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ORIGINAL RESEARCH ARTICLE

Direct targeting of hippocampal neurons for apoptosis by glucocorticoids is reversible by mineralocorticoid receptor activation

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An important question arising from previous observations *in vivo* is whether glucocorticoids can directly influence neuronal survival in the hippocampus. To this end, a primary postnatal hippocampal culture system containing mature neurons and expressing both glucocorticoid (GR) and mineralocorticoid (MR) receptors was developed. Results show that the GR agonist dexamethasone (DEX) targets neurons (microtubule-associated protein 2-positive cells) for death through apoptosis. GR-mediated cell death was counteracted by the MR agonist aldosterone (ALDO). Antagonism of MR with spironolactone ([7α-(acetylthio)-3-oxo-17α-pregn-4-ene,21 carbolactone] (SPIRO)) causes a dose-dependent increase in neuronal apoptosis in the absence of DEX, indicating that nanomolar levels of corticosterone present in the culture medium, which are sufficient to activate MR, can mask the apoptotic response to DEX. Indeed, both SPIRO and another MR antagonist, oxprenoate potassium ((7α,17α)-17-Hydroxy-3-oxo-7-propylpregn-4-ene-21-carboxylic acid, potassium salt (RU28318)), accentuated DEX-induced apoptosis. These results demonstrate that GRs can act directly to induce hippocampal neuronal death and that demonstration of their full apoptotic potency depends on abolition of survival-promoting actions mediated by MR.
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Disorders of mood and cognition are linked to hypersecretion of glucocorticoids (GCs) resulting from reduced efficacy of GC-negative feedback.[1–3] One enduring view is that a loss of neurons bearing glucocorticoid (GR) and mineralocorticoid (MR) receptors in the hippocampus is the primary cause of disinhibited hypothalamo-pituiatry-adrenal (HPA) activity; moreover, hippocampal cell loss is likely to have repercussions on cognition and the regulation of mood and anxiety.

Numerous previous experiments suggest that excessive corticosteroid secretion endangers the survival of hippocampal neurons by increasing their vulnerability to concomitant stimuli such as excitatory amino acids, calcium influxes and reactive oxygen species.[4,5] The detrimental effects of corticosteroids on the hippocampus are backed by imaging studies in humans: there is a strong negative correlation between cortisol levels and hippocampal volume in patients with major depression[6,7] and in subjects with Cushing's disease;[8] reduced hippocampal volumes were also recently found in rats treated with dexamethasone (DEX), a GR agonist.[9] While most of the hippocampal shrinkage observed may be due to neuritic atrophy, including dendritic impoverishment and synaptic loss,[10–12] other experiments have demonstrated that GR activation activates a molecular cascade, leading to significant levels of neuronal cell death through apoptotic mechanisms.[13] Besides apoptosis of mature hippocampal neurons,[14] there is also evidence that GRs interfere with proliferation of granule neurons of the dentate gyrus.[15]

MR and GR both bind cortisol/corticosterone, albeit with differing affinities: whereas GR become occupied by corticosteroid levels in the high physiological range, MR appear to be tonically active under basal levels of HPA activity, and there is evidence that activity of the two receptors is coordinated so as to maintain endocrinological and behavioral balance.[1,16,17] We previously proposed that the relative occupation of MR and GR also contributes to maintenance of hippocampal cell numbers, since activation of MR counteract deleterious GC actions on neuronal survival.[11,13,14] Indeed, the results of earlier studies suggest that MR occupation may be essential for the survival of dentate granule neurons.[18–20]

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The interpretation of previous data showing that DEX can induce neuronal cell death in animals has been questioned on the basis that because DEX poorly penetrates the brain and produces 'chemical adrenalectomy' by suppressing pituitary ACTH secretion, the observed cell loss may reflect abolition of MR activation rather than a direct effect of GR activation.[1] The work reported here readdresses this issue by using a pharmacologically malleable model, namely primary hippocampal cell cultures. Our results demonstrate the inherent apoptosis-inducing properties of DEX, which become better evident if neuroprotective actions mediated by MR are blocked.

Materials and methods

Experiments on animals were conducted in accordance with the European Communities Council Directive No. 86/609/EEC and local regulations. Hippocampal tissue was obtained from male rat pups, aged 4–5 days (Wistar rats; Charles River, Sulzfeld, Germany). Pilot *in vivo* studies in rats of this age showed an acute injection of DEX (100 μg/kg, i.p.) to result in a 60% increase in apoptotic cells in the hippocampus (measured by TUNEL histochemistry) within 24 h.

Tissue and cell dissociation

Rat pups were rapidly decapitated, their brains carefully dissected out and placed in ice-cold Neurobasal/B27 solution (Invitrogen, Karlsruhe, Germany). Hippocampi were dissected out and freed from meninges and vascular tissue excess under a dissecting microscope, before being sliced (ca. 250 μm thick) on a McIlwain tissue-chopper. Slices were briefly washed in ice-cold Neurobasal/B27 medium, centrifuged briefly ($70 \times g$; 20°C) and resuspended in a solution containing papain; to this end, the Papain Dissociation Kit (Worthington Biochemical Corp., Freehold, NJ, USA) was used with some minor modifications to the manufacturer's suggested protocol (see Hellbach *et al*[21]). Tissue digests were triturated gently and transferred to a medium consisting of Neurobasal/B27 medium, 1% fetal calf serum and 0.2% bovine serum albumin (BSA) at 37°C, filtered through a sterile nylon mesh (30 μm pore size) and centrifuged at $200 \times g$ (20°C; 5 min) before resuspension in Neurobasal/B27 medium containing enzyme inhibitor (ovomucoid/0.005% DNAse; Worthington). Aliquots of this suspension were run through a one-step ovomucoid/BSA density gradient (centrifugation at $70 \times g$ at 20°C for 5 min), taken up in Neurobasal/B27 medium containing basic fibroblast growth factor (bFGF) (10 ng/ml), Glutamax I (0.5 mM) and kanamycin (100 μg/ml) (all from Invitrogen), and plated onto gelatin/PDL-coated glass coverslips (Superior, Bad Mergentheim, Germany) at a density of 450 ± 33 cells/mm^2. Cultures were maintained at 37°C, under 5% CO_2 and 99% relative humidity. Cortisol levels in the B27 supplement added to the incubation medium were 15–30 ng/ml, as determined by radioimmunoassay.

Treatments

Cells were exposed to drugs after 6 days *in vitro*; exposure to experimental drugs was for 48 h. The GR agonist (DEX) was obtained as a sodium phosphate salt in aqueous form (Fortecortin®, Merk, Darmstadt, Germany) and used at doses of either 10^{-6} or 10^{-5} M. The prototypic MR agonist aldosterone (ALDO) was purchased from Sigma (Deisenhofen, Germany) and used at a dose of 10^{-5} M (survival studies) or 10^{-8} M (translocation experiments) after solution in ethanol. Two MR antagonists were used alone or as cotreatments with DEX: spironolactone ([7α-(acetylthio)-3-oxo-17α-pregn-4-ene,21 carbolactone] (SPIRO); Sigma) was used at between 10^{-9} and 10^{-5} M after solution in ethanol; oxprenoate potassium ((7α,17α)-17-Hydroxy-3-oxo-7-propylpregn-4-ene-21-carboxylic acid, potassium salt (RU28318)), purchased from Tocris Cookson (Bristol, UK), was used at dose ranging from 10^{-9} to 10^{-5} M after direct solution in growing medium. Final ethanol concentrations in the medium were 0.01%; drug-naive cultures were incubated in Neurobasal/B27 medium containing 0.01% ethanol for equivalent times. In all experiments, the position of wells subjected to control or drug treatments was randomized; each experiment was performed on at least three independent occasions.

Hoechst staining

Cultures were fixed in ice-cold 4% paraformaldehyde (10 min), washed in phosphate-buffered saline (PBS) and incubated with Hoechst 33342 (Phenol, 4-[5-(4-methyl-1-piperazinyl)[2,5′-bi-1H-benzimidazol]-2′-yl]-, trihydrochloride) (1 μg/ml; Molecular Probes, Leiden, The Netherlands) for 45 min in the dark. After rigorous washing (PBS), coverslips were mounted in antifading medium. The total number of cells per unit area was determined by counting all Hoechst-stained nuclei, that is, those with intact and disintegrating nuclei, using the cell counting parameters described below.

Immunocytochemistry

For characterization of cultures by light microscopy, cells were fixed in ice-cold 4% paraformaldehyde (10 min) and incubated with the following primary antibodies: mouse anti-microtubule-associated protein 2 (MAP2; Roche, Mannheim, Germany; 2 μg/ml), mouse anti-myelin basic protein (MBP) for oligodendrocytes (Chemicon, Temecula, CA, USA; 1 : 500) and rabbit anti-glial fibrillary acidic protein (GFAP; DAKO, Hamburg, Germany; 1 : 500). Peroxidase-conjugated antibodies (Sigma) and 3,3′-diaminobenzidine tetrahydrochloride (0.025%; Sigma) were used to visualize immunoreactive elements. Specimens were examined on an Olympus BX-60 microscope, video-linked to a computer equipped with image-processing software (ImagePro, Maryland, USA). Cell counts

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were performed on equally sized microscopic fields from five coverslips under $\times 400$ magnification; 10 fields (0.072 mm^2) were randomly chosen across two diameters of the coverslip in order to avoid potential confounding errors arising from periphery–center gradients in the distribution of cells; an average of 1000 cells were sampled on each coverslip, and the results shown represent values from five to eight coverslips per treatment. Data representing numbers of immunolabeled cells are expressed relative to total number of cells labeled with Hoechst dye (see above).

Electron microscopic analysis
For classical electron microscopic characterization of the cultures, cells were fixed in 1% paraformaldehyde/1% glutaraldehyde in 0.1 M PBS (pH 7.4) for 1 h. Thereafter, the cells were treated with 1% osmium tetroxide, dehydrated in a graded series of ethanol and flat-embedded in Epon resin.[22] Serial ultrathin sections were cut on an ultratome and contrasted with uranyl acetate and Reynold's lead citrate. Ultrastructural evaluation was carried out with a Hitachi transmission electron microscope.

Detection of apoptosis by TUNEL histochemistry
Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) histochemistry was performed on 4% paraformaldehyde-fixed cells, as described.[14,23] TdT was purchased from MBI Fermentas (Heidelberg, Germany). The above-detailed morphometric procedures were use to quantify numbers of apoptotic (TUNEL positive) as a proportion of Hoechst-stained cells.

Reverse-transcriptase PCR
To detect *GR and MR* mRNA transcripts in the cultures, total RNA, free from chromosomal DNA contamination, was isolated (RNAeasy kit, Qiagen, Hilden, Germany) and reverse transcribed with SUPERSCRIPT™ II RNA H-reverse transcriptase (Life Technologies) using custom-synthesized oligo-$dT_{12–18}$ primers (MWG Biotech, Ebersberg, Germany). Published primers[24] were used and amplication conditions were denaturation for 40 s at 94°C, annealing for 1 min at 62°C (*MR*) or 40°C (*GR*), and primer extension for 2 min at 72°C; optimal amplifications of *MR* and *GR* mRNA were achieved after 35 and 25 cycles, respectively.

Nuclear translocation of MR and GR
After 6 days *in vitro*, cells were exposed to either DEX or ALDO (both at 10^{-8} M) for 6 h. Cells were then either prepared for detection of MR and GR by immunofluorescence (fixation as before) or Western blotting, using rabbit antiglucocorticoid receptor (GR; Santa Cruz Biotechnology, Heidelberg, Germany; 1 : 1500) or rabbit antimineralocorticoid receptor (MR; Santa Cruz; 1 : 1500). Fluorescence images from DEX- or ALDO-treated cells were visually compared to those from nontreated cells; in the case of GR, the abundance of translocated receptor was obvious, whereas degree of MR occupation had to be based on subjectively judged increases in fluorescence signal intensities. For GR Western blots, cells were lysed, processed and evaluated using semi-quantitative densitometry as described previously.[25]

Statistics
All numerical data, shown as means $\pm$ SEM, were subjected to ANOVA and appropriate *post hoc* analyses (SigmaStat 3). The level of significance was preset at $P \leq 0.05$.

Results

Model validation
Studies in various species, including humans, primates, sub-primates and rats, have described correlations between elevated systemic levels of corticosteroids levels and reduced hippocampal volumes and increased hippocampal cell death or neuritic atrophy.[4–14,26] Corticosteroids have also been shown to increase the likelihood of neuronal death in rat embryonic hippocampal cultures.[27,28] In the present experiments, hippocampal cell cultures were prepared from postnatal rats, aged 4–5 days. Cells were grown in a serum-free, chemically defined medium, which contained nanomolar amounts of cortisol (see Materials and methods). Preliminary *in vivo studies* showed that the hippocampi of rats of this age are sensitive to the apoptosis-inducing actions of DEX, showing a 60% increase in cell death following an acute injection of the drug (data not shown).

Cells established dense networks *in vitro,* with some 25–30% of cells staining positively for MAP2, a marker of mature neurons (Figure 1a and b). GFAP-positive astrocytes (Figure 1c) or and myelin basic protein-positive oligodendrocytes (Figure 1d) made up some 10–15% of the culture population. Approximately 40% of cells in culture incorporated the mitosis marker bromodeoxyuridine, some 30% of which were found to be neuroblasts (doublecortin positive).[29] Electron microscopic analysis of the cultures revealed cells showing morphological features typical of neurons, astrocytes and oligodendrocytes (Figures 1e–j).

Consistent with results from *in vivo* studies,[30,31] cells in culture were seen to express *MR* and *GR* mRNA (detected by RT-PCR analysis; Figure 2a) as well as receptor protein (immunofluorescence images in Figures 2b and c). Under basal conditions, MR were localized in both the cytoplasmic and nuclear compartments, probably owing to the low levels of cortisol in the medium supplement (B27) or because of its apparent ligand-independent localization;[32] treatment with ALDO resulted in a greater intensity of immunoreactive signal in the nucleus. In contrast, GR in untreated cells were predominantly located in the cytoplasm, and were only found in the nucleus after exposure to DEX. The morphological localization of GR was corroborated by evidence obtained by

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Western blot analysis of cytoplasmic and nuclear fractions; Figure 2d shows that DEX treatment results in a conspicuous relocation of GR from the cytoplasmic to the nuclear compartment. Together, these data indicate that the receptors expressed *in vitro* are likely to serve their roles as ligand-activated transcription factors.

DEX induces neuronal cell death in an ALDO-reversible manner

Treatment of hippocampal cultures with the GR agonist DEX (10^{-5} M) doubled the relative number of TUNEL-positive (apoptotic) cells within 48 h ($P<0.05$; Figure 3a). A significant proportion of these apoptotic cells were mature neurons, as judged by the reduction in the MAP2 immunoreactive subpopulation ($P<0.05$; Figure 3b). Whereas exposure to ALDO (10^{-5} M) alone did not affect the incidence of apoptosis or the number of surviving neurons, the MR agonist significantly attenuated the apoptosis-inducing actions of DEX ($P<0.5$; Figure 3). Thus, consistent with our previous findings,[13,14] MR occupation counteracts the actions GR.

MR antagonism causes apoptosis and accentuates GR-mediated apoptosis

Blockade of MR with SPIRO (10^{-8}–10^{-5} M) resulted in a dose-dependent increase in levels of apoptosis (Figure 4a). The less-potent MR antagonist oxprenoate (RU28318)[33–35] only stimulated apoptosis when used at 10^{-5} M, the highest doses tested (Figure 4b). The apoptotic actions of SPIRO and RU28318 most probably result from their counteraction of the prosurvival effects of the nanomolar (MR-activating) levels of corticosterone in the B27/Neurobasal medium; these results indicate that tonic occupation of MR is essential for neuronal survival. The view that MR activation promotes cell survival was boosted by the finding that both MR antagonists synergized with a suboptimal dose of DEX (10^{-6} M; 10-fold lower than the dose used in the previous experiment) to induce apoptosis; our observations show that manifestation of the full apoptotic potential of GR agonists *in vitro* can be masked by MR agonists that are inadvertently present in the medium.

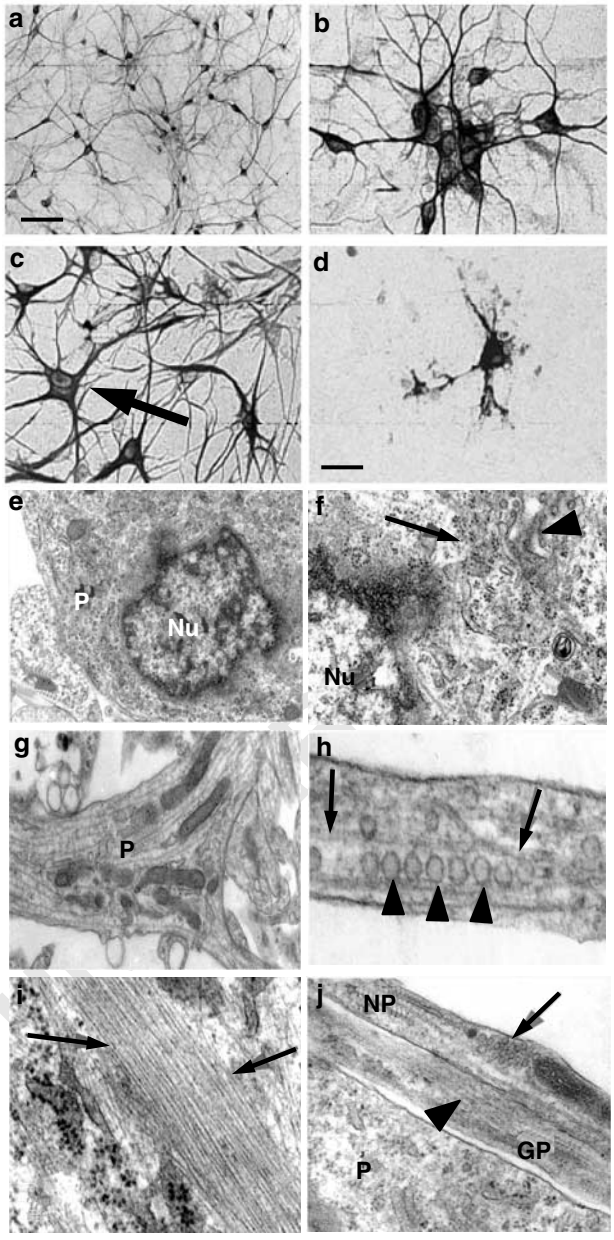

Figure 1 Immunocytochemical and ultrastructural characterization of primary postnatal rat hippocampal cultures. (a) Low-magnification field view of primary rat hippocampal cultures (5 days *in vitro*), immunostained with MAP2. (b) High-power micrography showing differentiated MAP2-immunolabeled neurons. (c) High-power micrograph of astrocytes immunostained for GFAP. (d) An immunoreactive oligodendrocyte labeled with anti-MBP. Scale bars represent 120 μm (a) and 30 μm (b–d). (e) Medium-power electron micrograph of a cultured cell showing typical features of a neuron. Note the large nucleus (Nu) within the perikaryon (P) richly endowed with free and membrane–bound ribosomes. ($\times$10 000). (f) Electron micrograph showing numerous ribosomes (arrow) and cysts of a Golgi complex (arrowhead) within the neuronal cytoplasm ($\times$28 000). (g) Demonstration of a growing neuronal process (P) exhibiting bundles of microtubules and mitochondria ($\times$13 000). (h) High-power view of a neuronal process with microtubules organized in a parallel fashion (arrow). Note the association of ovoid transit vesicles (arrowheads) with microtubules ($\times$32 000). (i) High-power micrograph of an astrocyte with a prominent bundle of glial filaments within its cytoplasm (arrows) ($\times$40 000). (j) Example of a neuronal perikaryon (P) lying adjacent to a glial process (GP) showing glial filaments (arrowhead). Also shown is a neuronal process (NP) growing along the surface of the glial element. The arrow points to a cluster of synaptic-like vesicles ($\times$30 000).

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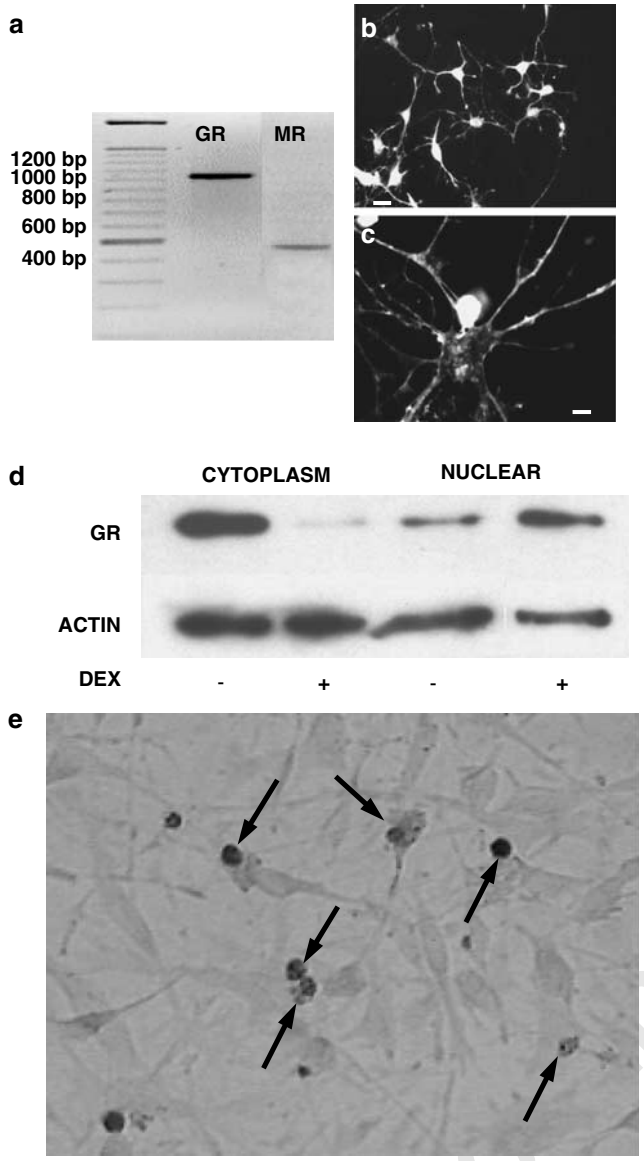

Figure 2 GR and MR receptor expression and translocation in hippocampal cell cultures. (a) RT-PCR demonstration of MR and GR expression in primary hippocampal cultures maintained for 5 days *in vitro*. The transcript size of each receptor corresponded with previously published data.[24] (b) Low-power photomicrograph showing neurons displaying GR immunoreactivity. Note that the cells were treated with DEX (10^{-7} M) for 6 h before fixation, and that the GR signal is mainly located in nuclei, indicating translocation of the receptor from its normal cytoplasmic (unliganded) location. (Scale bar represents 60 μm). (c) High-power view of a neuron displaying MR immunoreactivity in its nucleus following exposure to the prototypic MR agonist ALDO (10^{-8} M) for 6 h (scale bar represents 15 μm). Note that a small proportion of neurons and glia displayed MR immunoreactivity in their cytoplasm in the absence of exogenous ligand (data not shown), probably owing to the low concentrations of cortisol present in the growing (B27) medium. (d) Further demonstration (Western blot) that DEX treatment (10^{-6} M) mobilizes immunoreactive GR to the nucleus. (e) Cells showing some typical features of apoptosis as revealed by TUNEL histochemistry are indicated by arrows; culture was treated with DEX.

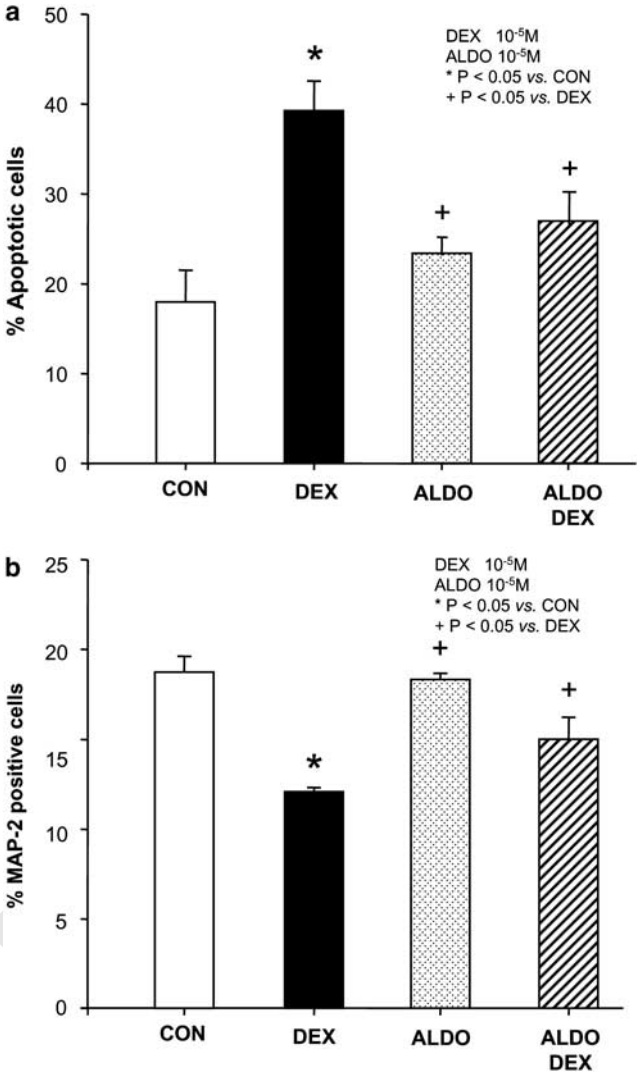

Figure 3 Opposing effects of GR and MR ligands on hippocampal neuron survival. (a) In contrast, the effects of the MR agonist ALDO (10^{-5} M), the GR ligand DEX (10^{-5} M) induces significant levels of apoptosis in hippocampal cultures. The apoptotic potential of DEX is significantly attenuated when cultures are concomitantly treated with ALDO and DEX. (b) The size of the neuronal (MAP2-positive) subpopulation of cells in the cultures is significantly reduced in the presence of DEX (10^{-5} M) and unaffected by ALDO (10^{-5} M); the number of MAP2-positive neurons is not significantly different from that observed in controls when cultures are exposed both ALDO and DEX. It is inferred from the mirror images of the data shown in (a) and (b) that DEX targets neurons for apoptotic death in an ALDO-reversible manner.

Discussion

While a number of potential molecular mechanisms may be proposed to explain the basis of impaired GR feedback in the hippocampus, the impact of GR-induced destruction of the neural substrate mediating this feedback has been a recurring theme in psycho-neuroendocrinology over the last decade. A series of

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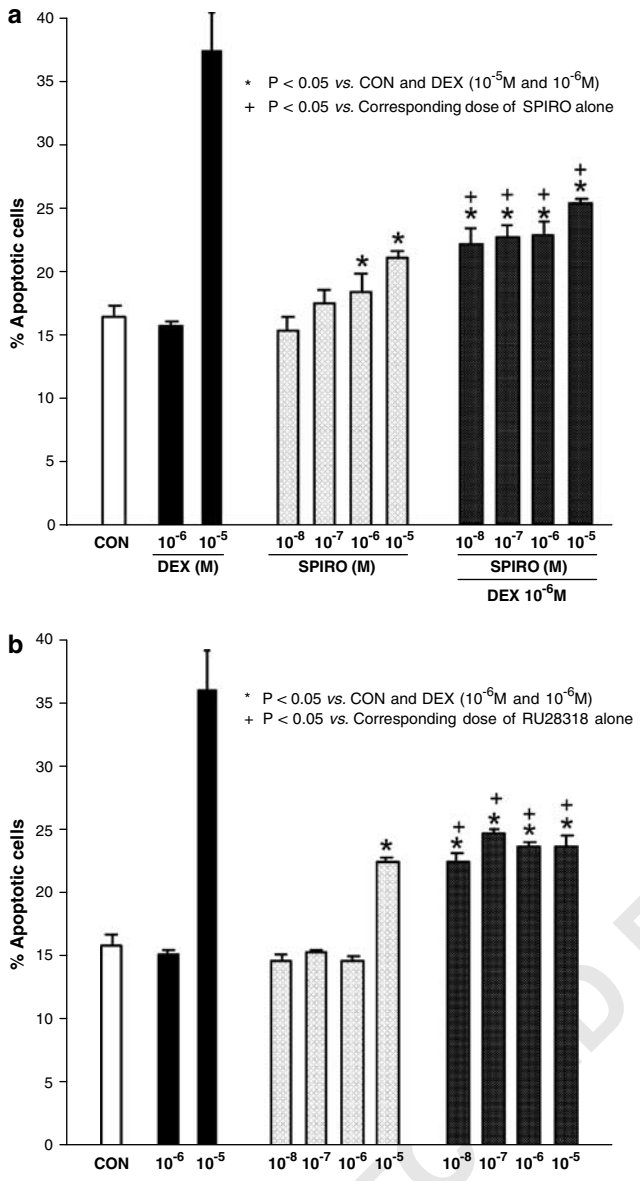

Figure 4 Blockade of MR compromises neuronal survival and predisposes neurons to DEX-induced apoptosis. As compared to the previously used dose of DEX (10^{-5} M; Figure 3), a 10-fold lower dose of the GR agonist does not cause significant apoptosis in hippocampal cultures (cf second and third bars in a and b). Reasoning that this may have resulted from nanomolar (MR-activating) concentrations of levels of corticosterone in the culture medium, cells were exposed to two MR antagonists, SPIRO (a) and RU28318 (b); these compounds display slightly different pharmacological profiles.[33–35] In the absence of DEX, addition of SPIRO (10^{-8}–10^{-5} M) dose dependently increased the incidence of apoptosis (a); RU28318 at a dose of 10^{-5} M also significantly stimulated apoptosis (b). In combination with the apoptosis noninducing dose of DEX (10^{-6} M; a and b), all doses of SPIRO and RU28318 led to levels of cell death that were significantly greater than those seen after treatment with the antagonist alone, that is, blockade of MR with either SPIRO or RU28318 increased neuronal sensitivity to the apoptotic actions of DEX.

in vivo studies in rats previously demonstrated that the GR agonist DEX stimulates cell death in the hippocampus as well as in other selected brain regions such as the striatum and substania nigra.[13,14,36,37] However, it remains unclear as to whether these effects occur directly or whether glucocorticoids merely exacerbate the neurotoxic effects of other more potent insults such as excitatory amino acids and reactive oxygen species.[27,28,38]

Direct neural actions of DEX have also been questioned on the basis of data showing that DEX has limited access to the brain.[1] Observations that adrenalectomy leads to apoptosis in the hippocampus,[18,19,39] together with the fact that DEX treatment leads to a state of 'chemical adrenalectomy',[1] boost the argument against direct effects of DEX on hippocampal cell survival. On the other hand, significant levels of apoptosis can be observed within 24 h of a single injection of DEX,[14] whereas the effects of adrenalectomy display different temporal and spatial dynamics.[39–41] Further, the apoptotic effects of DEX were previously shown to be blocked with a GR antagonist;[36] GR are transcription factors and we have previously shown that their activation by DEX can trigger a molecular death cascade in the hippocampus and cell cycle arrest in a neural cell line.[13,25]

The issue of whether DEX acts directly on neurons to stimulate their demise cannot be resolved easily *in vivo*. We therefore attempted to address this question in primary neuronal cultures derived from early postnatal rats. These cultures were carefully characterized and their suitability for our studies verified, especially with respect to ontogeny of elements involved in the regulation and responsiveness of the HPA axis,[30,31,42] as well as to age-related differences in the *in vivo* responses to the cell death-inducing actions of corticosteroids.[14,43] The majority of mature neurons (MAP2 immunoreactive) cells in culture displayed the typical size and shape of granule cells. Although immature neurons (doublecortin-positive) cells were also observed, our analysis only included cells staining for MAP2 since previous studies showed that newly born hippocampal cells do not express MR and GR.[44]

The exposure of cultures to DEX at a dose of 10^{-5} M led to a significant loss of mature (MAP2-positive) neurons, an event accompanied by a significant increase in the incidence of apoptosis. The results obtained in this isolated hippocampal neuron model, in which DEX can directly access individual cells, therefore show that DEX has the intrinsic potential to induce neuronal cell death, and that its effects occur independently of the HPA axis and other confounding factors. The results of previous studies have shown that DEX induces apoptosis by activating GR:[36] although some earlier reports have suggested that DEX may bind to MR under specific *in vitro* conditions, there is strong evidence that DEX is predominantly a GR agonist.[45]

The apoptotic actions of DEX (10^{-5} M) were shown to be significantly attenuated when the MR agonist

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ALDO (10^{-5} M) was added to the culture medium. This finding is consistent with previous results obtained in rats.[11,13,14] Since ALDO on its own did not alter apoptotic cell and neuronal numbers, our results add currency to the view that MR can trigger neuroprotective mechanisms; the latter concept emerged from a number of older studies, which showed that either ALDO or low, MR-activating levels of corticosterone could prevent or reverse adrenalectomy-induced apoptosis.[46,18,41] Notably, the effects of adrenalectomy cannot be ameliorated by the administration of DEX.[19,47]

Chemically defined, serum-free media of the sort used in these studies have significant advantages over serum-containing media. Nevertheless, our experiments were somewhat confounded by the presence of corticosterone (nanomolar range, sufficient to activate MR) in the medium. This factor most probably accounts for our observation that the MR antagonist SPIRO increased neuronal cell death in a dose-dependent (10^{-7}–10^{-5} M) manner, although another (weaker) MR antagonist (RU28318) did not alter basal levels of apoptosis. This finding supports the interpretation that tonic activation of MR is essential for the survival of hippocampal neurons.

In view of the above results, we hypothesized that blockade of 'medium-activated' MR would accentuate the effects of GR stimulation; the hypothesis was proven correct by analysis of apoptosis after concomitantly treating cultures with either SPIRO or DEX and a suboptimal dose of DEX: whereas DEX at a dose of 10^{-6} M did not induce cell death, its combination with a range of doses of SPIRO or RU28318 (10^{-8}–10^{-5} M) significantly exacerbated the occurrence of neuronal apoptosis. Besides acting on MR, SPIRO can also antagonize androgen and progesterone receptors;[48] however, since similar results were obtained with RU28318, a drug with greater selectivity for MR, our inference that neuroprotective effects are mediated by MR is warranted.

In summary, evidence gained in a cellular model shows that GRs can lead to hippocampal cell death without the participation of other aggravating factors, so long as their effects are not masked by previously activated MR. The presented results also bolster the view that hippocampal neuronal survival depends on the tonic occupation of MR. Further, insofar that these data demonstrate the opposing roles of MR and GR in hippocampal cell survival, they add credibility to the 'receptor balance hypothesis' that was generated from endocrinological and behavioral studies.[1,17] If GR-induced hippocampal cell losses do indeed contribute to impaired regulation of the HPA axis, eventually leading to mood, anxiety and cognitive impairments, therapeutic tools designed to activate selectively MR or to improve specifically their signaling efficiency in neurons[49,50] would be a worthwhile strategy. In closing, it however needs to be emphasized that, even if deserving of further investigation with respect to functional significance, the contribution of apoptotic events to hypercortisolemia-induced reductions in hippocampal size are unlikely to be major; as discussed elsewhere,[12] the fact that recovery of both the morphological and behavioral changes caused by elevated corticosteroid secretion are possible after nomocortisolemia has been re-established,[8,51] suggesting that the reported reductions in hippocampal volume are partially due to neuritic atrophy rather than irreversible cell death.

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