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## **Increased surface P2X4 receptor regulates anxiety and memory in P2X4 internalization-defective knock-in mice**

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Short/running title: Increased surface P2X4 alters plasticity and behavior

## Abstract

ATP signaling and surface P2X4 receptors are upregulated selectively in neurons and/or glia in various CNS disorders including anxiety, chronic pain, epilepsy, ischemia and neurodegenerative diseases. However, the cell-specific functions of P2X4 in pathological contexts remain elusive. To elucidate P2X4 functions, we created a conditional transgenic knock-in P2X4 mouse line (Floxed P2X4mCherryIN) allowing the Cre activity-dependent genetic swapping of the internalization motif of P2X4 by the fluorescent mCherry protein to prevent constitutive endocytosis of P2X4. By combining molecular, cellular, electrophysiological and behavioral approaches, we characterized two distinct knock-in mouse lines expressing non-internalized P2X4mCherryIN either exclusively in excitatory forebrain neurons or in all cells natively expressing P2X4. The genetic substitution of wild-type P2X4 by non-internalized P2X4mCherryIN in both knock-in mouse models did not alter the sparse distribution and subcellular localization of P2X4 but increased the number of P2X4 receptors at the surface of the targeted cells mimicking the pathological increased surface P2X4 state. Increased surface P2X4 density in the hippocampus of knock-in mice altered LTP and LTD plasticity phenomena at CA1 synapses without affecting basal excitatory transmission. Moreover, these cellular events translated into anxiolytic effects and deficits in spatial memory. Our results show that increased surface density of neuronal P2X4 contributes to synaptic deficits and alterations in anxiety and memory functions consistent with the implication of P2X4 in neuropsychiatric and neurodegenerative disorders. Furthermore, these conditional P2X4mCherryIN knock-in mice will allow exploring the cell-specific roles of P2X4 in various physiological and pathological contexts.

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**Introduction**

The release and extracellular action of ATP are a widespread mechanism for cell-to-cell communication in living organisms through activation of P2X and P2Y receptors expressed at the cell surface of most tissues, including the nervous system<sup>1</sup>. Several P2X receptors (P2X) are expressed in the central nervous system (CNS) with varying distributions in neurons as well as in glia<sup>2-4</sup>. P2X receptors are ATP-gated cation channels and their activation by ATP, co-released by neurons with other neurotransmitters<sup>5,6</sup> or released as a gliotransmitter by astrocytes<sup>7-9</sup>, has profound modulatory actions at synapses<sup>1, 10, 11</sup>. Among the seven P2X subunits, P2X4 displays high calcium permeability<sup>12, 13</sup> and a widespread distribution in CNS neurons and glial cells as well as in peripheral tissues<sup>14-16</sup>. Nevertheless P2X4 expression in the brain is sparse<sup>15</sup> and its contribution to the synaptic modulation in normal conditions remains debated<sup>17, 18</sup>. A growing body of evidence suggests that upregulated P2X4 expression plays important roles in various CNS disorders including chronic pain and neurodegenerative diseases such as Alzheimer's disease (AD) or amyotrophic lateral sclerosis (ALS)<sup>19-26</sup>. In the healthy organism, P2X4 is constitutively internalized by the interaction between the adaptor protein 2 (AP2) and a non-canonical endocytosis motif in the C-tail of P2X4 subunit<sup>27, 28</sup>. As a result, P2X4 is found preferentially in intracellular compartments ensuring a low surface expression not only in neurons, but also in microglia and macrophages<sup>29-32</sup>. Intracellular P2X4 may promote vesicle fusion of endosomes or lysosomes<sup>32, 33</sup>. Importantly, intracellular P2X4 pools can be mobilized and trafficked to the cell surface<sup>31, 34</sup>, consistent with the critical role of increased P2X4 surface expression in pathological states<sup>19, 21, 24-26, 35-37</sup>. The specific increase in P2X4 expression and surface trafficking in spinal microglia is critical for the pathogenesis of chronic pain<sup>24, 38-41</sup>. Increased P2X4 expression and surface density in neurons has



86 been observed in the hippocampus or in the spinal cord of AD or ALS mouse models, respectively,  
87 suggesting that upregulated P2X4 may contribute to synaptic dysfunction and/or cell death in AD or  
88 ALS<sup>23, 25, 26, 35</sup>. However, the extent of the upregulated surface P2X4 state and the cell-specific functions  
89 of P2X4 in the pathological context remain elusive.

90 Here we report the development of conditional knock-in (P2X4mCherryIN) mice mimicking a  
91 pathological increase of surface P2X4. We show that the increase of P2X4 at the surface of excitatory  
92 neurons decreases anxiety, impairs memory processing, and alters activity-dependent synaptic plasticity  
93 phenomena in the hippocampus suggesting that upregulation of neuronal P2X4 observed in AD<sup>25</sup> may  
94 have key roles in AD pathogenesis. Overall, we provide an innovative knock-in P2X4 model to study the  
95 functional contributions of upregulated P2X4 in specific cells of the nervous system but also in peripheral  
96 tissues throughout the body.

## 98 **Materials and methods**

### 100 **Experimental model**

101 The *P2rx4* conditional knock-in mouse line was established at the MCI/ICS (Mouse Clinical Institute,  
102 France). *Xenopus* oocytes were isolated as described<sup>42, 43</sup>. Cultures of hippocampal neurons were  
103 prepared as described<sup>44</sup> with some modifications. Peritoneal cells were isolated as described<sup>45</sup> (see  
104 Supplementary information).

### 106 **Ethics approval**

107 All experimental procedures complied with official European guidelines for the care and use of  
108 laboratory animals (Directive 2010/63/UE).

110 **Immunofluorescence and microscopy**

111 Immunofluorescence studies of neurons, macrophages, brain immunohistochemistry and image  
112 acquisition are described in the Supplement and electron microscopy was performed as previously  
113 described<sup>46</sup> (see Supplementary information).

114  
115 **Biotinylation assays and immunoblotting**

116 Surface biotinylation experiments were performed as described previously<sup>8, 43, 47</sup> from injected *Xenopus*  
117 oocytes, mouse peritoneal macrophages and hippocampal cell cultures (see Supplementary  
118 information).

119

120 **Electrophysiology**

121 Extracellular field recordings and induction of synaptic plasticity in hippocampal brain slices are  
122 described in the Supplement. Two-electrode voltage-clamp recordings from *Xenopus* oocyte were  
123 performed as previously described (see Supplementary information).

124

125 **Mouse behavior**

126 Open field procedure, novel object recognition test<sup>48, 49</sup>, elevated plus maze<sup>50</sup>, the Y-maze two-trial  
127 procedure<sup>51</sup> and spatial memory testing conducted in an 8-arm radial maze are described in the  
128 Supplementary information.

129

130 **Quantification and statistical analysis**

131 The number of independent experiments or animals (n), the statistical test used for comparison and the  
132 statistical significance (p values) are specified for each Figure panel in the corresponding figure legend.  
133 Data are presented as mean  $\pm$  s.e.m. Data were analyzed and graphs were generated using GraphPad  
134 Prism.

135

136

## 137 Results

138

### 139 Substitution of the internalization motif of P2X4 by mCherry protein increases surface density 140 of functional P2X4 receptors

141 Mutation or ablation of the endocytosis motif of P2X4 (Y<sup>378</sup>xxGL) was previously shown to increase the  
142 surface trafficking of P2X4 without altering its functional properties<sup>28, 43</sup>. We generated a  
143 P2X4mCherryIN construct in which the endocytosis motif of mouse wild-type (WT) P2X4 was  
144 suppressed by swapping the last 11 amino acids in the C-tail of P2X4 with the sequence coding for the  
145 red fluorescent protein mCherry (Fig. 1a). ATP-evoked currents recorded from P2X4WT or  
146 P2X4mCherryIN expressing *Xenopus* oocytes (Fig. 1b) showed that P2X4mCherryIN response  
147 amplitudes were significantly larger than those recorded from P2X4WT (Fig. 1b). We next examined the  
148 surface level of P2X4WT and P2X4mCherryIN by biotinylation assays and western blotting from oocytes  
149 (Fig. 1c,d). Anti-P2X4 antibodies allowed the detection of P2X4WT (70 kDa) but not P2X4mCherryIN  
150 (the anti-P2X4 epitope (amino acids 370-388) is deleted in P2X4mCherryIN constructs).  
151 P2X4mCherryIN was detected using anti-RFP antibodies showing a band at 100 kDa in agreement with  
152 the fusion of mCherry (30 kDa) to P2X4 (Fig. 1c). Surface/total protein ratio showed that  
153 P2X4mCherryIN is more effectively translocated to the surface than P2X4WT in *Xenopus* oocytes (Fig.  
154 1d,  $p < 0.05$ ).

155 We next transfected hippocampal neurons and Cos cells (Fig. 1e-h and supplementary Fig. S1a-d) with  
156 extracellularly tagged HA-P2X4WT and HA-P2X4mCherryIN to visualize surface P2X4 on living cells  
157 using anti-HA antibodies. Total HA-P2X4 was revealed after cell permeabilization using anti-P2X4  
158 antibodies while the endogenous fluorescence of mCherry fused to P2X4 allowed for direct visualization.  
159 HA-P2X4WT is weakly expressed at the surface and is found mainly in intracellular puncta restricted to  
160 the soma and proximal dendrites of neurons<sup>27</sup>. In contrast, HA-P2X4mCherryIN was ~10 times more  
161 expressed at the surface of neurons. Surface P2X4mCherryIN puncta are uniformly distributed at the  
162 surface including distal dendrites. Co-staining with the post-synaptic marker Homer-1c-GFP revealed  
163 that surface P2X4mCherryIN are equally distributed in small extrasynaptic clusters and in larger clusters

164 juxtaposed to synapses (Fig. 1g,h and supplementary Fig. S1d). Finally, we confirmed the predominant  
165 intracellular localization of untagged P2X4WT in mouse transfected hippocampal neurons using Nodu-  
166 246, a rat monoclonal antibody recognizing the native extracellular domain of mouse P2X4<sup>52</sup>, whereas  
167 P2X4mCherryIN is highly and uniformly distributed at the cell surface (Fig. 1i).

168

### 169 **Generation of knock-in mice with non-internalized P2X4mCherryIN and expression in the brain**

170 We generated the conditional floxed P2X4mCherryIN knock-in mice (Floxed) by homologous  
171 recombination using a targeting vector (see Fig. 2a) designed to flox the last 4 exons (exons 9 to 12) of  
172 *P2rx4* allele (P2X4 internalization motif being located on exons 11 and 12) and followed by the insertion  
173 of a DNA fragment corresponding to the fusion of exons 9, 10 and partially 11 with mCherry cDNA. We  
174 next generated CMVCre<sup>+</sup>-P2X4mCherryIN<sup>F/F</sup> (namely CMV) mice and CaMK2Cre<sup>+</sup>-P2X4mCherryIN<sup>F/F</sup>  
175 (CaMK2) mice by breeding Floxed mice with mice either expressing the Cre recombinase under the  
176 cytomegalovirus (CMV-Cre) or the calmodulin kinase 2 (CaMK2-Cre) promoter (supplementary Fig. S2).  
177 Cre-dependent excision of floxed P2X4mCherryIN allele would lead to the replacement of P2X4WT by  
178 non-internalized P2X4mCherryIN. It is important to note that excised *p2rx4* gene remains under the  
179 control of its own promoter, thus P2X4mCherryIN will replace P2X4WT only in cells expressing natively  
180 P2X4 without altering its distribution pattern or its expression level. In CMV mice, where a Cre-  
181 dependent excision occurs in all cells, P2X4mCherryIN would replace P2X4WT in all cells natively  
182 expressing P2X4 throughout the body. In CaMK2 mice, substitution of P2X4WT by P2X4mCherryIN is  
183 expected to be restricted to excitatory forebrain neurons natively expressing P2X4, while other P2X4  
184 expressing cells such as glial cells and other neuronal types express P2X4WT. Homozygous Floxed,  
185 CMV and CaMK2 mice were used in all the following experiments and were viable, normal in size or  
186 weight reproduced normally and displayed no obvious physical or behavioral abnormalities (see  
187 Supplementary information).

188 Reverse transcription (RT)-PCR in different brain regions from the different mice showed that Floxed  
189 mice solely expressed P2X4WT mRNA while CMV mice only expressed P2X4mCherryIN mRNA (Fig.  
190 2c). As expected, CaMK2 mice expressed both wild-type and knock-in mRNAs since only a subset of

191 P2X4 expressing cells (forebrain excitatory neurons) replace P2X4WT by P2X4mCherryIN. Next, we  
192 tested P2X4mCherryIN expression in the brain on a protein level (Fig. 2d-f). The sparse expression of  
193 P2X4 throughout the brain observed in previous works<sup>3, 53</sup> was recently confirmed using tdTomato *p2xr4*  
194 reporter mice in which the detection of cytosolic tdTomato-expressing cells required the use of anti-  
195 tdTomato antibodies<sup>15</sup>. Not surprisingly, endogenous fluorescence of mCherry fused to P2X4 was not  
196 directly visible on brain slices of the different knock-in mice but could be visualized using anti-RFP  
197 antibodies. P2X4mCherryIN was detected in the pyramidal neuron layer of the hippocampus and in the  
198 soma of neurons throughout the cortex<sup>3, 15</sup> in both CMV and CaMK2, but not in Floxed or P2X4 knockout  
199 (P2X4KO) mice (Fig. 2d). In addition, we confirmed the abundant expression of P2X4mCherryIN in the  
200 epithelial glomerular layer of the olfactory bulb<sup>15, 54</sup> as well as a moderate presence in cerebellar Purkinje  
201 cells<sup>3, 15</sup> of CMV mice but not of CaMK2 mice, as expected. These results showed that P2X4mCherryIN  
202 distribution in CMV mice is similar to that of P2X4 in wild-type mice<sup>15</sup> and the restricted detection of  
203 P2X4mCherryIN in forebrain neurons in CaMK2 mice confirmed the specificity of Cre-dependent  
204 excision. (supplementary Fig. S3a-b). The detection of a 100 kDa band with anti-RFP antibodies in  
205 western blots of total proteins from different brain regions confirmed the substitution of P2X4WT by  
206 P2X4mCherryIN in CMV mice (Fig. 2e). The absence of detection of P2X4mCherryIN in CaMK2 mice  
207 indicated that the replacement of P2X4WT by P2X4mCherryIN is more widespread in the CMV than in  
208 CaMK2 mice, as expected. Moreover, the absence of detection of P2X4WT in Floxed or CaMK2 mice  
209 with anti-P2X4 confirmed the sparse P2X4 expression in the brain tissues (Fig. 2e).

210 Electron microscopy (E.M.) of anti-RFP labeling in hippocampal CA1 region or cortex revealed that  
211 P2X4mCherryIN was present at postsynaptic sites of excitatory synapses, preferentially at the edge of  
212 the postsynaptic density (Fig. 2f). At some asymmetric excitatory synapses, P2X4mCherryIN was  
213 located at both pre- and post-synaptic sites (Fig. 2f) and also found intracellularly in association with the  
214 endoplasmic reticulum membranes or occasionally with mitochondria (supplementary Fig. S3c). This  
215 suggests that replacement of P2X4WT by P2X4mCherryIN does not alter its subcellular distribution<sup>3</sup>.  
216 The identification of symmetric synapses on morphological criteria being difficult, the presence of P2X4  
217 at GABAergic synapses was not confirmed in CMV mice. In contrast, E.M. analysis revealed the

218 presence of P2X4mCherryIN in astrocytes of CMV mice in the astrocyte end-feet encircling endothelial  
219 cells (Supplementary Fig. S3d).

220

221 **Endogenous P2X4mCherryIN fluorescence is directly observable in macrophages or after LPS-**  
222 **induced *de novo* expression in microglia of CMV mice**

223 In mice, *de novo* P2X4 expression was observed in spinal microglia after nerve injury or in the brain  
224 after intracerebral lipopolysaccharide (LPS) injection in tdTomato *P2xr4* reporter mice<sup>15, 38, 39, 41, 55</sup>. We  
225 performed *in vivo* LPS microinjections into the hippocampus of CMV, CaMK2, Floxed and P2X4KO mice  
226 and examined P2X4mCherryIN fluorescence (Fig. 3 and supplementary Fig. S4). In control saline-  
227 injected mice, no endogenous mCherry fluorescence was visible in the hippocampus of all mouse  
228 genotypes and only a faint staining was revealed for the microglial marker Iba1 (Fig. 3a). In contrast, a  
229 strong increase in Iba1 staining was detected in all LPS-injected mouse lines attesting the microglial  
230 activation (Fig. 3a,b and supplementary Fig. S4). Remarkably, endogenous fluorescence of  
231 P2X4mCherryIN was directly visible and colocalized with Iba1 in CMV mice, but not in Floxed or CaMK2  
232 mice. Anti-RFP confirmed the abundant and specific P2X4mCherryIN expression in Iba1-positive cells  
233 solely in LPS-treated CMV mice. Notably, using Beno-271, a camelid nanobody recognizing the  
234 extracellular domain of mouse P2X4<sup>52</sup>, we showed increased P2X4 surface expression in microglia of  
235 CMV, CaMK2 and Floxed but not of P2X4KO mice (Fig. 3b and supplementary Fig. S4). These results  
236 validate the cellular specificity of the Cre-mediated excision in both knock-in mice and show that *de*  
237 *nov*o expression of P2X4 induced by LPS can be directly monitored by the visualization of the  
238 fluorescence of mCherry fused to P2X4.

239 P2X4 has been shown to be highly expressed in macrophages<sup>41</sup>. Endogenous mCherry fluorescence  
240 was directly visualized in freshly isolated peritoneal macrophages from CMV mice (Fig. 3c), the only  
241 knock-in line expressing P2X4mCherryIN receptors in macrophages as confirmed by western blots of  
242 macrophages isolated from CMV, CaMK2, Floxed and P2X4KO mice (Fig. 3d). To assess surface/total  
243 ratio of P2X4 and P2X4mCherryIN by western blot analysis, biotinylation assays were performed using  
244 macrophages in suspension. Our results showed that the number of surface P2X4mCherryIN is

significantly higher in CMV (Fig. 3e,f) than in Floxed mice expressing P2X4WT, demonstrating that the substitution of P2X4 by P2X4mCherryIN leads to an increase in surface P2X4 density in macrophages from CMV mice.

## **P2X4mCherryIN is differentially upregulated at the surface of neurons and glia in CMV and CaMK2 mice**

To demonstrate surface upregulation of P2X4mCherryIN in the CNS, we performed immunofluorescence experiments using hippocampal cultures from pups of both CMV, CaMK2 Floxed or P2X4KO mice. We showed the distribution of surface *versus* intracellular P2X4 using antibodies targeting either the native extracellular domain of P2X4 (Nodu-246), or against the intracellular C-tail of either P2X4WT (anti-P2X4) or P2X4mCherryIN (anti-RFP, Fig. 4). In the Floxed mice, most P2X4WT was detected intracellularly (Fig. 4a) as described previously<sup>27, 28, 34</sup>. In contrast, in both CMV and CaMK2 cells, a strong surface staining was detected using Nodu-246, as well as the presence of RFP labeling, indicating the high density of P2X4mCherryIN on the surface of these hippocampal cells (Fig. 4a). In cells from P2X4KO mice, no P2X4 was detected on the cell surface or intracellular (Fig. 4a). Anti-MAP2 antibodies revealed the specific expression of P2X4mCherryIN in neurons in both CMV and CaMK2 transgenic lines (Fig. 4b and supplementary Fig. S5a). Using antibodies against astrocytic GFAP and microglial Iba1 markers combined with Nodu-246 and anti-RFP, revealed the presence of surface P2X4mCherryIN in microglia and astrocytes from CMV, but not CaMK2 mice (Fig. 4c-d and supplementary Fig. S5b). Consistently, Western blotting of proteins extracted from hippocampal cultures of the different knock-in lines allowed the identification of P2X4mCherryIN proteins in the CMV and CaMK2 exclusively, whereas P2X4WT was detected in Floxed and CaMK2 hippocampal cells (Fig. 4e). Next, we examined surface and total P2X4 levels using either anti-P2X4 or anti-RFP antibodies by biotinylation assays and western blotting of hippocampal cells from the different transgenic mice. Quantification of immunoblots (Fig. 4f) clearly indicates that P2X4WT in Floxed and CaMK2 mice is mainly intracellular (Fig. 4g), while the amount of P2X4mCherryIN on the cell surface is significantly higher in both CMV and CaMK2 cells (Fig. 4a-d). Altogether, these results confirm that substitution of

272 P2X4<sup>WT</sup> by P2X4mCherryIN is restricted to excitatory neurons of CaMK2 mice while in CMV mice, the  
273 substitution occurs in all cells endogenously expressing P2X4 in CMV mice. In addition, our results  
274 demonstrate that P2X4mCherryIN expression leads to a stronger surface localization of  
275 P2X4mCherryIN in both CMV and CaMK2 mice (Fig. 4g).

276

### 277 **Increased surface density of P2X4 alters LTP and LTD at CA1 hippocampal synapses**

278 We next examined the impact of non-internalized P2X4mCherryIN in hippocampal synaptic plasticity.  
279 Field excitatory postsynaptic potential (fEPSP) were recorded in the hippocampal CA1 region after  
280 stimulation of presynaptic Schaffer collateral axons in acute brain slices from control Floxed and both  
281 CaMK2 or CMV mice (Fig. 5a-f). The input/output curves obtained by plotting fEPSP slopes against the  
282 amplitude of stimulation (Fig. 5b) showed no significant differences between slices from CMV or CaMK2  
283 mice compared to control Floxed mice indicating that increased P2X4 surface receptors do not disrupt  
284 basal excitatory transmission at CA1 synapses. LTP and LTD were then evaluated. Robust LTP was  
285 induced in slices from Floxed mice with persistent potentiation (Fig. 5c,d). High frequency stimulation  
286 failed to trigger a potentiation in CaMK2 slices, while in CMV slices a potentiation was induced, but  
287 evoked responses returned rapidly to baseline after the induction. At 40 min post-induction, LTP was  
288 absent in slices from both CMV and CaMK2 mice (Fig. 5c,d). Increased surface P2X4 appears to block  
289 LTD as well (Fig. 5e,f). LTD was induced in slices from Floxed, CMV and CaMK2 mice but a significant  
290 persistent synaptic depression was recorded solely in Floxed mice only (Fig. 5e,f). LTD from CMV and  
291 CaMK2 slices was not significantly different from baseline. Altogether, these results demonstrate that  
292 increased surface P2X4 blocks LTP and alters LTD at hippocampal CA1 synapses. Moreover, the  
293 effects are more pronounced when surface P2X4 is increased exclusively in forebrain excitatory  
294 neurons.

295

### 296 **Increased surface density of P2X4 in forebrain neurons alters anxiety and memory functions**

297 Next, the impact of non-internalized P2X4mCherryIN expression on the behavior of the different  
298 transgenic mice was then examined using a battery of tests (Fig. 5g-p and Supplementary Fig. S6).



299 First, potential changes in their general activity were assessed during open field exploration (Fig. 5g-i).  
300 Floxed, CaMK2 and CMV mice showed similar velocity and total distance traveled in the arena,  
301 indicating that increased surface P2X4 does not modulate basal locomotor activity (Fig. 5g,h and  
302 Supplementary Fig. S6b). Interestingly, CaMK2 mice spent significantly more times in the central zone  
303 (red area) compared to the other groups of mice (Fig. 5i), suggesting that increased surface P2X4  
304 density may reduce anxiety-like behavior. Neophobia-related anxiety was additionally examined by  
305 placing a novel object in the center of the arena (Fig. 5j-l; <sup>56</sup>). The latency to first exploring the novel  
306 object was slightly lower for CaMK2 than for CMV and Floxed mice but this difference failed to reach  
307 significance (Fig. 5k). However, CaMK2 mice spent significantly more time exploring the object than  
308 Floxed or CMV mice (Fig. 5l), suggesting that anxiety is lower in these mice. To corroborate these  
309 findings, the three mouse lines were tested in the elevated plus maze (Fig. 5m). Compared to CMV and  
310 Floxed mice, CaMK2 mice spent a greater proportion of time in the open arms of the maze, confirming  
311 their anxiolytic phenotype. These results indicate that neuronal P2X4 is involved in the regulation of  
312 anxiety-like behavior, with an increased number of P2X4 on neuronal surfaces resulting in reduced  
313 anxiety.

314 Spatial recognition memory was then evaluated using a modified version of the Y-maze two-trial arm  
315 discrimination task (Fig. 5n and Supplementary fig. S6; <sup>57</sup>). After a single encoding phase of 10 min with  
316 only two accessible arms, an inter-trial interval of 4 h resulted in a robust and similar preference for the  
317 unexplored, previously inaccessible, arm during the test phase in CMV, CaMK2 and Floxed mice. Thus,  
318 mice from all genotypes were capable of processing visuo-spatial information and forming short-term  
319 spatial recognition memory.

320 We next asked whether impairments in learning and memory underlie the observed synaptic plasticity  
321 deficits. We examined spatial memory in a more cognitively-challenging situation by submitting CMV,  
322 CaMK2 and Floxed mice to spatial discrimination tests in the 8-arm radial maze. Mice were required to  
323 locate the 3 constantly baited arms of the maze (Fig. 5o). To provide a rigorous control over the time  
324 course of neuronal events induced by spatial learning, we trained the mice over one single day.  
325 Reference memory training consisted of four equivalent blocks of six trials separated by a 1 h interval.

Each trial started with all 8 arms opened and terminated when the mouse entered the third baited arm and returned to the central platform of the maze. While the number of reference memory errors (i.e., entries into unbaited arms) decreased significantly over the four training blocks in all three mouse lines (Fig. 5o), CaMK2 mice made significantly more errors than CMV and Floxed mice (Fig. 5k,  $p = 0.0094$ ). Poorer performance of CaMK2 was particularly apparent in training blocks 2 and 4 compared to Floxed and CMV mice. Subsequently recent and long-term memory were evaluated by submitting mice to retrieval testing 1 and 14 days after training completion (Fig. 5p). As expected, Floxed mice exhibited memory decay over time with an increased number of reference memory errors at day 14. A similar decay was observed in CMV mice, indicating that forgetting was unaffected in this mouse line. In contrast, the poorer performance of CaMK2 mice achieved upon training remained stable across delays and was not further exacerbated by the passage of time. At Day 14, the number of reference memory errors was elevated but similar for all three genotypes. Thus, selective expression of non-internalized P2X4mCherryIN in excitatory forebrain neurons translated into impaired spatial memory processing.

## Discussion

To address pathologically increased surface P2X4 functions, we created a floxed knock-in P2X4mCherryIN mouse line and generated two distinct conditional P2X4mCherryIN lines, expressing non-internalized P2X4mCherryIN either in all cells natively expressing P2X4 (CMV mice) or in excitatory forebrain neurons (CaMK2 mice), respectively. Both CMV and CaMK2 P2X4mCherryIN knock-in mice were viable, reproduced normally and displayed no manifest phenotypic issues. The key finding of this study is that the increased surface density of P2X4 in forebrain excitatory neurons is a major regulator of hippocampal synaptic plasticity, learning and memory and anxiety functions indicating that increased neuronal P2X4 observed in AD models may have key role in the pathogenesis of AD<sup>25</sup>.

Importantly, analysis of P2X4mCherryIN expression showed that substitution of P2X4WT by P2X4mCherryIN occurred in the expected brain regions in both strains. In CMV mice, the expression

352 pattern of P2X4mCherryIN is consistent with what was described for wild-type P2X4 in the CNS, *i.e.*  
353 high expression in the olfactory epithelium and a sparse expression in the hippocampus, cortex,  
354 cerebellum and spinal cord<sup>3, 15, 53</sup>. In addition, and in line with other findings<sup>41, 58</sup>, P2X4mCherryIN was  
355 also found expressed in peritoneal macrophages of CMV mice. In CaMK2 mice, P2X4mCherryIN  
356 expression was mainly restricted to hippocampal and cortical regions, in line with previous reports of  
357 CaMK2 promoter selectivity<sup>59</sup>. The *in vitro* expression of P2X4mCherryIN construct revealed that the  
358 substitution of the last 11 amino acids of the C-tail of mouse P2X4 by the red fluorescent protein mCherry  
359 increases surface trafficking without altering P2X4 function and subcellular targeting<sup>3, 27, 28, 30, 42, 43</sup>.  
360 Indeed, in both CMV and CaMK2 knock-in mice, electron microscopy confirmed that P2X4mCherryIN  
361 are localized at both pre- and post-synaptic specializations in hippocampal or cortical excitatory  
362 neurons, as expected<sup>2, 3, 15</sup>. Inhibitory symmetric synapses are difficult to identify based on morphology  
363 criteria, thus the presence of P2X4mCherryIN at the GABAergic synapse, where P2X4 was reported to  
364 be present, remains to be shown by including specific markers<sup>9, 10, 42</sup>. Surface and intracellular staining  
365 on hippocampal cultures as well as biotinylation experiments from the different knock-in mice  
366 demonstrate that substitution of P2X4WT by P2X4mCherryIN induced a significant increase in surface  
367 P2X4 in both CaMK2 and CMV neurons. Additionally, P2X4mCherryIN was found to be present and  
368 upregulated at the surface of hippocampal microglia and astrocytes as well as macrophages of CMV  
369 mice. P2X4 expression in astrocytes has been previously observed, but was debated<sup>60</sup>. Notably,  
370 P2X4mCherryIN was detected in hippocampal astrocytes *in vitro*, but was detected on brain slices by  
371 E.M. only.

372 In contrast to transfected cells over-expressing P2X4mCherryIN, direct fluorescence of P2X4mCherryIN  
373 was undetectable from brain slices and hippocampal cultures of both knock-in mice, preventing the  
374 direct identification and functional characterization of P2X4mCherryIN-expressing cells. Fluorescence  
375 of P2X4mCherryIN was directly visible solely in isolated peritoneal macrophages of CMV mice in  
376 agreement with its high basal expression in macrophages<sup>41</sup>. Interestingly, following LPS injection in the  
377 hippocampus to induce microglial activation and increase *de novo* P2X4 expression<sup>15</sup>, endogenous  
378 fluorescence of P2X4mCherryIN was revealed in Iba1-positive microglia exclusively in CMV mice.

379 These results indicate that the P2X4mCherryIN fluorescence may represent a unique tool to directly  
380 monitor increased P2X4 expression in pathological models such as chronic pain, AD, ALS, alcohol  
381 intake, inflammation, epilepsy, ischemia or brain trauma<sup>14, 19, 22, 23, 25, 26, 35-38, 55, 61</sup>.

382 LTP and LTD phenomena are widely recognized as crucial molecular mechanisms underlying cognitive  
383 functions such as learning and memory<sup>62</sup>. Although activation of P2X receptors by glial ATP was recently  
384 shown to directly modulate glutamatergic synaptic strength<sup>10</sup>, P2X receptors exert modulatory actions  
385 exclusively during activity-dependent plasticity at central synapses and do not influence basal synaptic  
386 transmission<sup>17, 18, 63</sup>. Previous studies have shown P2X4 modulating hippocampal LTP via N-methyl-  
387 D-aspartate receptor receptors (NMDAR), however these findings remained controversial. The first  
388 work using P2X4KO mice revealed that LTP in CA1 neurons was slightly reduced compared to wild-  
389 type mice and that potentiation of P2X4 by ivermectin enhanced LTP only in wild-type mice<sup>17</sup>. Results  
390 suggested that P2X4 may enhance the content of NR2B subunits in synaptic NMDARs<sup>63</sup>. In contrast,  
391 an other study showed that the pharmacological blockade of P2X4 facilitated the induction of NMDAR-  
392 dependent LTP indicating an inhibitory impact of P2X4<sup>18, 64</sup>. Furthermore, increased P2X4 expression  
393 was reported in AD models<sup>25</sup> suggesting that P2X4 might also contribute to synaptic dysfunction and  
394 memory deficits. Field potential recordings show that P2X4mCherryIN expression does not change the  
395 basal excitatory transmission at CA1 synapses but causes impairments in both LTP and LTD in  
396 agreement with a negative impact of P2X4 on synaptic plasticity. Surprisingly, but in full agreement with  
397 our behavioral results, these deficits were stronger in CaMK2 than in CMV mice. Impaired LTP observed  
398 in CaMK2 mice occurred immediately suggesting a deficit of LTP induction. In contrast, in CMV mice,  
399 LTP was induced but progressively returned to baseline, rather indicating a deficit in LTP maintenance.

400 Since basal excitation mediated by  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor  
401 (AMPA) is unchanged, and LTD or LTP in hippocampal CA1 neurons are mainly initiated post-  
402 synaptically by NMDAR<sup>62</sup>, our results suggest that increased P2X4 in CA1 neurons might alter NMDAR  
403 function. P2X4 are highly permeable to calcium and can mediate a strong calcium influx at the resting  
404 membrane potential that may induce NMDAR inactivation<sup>64</sup>. P2X4 interacts also dynamically with

405 various other ligand-gated ion channels such as GABA<sub>A</sub><sup>10, 42, 65</sup> and may lead to NMDAR inhibition by  
406 similar crosstalk.

407 Behavioral phenotyping of the CMV mice did not reveal any overt alterations. In contrast, CaMK2 mice  
408 exhibited a significant decrease in anxiety-like behaviors and impaired spatial learning and memory  
409 functions. No change in locomotor, anxiety-like or cognitive functions were previously observed in  
410 P2X4KO mice<sup>66</sup>. Together, these results suggest that, in contrast to the basal state, increased surface  
411 P2X4 density observed in neurons in pathological situations such as in AD<sup>25</sup> might play essential roles  
412 in the regulation of synaptic plasticity and behavior such as anxiety and learning and memory.

413 The absence of a phenotype of the CMV mice, although in agreement with the weaker effects on the  
414 synaptic plasticity of CMV compared to CaMK2 mice, is intriguing since substitution of P2X4WT by  
415 P2X4mCherryIN in all cells natively expressing P2X4 was expected to have more pronounced effects  
416 than specific P2X4mCherryIN expression solely in forebrain excitatory neurons. However, this could be  
417 explained by temporal differences between CMV and CaMK2 promoter activation. CMV is a ubiquitous  
418 early-gene promoter leading to the genetic excision of Floxed P2X4mCherryIN in the germ-line and  
419 consequently from the beginning of the embryonic development while the CaMK2 promoter is  
420 considered as an adult promoter with postnatal activity reaching its maximum around the third postnatal  
421 week<sup>67</sup>. Expression of P2X4mCherryIN in all cells natively expressing P2X4 during the developmental  
422 period and thereafter may have deleterious effects leading to developmental compensations. Another  
423 possibility is that the expression of P2X4mCherryIN in CMV mice in other types of neurons along with  
424 expression in glial cells may counterbalance the effect of its increased surface density specifically in  
425 excitatory neurons. Indeed, increased P2X4 expression in spinal cord microglia during neuropathic  
426 conditions triggers the release of BDNF by microglia resulting in neuronal hyperexcitability<sup>39, 40</sup>. In  
427 addition, microglia and BDNF have been shown to influence synaptic plasticity and learning in the brain  
428<sup>68</sup>. These results suggest that increased surface P2X4 in microglia of CMV mice may promote synaptic  
429 plasticity and counterbalance the negative impact of an increase of surface P2X4 specifically in  
430 excitatory neurons of CaMK2 mice.

431 P2X4 is present in multiple cell types in the peripheral or central nervous system and in various epithelial,  
432 endothelial or immune cells. After injury and during inflammation and cell damage, high levels of ATP  
433 are released and P2X4 are strongly upregulated on cell surfaces. This upregulation seems to  
434 orchestrate key events during neurodegenerative diseases, neuropathic and inflammatory pain,  
435 ischemia-induced inflammation, alcohol intake, airways inflammation in asthma, rheumatoid arthritis or  
436 postsurgical liver regeneration (see for review<sup>14</sup>).

437 These novel conditional knock-in mice defective for P2X4 internalization provide a valuable tool which  
438 will allow to further decipher the role of upregulated P2X4 state not only in the context of neurological  
439 diseases, but also of peripheral inflammation, infection as well as in lung, cardiac or liver functions<sup>69-73</sup>.

440

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452

## 453 **Author contributions**

454 EB, TD, KSP, AM, J-TP, ED, A-EA, ET, MR, FG, ON performed the experiments and analyzed the data.  
455 PS, BB, SL, FG, SB, ON and EB-G designed the experiments and analyzed the data. PB, EB, FK-N

456 contributed with key reagents. EB-G conceived the knock-in mice and the study. KSP, ON, and EB-G  
457 wrote the manuscript. All authors commented the manuscript.

458

459 **Conflict of interest**

460 The authors declare they have no conflict of interest.

461

462 **Supplementary information is available at MP's website**

463

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## 723 **Figure legends**

724

725 **Fig. 1** Substitution of the internalization motif of P2X4 by mCherry increases ATP current density and  
726 surface expression of P2X4 receptors. **a** Schematic representation of the mouse P2X4 subunit topology  
727 and C-terminal sequence of wild-type P2X4 (WT) and P2X4mCherryIN subunits. The AP2 binding site  
728 (blue circle) within the C-terminal sequence of mouse P2X4WT was exchanged for the red fluorescent  
729 protein mCherry sequence (P2X4) preventing clathrin-dependent internalization. **b** Representative  
730 superimposed currents evoked by applications of ATP (100  $\mu$ M, 5 s) from oocytes expressing wild-type  
731 P2X4 (WT) (black traces) or P2X4mCherryIN receptors (red traces). Bar graphs of the mean amplitude  
732 of ATP current recorded from oocytes expressing P2X4WT or P2X4mCherryIN as a function of the  
733 quantity of injected cDNAs. Recordings were performed the same day (2 or 3 days) post nuclear  
734 injection. Error bars represent s.e.m., number of cells is indicated in parentheses, \*\*p < 0.01, \*\*\*p <  
735 0.001, one-way ANOVA and Tuckey's post hoc test. Blockade of P2X4 internalization induced a ~7-fold  
736 increase in ATP current amplitudes. **c** Specific detection by Western blots of total proteins from oocytes  
737 expressing P2X4WT (70 kDa) or P2X4mCherryIN (100 kDa) with anti-P2X4 or anti-RFP antibodies,  
738 respectively. **d** Representative immunoblots of total and surface biotinylated proteins from oocytes  
739 expressing P2X4WT (WT) or P2X4mCherryIN (mCherryIN) using anti-P2X4 or anti-RFP antibodies.  
740 Bars represent mean  $\pm$  s.e.m. of the surface/total ratio of P2X4WT (grey bars,  $4.73 \pm 1.64\%$ , n = 5) and  
741 P2X4mCherryIN (red bars,  $27.05 \pm 6.2\%$ , n = 3). The number of independent experiments is indicated  
742 in parentheses, \*p < 0.05, unpaired t-test. **e** Confocal overlay images of hippocampal neurons  
743 transfected with extracellular HA-tagged P2X4WT or HA-P2X4mCherryIN revealed a strong increase in  
744 surface P2X4 in neurons expressing P2X4mCherryIN vs P2X4 WT. Surface P2X4 was revealed by  
745 labeling living cells with anti-HA antibodies (green). Total P2X4 was detected after fixation,  
746 permeabilization and staining with anti-P2X4 antibody (red) in P2X4 WT cells or by mCherry  
747 fluorescence (red) in P2X4mCherryIN cells. Scale bars, 20  $\mu$ m. **f** Mean fluorescence intensity of surface  
748 HA-P2X4WT and HA-P2X4mCherryIN illustrated in E and in supplementary Fig. S1. Error bars, s.e.m.,  
749 number of cells is indicated in parentheses, \*\*\*p < 0.001, unpaired Mann-Whitney test. **g,h** Enlarged

750 image of a dendrite, showing surface clustering of HA-P2X4mCherryIN. Postsynaptic regions are  
 751 indicated by detection of Homer 1c (blue). Scale bar, 5  $\mu$ m. 42% of clusters are localized in the vicinity  
 752 of excitatory glutamatergic synapses. **g** Quantification of the size, fluorescence intensity and density of  
 753 clusters at extrasynaptic site and in the vicinity of glutamatergic synapses showing P2X4 form larger  
 754 clusters near synapses. Error bars: s.e.m., two-way ANOVA and Tuckey's post hoc test, interaction F  
 755 (2, 341) = 11.38; \*\*\*p < 0.001. **i** Epifluorescence images of hippocampal neurons transfected with  
 756 P2X4WT or P2X4mCherryIN revealed the strong increase of surface P2X4mCherryIN compared to  
 757 P2X4WT. Surface P2X4WT and P2X4mCherryIN were revealed on living cells with Nodu-246 antibody  
 758 (green) recognizing the native extracellular domain of P2X4. Total P2X4WT or P2X4mCherryIN were  
 759 detected after fixation and permeabilization with anti-P2X4 antibodies (red) or directly by the red  
 760 fluorescence of mCherry protein, respectively. Scale bar, 10  $\mu$ m.

761

762 **Fig. 2** Generation of conditional P2X4mCherryIN knock-in mice and P2X4 expression in CMV-Cre or  
 763 CaMK2-Cre P2X4mCherryIN knock-in mice. **a** Schematic diagram of the mouse *P2xr4* gene and  
 764 targeting vector strategy used to generate floxed P2X4mCherryIN knock-in mice by homologous  
 765 recombination (Floxed). By breeding floxed P2X4mCherryIN with CMV-Cre mice or CaMK2-Cre mice,  
 766 we generated constitutive CMVCre-P2X4mCherryIN (CMV) and CaMK2Cre-P2X4mCherryIN (CaMK2)  
 767 to obtain a gain-of-function of P2X4 in all cells or in excitatory forebrain neurons natively expressing  
 768 P2X4, respectively. **b** Southern blot of genomic DNA of WT and targeted allele (Floxed) using external  
 769 probe (P) after digestion by *Mfe* I or *Ssp* I enzyme (E) indicated in **a**. **c** Expression of P2X4WT and  
 770 P2X4mCherryIN mRNAs by RT-PCR in cortex (Cx), cerebellum (Cb) and hippocampus (Hip) brain  
 771 regions from Floxed P2X4mCherryIN, CMVCre<sup>+</sup>/Floxed P2X4mCherryIN (CMV) and  
 772 CaMK2Cre<sup>+</sup>/Floxed P2X4mCherryIN (CaMK2) mice. **d-f** Protein expression of P2X4mCherryIN in the  
 773 mouse brain revealed using anti-RFP antibodies recognizing mCherry protein. **d** Brain sections  
 774 immunostained with anti-RFP antibodies show P2X4mCherryIN expression in the hippocampus and  
 775 cortex of CMVCre-P2X4mCherryIN and CaMK2Cre-P2X4mCherryIN while no signal is visible in Floxed  
 776 P2X4mCherryIN and P2X4 Knockout (KO) mice (see also supplementary Fig. S3). Scale bar, 20  $\mu$ m. **e**

777 Western blotting of total proteins extracted from distinct CNS regions isolated from CMV-  
778 P2X4mCherryIN (CMV), CaMK2-P2X4mCherryIN (CaMK2), Floxed P2X4mCherryIN and P2X4KO mice  
779 revealed with anti-RFP or anti-P2X4 antibodies. Anti-Tubulin antibody was used as a loading control.  
780 OB, olfactory bulb; Hip, hippocampus; Cx, cortex; Cb, cerebellum; Hypo, hypothalamus; SC, spinal cord.  
781 **f** Electron microscopy images showing pre-embedding anti-RFP immunostaining in CA1 region of the  
782 hippocampus or cortex from CMV-P2X4mCherryIN and CaMK2-P2X4mCherryIN mice (see also  
783 supplementary Fig. S3). Staining is close to the plasma membrane of pre- and post-synaptic  
784 specializations of excitatory asymmetric synapses (top). Examples of spines where P2X4mCherryIN is  
785 detected only at the postsynapse, mainly located at the edge of the postsynaptic density. t, presynaptic;  
786 sp, spine. Scale bar, 0.5  $\mu$ m.

787

788 **Fig. 3** Endogenous P2X4mCherryIN fluorescence in hippocampal microglia after LPS-induced de novo  
789 P2X4 expression and in peritoneal macrophages from CMV-P2X4mCherryIN mice. **a** Representative  
790 immunohistochemistry images of Iba1 and endogenous fluorescence of P2X4 mCherryIN (top, left) from  
791 the hippocampal region of the different mice injected with saline or 4  $\mu$ g LPS on each side (see also  
792 supplementary Fig. S4). The mCherry fluorescence increased in Iba1-positive cells after LPS specifically  
793 in CMV mice. **b** Immunodetection of P2X4mCherryIN using Beno-271 antibodies (a Camelid nanobody  
794 directed against the extracellular domain of native P2X4) and anti-RFP antibodies from CMV or CaMK2  
795 mice after LPS injections. An enlarged overlay reveals that the increase of P2X4mCherryIN is detected  
796 in Iba1-positive microglia cells by both antibodies in CMV mice while such increase is observed only  
797 with Beno-271 in CaMK2 mice. Scale Bar, 50  $\mu$ m. **c** Endogenous P2X4mCherryIN fluorescence of  
798 macrophages isolated from the peritoneal cavity of Floxed and CMV mice. Differential interference  
799 contrast (DIC) and red fluorescence images reveal the P2X4mCherryIN exclusively in CMV mice. Scale  
800 bar, 20  $\mu$ m. **d** Western blotting of total proteins from macrophages of CMV, CaMK2, Floxed and P2X4KO  
801 mice using anti-RFP and anti-P2X4 revealed that P2X4WT is substituted by P2X4mCherryIN in  
802 macrophages of CMV mice only. P2X4mCherryIN is not detected by anti-P2X4 directed against the C-  
803 terminal of P2X4. Anti-actin antibody was used as loading control. **e,f** Surface P2X4 expression is

804 increased in CMV mouse peritoneal macrophages. Representative immunoblots of total and  
805 biotinylated surface proteins from peritoneal macrophages isolated from Floxed and CMV mice revealed  
806 with anti-P2X4 and anti-RFP antibodies, respectively. As in **d**, total proteins of P2X4KO macrophages  
807 were used as a negative control. **f** Quantification of surface/total expression of P2X4WT and  
808 P2X4mCherryIN in macrophages shown representatively in **e**. Bars represent mean  $\pm$  s.e.m. of the  
809 surface/total ratio of P2X4WT (Grey bar,  $17.36 \pm 1.03\%$ ,  $n = 53$ ) in floxed mice and P2X4mCherryIN in  
810 CMV mice (red bar,  $50.79 \pm 2.71\%$ ,  $n = 51$ ) and show that P2X4 surface expression is strongly increased  
811 in cells expressing P2X4mCherryIN mice compared to those expressing P2X4WT. The number of  
812 independent experiments is indicated by clear and black circles. \*\*\*  $P < 0.001$ , unpaired t-test.

813

814 **Fig. 4** Surface P2X4mCherryIN expression is increased in hippocampal neurons of CaMK2 or CMV  
815 mice as well as in glial cells of CMV mice. **a** Representative images of surface and total P2X4WT and/or  
816 P2X4mCherryIN in Floxed, CMV and CaMK2 primary hippocampal cell cultures reveal that surface  
817 P2X4mCherryIN is strongly increased compared to P2X4WT. Extracellular surface P2X4 and  
818 P2X4mCherryIN are visualized using Nodu-246 (green) antibodies. Intracellular P2X4WT is revealed by  
819 anti-P2X4 antibodies (red) in Floxed and P2X4KO mice while intracellular P2X4mCherryIN is revealed  
820 using anti-RFP antibodies (red) from CaMK2 and CMV mice. Staining was performed at 21 *div*. Scale  
821 bars, 10  $\mu$ m. **b** High density of surface P2X4mCherryIN using Nodu-246 (green) is detected in CaMK2  
822 and CMV neurons identified using neuronal MAP2 marker (magenta). Surface P2X4WT is almost absent  
823 in floxed mice and not detected in P2X4KO neurons (see supplementary Fig. S5). **c,d** P2X4mCherryIN  
824 is expressed only in astrocytes and microglia of CMV mice solely. **c** Surface and total P2X4mCherryIN  
825 revealed using Nodu-246 and anti-RFP antibodies (red) respectively, in GFAP-positive astrocytes  
826 (green) from 7 *div* primary hippocampal CMV cells. Nuclear marker DAPI is shown in blue. **d** Surface  
827 and total P2X4mCherryIN using Nodu-246 or anti-RFP antibodies (red), respectively, in microglia  
828 stained using anti-Iba1 antibodies (green) from 21 *div* primary hippocampal cells from CMV mice.  
829 Nuclear marker DAPI is shown in blue. **e** Western blotting of total proteins from primary hippocampal  
830 cells from Floxed, CMV, CaMK2, and P2X4KO mice using anti-RFP and anti-P2X4 antibodies reveal



831 P2X4mCherryIN specifically in CMV and CaMK2 mice while P2X4WT is detected in CaMK2 and Floxed  
832 mice. **f** Representative immunoblots of total and biotinylated surface proteins from primary hippocampal  
833 cells isolated from Floxed, CMV and CaMK2 mice revealed with either anti-P2X4, anti-RFP or both  
834 antibodies, respectively. As in E, total proteins of P2X4KO macrophages were used as a negative  
835 control. **g** Quantification of surface/total P2X4WT and P2X4mCherryIN in hippocampal cells. Bars  
836 represent mean  $\pm$  s.e.m. of the surface/total ratio of P2X4WT (grey bar) in Floxed and CaMK2 mice and  
837 P2X4mCherryIN in CaMK2 (orange bar) or CMV mice (red bar) and show that P2X4 surface expression  
838 is strongly increased in cells expressing P2X4mCherryIN mice compared to those expressing P2X4WT.  
839 The number of independent experiments is indicated by circles. one-way ANOVA, and Bonferroni's post  
840 hoc test,  $F(3, 20) = 17.2$ ; \*  $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

841

842 **Fig. 5** Surface increase of neuronal P2X4 impairs LTP and LTD at CA1 hippocampal synapses and  
843 alters anxiety, spatial learning and memory. **a** Schematic drawing of the experimental protocol and  
844 example of a fEPSP recorded from the hippocampal CA1 region (Erec). fEPSP were induced by the  
845 electrical stimulation (stim) of the Schaffer collaterals (Sch) in the CA3 area and recorded in the stratum  
846 radiatum layer in brain slices of Floxed, CaMK2 and CMV P2X4mCherryIN mice. DG: dentate gyrus. **b**  
847 The relative field slope is plotted against stimulation intensity. The input/output curves for neurons in  
848 hippocampal slices from Floxed (gray circles,  $n = 7$ ), CaMK2 (orange circles,  $n = 7$ ) and CMV  
849 P2X4mCherryIN (red circles,  $n = 6$ ) mice show similar linear regression (mean  $\pm$  s.e.m., one-way  
850 ANOVA,  $F(2,18) = 0.15$ ;  $p = 0.85$ ) indicating that basal synaptic transmission is not affected by the  
851 increase of surface P2X4 expression. **c,d** Surface P2X4 increase impairs LTP. **c** Plots of normalized  
852 fEPSP slopes recorded in CA1 over time before and after the induction of LTP. LTP was induced by 3  
853 tetanic trains (100Hz, 1s) with a 20 sec interval) in control Floxed ( $n = 10$ ), CaMK2 ( $n = 10$ ) or CMV mice  
854 ( $n = 9$ ). **d** Bar graph summary showing the relative change of fEPSP slope 30-40 min after the induction  
855 of LTP in Floxed ( $144.7 \pm 11.3\%$  of baseline,  $n = 10$ ), CMV mice ( $113.2 \pm 5.27\%$  of baseline,  $n = 9$ ) or  
856 CaMK2 ( $115.6 \pm 3.25\%$  of baseline,  $n = 10$ ), one-way ANOVA and tuckey's post hoc test;  $F(3,25) =$   
857  $9.41$ ;  $p = 0.002$ , \*\*\* $p < 0.001$ ; Error bars: s.e.m.; number of mice is indicated in parentheses. **e,f**

858 Increased surface P2X4 impairs LTD. **e** Plots of normalized fEPSP slopes recorded in CA1 over the  
 859 time before and after the induction of LTD with a 1 Hz, 15 min train Floxed (n = 21), CaMK2 (n = 11) and  
 860 CMV mice (n = 9). **f** Bar graph summary showing the relative change of fEPSP slope 30-40 min after  
 861 the induction of LTD in Floxed ( $79.09 \pm 3.63\%$  of the baseline, n = 22,  $p < 0.001$ ), CMV ( $84.58 \pm 5.97\%$   
 862 of baseline, n = 8,  $p > 0.05$ ) and CaMK2 mice ( $94.24 \pm 3.89\%$  of baseline, n = 9,  $p > 0.05$ ). one-way  
 863 ANOVA and tuckey's post hoc test;  $F(3,44) = 5.44$ ;  $p = 0.0028$ ,  $**p < 0.01$ ; Error bars: s.e.m.; Number  
 864 of mice is indicated in parentheses. **g-i** Open field assessments of control (Floxed mice, n = 24) and  
 865 CaMK2 (n = 13) or CMV (n = 12) P2X4mCherryIN mice. **g** Examples of exploration trajectories (top  
 866 view) in the empty arena for each type of mice. Peripheral and central activities are indicated by yellow  
 867 and red traces, respectively. **h** Similar total travel distance by each mouse line in the empty arena during  
 868 10 min was similar. **i** CaMK2 mice spent more time in the central arena compared to Floxed and CMV  
 869 mice, one-way ANOVA and Bonferroni's post hoc test;  $F(2,46) = 5.139$ ;  $p = 0.0097$ ,  $**p < 0.01$ . **j-l** One  
 870 object exploration test in control (Floxed mice, n = 24) and CaMK2 (n = 13) or CMV (n = 12)  
 871 P2X4mCherryIN mice. **j** Examples of exploration trajectories (top view) for each type of mice. Activities  
 872 far away or at proximity of the object are indicated in yellow or red, respectively. **k** Latency to first  
 873 interaction with the novel object is shown for each type of mouse. One-way ANOVA and Bonferroni's  
 874 post hoc test,  $F(2,46) = 2.868$ ;  $p = 0.067$ . **l** CaMK2 mice spent more time exploring the novel object than  
 875 Floxed and CMV mice. One-way ANOVA,  $F(2,46) = 10.27$ ;  $p = 0.0002$ ;  $**p < 0.01$ ,  $***p < 0.001$ . **m**  
 876 Mean percent of time spent in the open arms of the elevated plus maze for Floxed mice (n = 22), CaMK2  
 877 (n = 14) and CMV (n = 11) mice. CaMK2 spent more time in the open arms, indicating reduced anxiety  
 878 compared to the other mouse lines. One-way ANOVA and Bonferroni's post hoc test,  $F(2,44) = 5.186$ ;  
 879  $p = 0.0095$ ;  $*p < 0.05$ . **n** Spatial recognition memory evaluated in the Y-maze. Floxed (n = 24), CaMK2  
 880 (n = 13) or CMV (n = 12) mice were able to recognize the novel arm of the maze that was rendered  
 881 accessible 4 h after encoding (one-way ANOVA,  $F(2,46) = 0.097$ ;  $p = 0.907$ ). **o,p** Spatial learning and  
 882 memory assessment using the 8-radial maze in Floxed mice (gray), CaMK2 (orange) and CMV (red)  
 883 mice. The number of mice in each group is indicated in parentheses. **o** Learning occurred over one  
 884 single day and consisted of 4 blocks of six trials separated by a 1 h interval. The location of the 3

885 constantly baited arms of the maze is indicated in red. CaMK2 mice committed more reference memory  
886 errors (visits to unbaited arms) compared to Floxed and CMV mice. \* $p < 0.05$ , \*\*  $p < 0.01$ . Paired two-  
887 way ANOVA and Bonferroni's post hoc test, Time effect,  $F(3,255) = 205.3$ ,  $p < 0.0001$ . Paired two-way  
888 ANOVA, genotype effect,  $F(2,85) = 4,927$ ,  $p = 0.0094$ . **p** Retrieval testing 1 (recent) or 14 (long-term)  
889 days after initial training in the 8-arm radial maze for all three mouse lines. Performance achieved on  
890 the fourth training block (D0) is shown for comparison. The poorer performance exhibited by CaMK2  
891 mice (higher number of errors) was not exacerbated by the passage of time. \* $p < 0.05$ , paired two-way  
892 ANOVA and Bonferroni's post hoc test, delay effect,  $F(1,35) = 11.70$ ;  $p=0.0016$ . All data are presented  
893 as mean  $\pm$  s.e.m.

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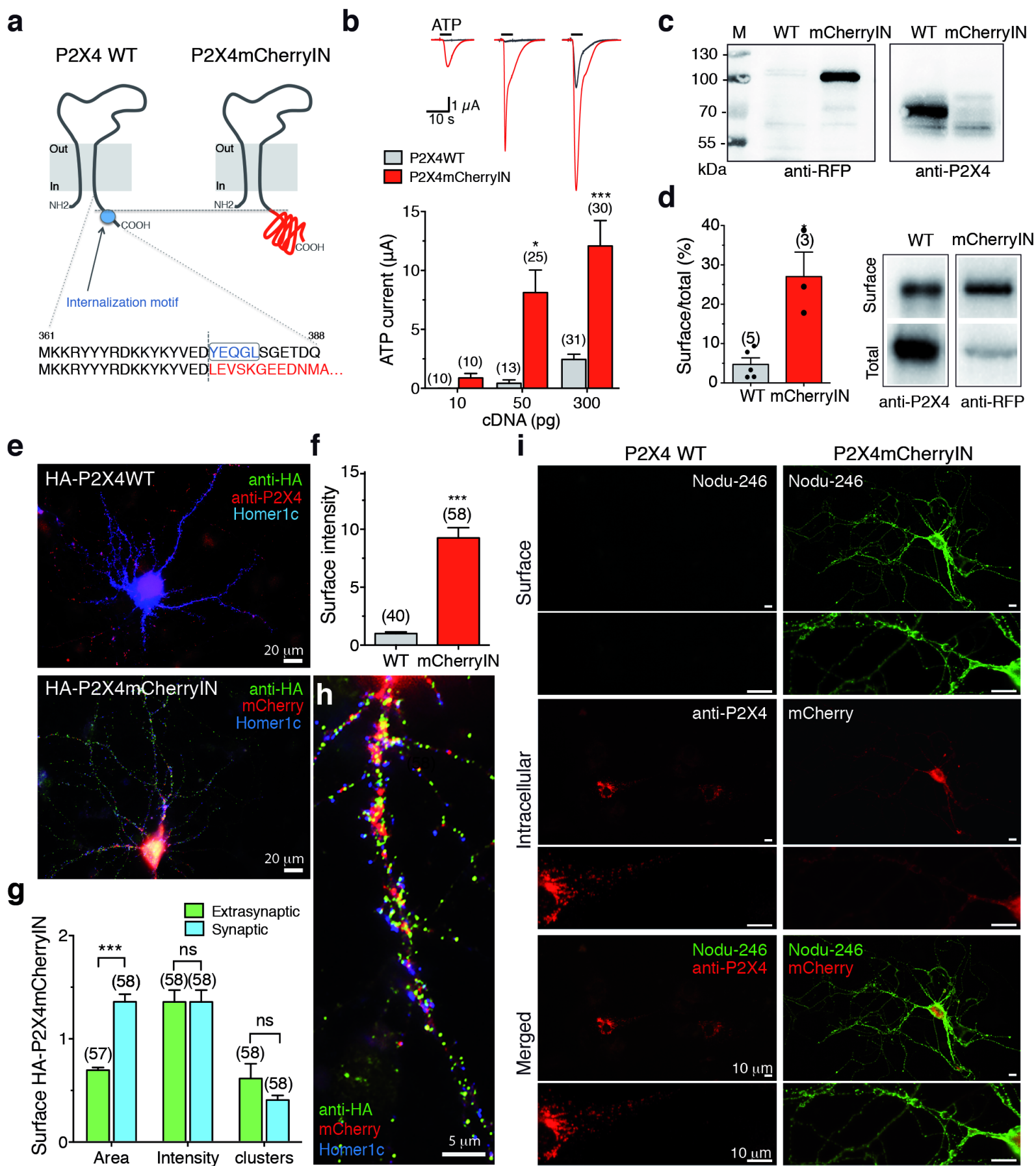


Fig. 1

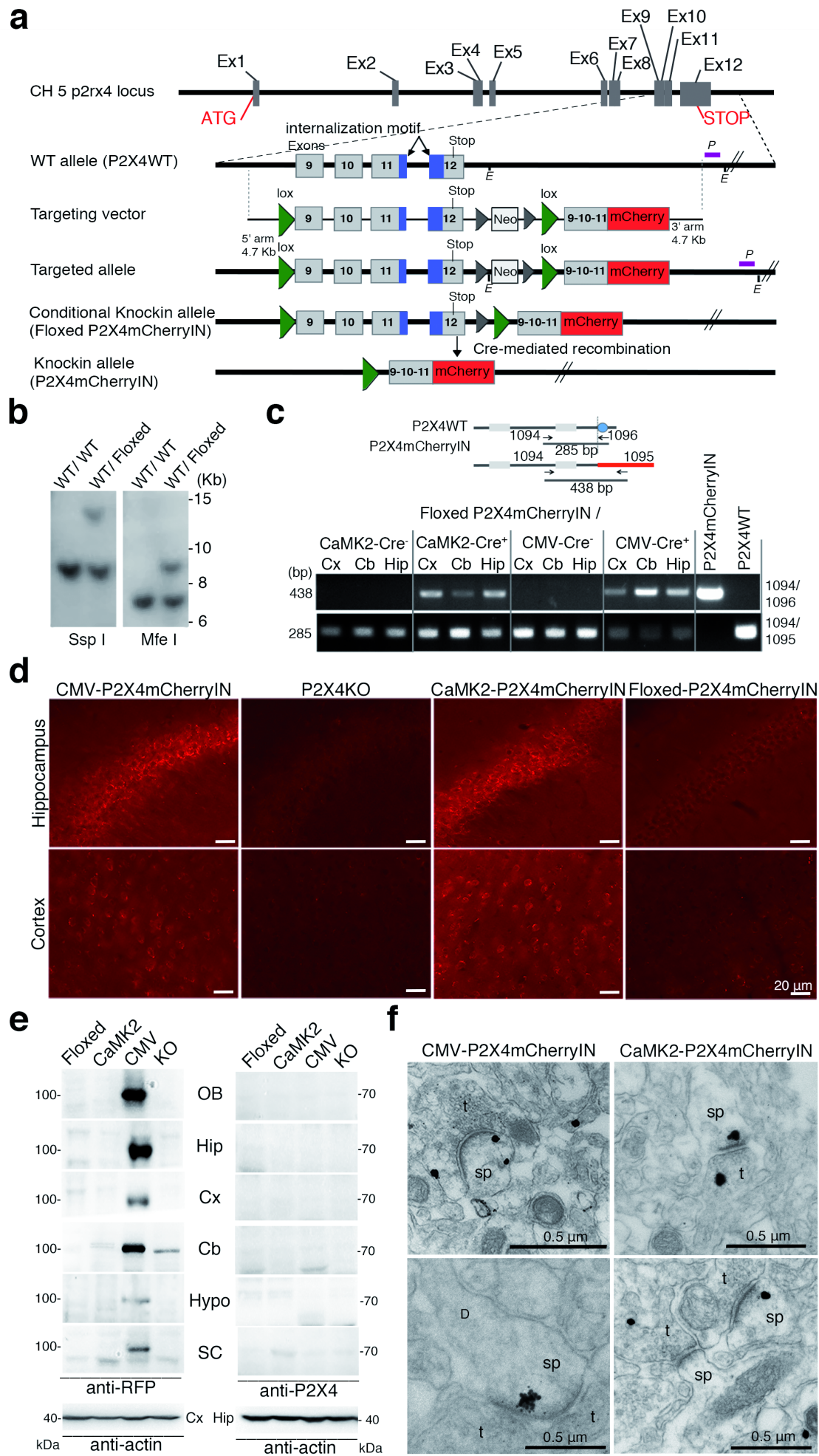


Fig. 2



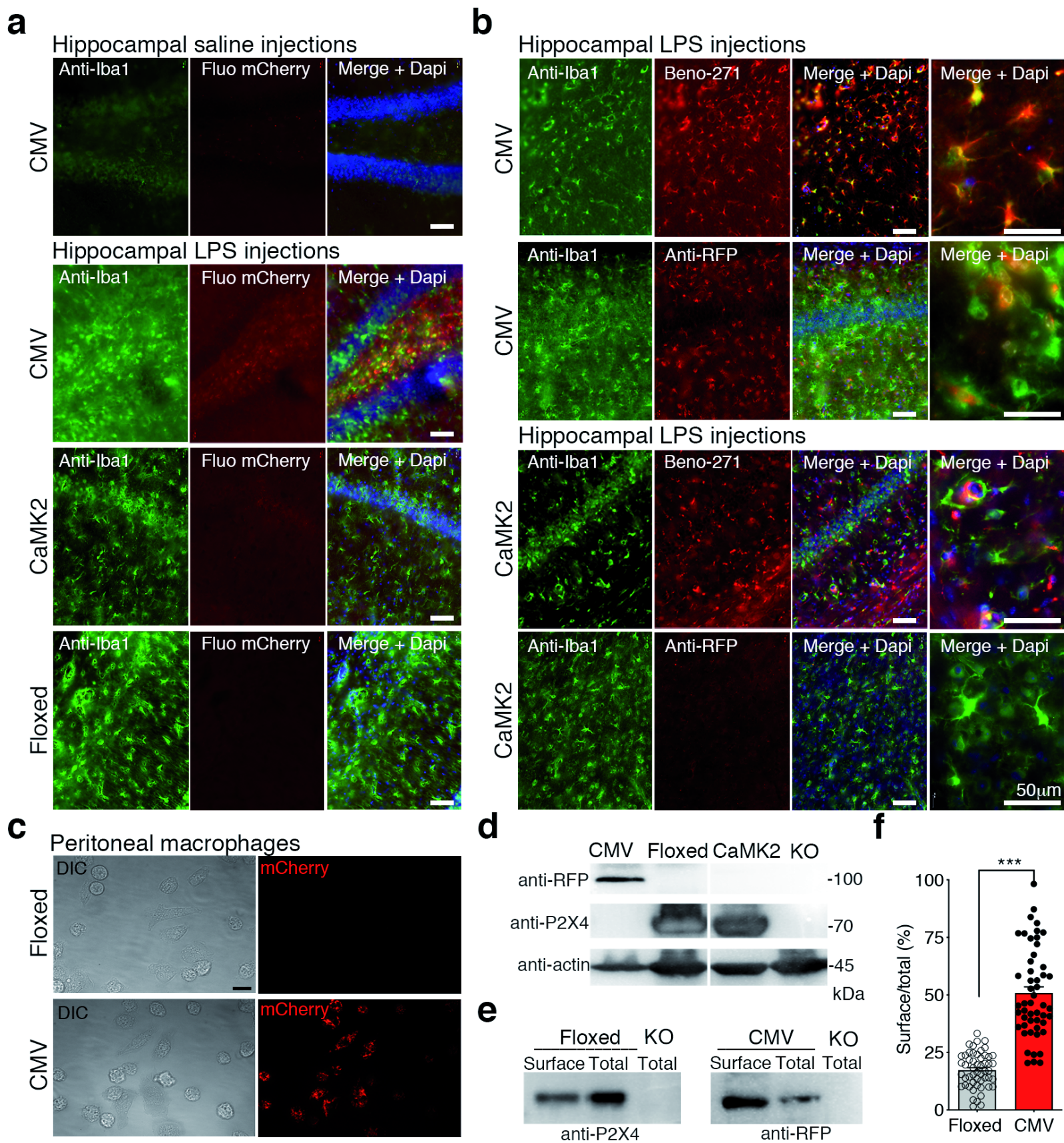


Fig. 3



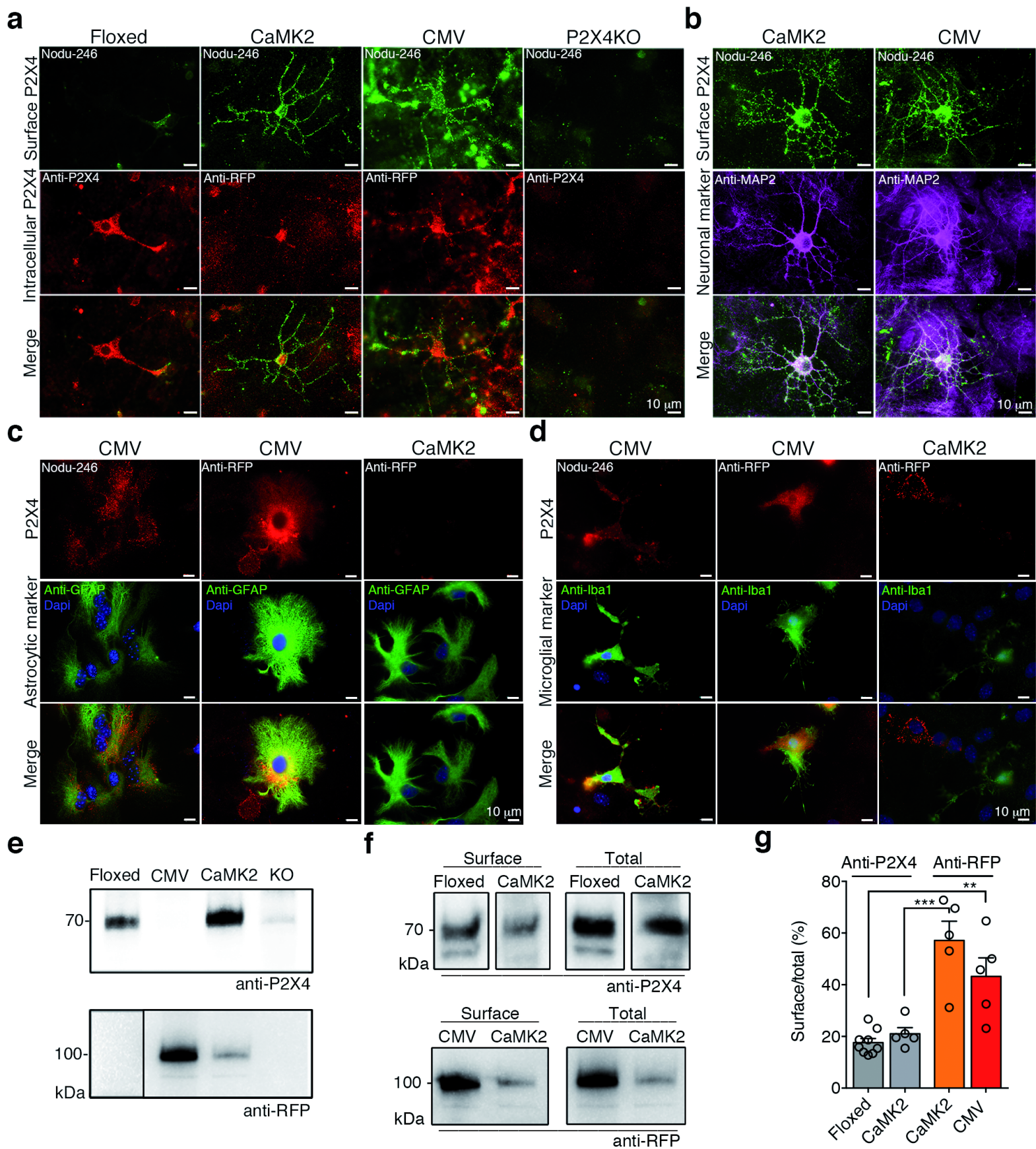


Fig. 4

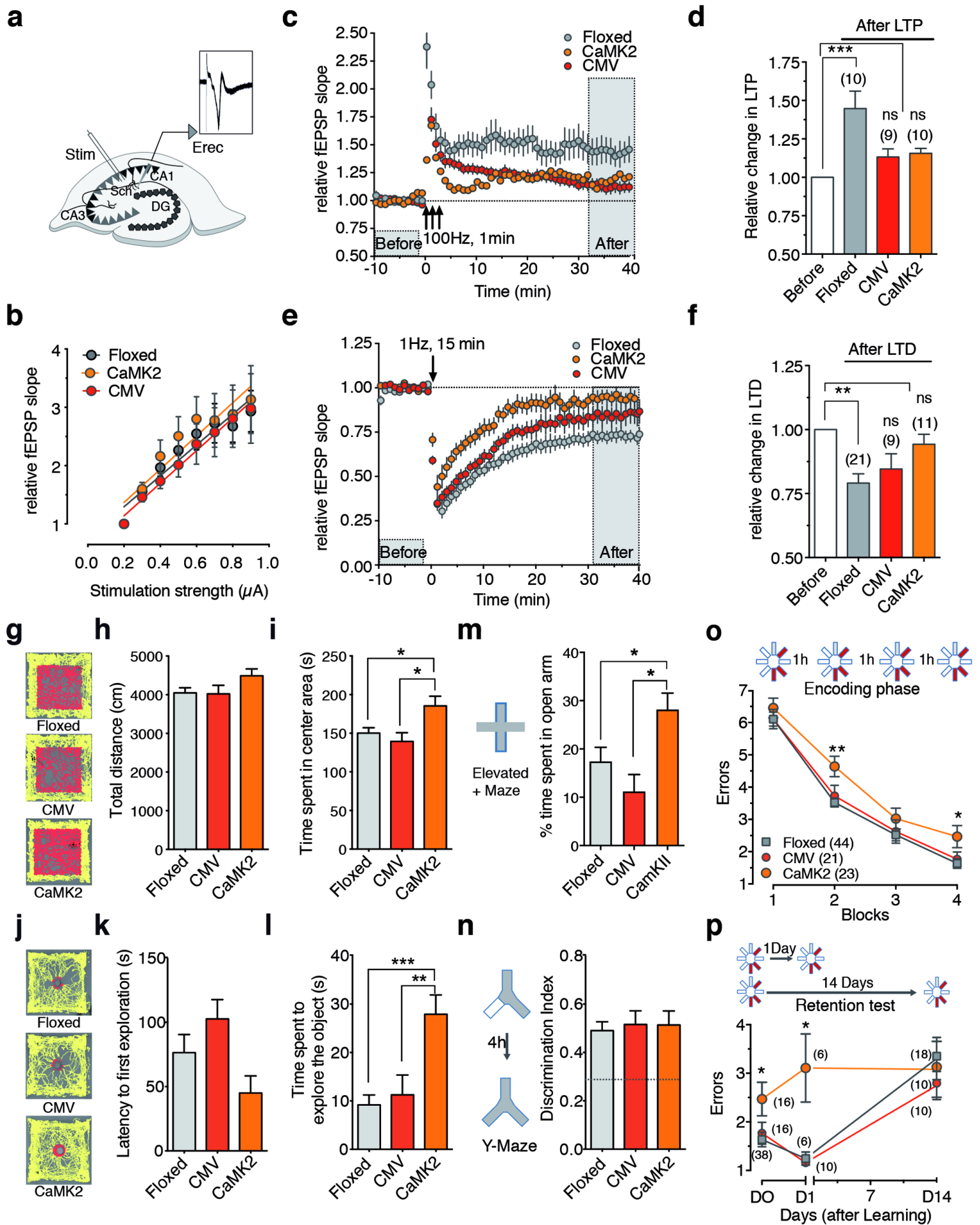


Fig. 5