu4 (Extended Data Fig. 8f). Notably, the cerebellum did not produce a specific signal between DN1 and DN42 (Fig. 4e,g), consistent with mGlu2 and mGlu4 subunits being expressed in different cell types<sup>1,19,46</sup> (Extended Data Figs. 4 and 6d). Surprisingly, the slope for the cerebellum was even negative (Fig. 3g and Extended Data Fig. 8g), most probably due to the relatively high amount of mGlu4 homodimers in this region that was sufficient to titrate the DN42 present at 1.6 nM in the assay, thus resulting in a slight but significant decrease in FRET. In agreement with this hypothesis, the slope was not negative for samples from mGlu4-KO mice, but it was negative in samples from mGlu2-KO mice (Extended Data Fig. 8g).

An mGlu2–mGlu4 heterodimer FRET-based biosensor. Although the above data provide strong evidence for the presence of mGlu2–mGlu4 heterodimers in various brain regions, one cannot exclude the possibility that the signal came from the proximity between mGlu2 and mGlu4 homodimers. To bring further evidence of the existence of the endogenous mGlu2–mGlu4 heterodimer, we developed a nanobody-based FRET conformational sensor for this heterodimer using DN10 and DN42, as DN10 can bind to the mGlu2–mGlu4 heterodimer in the active state only (Extended Data Fig. 9a,b and Supplementary Tables 1 and 2). Of note, L-AP4, a partial agonist of the mGlu2–mGlu4 heterodimer<sup>17,27</sup>, remained partial in promoting DN10 binding (Extended Data Fig. 9a,b), as
monitored by the FRET between the nanobody and the SNAP–mGlu4 subunits. Interestingly, DN10 binding to mGlu2–mGlu4 in the presence of the mGlu4 agonist L-AP4 (Extended Data Fig. 9a,c) was strongly potentiated by the mGlu2 agonist LY379268 (Extended Data Fig. 9c,e), whereas L-AP4 did not induce binding of DN10 to the control mGlu2 homodimer (Extended Data Fig. 9d).

By combining DN42, which specifically binds to the mGlu4 subunit, and DN10, which binds to the active form of mGlu2–mGlu4, we could detect mGlu2–mGlu4 activation by FRET (Fig. 5a–f and Extended Data Fig. 9f), a signal that could not result from either homodimers (Extended Data Fig. 9g). Similar data were obtained from cells expressing the controlled mGlu2–mGlu4 (Fig. 5a–c) and HEK293 cells20

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**Fig. 4 | Relative quantification of the mGlu2–mGlu4 heterodimer by FRET.**

**a,b,** Schematic representation of the FRET-based ‘detector’ for the mGlu2–mGlu4 heterodimer with mGlu2<sub>C1</sub> and mGlu4<sub>C2</sub> (a) or wild-type mGlu2 and mGlu4 coexpressed (b). This ‘detector’ is composed of DN42–Tb (1.6 nM) and DN1–d2 (25 nM). ER, endoplasmic reticulum; PM, plasma membrane. c,d, TR-FRET signal measured in HEK293 cells transiently transfected to express mGlu2<sub>C1</sub> and mGlu4<sub>C2</sub> (c) or cotransfected to express both wild-type mGlu2 and mGlu4 (d) or in mock cells in the presence of the ‘detector’ with LY379268 (1 μM) or LY341495 (10 μM). Data are mean ± s.e.m. of triplicate measurements from one representative experiment of three independent experiments. e–g, Relative expression of the mGlu2–mGlu4 heterodimer in the indicated brain tissues as shown by the ‘detector’. The TR-FRET signal indicates the slope values of the relative linear quantification experiments. Each dot represents a TR-FRET experiment performed on the indicated brain tissue of one mouse. For e, n = 5 for all samples of wild-type mice except for the olfactory bulb (n = 4) and the cerebellum of the mGlu4-KO mouse group (n = 3). For f, n = 3 for the olfactory bulb of wild-type, mGlu2-KO and mGlu4-KO mice. For g, n = 8 for the cerebellum of wild-type mice, n = 3 for the mGlu2-KO group and n = 6 for the mGlu4-KO group. Data are mean ± s.e.m. and were analyzed using one-way ANOVA followed by Dunnett’s post hoc test, compared with the cerebellum of the mGlu4-KO group (e), with ***P < 0.0001 for the olfactory bulb and the PFC, **P < 0.01 for the striatum (0.0054), the hippocampus (0.0062) and the cerebellum (0.0045) and n.s., P > 0.05 for the midbrain (0.1533); compared with the olfactory bulb of the mGlu4-KO group (f), with ***P < 0.0001 for the wild-type group (0.0002) and n.s., P > 0.05 for the mGlu2-KO group (0.8100); or compared with the cerebellum of the wild-type group (g), with n.s., P > 0.05 for the mGlu2-KO group (0.4120) and ***P < 0.0001 for the mGlu4-KO group (0.0005).

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**Fig. 5 |** Relative quantification of the mGlu2–mGlu4 heterodimer by FRET. a, Schematic representation of the FRET-based ‘detector’ for the mGlu2–mGlu4 heterodimer with mGlu2<sub>C1</sub> and mGlu4<sub>C2</sub> (a) or wild-type mGlu2 and mGlu4 coexpressed (b). This ‘detector’ is composed of DN42–Tb (1.6 nM) and DN1–d2 (25 nM). ER, endoplasmic reticulum; PM, plasma membrane. c,d, TR-FRET signal measured in HEK293 cells transiently transfected to express mGlu2<sub>C1</sub> and mGlu4<sub>C2</sub> (c) or cotransfected to express both wild-type mGlu2 and mGlu4 (d) or in mock cells in the presence of the ‘detector’ with LY379268 (1 μM) or LY341495 (10 μM). Data are mean ± s.e.m. of triplicate measurements from one representative experiment of three independent experiments. e–g, Relative expression of the mGlu2–mGlu4 heterodimer in the indicated brain tissues as shown by the ‘detector’. The TR-FRET signal indicates the slope values of the relative linear quantification experiments. Each dot represents a TR-FRET experiment performed on the indicated brain tissue of one mouse. For e, n = 5 for all samples of wild-type mice except for the olfactory bulb (n = 4) and the cerebellum of the mGlu4-KO mouse group (n = 3). For f, n = 3 for the olfactory bulb of wild-type, mGlu2-KO and mGlu4-KO mice. For g, n = 8 for the cerebellum of wild-type mice, n = 3 for the mGlu2-KO group and n = 6 for the mGlu4-KO group. Data are mean ± s.e.m. and were analyzed using one-way ANOVA followed by Dunnett’s post hoc test, compared with the cerebellum of the mGlu4-KO group (e), with ***P < 0.0001 for the olfactory bulb and the PFC, **P < 0.01 for the striatum (0.0054), the hippocampus (0.0062) and the cerebellum (0.0045) and n.s., P > 0.05 for the midbrain (0.1533); compared with the olfactory bulb of the mGlu4-KO group (f), with ***P < 0.0001 for the wild-type group (0.0002) and n.s., P > 0.05 for the mGlu2-KO group (0.8100); or compared with the cerebellum of the wild-type group (g), with n.s., P > 0.05 for the mGlu2-KO group (0.4120) and ***P < 0.0001 for the mGlu4-KO group (0.0005).
Fig. 5 | A conformational biosensor detects activation of the endogenous mGlu2–mGlu4 heterodimer. a–d, Schematic representation of the conformational biosensor for the mGlu2–mGlu4 heterodimer when the mGlu2C1 and mGlu4C2 constructs are used to have only the heterodimer at the surface (a) or when wild-type mGlu2 and mGlu4 are coexpressed (d) in the presence of the indicated mGlu2 and mGlu4 agonists and antagonist. This biosensor is composed of DN42–Tb (1.6 nM) and DN10–d2 (25 nM). The large increase in L-AP4 potency caused by a low concentration of LY379268 (1 μM) or L-AP4 (100 μM) or the antagonist LY341495 (100 μM) and/or the antagonist LY341495 (10 μM). Data were analyzed using one-way ANOVA followed by Tukey’s post hoc test, with ****P ≤ 0.0001 and n.s., P > 0.05. For b, data are mean ± s.e.m. of four independent experiments performed in triplicate and normalized to the response to LY341495 (100%) and LY379268 (100%). For h, data are mean ± s.e.m. of triplicate measurements from a representative experiment of three independent experiments. c, f, i. Dose-dependent effects of the indicated ligands on the TR-FRET signal of the biosensor measured in HEK293 cells cotransfected to express mGlu2C1 and mGlu4C2 (c) or wild-type mGlu2 and mGlu4 (f) or in the dissociated cells from the olfactory bulb (i). Data are mean ± s.e.m. of n independent experiments performed in duplicate (c, f, n = 3) or in triplicate (i, n = 5) and were normalized to the response to LY379268. g, TR-FRET signal of the biosensor when the ratio in the presence of LY379268 (1 μM) and LY341495 (10 μM) was measured. Each dot represents one sample. In addition, in the olfactory bulb (Fig. 5h), L-AP4 potency was 0.9455 P < 0.0001. For e, data are mean ± s.e.m. of four independent experiments performed in triplicate and normalized to the response to LY341495 (10% and LY379268 (100%). For h, data are mean ± s.e.m. of triplicate measurements from a representative experiment of three independent experiments. c, f, i. Dose-dependent effects of the indicated ligands on the TR-FRET signal of the biosensor measured in HEK293 cells cotransfected to express mGlu2C1 and mGlu4C2 (c) or wild-type mGlu2 and mGlu4 (f) or in the dissociated cells from the olfactory bulb (i). Data are mean ± s.e.m. of n independent experiments performed in duplicate (c, f, n = 3) or in triplicate (i, n = 5) and were normalized to the response to LY379268. g, TR-FRET signal of the biosensor when the ratio in the presence of LY379268 (1 μM) and LY341495 (10 μM) was measured. Each dot represents one measurement of the indicated brain tissue of wild-type or mGlu4-KO mice (n = 4, except the striatum group of mGlu4-KO mice (n = 3)). Data were analyzed using two-tailed Student’s t-test, with ****P ≤ 0.0001, **P ≤ 0.01, *P ≤ 0.05 and n.s., P > 0.05, and the exact P values are indicated.

from cells expressing both mGlu2 and mGlu4 subunits (Fig. 5d–f). Under the latter conditions, both homodimers were present on the cell surface along with the heterodimer (Fig. 5d), demonstrating that they do not interfere with the specific signal generated by the active heterodimer. Under these conditions, treatment with the agonist LY379268 generated a large signal that was largely inhibited by the antagonist LY341495, while treatment with L-AP4 generated a smaller signal (Fig. 5b,e). Moreover, as previously reported17,27,34, a strong positive cooperativity was observed between the mGlu2 and the mGlu4 agonists on the heterodimer, illustrated here with the large increase in L-AP4 potency caused by a low concentration of LY379268 (Fig. 5c,f and Extended Data Fig. 10d). These data are also consistent with the IP3 production data obtained from cells expressing controlled mGlu2–mGlu4 or both mGlu2 and mGlu4 (Extended Data Fig. 10a–c). Altogether, these results show that DN10 and DN42 can be used to detect the active form of the mGlu2–mGlu4 heterodimer.

This mGlu2–mGlu4 biosensor also detected activation of endogenous mGlu2–Glu4 heterodimer in dissociated brain cells as revealed by the synergy between the mGlu2 and mGlu4 agonists. Activation of endogenous mGlu2–mGlu4 was revealed by the large FRET signal induced by LY379268 in all regions where the mGlu2–mGlu4 heterodimer was detected (that is, the olfactory bulb, PFC, striatum and hippocampus) but not in the cerebellum (Fig. 5g). As expected, the LY379268 effect disappeared in mGlu4-KO brain samples. In addition, in the olfactory bulb (Fig. 5h), L-AP4 potency...
Our study describes an innovative and general method for the quantification and analysis of endogenous multi-subunit membrane proteins using nanobody-based optical sensors. Using this method, we provide direct evidence for the existence of mGlu2–mGlu4 heterodimers in different brain areas. Surprisingly, our results revealed that most mGlu4 subunits are likely associated with another subunit, such as mGlu2, in most brain regions outside the cerebellum.

Our method combines the high spatial resolution of TR-FRET technology (<15 nm) with the small size of single-domain nanobodies (~2.5 nm) to detect low amounts of endogenous subunits in native tissues. Obtaining such information in native membranes is essential, as lipid composition and ions likely play an important role in stabilizing protein complexes. No chemical fixation or biochemical treatment of the biological sample is required, in contrast to other analyses, thus preventing conformational changes of the complex. In addition, our results prove that nanobodies have great potential as TR-FRET probes, which help to solve the shortcomings of small molecules in terms of specificity, which limits their use in TR-FRET experiments. Nanobodies have hydrophilic properties, in contrast to small molecules that can be hydrophobic, and help overcome the limitations of classical antibodies in recognizing specific protein conformations. Nanobodies are small antibodies (ten times smaller than immunoglobulin G (IgG) proteins) and easy to engineer and display good and rapid tissue penetration. They often recognize conformational and cryptic epitopes not accessible to classical antibodies. Our method can be applied to any cell surface protein, including ligand-gated ion channels or transporters.

In addition, our method is versatile, as the fluorophores can be covalently attached to small ligands, antibody fragments or common antibodies. Finally, our method does not require a high level of expertise or expensive equipment. It only entails working in microplates with standard biochemical protocols and a standard commercial TR-FRET reader. However, the TR-FRET approach may not be appropriate for the detection of heterodimers using microscopy of brain slices due to the low quantum yield of the donor and the need for special equipment for the time delay between the excitation and the measurement of the emission signal. It can however be used for cultured neurons (Extended Data Fig. 2). However, the use of fluorophores compatible with conventional FRET microscopy may allow the detection of dimers in neuronal subcompartments using microscopy of cultured neurons with better precision.

Our approach has two major advantages in investigating endogenous mGlu heterodimers. First, it analyzes the heterodimer entity directly and not its downstream signaling that could result from cross-talk between signaling pathways. Second, our biosensors are good reporters of the conformational change of the receptor during activation, as are other sensors of the mGlu receptors. However, whether this approach can be used to detect mGlu receptor activation in real time remains to be tested. For this, the use of fluorophores compatible with conventional FRET microscopy will be necessary. It will also be essential to take into consideration the ‘ON’ rate of binding of the nanobody that recognizes the active form of the receptor, as this may be much slower than the ‘ON’ rate of mGlu4 receptor activation that occurs in the submillisecond time scale. It is clear that this second point will generate limitations for such analysis. Finally, the pharmacological signature of our new sensors using orthosteric ligands could be defined in transfected cells and could then be observed in native brain samples.

Our study reveals an intriguing distribution of mGlu4 heterodimers, mainly found in the cerebellum, where they do not form detectable heterodimers with mGlu2 as expected, as these two subunits are expressed in different types of neurons in the cerebellum. These data appear to be an excellent control for our assay. The absence of significant detection of the mGlu4 homodimer in most brain regions does not exclude the fact that some homodimers may be present. Indeed, mGlu4 homodimers were proposed at hippocampal–medial PFC (mPFC) and amygdala–mPFC synapses and at corticostriatal synapses, suggesting that our approach is not
sensitive enough to detect these homodimers. However, this conclusion was based on the use of mGlur4 positive allosteric modulators inactive at mGlur2–mGlur4, such as N-phenyl-7-(hydroxyimino) cyclopropyl[b]chromen-1a-carboxamide (PHCCC). As the effect of these compounds on mGlur4–mGlur3, mGlur4–mGlur7 or mGlur4–mGlur8 heterodimers is not known, further pharmacological studies of these heterodimers will be necessary to clarify this issue. Regardless, our data clearly show that, in many brain regions, there are more mGlur2–mGlur4 dimers than mGlur4–mGlur4 dimers, as a larger signal could be detected with the mGlur2–mGlur4 detector, despite a very similar FRET efficacy per dimer. Interestingly, an astonishing distribution of the mGlur2–mGlur4 heterodimer was observed, with high expression in the olfactory bulb and the PFC, in agreement with the demonstration of mGlur2–mGlur4 at thalamo–mPFC synapses, where they would coexist with mGlur2 homodimers without excluding low amounts of mGlur4 homodimers. This is consistent with the link between the mGlur2 subunit and psychiatric diseases involving the PFC.

Future studies on the existence of other mGlur heterodimers are crucial for assessing the physiological role of mGlur receptors in the brain, potential new drug targets. Indeed, mGlur4 heterodimers could explain the effect of an mGlur4 allosteric modulator acting in the basal ganglia, which had no effect on the mGlur4 homodimer. In addition, mGlur7 heterodimers could also contribute to the enigmatic function of the mGlur7 subunit due to its very low glutamate potency and the effect of mGlur7 negative allosteric modulators with context-dependent activity. Further studies are necessary to clarify these issues, as well as the functional role and therapeutic potential of these mGlur heterodimers, a step that will require the development of specific ligands for these receptor species.

In conclusion, we have reported a general and versatile approach compatible with the quantification and functional analysis of membrane proteins from endogenous native tissues without disrupting the membrane environment, but the availability of specific ligands is a major limitation. However, the number of antibodies targeting these proteins, including nanobodies, is rapidly expanding, even those selective for a conformational state.

Online content
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Methods

Animal ethics. This project followed the Animal Welfare Body guidelines and was approved by the internal ethics committee of the Institut de Génomique Fonctionnelle. Wild-type mice were purchased from Janvier Labs, and mGlu2-KO micewere kindly provided by C. Maesso (Virginia Commonwealth University School of Medicine), while mGlut4-KO mice were also available at the Institut de Génomique Fonctionnelle. Animals were housed under a 12-h light-dark cycle at 23 ± 2 °C with a relative humidity of 53% ± 10%. Mice had access to water and food ad libitum.

Reagents, cell lines and plasmids. DCC-G IV, LY314495, LY379268 and LY53470 were purchased from Tosics Bioscience. Glutathione was purchased from Sigma-Aldrich. LSP-2004 was provided by F. Acher (Paris, France). Anti-c-Myc-d2, anti-6His-d2, SNAP-Lumi4-Tb, Lumi4-Tb–NHS and SNAP-d2–NHS and Tag-lite buffer were kindly supplied by PerkinElmer Cisbio. HEK293 cells (ATCC, CRL-1573, lot 3449004) were cultured in DMEM (Thermo Fisher Scientific) supplemented with 10% (vol/vol) FBS (Sigma-Aldrich) at 37 °C with 5% CO2. HEK293F cells were cultured in suspension in SMM-293-TI animal-free and serum-free medium at 37 °C with 5% CO2 and shaking at 200 r.p.m. Of the cells, 2–4 l of the culture medium. The sequence encoding the C terminus of the nanobody was fused with the sequence encoding the Fc region of human immunoglobulin IgG1.

Production and purification of nanobodies. mGlu4 subunit was selected from the VHH library after all procedures including purification and separation of nanobodies by affinity chromatography with metal ion afﬁnity chromatography. Bacteria were then infected with the helper phage, and phage-containing buffers to extract the periplasmic proteins. Nanobody in the periplasmic space was obtained by detergent lysis of E. coli BL21DE3 colony transformed with the pHEN phagemid carrying cDNA the mGlu4 receptor. Each round was preceded by a depletion step for cells that were not transfected, and positive selection was performed in the presence of an excess of antibodies reacting on control HEK293 cells. Escherichia coli TG1 bacteria were infected with eluted phages and used for sequencing and production of the nanobody.

Library construction and DN42 selection. The DN42 nanobody targeting the mGlut4 subunit was selected from the VHH library after all procedures including llama immunization, library construction and selection of nanobodies targeting mGlut4 were performed as described previously. Bacteria were then infected with the KM13 helper phage, and phage-containing pellets were purified by two selection rounds with 2 × 108 HEK293F cells transferred to express rat mGlut4 receptor. Each round was preceded by a depletion step for cells that were not transfected, and positive selection was performed in the presence of an excess of antibodies reacting on control HEK293 cells. Escherichia coli TG1 bacteria were infected with eluted phages and used for sequencing and production of the nanobody.

Production and purification of nanobodies. For the nanobodies DN1 and DN10, production and purification were performed as described previously. Briefly, an E. coli BL21DE3 colony transformed with the pHEN phagemid carrying cDNA for the nanobody of interest was grown in LB medium. The bacteria were cultured in large scale at 37 °C, and nanobody expression was induced with 1 mM IPTG at 28 °C. Bacteria were then collected after centrifugation and washed with phosphate-buffered saline (PBS). The supernatant was collected from the supernatant after centrifugation at 4 °C.

For large-scale production of the DN42 and Fc–GFP nanobodies, HEK293F cells were cultured at a density of 0.6 × 106 cells/ml with 180 ml fresh medium in a 2-L culture bottle at 37 °C with 5% CO2 and shaken at 200 r.p.m. Of the cells, 1–1.5 × 106 ml were transferred with a mixture of Fc–DN42 or Fc–GFP plasmids (225 μg in 12 ml OMEM) and PEI (675 μg in 12 ml OMEM). Cells were cultured for 4–7 days at 37 °C with 5% CO2 and shaking at 200 r.p.m. The supernatant was collected after a 10-min centrifugation at 2,000g and 4 °C.

The His-tagged nanobodies from both bacteria and HEK293F cells were then purified from the supernatant by Ni-NTA purification (Qiagen) in accordance with the manufacturer’s instructions. Finally, the nanobodies were purified by size-exclusion chromatography on a Superdex 200 10/300 column for DN42 and Fc–GFP and a Superdex 75 10/300 column for DN1 and DN10 (GE Healthcare) in PBS (pH 7.4).

Nanobody labeling. Nanobodies were dialyzed overnight at 4 °C and incubated (250 μg nanobody per 2 ml) at 20 °C with d2–NHS (PerkinElmer Cisbio) in 0.1 M carbonate buffer (pH = 9) and Lumi4-Tb–NHS in 50 mM phosphate buffer (pH = 8) at a molar ratio of 6 or 12 for 45 or 30 min, respectively. The nanobodies were then purified using a gel filtration column (in 100 mM phosphate buffer (pH = 7). The final molar ratio, namely, the number of fluorophores per nanobody, was calculated as the fluorophore concentration/conjugated nanobody concentration, and conditions were set for a ratio between 2 and 3 (for DN1 and DN10), 2 and 4 (for DN42 labeled with d2) or 5 and 8 (for DN42 labeled with Lumi4-Tb). The concentration of fluorophores in the labeled fraction was calculated as optical density (OD)−1 for each fluorophore (OD at 340 nm and ε = 26,000 M−1 cm−1 for Lumi4-Tb, OD at 650 nm and ε = 225,000 M−1 cm−1 for d2), while the concentration of the nanobodies was determined by the OD at 280 nm (OD of 1). The conjugated concentration was calculated as OD−1(ODmax−Rζmax)−1. After purification, labeled nanobodies were supplemented with 0.1% BSA and stored at −20 °C.

TR-FRET binding measurements. The FRET signal was determined by measuring the ratio of d2 acceptor emission at 620 nm versus 655 nm and Lumi4-Tb donor emission (emission, 620 nm) using a 50-μs delay and 450-μs integration upon excitation at 337 nm. All data were obtained using a PHERAstar FS reader (BMG LABTECH). The TR-FRET ratio was calculated as emission at 665 nm emission at 620 nm x 10, as previously described.

HEK293 cells were cotransfected to express rat SNAP-tagged mGlut4 and EAC1 (unless otherwise indicated) using Lipofectamine in a 100-mm cell culture dish in accordance with the manufacturer’s instructions. Twenty-four hours after transfection, cells were plated in polystyrene-coated, white 96-well plates (Greiner Bio-One) at 105 cells per well and cultured overnight at 37 °C with 5% CO2 for adherent cell experiments. Cells were labeled with 100 nM SNAP-Lumi4-Tb in DMEM GlutaMAX (Thermo Fisher Scientific) for 2 h at 37 °C and then washed three times with Krebs buffer (10 mM HEPES, pH 7.4, 1.46 mM NaCl, 4.2 mM KCl, 1.0 mM CaCl2, 0.5 mM MgCl2, 5.6 mM glucose and 0.1% BSA). For suspension experiments, cells were frozen at −80 °C with 10% DMSO and then washed three times with Krebs buffer before use. Five microliters of cells were plated in a white, small-volume 384-well plate (Greiner Bio-One) at 2 x 103 cells per well.

To determine the selectivity of DN42 for the mGlut4–mGlut8 receptors, 100 nM nanobody and 200 nM anti-His antibody labeled with d2, and the agonists (1 μM quisqualic acid for mGlut1; 5 or 100 μM L-AP4 for mGlut4, mGlut6, mGlut7 and mGlut8; 100 nM LY379268 for mGlut4 and mGlut3) or the antagonist (10 μM DCG-IV) were added to labeled cells in adherent cell experiments, with a total volume of 60 μl per well. After overnight incubation at 25 °C, the TR-FRET signal between Lumi4-Tb and d2 was determined.

To determine the affinity of the c-Myc-tagged nanobodies (DN1 and DN10) and the His-tagged nanobody DN42, 200 nM anti-c-Myc and 100 nM anti-His antibodies, both labeled with d2, were used. The different reagents were applied to labeled cells in adherent or suspension experiments, with a total volume of 20 μl per well incubated overnight at 25 °C. The TR-FRET signal between Lumi4-Tb and d2 was determined.

SNAP subunit quantification. Transfected HEK293 cells were plated in polystyrene-coated, white 96-well plates (Greiner Bio-One) at 105 cells per well and cultured overnight at 37 °C with 5% CO2. The cells were then labeled with 100 nM SNAP-Lumi4-Tb in DMEM GlutaMAX for 2 h at 37 °C, followed by three washes with Krebs buffer. The signal was determined by measuring the emission intensity of Lumi4-Tb at 620 nm with a 50-μs delay and 450-μs integration upon excitation at 337 nm. All data were obtained using a PHERAstar FS reader (BMG LABTECH).

Tissue sample preparation. To obtain dissociated cells from brain tissue, 6–8-week-old C57BL/6 mice (including wild-type or Grm4−/− male and female mice) were euthanized; the whole brain was dissected in cold PBS (pH = 7.4) to obtain the regions of interest, according to the Allen Mouse Brain Atlas (https://mouse.brain-map.org/3). Tissues were quickly cut into small pieces using a scalpel, collected in a 1.5-mL cryotube (Thermo Fisher Scientific), then transferred into a new 2-mL cryotube. The remaining precipitate was resuspended in 300 μl DMEM GlutaMAX supplemented with 10% FBS. After 20 min at 37 °C, the cells were dissociated by pipetting. Next, 200 μl DMEM GlutaMAX supplemented with 10% FBS was added. After 8 min of incubation at 37 °C, the mixture was transferred into a new 2-mL tube. The resulting precipitate was resuspended in 300 μl DMEM GlutaMAX supplemented with 10% FBS. After 3 min of incubation, the supernatants were transferred and combined with the first supernatant. This step was repeated two more times to obtain the largest number of dissociated cells. Finally, the total dissociated cells were centrifuged at 3,000g for 5 min, and the cell pellet was resuspended in 400μl cold PBS.

TR-FRET measurement with the nanobody-based sensors. For TR-FRET measurements in attached cells, transfected HEK293 cells were plated in...
polystyrene-coated, white 96-well plates (Greiner Bio-One) at 105 cells per well and cultured overnight at 37 °C with 5% CO2. Cells were starved in DMEM GlutaMAX for 2 h at 37 °C and then washed once with Krebs buffer to reduce ambient glutamate levels. Donor and acceptor nanobodies and ligands were prepared in Krebs buffer and added to the plate to reach a total volume of 60 µl per well. For TR-FRET measurements in cell suspensions, 5 µl HEK293 cells alone or transfected with plasmids encoding mGlu receptors were labeled in Tris–Krebs buffer with the appropriate nanobody: 200 nM DN1–d2 (overnight at 4 °C) or 1.6 nM DN42–d2 for the SNAP-tagged mGlur4 homodimer; 1.6 nM DN24–Tb and 25 nM DN1–d2 for the coexpressed wild-type CLIP-tagged mGlur2 and SNAP-tagged mGlur4 to obtain the mGlur2–mGlur4 heterodimer or the SNAP or CLIP substrates (100 nM SNAP–Lumi4–Tb and 100 nM SNAP–d2 for the SNAP-tagged mGlur4 homodimer; 100 nM CLIP–Lumi4–Tb and 100 nM SNAP–d2 for the coexpressed wild-type CLIP-tagged mGlur2 and SNAP-tagged mGlur4). After labeling, the cells were washed three times with Tris–Krebs buffer, and the signal was recorded with 100 µl Tris–Krebs buffer per well. The TR-FRET signal was measured using a PHERAstar FS reader, by the emission of d2 at 665 nm with a 50-µs delay and an integration time of 450 µs after excitation at 337 nm.

**Bicinoclinic acid assay for protein quantification.** The dissociated cells (30 µl) in PBS were diluted twice with PBS containing 2% Triton X-100 and incubated at 20 °C for 1 h. The total protein quantity was measured in triplicate using a bicinoclinic acid kit (BCA, Sigma-Aldrich) in accordance with the manufacturer’s instructions using the Infinite F500 microplate reader (Tecan).

**Relative quantification of the mGlur4–mGlur4 homodimer and the mGlur2–mGlur4 heterodimer by TR-FRET.** Twenty-four hours after transfection, cells (100,000 cells per well in a 96-well microplate) were labeled in Tris–Krebs buffer for 1 h with the donor ‘magnetic’ nanobodies (1 µM DN1–d2 and 1.6 nM DN42–d2 for the SNAP-tagged mGlur4 homodimer; 1.6 nM DN24–Tb and 25 nM DN1–d2 for the coexpressed wild-type CLIP-tagged mGlur2 and SNAP-tagged mGlur4) and the ‘detector’ nanobodies (1 µM DN2–d2 and 100 nM SNAP–d2 for the SNAP-tagged mGlur4 homodimer; 100 nM CLIP–Lumi4–Tb and 100 nM SNAP–d2 for the coexpressed wild-type CLIP-tagged mGlur2 and SNAP-tagged mGlur4). After labeling, the cells were washed three times with Tris–Krebs buffer, and the signal was recorded with 100 µl Tris–Krebs buffer per well. The TR-FRET signal was measured using a PHERAstar FS reader, by the emission of d2 at 665 nm with a 50-µs delay and an integration time of 450 µs after excitation at 337 nm.

**Measurements of inositol phosphate concentration.** HEK293 cells were transiently cotransfected to express mGlur4 receptors, EAAC1 and the chimeric C14 protein using Lipofectamine 2000. Sixteen hours after transfection, the cells were incubated with the indicated ligands and 10 mM LiCl for 30 min. The accumulated IP1 concentration was quantified using a PHERAstar FS reader and the IP-One HTTRF assay kit (PerkinElmer Cabiso) in accordance with the manufacturer’s instructions.

**Neuronal culture and TR-FRET microscopy imaging.** The primary hippocampal neurons were cultured following the procedures described previously, and, after 17 d of culture, neurons were imaged. Neurons were labeled with 120 nM DN1–d2 and 25 nM DN10–d2 (1.5 h at 20 °C). Sections were fixed at 30% sucrose solution, included in an optimal cutting temperature compound (OCT, VWR) and 16-μm sagittal sections were obtained. Sections were rinsed with PBS and incubated for 1 h at 4 °C with the ‘antibody’ nanobody: 200 nM DN1–d2 (overnight at 4 °C) or 1.6 nM DN42–d2 for the SNAP-tagged mGlur4 homodimer; 1.6 nM DN24–Tb and 25 nM DN1–d2 for the coexpressed wild-type CLIP-tagged mGlur2 and SNAP-tagged mGlur4. After labeling, the cells were washed three times with Tris–Krebs buffer, and the signal was recorded with 100 µl Tris–Krebs buffer per well. The TR-FRET signal was measured using a PHERAstar FS reader, by the emission of d2 at 665 nm with a 50-µs delay and an integration time of 450 µs after excitation at 337 nm.

**Statistical analysis.** All data are presented as mean ± s.e.m. and were initially analyzed using GraphPad Prism (version 9.1.2 for Windows, GraphPad software) using a two-way ANOVA with Tukey's post hoc test. Normally distributed datasets (P > 0.05) were analyzed using parametric tests, two-tailed Student’s t-tests or one-way ANOVA followed by Dunnett’s or Tukey’s post hoc analysis or two-way ANOVA followed by Tukey's post hoc test, depending on the experiments analyzed. For data analyzed using one-way ANOVA with a significant Brown–Forsythe test (P < 0.05, meaning that there was unequal variance between the different groups), datasets were analyzed using Welch’s ANOVA test followed by Dunnett’s T3 post hoc test (recommended for n < 50 per group). For all statistical analyses, a probability of 0.05 was defined as a significant difference. The exact P values are indicated in figures or in figure legends.

**Brain collection and fixation.** Mice were euthanized with 140 mg per kg sodium pentobarbital (Euthasol Vet, Dômes Pharma Véterinaire TVM) followed by cardiac perfusion with PBS. Brains were extracted and incubated overnight at 4 °C in a 4% paraformaldehyde solution (EuroMedex), cryoprotected for 4 d at 4 °C with a 30% sucrose solution, included in an optimal cutting temperature compound (Tissue-Tek O.C.T., Sakura Finetek) and quickly frozen in ethanol cooled on dry ice. Brains were stored at −80 °C until use. Frozen brains were mounted on a cryostat (Leica Biosystems), and 16-μm sagittal sections were obtained. Sections were mounted on Superfrost Plus glass slides (Microm France) and kept at −20 °C until use.

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Author contributions
J.M. developed the FRET-based detectors and the FRET-based conformational change sensors and performed sensor experiments, IP1 assays, co-immunoprecipitation and immunoblotting. C.X. proposed the idea and set up the protocol for the nanobody-based sensors for the detection of mGlu2 expression and activation in dissociated brain cells and performed the DN1 and DN10 sensor experiments in HEK293 cells and dissociated brain cells and IP1 assays. P.-A.L. performed tissue immunofluorescence assays. S.R. designed and performed experiments with the FRET-based detectors and conformational change sensors. M.M. performed TR-FRET microscopy imaging, nanobody production, purification and labeling. B.Z. performed nanobody production, purification, labeling and co-immunoprecipitation. P.S. and E.B. developed the FRET-based conformational change biosensor for the mGlu2 homodimer in HEK293 cells. J.A., I.B. and J.L.M. provided brain samples and trained J.M. for brain sample preparation. J.G.-M. prepared brain samples for wild-type and mGlu2-KO mice. P.C. screened for anti-mGlu2 and anti-mGlu4 nanobodies and prepared the Fc versions. J.L., J.-P. and P.R. conceived experiments, supervised the work and wrote the manuscript.

Competing interests
P.R. and J.-P.P. are funded by PerkinElmer Cisbio through the collaborative laboratory Eidos. The remaining authors declare no competing interests.

Additional information
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Correspondence and requests for materials should be addressed to Jianfeng Liu, Jean-Philippe Pin or Philippe Rondard.
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Extended Data Fig. 1 | DNI/DN10 FRET-based biosensor detects mGlu2 homodimers on transfected HEK-293 cells. (a) TR-FRET signal measured on HEK-293 cells transiently transfected with mGlu2 receptors in the presence of donor nanobody DNI-Tb (7.5 nM) and LY379268 (1 μM), with or without acceptor nanobody DN10-d2 (15 nM). (b) Specific TR-FRET signal on HEK-293 cells transiently transfected with mGlu2 receptors in the presence of DNI-Tb (7.5 nM) with increasing concentrations of DN10-d2. For each concentration of acceptor nanobody, specific FRET signal was the difference between samples in the presence of LY379268 (1 μM) and LY341495 (10 μM). Data are mean ± SEM of three independent experiments performed in triplicates and normalized to the maximum. (c) TR-FRET signal measured on HEK-293 cells transiently transfected with indicated mGlu receptors or on mock cells with LY379268 (1 μM). In a and c, data are mean ± SEM of triplicate determinations from one representative out of three experiments.
Extended Data Fig. 2 | TR-FRET confocal imaging on primary cultured hippocampal neurons. DN10-Tb (80 nM) and DN1-d2 (120 nM) were applied on the primary cultured hippocampal neurons. Fluorescence signals of different channels, TR-FRET channel (left), donor channel (middle) or acceptor channel (right) were obtained under agonist (150 nM LY379268, top) or antagonist (1μM LY341495, bottom). Scale bar: 20 μm.
Extended Data Fig. 3 | DN1/DN10 biosensor measures endogenous mGlu2 on cells from brain regions. (a) Diagram shows flow of the tissue samples experiments. (b) Schematic diagram of adult mouse brain removed from most of the cortex, in top view. Brain regions analyzed are olfactory bulb (blue), prefrontal cortex (red), striatum (green), hippocampus (purple), midbrain (orange) and cerebellum (gray). (c) Relative quantification of the endogenous mGlu2 receptor in the indicated brain regions in the same mouse using the indicate FRET-based biosensor in presence of 1 μM LY379268. Data are mean ± SEM of triplicate determinations from one representative out of three experiments.
Extended Data Fig. 4 | DN1-d2 immunofluorescence staining of a saggital section in wild-type and mGlu2 knock out mouse brains. The scale bars are indicated.
Extended Data Fig. 5 | FRET-based detector mainly detects mGlu2 homodimer. (a) TR-FRET signal measured on HEK-293 cells transiently transfected with mGlu2 receptors in the presence of DN1-Tb (25 nM) and LY341495 (10 μM), with or without DN1-d2 (25 nM). (b) Specific TR-FRET signal on HEK-293 cells transiently transfected with mGlu2 receptors in the presence of DN1-Tb (25 nM) and LY341495 (10 μM) with increasing concentrations of DN1-d2. For each concentration of acceptor nanobody, specific FRET signal was the difference between samples transfected with mGlu2 receptor or not. Data are mean ± SEM of three independent experiments performed in triplicates and normalized to the maximum. (c and d) TR-FRET intensity and cell surface expression were measured on various expression levels of the indicated cell samples. Experiments were performed with HEK-293 cells transiently transfected with SNAPmGlu2 which fused a SNAP tag at the N terminus of receptors (c) or transiently co-transfected with SNAPmGlu2C1 and mGlu2C2 or mGlu4C2 (d). The surface expression of receptors was measured as the specific Tb emission at 620 nm after labeling by substrate SNAP-Lumi4-Tb and exciting at 337 nm. TR-FRET intensity was measured in the presence of 10 μM LY341495. (e) Relative quantification of mGlu2 receptor in the indicated brain tissues from a same mouse in the presence of 10 μM LY341495. For a and c-e, Data are mean ± SEM of triplicate determinations from one representative out of three experiments.
Extended Data Fig. 6 | Nanobody DN42 specifically targets mGlu4 receptors. (a) Cartoon illustrating the principle of the TR-FRET binding assay. The receptor fused to a SNAP-tag (dark gray circled labeled 'S') is labeled with donor fluorescent dye Lumi4-Tb (blue circled ‘D’) while the nanobody DN42 (dark blue) bearing a 6xHis tag epitope at its C-terminus is labeled with 100 nM of anti-His antibody (bright blue) coupled to d2 fluorophores (red circled ‘A’). Binding of the nanobody to the receptor is then measured by TR-FRET. (b) Specific TR-FRET binding data obtained with the indicated mGlu receptor and either 100 nM Fc-DN42 or a control irrelevant nanobody (100 nM) in cells under indicated drug conditions, in the presence of the agonist 1 μM quisqualic acid (mGlu1 and 5) or 100 μM L-AP4 (mGlu4, 6, 7 and 8) or 100 nM LY379268 (mGlu2 and 3) or the antagonist LY341495 (10 μM). Data are mean ± SEM of triplicate determinations from one representative out of three experiments. (c) Saturation binding curves obtained with DN42 on SNAPmGlu4 receptors under indicated drug conditions, in the presence of the agonist L-AP4 (100 μM), or the antagonist LY341495 (10 μM). Data are mean ± SEM of three independent experiments performed in triplicates and normalized to the maximum. (d) DN42-d2 immunofluorescence staining of a sagittal section in wild-type and mGlu4 KO mouse brains. The scale bars are indicated.
Extended Data Fig. 7 | FRET-based detector mainly detects mGlu4 homodimer. (a) TR-FRET signal measured on HEK-293 cells transiently transfected with mGlu4 receptors in the presence of DN42-Tb (1.6 nM) and LY341495 (10 μM), with or without DN42-d2 (1.6 nM). (b) Specific TR-FRET signal on HEK-293 cells transiently transfected with mGlu4 receptors in the presence of DN42-Tb (1.6 nM) and LY341495 (10 μM), with increasing concentrations of DN42-d2. For each concentration of acceptor nanobody, specific FRET signal was the difference between samples transfected with mGlu4 receptors or not. Data are mean ± SEM of three independent experiments performed in triplicates and normalized to the maximum. (c and d) TR-FRET intensity and cell surface expression were measured on various expression levels of the indicated cell samples. Experiments were performed with HEK-293 cells transiently transfected with SNAPmGlu4 which fused a SNAP tag at the N terminus of receptors (c) or transiently co-transfected with SNAPmGlu4C2 and mGlu4C1 or mGlu2C1 (d). The surface expression of receptors was measured as the specific Tb emission at 620 nm after labeling by substrate SNAP-Lumi4-Tb and exciting at 337 nm. TR-FRET intensity was measured in the presence of 10 μM LY341495. (e) Relative quantification of mGlu4 receptor in the indicated brain tissues from a same mouse in the presence of 10 μM LY341495. (a and c-e) Data are mean ± SEM of triplicate determinations from one representative out of three experiments.
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | FRET-based detector only detects mGlu2-4 heteromer. (a) TR-FRET signal measured on HEK-293 cells transiently expressing mGlu2<sub>C1</sub>-4<sub>C2</sub> heterodimer in the presence of DN42-Tb (1.6 nM) and LY341495 (10 µM), with or without DN1-d2 (25 nM). (b) Specific TR-FRET signal on HEK-293 cells transiently expressing mGlu2<sub>C1</sub>-4<sub>C2</sub> heterodimer in the presence of DN42-Tb (1.6 nM) and LY341495 (10 µM) with increasing concentrations of DN1-d2. For each concentration of acceptor nanobody, specific FRET signal was the difference between samples expressing mGlu2<sub>C1</sub>-4<sub>C2</sub> heterodimer or not. Data are mean ± SEM of three independent experiments performed in triplicates and normalized to the maximum. (c) TR-FRET intensity and cell surface expression were measured for various expression levels of the indicated cell samples. Experiments were performed with HEK-293 cells transiently co-transfected with SNAPmGlu2<sub>C1</sub> and mGlu4<sub>C2</sub> (red) or SNAPmGlu2<sub>C1</sub> and mGlu2<sub>C2</sub> (blue). The surface expression of receptors was measured as the specific Tb emission at 620 nm after labeling by substrate SNAP-Lumi4-Tb and exciting at 337 nm. TR-FRET intensity was measured in the presence of 10 µM LY341495. (d) TR-FRET signal measured on HEK-293 cells transiently transfected with indicated the mGlu receptors or on mock cells with LY341495 (10 µM). (e and g) Relative quantification of mGlu2-4 heterodimer in the indicated brain tissues in the presence of 10 µM LY341495. (f) mGlu2 and mGlu4 are co-immunoprecipitated from mouse olfactory bulb. FcDN42 was used as probe antibody in pull down experiments. FcGFP was used as a negative control (IgG control). Results are one representative out of three independent experiments performed. For a, c-e and g, Data are mean ± SEM of triplicate determinations from one representative out of three experiments.
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | Nanobody DN10 allows to develop a FRET-based conformational biosensor for mGlu2-4 heterodimer. (a) Schematic representation of the TR-FRET binding assay for the mGlu2-4 controlled heterodimer. The receptor fused to a SNAP-tag (dark gray circled labeled ‘S’) at the N-terminus of mGlu4 subunit is labeled with donor fluorescent dye Lumi4-Tb (blue circled ‘D’), while the nanobody DN10 (purple) coupled to a d2 fluorophore (red circled ‘A’). Binding of the nanobody to the receptor is then measured by TR-FRET. (b) Saturation binding experiments with DN10-d2 on the cell surface mGlu2-4 controlled heterodimer using the constructs mGlu2c2 and SNAPmGlu4c2 transiently co-transfected in HEK-293 cells. Binding of DN10-d2 to the receptor was measured by TR-FRET under the indicated conditions, buffer (gray, n = 5) or in the presence of the mGlu2 agonist LY379268 (1 μM, blue, n = 5), the mGlu4 agonist L-AP4 (100 μM, red, n = 3), or the antagonist LY341495 (10 μM, black, n = 5). Data are mean ± SEM of at least three individual experiments each performed in triplicates and normalized to the maximum. (c and d) Dose-dependent effects of the indicated ligands on TR-FRET signal between SNAP-tag labeled with donor fluorescent dye Lumi4-Tb (blue circled ‘D’) and 25 nM DN10-d2. Signals were measured on HEK-293 cells transiently co-transfected with mGlu2c1 and SNAPmGlu4c2 (c, n = 3) or co-transfected with mGlu2c1 and SNAPmGlu2c2 (d, n = 4). Data are mean ± SEM of at least three independent experiments performed in triplicate and normalized to the response of LY379268. (e) The FRET potencies (pEC50) in the indicated conditions. Data are mean ± SEM from HEK-293 cells transiently co-transfected with mGlu2c1 and SNAPmGlu4c2 (c, n = 3) or with mGlu2c2 and SNAPmGlu2c2 (d, n = 4). One-way ANOVA with Tukey’s multiple comparisons test (mGlu2 under the LY379268 is not included), with **P = 0.0041. (f) TR-FRET signal measured on HEK-293 cells transiently transfected with mGlu2c2 and mGlu4c2 or on mock cells in presence of DN42-Tb (1.6 nM) and DN10-d2 (25 nM) with 1 μM LY379268 (LY37) or 10 μM LY341495 (LY34). (g) TR-FRET signal measured on HEK-293 cells transiently transfected with the indicated mGlu receptors or on mock cells with LY379268 (1 μM). For f - g, Data are mean ± SEM of triplicate determinations from one representative out of three experiments.
Extended Data Fig. 10 | IP1 accumulation of mGlu2-4 heterodimer shows its specific pharmacological fingerprints. (a and b) Dose-dependent effects of the indicated ligands on IP1 accumulation. Signals were measured on HEK-293 cells transiently co-transfected with mGlu2C1 and mGlu4C2 (a) or with mGlu2 and mGlu4 (b) and EAAC1 and the chimeric G protein Gqi9. Data are mean±SEM of three independent experiments performed in triplicate and normalized to the response of LY379268. (c) IP1 accumulation potencies (pEC50) of the indicated drug on the receptor conditions in a and b. Data are mean±SEM of three independent experiments. Two-way ANOVA with Tukey’s multiple comparisons test, with ***P < 0.001 and *P ≤ 0.05. (d) TR-FRET potencies (pEC50) for the indicated ligands on the conformational sensor with the constructs mGlu2C1 and mGlu4C2, or when both wild-type mGlu2 and mGlu4 subunits are co-transfected. Data are presented as the mean±SEM of four independent experiments. Two-way ANOVA followed by a Tukey’s post-hoc test, with ***P < 0.001, **P ≤ 0.01, and ns P > 0.05. (e) TR-FRET potencies (pEC50) of the ligands on the conformational sensor measured in dissociated olfactory bulb cells. Data are presented as the mean±SEM of four independent experiments. One-way ANOVA followed by a Tukey’s post-hoc test, with ****P ≤ 0.0001 and ***P ≤ 0.001. (f) Correlation between the potencies (pEC50) determined with the indicated agonist conditions by the heterodimer conformational sensor and IP1 assay. Data are mean±SEM of at least three independent experiments (TR-FRET of olfactory bulb (n = 4), mGlu2 + mGlu4 (n = 3) and mGlu2C1 + mGlu4C2 (n = 3) and IP1 accumulation of mGlu2C1 + mGlu4C2 (n = 3)). LY37 means LY379268. All exact P values are indicated in the panels c–e.
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*Our web collection on statistics for biologists contains articles on many of the points above.*

Software and code

Policy information about availability of computer code

| Data collection | All TR-FRET measurements were acquired using PHARStar control software (BMG LabTech, Ortenberg, Germany) in Excel (Microsoft, version 15.22).
| Data analysis | Protein concentration determination by microplate reader experiments were acquired using Tecan F500 control software [Version V2.1, Tecan Group Ltd.] in Excel (version 15.22). Data were plotted and statistically analyzed using Prism (Version 9.1.2, GraphPad Software); Fluorescent images were analyzed in ImageJ (Version 1.440, National Institutes of Health); Gel images were acquired through an imager system (Tanon 5200 Mult) and analyzed in Adobe Photoshop (Version 2020, Adobe). |

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All raw data of the main and extended Figures and Supplementary information are all included in the Data Source files. Materials and protocols are available on request. No deposit in publicly available datasets has been made since we want to control the access to our nanobodies. Link to the Allen Mouse Brain Atlas
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
Sample sizes were not predetermined using statistical analyses. The sample size varied between 3-4 for in vitro experiments. This sample size was used to verify experimental reproducibility and to obtain mean +/- SEM where applicable. Because of the practical condition restrictions, samples sizes were limited, e.g. cerebellum from mGlu2 KO and mGlu4 KO mice related to Fig. 2c, Fig. 3g and Fig. 3h. But all the presented data are representative results for at least three experiments that were performed independently. Information on the number of replicates and independent experiments that were performed for each measurement is provided in the manuscript.

Data exclusions
No data was systematically excluded. In TR-FRET experiments, few individual outliers within a triplicate were excluded from the analysis (this datapoints have been clearly highlighted in the Data Sources) when their values was above 30% in the triplicate (an exclusion criteria pre-established).

Replication
Number of independent experiments and replicates are indicated in the legends to the main figures, Extended data and Supplementary information.

Randomization
No randomization was attempted or needed. Randomization was not necessary as the independent variables to be tested were sufficient for the functional interpretation within this study, i.e. wild-type vs KO mice or dose-response determination.

Blinding
Blinding was not applicable to this study. All experimental data were acquired using automated equipment and analyzed using computational softwares, eliminating human error and bias.

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| n/a  | Involved in the study |
|------|------------------------|
| Antibodies | |
| Eukaryotic cell lines | X |
| Palaeontology and archaeology | X |
| Animals and other organisms | X |
| Human research participants | X |
| Clinical data | X |
| Dual use research of concern | X |

Methods

| n/a  | Involved in the study |
|------|------------------------|
| ChIP-seq | |
| Flow cytometry | |
| MRI-based neuroimaging | |

Antibodies

Antibodies used

Home made nanobodies: DN1, DN10 and DN42 with Fc fragment or GFP nanobody with Fc fragment. Commercial antibodies: mGluR2 antibody (Abcam, ab15672); mGluR4 antibody (Abcam, ab184302); HRP-conjugated goat anti-mouse IgG secondary antibody (Cell Signaling, 7076S) and HRP-conjugated goat anti-rabbit IgG secondary antibody (Cell Signaling, 7074S); d2-labeled anti-c-Myc antibody (61MYCDAF) and d2-labeled anti-6His antibody (61HIS6LF) are from PerkinElmer Cisbio (Codolet, France) and were used as the secondary antibody in TR-FRET binding experiments of non-labeling nanobodies.

Validation

The specificity and affinity of the nanobodies were described in this study and the previous work (Scholler et al., Nature Communications, 2017). The specificity of DN2 for different rat mGlu receptors was confirmed by TR-FRET binding experiments (Extended data Fig 6b), and the specificity of DN1 and DN10 for different rat mGlu receptors were confirmed by TR-FRET binding experiments in the previous work (Scholler et al., Nature Communications, 2017). The specificity of DN2 and DN1 for mouse receptors were confirmed by immunofluorescence in WT and KO mice brain slices (Extended data Fig 4 and Extended data Fig 6d). The affinity of the nanobodies were described in Supplementary Table 1 and 2. Commercial antibodies used in this study are widely used and have been validated by the respective manufacturer. mGluR2 antibody (Abcam, ab15672) https://www.abcam.com/metabotropic-glutamate-receptor-2mglur2-antibody-mg2na-s-
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)  HEK-293 cells (ATCC, CRL-1573, lot: 3449904) were used in all experiments that involved the expression of mGluRs in heterologous cells; FreeStyle™ 293 F Cells (Catalog Number: R790-07, ThermoFisher) were used only to produce nanobodies with Fc fragment (DN42 for mGlu4 and GFP nanobody). HEK-293T cells were used only to the preparation of cells for the llama immunization.

Authentication  All cell lines were used without further authentication.

Mycoplasma contamination  HEK-293 cells were tested monthly in laboratory and no mycoplasma contamination was found. It was described in the manuscript. HEK-293T and FreeStyle™ 293-F Cells were used without further mycoplasma contamination test.

Commonly misidentified lines (See ITAC register)  No commonly misidentified cell lines were used in this study.

Animals and other organisms

Policy information about studies involving animals, ARRIVE guidelines recommended for reporting animal research

Laboratory animals  6-8 weeks C57BL/6 mice (including wild-type, Grm4-/- and Grm2-/-). Both males and females were used (no need for gender analysis) 18 days pregnant Sprague-Dawley rat (wild-type) were used to primary hippocampal neurons culture (age was not clear).

Wild animals  The study did not involve wild animals.

Field-collected samples  The study did not involve samples collected from field.

Ethics oversight  This project followed the Animal Welfare Body and was approved by the Internal Ethic Committee of the Institut de Génémique Fonctionelle (Montpellier, France).

Note that full information on the approval of the study protocol must also be provided in the manuscript.