

1 **Mineralocorticoid receptor overexpression facilitates differentiation and**  
2 **promotes survival of embryonic stem cell-derived neurons**

3 Abbreviated title: Mineralocorticoid receptor as neuroprotective factor

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24

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26

27

28 **Abbreviations:** EB, embryoid bodies; ES, embryonic stem (cell); hMR, human mineralocorticoid  
29 receptor; GC, glucocorticoid; GR, glucocorticoid receptor; MAP2, microtubule associated protein 2;  
30 MR, mineralocorticoid receptor; PCNA, proliferating cell nuclear antigen; *t*-BHP, *tert*-  
31 butylhydroperoxide; WT, wild-type.

32

33 **Abstract**

34 Mineralocorticoid receptor (MR), highly expressed in the hippocampus, binds corticosteroid hormones  
35 and coordinately participates, with the glucocorticoid receptor (GR), to the control of stress responses,  
36 memorization and behavior. To investigate the impact of MR in neuronal survival, we generated  
37 murine embryonic stem (ES) cells that overexpress human MR (P1-hMR) and are induced to  
38 differentiate into mature neurons. We showed that recombinant MR expression increased throughout  
39 differentiation and is 2-fold higher in P1-hMR ES-derived neurons compared to wild type (WT)  
40 controls while GR expression was unaffected. Although proliferation and early neuronal  
41 differentiation were comparable in P1-hMR and WT ES cells, MR overexpression was associated with  
42 higher late neuronal marker expression (MAP2,  $\beta$ -tubulin III). This was accompanied by a shift  
43 towards neuron survival with an increased ratio of anti- vs pro-apoptotic molecules and 50% decreased  
44 caspase 3 activity. Knocking down MR overexpression by small interfering RNAs drastically reversed  
45 neuroprotective effects with reduced Bcl<sub>2</sub>/Bax ratio and decreased MAP2 expression. P1-hMR  
46 neurons were protected against oxidative stress-induced apoptosis through reduced caspase 3  
47 activation and drastically increased Bcl<sub>2</sub>/Bax ratio and  $\beta$ -tubulin III expression. We demonstrated the  
48 involvement of MR in neuronal differentiation and survival and identify MR as an important  
49 neuroprotective mediator opening potential pharmacological strategies.

50

51

## 52 **Introduction**

53 The mineralocorticoid receptor (MR), a ligand-dependent transcription factor, is highly expressed in  
54 the brain, notably in the hippocampus, where it is physiologically occupied and activated by  
55 glucocorticoid hormones (GC) (1). MR plays an important role in the neuroendocrine and behavioral  
56 responses to stress and in establishing cognitive functions (2). The classical nuclear MR is involved in  
57 the stability and integrity of neuronal networks (3). However, recent evidences suggest that rapid  
58 effects of GC depend on a membrane-located MR that modulates neuronal excitability (4-5). The  
59 central actions exerted by GC are also mediated by the lower affinity glucocorticoid receptor (GR).  
60 Thus, the MR/GR balance is of crucial importance to normalize brain activity and to regulate  
61 hippocampal plasticity (6).

62 Several pharmacological studies and analyses of mouse models have shown that MR activation, in  
63 contrast to GR activation, is required for neuronal survival in the hippocampus (7-9). While  
64 stimulation of anti-apoptotic pathways by MR may partially explain its neuroprotective role (10-11), a  
65 rapid increase of MR expression following neuronal injury was reported (12), thus establishing a  
66 positive relationship between MR expression and neuroprotection. We have recently demonstrated that  
67 MR expression via transcriptional activation of its two promoters increase during neuronal  
68 differentiation of murine embryonic stem (ES) cells (13). However, the mechanisms by which MR  
69 promotes neuronal differentiation and maintains neuron survival remain unclear.

70 The hippocampus is a major site of neurogenesis in adulthood. Specific MR activation enhances  
71 neonatal neurogenesis (14) thus promoting cognitive processes. Forebrain MR over-expression  
72 improves memory processes in mice (9), while MR knockout animals exhibit impaired learning  
73 abilities (15). Moreover, hippocampal neurons greatly decrease with age (16) in parallel with the  
74 hippocampal MR expression (17), indicating that reduced MR expression is associated with neuronal  
75 dysfunction in the hippocampus of older individuals.

76 To investigate the impact of MR on neuronal survival and/or differentiation and better elucidate the  
77 molecular mechanisms involved, we exploited an ES cell model that could be committed to neuronal  
78 differentiation (13) and compared wild-type and hMR over-expressing ES cell lines derived from mice

79 overexpressing hMR (18-19). These cell-based systems offer a unique opportunity to examine the  
80 functional consequences of MR over-expression on the regulation of the apoptosis signaling pathway  
81 during neuronal differentiation and in mature neurons. We showed that MR over-expression increases  
82 expression of the late neuronal markers that in turn, is associated with an increase in the ratio between  
83 anti- and pro-apoptotic molecules, providing direct evidence for an anti-apoptotic impact of neuronal  
84 MR.

85

## 86 **Materials and Methods**

### 87 **Cell Culture**

88 A murine hMR-overexpressing ES cell line, in which the P1 promoter drives hMR cDNA expression,  
89 was derived as described (19). The wild-type D3 ES cell line (ATCC no. CRL-11693) and the hMR  
90 ES cells were grown on 0.1% gelatin-coated plates (Sigma-Aldrich, Lyon, France) and on feeder cells  
91 (STO Neomycin LIF, kindly provided by Dr Alan Bradley, The Wellcome Trust Sanger Institute, UK)  
92 pretreated with 15 µg/ml mitomycin C (Sigma-Aldrich) for 4 h. Cells were cultured at 37°C in a  
93 humidified incubator in presence of 5% CO<sub>2</sub>.

94 *Reagents* - ES medium was composed of DMEM (PAA, Les Mureaux, France) containing 15% fetal  
95 calf serum (FCS specifically tested for ES culture (AbCys SA, Paris, France), 1X non-essential amino  
96 acids (PAA), 2 mM glutamine (PAA), 100 U/ml penicillin (PAA), 100 µg/ml streptomycin (PAA), 20  
97 mM HEPES (PAA) and 100 µM β-mercaptoethanol (Sigma-Aldrich). Embryoid Bodies (EB) medium  
98 had a similar composition but contained 10% FCS without β-mercaptoethanol. Cortisol and  
99 aldosterone concentrations in the serum batch used for all experiments were measured at 30.25 nM  
100 and 41 pM , respectively. Neuron medium was similar to EB medium but was supplemented with 5  
101 µg/ml insulin (Sigma-Aldrich), 5 µg/ml transferrin (Sigma-Aldrich), and 29 nM sodium selenate  
102 (Sigma-Aldrich).

103 *Differentiation of ES cells into Neuronal-like cells* – Neuronal differentiation was induced in ES  
104 medium containing 15% FCS with retinoic acid (RA), as previously described, via embryoid bodies  
105 (EB) formation (13). Of note, we were unable to achieve neuronal differentiation of ES cells when  
106 cultivated during two weeks with medium containing Dextran-Charcoal Coated (DCC) serum.  
107 Briefly, ES cells formed EB when exposed to 10<sup>-6</sup> M Retinoic acid (Sigma-Aldrich) for 5 days in non-  
108 adhesive bacterial dishes. At day 7, EB were dissociated and incubated in neuron medium until day 14  
109 in adherence in tissue culture dishes. Cells were washed in PBS and froze before RNA or protein  
110 extraction.

111 *Cell Treatment* – For hormonal treatment, after 24 h incubation in DCC medium, aldosterone (Acros  
112 Organics, Halluin, France), or corticosterone (Sigma-Aldrich), and/or RU486 (Mifepristone) (Sigma-  
113 Aldrich) were added to the culture medium at day 13 of the neuronal differentiation. After 6 h, total

114 RNA was extracted with Trizol and gene expression was measured by quantitative real-time PCR. For  
115 apoptosis induction, cells were treated at day 14 with 400  $\mu$ M *tert*-butylhydroperoxide (*t*-BHP)  
116 (Sigma-Aldrich) for 3 h in neuron medium containing 10% FCS. Successively, proteins were extracted  
117 and quantified by Western blot.

118

### 119 **Flow cytometry**

120 Cells were fixed and permeabilized using the Foxp3 Staining Buffer Set (eBioscience). Cells were  
121 then stained with anti-Ki67 antibody or with isotype control (BD Bioscience) for 30 min on ice. Flow  
122 cytometry was performed with a FACSCanto cytometer (BD Biosciences) and data files were  
123 analyzed using FlowJo software (Tree Star Inc.).

124

### 125 **Quantitative Real Time PCR**

126 Gene expression was quantified by real time PCR. Total RNA was processed for real time PCR on an  
127 ABI 7300 Sequence Detector (Applied Biosystems, Courtaboeuf, France). Briefly, 1  $\mu$ g of total RNA  
128 was treated using the DNase I Amplification Grade procedure (Invitrogen). RNA was then reverse-  
129 transcribed with 50 U MultiScribe reverse transcriptase (Applied Biosystems). After 10-fold dilution,  
130 1/20th of the reverse transcription reaction was used for PCR using the Fast SYBR® Green PCR  
131 master mix (Applied Biosystems). Final primer concentrations were 300 nM for each primer (see  
132 Supplemental Table 1 for sequences). Reaction parameters were 50 °C for 2 min followed by 40  
133 cycles at 95°C for 15 s, and 60 °C for 1 min. For standard preparation amplicons were purified from  
134 agarose gel and subcloned into pGEMT-easy plasmid (Promega), then sequenced to confirm the  
135 identity of each fragment. Standard curves were generated using serial dilutions of linearized standard  
136 plasmids, spanning 6 orders of magnitude and yielding correlation coefficients >0.98 and efficiencies  
137 of at least 0.95, in all experiments. Standard and sample values were determined in duplicate from  
138 independent experiments. Relative expression within a given sample was calculated as the ratio:  
139 attomol of specific gene/femtomol of 18S. Results are mean  $\pm$  S.E.M and represent the relative  
140 expression compared with that obtained with control cells, which was arbitrary set at 1.

141

142 **Western blot**

143 Total protein extracts were prepared from ES cells and neuron cultures. Cells were lysed, in lysis  
144 buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 30 mM Na pyrophosphate, 50 mM Na  
145 fluoride, 1% Triton X100, 1X protease inhibitor from Sigma) on ice. Immunoblots were incubated 1 h  
146 at room temperature in 5% fat free milk-Tris buffer saline – 0.1% Tween 20 (TBS-T) before overnight  
147 incubation at 4°C with one of the following antibodies: rabbit anti-MR 39N (1/1,000), mouse anti-β-  
148 tubulin III TU-20 (1/1,000) (Millipore, Molsheim, France), rabbit anti-Bcl<sub>2</sub> (1/500) (Ozyme, Saint-  
149 Quentin-en-Yvelines, France), mouse anti-PCNA (1/1,000) (Dako, Trappes, France), rabbit anti-  
150 caspase 3 (1/1,000) (Ozyme), rabbit anti-Bax (1/15,000) (Ozyme) and mouse anti-GR (clone FIGR,  
151 Millipore, 1/500). After extensive washing, membranes were incubated for 30 min at room  
152 temperature with peroxidase-conjugated goat anti-rabbit (1/15,000) or anti-mouse (1/15,000)  
153 secondary antibodies (Vector Laboratory, Burlingame, CA). After washing, the antigen-antibody  
154 complex was visualized by the ECL<sup>+</sup> detection kit (GE Healthcare Europe, Orsay, France). For loading  
155 normalization, membranes were incubated with rabbit anti-GAPDH (1/5,000) (Sigma-Aldrich) or  
156 mouse anti-α-tubulin (1/10,000) (Sigma-Aldrich). Signal intensities were quantified with QuantityOne  
157 software (Bio-Rad, Marnes-la-Coquette, France). Alternatively, the Odyssey imaging system (LI-COR  
158 Biosciences, Bad Homburg, Germany) was used for quantification with IRDye© 800CW or 680LT  
159 near-infrared fluorescent secondary antibodies.

160

161 **Confocal Immunofluorescence Microscopy**

162 Cells grown on sterile coverslips were fixed with methanol for 10 min, rinsed with PBS-0.1% Tween  
163 20 and incubated with a PBS, 5% BSA, 0.1% casein block for 20 min followed by overnight  
164 incubation at 4°C with the anti-MR 39N polyclonal antibody (4 µg/ml) then with Alexa Fluor 555 goat  
165 anti-rabbit (1/1,000) (Molecular Probes) for 1 h at room temperature. The cells were next rinsed in  
166 PBS, and incubated with the anti-β-tubulin III TU-20 monoclonal antibody (1/100) (AbCys) for 2 h at  
167 room temperature followed by washing and incubation with Alexa Fluor 488 goat anti-mouse antibody  
168 (1/1,000) (Molecular Probe) for 1 h at room temperature. The coverslips were then mounted with



169 Fluorescence Mounting Medium (Dako), before analysis and imaging by confocal fluorescence  
170 microscopy (Zeiss HAL confocal microscope).

171

### 172 **MR knockdown by siRNA**

173 Neurons were transiently transfected at day 11 with 100 nM siRNA (Invitrogen; see Supplemental  
174 Table 1 for sequences), using Lipofectamine RNAiMAX (Invitrogen) in Opti-MEM Reduced Serum  
175 Medium (Invitrogen) according to the manufacturer's recommendations. Six hours post-transfection,  
176 cells were incubated in neuron medium for 48 h. At day 14, total RNA were extracted and gene  
177 expression was measured by qPCR.

178

### 179 **Statistical Analyses**

180 Results represent mean  $\pm$  SEM of at least 6 samples for each condition unless stated otherwise.  
181 Statistical analyses were performed using a non parametric Mann-Whitney test (Prism4, Graphpad  
182 Software, Inc., San Diego, CA).

183

184

185 **Results**

186 **MR over-expression during neuronal differentiation of ES cells**

187 The hMR over-expressing ES cell line was established from transgenic P1-hMR mouse blastocysts  
188 (19). The transgenic mice were generated using 1.2 kb of the human proximal MR promoter, named  
189 P1, to drive hMR cDNA expression (18). To investigate the impact of MR over-expression during  
190 neuronal differentiation, we first examined the expression of hMR transgene mRNA in the  
191 recombinant ES cells by quantitative real-time PCR and showed that hMR transcript levels rose  
192 approximately 3.5-fold in mature neurons compared to undifferentiated ES cells (Fig. 1A). We next  
193 analyzed MR protein expression during neuronal differentiation in transgenic ES cells (P1-hMR)  
194 compared with wild-type (WT) using an anti-MR antibody recognizing both the endogenous murine  
195 MR and recombinant human MR (20). Western blot analyses revealed an approximately 1.6-fold  
196 increase of MR expression in the P1-hMR ES cells compared to WT ES cells and 1.7-fold increase in  
197 the P1-hMR neurons compared to WT neurons (Fig. 1B). In parallel, we showed that while  
198 endogenous mMR mRNA expression remains identical in undifferentiated P1-hMR and WT ES cells,  
199 differentiated neurons of both genotypes under the same experimental conditions exhibit a 3-fold  
200 increase in mMR transcripts without significant difference between transgenic and WT ES cell lines  
201 (Fig. 1C). Similarly, the presence of the transgene did not modify the expression of the closely related  
202 glucocorticoid receptor (GR) both at the mRNA and protein levels as measured by real-time qPCR  
203 during neuronal differentiation and western blot at d14 (Fig. 1D and E). This indicated that hMR  
204 overexpression does not affect endogenous corticosteroid receptor abundance in mature neurons.  
205 Double-immunolabeling experiments with the anti-MR and anti- $\beta$ -tubulin III antibodies clearly reveal  
206 a colocalization of MR and  $\beta$ -tubulin III (Merge panel Fig. 1F) showing that MR is almost exclusively  
207 expressed in mature,  $\beta$ -tubulin III-positive neurons. Altogether, these results demonstrate that ES cell-  
208 derived neurons provide an effective cell-based system to investigate the functional consequences of  
209 hMR over-expression during neuronal differentiation.

210

211 **Impact of MR over-expression on neuronal differentiation**

212 In order to examine the impact of MR over-expression, transgenic and WT ES cells were  
213 differentiated into neurons, and the variations of the expression levels of several specific neuronal  
214 markers were analyzed by quantitative real-time PCR. Under our experimental conditions where the  
215 ligand-dependent transcription factor MR was activated by corticosteroid hormones present in the  
216 serum containing medium, the expression profile of the neuronal progenitor marker nestin was similar  
217 in the ES cell lines of both genotypes during neuronal differentiation (Fig. 2A), suggesting that MR  
218 over-expression does not affect early neuronal commitment. Besides, the expression of the mature  
219 neuronal marker Microtubule-Associated Protein 2 (MAP2) was very low in undifferentiated ES cells  
220 but readily increased, as expected, in mature neurons. We performed neuronal differentiation of  
221 another WT ES cell line (19), assessing the expression of two late neuronal markers MAP2 and  
222 synaptophysin compared to the WT D3 ES cell line and did not find any significant difference (see  
223 supplemental Fig. S1). Interestingly, we showed that the MAP2 mRNA level was 4.5-fold higher in  
224 P1-hMR neurons than in WT controls (Fig. 2B). In addition, western blot analysis showed a 1.7-fold  
225 increase of another late neuronal marker  $\beta$ -tubulin III in P1-hMR neurons compared to WT neurons  
226 (Fig. 2C). Several hypotheses could account for these observations: MR over-expression might  
227 facilitate the differentiation of precursors into neuronal lineage and could promote the growth of  
228 mature neurons. An alternative and not mutually exclusive hypothesis is that MR-over-expression is  
229 associated with an increased survival of newly differentiated neurons.

230

### 231 **MR over-expression favors the increased survival of neurons**

232 The increased expression of late neuronal markers reflects an increase of neuronal proliferation or  
233 survival. We thus examined by Western blot the expression of the proliferation marker, PCNA  
234 (Proliferating Cell Nuclear Antigen) in neurons and did not detect any significant difference between  
235 WT or P1-hMR neurons (Fig. 3A). This result was confirmed by Fluorescence Activated Cell Sorting  
236 method, using an anti-Ki67 (another proliferation marker) antibody, (56.3% Ki67 positive WT cells vs  
237 57.0% Ki67 positive P1.hMR cells at d13 of differentiation, see supplemental Fig. S2), thus indicating  
238 that MR over-expression has no major impact on neuron proliferation. We then examined by Western  
239 blot the cleavage of caspase 3, as an index of caspase 3 activation and an indirect marker of apoptosis,

240 and showed a 57% reduction of caspase 3 cleavage in MR over-expressing neurons compared to WT  
241 (Fig. 3B), suggesting that MR over-expression may confer resistance to apoptotic cell death thus  
242 facilitating neuron survival.

243

#### 244 **Functional consequences of MR over-expression on neuron survival**

245 To determine the impact of MR over-expression on neuron survival, we studied the expression of  
246 transcripts and proteins encoded by the *Bcl<sub>2</sub>* gene family during neuronal differentiation of ES cell  
247 lines of both genotypes. The *Bcl<sub>2</sub>* gene family is a major regulatory component of the apoptotic  
248 pathway comprising death inducers and death repressors. These proteins are activated by different  
249 stimuli and represent upstream events leading to the conclusive phase of the apoptotic process  
250 involving caspase 3 activation. The ratio between death inducers and repressors is a key element  
251 determining cell survival or death (21-22). Specifically we examined the expression of two anti-  
252 apoptotic markers: *Bcl<sub>2</sub>* and *Bcl<sub>xL</sub>*, and two pro-apoptotic markers: Bax and Bak. Quantitative real-  
253 time PCR analysis indicated that the expression of *Bcl<sub>2</sub>* transcripts increases 6.5- and 23.7-fold during  
254 neuronal differentiation of WT and P1-hMR ES cell lines, respectively (Fig. 4A), *Bcl<sub>2</sub>* mRNA  
255 expression being significantly and reproducibly higher in P1-hMR than in WT neurons. Moreover, P1-  
256 hMR neurons exhibited a 2.5-fold rise of *Bcl<sub>xL</sub>* transcript levels compared to undifferentiated P1-hMR  
257 ES cells, whereas no statistical difference in *Bcl<sub>xL</sub>* expression was detected during neuronal  
258 differentiation of the WT ES cell line (Fig. 4B). Taken together, these findings strongly support a  
259 positive relationship between MR over-expression and an increase in the expression of anti-apoptotic  
260 markers. In contrast, steady state levels of Bax and Bak mRNA decreased by approximately 50% in  
261 WT ES cell-derived neurons but remained constant in P1-hMR neurons during neuronal differentiation  
262 (Figure 4C and 4D). Finally, of major interest, the ratios of both mRNA (Fig. 4E) and protein (Fig.  
263 4F) between anti-apoptotic and pro-apoptotic markers were always higher in P1-hMR than in WT  
264 neurons, providing strong evidence that MR over-expression promotes anti-apoptotic factors  
265 expression, thus facilitating neuronal survival.

266

#### 267 **MR knockdown inhibits neuronal-specific increase in anti-apoptotic markers**

268 To confirm that MR over-expression enhances neuronal differentiation and stimulates neuronal  
269 survival, a small interfering RNA (siRNA) strategy was exploited using two unrelated MR specific  
270 siRNAs in P1-hMR ES-derived neurons. In Fig. 5 is illustrated the decrease of mMR and hMR mRNA  
271 expression (approximately 67 % and 57 %, respectively), obtained 48h post-transfection with the  
272 respective siRNA compared with scrambled siRNA (Fig. 5A-B). This reduction was accompanied not  
273 only by a significant and concomitant diminution of the mRNA levels of the late neuronal marker  
274 MAP2 (98% and 86 %) but also by a decrease of the anti-apoptotic marker Bcl<sub>2</sub> (Fig. 5C-D). In  
275 parallel, the two MR siRNAs induced about a 50% increase of the relative expression of the pro-  
276 apoptotic marker Bax (Fig. 5E). Finally, of major interest, the two MR siRNAs caused a marked  
277 reduction of the anti-apoptotic to pro-apoptotic ratio (66%) (Fig. 5F). Collectively, these findings  
278 bring additional support for MR involvement in the increased expression of late neuronal markers. We  
279 also provide evidence that MR knock-down blunts the increase of anti-apoptotic markers associated  
280 with MR over-expression while facilitating the decrease of pro-apoptotic markers expression,  
281 validating the anti-apoptotic role of this receptor.

282

### 283 **The relative level of MR is crucial for the anti-apoptotic effect**

284 We decided then to investigate the impact of steroid hormones on the ratio of anti-apoptotic to pro-  
285 apoptotic markers, in ES-cell derived neurons. Cells were incubated with aldosterone or corticosterone  
286 at d13 of differentiation. Steroid-induced modification of Bcl<sub>2</sub> and Bax mRNA expression was  
287 measured after 6 h treatment using quantitative real-time PCR. As shown in Fig. 6, 100 nM  
288 aldosterone had no consequence on the ratio of anti-apoptotic to pro-apoptotic marker on both  
289 genotypes. In sharp contrast, corticosterone had a differential effect on P1-hMR neurons compared to  
290 WT neurons. A 35% decrease of Bcl<sub>2</sub> to Bax ratio was observed in WT neurons, whereas a 2.3-fold  
291 increase was observed in P1-hMR neurons. Corticosterone-induced effects were not affected by  
292 RU486, a GR antagonist, unambiguously demonstrating MR involvement in controlling the anti-  
293 apoptotic/pro-apoptotic signal balance. Collectively, these findings show that MR not only controls  
294 gene expression of death repressors and inducers but more importantly that neuronal MR abundance  
295 also dictates the extent and the direction towards pro- or anti-apoptotic phenotype.

296

### 297 **Neuronal MR over-expression reduces *t*-BHP-induced cell death**

298 To examine the effect of MR over-expression on oxidative stress-induced apoptosis, we compared the  
299 survival of WT and of MR over-expressing neurons after 3 h exposure to 400  $\mu$ M *tert*-  
300 butylhydroperoxide (*t*-BHP). *t*-BHP treatment led to characteristic WT cell morphological changes  
301 including round shape of neurons, beading followed by extensive degeneration of the neurites and lost  
302 of neuronal integrity, many cells detaching from the culture plate. In contrast, under similar  
303 experimental conditions, P1-hMR neurons appeared almost normal with only few floating cells.  
304 Western blot analyses show that the *t*-BHP-induced caspase 3 cleavage is 3-fold higher in WT than in  
305 P1-hMR neurons (Fig. 7A). Likewise, the ratio between anti-apoptotic and pro-apoptotic markers is 5-  
306 fold higher in P1-hMR than in WT neurons (Fig. 7B). Additionally, exposure of cultures to *t*-BHP  
307 induces to a drastic reduction of  $\beta$ -tubulin III protein expression in WT compared to P1-hMR neurons  
308 (Fig. 7C), supporting the morphological changes. Altogether, these data demonstrate that MR over-  
309 expression is associated with a significant protection against *t*-BHP-induced neuronal death.

310

### 311 **Discussion**

312 In this present work, we investigated whether and how MR controls neuronal differentiation and/or  
313 survival using a model of MR over-expression in ES cell-derived neurons obtained from P1-hMR  
314 transgenic mice (18-19). In this cell-based system, P1-hMR neurons exhibit a 2-fold increase in MR  
315 protein expression compared to differentiated WT neurons while GR expression level remains  
316 unchanged, leading to a moderately enhanced MR/GR ratio. To our knowledge, this is the first report  
317 that directly quantified the extent of neuronal MR overexpression at the protein level. This parameter  
318 is lacking in other brain-specific MR overexpression transgenic models (9, 23).

319 Given that the relative receptor density and their occupancy by corticosteroid hormones are known to  
320 greatly affect neuronal maintenance, transmission and damage (2), our ES-derived neurons in which  
321 expression of one component of the corticosteroid signaling is specifically modified, constitute an  
322 appropriate experimental system. **Even though one limitation of our model is that we could not**

323 directly control the concentration of corticosteroid hormones provided by the serum during the initial  
324 steps of neuronal differentiation, our cell based model remains suitable to clarify neuronal MR  
325 influence on cell differentiation, proliferation and susceptibility to cell apoptosis. Herein, we show that  
326 MR over-expression from early neuronal developmental stages and onwards is associated with  
327 increased expression of late neuronal differentiation markers. We unambiguously establish the pivotal  
328 role of MR in controlling the balance between anti- and pro-apoptotic signals as confirmed by  
329 knocking down MR expression with small interfering RNA strategy. We finally demonstrate the  
330 importance of MR abundance in conferring relative resistance to oxidative stress-induced cell  
331 apoptosis thus facilitating neuronal survival.

332 MR and GR are abundantly expressed in neurons of the limbic areas where they mediate quite  
333 opposite effects. MR and GR exhibit distinct functional properties notwithstanding their similarities of  
334 structure, and mechanisms of action. Most notably both receptors bind glucocorticoid hormones  
335 (cortisol in humans, corticosterone in rodents) but GR presents a low affinity while MR has a 10 fold  
336 higher affinity for glucocorticoids (24). In addition, as ligand-dependent transcription factors, MR and  
337 GR recruit similar but also distinct coregulators which may partially account for the diversity of  
338 neuronal responses to glucocorticoids (25-27). Recent accumulating evidences show that acutely or  
339 chronically unbalanced glucocorticoid concentrations differentially affect neuronal function. Under  
340 rest conditions, basal levels of glucocorticoids which predominantly activated MR are essential for  
341 neuronal development, integrity and function. On the other hand, under stress exposure, high levels of  
342 glucocorticoids, which fully occupied and activated GR, are detrimental and induce neuronal death (8)  
343 though cell cycle arrest and activation of apoptosis signaling pathways (11, 28-29). Repeated stressful  
344 events trigger the damaging effects of GR on neurons and brain functions (6, 30). Thus, the  
345 coordinated activation of MR/GR pathways appears to be a major and critical regulator of neuronal  
346 function. Yet, the extent of MR signaling activation in the central nervous system seems to depend on  
347 the MR abundance beside corticosteroid hormone levels. In this respect, our model of MR over-  
348 expressing ES derived neurons conveys important new informations concerning MR influence in  
349 neuronal determination and survival.

350 It is well established that the ratio between death repressors or anti-apoptotic molecules (e.g. Bcl<sub>2</sub>,  
351 Bcl<sub>xL</sub>) and death inducers (e.g. Bax, Bak) or pro-apoptotic markers is determinant for cell fate (21-22).  
352 Under basal conditions, Bcl<sub>2</sub> and Bcl<sub>xL</sub> sequester by dimerization Bax and Bak in the cytosol, thus  
353 preventing their migration to mitochondria and apoptosis. However, when the amount of repressors is  
354 insufficient to neutralize all the pro-apoptotic molecules, apoptotic signals prevail leading to caspase  
355 activation and apoptosis (31-32).

356 We demonstrated that the anti-apoptotic/pro-apoptotic molecule ratio is much higher in MR over-  
357 expressing neurons than in WT neurons, supporting a neuroprotective role of MR. As previously  
358 reported on other models (10-11), the involvement of neuronal MR in regulating apoptosis signaling  
359 pathways is corroborated by several lines of evidence.

360 First, we show that corticosterone, but not aldosterone exposure of WT neurons significantly increases  
361 the pro-apoptotic potential while corticosterone exerts an anti-apoptotic effect on P1-hMR neurons.  
362 These opposite actions of corticosterone persist in presence of the GR antagonist RU486, identifying  
363 MR as a pro-survival factor and underlying the role of MR over-expression in conferring neuronal  
364 resistance to apoptotic signals. These findings are in agreement with previous *in vitro* and *in vivo*  
365 studies that reported a rapid upregulation of MR (mRNA and protein) associated with an increased  
366 survival of rat primary cortical neurons in response to mild injury and in rat hippocampus following  
367 hypothermic transient global ischemia (12). The *in vivo* neuroprotective effect of MR was further  
368 demonstrated by transgenic mice presenting specific forebrain MR over-expression. These animals  
369 exhibited a decreased sensitivity to stress, anxiety-like behavior and enhanced memory (9, 23). More  
370 importantly, these transgenic mice presented with attenuated hippocampal neuron loss after cerebral  
371 ischemia, consistent with the increased survival of MR over-expressing ES-derived neurons we  
372 described.

373 Second, to validate the assumption the MR over-expression confers apoptosis resistance, we  
374 performed MR knockdown in P1-hMR neurons by a siRNA strategy. Along with the marked  
375 reduction of MR expression, a significant decrease of MAP2 expression was observed consistent with  
376 a massive loss of mature neurons associated with a reduced anti/pro-apoptotic Bcl<sub>2</sub>/Bax ratio. Taken  
377 together, there is a clear positive relationship between MR abundance, anti-/pro-apoptotic factor



378 expression ratio and neuronal marker level. This observation is in agreement with the forebrain  
379 specific genetic disruption of MR in mice, which associates altered learning processes and dentate  
380 granule cell degeneration (7, 15). Taken together, these findings provide strong evidence that  
381 increased *in vitro* and *in vivo* MR expression is directly and causally linked to the promotion of  
382 neuronal survival.

383 Third, additional results corroborate the prominent role of activated MR signaling in preventing  
384 neuronal cell death-signaling cascade. We explored cell viability after acute exposure of neurons to *t*-  
385 BHP, a strong inducer of oxidative stress. We show that MR overexpressing neurons were resistant to  
386 oxidative injury as revealed by the reduction in caspase 3 cleavage and the sharp increase in b-tubulin  
387 III protein expression monitoring neuronal survival. Interestingly, several physiological or  
388 pathophysiological conditions are clearly associated with an increased expression of brain MR such as  
389 during aging (17), after antidepressant imipramine treatment (33), in depressed patients (34) or after  
390 cerebral ischemia (35).

391 The molecular mechanisms by which MR may regulate gene expression of the Bcl<sub>2</sub> family members  
392 remain to be established. As a transcription factor, MR may directly or indirectly interact with the  
393 regulatory sequences of anti-apoptotic genes to modulate their transcription. Several groups have  
394 identified potential hormone responsive elements in Bcl<sub>xL</sub> and Bcl<sub>2</sub> gene promoters which specifically  
395 bind PR and GR *in vitro* and *in vivo* (36-38). Given that MR binds to the same consensus HRE  
396 sequence, it is tempting to speculate that MR may regulate Bcl<sub>2</sub> and Bcl<sub>xL</sub> gene expression by acting  
397 directly on the HRE sequences located at their promoters, in the context of neuronal survival in  
398 rodents (39). This does not exclude that MR activates or represses other specific sets of target genes  
399 essential for neuronal survival program.

400 We also surmise that the shift of the pro-apoptotic/anti-apoptotic balance towards neuron survival may  
401 account for the higher expression of late neuronal markers in P1-hMR neurons. Indeed, it has been  
402 previously proposed that anti-apoptotic factors facilitate neuronal differentiation, whereas a reduction  
403 of pro-apoptotic factors expression was observed by several groups (40-44). Therefore, besides their  
404 role in cell death, the proteins of Bcl<sub>2</sub> family are largely implicated in neurogenesis.

405 An additional layer of complexity is given by the putative membrane-located MR which exerts rapid  
406 non-genomic actions resulting in the stimulation of the frequency of excitatory postsynaptic glutamate  
407 currents in the mouse hippocampus. This effect was blocked by MR specific antagonist  
408 spironolactone, and did not occur in brain specific MR knockout mice (5). Surprisingly, this  
409 membrane-located MR seems to have a 10 to 20 fold lower affinity for GC than the intracellular MR.  
410 Interestingly, MR has been recently detected in the membranes of rat amygdala glutamatergic and  
411 GABAergic neurons, with a presynaptic and postsynaptic localization (45). Whether this membrane  
412 MR is involved in neuronal survival remains to be elucidated.

413 In conclusion, we have successfully established a novel model of MR over-expression using the  
414 neuronal differentiation of ES cells that was proven to be a suitable cell-based system to investigate  
415 many functions of neuronal MR. This alternative approach fully complementing previous cellular and  
416 animals models should facilitate the development of therapeutic strategies designed to improve  
417 neuronal MR signaling efficiency and thereby opening new means to prevent or attenuate neuronal  
418 cell apoptosis in neurodegenerative diseases.

419

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423

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554 **Figures and Legends**

555

556 **Figure 1: MR over-expression during neuronal differentiation**

557 **A)** Relative hMR mRNA expression levels were determined using qPCR in undifferentiated ES cells  
558 and neurons. Results are means  $\pm$  SEM of two independent experiments of six samples performed in  
559 duplicate for each developmental stage indicating the relative expression compared with basal levels  
560 of ES (arbitrarily set at 1). \*\*  $P < 0.01$ . Mann Whitney test. Relative mRNA expression is normalized to  
561 18S rRNA expression (see Materials and Methods section). **B)** Western blot analyses of MR protein  
562 expression in WT and P1-hMR ES cell lines. Undifferentiated ES and neurons lysates from each ES  
563 cell line were processed for immunoblotting with anti-MR antibody. GAPDH was used as loading  
564 control. MR was normalized to GAPDH protein levels after digitalization on a gel scanner with  
565 QuantityOne software (Bio-Rad, Marnes-la-Coquette, France). Results are presented as MR/GAPDH  
566 ratio and as compared with basal levels of WT ES (arbitrarily set at 1). **C-D)** Relative mMR and mGR  
567 mRNA expression levels were determined using qPCR in undifferentiated ES cells and neurons from  
568 WT and P1-hMR ES cell lines. Results are means  $\pm$  SEM of two independent experiments on six  
569 samples performed in duplicate for each developmental stage and represent the relative expression  
570 compared with basal levels of ES (arbitrarily set at 1). Mann Whitney test. Relative mRNA expression  
571 is normalized to 18S rRNA expression (see Materials and Methods section). **E)** Western blot analysis  
572 of GR expression in WT and P1.hMR neurons and signal quantification of the GR/GAPDH ratio ( $n =$   
573 6), ns: non significant. WT mean value arbitrarily set at 1. **F)** Double-immunolabeling of P1-hMR  
574 neurons with antibodies against  $\beta$ -tubulin III (green) (*left panel*) and MR (red) (*middle panel*); merged  
575 images are shown on the *right*. Original magnification  $\times 40$ .

576

577 **Figure 2: MR over-expression stimulates late neuronal markers without increasing neuronal**  
578 **proliferation**

579 **A-B)** Relative nestin and MAP2 mRNA expression levels were determined using qPCR in  
580 undifferentiated ES cells and neurons. Results are means  $\pm$  SEM of two independent experiments of  
581 six samples performed in duplicate for each developmental stage and represent the relative expression

582 compared with levels of WT. P1-hMR and WT undifferentiated ES cell set arbitrarily at 1. \*\*\*  
583  $P < 0.001$  Mann Whitney test. Relative mRNA expression is normalized to 18S rRNA expression (see  
584 Materials and Methods section). **C)** Western blot analyses of  $\beta$ -tubulin III protein expression in WT  
585 and P1-hMR ES cell lines. Undifferentiated ES and neurons lysates from each ES cell line were  
586 processed for immunoblotting with anti- $\beta$ -tubulin III antibody. GAPDH was used as loading control.  
587  $\beta$ -tubulin III levels were normalized to GAPDH protein levels after digitalization on a gel scanner with  
588 QuantityOne software (Bio-Rad, Marnes-la-Coquette, France). Results are presented as  $\beta$ -tubulin III  
589 /GAPDH ratio and as compared with basal levels of WT neurons (arbitrarily set at 1).

590

591 **Figure 3: MR over-expression does not affect cell proliferation but reduces Caspase 3 activity in**  
592 **neurons**

593 **A)** Western blot analyses of PCNA protein expression in WT and P1-hMR neurons. Neurons lysates  
594 from each ES cell line were processed for immunoblotting with anti-PCNA antibody. GAPDH was  
595 used as loading control. PCNA was normalized to GAPDH protein levels after digitalization on a gel  
596 scanner with QuantityOne software (Bio-Rad, Marnes-la-Coquette, France). Results are presented as  
597 ratio PCNA/GAPDH and as compared with basal levels of WT neurons (arbitrarily set at 1). **B)**  
598 Caspase 3 activity was analyzed by western blot expression in WT and P1-hMR neurons. Lysates were  
599 processed for immunoblotting with an antibody recognizing both caspase 3 and cleaved-caspase 3.  
600 Protein levels were quantified after digitalization on a gel scanner using QuantityOne software (Bio-  
601 Rad, Marnes-la-Coquette, France). Results are presented as ratio cleaved-caspase 3 /caspase 3 of 6  
602 samples and as compared with basal levels of WT neurons (arbitrarily set at 1). \*\*  $P < 0.01$ . Mann  
603 Whitney test.

604

605 **Figure 4: Anti-apoptotic factors expression is increased in P1-hMR neurons**

606 **A-B-C-D)** Relative Bcl<sub>2</sub>, Bcl<sub>XL</sub>, Bax, and Bak mRNA expression levels were determined using qPCR  
607 in undifferentiated ES cells and neurons. Results are means  $\pm$  SEM of two independent experiments of  
608 six samples performed in duplicate for each developmental stage and represent the relative expression  
609 compared with levels of undifferentiated ES cell set arbitrarily at 1. \*\*\*  $P < 0.001$  Mann Whitney test.

610 Relative mRNA expression is normalized to 18S rRNA expression (see Materials and Methods  
611 section). **E)** The table represents the ratio between the mean of each anti-apoptotic marker expression  
612 to the mean of each pro-apoptotic marker expression in neuronal state. **F)** Western blot analyses of  
613 Bcl<sub>2</sub> and Bax protein expression in WT and P1-hMR neurons. Lysates were processed for  
614 immunoblotting with anti-Bcl<sub>2</sub> or Bax antibody. GAPDH was used as loading control. Bcl<sub>2</sub> and Bax  
615 were normalized to GAPDH protein levels after digitalization on a gel scanner by using QuantityOne  
616 software (Bio-Rad, Marnes-la-Coquette, France). The table represents the ratio between Bcl<sub>2</sub> to Bax  
617 expression in neuronal state.

618

619 **Figure 5: MR down-regulation inhibits MR-mediated neuroprotective effects**

620 P1-hMR neurons were transfected with either the control scrambled siRNA (scr MR) or by two  
621 unrelated MR siRNA (si1 MR, si2 MR). **A-E)** Relative mMR, hMR, MAP2, Bcl<sub>2</sub>, Bax mRNA  
622 expression levels were determined using qPCR. Results are means ± SEM of six samples performed in  
623 duplicate and represent the relative expression compared with basal levels of control scrambled siRNA  
624 transfected neurons (scr MR). \*\*P<0.01, \*\*\*P<0.001. Mann Whitney test. Relative mRNA expression  
625 is normalized to 18S rRNA expression (see Materials and Methods section). **F)** The graph represents  
626 the ratio between the mean of anti-apoptotic marker Bcl<sub>2</sub> expression to the mean of pro-apoptotic  
627 marker Bax expression in each experimental condition.

628

629 **Figure 6: Opposite effects of corticosterone-activated MR signaling on Bcl<sub>2</sub>/Bax ratio in WT and**  
630 **P1-hMR neurons.**

631 WT and P1-hMR neurons were exposed to 100 nM aldosterone (ALDO) and corticosterone (CORT)  
632 in the absence or presence of 1 μM RU486. **A-B)** Results are means ± SEM (six samples performed in  
633 duplicate), of ratio between the mean of anti-apoptotic marker Bcl<sub>2</sub> expression to the mean of pro-  
634 apoptotic marker Bax expression in neurons. \*\* P<0.01, \* P<0.05. Mann Whitney test.

635

636 **Figure 7: Neuronal MR over-expression confers resistance to oxidative stress-induced cell death**



637 WT and P1-hMR neurons were exposed to 400  $\mu$ M *t*-BHP for 4 hours. **A)** Caspase 3 activity was  
638 analyzed by western blot expression in WT and P1-hMR neurons. Lysates were processed for  
639 immunoblotting with antibody recognizing both caspase 3 and cleaved-caspase 3. Protein levels after  
640 digitalization on a gel scanner by use QuantityOne software (Bio-Rad, Marnes-la-Coquette, France).  
641 Results are presented as cleaved-caspase 3 /caspase 3 ratio (n = 9) and compared to ratio detected in  
642 WT neurons (arbitrarily set at 1). \* P<0.05. Mann Whitney test. **B)** Western blot analyses of Bcl<sub>2</sub> and  
643 Bax protein expression in WT and P1-hMR neurons. Neuron lysates from each ES cell line were  
644 processed for immunoblotting with anti-Bcl<sub>2</sub> or anti-Bax antibody. Results are presented as ratio Bcl<sub>2</sub>  
645 /Bax (n=9) and compared with basal levels of WT neurons (arbitrarily set at 1). \*\*\* P<0.001. Mann  
646 Whitney test.

647 **C)** Western blot analyses of  $\beta$ -tubulin III protein expression in WT and P1-hMR neurons. Neurons  
648 lysates from each ES cell line were processed for immunoblotting with anti-  $\beta$ -tubulin III antibody.  
649 GAPDH was used as loading control.  $\beta$ -tubulin III was normalized to GAPDH protein levels after  
650 digitalization on a gel scanner by use QuantityOne software (Bio-Rad, Marnes-la-Coquette, France).  
651 Results are presented as ratio  $\beta$ -tubulin III /GAPDH (n = 6, \*\*P<0.01. Mann Whitney test) and as  
652 compared with basal levels of WT neurons (arbitrarily set at 1).