

# 1 Regulation of Mineralocorticoid Receptor Expression during Neuronal 2 Differentiation of Murine Embryonic Stem Cells

3

4 **Abbreviated title:** MR in ES-derived Neurons

5

## 6 **Précis:**

7 Mineralocorticoid receptor expression increases during embryonic stem cell-derived neuronal  
8 differentiation through aldosterone-stimulated activation of its alternative promoters.

9

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26

## 27 **Disclosure information:**

28 M. M., G.M., S.V., P.L., D.L., M.L. have nothing to disclose.

29

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33

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39

**40 ABSTRACT**

41 Mineralocorticoid receptor (MR) plays a critical role in brain function. However, the regulatory  
42 mechanisms controlling neuronal MR expression that constitutes a key element of the hormonal  
43 response are currently unknown. Two alternative P1 and P2 promoters drive human MR gene  
44 transcription. To examine promoter activities and their regulation during neuronal differentiation and  
45 in mature neurons, we generated stably transfected recombinant murine embryonic stem (ES) cell  
46 lines, namely P1-GFP and P2-GFP, in which each promoter drove the expression of the reporter gene  
47 Green Fluorescent Protein (GFP). An optimized protocol, using embryoid bodies and retinoic acid,  
48 permitted to obtain a reproducible neuronal differentiation as revealed by the decrease in phosphatase  
49 alkaline activity, the concomitant appearance of morphological changes (neurites) and the increase in  
50 the expression of neuronal markers (nestin,  $\beta$ -tubulin III, MAP2) as demonstrated by  
51 immunocytochemistry and qPCR. Using these cell-based models, we showed that MR expression  
52 increased by 5-fold during neuronal differentiation, MR being preferentially if not exclusively  
53 expressed in mature neurons. Although the P2 promoter was always weaker than the P1 promoter  
54 during neuronal differentiation, their activities increased by 7- and 5-fold, respectively and correlated  
55 with MR expression. Finally, while progesterone and dexamethasone were ineffective, aldosterone  
56 stimulated both P1 and P2 activity and MR expression, an effect that was abrogated by knockdown of  
57 MR by siRNA. Concluding, we provide evidence for a tight transcriptional control of MR expression  
58 during neuronal differentiation. Given the neuroprotective and antiapoptotic role proposed for MR, the  
59 neuronal differentiation of ES cell lines opens potential therapeutic perspectives in neurological and  
60 psychiatric diseases.

61

## 62 INTRODUCTION

63 The mineralocorticoid receptor (MR), a transcription factor belonging to the nuclear receptor  
64 superfamily, is expressed both in epithelial tissues, where it plays a critical role in mediating  
65 aldosterone-regulated transepithelial sodium transport, and in non-epithelial tissues such as the  
66 cardiovascular system and central nervous system where it controls important cardiovascular functions  
67 and behavioral processes (1). MR binds both aldosterone and glucocorticoid hormones with a similar  
68 affinity. In the brain, although aldosterone-selective MR is expressed in hypothalamic sites involved in  
69 the regulation of salt appetite (2), the highest MR expression is found in the limbic structures  
70 particularly in hippocampal neurons, where it is physiologically occupied by glucocorticoids.

71 In the central nervous system, MR is involved in the regulation of the Hypothalamic-Pituitary-  
72 Adrenocortical (HPA) axis activity under basal and stress conditions (3). MR activity, at low  
73 glucocorticoid levels, is associated with cell excitability and long-term potentiation, a cellular model  
74 for learning and memory formation (4). Animal models of MR overexpression or inactivation have  
75 also brought important information on MR function in the brain. Indeed, mice specifically  
76 overexpressing MR in the forebrain exhibited a reduction of anxiety-like behavior (5, 6), while mice  
77 lacking limbic MR had a normal anxiety-like behavior but improved in learning processes (7, 8).  
78 Moreover, studies on hippocampal neurons recently proposed an anti-apoptotic role for MR (9, 10),  
79 confirming studies using a MR knockout mouse model, which showed degeneration of hippocampal  
80 granule cells in adulthood (11), while reduced neuronal death was observed in MR overexpressing  
81 transgenic mice after transient cerebral global ischemia (6).

82 A key step in the mineralocorticoid signaling is the MR expression level. Differential brain MR  
83 capacities correlating with distinct responses of the HPA axis in two rat strains initially pointed out to  
84 the importance of MR expression level as a determinant factor of its action (12). This was further  
85 exemplified by knockout or overexpressing MR animals models (5-8). Even though MR expression  
86 levels constitute an essential element governing mineralocorticoid signaling in target tissues, very little  
87 is known concerning the regulatory mechanisms controlling neuronal MR abundance. It is well  
88 established that MR expression is regulated by transcriptional and post-transcriptional mechanisms  
89 (1). At the level of transcription, we have previously identified two functional promoters in the human

90 MR gene, referred to as P1 and P2, corresponding to the 5'-flanking regions of the two first  
91 untranslated exons 1 $\alpha$  and 1 $\beta$  (13). Functional characterization revealed that the P1 had a stronger  
92 basal transcriptional activity than the P2 promoter. While both promoters were stimulated by  
93 glucocorticoids, only P2 was activated by aldosterone. Moreover, as demonstrated in transgenic  
94 animals, P1 promoter was active in all MR-expressing tissues including the brain, whereas P2  
95 promoter activity was weaker and appeared to be restricted during development (14).

96 To define the molecular mechanisms governing MR expression during neuronal differentiation, we  
97 have developed stably transfected recombinant murine embryonic stem (ES) cell lines, namely P1-  
98 GFP and P2-GFP, in which each promoter drives the expression of the Green Fluorescent Protein  
99 (GFP) used as a reporter gene. These models offer the possibility to investigate the regulation of MR  
100 expression and of the promoter activities during the early stages of neuronal differentiation and in  
101 mature neurons. We show that MR expression increases during neuronal differentiation which is  
102 associated with a parallel aldosterone-regulated control of P1 and P2 promoter activities.

103

## 104 **MATERIALS AND METHODS**

### 105 *P1- and P2-GFP Constructs*

106 P1- and P2-GFP constructs were obtained from the pGL3-Enhancer Vector (Promega,  
107 Charbonnières, France), in which the luciferase gene was replaced by the GFP cDNA from pEGFP  
108 plasmid (Ozyme, Saint Quentin en Yveline, France). The *HindIII-AvaII* fragment of the hMR gene,  
109 referred to as P1, was amplified from the pGL2-HA plasmid (13) by PCR with GL1 and GL2 primers.  
110 This amplicon was thereafter ligated into the *SmaI-HindIII* sites of pGL3-Enhancer-GFP vector. The  
111 *SspI-SspI* fragment was extracted from the H31-P2 plasmid (15) by *EcoRI* and *EcoRV* digestion and  
112 was inserted into the unique *SmaI* site of pGL3-Enhancer-GFP plasmid after filling in all recessive  
113 ends with Klenow (Ozyme) treatment and dephosphorylation of pGL3-Enhancer by the Shrimp  
114 Alkaline Phosphatase (Promega). Transgenes have been subsequently sequenced to verify their  
115 integrity (*Service de Génétique Moléculaire, Pharmacogénétique, Hormonologie, CHU Bicêtre,*  
116 *France*).

117 Other constructs using the same *HindIII-AvaII* fragment and the same *SspI-SspI* fragment of hMR  
118 gene have been generated. They were obtained from H31-P1 and H31-P2 plasmids (15, 16), in which  
119 we have inserted the entire coding sequence of the GFP.

120

### 121 ***Transfection Procedures***

122 For stable transfection, the 7241d embryonic stem (ES) cell line was used. This cell line has been  
123 derived in Cooney's laboratory (Baylor College of Medicine, Houston Texas), from a blastocyst  
124 obtained from the breeding of LRH +/- heterozygote mice (17) and was genotyped, phenotyped and  
125 characterized as a standard wild type ES cell line (Le Menuet and Cooney, unpublished data). The  
126 undifferentiated cells 7241d were seeded in 100 mm-Petri dishes one day before transfection. Five µg  
127 of P1- or P2-GFP constructs were co-transfected with 1 µg of pcDNA3 plasmid, containing the  
128 neomycin gene (Invitrogen, Cergy-Pontoise, France), using 15 µl Lipofectamine 2000 reagent  
129 (Invitrogen). Forty-eight hours post-transfection, selection was initiated with 400 µg/ml of G418  
130 (PAA, Les Mureaux, France). Neo-resistant clones were picked up after 5 days of G418 selection and  
131 propagated using the same medium. Two transfections, for each construct, were carried out, resulting  
132 in 11 neo-resistant clones having integrated the P1-GFP construct whereas 4 integrated the P2-GFP  
133 construct. In addition, the constructs obtained from H31-P1 and H31-P2 plasmids were stably  
134 transfected in undifferentiated ES cells.

135

### 136 ***Cell Culture***

137 Mouse ES cells were grown on 0.1% gelatin-coated plates (Sigma-Aldrich, Lyon, France) and on  
138 feeder cells (STO Neomycin LIF, kindly provided by Dr Alan Bradley, The Wellcome Trust Sanger  
139 Institute, UK) treated with 15 µg/ml mitomycin C (Sigma-Aldrich) for 4 hours. Cells were cultured at  
140 37°C in a humidified incubator gassed with 5% CO<sub>2</sub>.

141 ***Reagents*** - ES medium was composed of DMEM (PAA) containing 15% fetal calf serum (FCS  
142 specifically tested for ES culture (AbCys SA, Paris, France), 1X non-essential amino acids (PAA), 2  
143 mM glutamine (PAA), 100 U/ml penicillin (PAA), 100 µg/ml streptomycin (PAA), 20 mM Hepes  
144 (PAA) and 100 µM β-mercaptoethanol (Sigma-Aldrich). Embryoid Bodies (EB) medium was similar

145 to ES medium except that it contained 10% FCS without  $\beta$ -mercaptoethanol. Neuron medium was  
146 similar to EB medium but was supplemented with 5  $\mu\text{g/ml}$  insulin (Sigma-Aldrich), 5  $\mu\text{g/ml}$   
147 transferrin (Sigma-Aldrich), and 29 nM sodium selenate (Sigma-Aldrich).

148 ***Differentiation of ES cells into Neuronal-like cells*** - ES cells were cultured on feeder cells for two  
149 days after thawing. Subsequently, they were cultured without feeder cells in ES medium with  
150 Leukemia Inhibitory Factor (LIF) 1,000 U/ml (AbCys) and differentiation was started after 2 days. To  
151 induce EB formation, ES cells were detached, dissociated into single cells with 0.25% trypsin  
152 (Invitrogen) and then  $1.10^6$  ES cells were plated onto non-adherent bacterial Petri dishes (Greiner Bio-  
153 one SAS, Courtaboeuf, France) in ES medium without LIF. EB medium with 1  $\mu\text{M}$  all *trans*-retinoic  
154 acid (Sigma-Aldrich) was added after 2 days and was changed every other day. At day 7, EB were  
155 washed twice with PBS (PAA) and trypsinized for 10 min in a water bath at 37 °C. EB were then  
156 gently but thoroughly resuspended in 10 ml EB medium, then centrifuged for 3 min at 400 g at room  
157 temperature. The pellet was resuspended in neuron medium and the cell suspension was filtered  
158 through a 40- $\mu\text{m}$  nylon cell strainer (BD Biosciences, USA). The cell suspension was immediately  
159 seeded at a density of  $2.10^5$  cells per  $\text{cm}^2$ . Neuron medium was changed every 2 days.

160 ***Cell Treatment*** - After 24 h incubation in Dextran-Charcoal Coated (DCC) medium, aldosterone  
161 (Acros Organics, Halluin, France), dexamethasone or progesterone (Sigma-Aldrich), was added to the  
162 culture medium at day 13 of the neuronal differentiation. At day 14, total RNA was extracted by the  
163 Trizol reagent or cells were fixed.

164

#### 165 ***Alkaline Phosphatase Assay***

166 Cells were fixed for 2 min in 4% PBS-buffered paraformaldehyde (Sigma-Aldrich) and were rinsed  
167 once with PBS. During fixation, fresh staining solution was prepared by mixing Fast Red Violet,  
168 Naphtol AS-BI phosphate and water (2:1:1), according to the Quantitative Alkaline Phosphatase ES  
169 Characterization Kit protocol (Millipore, Saint Quentin en Yvelines, France). Fixed cells were  
170 incubated for 15 min with the staining solution at room temperature in the dark. Cells were rinsed with  
171 PBS and then photographed (microscope Motic AE21 and camera Olympus SP-51OUZ).

172

### 173 ***Immunocytochemistry***

174 Cells were fixed with methanol for 10 min, rinsed with PBS-Tween 20 and incubated with 4%  
175 horse immunoglobulins for 20 min, prior to overnight incubation at 4°C, with the primary monoclonal  
176 antibodies diluted as follows: anti-tubulin beta III TU-20 (1/50) (AbCys), anti-nestin 4D4 (5 µg/ml)  
177 (AbCys), anti-APC CC1 (1/20) (Calbiochem, Nottingham, UK), and anti-GFAP G-A-5 (1/200)  
178 (Sigma-Aldrich). After endogenous peroxidase quenching with 3% H<sub>2</sub>O<sub>2</sub> in PBS and washing,  
179 immunodetection was performed using the anti-mouse ImmPress reagent kit (Vector, Burlingame,  
180 CA) according to the manufacturer's instructions. Negative mouse ascites and pre-immune mouse  
181 immunoglobulins of the same sub-class were used as negative controls.

182

### 183 ***Confocal Immunofluorescence Microscopy***

184 Cells were fixed with methanol for 10 min, rinsed with PBS-Tween 20 and incubated with PBS  
185 5%-BSA 0.1% casein block for 20 min. Cells were then incubated overnight at 4°C, with anti-MR  
186 39N polyclonal antibody (4 µg/ml) followed by incubation with Alexa Fluor 555 goat anti-rabbit  
187 (1/1000) (Molecular Probes) for 1 h at room temperature. The cells were next rinsed in PBS, and  
188 incubated with the monoclonal antibodies anti-tubulin beta III TU-20 (1/100) (AbCys), or anti-nestin  
189 4D4 (5 µg/ml) (AbCys), or anti-GFAP G-A-5 (1/200) (Sigma-Aldrich), or anti-GFP (1/30) (AbCys)  
190 for 2 h at room temperature followed by washing and incubation with Alexa Fluor 488 goat anti-  
191 mouse (1/1000) (Molecular Probe) for 1 h at room temperature. After washing with PBS and mounting  
192 with Fluorescence Mounting Medium (Dako, Trappes, France), cells were analyzed and imaged by  
193 confocal fluorescence microscopy (Zeiss HAL confocal microscope). The anti-MR antibody (39N)  
194 was generated in rabbits immunized with the human MR 1-18 peptide and purified by affinity  
195 chromatography (Double X/XP boosting antibody production program, Eurogentec, Seraing,  
196 Belgium). The specificity of the anti-MR antibody was previously characterized (18,19).

197

### 198 ***DNA extraction***

199 DNA was extracted from cells with lysis buffer composed of 50 mM Tris pH8, 10 mM EDTA,  
200 10% SDS, 400 mM NaCl, and supplemented with proteinase K (Euromedex, Souffelweyrshheim,

201 France) 50 mg/ml, incubated overnight at 37°C, precipitated with isopropanol, rinsed with ethanol and  
202 dissolved in water.

203

#### 204 ***RT-PCR***

205 Total RNA was extracted with TRIZOL reagent (Invitrogen) according to the manufacturer's  
206 recommendations and RNA were processed for RT-PCR analysis. Briefly, 2 µg of total RNA was  
207 treated using the DNase I Amplification Grade procedure (Invitrogen). RNA was then reverse-  
208 transcribed with 200 units of reverse transcriptase using the Superscript™ II kit (Invitrogen)  
209 according to the manufacturer's recommendations using random hexamers (Promega). PCR were  
210 performed with a thermocycler (Stratagene, Paris, France) in a final volume of 25 µl, in 1X PCR  
211 buffer containing 1.5 mM MgCl<sub>2</sub>, 10 pmol of sense and antisense primers (see supplemental table),  
212 200 µM dNTP, 1 unit of *Taq* polymerase, and 2 µl of the reverse transcription reaction. The PCR  
213 cycles were as followed: 95 °C for 5 min, specific hybridization temperature for 1 min, 72 °C for 1  
214 min, during 1 cycle; 95 °C for 45 s, specific hybridization temperature for 45 s, 72 °C for 45 s, during  
215 30 cycles; 72 °C for 7 min, during 1 cycle. Amplicons were thereafter separated onto agarose gels and  
216 visualized under UV excitation.

217

#### 218 ***Quantitative Real Time PCR***

219 Gene expression was quantified by real time PCR. Total RNA was processed for real time PCR  
220 carried out on an ABI 7300 Sequence Detector (Applied Biosystems, Courtaboeuf, France). Briefly, 1  
221 µg of total RNA was treated using the DNase I Amplification Grade procedure (Invitrogen). RNA was  
222 then reverse-transcribed with 50 units of MultiScribe reverse transcriptase (Applied Biosystems).  
223 Samples were diluted 10-fold, and then 1/20 of the reverse transcription reaction was used for PCR  
224 using the Power SYBR® Green PCR master mix (Applied Biosystems). Final primer concentrations  
225 were 300 nM for each primer (see supplemental table). Reaction parameters were 50 °C for 2 min  
226 followed by 40 cycles at 95°C for 15 s, and 60 °C for 1 min. For preparation of standards, amplicons  
227 were purified from agarose gel and subcloned into pGEMT-easy plasmid (Promega), then sequenced  
228 to confirm the identity of each fragment. Standard curves were generated using serial dilutions of

229 linearized standard plasmids, spanning 6 orders of magnitude and yielding correlation coefficients  
230 >0.98 and efficiencies of at least 0.95, in all experiments. Standard and sample values were  
231 determined in duplicate from independent experiments. Relative expression within a given sample was  
232 calculated as the ratio: attomol of specific gene/femtomol of 18S. Results are mean  $\pm$  S.E.M and  
233 represent the relative expression compared with that obtained with control cells, which was arbitrary  
234 set at 1.

235

### 236 ***MR knockdown by siRNA***

237 Neurons were transiently transfected at day 12 with 100 nM siRNA (Eurogentec; sequences were  
238 reported in Supplemental Table), using Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM  
239 Reduced Serum Medium (Invitrogen) according to the manufacturer's recommendations. Six hours  
240 post-transfection, cells were incubated in DCC medium for 24 h followed by an additional 24h  
241 exposure to aldosterone. At day 14, total RNA was extracted and MR and GFP expression were  
242 measured by qPCR.

243

### 244 ***Statistical Analyses***

245 Results represent mean  $\pm$  SEM with at least 6 samples for each condition. Statistical analyses were  
246 performed using a non parametric Mann-Whitney test (Prism4, Graphpad Software, Inc., San Diego,  
247 CA).

248

## 249 **RESULTS**

### 250 ***Characterization of stably transfected embryonic stem cell lines P1-GFP and P2-GFP***

251 P1 and P2-GFP ES cell lines have been generated to examine the two hMR promoter activities  
252 during neuronal differentiation. As shown in Figure 1A, the proximal P1 promoter contained the 5'-  
253 flanking region (969 bp) of the exon 1 $\alpha$  and part of this first untranslated exon (239 bp). The P2  
254 promoter was composed of the 5'-flanking region (1682 bp) of the untranslated exon 1 $\beta$  and the first  
255 123 bp of this exon. These promoter regions were inserted into the pGL3-Enhancer Vector, which  
256 contains the SV40 enhancer, in order to enhance the basal activity of these promoters and thereby

257 facilitate detection of the reporter gene GFP. After stable transfection of murine ES cells, neomycin-  
258 resistant clones were isolated and genomic integration of the constructs was determined by PCR  
259 (Figure 1B). Several recombinant ES cell lines, namely P1-GFP and P2-GFP, were established. We  
260 demonstrated the expression of GFP mRNA and endogenous MR mRNA by RT-PCR, in 7 days EB,  
261 thus confirming the functionality of the constructs in these ES cell lines (Figure 1C). The GFP protein  
262 was expressed in these EB, as visualized by fluorescence microscopy (Figure 1D). Similar results were  
263 obtained using two other independent stably transfected cell lines P1 and P2-GFP, generated with  
264 identical constructs except that they were devoid of enhancer, thereby validating our strategy (see  
265 *Materials and Methods section*, data not shown). Altogether, these cell-based systems offer the  
266 possibility to study MR promoter activities as well as the regulation of MR expression during the first  
267 steps of neuronal differentiation and in mature neurons.

268

### 269 ***Neuronal differentiation***

270 The neuronal differentiation protocol used in the present study, essentially followed one previously  
271 described (20). Briefly, after withdrawal of LIF, ES-cells formed EB, which were incubated in non-  
272 adhesive bacterial dishes with retinoic acid (RA) for 5 days. EB were dissociated and incubated in the  
273 neuron specific medium, and after 3 days originated differentiated cells harbouring a neuron like  
274 phenotype. While a strong alkaline phosphatase activity was detected in undifferentiated ES cells, this  
275 activity dramatically decreased in RA-stimulated cells, thus confirming their commitment to neuronal  
276 differentiation (Figure 2A). After 5 days treatment with RA, the expression of the neuronal progenitor  
277 marker nestin and the mature neuronal marker Microtubule-Associated Protein-2 (MAP2) was already  
278 detected by quantitative real time PCR in EB. Incubation with neuron specific medium readily  
279 increased mRNA expression levels of these neuronal markers (Figure 2B-2C). The two other  
280 independent stably transfected cell lines P1 and P2-GFP, the parental line and the D3 ES cell line  
281 (ATTC number: CRL-11693), used as a wild type control, were differentiated into neurons according  
282 to the same protocol. No appreciable differences in the expression profile of neuronal markers were  
283 observed between the different cell lines. Therefore, the results represent the mean of all cell lines.  
284 Immunocytochemical studies, performed at the end of the neuronal differentiation procedure, showed

285 that most cells expressed the neuronal markers nestin and  $\beta$ tubulin III (another marker of mature  
286 neurons) and displayed elongated neuritic processes. Of note, few cells, clustered in some areas,  
287 expressed the astrocyte marker GFAP (Glial Fibrillary Acidic Protein) or the oligodendrocyte marker  
288 APC (Adenomatous Polyposis Coli) (Figure 2D). These results conclusively demonstrate that this  
289 neuronal differentiation protocol allows to generate, in a reproducible manner, cultures mainly  
290 composed of cells expressing immature and mature neuronal markers, but also comprising a minority  
291 of cells expressing astroglial markers.

292

### 293 ***Regulation of MR expression during neuronal differentiation***

294 To investigate the molecular mechanisms regulating MR expression, we first examined endogenous  
295 MR mRNA expression during neuronal differentiation by quantitative real-time PCR. We observed  
296 that MR expression rose approximately by 3-fold in EB and by 5-fold in mature neurons (Figure 3).  
297 As described above, a similar MR expression pattern was observed in all ES cell lines. Thus, we were  
298 able to define the temporal expression pattern of MR during ES differentiation and to demonstrate a  
299 progressive increase of MR abundance along with neuronal commitment. Double immunolabeling  
300 experiments using anti-MR (A) associated with anti-nestin (B) antibodies have been performed  
301 (Figure 4). The merged panel (C) shows the absence of MR and nestin colocalization, indicating that  
302 nestin-positive cells did not express the MR at a detectable level. On the contrary, double  
303 immunolabelling with anti- $\beta$ tubulin III revealed a specific MR and  $\beta$ tubulin III colocalization (D-E-F).  
304 GFAP-positive cells did not express the MR (G-H-I), similarly to the nestin-positive cells. For each  
305 experiment, the negative control was performed using pre-immune mouse immunoglobulins of the  
306 same sub-class or non reactive mouse ascites (data not shown). Of note, MR localization is nucleo-  
307 cytoplasmic under these experimental conditions. Altogether, these data suggest that MR is  
308 preferentially if not exclusively expressed in mature neurons.

309 We next examined the variation of P1 and P2 promoter activities during neuronal differentiation of  
310 P1 and P2-GFP ES cell lines. The relative GFP expression was measured by quantitative real-time  
311 PCR, and was considered as a direct index of promoter activity. Figure 5A shows a 7-fold increase in  
312 GFP transcripts in P1-GFP derived differentiated neurons compared to that measured in the

313 corresponding EB stage. Similarly, a 5-fold increase in GFP mRNA levels was observed in P2-GFP  
314 derived neurons, indicating that both P1 and P2 promoters were activated during neuronal  
315 differentiation. We also compared the relative GFP expression driven by either P1 or P2 promoter in  
316 undifferentiated ES cells as well as in neurons (Figure 5B). We thus demonstrated that the P1 activity  
317 was always higher than P2 activity by a 46-fold and a 6-fold factor in undifferentiated ES cells and in  
318 mature neurons, respectively. These findings were reproducibly obtained with the other recombinant  
319 ES cell lines. These results confirmed previous data obtained by transient transfection assays  
320 performed in fibroblast-like cells and in transgenic mice (13, 14) which indicated that the P1 promoter  
321 basal transcriptional activity is also stronger than that of P2 promoter, irrespective of the model  
322 studied. Double staining showed that GFP was present in P1-GFP MR-positive cells (Figure 5C),  
323 indicating that P1 promoter was transcriptionally active in neurons. In P2-GFP cells, GFP expression  
324 was most of the times below the immunodetection threshold, confirming that P2 activity is weaker  
325 than P1 activity. Altogether, our results clearly demonstrate that P1 and P2 promoters are both  
326 effective in driving MR expression throughout neuronal differentiation but with different strengths.

327

### 328 ***Hormonal regulation of P1 and P2 activity and MR expression in ES cell-derived neurons.***

329 Previously, *in vitro* characterization of the P1 and P2 promoters revealed that both promoters were  
330 activated by dexamethasone, *via* the glucocorticoid receptor (GR), whereas only P2 was activated by  
331 aldosterone, *via* MR (13). Moreover, it has been also demonstrated that progesterone, *via* the  
332 progesterone receptor (PR), increases MR mRNA levels in primary hippocampal neuron cultures (21).  
333 We thus decided to investigate the impact of these steroid hormones on MR expression in ES cell-  
334 derived neurons. First, we examined the expression pattern of GR and PR and demonstrated that their  
335 transcript levels also increased by 7-fold and 20-fold, respectively during neuronal differentiation  
336 (*Supplemental figure*). These results provide evidence that GR and PR are progressively and more  
337 intensively expressed during the first steps of neuronal differentiation, thus suggesting that they could  
338 represent key players in ES cell-derived mature neuron function.

339 We next exposed ES cell-derived neurons to aldosterone, dexamethasone or progesterone at day 13  
340 of differentiation. Steroid-induced modification of P1 and P2 activity and MR mRNA expression were

341 determined after 24 h treatment using quantitative real-time PCR. As shown in Figure 6A, 100 nM  
342 aldosterone increased GFP mRNA expression in P1-GFP neurons by 1.4-fold, whereas dexamethasone  
343 and progesterone failed to exert any significant effect. Dose-dependent curves demonstrated that the  
344 P1 promoter was activated only at the highest aldosterone concentration (Figure 6B). On the other  
345 hand, the P2 promoter was highly sensitive to aldosterone action since GFP mRNA levels already  
346 increased by a 1.5-fold factor in P2-GFP neurons at 1 nM aldosterone concentration (Figure 6C and  
347 6D). Similarly, neuronal MR expression was significantly enhanced in the presence of 100 nM  
348 aldosterone, following the evolution of aldosterone-induced P1 promoter activity (Figure 6E and 6F).  
349 In sharp contrast, dexamethasone and progesterone had no significant effect on GFP and MR mRNA  
350 levels in these ES cell-derived neurons, suggesting that these steroid hormones did not regulate MR  
351 expression in neurons. In order to examine the involvement of MR in the regulation of its own  
352 promoters' activity, we decided to knockdown MR expression by a siRNA strategy with two unrelated  
353 MR-specific siRNA in the P2-GFP ES-derived neurons since they exhibited the highest sensitivity to  
354 aldosterone action. As shown in Figure 7, compared with scrambled siRNA, a decrease in MR mRNA  
355 expression was obtained by each of two MR siRNA (Figure 7A) and was accompanied by the  
356 disappearance of aldosterone-stimulated GFP expression (Figure 7B). Altogether, these findings  
357 provide support for the involvement of MR, but not of GR, in the activation of both P1 and P2  
358 promoters, pointing to the implication of the mineralocorticoid signaling pathway in the regulatory  
359 mechanisms governing MR expression in mature neurons.

360

## 361 **DISCUSSION**

362 In the present study, we have established a novel ES cell model of neuronal differentiation to assess  
363 the molecular mechanisms involved in MR expression using stably transfected P1-GFP and P2-GFP  
364 ES cell lines. We found that MR expression gradually increases during neuronal differentiation and  
365 demonstrated that MR is preferentially, if not exclusively, expressed in mature neurons. This is  
366 concomitant with the increase of GR and PR expression, underlying the potential importance of these  
367 steroid receptors during neurogenesis and differentiation. We showed that both P1 and P2 promoter  
368 drive neuronal MR expression, the former being always stronger than the latter. Finally, we provided

369 evidence that aldosterone treatment significantly induces both MR expression and P1 and P2 activity  
370 in mature neurons. These findings suggest the involvement of MR activation in the stimulation of P1  
371 and P2 activities and on MR expression in mature neurons, a hypothesis supported by MR knockdown  
372 experiments. However, the precise molecular mechanisms responsible for the aldosterone-induced MR  
373 expression, and how P1 and P2 promoters are specifically activated by aldosterone remain to be  
374 determined.

375 The present work constitutes a detailed study characterizing MR expression during neuronal ES  
376 cell differentiation. Indeed, the ES cell model has been shown to be quite relevant in the neuronal field  
377 and widely used to decipher the activity or function of genes of interest and to better understand the  
378 pathophysiological mechanisms involved in some neurodegenerative diseases (22). A previous initial  
379 report demonstrated an increase of MR mRNA expression associated with a decrease of GR and PR  
380 expression during the first six days of spontaneous differentiation of human and murine ES cells (23).  
381 We provide evidence that the level of steroid receptors including MR, GR and PR increases during  
382 neuron commitment of murine ES cells in accordance with the increase of PR content demonstrated  
383 during differentiation of dopamine neurons derived from mouse ES cells (24).

384 It is well established that MR is expressed in the limbic system most notably in the hippocampus  
385 (25). Within the hippocampus, the highest density occurs in pyramidal cells of the CA1 region and in  
386 granule neurons of the dentate gyrus but in the adult hippocampus MR is not expressed in the neuronal  
387 stem cells (26). As opposed to GR, MR seems to be specifically expressed in mature neurons and is  
388 absent in glial cells (26), consistent with our findings which reveal that the majority of ES-derived  
389 mature neurons are MR-positive. In contrast, MR does not colocalize with GFAP-positive cells even  
390 though an increase of MR-expressing astrocytes was been reported in the striatum after ischemic  
391 conditions (27).

392 The question on the precise subcellular localization of neuronal MR is still debated, owing to the  
393 dual possibility of the classical nuclear tropism of MR acting as a hormone-dependent transcription  
394 factor (28, 29) and of the putative membrane MR mediating rapid, nongenomic effects *via* pre- as well  
395 as postsynaptic pathways (30). To further investigate this subcellular trafficking, we used ES-  
396 differentiated mature neurons which were submitted to aldosterone or glucocorticoid exposure. Under

397 basal conditions, MR mostly resided in the cytoplasm of neurons with no obvious nuclear  
398 translocation after aldosterone treatment, supporting the possibility of a membrane-bound MR. In  
399 contrast, dexamethasone or corticosterone induced a clear nucleocytoplasmic redistribution (data not  
400 shown). Given the coexpression with neuronal GR, we formulate the hypothesis that GR plays an  
401 indispensable role in MR nuclear trafficking, via GR-MR heterocomplexes, in agreement with a recent  
402 study demonstrating the importance of the presence of GR in mediating MR-dependant transcription  
403 (31).

404 Another important issue, given the potential neuroprotective effect of MR (32, 33) is a better  
405 understanding of the regulatory mechanisms that control neuronal MR expression. Beside  
406 transcriptional control, the promoter activity during neuronal differentiation could also be regulated by  
407 epigenetic mechanisms. Indeed, some transcription factors (Oct4, Nanog, and Sox2) or histone  
408 modification and DNA methylation are coordinated to control the expression of pluripotent versus  
409 developmental genes in ES cells and during *in vitro* differentiation. A recent study shows that the  
410 promoters of a third of the genes expressed in undifferentiated ES cells are methylated and that these  
411 genes are implicated in development (34). Along this line, *in silico* analysis of the P2 promoter  
412 sequence revealed the presence of several GC-rich regions likely to be methylated, which could  
413 account for its weak activity. In a recent study, pyrosequencing of two CpG islands within rat MR  
414 gene promoter demonstrated a correlation between methylation and MR expression (35). Weaver et al  
415 showed that the methylation of CpG on the transcription factor NGFI-A (Nerve Growth Factor-  
416 Inductible protein A) response elements located in the GR promoter resulted in a transcriptional  
417 repression in hippocampal neurons (36). Interestingly, P2 promoter exhibited two NGFI-A response  
418 elements at position -834 bp and -857 bp, which could explain the modulation of this promoter  
419 activity observed during ES cells neuronal differentiation. Recently, a novel role for the  $\beta$  promoter of  
420 rat MR gene was defined (37). This promoter has a weak activity under basal conditions, but its  
421 activity increases under hypothermic anoxia in neonatal rat hippocampus. Given that the rat  $\beta$   
422 promoter is the homologous of the human P2 promoter gene, this finding suggests a potential role for  
423 this otherwise weak promoter and opens interesting pathophysiological perspectives.

424 It has been proposed that neuronal MR expression is subjected to a tight regulatory control by  
425 different stimuli. For instance, MR expression increases in primary hippocampus neuron cultures after  
426 serotonin exposure (38) or after antidepressant treatment in adrenalectomized male mice (39).  
427 Dexamethasone and progesterone also increase rat MR gene transcription in primary hippocampal  
428 neurons (21, 40). Using ES-derived mature neurons, we demonstrated that aldosterone but not  
429 glucocorticoid or progesterone significantly increases both neuronal MR expression as well as P1 and  
430 P2 activity. Moreover, we showed that down-regulation of MR expression by siRNA in ES-derived  
431 neurons leads to a disappearance of aldosterone-stimulated GFP expression, providing evidence that  
432 MR is at least in part implicated in the regulation of its own promoter activity. This does not exclude  
433 that other mechanisms as recently proposed (41) might be involved in the aldosterone-regulated P1  
434 and P2 promoter activities. However, the mechanisms by which aldosterone-activated MR controls  
435 MR promoter activity and whether this occurs through direct or indirect interaction with others  
436 transcription factors remain to be established. Collectively, these findings suggest a pivotal role of  
437 neuronal MR abundance and of its subcellular localization which are likely important players involved  
438 in the complex developmental regulatory pattern (42, 43), the corticosteroid-regulated synaptic  
439 plasticity (30) and neuronal excitability (4). The importance of MR function in the brain is largely  
440 demonstrated by animal models of MR overexpression or inactivation. No major morphological  
441 alteration of the CNS was detected in adult MR<sup>-/-</sup> mice, suggesting that MR is mainly involved in the  
442 maintenance of cellular and structural integrity of neurons rather than in CNS development itself. This  
443 is in accordance with the neuroprotective and antiapoptotic role proposed for MR (10, 32). Taken  
444 together, these experimental data emphasize the physiological and pathophysiological importance of  
445 MR in the limbic brain. In this context, neuronal differentiation of ES cell lines proves to be a useful  
446 model to investigate novel signaling pathways involved in modulation of neuronal MR expression,  
447 opening some potential therapeutic perspectives in neurological and psychiatric diseases.

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456

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## 589 **FIGURE LEGENDS**

### 592 **Figure 1: Generation of hMR promoter-GFP expressing stable ES cell lines**

593 **A)** Schematic representation of the two P1-GFP and P2-GFP constructs. The 5'-flanking regions of  
594 hMR gene contains the two first untranslated exons 1 $\alpha$  and 1 $\beta$  (*solid boxes*). The *Hind III-Ava II* (-969,  
595 +239) P1 fragment and *SspI-SspI* (-1682, +123) P2 fragment were used to generate P1-GFP and P2-  
596 GFP plasmids, respectively. The GFP (*hatched box*) was used as a reporter gene. As described in the  
597 "Materials and Methods" section the pGL3-Enhancer Vector contains the SV40 Enhancer (*black box*).

598 **B)** PCR genotyping of undifferentiated resistant cell lines (numbered 1 to 7). Rapsn was used as  
599 internal genomic control. **C)** RT-PCR analyses of GFP, MR,  $\beta$ -actin (used as internal control) mRNA

600 expression in 7 day embryoid bodies (from  $1.10^6$  cells in suspension in 15% FCS supplemented  
601 medium). **D)** GFP protein expression was observed in 7 day embryoid bodies (from  $1.10^6$  cells in  
602 suspension in 15% FCS supplemented medium). Original magnification: x20

603

#### 604 **Figure 2: Neuronal differentiation of ES cell lines**

605 **A)** Alkaline Phosphatase activity of ES cells and neurons. Alkaline phosphatase activity was measured  
606 using the colorimetric Alkaline Phosphatase Detection assay kit. Original magnification: x10 **B, C)**  
607 Relative Nestin and MAP2 mRNA expression levels were determined using qPCR at different stages  
608 of differentiation: undifferentiated ES cells, embryoid bodies (EB) and neurons. Results are means  $\pm$   
609 SEM of six independent experiments of three samples performed in duplicate for each developmental  
610 stage, and represent the relative expression compared with basal levels of ES (arbitrarily set at 1). \*\*\*  
611  $P < 0.001$ . Mann Whitney test. Relative mRNA expression is normalized to 18S rRNA expression (see  
612 Materials and Methods section). **D)** Immunocytochemical detection of Nestin,  $\beta$ -tubulin III, GFAP and  
613 APC in neurons. Original magnification: x40.

614

#### 615 **Figure 3: MR expression during neuronal differentiation**

616 Relative MR mRNA expression levels were determined using qPCR at different stages of  
617 differentiation: undifferentiated ES cells, embryoid bodies (EB) and neurons. Results are means  $\pm$   
618 SEM of six independent experiments of three samples performed in duplicate for each developmental  
619 stage and represent the relative expression compared with basal levels of ES (arbitrarily set at 1). \*\*\*  
620  $P < 0.001$ . Mann Whitney test. Relative mRNA expression is normalized to 18S rRNA expression (see  
621 Materials and Methods section).

622

#### 623 **Figure 4: MR is exclusively expressed in neurons**

624 Double immunolabelling analyses of neurons with antibodies against MR (red) (*left panel*), Nestin  
625 (green),  $\beta$ -tubulin III (green), and GFAP (green) (*middle panel*), and merge (*right panel*) . Original  
626 magnification: x40.

627

628 **Figure 5: Comparison of P1 and P2 activity during neuronal differentiation**

629 **A)** Relative GFP mRNA expression levels, which represent P1 and P2 activity, were determined using  
 630 qPCR at different stages of differentiation: embryoid bodies (EB) and neurons. Results are means  $\pm$   
 631 SEM of at least four independent experiments of three samples performed in duplicate and represent  
 632 the relative expression compared with basal levels in embryoid bodies (arbitrarily set at 1). \*\*  $P < 0.01$ .  
 633 Mann Whitney test. Relative mRNA expression is normalized to 18S rRNA expression (see Materials  
 634 and Methods section). **B)** Relative GFP mRNA expression levels were determined using qPCR at  
 635 different stages of differentiation: ES cells and neurons. Results are means  $\pm$  SEM of at least four  
 636 independent experiments of three samples performed in duplicate and represent the relative P2 activity  
 637 compared with basal levels of P1 activity (arbitrarily set at 1). \*\*  $P < 0.01$ . Mann Whitney test. Relative  
 638 mRNA expression is normalized to 18S rRNA expression (see Materials and Methods section). **C)**  
 639 Double immunolabelling analyses of neurons with antibodies against MR (red) (*left panel*), and GFP  
 640 (green) (*middle panel*), and merge (*right panel*). Original magnification: x40.

641  
 642 **Figure 6: Hormonal regulation of P1 and P2 activity and MR expression.**

643 Neurons were exposed to 100 nM aldosterone (ALDO), dexamethasone (DXM) or progesterone  
 644 (PROG) (**A,C,E**) or to increasing concentrations of aldosterone (1 to 100 nM) (**B,D,F**).  
 645 **A, B, C, D)** Relative GFP mRNA expression levels were determined using qPCR. Results are means  $\pm$   
 646 SEM of four independent experiments of six samples performed in duplicate and represent the relative  
 647 expression compared with basal levels of control untreated cells (C) (arbitrarily set at 1). \*\*\*  $P < 0.001$   
 648 \*\*  $P < 0.01$  \*  $P < 0.05$ . Mann Whitney test. Relative mRNA expression is normalized to 18S rRNA  
 649 expression (see Materials and Methods section). **E, F)** Relative MR mRNA expression levels were  
 650 determined using qPCR. Results are means  $\pm$  SEM of four independent experiments of six samples  
 651 performed in duplicate and represent the relative expression compared with basal levels of control  
 652 untreated cells (C) (arbitrarily set at 1). \*\*\*  $P < 0.001$ . Mann Whitney test. Relative mRNA expression  
 653 is normalized to 18S rRNA expression (see Materials and Methods section).

654

655

656 **Figure 7: MR down-regulation inhibits aldosterone-induced P2 promoter activity.**

657 Neurons were transfected with either the control scrambled siRNA (scr MR) or by two unrelated MR  
658 siRNA (si1 MR, si2 MR). After transfection, neurons were incubated or not with 100 nM aldosterone  
659 (ALDO).

660 **A)** Relative MR mRNA expression levels were determined using qPCR. Results are means  $\pm$  SEM of  
661 six samples performed in duplicate and represent the relative expression compared with basal levels of  
662 control scrambled siRNA transfected (scr MR) (arbitrarily set at 1). \*\* P<0.01. Mann Whitney test.  
663 Relative mRNA expression is normalized to 18S rRNA expression (see Materials and Methods  
664 section).

665 **B)** Relative GFP mRNA expression levels were determined using qPCR. Results are means  $\pm$  SEM of  
666 six samples performed in duplicate and represent the relative expression compared with basal levels of  
667 control untreated cells (C) (arbitrarily set at 1). \*\*\* P<0.001 \*\* P<0.01. Mann Whitney test. Relative  
668 mRNA expression is normalized to 18S rRNA expression (see Materials and Methods section).

669