



HAL
open science

Allosteric nanobodies uncovered a role of hippocampal mGlu2 receptor homodimers in contextual fear consolidation.

Pauline Scholler, Damien Nevoltris, Dimitri de Bundel, Simon Bossi, David Moreno-Delgado, Xavier Rovira, Driss El Moustaine, Michaël Mathieu, Emilie Blanc, Heather Mclean, et al.

► **To cite this version:**

Pauline Scholler, Damien Nevoltris, Dimitri de Bundel, Simon Bossi, David Moreno-Delgado, et al.. Allosteric nanobodies uncovered a role of hippocampal mGlu2 receptor homodimers in contextual fear consolidation.. Nature Communications, 2017. hal-01672805v1

HAL Id: hal-01672805

<https://hal.science/hal-01672805v1>

Submitted on 17 May 2018 (v1), last revised 8 Jun 2021 (v2)

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Allosteric nanobodies uncovered a role of hippocampal mGlu2 receptors in context**
2 **fear consolidation**

3
4
5
6
7 Pauline Scholler^{*.1,2}, Damien Nevoltris^{*.2,3}, Dimitri de Bundel¹, Simon Bossi⁴, David Moreno-
8 Delgado¹, Xavier Rovira¹, Driss El Moustaine¹, Michaël Mathieu¹, Emilie Blanc¹, Heather
9 McLean⁴, Elodie Dupuis², Gérard Mathis², Eric Trinquet², Hervé Daniel⁴, Emmanuel Valjent¹,
10 Daniel Baty³, Patrick Chames^{3,#}, Philippe Rondard^{1,#}, Jean-Philippe Pin^{1,#}

11
12
13
14 ¹ Institut de Génomique Fonctionnelle, CNRS UMR5203, INSERM U1191, Univ. Montpellier,
15 Montpellier, France

16 ² Cisbio Bioassays, Codolet, France

17 ³ Aix Marseille Univ, CNRS, INSERM, Institut Paoli-Calmettes, CRCM, Marseille, F-13009,
18 France

19 ⁴ CNRS UMR9197, Université Paris-Sud, Institut des Neurosciences Paris-Saclay, France.

20
21
22 * Equal contribution

23 # To whom correspondence should be addressed.

24
25
26

27 **Abstract**

28

29 Antibodies have enormous therapeutic and biotechnology potential. G protein-coupled
30 receptors (GPCRs), one of the main targets in drug development, are of major interest in
31 antibody development programs. Metabotropic glutamate receptors are dimeric GPCRs that
32 can control synaptic activity in a multitude of ways. Here we identify llama nanobodies that
33 specifically recognize mGlu2 receptors, among the eight subtypes of mGluR subunits. Two of
34 these nanobodies act as positive allosteric modulators (PAMs) on homodimeric mGlu2. One
35 of them potentiates agonist actions on activated mGlu2, but has no effect on mGlu2-4
36 heterodimers. This PAM enhances the inhibitory action of the orthosteric mGlu2/mGlu3
37 agonist, DCG-IV, at mossy fiber terminals in the CA3 region of hippocampal slices. It also
38 impairs contextual fear memory demonstrating for the first time the functional role of mGlu2
39 homodimers in living animals. These data also highlight the potential of developing
40 antibodies with allosteric actions on GPCRs to better define their roles *in vivo*.

41

42

43

44 There is growing interest in developing either activating or inactivating antibodies with
45 therapeutic potential^{1,2}, but also as innovative tools to decipher the functional roles of cell
46 surface proteins³. G protein-coupled receptors (GPCRs), that are the main targets for small
47 therapeutic molecules, are now considered as promising targets for therapeutic antibodies⁴⁻⁷.
48 Single chain antibodies from camelids, such as llamas, are particularly well suited for such
49 purposes, being more prone to target specific conformations of their targets^{6,8,9}. Such tools
50 have already proven their potential for pharmacological actions^{6,10}, structural studies^{8,11}, and
51 use as biosensors³.

52 In the central nervous system, glutamate, the main excitatory neurotransmitter, exerts its fast
53 actions via ionotropic receptors, but also modulates synaptic activity via GPCRs, so called
54 metabotropic glutamate receptors (mGluRs)¹²⁻¹⁴. Eight genes encoding mGluRs are found in
55 mammalian genomes, and are classified into three groups. While group-I receptors (mGlu1
56 and mGlu5) are mainly post-synaptic receptors that contribute to glutamatergic synaptic
57 responses, group-II (mGlu2 and 3) and -III (mGlu4, 6, 7 and 8) are mainly pre-synaptically
58 located, and inhibit transmitter release at various types of synapses¹². As such, mGluRs are
59 considered to be interesting targets for the treatment of various brain diseases including
60 psychiatric or neurodegenerative diseases^{12,13}.

61 Among the various mGluR subtypes, mGlu2, but also mGlu3 and 5, open new possibilities
62 for novel antipsychotic drugs^{13,15}. However studies on the roles of mGlu2 are made difficult
63 by the limited number of specific tools. Indeed, there are no specific mGlu2 antibodies to
64 determine their precise localization in the brain¹⁶. Moreover, because of the high
65 conservation of the orthosteric glutamate binding site located in the Venus flytrap
66 extracellular domain (VFT) of these receptors¹⁷, only very few selective agonists have been
67 reported^{18,19}. Efforts were concentrated on the development of positive allosteric modulators
68 (PAMs) interacting with the less conserved 7 transmembrane domains (7TM)¹⁷. Although
69 subtype selective PAMs have been identified, a number of limitations for their development
70 have been observed^{20,21}. Although knock out lines are available^{12,13}, one cannot exclude
71 compensation during development. Eventually, mGluRs, and especially mGlu2 have been
72 reported to associate with other mGlu subunits to form heterodimers²²⁻²⁴, and evidence for
73 mGlu2-4 heterodimers in cortico-striatal terminals have recently been provided²³. These
74 observations strengthen the need for more specific tools to better characterize the functional
75 roles of homo or heterodimeric mGluRs containing the mGlu2 subunit.

76 In the present study, we aimed at identifying nanobodies^{25,26} that recognize specific
77 conformations of the mGlu2 receptor. This led us to identify two nanobodies that specifically
78 bind to the active form of the mGlu2. Accordingly, these nanobodies act as PAMs, enhancing
79 the agonist action at mGlu2 receptors in transfected cells and in brain slices. When injected
80 in the hippocampus, these nanobodies also enhance the effect of a group-II mGluR agonist
81 in the fear-conditioning test, demonstrating their possible use to decipher the physiological
82 role of mGlu2 receptors in the brain. These data nicely illustrate novel possibilities to develop
83 mGlu allosteric modulators for numerous therapeutic actions, and exemplify the use of
84 nanobodies to allosterically modulate GPCRs.

85

86

87 **Results:**

88 **Identification of mGlu2 selective nanobodies**

89 To identify nanobodies recognizing mGlu2 receptors, HEK-293 cells transiently expressing
90 both rat and human mGlu2 were injected in llamas, and VHH encoding sequences were
91 amplified to generate a phage display library²⁷. By screening the later using a purified mGlu2
92 receptor reconstituted into nanodiscs, several positive clones were isolated and three of
93 them, DN1, DN10 and DN13 were retained for analysis. FRET based binding data (Fig. 1a)
94 revealed that all three nanobodies bind to mGlu2 in the presence of ambient glutamate
95 produced by the cells, and not to any other mGluR (Fig. 1b).

96 Because glutamate concentration in the blood is sufficient to fully activate mGlu2 receptors,
97 we expected that some of our identified nanobodies could preferentially bind to the active
98 form of the receptor (Fig 1c). Indeed, whereas DN1 displays the same affinity for the active
99 and inactive forms of mGlu2 (Fig. 1d and Supplementary Table 1), DN10 and DN13
100 specifically bind to the active form stabilized by the orthosteric agonist, LY379268 (Fig. 1e,f
101 and Supplementary Table 1). No binding was detected on the inactive form of the receptor in
102 the presence of the antagonist LY341495 (Fig. 1e,f). Note that in the absence of any added
103 ligand, and under conditions leading to very low extracellular glutamate concentrations in the
104 assay medium through the co-transfection of the receptor with the high affinity glutamate
105 transporter EAAC1, no binding of DN10 and DN13 to mGlu2 could be detected
106 (Supplementary Fig. 1a). This is in contrast to the conditions used for the screening and first
107 characterization of the nanobodies where binding could be detected under basal condition,
108 likely due to the presence of enough glutamate produced by the cells in the assay (Fig. 1b).
109 According to these data, the specificity of both DN10 and DN13 was further examined on the
110 eight mGlu subtypes in the presence of a saturating concentration of either agonists or
111 antagonists (Supplementary Fig. 1b and 1c, respectively). It demonstrated that these two
112 nanobodies only bind on the active form of mGlu2 receptor.

113 **DN10 and DN13 are positive allosteric modulators of mGlu2**

114 A possible effect of both DN10 and DN13 was first examined using a mGlu2 biosensor²⁸.
115 This sensor makes use of the large movement between the VFTs that occurs upon receptor
116 activation, leading to an increase in distance between the N-terminal SNAP tags carried by
117 each subunit²⁸. Such movement lead to a large decrease in lanthanide-based resonance
118 energy transfer (LRET) measured in a time resolved manner (TR-FRET) (Fig. 2a). In the
119 presence of an EC₂₀ concentration of LY379268, both DN10 and DN13 were found to
120 activate the biosensor in the high nanomolar range (Fig. 2b). A similar agonist effect of both
121 DN10 and DN13 was observed using a functional assay based on the activation of
122 phospholipase C by this Gi/o-coupled receptor using a chimeric Gqi protein (Supplementary
123 Fig. 2). When the extracellular glutamate concentration was maintained as low as possible
124 using EAAC1, both DN10 and DN13 clearly increased mGlu2 agonist LY379268 potency
125 (Fig. 2c,d), revealing their positive allosteric effect. However, whereas DN13 retains minimal
126 agonist activity under such conditions (Fig. 2d), DN10 was still able to activate mGlu2 (Fig.
127 2c). Such observations were confirmed using the inositol-phosphate accumulation assay
128 (Fig. 2e,f). These data demonstrate that both DN10 and DN13 act as PAMs on mGlu2, but
129 that DN10 has, in addition, an intrinsic agonist activity (known as ago-PAM²⁹).

130

131 **DN13 binds at an epitope specific of the active conformation**

132 The binding ability of the nanobodies on various mGlu2 constructs lacking the 7TM domains,
133 the cysteine-rich domain, or the entire extracellular domain, revealed that all three
134 nanobodies bind to the VFT (Supplementary Fig. 3). Moreover, competition studies revealed
135 that DN10 and DN13 share part of their binding epitope, while DN1 binds at a different site
136 (Supplementary Fig. 4). To identify the epitope recognized by DN13, we made use of the
137 inability of these nanobodies to bind to the homologous mGlu3 receptor, and their lack of
138 affinity for the inactive form of the receptor. The major conformational change in the VFT
139 dimer occurring upon receptor activation is the relative reorientation of the VFTs leading to a
140 close apposition of the second lobes, which are distant in the inactive form (Fig. 1c)²⁸.
141 Accordingly, a specific crevice at the interface of the second lobes is formed in the active
142 form of the dimer only, and a few residues in that area were found to be different between
143 mGlu2 and mGlu3 receptors (Fig. 3a-c and Supplementary Table 2). These include Leu226,
144 Arg445 and Ile450 in protomer A (Fig. 3c) (Gln, Thr and Met in mGlu3, respectively), and
145 Ser246, Ala248, Ala249, Glu251 and Gly252 in protomer B (Ile, Lys, Ser, Asp and Ser in
146 mGlu3, respectively). Docking experiments conducted with a 3D model of DN13 and a
147 mGlu2 VFT dimer model suggest that DN13 interacts at that site (Fig. 3a-c). This is further
148 demonstrated by our observation that DN13 does not interact with a mGlu2 mutant in which
149 these residues were replaced by their mGlu3 equivalent (Fig. 3d and Supplementary Table
150 3) despite their normal expression and coupling properties (Supplementary Fig 5). In
151 contrast, DN13 binds with a nanomolar affinity on the mGlu3 mutant bearing these residues
152 from mGlu2 (Fig. 3e and Supplementary Table 3).

153 There is increasing evidence suggesting that mGlu2 receptors can exist not only as
154 homodimers, but also as heterodimers, associated with any group-III mGluRs^{22,23}. Because
155 the epitope recognized by DN13 involves both subunits in the mGlu dimer, we predicted that
156 it would be specific for the mGlu2 homodimer relative to the heterodimers made of mGlu2
157 and a group-III mGlu subunits. Indeed, DN13 did not bind to the mGlu2-4 heterodimer
158 (Supplementary Fig. 6a), nor did it potentiate agonist action on this heterodimer as revealed
159 using either a mGlu2-4 specific biosensor, and a functional assay using subunit combination
160 that ensure targeting to the cell surface of the heterodimer only³⁰ (Supplementary Fig. 6b,c).

161 **DN13 potentiates the pre-synaptic effect of mGlu2**

162 The mGlu2 gene is well expressed in hippocampal dentate gyrus granule neurons, where the
163 receptors are targeted to mossy fiber terminals that contact pyramidal neurons in the CA3
164 area (Fig. 4a). These terminals may also contain mGlu3, mGlu4 and mGlu7 that are also
165 expressed by granule neurons^{31,32}. In acute hippocampal slices, we examined the effect of
166 nanobodies on mossy fiber terminal mGlu2 activation by quantifying presynaptic calcium
167 transients evoked by electrical stimulation of mossy fibers, using photometric measurements
168 of the fluorescent Ca²⁺ sensitive dye, magnesium green-AM³³. We found that saturating
169 concentrations of DN13 (2.5 μM), did not affect the amplitude of Ca²⁺ transients in mossy
170 fiber terminals when applied alone but clearly enhanced the inhibitory effect of low
171 concentrations (100 nM) of the group-II mGluR agonist DCG-IV (24 ± 1.4 % and 8.3 ± 0.9 %
172 inhibition with and without DN13, respectively, p < 0.001, n = 9) (Fig. 4b). In the presence of
173 DN13, the effect of a low concentration of DCG-IV nearly reached the maximal effect
174 observed with saturating concentrations of the drug (30.5 ± 1.9 %, n = 6). In addition, and as

175 expected for a PAM effect, the off rate of the inhibitory action of a saturating concentration of
176 DCG-IV (5 μ M) is prolonged in the presence of DN13 (Supplementary Fig. 7).

177 The hippocampal mossy fiber pathway projecting from the dentate gyrus to the CA3 is
178 critically involved in memory processing. Infusion of the group-II mGluR agonist DCG-IV into
179 the CA3 area was previously shown to block contextual fear memory consolidation in mice³⁴.
180 Consistent with this observation we found that DCG-IV infused into the CA3 area (Fig. 4d
181 and supplementary Fig. 8) specifically disrupted contextual fear memory consolidation (Fig.
182 4e) without affecting cued fear memory consolidation (Fig. 4f) when immediately following
183 conditioning (Fig. 4c). DN13 did not affect fear memory consolidation when applied alone,
184 but potentiated the effect of low concentrations of DCG-IV, demonstrating the involvement of
185 mGlu2 (Fig. 4e).

186

187 **Discussion**

188

189

190

191

192

193

194

195

196

197

198

199

In the present study, we describe three nanobodies that specifically recognize mGlu2 in the nanomolar range, being then the first mGlu2 selective antibodies. Most interestingly, while one of these does not discriminate between the different conformations of the receptor, the two others, DN10 and DN13 exclusively bind to the active form, interacting at a site exclusively found in the active form of the dimer. In the study we have further characterized the DN13 nanobody that behaves as a PAM with no intrinsic agonist activity. Indeed, DN13 potentiated the action of mGlu2 agonists not only in heterologous expression systems, but also in brain slices and *in vivo*. Because the binding epitope of DN13 involves residues from both subunits, it is indeed inactive on mGlu2-4 heterodimers, indicating that the effects observed both in brain slices and *in vivo* at the level of the hippocampal CA3 area likely involves mGlu2 homodimers.

200

201

202

203

204

205

206

207

208

209

210

211

212

213

214

215

Despite the identification of both mGlu2 and mGlu3 in the early 90's, and the interest they have generated for the development of anxiolytic and antipsychotic drugs, studies of these two receptors have been hampered by the lack of specific pharmacological tools. Until now, available antibodies have not been able to discriminate between mGlu2 and mGlu3¹⁶, and only few selective ligands have been developed^{18,19}. However, the availability of mGlu2 and mGlu3 knockout mice (or mouse lines) coupled with more sophisticated pharmacology has strengthened the interest in targeting mGlu2 specifically for antipsychotic effects³⁵. Today, the most selective and promising ligands are mGlu2 PAMs that bind to a hydrophobic cavity in the 7TM³⁶. Accordingly, such molecules show high hydrophobicity enabling them to pass through the blood brain barrier. However, this also limits their effective concentration in the cerebrospinal fluid, and increases the chance for off target activity²⁰. Despite the therapeutic potential of PAMs, so far only orthosteric mGlu2-3 agonists have reached phase 3 clinical trails for anxiety and schizophrenia³⁵ but have had very limited success. Our nanobodies are the first mGlu2 selective PAMs that do not target the 7TM domain, but rather the VFT domain. These molecules reveal new possibilities to develop selective mGlu2 PAMs that are designed to target this new site, without the limiting hydrophobic properties.

216

217

218

219

220

221

222

223

Although mGluRs were thought to exist exclusively as homodimers, recent data has revealed that both group-I, and group-II/III mGluRs can associate to form multiple types of heterodimers²². Among these, the heterodimeric mGlu2-4 receptor is likely present in striato-cortical terminals, as illustrated by co-immunoprecipitation experiments, and the selective action of one mGlu4 PAM²³. This is in agreement with our observation that DN13 interacts with both subunits in the mGlu2 VFT dimer and was found to be inactive on the mGlu2-4 heterodimer. This finding will lead to multiple possibilities to develop selective nanobodies for mGlu homo and heterodimers comprised of specific mGlu subunits.

224

225

226

227

228

229

230

231

232

Although group-II mGluR agonists are well known for their anxiolytic and antipsychotic properties, recent data also suggest that they act to consolidate context fear memory³⁴. This may result from a reduction in pre-synaptic glutamate release following activation of group-II mGluRs located at the mossy fiber terminals in the CA3 area of the hippocampus. These terminals originate from the granule neurons of the dentate gyrus that also express mGlu3¹⁶ and as such, that the inhibitory action of the group-II agonist DCG-IV reported at these synapses may involve either mGlu2 or mGlu3. Our data using DN13, an mGlu2 selective PAM, suggest that only mGlu2 receptors are involved in the DCG-IV effect. Eventually, the DN13-mediated potentiation of the DCG-IV effect confirmed that a selective

233 activation of mGlu2 can prevent the consolidation of context fear memory. These data also
234 argue against the involvement of an mGlu2 receptor heterodimer containing mGlu4, and
235 likely the other group-III mGlu7 subunit both expressed in hippocampal granule
236 neurons^{16,31,32}.

237 Taken together, our data are the first to report the development of PAM nanobodies
238 acting at a GPCR. Antibodies show increasing potential in therapeutics, although mainly by
239 targeting proteins other than GPCRs¹. Since GPCRs still represent important targets for
240 therapeutic interventions, these membrane receptors have only recently been highlighted as
241 possible targets for antibody-based biologics^{4,5}. So far, such possibilities have been validated
242 through the identification of antibodies inhibiting chemokine receptors^{4,6}. Here we extend the
243 use of this approach revealing the feasibility to develop nanobodies with very selective PAM
244 activity at mGlu2 GPCRs, thus offering a way to better identify their actions *in vivo*, as well as
245 localizing activated receptors within the brain. Although an access to the brain would be
246 needed for targeting these central receptors for therapeutic intervention, conditions have
247 been reported to facilitate brain penetration of nanobodies³⁷. Moreover, mGlu receptors are
248 not only expressed in the CNS, but also at the periphery where they have a role in the
249 regulation of cardiovascular³⁸ and immune systems³⁹, as well as in cancer⁴⁰. Taken together,
250 mGluR targeting nanobodies offer interesting possibilities for therapeutic intervention.

251

252

253 **Acknowledgements**

254 We thank L Prézeau, D Maurel and C Vol from ARPEGE (Pharmacology Screening-
255 Interactome) facility at the Institut de Génomique Fonctionnelle (Montpellier, France) for their
256 help in various microplate assays. Funding was provided by the Centre National de la
257 Recherche Scientifique (CNRS), the Institut National de la Santé et de la Recherche
258 Médicale (INSERM), the University of Montpellier, Cisbio Bioassays, the Fondation
259 Recherche Médicale (FRM DEQ20130326522) and the Fondation Bettencourt Schueller to
260 JPP; the Fond Unique Interministériel of the french government (FUI, Cell2Lead project) to
261 GM, JPP and DB; the Agence Nationale de la Recherche (ANR-15-CE18-0020-01) to PR.

262

263 **Author Contributions:**

264 JPP, PR, PC, DB, EV, HD, HML, ET, GM, ED designed the research.

265 DN generated the nanobody phage display library and performed the screening and primary
266 characterization of the nanobodies, DEM set up the conditions and prepared the purified
267 mGlu2 receptor in nanodiscs, PS, DMD, MM, EB performed the *in vitro* characterization of
268 the nanobodies, XR performed the *in silico* studies, SB performed the experiments on
269 hippocampal slices, DDB performed the *in vivo* experiments.

270 JPP, PR and PS wrote the paper, with inputs from HML, PC and EV.

271

272 **Author Information**

273 The authors declare competing financial interests. Correspondence and requests for
274 materials should be addressed to JPP (jean-philippe.pin@iqf.cnrs.fr), PR
275 (philippe.rondard@iqf.cnrs.fr) or PC (patrick.chames@inserm.fr)

276

277

278

279 **Figure legends:**

280

281 **Figure 1:** Nanobodies DN1, DN10 and DN13 specifically interact with mGlu2 receptors. **a)**
282 Cartoon illustrating the principle of the TR-FRET binding assay. The receptor fused to a
283 SNAP-tag (black circled labeled "S") is labeled with Lumi4-Tb (light blue ball) while the
284 nanobody (purple) bearing a c-Myc epitope at its C-terminus is labeled with 200 nM of anti-c-
285 Myc antibody (green) coupled to d2 fluorophores (orange). Binding of the nanobody to the
286 receptor is then measured by a TR-FRET signal. **b)** Specific TR-FRET binding data obtained
287 with the indicated mGlu receptor and either DN1, DN10, DN13 or a control irrelevant
288 nanobody in cells under basal condition with ambient glutamate. Data are mean \pm SD of
289 triplicates from a typical experiment representative of three experiments. **c)** Cartoon
290 illustrating the main active (left), and inactive (right) conformation of an mGlu2 dimer,
291 stabilized by an agonist (glutamate or LY379268) or the antagonist LY341495, respectively.
292 **d,e,f)** Saturation binding curves obtained with DN1 (**d**), DN10 (**e**) and DN13 (**f**) on mGlu2
293 receptors under control conditions with low extracellular glutamate thanks to the co-
294 expression of the high affinity glutamate transporter EAAC1 (buffer), in the presence of the
295 agonist LY379268 (1 μ M), or the antagonist LY341495 (10 μ M). Data are mean \pm sem of
296 three individual experiments each performed in triplicates.

297

298

299 **Figure 2:** DN10 and DN13 are positive allosteric modulators of mGlu2. **a)** Cartoon illustrating
300 the principle of the mGlu2 sensor assay, where the VFT movement associated with receptor
301 activation results in a decrease in TR-FRET signal measured between Lumi4-Tb and SNAP-
302 Green labeled SNAP tags. **b)** DN10 and DN13 concentration dependent decrease the sensor
303 signal in the presence of EC₂₀ concentration of agonist LY379268 (0.1 nM), indicative of
304 receptor activation. DN10 (**c**) and DN13 (**d**) dose-dependently potentiate the effect of
305 LY379268 on the mGlu2 sensor. DN10 (**e**) and DN13 (**f**) dose-dependently potentiate the
306 effect of LY379268 on the production of inositol phosphate in mGlu2-transfected cells. Note
307 that the lower the IP-One Gq HTRF® ratio, the higher the amount of inositol monophosphate
308 produced (IP1). Data are means \pm sem of three individual experiments each performed in
309 triplicates.

310

311 **Figure 3:** DN13 interacts at the lobe 2 crevice on the activated mGlu2 VFT dimer. **a-b)** View
312 of the proposed docking of DN13 (orange) on the mGlu2 extracellular domain dimer (*a*,
313 lateral view, *b*, top view). **c)** Detailed view of the proposed docking of DN13 illustrating
314 proposed residues involved in selectivity, shown are Leu226 and Arg445 in protomer A, and
315 Ser246, Ala248 (yellow), Ala249, and Glu251 from protomer B. **d)** Saturation binding curves
316 of DN13 on mGlu2 WT, mGlu2 bearing mGlu3 specific residues from protomer A (mut A),
317 mGlu2 bearing mGlu3 residues from protomer B (mut B), mGlu2 A248K mutant. **e)**
318 Saturation binding curves of DN13 on mGlu3 WT, mGlu3 bearing the mGlu2 residues on
319 protomer A (mut A), and mGlu3 bearing all identified residues of mGlu2 (mut AB). Data are
320 mean \pm SD of triplicates from a typical experiment representative of three experiments.

321

322 **Figure 4:** mGlu2 receptors in the CA3 area of the hippocampus control contextual fear
323 consolidation. **a)** A schematic view of the hippocampus illustrating the granule neurons of the
324 dentate gyrus (DG) projecting to the pyramidal neurons in the CA3 area via the mossy fibers.
325 **b)** DN13 potentiates the inhibitory effect of a mGlu2-mGlu3 agonist (DCG-IV, 100 nM) on
326 presynaptic evoked calcium transients in the CA3 area (red box in panel *a*) in response to

327 electrical stimulation of the mossy fibers (blue arrow head in panel a). Data are normalized
328 amplitudes of peak fluorescence transients ($\Delta F/F$) evoked by five stimulations of mossy
329 fibers (delivered at 100 Hz). Insert on the right shows the average normalized area
330 corresponding to the depressant effect of DCG-IV alone (black bar, $100 \pm 5.4\%$, $n=10$) and in
331 the presence of DN13 (white bar, $578.5 \pm 69\%$, $n=9$), $p<0.001$. Insert on the left displays
332 superimposed fluorescence changes in one of these experiments recorded at the indicated
333 times. Each trace is an average of 10 consecutive trials. Since the variance was different
334 between DCG-IV and DCG-IV + DN13 groups, the Welch test was applied for statistical
335 analysis. **c)** Schematic of the experimental protocol used for the contextual fear consolidation
336 examination in mice, and drug infusion site **(d)**. **(e)** Contextual fear memory expression
337 ANOVA: $F_{5,35} = 6.025$, $P = 0.0004$. **(f)** Cued fear memory. ANOVA: $F_{5,35} = 0.3053$, $P =$
338 0.9066 . * $P < 0.05$ vs PBS and ** $P < 0.01$ vs PBS.
339
340
341
342

343 **Methods**

344

345 **Reagents, cell lines, antibodies and plasmids**

346 HEK293 cells were cultivated in DMEM (Thermo Fischer Scientific, Courtaboeuf, France)
347 complemented with 10% (v/v) fetal bovine serum. All drugs (DCG-IV, LY341495, LY379268,
348 LY487379) were from Tocris Bioscience (Bristol, UK). All HTRF® reagents, labeled
349 monoclonal antibodies Anti-c-myc-d2 and Anti-6His-d2, labeled ligands (SNAP-Lumi4-Tb,
350 SNAP-Red and CLIP-Red) and SNAP-tag mGluR plasmids, were a kind gift from Cisbio
351 Bioassays (Codolet, France). The pRK5 plasmids encoding wild-type rat mGluR subunits,
352 with a HA-tag and with SNAP or CLIP inserted just after the signal peptide, were previously
353 described (Scholler et al Nat Chem Biol 2017). Point mutations were introduced in the SNAP-
354 tag mGlu2 or mGlu3 plasmids according to the QuikChange mutagenesis protocol (Agilent
355 Technologies, Santa Clara, CA, USA).

356

357 **Llama immunization and library construction**

358 Two llamas (*Lama glama*) were immunized subcutaneously 4 times with 5×10^7 HEK293T
359 cells transfected with rat mGluR2 and human mGluR2. VHH library constructions were
360 performed in *E. coli* TG1 strain as previously described^{27,41}. Library diversities were above
361 10^9 transformants.

362

363 **Selection of nanobodies by phage display**

364 20 μ L of the bacteria library was grown in 50 mL of 2YTAG (2YT/ampicillin 100 μ g/mL/2%
365 glucose) at 37°C with shaking (250 rpm) to an OD₆₀₀ between 0.5 and 0.7. Bacteria were
366 infected by KM13 helper phage using a multiplicity of infection of 20 during 30 min at 37°C
367 without shaking. The culture was centrifuged for 15 min at 3000 g, and bacterial pellet was
368 re-suspended in 250 mL of 2YTA / kanamycine (50 μ g/mL) for an overnight phage-
369 nanobodies production at 30°C with shaking. The overnight culture was split in 10 vials and
370 centrifuged for 20 min at 3000 g. Five mL of 80% PEG8000, 2.5 mM NaCl were added to the
371 supernatant in a new clean vial and incubated for 1 h on ice to induce phage particle
372 precipitation. The solution was centrifuged for 20 min at 3000 g at 4°C and the phage-
373 containing pellet was re-suspended in 1 mL PBS. Another centrifugation step (2 min, 14000
374 g) was performed to eliminate bacterial contaminant, and 200 μ L of PEG8000 NaCl was
375 added to supernatants in a new vial. After 30 min on ice and a last centrifugation (5 min,
376 14000 g), phage-containing pellets were re-suspended in 1 mL PBS to obtain ready to used
377 phage-nanobodies for selections.

378 To obtain mGluR2 specific clones, a first round of selection (S1) was performed on Maxisorp
379 plates (Nunc, Maxisorp®) coated 24 h at 4°C with purified human mGlu2 receptor
380 reconstituted in nanodiscs⁴². Before selection on purified mGluR2, phage-nanobodies library
381 was depleted by incubation with empty nanodiscs (without receptor) to eliminate anti-
382 nanodisc antibodies and to reduce non-specific binding. Remaining phages and purified
383 mGluR2 coated wells were saturated with 2% milk/PBS during 1 h at 4°C, and then phages
384 and antigen were incubated together during 2 h at 4°C for selection with shaking. Wells were
385 then washed 10 times with PBS, and bound phages were finally eluted with 1 mg/ml trypsin
386 solution (Sigma-Aldrich, Saint-Quentin Fallavier, France) during 30 min at room temperature
387 with shaking. Phages were rescued and reamplified by infection of TG1 and phage
388 production as above, yielding S1 polyclonal phage population.

389 To avoid non-specific selection against proteins that composed nanodiscs and to select
390 antibodies against mGlu2 receptor in a cellular context, a second round of selection (S2) was

391 performed on HEK293T cells transfected with rat mGluR2 (2×10^7 cells). S1 polyclonal phage
392 population and cells were saturated in 2% milk/PBS during 1 h at 4°C, and incubated
393 together in the same conditions as described previously. After five PBS washes, bound
394 phages were eluted using trypsin solution (1 mg/ml) during 30 min at room temperature.
395 Phages were again rescued in TG1 and infected bacteria corresponding to S2 were plated.
396 Individual TG1 colonies from S2 were picked and grown in two different 96-deep-well plates
397 in 400 μ L of 2YTAG. After overnight growth, half of the culture was frozen at -80°C in 20%
398 glycerol for backup, and the rest of culture was used for soluble nanobodies production
399 induced by isopropyl- β -26-D-thiogalactopyranoside (IPTG). Nanobody concentrations in
400 supernatant were estimated at 100-500 nM using the DoubleTag check kit (Cisbio
401 Bioassays) according to manufacturer's recommendations.

402

403 **Production and purification of nanobodies**

404 For large-scale nanobody production, positive phagemids from screening step were
405 transformed in *E. coli* BL21DE3 strain. A single colony was grown into 10 ml of LB
406 supplemented with 100 μ g/mL ampicillin, 1% (wt/vol) glucose and 1 mM $MgCl_2$ overnight at
407 37°C with shaking. Then 1 L of LB supplemented with 100 μ g/mL ampicillin, 0.1% (wt/vol)
408 glucose and 1 mM $MgCl_2$ was inoculated with 10 ml of the preculture and incubated until an
409 OD_{600} of 0.7. The nanobody expression was then induced with 1 mM IPTG (final
410 concentration) and bacteria were grown overnight at 28°C with shaking. Bacteria were then
411 collected by centrifugation for 10 min at 5,000 g, re-suspended in 15 mL of ice-cold TES
412 buffer (0.2 M Tris, 0.5 mM EDTA, 0.5 M sucrose, pH 8), and incubated for at least 1 h at 4°C
413 on a shaking platform. 30 mL of TES/4 buffer (TES buffer diluted 4 times in water) were then
414 added to the solution and further incubated for at least 45 min at 4°C on a shaking platform.
415 The periplasmic extract was recovered by collecting the supernatant after centrifugation of
416 the suspension for 30 min at 10,000 g at 4°C. The His-tagged nanobodies were then purified
417 from the periplasmic extract by using Ni-NTA purification (Qiagen, Hilden, Germany)
418 according to the manufacturer's instructions.

419

420 **Nanobody labeling**

421 Nanobodies were dialysed overnight at 4°C and incubated (250 μ g of nanobodies at 2
422 mg/ml) with the fluorophore-NHS (d2-NHS (Cisbio Bioassays, Codolet, France) into
423 carbonate buffer pH 9, and Lumi4-Tb-NHS (Cisbio Bioassays, Codolet, France) in phosphate
424 buffer 50 mM at pH 8, or acceptor and donor labeling, respectively) at a molar ratio of 6, for
425 45 min at room temperature. Nanobodies were then purified by gel filtration column (NAP-5)
426 in phosphate buffer 100 mM pH 7. The final molar ratio (corresponding to the number of
427 fluorophore per nanobodies) was calculated as the fluorophore concentration/conjugated
428 nanobody concentration, and the conditions set up for a ratio between 2 and 3. The
429 concentration of fluorophores in the labeled fraction was calculated as the OD/ϵ for each
430 fluorophore (OD at 340 nm and $\epsilon=26,000 M^{-1}.cm^{-1}$ for Lumi4-Tb, and OD at 650 nm and
431 $\epsilon=225,000 M^{-1}.cm^{-1}$ for d2), while that of nanobodies was determined by the OD_{280} . The
432 conjugated concentration calculated as $OD_{280}-(OD_{fluor}/R_z,max)/\epsilon$ nanobody, with R_z
433 $max=OD_{fluor}/OD_{280}$. Purified labeled fractions were supplemented with 0.1% BSA and kept at -
434 20°C.

435

436 **Time resolved fluorescence resonance energy transfer (TR-FRET) assay**

437 Binding and competition experiments assays were performed using HEK-293 cells
438 transfected with rat SNAP-tagged mGluR by electroporation as previously described²². 24 h

439 after transfection, cells were labeled with 300 nM SNAP-Lumi4-Tb in DMEM-GlutaMAX
440 (Thermo Fischer Scientific) for 1 h at 37°C, and then washed three times with Krebs buffer.
441 Depending on the experiments, either 100,000 cells per well were seeded in white 96-well
442 plates (Greiner, Kremsmünster, Austria). For epitope mapping, cells were transfected with
443 Lipofectamine 2000 (Thermo Fischer Scientific) according to the manufacturer's instructions.
444 20,000 cells/well were used for a white 384SV-well plates (Greiner). The His₆- and c-Myc-
445 tagged nanobodies were incubated with the drugs and transfected cells and revealed by 200
446 nM of Anti-6His-d2 or Anti-c-myc-d2. When using d2 labeled nanobody, the anti-tag antibody
447 was replaced by 5 µL of Krebs buffer. After 2h incubation at 4°C, d2 acceptor TR-FRET
448 signal (665 nm) and Tb donor signal (620 nm) were measured using a 50 µs delay and a 450
449 µs integration upon excitation at 337 nm on a PHERAstar FS (BMG LabTech). TR-FRET
450 ratio (665 nm / 620 nm x 10⁴, Cisbio Bioassays patent US5,527,684) was calculated for
451 preventing interference due to medium variability and chemical compound or to normalize
452 experiments when using cells expressing different receptors levels.
453 For the mGlu2 TR-FRET biosensor, the SNAP-tagged mGlu2 homodimer was labeled with
454 SNAP-Lumi4-Tb and SNAP-Red substrates as previously reported^{28,30}.
455 Measurement of inositol phosphate accumulation in HEK293 cells transiently expressing the
456 mGlu receptors and a chimeric Gq_{i9} protein (enabling the coupling of mGlu2 to the Gq
457 pathway) after a 24 h transfection with Lipofectamine 2000 was determined using the IP-One
458 Gq kit (Cisbio Bioassays) according to manufacturer's recommendations as previously
459 described⁴³.

460

461 **In silico analysis of DN13 binding site**

462 The homology models of DN13 nanobody and the extracellular domain of mGlu₂ were
463 generated with Modeller 9.12⁴⁴ based on the crystal structure of β₂-adrenoceptor bound
464 nanobody (PDB 3P0G) and the mGlu₃ amino terminal domain as a template (PDB Code
465 2E4U)⁴⁵, respectively, using the loop optimization method. The sequences of template and
466 modeled proteins were aligned with ClustalW2⁴⁶. From 100 models generated, the top ten
467 classified by DOPE score⁴⁷ were visually inspected and the best scored structure with
468 suitable loops was chosen. The closed-closed mGlu₂ dimeric state was constructed by
469 superimposition with the crystal structure of the active state of the extracellular domain of
470 mGlu₁ (PDB code 1ISR)⁴⁸. A comparison with the very recently published structure of mGlu₂
471 in active state (PDB code 4XAS)⁴⁹ demonstrates a close similarity with a Cα RMSD of 1.36
472 for the dimer and 0.86 for the monomer. The maximum structural divergence is found in the
473 loops whereas the parts analyzed in the mutational study are very accurately located in the
474 model.

475 A docking based approach was used to find the binding site of DN13 nanobody in mGlu2
476 according to a previously described methodology⁵⁰. Briefly, ZDOCK 3.0 program⁵¹ was used
477 to perform an exhaustive rigid-body search in the six-dimensional rotational and translational
478 space. The three rotational angles were sampled with 6° spacing, and the three translational
479 degrees of freedom were sampled with a 1.2 Å spacing. For each set of rotational angles,
480 only the best translationally sampled prediction was retained resulting in 54,000 predictions.
481 The 2,000 first ranked predictions were clustered with MMTSB Tool Set⁵² using K-means and
482 a radius of 2.5 Å. The ten most populated clustered were visually inspected to avoid
483 structural violations and symmetric results. Discovery Studio 4.0 (BIOVIA – A Dassault
484 Systèmes brand – 5005 Wateridge Vista Drive, San Diego, CA 92121 USA) was used for
485 protein structure visualization and PDB file editing purposes. Images were generated with

486 UCSF Chimera software⁵³. The multiple sequence alignment visualization and analysis was
487 performed with Jalview2 software⁵⁴.

488

489 **Slice preparation and calcium transient recordings**

490 Experiments were performed using hippocampal slices prepared from twenty six 21-25 day-
491 old male Sprague-Dawley rats. No experiments were excluded from the analysis. In
492 accordance with guidelines from the Centre National de la Recherche scientifique (CNRS,
493 France), animals were killed by decapitation after anesthesia with 2-bromo-2-chloro-1, 1, 1-
494 trifluoroethan, and the brain was removed rapidly and put in an ice-cold cutting solution (75
495 mM sucrose, 25 mM glucose, 25 mM NaHCO₃, 2.5 mM KCl, 87 mM NaCl, 1.25 mM KH₂PO₄,
496 7mM MgCl₂, 0.5 mM CaCl₂). Parasagittal hippocampal slices, 350 μm thick, were prepared
497 using a Vibroslicer (Motorised Advance Vibroslice MA752, Campden Instruments) according
498 to ⁵⁵. Slices were then placed in oxygenated (saturated with 95% O₂ and 5% CO₂) artificial
499 CSF (138.6 mM NaCl, 3 mM KCl, 1.15 mM KH₂PO₄, 1.15 mM MgSO₄, 24 mM NaHCO₃, 2
500 mM CaCl₂, 10 mM glucose) and left to recover at room temperature for at least 1 h. Slices
501 were then transferred to the recording chamber, where they were maintained at 29–30°C and
502 perfused with oxygenated artificial CSF as above.

503 Presynaptic calcium transients were recorded by photometry according to Regehr and
504 collaborators^{33,56}. A solution of 100 μM of the membrane-permeant calcium dye Magnesium
505 Green-AM was delivered during 40 min at the level of the stratum lucidum in CA3, where
506 mossy fibers contact proximal dendrites from pyramidal cells. After loading of the mossy
507 fiber, slices were left for at least 30 min to allow diffusion of the fluorochrome in the fibers. A
508 stimulation electrode filled with artificial CSF was placed between the fluorochrome loading
509 site and the measurement window. To measure the effect of mGlu2 ligands on evoked
510 presynaptic calcium influx, a train of five 100 Hz stimulations is delivered every 30 s to the
511 mossy fiber to induce presynaptic calcium transients, in the presence or absence of the
512 indicated drugs or nanobodies. During acquisition, a GABA_A receptor antagonist (bicuculline
513 methiodide) was added to the artificial CSF to block any GABAergic component.

514 Measurements of intracellular calcium variations were performed on an epifluorescence
515 microscope (Zeiss axioskop 2), with a mercury lamp (Mercury short Arc HBO, 103 W) for
516 excitation (485 nm excitation filter), and a 530 nm emission filter. The measurement window
517 was localized at some distance from the loading site, allowing the selective recording of
518 loaded mossy fiber with a high signal to noise ratio. The basal fluorescence (F) and the
519 amplitude of the fluorescence peak after mossy fiber stimulation (ΔF), and the ΔF/F ratio
520 were measured in real time. Each recording was then analyzed individually using Microsoft
521 Excel before pooling them all together. Statistical significance was assessed by either an
522 unpaired Student's t test or a Welch's t. The similarity of variance between each group of
523 results was tested using Fisher's test with $\alpha = 0.02$. (n) indicates the number of cells included
524 in the statistics.

525

526

527 **Contextual fear memory**

528 *Cannula implantation.* Mice were bilaterally implanted with an infusion cannulae (26 gauge,
529 2.5 mm, Plastics One, Roanoke, VA, USA) aimed at the dorsal hippocampal CA3 using flat
530 skull coordinates: AP:-1.6 mm, ML ±2.5 mm, DV:-1.5 mm. The cannulae were fixed to the
531 skull using anchor screws and acrylic dental cement (AgnTho's, Lidingö, Sweden). Following
532 surgery, mice were placed on a heating mat and a dummy cannula was inserted into each
533 guide cannula to seal off the opening. Mice were allowed to recover from surgery for a

534 minimum of one week during which time they were handled and habituated to the drug
535 infusion procedure on a daily basis.

536 *Fear conditioning.* Pavlovian fear conditioning was performed in a conditioning box (20 cm
537 width × 20 cm length × 20 cm height) placed within a sound proof chamber (Panlab,
538 Barcelona, Spain). Different contexts were used: (A) white walls, metal grid on black floor,
539 washed with 1% acetic acid, or (B) black walls, white rubber floor, washed with 70% ethanol).
540 Mice were conditioned in context A. After 2 min habituation, mice received three pairings
541 (60–120 s variable pairing interval) of a conditioned stimulus (CS: 4 kHz, 80 dB, 30 s tone)
542 with an unconditioned stimulus (US: 2 s, 0.6 mA scrambled footshock) using a freezing
543 system (Panlab). After 24 hours, contextual fear was tested by placing the mice in context A
544 for 5 min and after another 24 hours, cued fear was tested by first placing the mice in context
545 B for 2 min and after which the CS was presented twice (120 s intertrial interval). Freezing
546 was measured using a load cell coupler (Panlab) and was defined as the lack of activity
547 above a body weight-corrected threshold for a duration of 1 s or more as analyzed using
548 Freezing software (Panlab).

549 *Drug infusions.* Drug infusions were made using an injection cannula (33 gauge, 3.5 mm,
550 Plastics One). Immediately following fear conditioning, mice were gently scruffed and an
551 injection cannula was inserted into each guide cannula. The injection cannulae were
552 designed to protrude 1.0 mm from the tip of the guide cannula and effectively penetrated into
553 the hippocampal CA3. Drugs or vehicle were infused at a flow rate of 0.10 µl per min and in a
554 total volume of 0.25 µl per infusion site. Following infusion, the injection cannula was left in
555 place for 1 min to allow drugs to diffuse from the cannula tip. Dummy cannulae were then
556 inserted into each of the guide cannula and mice were returned to the homecage. At the end
557 of each experiment, correct implantation of the guide cannulae was histologically verified on
558 40 µm slices obtained from brains fixed in 4% paraformaldehyde.

559

560 **References**

- 561 ¹ Chames, P., Van Regenmortel, M., Weiss, E., & Baty, D. *Br J Pharmacol* 157 (2),
562 220-233 (2009).
563 ² Seigniny, J. *et al. Nature* 537 (7618), 50-56 (2016).
564 ³ Irannejad, R. *et al. Nature* 495 (7442), 534-538 (2013).
565 ⁴ Hutchings, C.J., Koglin, M., & Marshall, F.H. *MAbs* 2 (6), 594-606 (2010).
566 ⁵ Webb, D.R., Handel, T.M., Kretz-Rommel, A., & Stevens, R.C. *Biochem Pharmacol*
567 85 (2), 147-152 (2013).
568 ⁶ Mujic-Delic, A., de Wit, R.H., Verkaar, F., & Smit, M.J. *Trends Pharmacol Sci* 35 (5),
569 247-255 (2014).
570 ⁷ Jo, M. & Jung, S.T. *Exp Mol Med* 48, e207 (2016).
571 ⁸ Steyaert, J. & Kobilka, B.K. *Curr Opin Struct Biol* 21 (4), 567-572 (2011).
572 ⁹ Staus, D.P. *et al. Nature* 535 (7612), 448-452 (2016).
573 ¹⁰ Jahnichen, S. *et al. Proc Natl Acad Sci U S A* 107 (47), 20565-20570 (2010).
574 ¹¹ Rasmussen, S.G. *et al. Nature* 477 (7366), 549-555 (2011).
575 ¹² Nicoletti, F. *et al. Neuropharmacology* 60 (7-8), 1017-1041 (2011).
576 ¹³ Niswender, C.M. & Conn, P.J. *Annu Rev Pharmacol Toxicol* 50, 295-322 (2010).
577 ¹⁴ Pin, J.-P. & Bettler, B. *Nature* 540 (7631), 60-68 (2016).
578 ¹⁵ Moghaddam, B. & Adams, B.W. *Science* 281 (5381), 1349-1352 (1998).
579 ¹⁶ Ferraguti, F. & Shigemoto, R. *Cell Tissue Res* 326 (2), 483-504 (2006).

580 17 Rondard, P., Goudet, C., Kniazeff, J., Pin, J.-P., & Prezeau, L. *Neuropharmacology*
581 60, 82-92 (2011).
582 18 Johnson, M.P. *et al. Psychopharmacology (Berl)* 179 (1), 271-283 (2005).
583 19 Nielsen, C.K. *et al. Med. Chem. Commun.* 2, 1120-1124 (2011).
584 20 Flor, P.J. & Acher, F.C. *Biochem Pharmacol* 84 (4), 414-424 (2012).
585 21 Gregory, K.J. & Conn, P.J. *Mol Pharmacol* 88 (1), 188-202 (2015).
586 22 Doumazane, E. *et al. FASEB J* 25 (1), 66-77 (2011).
587 23 Yin, S. *et al. J Neurosci* 34 (1), 79-94 (2014).
588 24 Pandya, N.J. *et al. Proteomics* (2016).
589 25 Hamers-Casterman, C. *et al. Nature* 363 (6428), 446-448 (1993).
590 26 Lauwereys, M. *et al. EMBO J* 17 (13), 3512-3520 (1998).
591 27 Behar, G. *et al. FEBS J* 276 (14), 3881-3893 (2009).
592 28 Doumazane, E. *et al. Proc Natl Acad Sci (USA)* 110 (15), 5754-5755 (2013).
593 29 Christopoulos, A. *et al. Pharmacol Rev* 66 (4), 918-947 (2014).
594 30 Scholler, P. *et al. Nat Chem Biol*, in press (2017).
595 31 Shigemoto, R. *et al. J Neurosci* 17 (19), 7503-7522 (1997).
596 32 Wright, R.A. *et al. Neuropharmacology* 66, 89-98 (2013).
597 33 Regehr, W.G. & Atluri, P.P. *Biophys J* 68 (5), 2156-2170 (1995).
598 34 Dumas, S., Ceccom, J., Halley, H., Frances, B., & Lassalle, J.M. *Learn Mem* 16 (8),
599 504-507 (2009).
600 35 Patil, S.T. *et al. Nat Med* 13 (9), 1102-1107 (2007).
601 36 Schaffhauser, H. *et al. Mol Pharmacol* 64 (4), 798-810 (2003).
602 37 Li, T. *et al. FASEB J* 26 (10), 3969-3979 (2012).
603 38 Moore-Morris, T. *et al. Mol Pharmacol* 75 (5), 1108-1116 (2009).
604 39 Fallarino, F. *et al. Nat Med* 16 (8), 897-902 (2010).
605 40 Nicoletti, F. *et al. Trends Pharmacol Sci* 28 (5), 206-213 (2007).
606 41 Alvarez-Rueda, N. *et al. Mol Immunol* 44 (7), 1680-1690 (2007).
607 42 El Moustaine, D. *et al. Proc Natl Acad Sci U S A* 109 (40), 16342-16347 (2012).
608 43 Trinquet, E. *et al. Anal Biochem* 358 (1), 126-135 (2006).
609 44 Sali, A. & Blundell, T.L. *J Mol Biol* 234 (3), 779-815 (1993).
610 45 Muto, T., Tsuchiya, D., Morikawa, K., & Jingami, H. *Proc Natl Acad Sci U S A* 104
611 (10), 3759-3764 (2007).
612 46 Larkin, M.A. *et al. Bioinformatics* 23 (21), 2947-2948 (2007).
613 47 Shen, M.Y. & Sali, A. *Protein Sci* 15 (11), 2507-2524 (2006).
614 48 Tsuchiya, D., Kunishima, N., Kamiya, N., Jingami, H., & Morikawa, K. *Proc Natl Acad*
615 *Sci U S A* 99 (5), 2660-2665 (2002).
616 49 Monn, J.A. *et al. Journal of Medicinal Chemistry* 58 (4), 1776-1794 (2015).
617 50 Casciari, D., Seeber, M., & Fanelli, F. *BMC Bioinformatics* 7, 340 (2006).
618 51 Pierce, B.G., Hourai, Y., & Weng, Z. *PLoS One* 6 (9), e24657 (2011).
619 52 Feig, M., Karanicolas, J., & Brooks, C.L., 3rd. *J Mol Graph Model* 22 (5), 377-395
620 (2004).
621 53 Pettersen, E.F. *et al. J Comput Chem* 25 (13), 1605-1612 (2004).
622 54 Waterhouse, A.M., Procter, J.B., Martin, D.M., Clamp, M., & Barton, G.J.
623 *Bioinformatics* 25 (9), 1189-1191 (2009).
624 55 Bischofberger, J., Engel, D., Li, L., Geiger, J.R., & Jonas, P. *Nat Protoc* 1 (4), 2075-
625 2081 (2006).
626 56 Regehr, W.G. & Tank, D.W. *J Neurosci Methods* 37 (2), 111-119 (1991).
627

Figure 1

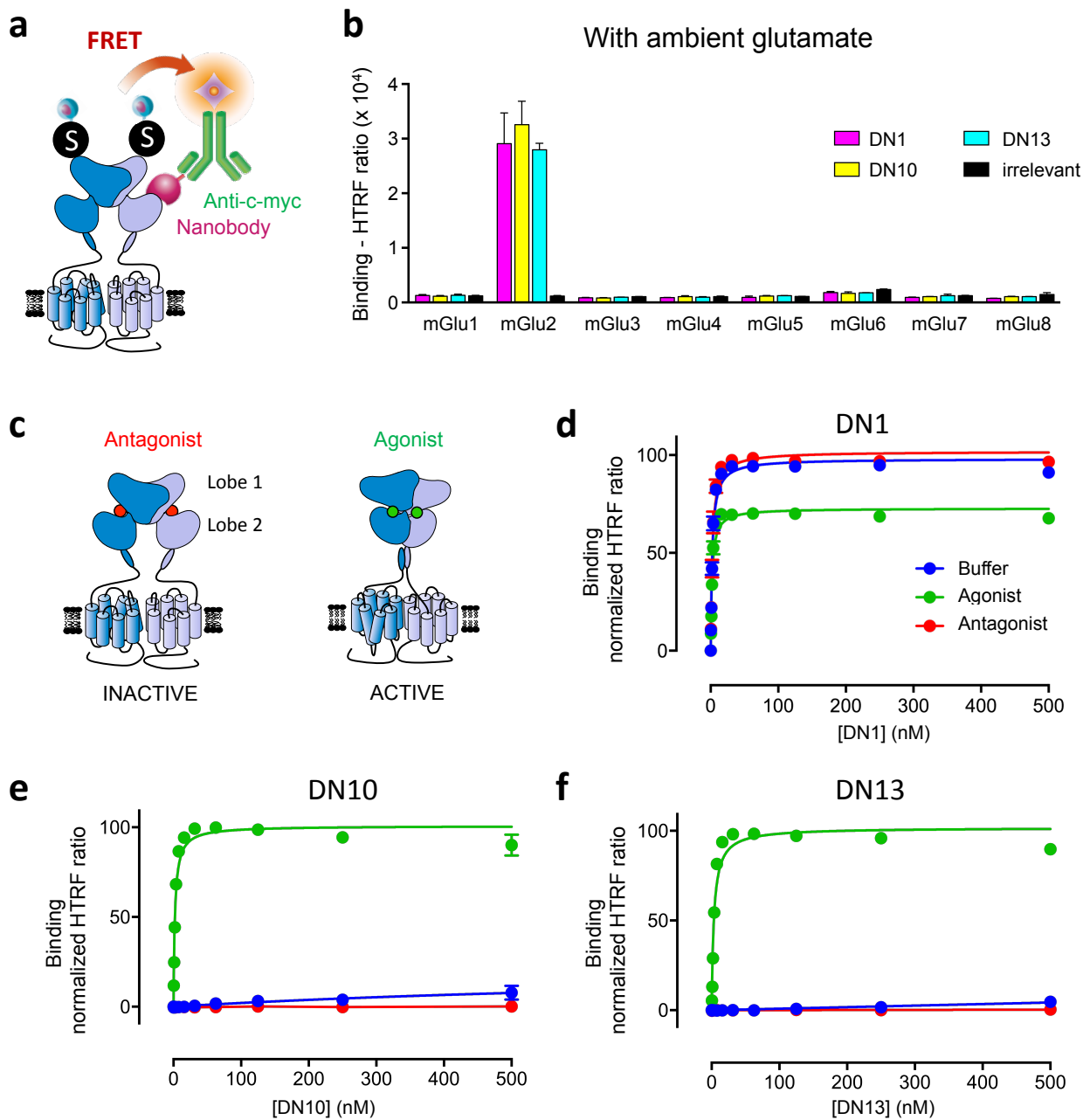


Figure 1: Nanobodies DN1, DN10 and DN13 specifically interact with mGlu2 receptors. **a)** Cartoon illustrating the principle of the TR-FRET binding assay. The receptor fused to a SNAP-tag (black circled labeled "S") is labeled with Lumi4-Tb (light blue ball) while the nanobody (purple) bearing a c-Myc epitope at its C-terminus is labeled with 200 nM of anti-c-Myc antibody (green) coupled to d2 fluorophores (orange). Binding of the nanobody to the receptor is then measured by a TR-FRET signal. **b)** Specific TR-FRET binding data obtained with the indicated mGlu receptor and either DN1, DN10, DN13 or a control irrelevant nanobody in cells under basal condition with ambient glutamate. Data are mean \pm SD of triplicates from a typical experiment representative of three experiments. **c)** Cartoon illustrating the main active (left), and inactive (right) conformation of an mGlu2 dimer, stabilized by an agonist (glutamate or LY379268) or the antagonist LY341495, respectively. **d,e,f)** Saturation binding curves obtained with DN1 (**d**), DN10 (**e**) and DN13 (**f**) on mGlu2 receptors under control conditions with low extracellular glutamate thanks to the co-expression of the high affinity glutamate transporter EAAC1 (buffer), in the presence of the agonist LY379268 (1 μ M), or the antagonist LY341495 (10 μ M). Data are mean \pm sem of three individual experiments each performed in triplicates.

Figure 2

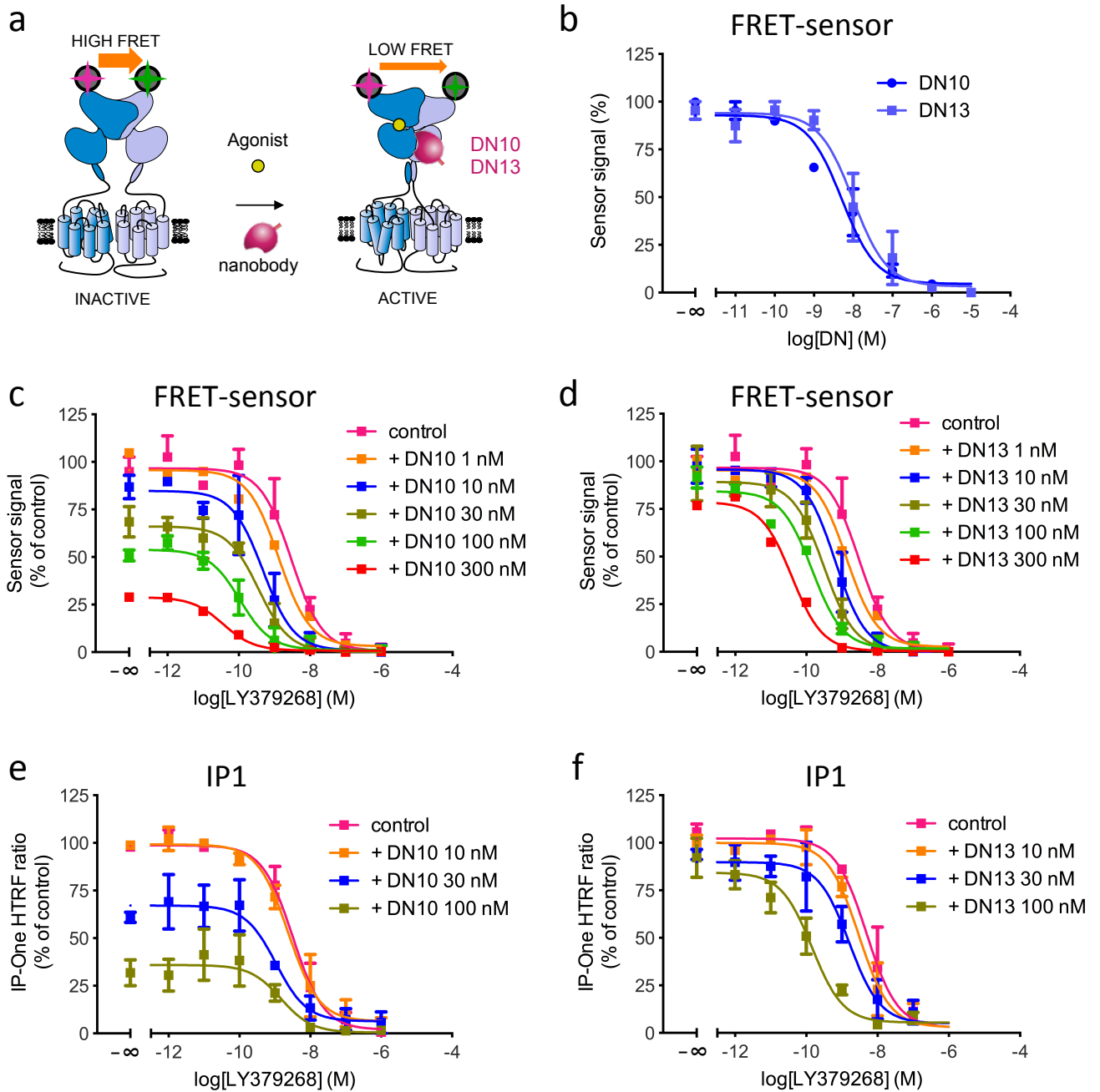


Figure 2: DN10 and DN13 are positive allosteric modulators of mGlu2. **a)** Cartoon illustrating the principle of the mGlu2 sensor assay, where the VFT movement associated with receptor activation results in a decrease in TR-FRET signal measured between Lumi4-Tb and SNAP-green labeled SNAP tags. **b)** DN10 and DN13 concentration dependent decrease the sensor signal in the presence of EC_{20} concentration of agonist LY379268 (0.1 nM), indicative of receptor activation. DN10 **(c)** and DN13 **(d)** dose-dependently potentiate the effect of LY379268 on the mGlu2 sensor. DN10 **(e)** and DN13 **(f)** dose-dependently potentiate the effect of LY379268 on the production of inositol phosphate in mGlu2-transfected cells. Note that the lower the IP-One HTRF ratio, the higher the amount of inositol monophosphate produced (IP1). Data are means \pm sem of three individual experiments each performed in triplicates.

Figure 3

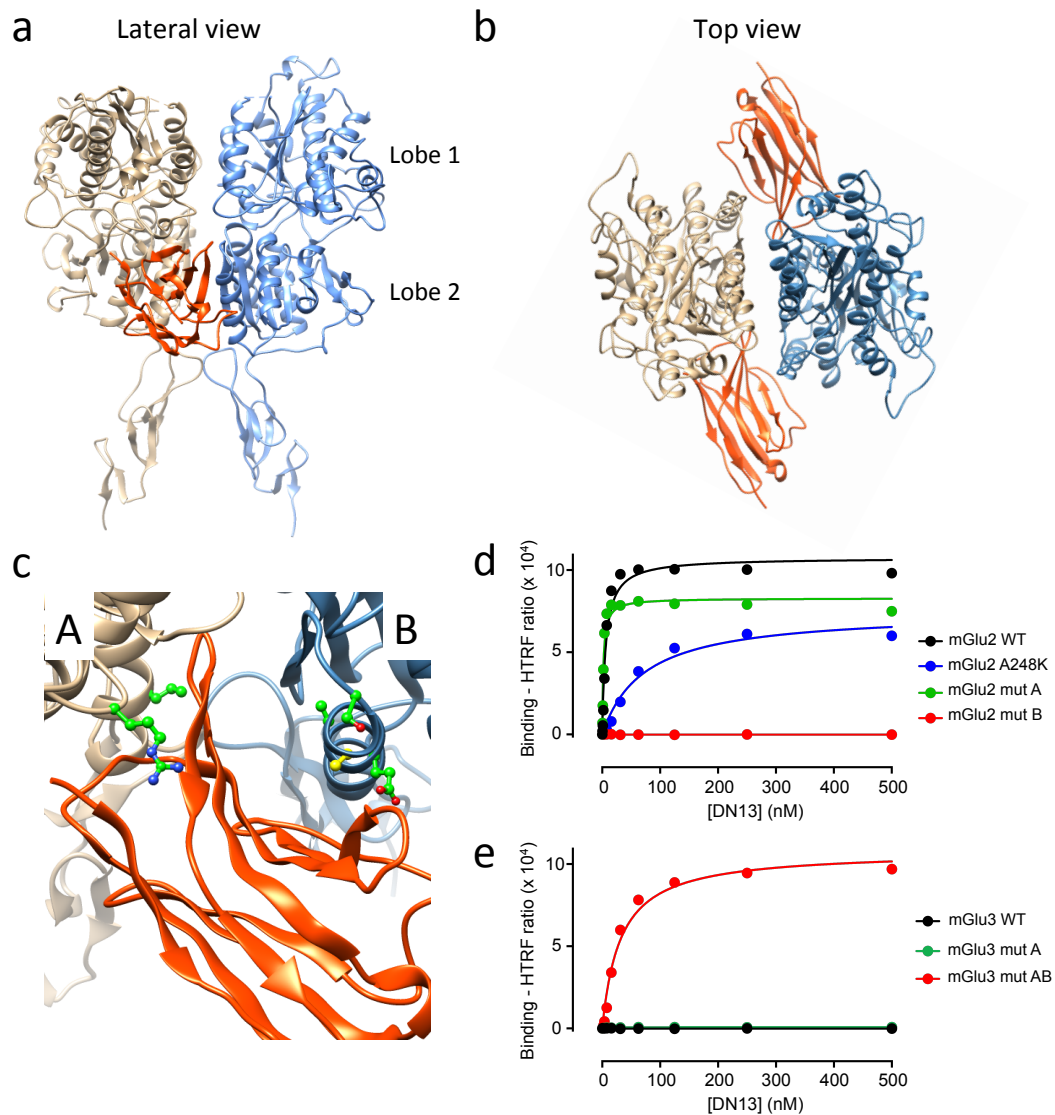


Figure 3: DN13 interacts at the lobe 2 crevice on the activated mGlu2 VFT dimer. **a-b)** View of the proposed docking of DN13 (orange) on the mGlu2 extracellular domain dimer (*a*, lateral view, *b*, top view). **c)** Detailed view of the proposed docking of DN13 illustrating proposed residues involved in selectivity, shown are Leu226 and Arg445 in protomer A, and Ser246, Ala248 (yellow), Ala249, and Glu251 from protomer B. **d)** Saturation binding curves of DN13 on mGlu2 WT, mGlu2 bearing mGlu3 specific residues from protomer A (mut A), mGlu2 bearing mGlu3 residues from protomer B (mut B), mGlu2 A248K mutant. **e)** Saturation binding curves of DN13 on mGlu3 WT, mGlu3 bearing the mGlu2 residues on protomer A (mut A), and mGlu3 bearing all identified residues of mGlu2 (mut AB). Data are mean \pm SD of triplicates from a typical experiment representative of three experiments.

Figure 4

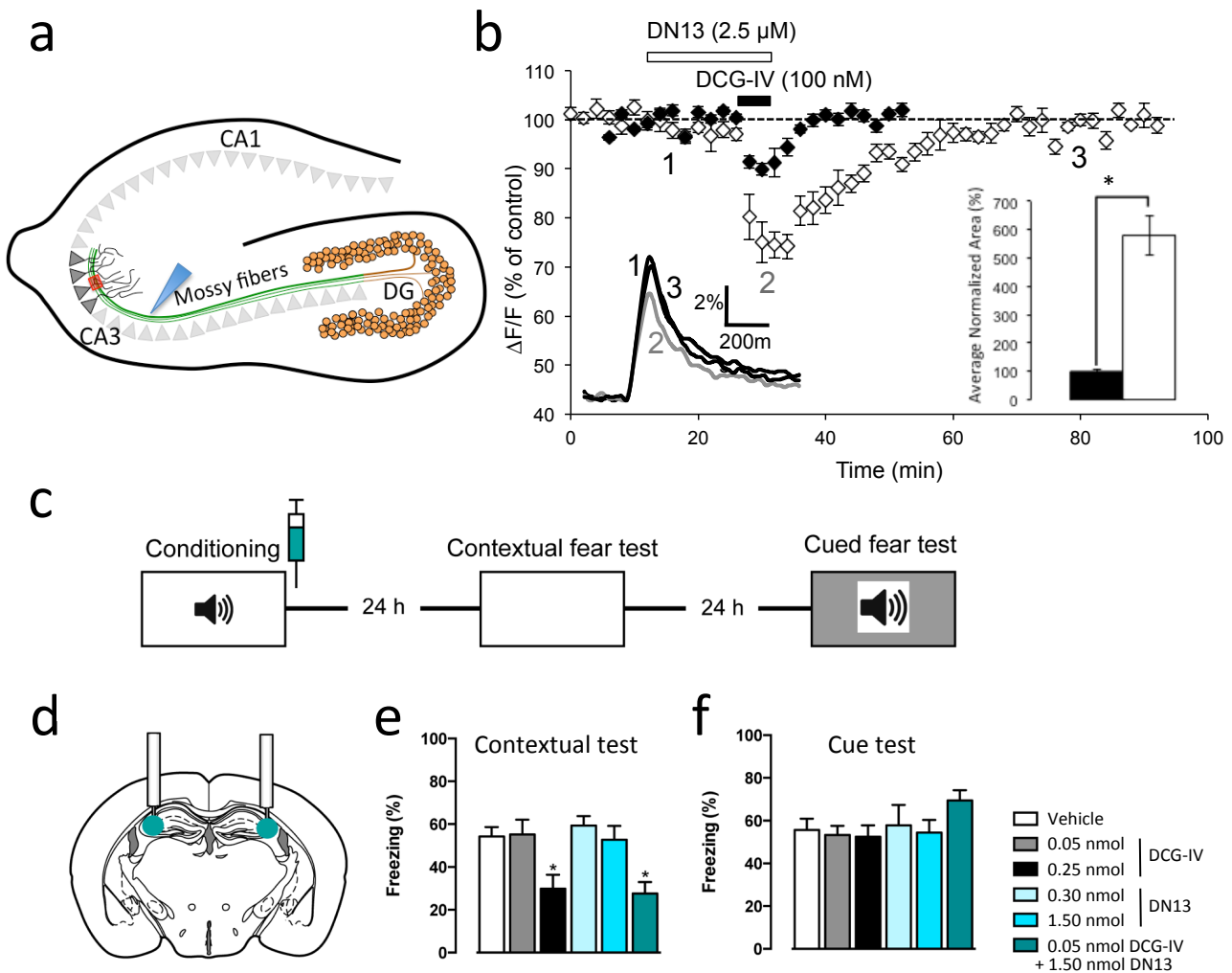


Figure 4: mGlu2 receptors in the CA3 area of the hippocampus control contextual fear consolidation. **a)** A schematic view of the hippocampus illustrating the granule neurons of the dentate gyrus (DG) projecting to the pyramidal neurons in the CA3 area via the mossy fibers. **b)** DN13 potentiates the inhibitory effect of a mGlu2-mGlu3 agonist (DCG-IV, 100 nM) on presynaptic evoked calcium transients in the CA3 area (red box in panel *a*) in response to electrical stimulation of the mossy fibers (blue arrow head in panel *a*). Data are normalized amplitudes of peak fluorescence transients ($\Delta F/F$) evoked by five stimulations of mossy fibers (delivered at 100 Hz). Insert on the right shows the average normalized area corresponding to the depressant effect of DCG-IV alone (black bar, $100 \pm 5.4\%$, $n=10$) and in the presence of DN13 (white bar, $578.5 \pm 69\%$, $n=9$), $p < 0.001$. Insert on the left displays superimposed fluorescence changes in one of these experiments recorded at the indicated times. Each trace is an average of 10 consecutive trials. Since the variance was different between DCG-IV and DCG-IV + DN13 groups, the Welch test was applied for statistical analysis. **c)** Schematic of the experimental protocol used for the contextual fear consolidation examination in mice, and drug infusion site **(d)**. **(e)** Contextual fear memory expression ANOVA: $F_{5,35} = 6.025$, $P = 0.0004$. **(f)** Cued fear memory. ANOVA: $F_{5,35} = 0.3053$, $P = 0.9066$. * $P < 0.05$ vs PBS and ** $P < 0.01$ vs PBS.