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## Endocytosis of Activated Muscarinic m2 Receptor (m2R) in Live Mouse Hippocampal Neurons Occurs via a Clathrin-Dependent Pathway

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1 **Endocytosis of activated muscarinic m2 receptor (m2R) in live mouse**  
2 **hippocampal neurons occurs via a clathrin-dependent pathway**

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34 **Running title** : Muscarinic m2 receptor clathrin-dependent endocytosis in live neurons

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37

38 **ABSTRACT**

39

40 Our aim was to examine the dynamics of the muscarinic m2 receptor (m2R), a G-protein  
41 coupled receptor (GPCR), after agonist activation in living hippocampal neurons, and  
42 especially clathrin dependency endocytosis. We have previously shown that the m2R  
43 undergoes agonist-induced internalization *in vivo*. However, the dynamics and the nature of  
44 the endocytotic pathway used by m2R after activation are still unknown in living neurons.  
45 Using live cell imaging and quantitative analyses, we have monitored the effect of  
46 stimulation on the fate of the membrane-bound m2R and on its redistribution in  
47 intraneuronal compartments. Shortly (6 min) after activation, m2R is internalized in  
48 preexisting clathrin-coated pits and not in newly-formed pits. Furthermore, after clathrin-  
49 dependent endocytosis, m2R associates with early and late endosomes and with subcellular  
50 organelles involved in degradation.  
51 Together, these results provide, for the first time, a description of m2R dynamics and  
52 trafficking in living neurons and prove unambiguously that m2R undergoes clathrin-  
53 dependent endocytosis before being degraded.

54

55 **KEYWORDS**

56

57 Internalization ; G protein-coupled receptor ; Mouse ; Trafficking ; Time lapse confocal  
58 microscopy.

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61 **ABBREVIATIONS**

62

63 GPCR, G protein-coupled receptor; m2R, m2 receptor; ACh, acetylcholine; LM, light  
64 microscopy; CCh : carbamylcholine ; CCP : clathrin coated pits, Tf, Alexa Fluor® 594  
65 Conjugated Tf; GFP : green fluorescent protein ; ICC : immunocytochemistry ; NHS,  
66 normal horse serum ; SEP : super ecliptic pHluorin ; PBS : phosphate-buffered sodium.

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73 **INTRODUCTION**

74  
75 Most neurotransmitter and neurotransmitter-related drugs modulate neuronal activity  
76 through G-protein-coupled receptors (GPCRs). Mechanisms that control GPCR  
77 compartmentalization, including membrane availability, enable a neuron to adapt its  
78 response to local changes in neurotransmitter environment.  
79

80 Ligand-induced endocytosis is characterized by the internalization of membrane  
81 molecules, including GPCRs, from the cell surface into internal membrane compartments.  
82 Endocytosis is a complex process that involves different steps. First, endocytosis of GPCRs,  
83 classically involves recruitment of agonist-occupied receptor into vesicles for entry into the  
84 endocytic pathway. This early vesicular trafficking can be divided into two main pathways :  
85 the classic, clathrin-mediated endocytic pathway and the atypical, clathrin-independent, that  
86 may be caveolin-1 or *flotillin-1*-enriched lipid-raft-dependent (Hansen & Nichols, 2009).  
87 Second, the cell may lead receptor containing vesicles to further endosomal processing  
88 through different subcellular compartments and may either recycle the GPCR back to the  
89 plasma membrane and/or degrade them. These early and late trafficking events mediate  
90 important functions for the neuron, tuning its responsiveness to ligands over both short-term  
91 and long-term periods and regulating receptor coupling to signal transduction pathways.  
92 The molecular mechanisms underlying the endocytotic processing are still not clearly  
93 defined but are receptor-specific and may vary between cell types. For example, the highly  
94 related dopamine D1 or D2 receptors may have different internalization pathways (Vickery  
95 & von Zastrow, 1999). Intracellular signaling pathway may also be dependent of the cell  
96 type as shown for ErbB2 or 5-HT<sub>1A</sub> receptor (Carrel *et al.*, 2006; Hashizume *et al.*, 2008).  
97 GPCR endocytosis studies have mostly been performed in cell lines and rarely in neurons.  
98 Yet, as polarized and arborized cells, neurons may display endocytosis features that serve  
99 their specific physiological functions, including receptor targeting to distinct subcellular  
100 compartments (McDonald *et al.*, 2007a).  
101

102 Our work focuses on the muscarinic receptor m2R, a metabotropic acetylcholine  
103 receptor involved in autoregulation of ACh release especially in the hippocampus and cortex  
104 (Zhang *et al.*, 2002). In the present study, we have investigated the dynamics of the early  
105 endocytosis steps of the acetylcholine muscarinic m2 receptor (m2R) in live neurons.  
106 Indeed, the subcellular events after the stimulation of m2R may play a key role in the  
107 function of cholinergic neurons, especially in the regulation of their neuronal activity and/or  
108 in the inhibition of ACh release. We have previously shown that m2R displays endocytosis  
109 *in vivo* in striatal cholinergic neurons after acute stimulation (Bernard *et al.*, 1998; Liste *et*  
110 *al.*, 2002; Decossas *et al.*, 2003; Bernard *et al.*, 2006). However, the precise endocytotic  
111 pathways used by m2R in living neurons are still unknown. One of the aims of our work was  
112 to determine whether m2R internalization occurs via clathrin-coated pits.  
113

114 The m2R dynamics was investigated in hippocampal neurons after agonist activation  
115 using new fluorescent m2R fusion proteins N-terminally tagged with green fluorescent  
116 protein (GFP) or super-ecliptic pHluorin (SEP), a pH-sensitive chimera which facilitate the  
117 detection of surface receptor expression in live cells (McDonald *et al.*, 2007b). Live-cell  
118 confocal imaging was used to visualize, analyse and quantify m2R dynamics. Real-time  
119 early trafficking events of the m2R were especially examined with regard to clathrin, a key  
120 protein of the endocytic pathway, and to other intraneuronal post-endocytic compartments.  
121

122 **MATERIALS AND METHODS**

123

124 All relevant experimental procedures followed the guidelines of the European Communities  
125 Council Directive (86/809/EEC) regarding the care and use of animals for experimental  
126 procedures, and the Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé  
127 et de la Protection Animale (permission no. A 94-028- 21), and were approved by the  
128 Regional Ethics Committee no. 3 of Ile-de-France region on Animal Experiments.

129

### 130 **DNA constructs**

131

132 Two plasmids, mM2-pcDPS and pRK-ssGFP-NK3 encoding the m2R and GFP-neurokinin  
133 3 receptor, were used to generate the GFP-m2R construct. The m2R fragment was amplified  
134 from the mM2-pcDPS plasmid by PCR and introduced as a Sall/XbaI fragment in the pRK-  
135 ssGFP-NK3 plasmid to replace NK3 and to generate a pRK-ssGFP-m2R plasmid and obtain  
136 the N-terminal labeled version of the receptor. The GFP-m2R fragment was flanked  
137 upstream of GFP by an optimized artificial signal sequence derived from the human growth  
138 hormone (hGH1; a signal sequence (ss)) (McDonald *et al.*, 2007b). This plasmid is  
139 designated as GFP-m2R throughout this paper. Another plasmid encoding for m2R tagged  
140 with the super-ecliptic pHluorin, a pH dependent fluorochrome, was generated (SEP-m2R).  
141 The SEP fragment was amplified from the SEP-TOPO plasmid by PCR and introduced as a  
142 BglII/SalI fragment in the GFP-m2R plasmid to replace GFP and to generate a pRK-ssSEP-  
143 m2R plasmid. This plasmid is designated as SEP-m2R throughout this paper. The mM2-  
144 pcDPS, pRK-ssGFP-NK3 and SEP-TOPO were generous gifts from J. Wess (NIH,  
145 Bethesda, USA), A. Irving (University of Dundee, UK) and J. Henley (University of Bristol,  
146 UK), respectively. Alternatively, we have removed GFP from the pRK-ssGFP-m2R plasmid  
147 to produce a pRK-ss-m2R plasmid that coded for the wild-type m2R that was used to check  
148 the absence of negative effects of GFP in the endocytotic processes. This plasmid is  
149 designated as WT-m2R throughout this paper. The integrity of the constructs was confirmed  
150 by sequencing. CAV1-mCherry was a gift from Ari Helenius (Addgene plasmid # 27705,  
151 Hayer et al, 2010).

152

153

### 154 **Neuronal cultures and transfections**

155

156 Post-natal day 0 C57BL/6 mice were euthanized by decapitation. Hippocampi were  
157 dissected from mouse brains and dissociated in *Hanks' Balanced Salt Solution* (HBSS) with  
158 papaine (Worthington Biochemical Corp. Lakewood, NJ, USA; 9001-73-4; 25U/ml).  
159 Hippocampal neurons were plated on glass coverslips previously coated with poly-L-Lysine  
160 0.01% (Sigma-Aldrich, St. Louis, MO, USA). Neurons were grown in Neurobasal A  
161 medium supplemented with 2% B27, 1% glutamax and 0.5% penicillin-streptomycin (Life  
162 technologies; 10888022; 35050038; 17504044; 15140122; respectively) and maintained in  
163 an incubator with 5% CO<sub>2</sub>. Hippocampal neurons were transfected at day *in vitro* (DIV) 7  
164 with the appropriate cDNA (WT-m2R, GFP-m2R, SEP-m2R or DsRed-clathrin) using  
165 Lipofectamine 2000 (Life technologies; 11668019) in OptiMEM medium (Life  
166 technologies; 31985-062). All experiments were performed the day after transfection  
167 (DIV8). The m2R is not constitutively expressed by hippocampal neurons in culture.

168

### 169 **Pharmacological treatments.**

170

171 The effect of a muscarinic receptor agonist carbamylcholine, further referred to as  
172 “carbachol” (CCh) (Sigma, St. Louis, MO, USA), on m2R trafficking was observed in  
173 hippocampal neurons.

174 For real-time experiments, the imaging chamber was perfused 1–120 min with 30 or 100 $\mu$ M  
175 CCh diluted in the isotonic medium. In some experiments, neurons were perfused with  
176 10nM of the muscarinic receptor antagonist atropine (Sigma-Aldrich, St. Louis, MO, USA),  
177 10 min prior to 100 $\mu$ M CCh. CCh was added then together with atropine. In order to reveal  
178 receptors associated with acidic intraneuronal organelles after SEP-m2R transfection,  
179 NH<sub>4</sub>Cl (50mM) was added in the perfusion bath.

180

181 For other experiments, neurons were incubated with 30 or 100 $\mu$ M CCh in  
182 Neurobasal medium for 3, 6, 20min, 1 or 2 hrs and fixed with 2% paraformaldehyde for 5  
183 min. In some experiments, neurons were perfused with 10nM of the muscarinic receptor  
184 antagonist atropine (Sigma, St. Louis, MO, USA) 10 min prior to CCh and then during  
185 endocytosis 15 min together with 30 $\mu$ M CCh.

186

### 187 **Clathrin-dependent endocytosis blockade**

188

189 The clathrin-dependence endocytosis of was investigated by blocking this pathway using  
190 different biochemical and molecular means.

191

192 ***Molecular manipulation of a selected clathrin-dependent endocytosis pathway protein.*** To  
193 block the clathrin-dependent route in the endocytic pathway, we blocked the function of a  
194 key protein in the endocytic pathway, Eps15, by expressing dominant-negative proteins,  
195 fused to GFP. DIII and EH29 mutants were generated by deleting distinct parts of the DNA  
196 coding for Eps15 (Benmerah *et al.*, 1999). Plasmid constructs of dominant negative Eps15  
197 (DIII and EH29) and control (D3 $\Delta$ 2) were kindly provided by A. Benmerah (Hôpital  
198 Necker-Enfants Malades, Paris, France). Neurons were transiently co-transfected with  
199 dominant negative plasmids and the WT-m2R plasmid using Lipofectamine 2000 (Life  
200 Technologies, Saint Aubin, France). Neurons expressing simultaneously Eps15 mutants or  
201 control (identified by GFP staining) and m2R (identified by m2R ICC) were analysed.

202

203 **A1594-Tf uptake used as a marker of clathrin-mediated endocytosis in hippocampal**  
204 **neurons.**

205

206 Transferrin uptake occurs through a clathrin-mediated endocytosis (Benmerah *et al.*, 1999).  
207 In order to know whether m2R is internalized with the same pathway, we have studied the  
208 colocalization of fluorescence for m2R and A1594-Tf in untreated neurons and after CCh  
209 stimulation. For that, the neurons were incubated with A1594-Tf alone for 10 min, then with  
210 or without CCh for 15 min. After fixation, neurons were observed under the confocal  
211 microscope and the colocalization of fluorescent m2R ICC signal and A1594-Tf was  
212 analyzed using the Jacop ImageJ Plugin (see below).

213

214

### 215 **Antibodies and immunocytochemistry**

216

217 ***Antibodies.*** The m2R expressed after transfection with the WT-m2R plasmid was  
218 immunolocalized using a monoclonal anti-m2R antibody raised in rat against an intracellular  
219 epitope of the receptor (rat, Chemicon, Cat# MAB367, Lot# RRID: AB\_94952). The  
220 antibody recognized a single band on Western blots corresponding to the m2i3-GST fusion

221 protein (Levey et al., 1995). In immunohistochemistry, it exhibited a pattern identical to that  
222 seen previously with polyclonal antibodies against the same antigen (Levey et al., 1991,  
223 1995). No immunoreactivity was seen when the antibody was used on tissue from m2  
224 receptor knockout mice (Duttaroy et al., 2002).

225 In some experiments, GFP or SEP expressed after transfection with the GFP-m2R or  
226 SEP-m2R plasmids was detected using a anti-GFP antibody (mouse, Roche Applied Science  
227 Cat# 11814460001, Lot# RRID:AB\_390913). To identify subcellular organelles associated  
228 with m2R after stimulation with CCh for 3, 6 or 20min, 1 or 2hrs, the following antisera  
229 were used: anti-Clathrin heavy chain (CHC ; mouse ; BD Biosciences Cat# 610499 Lot#  
230 RRID:AB\_397865) ; anti-Golgi matrix protein of 130kDa (GM130; mouse; BD Biosciences  
231 Cat# 610822 Lot# RRID:AB\_398141); anti-Rab5 (mouse; BD Biosciences Cat# 610281  
232 Lot# RRID:AB\_397676); anti-Rab9 (mouse; Thermo Fisher Scientific Cat# MA3-067 Lot#  
233 RRID:AB\_2175599); anti-protein disulphide isomerase (PDI; mouse; Thermo Fisher  
234 Scientific Cat# MA3-019 Lot# RRID:AB\_2163120); anti-cathepsin D (CathD (G-19);  
235 mouse; Santa Cruz Biotechnology Cat# sc-6494 Lot# RRID:AB\_2087097). Secondary  
236 antibodies used were donkey anti-rat Alexa568-conjugated or goat anti-rat Alexa488- (m2R,  
237 Thermo Fisher Scientific Cat# A-11077, RRID:AB\_2534121 or Molecular Probes Cat# A-  
238 11006, RRID:AB\_141373; respectively) and goat anti-mouse Alexa488-conjugated (GFP,  
239 (Thermo Fisher Scientific Cat# A32723, RRID:AB\_2633275) or goat anti-mouse Alexa688-  
240 conjugated (GFP, CHC, GM130, Rab5, Rab9, PDI, Molecular Probes Cat# A-11004,  
241 RRID:AB\_141371).

242 .

243 **Immunocytochemistry.** Neurons were fixed with 2% paraformaldehyde for 5 min at room  
244 temperature. The cells were washed in PBS and incubated 30min with 4% normal donkey  
245 serum (Sigma, St. Louis, MO, USA). Primary antibodies were diluted in PBS with 1%  
246 normal donkey serum and 0.075% saponin and incubated overnight at 4°C. Neurons were  
247 washed in PBS and subsequently incubated with fluorescence-coupled secondary antibodies  
248 diluted in PBS with 0.075% saponin for 1 hr at room temperature. Finally, cells were  
249 washed in PBS and mounted in Prolong gold (ThermoFisher Scientific).

250

### 251 **Time-lapse imaging of cultured hippocampal neurons**

252

253 Time-lapse imaging was used to analyse 1) the pH-dependence of the SEP-m2R construct,  
254 2) the effect of CCh on the membrane associated m2R and 3) clathrin-dependence of m2R  
255 endocytosis and its dynamics.

256

257 Time-lapse sequences from cultured hippocampal neurons transfected with selected  
258 plasmids were collected using a Leica DMI6000B inverted microscope (Leica  
259 Microsystems, Deerfield, IL; USA) equipped with a Yokogawa CSU-X1 spinning disc  
260 confocal head (Roper Scientific, Lisses, France) and a 100 mW 491 and 561 nm laser  
261 controlled by MetaMorph (Molecular Devices, St. Grégoire, France). The setup was  
262 enclosed in a thermal incubator set to 37°C under 5% CO<sub>2</sub>. Images were collected through a  
263 63×/1.4 numerical aperture oil-immersion objective and an additional 2× lens on a  
264 QuantEM:512SC EMCCD (Photometrics, Tucson, AZ).

265

266 For validation of SEP pH-dependence, hippocampal neurons transfected with the  
267 SEP-m2R were observed under the spinning disk microscope for 30min and the pH of the  
268 medium was changed (7.4 to acidic pH (around 6.0)) time to time with or without NH<sub>4</sub>Cl  
269 (50mM). Stacks of images were collected every 30s for 30min. In order to analyse the effect

270 of CCh on the membrane associated m2R, hippocampal neurons transfected with the SEP-  
271 m2R were observed under the spinning disk microscope for 120min and stacks of images  
272 were collected. The clathrin-dependence of m2R endocytosis was analysed from double  
273 transfected neurons with and GFP-m2R plasmids. Stacks of images were acquired every 30s  
274 for 30min. Images were treated using Fiji (Schindelin *et al.*, 2012) and Adobe Photoshop  
275 softwares.

276

### 277 **Imaging of fixed cultures by confocal microscopy**

278

279 Images were acquired on a Leica SP5 confocal system (Leica Microsystems, Deerfield, IL;  
280 USA). z-Series stacks of confocal images were acquired at 1024 × 1024 pixel resolution,  
281 with a pinhole setting of one Airy unit and optimal settings for gain and offset. For double  
282 immunolabeling quantifications, images were taken with a 63×/1.4 numerical aperture  
283 (N.A.) Plan-Apochromat, an argon laser at an excitation wavelength of 488 nm, and a diode  
284 561 nm or two diodes at 561 and 633nm. Images were treated using Fiji (Schindelin *et al.*,  
285 2012) and Adobe Photoshop softwares.

286

### 287 **Quantification and statistical analyses**

288

#### 289 *Quantification of colocalization of fluorescence*

290 The quantification of colocalization of m2R with GFP or SEP (for validation of the  
291 constructs) and m2R with clathrin or organelle markers was analyzed with the ‘Just Another  
292 Colocalization Program’ (JACoP) Plugin (ImageJ, National Institutes of Health), and  
293 statistical data are reported from the Costes’s randomization-based colocalization module  
294 (Bolte and Cordelieres 2006). Costes’s randomization method for measurement of  
295 colocalization was used to confirm, with > 95% certainty, that the colocalization observed  
296 between the m2R and clathrin or organelle immunofluorescent signals was not caused by  
297 chance coincidence (Costes *et al.* 2004). A Pearson’s coefficient (*pc*) was calculated.  
298 Costes’s randomization was applied on five neurons from four mice of each genotype using  
299 at least 150 iterations per image. For validation of the constructs, analyses were performed  
300 on somatic areas and on the neuropile. For the colocalization of m2R with clathrin and  
301 organelles markers, analyses have been restricted to the somatic area. Just individual images  
302 (and not stacks of images) were analyzed. The quantification of colocalization was  
303 performed from the labeling on images observed with the 63x objective (surface of the field:  
304 655  $\mu\text{m}^2$ ).

305 The *pc* calculated in colocalizations analyses in WT and stimulated neurons were compared  
306 using a Mann-Whitney U test or the Kruskal-Wallis test followed by Dunn's Multiple  
307 Comparison Test when more than two groups had to be compared. All data are shown as the  
308 means  $\pm$  SEM; NS : not significant; \*\*\*:  $p < 0.0001$ .

309

#### 310 *Quantification of the density of m2R clusters in mutants of Eps 15*

311 Hippocampal neurons were observed using the 63x objective and acquisitions were  
312 performed under the confocal microscope. Intracellular immunofluorescent clusters,  
313 representing m2R in endosomes, were segmented and counted using the FIJI/ImageJ  
314 software. Results are expressed as intracellular immunofluorescent clusters per  $\mu\text{m}^2$   
315 cytoplasmic surface in Eps15 dominant negative-treated and control neurons.

316

317 ***Quantification of variation of fluorescence in time lapse experiments.*** The quantification  
318 of variation of fluorescence levels with time was automatically performed using the Fiji  
319 software. Mean intensity measurements following background subtraction from the whole  
320 neuron were pooled for each cell. Control values corresponded to the mean fluorescence



321 intensity immediately at the beginning of the experiment or before addition of the  
322 muscarinic agonist. Data were compared using the repeated ANOVA test followed by the  
323 Dunnet post-hoc test comparing each value to the value at the beginning of CCh treatment.  
324 The  $p$  values values are : \*  $p < 0.05$ ; \*\*:  $p < 0.001$ ; \*\*\*:  $p < 0.0001$ . The quantification of the  
325 number of clusters of internalized m2R per surface of neuron after CCh treatment was  
326 performed on projections of the stacks images. Data were compared using the repeated  
327 ANOVA test followed by the Dunnet post-hoc test comparing each value to the value at  
328 0min or the Mann-Whitney U test when the data were unpaired. The  $p$  values values are : \*  
329  $p < 0.05$ ; \*\*:  $p < 0.001$ ; \*\*\*:  $p < 0.0001$ .

330

331 All the experiments have been replicated at least three times. For quantitative studies, 15 to  
332 25 neurons per group were analyzed.

333

334

335

336 **RESULTS**

337

338 **Validation of the GFP-, SEP- and WT-m2R constructs**

339

340 **Expression of GFP-m2R and SEP-m2R in living and fixed neurons.** To analyse the  
341 dynamics of GPCRs with high resolution in fixed and living neurons, the m2R was tagged at  
342 the N terminus with GFP or SEP. By live or fixed-cell confocal microscopy, we have  
343 detected a predominant plasma membrane distribution of GFP-m2R or SEP-m2R  
344 fluorescence (Fig. 1A,A'',C,C'',D,D'',E,G) in the cell body and the proximal and distal  
345 dendrites of hippocampal neurons. This distribution was similar to that detected for the m2R  
346 using a third intracellular loop directed antibody (Fig. 1A',A'',D',D'') or to the endogenous  
347 receptor in hippocampal neurons (Bernard *et al.*, 2003). Cytoplasmic fluorescence signal  
348 was low. Similar labelings were observed after transfection with the WT-m2R plasmid and  
349 immunocytochemistry with an m2R antibody (Fig. 1H). The analysis of the colocalization of  
350 GFP with m2R-ICC and SEP with m2R-ICC was performed on fixed neurons using the  
351 Jacop Plugin of ImageJ are reported from the Costes's randomization-based colocalization  
352 module (see Materials and methods). The high Pearson's coefficient at or higher than 0.8  
353 ( $0.8707 \pm 0.0171$ ,  $n=22$ ;  $0.8489 \pm 0.0199$ ,  $n=25$ ; respectively) confirmed the validation of  
354 the GFP-, SEP- and WT-m2R constructs.

355

356 **GFP and SEP are correctly addressed to the external plasma membrane.** In order to check  
357 that the m2R was correctly folded and addressed as expected to the plasma membrane with  
358 its N terminus at its extracellular side, we have co-detected GFP and SEP by their native  
359 fluorescence and by immunocytochemistry using an anti-GFP antibody. GFP  
360 immunofluorescence on fixed and unpermeabilized neurons, was restricted to the plasma  
361 membrane in perikarya and dendrites (Fig. 1C',C''). The analysis of the colocalization of  
362 GFP with GFP-ICC and SEP with GFP-ICC using the Jacop Plugin of ImageJ gave a high  
363 Pearson's coefficient ( $0.8006 \pm 0.0266$ ,  $n=14$  and  $0.7696 \pm 0.0612$ ,  $n=7$ ; respectively)  
364 confirmed the validation of the GFP-, SEP- and WT-m2R constructs.

365

366 **Surface expression of GFP-m2R and SEP-m2R is dynamically regulated by agonist**  
367 **exposure.** As for other GPCRs, the m2R is internalized from the cell surface following  
368 agonist binding (Bernard *et al.*, 1997; Bernard *et al.*, 1998; Bernard *et al.*, 2003; Bernard *et al.*,  
369 2006). We especially checked that GFP or SEP did not disturb m2R internalization. The  
370 GFP-m2R and SEP-m2R underwent a time-dependent loss of cell surface receptors  
371 following CCh (100  $\mu$ M) exposure in agreement with data for endocytosis of wild-type m2R  
372 expressed in neurons (Fig. 1B,B',B'',F) (Bernard *et al.*, 1998; Bernard *et al.*, 2003; Bernard  
373 *et al.*, 2006). Internalization was also observed when neurons were transfected with the WT-  
374 m2R plasmid and the m2R detected by immunocytochemistry with an m2R antibody (Fig.  
375 1I).

376

377 **Validation of pH-dependence of SEP-m2R.** Although GFP is useful to report receptor  
378 localization, it is not possible to distinguish between surface and intracellular receptors in  
379 live cells using fusion proteins incorporating GFP. However, SEP, a pH-sensitive variant of  
380 GFP can be used to report surface expression, when expressed at an extracellular site  
381 (Miesenbock *et al.*, 1998; Ashby *et al.*, 2006; McDonald *et al.*, 2007b). Genetically  
382 encoding SEP into the extracellular domain of a membrane protein of interest positions the  
383 fluorophore on the luminal side of the endoplasmic reticulum (ER) and in the extracellular  
384 region of the cell. SEP is fluorescent when the pH is greater than 6, but remains in an off  
385 state at lower pH values. Therefore, receptors tagged with SEP fluoresce when residing in

386 the ER or upon insertion in the plasma membrane (PM) but not when confined to a  
387 trafficking vesicle. We therefore generated a SEP-m2R chimera by switching GFP for SEP  
388 in the original GFP-m2R construct. The pH dependence of SEP-m2R fluorescence was  
389 characterized in transfected hippocampal neurons. At physiological pH 7.4, SEP exhibits  
390 similar fluorescence to wild-type GFP (Fig. 2A). A strong membrane-associated  
391 fluorescence was observed in the whole dendritic arborization and in the cell body. A faint  
392 staining was seen in the cytoplasm. When the pH is decreased to 6, the dendritic and cell  
393 body membrane labeling strongly decreases as well as the intracytoplasmic staining (-81%  
394 and -98%, respectively) (Fig. 2B,E). Changes in fluorescence levels are reversible when the  
395 pH is back to 7.4 (Fig. 2C,E). Also, SEP-m2R fluorescence is visible again at the plasma  
396 membrane of dendrites and cell body (Fig. 2C). The quantification shows that SEP-m2R  
397 fluorescence is 10% under control (pH 7.4) values in the whole neuron (Fig. 2E).  
398 Administration of NH<sub>4</sub>Cl (50mM), a compound that equilibrates luminal pH of acidic  
399 intracellular vesicles to the extracellular neutral pH, reveals labeling in some  
400 intracytoplasmic organelles when the pH is back around 6.0 to 7.4, and the SEP-m2R  
401 fluorescence is 59% above control values (Fig. 2C,E). In contrast, when the extracellular  
402 medium, including intravesicular pH (due to NH<sub>4</sub>Cl presence) is set to a more acidic pH  
403 (around 6.0), SEP-m2R labeling disappears at membranes and in the soma (-87% and -84 %  
404 (Fig.2D,E)).

405

406 ***Specificity of activation of muscarinic receptors by carbachol.*** Receptor internalization  
407 induced by CCh was totally blocked in the presence of the muscarinic receptor antagonist  
408 atropine (10nM) (Fig. 1J).

409

410 All validation experiments were performed on three independent cultures, and in each  
411 culture, at least ten neurons were analysed.

412

### 413 **Dynamics of m2R internalization**

414

415 To investigate the dynamic properties of the m2R after agonist activation, we have  
416 developed a combination of experiments using individual live hippocampal neurons  
417 transfected with the GFP-m2R or SEP-m2R plasmids and exposed to 100µM CCh for 100-  
418 120 min and monitored by time-lapse confocal microscopy. SEP-m2R staining allowed us to  
419 analyse and quantify the dynamics of membrane m2R disappearance only. GFP-m2R  
420 labeling experiments were useful to analyse the dynamics of m2R internalization.

421

422 SEP-m2R fluorescence slowly drops to reach a minimum of 62% of initial levels  
423 54min after the application of the drug (Fig. 3B,D). Then SEP-m2R labeling rises back to  
424 reach control values at 84min after exposure to CCh. The statistical analysis shows a  
425 significant decrease of the m2R, 39, 54 and 69min after the initiation of the treatment  
426 (Fig.3D). However, m2R fluorescence levels are not different from 39 to 69min, revealing a  
427 steady state of the m2R intensity levels. Addition of NH<sub>4</sub>Cl reveals that m2R is associated  
428 with acidic compartments, by inducing the appearance of a strong punctiform labelling in  
429 the cytoplasm at the level of the soma and dendrites (Fig. 3C,D).

430

431 While SEP-m2R fluorescence decreases at the plasma membrane, a punctiform GFP-m2R  
432 labeling appears close to the membrane and in the cytoplasm as early as 6 min after  
433 administration of CCh (Fig. 4C). The statistical analysis demonstrates that the number of  
434 m2R clusters significantly increases as early as 10 min after the beginning of CCh treatment  
435 (Fig. 4G). Time-lapse analysis shows that the number of internalized clusters increases with

436 agonist exposure during the first 30 min and stabilizes afterwards.

437

### 438 **m2R endocytosis in clathrin-coated pits (CCP)**

439

440 Plasma membrane proteins, especially GPCRs, internalize following agonist stimulation  
441 through a variety of distinct endocytic pathways (Doherty & McMahon, 2009). The best-  
442 characterized pathway is the well-known clathrin-dependent endocytosis, although other  
443 clathrin-independent pathways also exist (Roseberry & Hosey, 2001). The nature of the  
444 m2R endocytotic pathway, especially in neurons, is still under debate. We have tested here  
445 the m2R clathrin-dependent endocytosis hypothesis.

446

447 In order to know if m2R clusters after stimulation in native CCPs, we have identified  
448 these compartments in fixed neurons using an anti-clathrin heavy chain antibody in cells  
449 transfected with WT-m2R. In control neurons, no obvious colocalization of fluorescent m2R  
450 and clathrin was detected (Fig. 5A-A''). In contrast, m2R is highly colocalized with clathrin  
451 in neurons treated with CCh (Fig.5B-B''; arrows). The statistical analysis confirmed that  
452 Pearson's coefficients are significantly different in control compared to treated neurons  
453 (Mann-Whitney U test :  $p < 0.0001$ ; Fig. 5C).

454

455 Live imaging allowed us to determine that activated m2R clusterized to preexisting CCPs in  
456 neuronal cells. For that, live hippocampal neurons were co-transfected with GFP-m2R and  
457 DsRed-Clathrin and observed by spinning disk confocal microscopy before and during a 30-  
458 min-long CCh (30 $\mu$ M) treatment. Our observations revealed that agonist treatment clearly  
459 induced appearance of green fluorescence clusters in both cell bodies and dendrites (Fig.  
460 6D-E'') as early as 6 min after administration of CCh. These clusters co-localized in CCP  
461 identified by DsRed-Clathrin expression (arrows in Fig. 6D-E''). These results suggest that  
462 membrane-associated receptors move to preexisting CCPs shortly (6 min) after activation.

463

### 464 **Al594-Tf uptake as a marker of m2R clathrin-mediated endocytosis**

465

466 In order to know if m2R internalization involves clathrin-mediated endocytosis (CME), we  
467 analyzed uptake of a protein that is well known to display constitutive CME (Benmerah *et*  
468 *al.*, 1999) in control neurons and in neurons treated with CCh. For that, the same neurons were  
469 incubated with Al594-Tf all along the experiment (see Materials and methods). In a control  
470 neuron, Al594-Tf is detected in the cytoplasm (Fig. 7A',A''), whereas m2R is present at the  
471 plasma membrane (Fig. 7A,A''). In contrast, in a neuron treated by CCh, m2R and Al594-Tf  
472 often colocalized (Fig. 7B''). The quantitative analyses of Pearson's coefficients,  
473 demonstrated that colocalization of m2R immunofluorescence and Al594-Tf significantly  
474 increased after CCh stimulation (Kruskal-Wallis test, followed by Dunn's Multiple  
475 Comparison Test: \*\*\*: $p < 0,0001$ ; Fig.7C). Atropine prevented the increased colocalization  
476 of m2R and Al594-Tf (Fig.7C).

477

### 478 **Blockade of m2R clathrin-mediated endocytosis in hippocampal neurons**

479

480 In order to determine if m2R internalization is strictly clathrin-dependent or whether it may  
481 involve other endocytotic pathways, we have used dominant-interfering mutant proteins  
482 (Eps15).

483

484 *Expression of Eps15 mutants disrupts m2R trafficking.* Eps15 is a constitutive  
485 component of plasma membrane CCP (Benmerah *et al.*, 1999). To determine if disruption of

486 Eps15 function influences m2R trafficking, we co-expressed in living hippocampal neurons  
487 two different Eps15 mutants (GFP-EH29 or GFP-DIII) or a control mutant, GFP-D3Δ2,  
488 with WT-m2R. We then analysed m2R post-endocytic trafficking by confocal microscopy  
489 after a 15-min-long CCh (30μM) treatment. The expression of the mutants was checked by  
490 the detection of GFP staining in neurons (Fig. 8A', B' and C'). Expression of GFP-EH29 or  
491 GFP-DIII mutants completely prevents the punctate staining characteristic of m2R  
492 endocytosis induced by CCh. As shown in Fig. 8A and 8B, m2R immunostaining is located  
493 mostly at the plasma membrane of soma and dendrites 15 min after the initiation of CCh  
494 treatment. In contrast, m2R is still internalized when the control mutant, GFP-D3Δ2, is  
495 expressed (Fig. 8C). The statistical analysis (Fig. 8D) confirmed that 1) GFP-EH29 or GFP-  
496 DIII mutants block m2R endocytosis (Mann-Whitney U test : NS; Fig. 8D) and 2) the  
497 control mutant has no effect on m2R internalization (Mann-Whitney U test :\*\*\*: p<0.0001;  
498 Fig. 8D).

499

### 500 **Role of caveolin 1 in m2R endocytosis**

501

502 Proteins may also internalize through clathrin-independent pathways (Doherty & McMahon,  
503 2009). One of these pathways involves caveolae. To address the question of a role of  
504 caveolae-dependent pathway in m2R internalization, we have co-transfected the Cav1-  
505 mCherry plasmid with WT-m2R, and we have quantified colocalization of Cav1 and m2R  
506 fluorescent signals without or after 6 min, 12 min or 15 min of treatment with CCh (Fig. 9).  
507 We did not find any difference in Pearson's coefficients between control and treated neurons  
508 (Mann-Whitney U test : NS; Fig. 9C).

509

### 510 **Post-endocytotic fate of m2R**

511

512 Shortly after activation (6 min), m2R immunoreactivity is detected in numerous vesicles  
513 positive for CHC, a marker of CCP (CHC), EEA1 markers of early (EEA1) and late  
514 endosomes (Rab9) (Fig. 10A-C''') and cathepsin D, a marker of lysosomes (Fig. 10F-F'').  
515 After 20 min of CCh exposure, m2R immunoreactivity is identified in some vesicles  
516 positive for PDI, a marker of endoplasmic reticulum and in GM130, a marker of Golgi  
517 apparatus (Fig. 10D-E'').

518

519

## 520 **DISCUSSION**

521

522 In the present study, we have developed a live-cell imaging approach to gain insights into  
523 the dynamics of a GPCR in living neurons, the muscarinic m2R. We have produced and  
524 validated different DNA constructs to allow expression of m2R in hippocampal neurons *in*  
525 *vitro*. We have especially studied the early steps of m2R endocytosis triggered by the  
526 stimulation. We have demonstrated, for the first time, that m2R is internalized in live  
527 neurons, after stimulation by an agonist, through a clathrin-mediated endocytotic pathway.

528

### 529 **Methodological aspects**

530

531 **Structural validation of constructions** : Consideration of receptor structure and function are  
532 important factors in the generation of fluorescent tag/GPCR chimeras. The construction has  
533 to preserve the native ability to address the m2R to the plasma membrane, to bind its ligands  
534 and not to modify intracellular receptor signaling. We have thus chosen to attach GFP at the  
535 extracellular N-terminus of the m2R, since this site is commonly used as a tagging site for  
536 many other GPCRs (McDonald *et al.*, 2007b; Lelouvier *et al.*, 2008). Several  
537 complementary experiments argue for the fact that our m2R-GFP and m2R-SEP constructs  
538 are valuable tools for such dynamic studies. Indeed, the m2R-GFP and m2R-SEP displayed  
539 the same subcellular localization as the wild type or the endogenous m2R (Bernard *et al.*,  
540 1998); i.e., is homogeneously distributed at the plasma membrane of the somatodendritic  
541 domain. With or without tag, most of m2Rs, revealed by GFP, or SEP or ICC, are detected  
542 at the plasma membrane, suggesting that the m2R is correctly addressed to the plasma  
543 membrane of soma and dendrites. Moreover, the easy detection of GFP and SEP at the  
544 plasma membrane using an anti-GFP antibody, in a non-permeabilized condition,  
545 demonstrates that the tag is correctly fused to the extracellular N-terminus of m2R protein.  
546 The colocalization of GFP or SEP fluorescence with anti-m2R ICC shows that GFP and SEP  
547 are faithful markers of m2R.

548

549 **Validation of pH-dependence of SEP-m2R** : We used the SEP-m2R constructs to monitor  
550 and quantify variations of m2R at the plasma membrane upon agonist stimulation. Since  
551 SEP-m2R is tagged at the extracellular N-terminus, SEP will be present in the lumen of  
552 intracellular organelles during receptor endocytosis. Since these organelles have acidic pH  
553 (Demaurex, 2002), the fluorescence of endocytosed SEP-tagged receptors will be obscured.  
554 In agreement with these data, we have indeed demonstrated that 1) SEP-m2R fluorescence is  
555 quenched at acidic pH and 2) neutralization of intraneuronal vesicles medium by NH<sub>4</sub>Cl  
556 reveals their content in m2R. The SEP-m2R construct is therefore well suited to studying  
557 dynamic changes in surface receptor expression in live cells.

558

### 559 **Muscarinic receptor stimulation induces m2R internalization in hippocampal neurons**

560

561 We have shown here that agonist stimulation of m2R induces internalization of this receptor  
562 in neurons *in vitro*. This is in agreement with previous data observed *in vivo* for the native  
563 receptor (Bernard *et al.*, 1998; Liste *et al.*, 2002; Decossas *et al.*, 2003; Decossas *et al.*,  
564 2005). Our results demonstrate that the incorporation of GFP into the m2R protein does not  
565 modify its ability to bind its ligands and internalize upon agonist stimulation. Indeed, m2R  
566 internalization induced by CCh stimulation was observed with the same timing (6 min after  
567 the initiation of activation) when GFP- and WT-m2R constructions are transfected.  
568 Moreover, we have checked that m2R internalization was actually due to specific activation  
569 of muscarinic receptors since it was blocked by atropine.

570

571 The study of receptor internalization phenomena requires the monitoring of two critical  
572 parameters : 1) the variation in receptor availability at the plasma membrane and 2)  
573 appearance of these receptors in intraneuronal compartments. The use of the SEP-m2R  
574 construct allowed the identification of three steps in the dynamics of membrane m2R  
575 density changes induced by agonist stimulation. First, m2R membrane density regularly  
576 decreases during the first forty minutes. In the same time, GFP-m2R experiments  
577 demonstrates m2R clusters appearance in the somatic and dendritic cytoplasm. The use of  
578 NH4Cl on SEP-m2R expressing neurons reveals that the compartments containing m2R are  
579 acidic and thus probably correspond to endosomes. Taken together, our results suggest that  
580 this first step is mainly operated through endocytosis of membrane m2R into endosomes.  
581 Second, surface and internalized m2R densities stabilize (as measured by both SEP- and  
582 GFP-m2R). This suggests that the bulk internalization process is over. This may reveal a  
583 saturation of the endocytosis machinery, especially saturation of binding to protein involved  
584 in endocytosis. Lou *et al* (2008) have shown that saturation of the endocytosis process  
585 occurs in absence of dynamin 1, a predominant component of the endocytic response. We  
586 may also assume that clathrin-dependent endocytosis has limited capacity and is saturated  
587 when m2R are saturated themselves as demonstrated for the EGF receptor (Schmidt-  
588 Glenewinkel *et al.*, 2008). The third step is characterized by the recovery of m2R at the  
589 plasma membrane. At the same time, m2R is still accumulated in acidic compartments in the  
590 cytoplasm as demonstrated by NH4Cl application on SEP-m2R neurons and by the  
591 appearance of cytoplasmic GFP-m2R clusters. Recycling and/or neosynthesis of m2R may  
592 contribute to restore a normal receptor density at membranes. Recycling has been well  
593 studied for some GPCRs (Hanyaloglu & von Zastrow, 2008 ; Lelouvier *et al.*, 2008; Zenko  
594 & Hislop, 2017) and was shown to be a key phenomenon in the recovery of cell function.  
595 Interference with each process through exposure to monensin (recycling) or cycloheximide  
596 (neosynthesis) should help to determine what is the mechanism leading to the normalization  
597 of surface m2R density.

598

### 599 **The m2R endocytosis is clathrin-dependent**

600

601 We have demonstrated here unambiguously that the m2R was endocytosed in a clathrin-  
602 dependent way in neurons. First, we have shown that, shortly after stimulation, the m2R  
603 colocalized with clathrin-coated pits. Second, we have shown that m2R is partly internalized  
604 together with A1594-Tf, a molecule known to be internalized through a CME pathway.  
605 Third, disruption of CCP using over-expression of Eps15 negative dominants abolished  
606 m2R endocytosis.

607

608 The clathrin-dependence of m2R endocytosis is still under debate (Zenko & Hislop,  
609 2017). Some studies demonstrated that m2R internalization pathway involves CCP only  
610 (Pals-Rylaarsdam *et al.*, 1997 ; Jones *et al.*, 2006; Yamanushi *et al.*, 2007). Other claimed  
611 that m2R is internalized through a clathrin-independent process (Vogler *et al.*, 1999;  
612 Delaney *et al.*, 2002; Wan *et al.*, 2015). Ockenga and Tikkanen (2015) propose that m2R  
613 endocytosis takes place by means of an atypical clathrin-mediated pathway that may involve  
614 a specific subset of CCP. Finally, some authors showed that the internalization of the m2R  
615 utilizes neither clathrin-coated pits nor caveolae (Roseberry & Hosey, 2001). These  
616 discrepancies may be explained in different ways. The signalling and trafficking properties  
617 of GPCRs may depend on the cell and cellular context (Ritter & Hall, 2009). Non-neuronal  
618 cells may not natively produce all the proteins involved in the clathrin mediated endocytotic  
619 machinery. In contrast, we have demonstrated that in neurons, m2R endocytosis is clathrin-

620 dependent, even without overexpression of any endocytotic complex proteins. Indeed, we  
621 have shown agonist-induced m2R internalization in native CCP, i.e. detected by clathrin  
622 immunocytochemistry.

623 Many studies have demonstrated the essential role of CCP in endocytosis and  
624 cellular signalling processes at the plasma membrane. CCP have also been shown to play a  
625 role in the transport of hydrolases from the Golgi complex to the lysosome and for polarity  
626 of the basolateral plasma membrane proteins in the epithelial cell line MDCK, and from the  
627 somato-dendritic membrane to axonal membrane in neurons (Deborde *et al.*, 2008). We may  
628 hypothesize that CCP may play a role in the transport of endocytosed m2R from one  
629 subcellular compartment to another. Alternatively, clathrin was also shown to participate in  
630 rapid recycling after cargo accesses early endosomes (Zhao & Keen, 2008). CCP containing  
631 m2Rs may thus contribute to recycling of m2R at the membrane, as we have suggested  
632 above.

633

### 634 **The m2R is endocytosed at pre-existing CCP**

635

636 One important issue in cell biology of CCP is to know whether the agonist stimulation  
637 initiates the formation of clathrin-coated domains that are specialized for endocytosis of  
638 activated m2R or whether they are simply mobilized to pre-existing CCP. Time lapse  
639 experiments on living neurons allowed us to answer that muscarinic receptor activation  
640 induces m2R accumulation in already pre-existing CCP. Indeed, when m2R and clathrin  
641 were co-expressed in the same living neuron, we were able to show that m2R clusters co-  
642 localized with Ds-Red-clathrin spots that were already present in dendrites or in cell bodies  
643 before stimulation. We hypothesize that, upon activation, phosphorylated m2Rs may shift in  
644 mobility and be trapped at preformed CCP. Similar evidence, i.e. activated receptor clathrin-  
645 mediated endocytosis in preexisting CCP, have been obtained previously in non-neuronal  
646 cell cultures (Santini *et al.*, 2002; Scott *et al.*, 2002). Our results with those obtained for  
647 somatostatin type 2A receptors are the only ones to our knowledge to reveal this behaviour  
648 in neurons (Lelouvier *et al.*, 2008). Further experiments are needed in order to determine  
649 whether this feature can be generalized to all GPCRs in neurons.

650 The study of arrestin translocation and redistribution of receptor-arrestin complexes in CCPs  
651 like  $\beta$ -arrestin 2 and chimera between Eps15, a constitutive component of CCPs, during the  
652 early stages of ligand-mediated endocytosis as shown by Scott *et al.* (2002) may help to  
653 confirm our data. However, due to the fact that current microscopy techniques cannot  
654 distinguish between single pre-formed clathrin structures at the plasma membrane and  
655 clusters of dynamic clathrin-coated pits, some of which are formed in close proximity to  
656 other clathrin structures, we cannot exclude that part of m2R endocytosis occurs through *de*  
657 *novo* CCPs in response to the m2R agonist binding.

658

### 659 **The m2R does not involve caveolae-mediated endocytosis.**

660

661 When m2R and CAV1-mCherry were co-expressed in the same neuron, we did not find that  
662 m2R clusters colocalized with CAV1-mCherry. This suggests that m2R endocytosis does  
663 not use the clathrin-independent pathway involving caveolae. Further experiments are  
664 required to determine whether other m2R undergoes other clathrin-independent endocytotic  
665 pathways.

666

### 667 **Post-endocytotic fate of m2R**

668



669 We have identified to which subcellular organelles m2R is targeted in order to identify the  
670 post-endocytotic pathway where the activated receptors are sorted (Fig. 10). We have  
671 detected m2R in vesicles expressing Clathrin heavy chain (CHC), EEA1 and Rab9, as soon  
672 as 6 min after stimulation (Fig. 10A-C''). This suggests that, after endocytosis in clathrin-  
673 coated pits (identified by CHC immunohistochemistry, Fig. 10A-A''), m2R is sorted to  
674 early, then late endosomes (Fig. 10B-C''). Early endosomes are considered as the first sites  
675 where internalized proteins, including GPCRs, are targeted before being either recycled, or  
676 degraded (Lakadamyali *et al.* 2006).

677

678 The colocalization of m2R with cathepsin D, a marker of lysosomes (Fig. 10G-G''),  
679 confirms the hypothesis of m2R degradation after activation and endocytosis. This is in  
680 agreement with earlier data showing the accumulation of m2R into multivesicular bodies,  
681 which are organelles resulting of the fusion of lysosomes (Bernard *et al.* 1998; Tsuga *et al.*  
682 1998).

683 The fact that m2R content is not increased in endoplasmic reticulum, revealed by PDI ICC  
684 in fixed neurons suggests that m2R is not targeted to compartments involved in m2R  
685 neosynthesis (Fig. 10C-C''). This is in agreement with the absence of m2R-SEP labeling in  
686 live experiments. Indeed, if m2R-SEP is present in the endoplasmic reticulum, a neutral  
687 compartment, SEP should emit light. This is in agreement with earlier studies (Bernard *et al.*  
688 1998).

689

690

## 691 **CONCLUSION**

692

693 We have demonstrated here for the first time that m2R is endocytosed into living neurons  
694 via a clathrin-dependent pathway and through pre-existing clathrin coated pits. The role of  
695 clathrin-mediated endocytosis in signal transduction has yet to be fully understood. It is  
696 known that m2R, as an autoreceptor, modulates acetylcholine release in hippocampus and  
697 cortex (Zhang *et al.*, 2002). How does m2R endocytosis alters acetylcholine release and is  
698 clathrin-mediated endocytosis involved in this alteration are still open questions. It is likely  
699 that clathrin-mediated endocytosis plays a key role in the regulation of signal transduction  
700 by physically removing activated m2R from the cell surface, that would have as a  
701 consequence to terminate the signal. Unless m2Rs recycle from endosomes. Another role of  
702 clathrin-mediated endocytosis may be to produce transport vesicle to convey m2R to axonal  
703 varicosities where it is involved in the regulation of acetylcholine release. This phenomenon  
704 called transcytosis has been reported for Trk receptors (Ascaño *et al.* 2009) but never for a  
705 muscarinic receptor. Analysis of m2R redistribution at the axonal levels may help to  
706 consider this hypothesis. If our work demonstrates that m2R is internalized through clathrin-  
707 mediated endocytosis, we cannot exclude that another endocytotic pathway contributes to  
708 m2R internalization.

709

710 The regulation of m2R membrane availability may thus contribute to regulate  
711 neuronal sensitivity to acetylcholine and relative drugs in physiological or pathological  
712 conditions displaying abnormalities in acetylcholine transmission such as Alzheimer's  
713 disease or schizophrenia (Wess *et al.*, 2007).

714

715

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717

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727

728

729 **AUTHOR CONTRIBUTIONS STATEMENT**

730

731 LL has performed neuronal cultures, immunocytochemistry and imaging. DD participated in  
732 immunocytochemistry experiments and analysis. AF participated to neuronal cultures. ZC  
733 and PD contributed to data interpretations. VB conceptualized the research, designed the  
734 project, participated in the analysis and data interpretation and drafted the work.

735

736 **CONFLICT OF INTEREST STATEMENT**

737

738 The submitted work was not carried out in the presence of any personal, professional or  
739 financial relationships that could potentially be construed as a conflict of interest.

740

741

742

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942 **FIGURE LEGENDS**

943

944 **Figure 1**

945 **Validation of GFP-m2R, SEP-m2R and WT-m2R expressing vectors and localization**  
946 **of transfected m2R in hippocampal neurons.** Hippocampal neurons were transfected with  
947 a plasmid encoding the GFP-tagged receptor (A-C''), SEP-tagged receptor (D-G) or wild-  
948 type receptor (WT-SS-m2R : H-J), fixed, and processed for visualization of the receptor by  
949 confocal microscopy. Equatorial images of neurons (0.5µM in depth) were selected and  
950 illustrated on this panel. The m2R localization was identified by GFP (A,A'',B,B'',C,C'') or  
951 SEP (D,D'',E,F,G) native fluorescence or by fluorescent ICC using an anti-m2R  
952 (A',B',D',H-J) or anti-GFP antibody (C',G). Whatever the construction, the fluorescent  
953 signal is localized at the membrane of the soma and the dendrites. A faint signal is detected  
954 in the cytoplasm. B,F,I. The stimulation with a muscarinic receptor agonist (Carbachol  
955 (CCh), 100µM) induces a huge decrease of the signal at the somatic and dendritic  
956 membranes and a the appearance of a punctiform labeling in the cytoplasm when using a  
957 GFP (B) or SEP-tagged (F) or WT (I) construction. J : A neuron, that has been pre-incubated  
958 with Atropine (10nM), a muscarinic receptor antagonist, display a membrane labeling at the  
959 soma and dendrites similar to a control staining. A-B'',D-D'': Fluorescent signals detected  
960 by direct visualisation of GFP (A,B) or SEP (D) and by m2R ICC (A',B',D') in a same  
961 neuron perfectly colocalize (A'',B'',D''). C-C'': GFP or SEP detection by ICC (C',G)  
962 display a membrane labeling in a non-permeabilized neuron that colocalizes with the direct  
963 GFP or SEP fluorescence (C,C'',G).

964

965 **Figure 2**

966 **Validation of pH-dependence of SEP-m2R.** A living hippocampal neuron transfected with  
967 SEP-m2R was observed by spinning disk confocal microscopy for 30min. A stack of 20  
968 images (0.5µM in depth) were collected at 30s intervals. A projection of the stack images  
969 was performed and an equatorial image was extracted (insert) and illustrated on this panel  
970 (A-D). The effect of pH was observed on the fluorescence level with or without NH4Cl. A :  
971 At pH7.4, the SEP-m2R is detected at the membrane of cell body and proximal dendrites.  
972 B : At acidic pH, SEP-m2R labeling strongly decreases. C : The SEP-m2R labeling is seen  
973 again at the plasma membrane when the medium is back to pH7.4. When NH4Cl (50mM)  
974 that is known to reveal receptors associated with acidic intraneuronal organelles is added in  
975 the medium, punctiform m2R labeling was also seen in the cytoplasm. D : At acidic pH with  
976 NH4Cl, the SEP-m2R labeling is very weak again. E : At pH7.4, the SEP-m2R is detected  
977 again. F : Quantification of the fluorescence level. Fluorescence was measured at the level  
978 of the whole neuron and in soma using the Fiji software. Data are expressed as normalized  
979 values compared to the fluorescence level at pH7.4 at 0min. SEP-m2R labeling strongly  
980 decreases at acidic pH at plasma membranes and in the cytoplasm. NH4Cl at pH7.4 induces  
981 an increase of the staining close to the control values at the plasma membranes and much  
982 higher in soma. The acidic pH with NH4Cl strongly decreases fluorescence at membranes  
983 and in soma. The recovery of SEP-m2R fluorescence is shown in both compartments when  
984 the medium is back to pH7.4.

985

986 **Figure 3**

987 **Time lapse imaging and quantification of SEP-m2R membrane labeling in a living**  
988 **neuron after stimulation by CCh, a muscarinic receptor agonist.** A living hippocampal  
989 neuron transfected with SEP-m2R was observed by spinning disk confocal microscopy for  
990 75min. A stack of 20 images were collected at 30s intervals. An equatorial image (0.5µM in  
991 depth) was selected and illustrated on this panel (A-C). A : In control condition, the SEP-



992 m2R staining is detected at the membrane of the cell body and proximal dendrites. A faint  
993 signal is also shown in the cytoplasm. B : CCh (100 $\mu$ M) induces a decrease of SEP-m2R  
994 labeling at cell body and dendrites levels. C : Application of NH<sub>4</sub>Cl (50mM) that reveals  
995 receptors associated with acidic intraneuronal organelles induces an abundant and intense  
996 punctiform staining in the cytoplasm. D : Quantification of the effect of CCh on the  
997 fluorescence level +/- SEM in 4 neurons using the Fiji software. Fluorescence was measured  
998 on three different neurons on a projection of the stack images at the level of the whole  
999 neuron using the Fiji software. Data are expressed as normalized values compared to the  
1000 fluorescence level at 9 min before CCh application. The quantification shows a significant  
1001 difference of m2R fluorescence with time. The statistical analysis (Repeated measures  
1002 ANOVA test followed by the Dunnett post-hoc test), performed on raw data, shows that  
1003 CCh induces a significant decrease of fluorescence 39, 54 and 69min after the beginning of  
1004 the treatment. Post hoc analyses were performed on two segments of the slope to analyse 1)  
1005 the effect of CCh (from T=0min until 84min) and 2) the effect of NH<sub>4</sub>Cl (from T=84 min  
1006 until 120 min) on fluorescence levels. The values are compared to the values at T=9 min,  
1007 the initiation point of CCh application for the CCh effect and at T=84 min, the initiation of  
1008 NH<sub>4</sub>Cl application, for NH<sub>4</sub>Cl effect. Results show a significant decrease of the fluorescent  
1009 level from 39 to 54 min after CCh stimulation. From 54 min, fluorescence slowly returns to  
1010 normal values. In contrast, NH<sub>4</sub>Cl, which reveals m2R attached to acidic vesicles, induces a  
1011 significant increase of SEP-m2R fluorescence levels. NS : not significant, \* : p<0.05 ; \*\* :  
1012 p<0.001 ; \*\*\* : p<0.0001.

1013

#### 1014 **Figure 4**

1015 **Time lapse imaging and quantification of internalization of m2R in a living neuron**  
1016 **after stimulation by CCh, a muscarinic receptor agonist.** A living hippocampal neuron  
1017 transfected with GFP-m2R was observed by spinning disk confocal microscopy before and  
1018 during a 30-min-long carbachol (100 $\mu$ M) treatment. A stack of 20 consecutive confocal  
1019 images (0.5 $\mu$ m in depth) were acquired every 30 s. A projection of the stack images was  
1020 performed and illustrated on this panel (A-F). An equatorial image was selected and an  
1021 enlargement of a dendritic shaft is shown at the bottom of each image (insert). Here is  
1022 shown the GFP-m2R labeling in this neuron 3 min before CCh and every 3min for 30min.  
1023 Before CCh addition (A) and 3min after the beginning of agonist treatment (B), m2R was  
1024 detected mainly at the plasma membrane of soma and dendrites. Agonist induces  
1025 internalization of membrane-associated m2R and clusterization 6 min after treatment  
1026 initiation in the cytoplasm of soma and dendrites (arrows in C). G : Quantification of the  
1027 effect of CCh on the density of fluorescent clusters +/- SEM in the cytoplasm of 3 neurons.  
1028 Fluorescence was measured on a projection of the stack images using the Fiji software. The  
1029 clusters density increases during the first 30min and stabilized afterwards. The statistical  
1030 analysis (Repeated measures ANOVA test followed by the Dunnett post-hoc test) shows a  
1031 significant increase of the number of clusters as early as 10min after the beginning of CCh  
1032 stimulation.

1033

1034

#### 1035 **Figure 5**

1036 **Internalization of m2R in native clathrin-coated pits in neurons after stimulation by**  
1037 **CCh.** Hippocampal neurons were transfected with a plasmid encoding the WT-m2R,  
1038 stimulated with CCh (30 $\mu$ M) for 9 min and fixed. A stack of 10 images were collected and  
1039 an equatorial image was selected and an enlargement of a cell body is shown at the bottom  
1040 of each image (insert). (A-B''). (A-B'') : Native CCP where detected by  
1041 immunocytochemistry using an anti-clathrin heavy chain antibody (A',B'). A-A'' : A

1042 control neuron displays no m2R and clathrin colocalization. Nine minutes after the initiation  
1043 of CCh treatment, some m2R clusters colocalize with native CCP (arrows). C : The analysis  
1044 of the colocalization of clathrin and m2R-ICC was performed on fixed neurons using the  
1045 Jacop Plugin of ImageJ are reported from the Costes's randomization-based colocalization  
1046 module (see Materials and methods). The quantification of m2R and clathrin colocalization  
1047 and the statistical analysis demonstrates a significant increase of the Pearson's coefficient in  
1048 treated neurons (n=20) compared to cells treated with CCh (n=20) (Mann Whitney U test;  
1049 \* : p<0.0001). ). Control neurons : n=20; CCh-treated neurons : n=21.

1050

1051 **Figure 6 :**

1052 **Internalization of m2R in clathrin-coated pits in a living neuron after stimulation by**  
1053 **CCh.** A living hippocampal neuron transfected with GFP-m2R and Ds-Red clathrin was  
1054 observed by spinning disk confocal microscopy before and during a 30-min-long carbachol  
1055 (30 $\mu$ M) treatment. A stack of 10 images was collected and selected images at the indicated  
1056 times show a representative dendrite and a selected area in a cell body (insert) of a neuron.  
1057 Six minutes after the beginning of CCh treatment, m2R clusters appear at loci of clathrin  
1058 coated-pit (CCP) spots (arrows) in the dendrite and the cell body (A''-E''). Note that the  
1059 m2R clusters form in preexisting CCP.

1060

1061 **Figure 7**

1062 **Al594-Tf uptake and m2R clathrin-dependent endocytosis in hippocampal neurons.** A-  
1063 D' : Hippocampal neurons were transfected with m2R-WT, pre-incubated with Tf-Al594, 10  
1064 min before CCh treatment. Cells were fixed after 15min treatment. In control neurons,  
1065 Al594-Tf is detected in the cytoplasm as a punctiform labelling (A'-A''). CCh treatment  
1066 induces a strong decrease of membrane m2R labeling and the appearance of m2R  
1067 punctiform staining (B',B''). Al594-Tf and m2R-ICC signal often colocalize (B',B'', arrows).  
1068 The quantitative analysis of the colocalization of m2R and Al594-Tf in neurons was  
1069 performed using the Jacop Plugin of ImageJ and statistical data are reported from the  
1070 Costes's randomization-based colocalization module (see methods). Data are expressed as a  
1071 Pearson's coefficient (pc) and pc were compared using the Kruskal-Wallis test followed by  
1072 the Dunn's Multiple Comparison Test. Our analysis shows that the colocalization observed  
1073 between the m2R immunofluorescent signal and Al594-Tf is higher after treatment with  
1074 CCh compared to untreated neurons (\*\*\*: p<0.0001). Atropine prevents the increase of m2R  
1075 and Al594-Tf colocalization (Atropine treatment vs Control : NS : not significant). Control  
1076 neurons : n=25; CCh-treated neurons : n=19; CCh-treated + atropine neurons: n=9.

1077

1078

1079 **Figure 8**

1080 **Blockade of m2R clathrin-dependent endocytosis in hippocampal neurons with**  
1081 **negative dominant of Eps15.** Hippocampal living neurons were co-transfected with WT-  
1082 m2R plasmid and GFP-tagged EH29 and DIII mutants or their control (D3 $\Delta$ 2). The day after  
1083 transfection, neurons were treated with CCh (30 $\mu$ M) for 15min and fixed. The m2R was  
1084 detected by ICC. When the mutants are expressed (labeling in A', B'), m2R labeling is seen  
1085 at the plasma membrane of neurons (A,B). The expression of the control plasmid (labeling  
1086 in C') does not block m2R internalization (C). D : Intracellular immunofluorescent clusters,  
1087 representing m2R in endosomes, were segmented and counted using the FIJI/ImageJ  
1088 software. Results are expressed as intracellular immunofluorescent clusters per  $\mu$ m<sup>2</sup>  
1089 cytoplasmic surface in Eps15 dominant negative-treated and control neurons. The statistical  
1090 analysis shows that the expression of the EH29 and DIII mutants blocks m2R clusterization  
1091 (Mann-Whitney U test: NS : Not significant; EH29 : Control neurons : n=19; CCh-treated

1092 neurons : n=19; DIII : Control neurons : n=19; CCh-treated neurons : n=30). In contrast, the  
1093 control mutant (D3Δ2) does not inhibit m2R clusterization (Mann-Whitney U test:  $p <$   
1094 0.0001; Control neurons : n=19; CCh-treated neurons : n=15).

1095

### 1096 **Figure 9**

#### 1097 **Absence of internalization of m2R in caveole in fixed neuron after stimulation by CCh.**

1098 Hippocampal neurons were co-transfected with a plasmid encoding the wild-type receptor  
1099 (WT-SS-m2R : A,B) and CAV1-mCherry (A',B') fixed, and processed for visualization by  
1100 confocal microscopy. In control and treated neurons, CAV1-mCherry is detected in the  
1101 cytoplasm as a punctiform labelling (A',A'', B,B''). Some m2R and CAV1-mCherry  
1102 clusters colocalize (arrows) in both treated and untreated neurons. The quantitative analysis  
1103 of the colocalization of m2R and CAV1-mCherry in neurons was performed using the Jacop  
1104 Plugin of ImageJ and statistical data are reported from the Costes's randomization-based  
1105 colocalization module (see methods). Data are expressed as a Pearson's coefficient (pc) and  
1106 pc were compared using the Kruskal-Wallis test followed by the Dunn's Multiple  
1107 Comparison Test. Our analysis shows that pc values do not significantly differ in control  
1108 neurons and neurons treated with CCh for 6, 12 and 15 min (NS : not significant).

1109

### 1110 **Figure 10**

#### 1111 **Immunohistochemical localization of m2R in neuronal compartments involved in endocytosis, synthesis, maturation and degradation in fixed hippocampal neurons.**

1112 Hippocampal neurons were transfected with m2R-WT. Neurons were stimulated with CCh  
1113 at 30μM for 6, 20 min and 1hr fixed, and processed for visualization of m2R together with  
1114 markers of intraneuronal compartments and observed by confocal microscopy. A-C'': 6min  
1115 after CCh stimulation (30μM), some m2R immunopositive punta colocalize with CHC in  
1116 clathrin-coated pits, EEA1 in early endosomes and Rab9 in late endosomes (arrow heads).  
1117 D-F'': 20min after CCh stimulation (30μM), we failed to detect no colocalization of m2R  
1118 with PDI, a marker of endoplasmic reticulum and GM130 and TGN38, markers of Golgi  
1119 apparatus. G-G'': 1hr after CCh stimulation (30μM), some m2R immunopositive punta  
1120 colocalize with CathD, a marker of lysosomes (arrow heads). The quantitative analysis of  
1121 the colocalization of m2R and markers of subcellular compartment in neurons was  
1122 performed using the Jacop Plugin of ImageJ and statistical data are reported from the  
1123 Costes's randomization-based colocalization module (see methods). Data are expressed as a  
1124 Pearson's coefficient (pc) and pc were compared using the Mann-Whitney U test. Our  
1125 analysis shows that the colocalization of the immunofluorescent signals for m2R with CHC,  
1126 EEA1, Rab9 and CathD is higher after treatment with CCh compared to untreated neurons  
1127 (CHC, Rab9 and CathD : \*\*\*:  $p < 0.0001$ ; EEA1 : \*\* :  $p < 0.01$ ). In contrast, the colocalization  
1128 of the immunofluorescent signals for m2R with PDI and GM130 do not significantly differ  
1129 in CCh-treated neurons compared to untreated Control neurons : CHC n=20, EEA1 n=16,  
1130 Rab9 n=15, PDI n=21, GM130 n=17, CathD n=17; CCh-treated neurons : CHC n=12,  
1131 EEA1 n=18, Rab9 n=15, PDI n=20, GM130 n=18, CathD n=15.

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