Adrenalectomy Regulates Apoptotic-Associated Genes in Rat Hippocampus

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Morphological studies of granular neurons of the hippocampus have shown that adrenalectomy (ADX) induces the cell death of granular neurons, an effect prevented by corticosterone replacement. We addressed the hypothesis that corticosterone regulates the expression of the apoptotic *bcl*-2 gene family. Five days after adrenalectomy, we observed morphological changes related to hippocampal granule cell apoptosis that was accompanied by terminal dUTP nick and labeling (TUNEL) labeling in nuclei located in the hilus region. Corticosterone replacement prevented the cell death induced by ADX. Using RT-PCR we found a reduction in mRNA levels of the antiapoptotic gene *bcl*-2 in whole hippocampus, an effect which was prevented by corticosterone administration to ADX rats. However, Bcl-2 protein levels were not altered by this treatment. We did not observe modifications in the level of *bcl*-X_L mRNA however, we did find a 40% reduction in Bcl-X_L protein levels, an effect not reversed by corticosterone. In contrast, we found a reduction in the mRNA of the antiapoptotic gene *bax* and Bax levels after ADX; both effects were prevented by corticosterone. The reduction in proapoptotic *bax* and in antiapoptotic *bcl*-2 mRNA levels in the whole hippocampus, suggests that local variations in these molecules could account for both neuronal viability of the CA1- CA3 and granular cell death detected by morphological means and observed after ADX.

Key Words: Adrenalectomy; corticosterone; apoptosis; Bax; Bcl-X_L; Bcl-2.

Introduction

The hippocampal formation of the brain is an important target for adrenocorticosteroid hormones, as reflected by its high abundance of corticosteroid receptors *(1–5)*. Two receptor sub-types for corticosterone have been identified; the high affinity and low capacity mineralocorticoid receptor (MR), which binds corticosterone (CORT) and aldosterone (ALD), and the low affinity and high capacity glucocorticoid receptor (GR), which binds CORT and dexamethasone *(6)*. These differences in binding efficiency allow both receptors to regulate gene expression in a ligand-concentration dependent manner. MR is principally active at low glucocorticoid levels whereas GR becomes active at higher glucocorticoids (GC) levels. Corticosteroids exert neurotrophic actions, involving the regulation of cognitive, behavioral, and neuroendocrine processes, and several studies have suggested that some affective disorders and cognition alterations, especially during aging, result from corticosteroid-induced neuronal loss during hippocampal formation *(7,10)*. Treatment of adult rats with supraphysiological CORT doses for 12 wk, equivalent to that seen after chronic stress, induces a reversible loss of pyramidal neurons in the hippocampus, producing more damage in CA3 than in CA1 fields *(1)*. On the other hand, the reduction of corticosteroid circulating levels by adrenalectomy (ADX), results in a destruction of the granule cell layer of the dentate gyrus (GD) with no apparent degeneration in pyramidal cell layers *(2,11,12)*. Morphological analyses have demonstrated that ADX-induced cell death of granular cells seems to be related to an apoptotic process which can be prevented by CORT *(2,11)* or ALD administration *(12)*, indicating a trophic influence of adrenal steroids on granular cell survival. Thus, MR and GR seem to promote differential cellular effects; MR appears to enhance and GR appears to compromise neuronal viability. Although the molecular events that result in a delayed death of neurons after ADX remains unknown, it is probable that CORT influences granular cell survival through gene regulation. Many neuropathological disorders share common cell death pathways, including the activation of genes linked to apoptosis, such as the bcl-2 family. Among the known regulators of apoptosis, Bcl-2 stands out for its ability to suppress neuronal cell death induced by a wide variety of insults and stimuli *(13)*. The bcl-x gene encodes two proteins, Bcl-X_L and Bcl-X_S, which have opposite apoptotic functions *(14)*. The expression of the L form blocks cell death in a similar way as Bcl-2 does, whereas

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Table 1
Serum Corticosterone Level
After Adrenalectomy and Hormonal Replacement

Animals	n	CORT (μg/100 mL)
ADX	9	$0.27 \pm 0.04^{\neq}$
ADX-C	6	6.28 ± 1.31*
Sham	10	12.07 ± 1.9***

The serum corticosterone level was measured five days after ADX or in adrenalectomized animals with corticosterone replacement (ADX-C) and compared with Sham animals. Data represent the mean values ± SEM.

$^{\neq}p < 0.05$ vs ADX-C; $*p < 0.05$ vs ADX; $***p < 0.001$ vs ADX (ANOVA).

the S form inhibits Bcl-2 and Bcl-X_L action, thus triggering apoptosis *(14)*. Another gene product, Bax, renders cells more sensitive to apoptotic stimuli *(15–19)*. Thus, Bax functions as a cell death inducer unless it is neutralized by either Bcl-2 or Bcl-X_L *(20,21)*, implying that the balance between death-inducer and death-repressor proteins controls whether an individual cell will ultimately survive or die. The main focus of this paper is to demonstrate that some genes and proteins related to the *bcl*-2 family are regulated by CORT, perhaps explaining the viability of CA3-CA1 hippocampal neurons and granular cell apoptosis observed following ADX.

Results

Adrenalectomy

In order to evaluate the effectiveness of ADX and CORT replacement, serum corticosterone concentrations were measured by radioimmunoassay (RIA) in all animals. Table 1 shows that the hormone serum level was 0.27 ± 0.04 μg/dL in the ADX group, animals which also displayed a reduction in body wt gain with respect to Sham controls (data not shown). ADX animals treated with CORT (ADX-C) had serum levels 20-fold higher (6.28 μg/dL ± 1.31) than ADX animals, lower than the basal level observed in Sham animals (12.07 μg/dL ± 1.9), yet providing an appropriate hormonal return to a physiological range.

Morphological Analysis of Apoptotic Granule Cells

Several studies have described the morphological changes typically associated with apoptotic cells, which present the following sequence: Clumping of chromatin against the nuclear envelope, condensation of the nucleus and cytoplasm, breaking up of pyknotic cells into apoptotic fragments, and digestion of apoptotic bodies *(11,27,28)*. To evaluate apoptotic death in the hippocampal tissue from ADX, ADX–C and Sham animals, we measured the number of degenerating cells in the whole hippocampus. Figure 1A shows that nuclear staining revealed a series of morphological changes associated with apoptosis induced during the five days after ADX. We found cells with morphologic breaking up of the condensed nucleus (Fig. 1B) and also the disassembly of pyknotic cells (Fig. 1C). We counted only those cells with the morphological changes clearly related to the last stage of apoptosis in order to avoid false positives. A quantitative analysis is shown in Fig. 1C and revealed that after ADX, there appeared a 12-fold increase in the number of cells presenting morphological alterations related to apoptosis in the whole GD (left and right side pooled) ($p < 0.001$). Within the GD, the alteration is more prominent in the suprapyramidal blade ($p < 0.001$) and infrapyramidal zones ($p < 0.001$), with an increase in 8- and 14-fold respectively, compared to Sham animals; there was no significant change in the polymorphic hilus region of the GD (Fig. 1C). These changes were prevented by CORT administration to ADX animals; indicating that the effect was dependent on this hormone (Fig. 1B). We did not find any significant alteration in other subfields of the hippocampus (CA1-CA4) by ADX (data not shown).

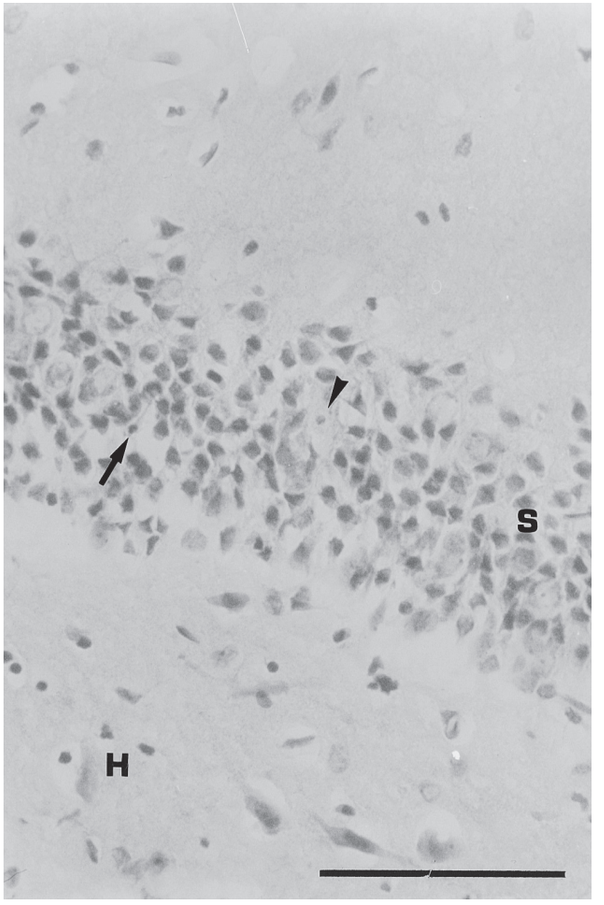

Fig. 1. (**A**) Morphological alterations after ADX are present within the granule cell layer in the same section. Most apoptotic cells display an extensive and tight condensation of nuclear materials into a darkly stained ball (arrow) and disassembly of the pyknotic cells into fragments (arrowhead). Scale bar 100 μm.

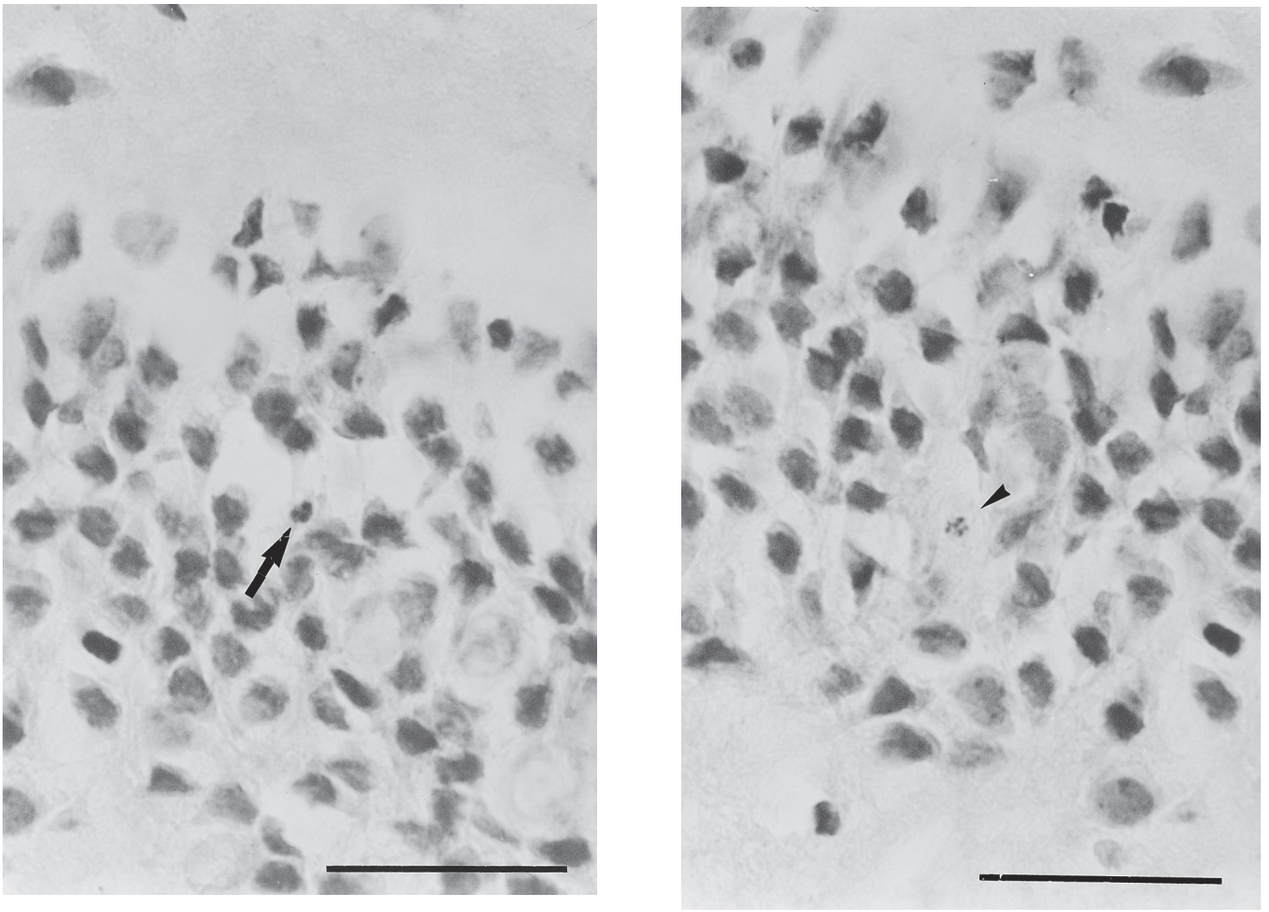

Fig. 1. (**B**) and (**C**) A higher magnification of (**A**). Scale bar = 40 μm.

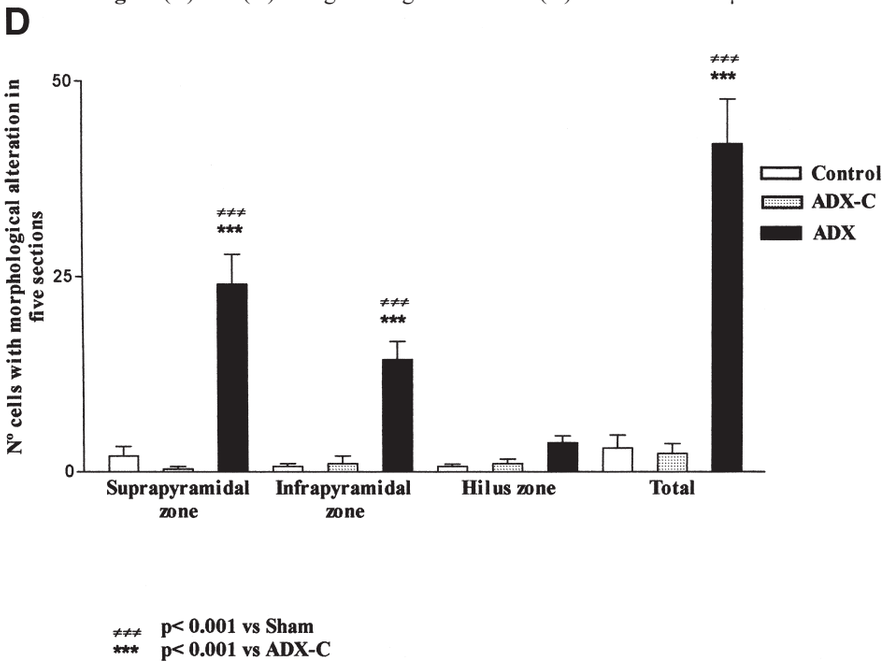

Fig. 1. (**D**) Number of cells displaying morphological alterations in the GD and hilus after ADX. Values indicate the mean value of the number of damaged cells present in five sections ± SEM. from 3–4 animals. H = hilus, S = suprapyramidal blade of the granule cell layer. $^{\neq\neq\neq}p < 0.001$ vs Sham $^{***}p < 0.001$ vs ADX-C.

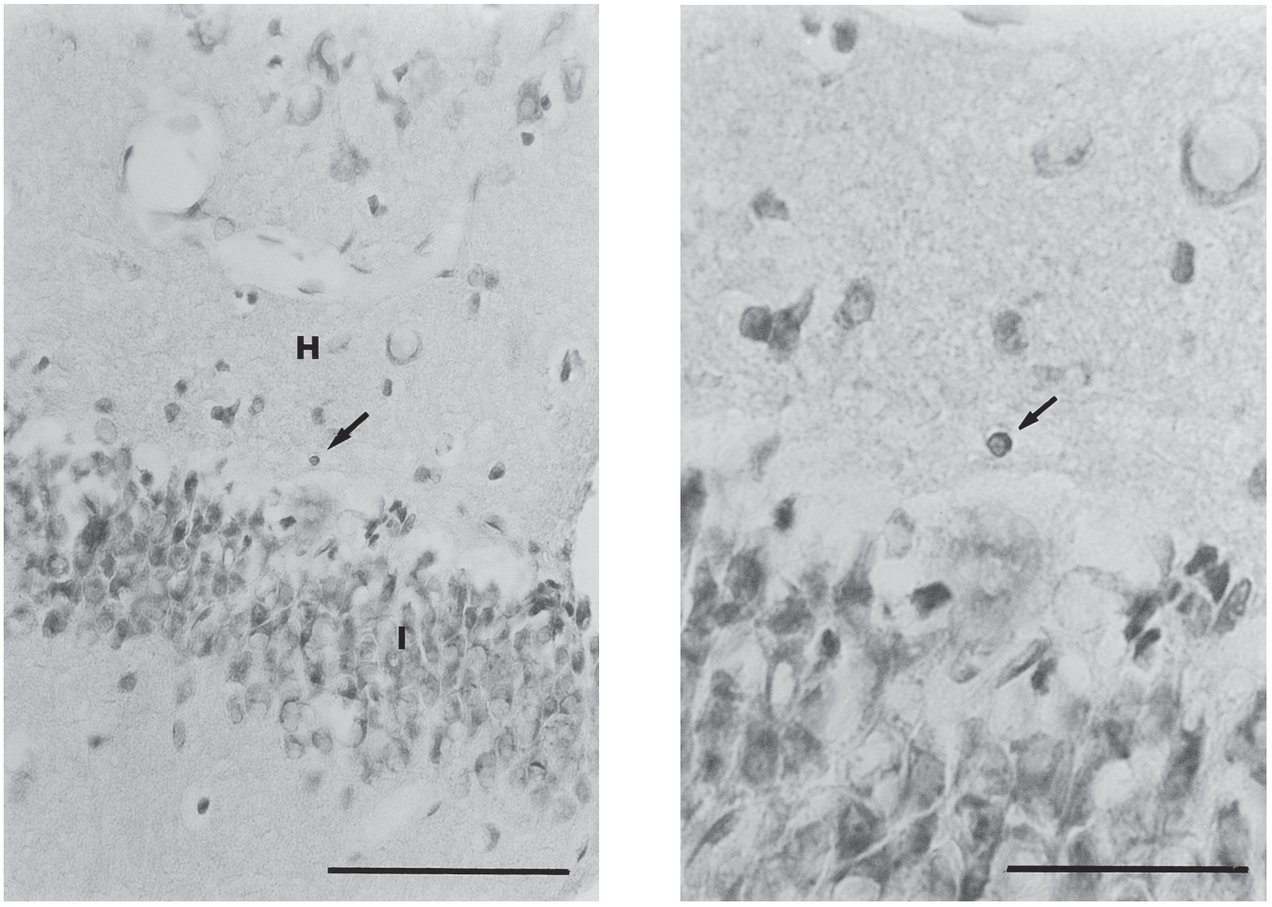

Fig. 2. (**A**) Photomicrograph illustrating TUNEL-positive nucleus within the hilus region of the GD (arrow). Scale bar = 100 μm. (**B**) A higher magnification of (**A**). Scale bar = 40 μm .

TUNEL-Studies

To correlate the morphological alterations observed with the biochemical changes that occur in apoptosis, we examined the TUNEL-based staining of brain slices to visualize nuclear DNA fragmentation, through free 3'-OH DNA end labeling. Thin sections taken from ADX animals revealed the presence of dark brown diaminobenzidine (DAB) stains in the hippocampus (Fig. 2A) and a higher magnification showed its nuclear localization (Fig. 2B). The quantitative analysis is shown in Fig. 2C and indicates that stained nuclei were preferentially located along the hilar border of the dorsal and ventral blade around the GD granule cell layer ($p < 0.001$); an effect prevented by CORT tratment. In contrast, we observed low nuclear staining within the suprapyramidal and infrapyramidal blades of the granule cell layer of GD (Fig. 2C). If we consider the overall GD (including the suprapyramidal, infrapyramidal, and hilus zones), the number of apoptotic cells did not increase further compared to controls, revealing a small contribution of the granule cell layer of GD to the total cell population affected by ADX-induced apoptosis (Fig. 2C). We did not observe any significant nuclear DAB stain in other subfields of the hippocampus (CA1-CA4) by ADX (data not shown).

Changes in mRNA Levels of Genes Regulating Cell Death Five Days after ADX

To determine whether ADX promotes changes in the bcl-2 family mRNA levels which would account for the apoptotic death observed in GD, we measured the changes in *bcl*-X_L, *bcl*-2 and *bax* mRNA content by RT-PCR. As shown in Fig. 3, there was no evident change in the steady-state level of antiapoptotic *bcl*-X_L mRNA. However, ADX induced a significant reduction ($p < 0.05$) to 60% in *bcl*-2 mRNA levels in relation to β-actin an effect which was slightly reversed toward control values after CORT replacement. After five days of ADX (the condition in which we demonstrated hippocampal granular cell death by apoptosis) proapoptotic *bax* mRNA levels were significantly reduced in relation to Sham animals ($p < 0.05$). Furthermore, the administration of CORT to ADX animals reversed *bax*

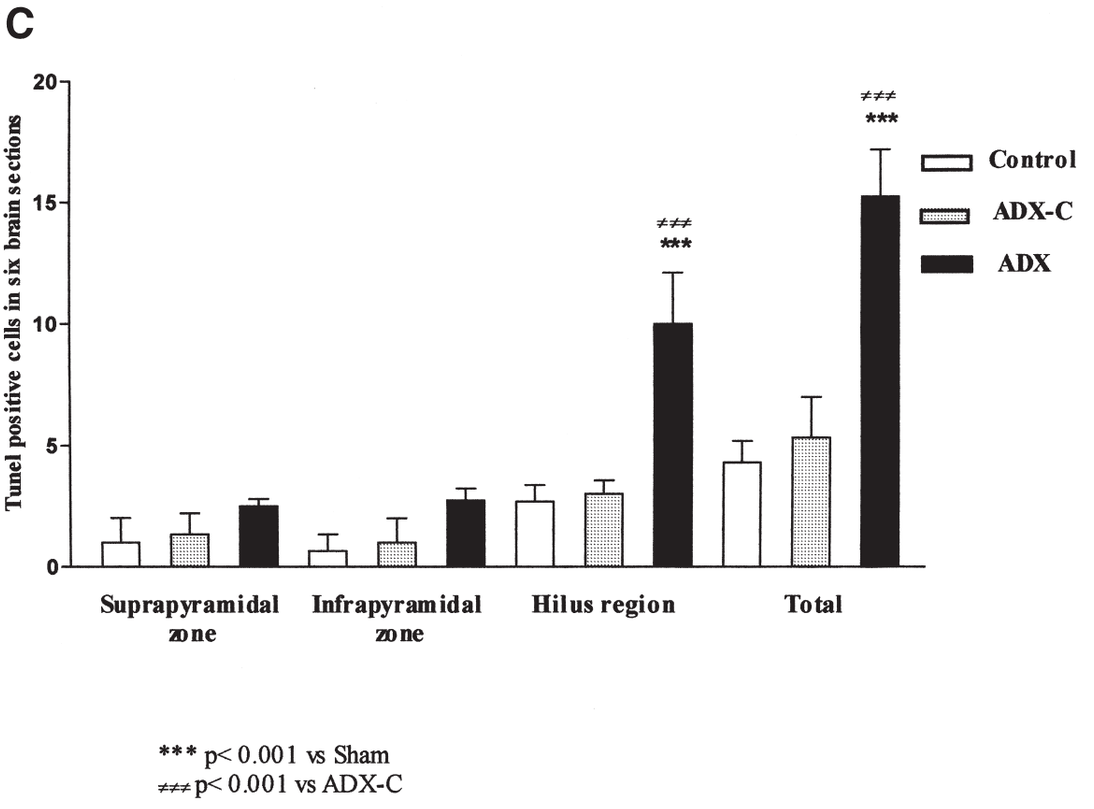

Fig. 2. (**C**) Number of positive nuclei for fragmented DNA as assessed by TUNEL-based technique in the GD and hilus after ADX. Values indicate the mean value of the number of damaged cells present in five sections ± SEM. from 3–4 animals. H = hilus, I = infrapyramidal blade of the granule cell layer, ADX = adrenalectomized animals, ADX-C = adrenalectomized animals with hormonal replacement. ***$p < 0.001$ vs Sham, $^{\neq\neq\neq}p < 0.001$ vs ADX-C.

mRNA levels to the values of Sham animals (Fig. 3), indicating that changes induced by ADX can be prevented by CORT replacement.

Effect of ADX on Pro- and Antiapoptotic Protein Levels

Immunoblot analyses were used to examine whether the changes induced by ADX in Bcl-X_L, Bcl-2, and Bax mRNA levels are also reflected in similar changes in protein expression; they provided additional evidence that proteins related to apoptosis control were regulated after ADX. A representative immunoblot shows Bax, Bcl-2, and Bcl-X_L content under different hormonal conditions (Fig. 4A). The results from independent experiments, where protein content detected by immunoblot was quantified by scanning densitometry (and normalized as percentage of the relative intensity of the bands respect to Sham), are shown in Fig. 4B. Bcl-X_L content in ADX rats was reduced to 60% with respect to the Sham animals but CORT treatment did not restore the control pattern. In contrast, the content of the other antiapoptotic protein Bcl-2 α, was not reduced, indicating that the expression of this protein does not depend on CORT levels. The extracts of control hippocampus present immunoreactivity for the 21-kDa band corresponding to the proapoptotic Bax α protein; ADX induced a significant reduction of 60% in its level which reverted to Sham levels with CORT replacement, showing a correspondence with those changes observed in mRNA levels.

Discussion

The apoptotic response of GD neurons to ADX is highly complex and poorly understood. In the present study, we found that ADX induces:

1. Morphological alterations around the GD with features similar to apoptosis.
2. An increase in the number of cells displaying positive TUNEL.
3. A reduction in the steady-state level of *bax* and *bcl*-2 mRNA in whole hippocampus.
4. A reduction in proapoptotic Bax protein as well as the antiapoptotic Bcl-X_L.

ADX Induces an Apoptotic Profile in the GD

Because ADX increases the proliferation rate of granule cell progenitors in the hippocampus of adult rats and also the number of cells displaying apoptosis *(9,10,29–31)*, it

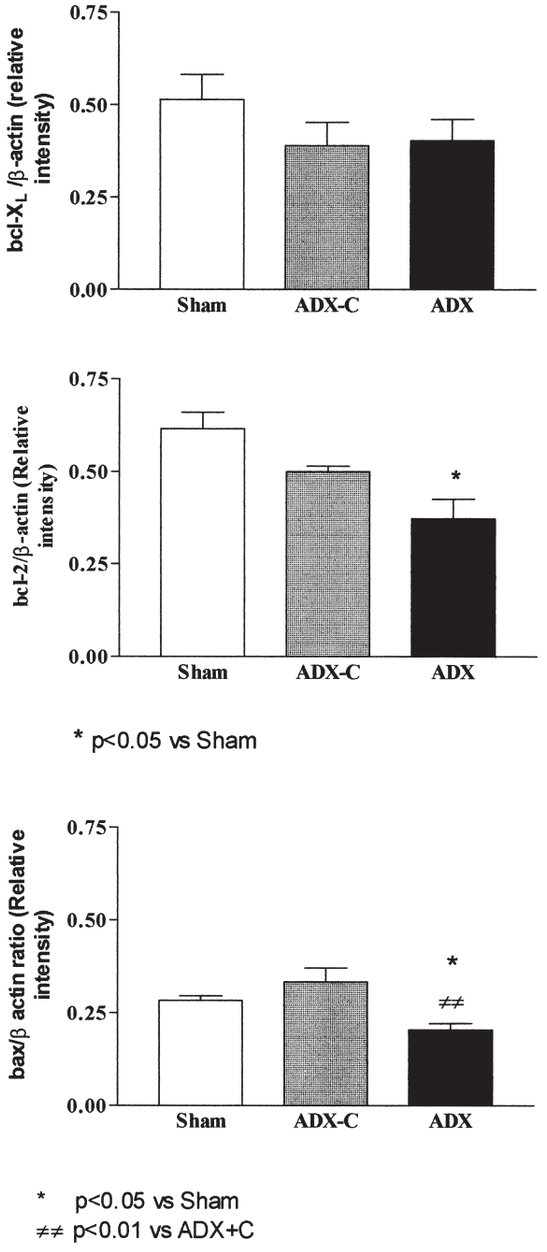

Fig. 3. Changes in the level of three genes belonging to the Bcl-2 protein family: *bax*, *bcl*-2, and *bcl*-X_L in whole hippocampus after ADX. Five µg total RNA from samples were reverse-transcribed and amplified by PCR as described in the text. The PCR products were electrophoresed through a 1.2% agarose gel, stained with ethidium bromide and photographed. The bands were scanned and their intensities were measured by UN-SCAN-IT program. The data were normalized according to the intensity of the β-actin bands and represent the mean ± SEM (n = 4-7). *$p < 0.05$ vs Sham $^{\neq\neq}p < 0.01$ vs ADX-C.

has been assumed that CORT has a dual effect, one inhibiting cell rate proliferation and the other having a trophic influence on granular cell survival *(9,10,29–31)*.

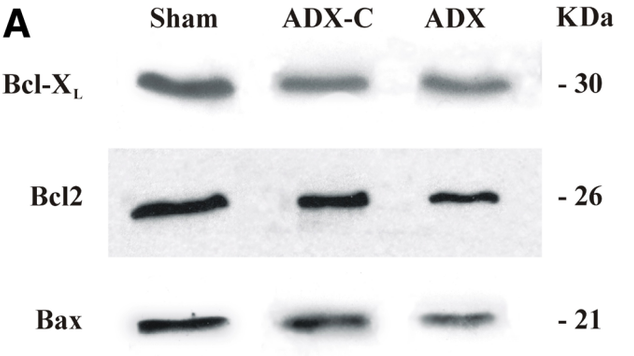

Fig. 4. (**A**) Representative immunoblot showing Bcl-X_L, Bcl-2 and Bax protein levels after ADX. Protein samples (100 µg protein/lane) were resolved by SDS-PAGE, and immunoblotting was performed with Bcl-X_L, Bcl-2 or Bax antibodies as described in Materials and Methods.

Our study demonstrated that ADX induced apoptosis with morphological alterations in granular cells of whole GD that seemed to correspond to mature cells because of their topological localization within this structure. This has also been demonstrated by others *(32)*. Using specific *in situ* labeling of DNA fragmentation, our study demonstrates apoptosis of a subset of cells confined to the hilus region of the GD. These might represent newly formed cells that have migrated to the GD and—due to their dependence on CORT for survival—ADX triggered apoptosis in accordance with other observations *(30,33,34)*. In addition, the fact that the morphological degeneration and nuclear TUNEL stains induced by ADX are prevented by CORT treatment, gives further support for a protective effect of this hormone, probably acting through MR receptors as has been demonstrated *(2,11,12)*. Although we did not identify the specific cell type(s) that are rescued by this adrenal hormone from undergoing apoptosis, it is likely that they are neuronal, as previous reports have shown that more than 80% of the cells found in GD are neurons *(35)*.

ADX Alters the mRNA Levels of Representative bcl-2 Gene-family Members

An important observation of this study is that ADX-induced apoptosis is restricted to cells of the GD, suggesting that neurons of the CA3-CA1 subfields do not require CORT to survive or, more likely, express molecules that protect them from ADX-induced injury. In this context, molecules related to the Bcl-2 family—which control the apoptotic process—are expressed normally in the central nervous system (CNS). Bcl-2 protein, a repressor of apoptosis, is widely distributed throughout the CNS during embryonic development, but declines with age, which suggests a role in neurogenesis *(36)*. However this protein is induced under neuronal insults in cells resistant to apoptosis, indicating a protective role in mature neurons *(13,15,16)*. In contrast, Bcl-X_L, with actions similar to Bcl-2 *(14)*, is expressed in both embryonic *(37–39)* and adult neurons

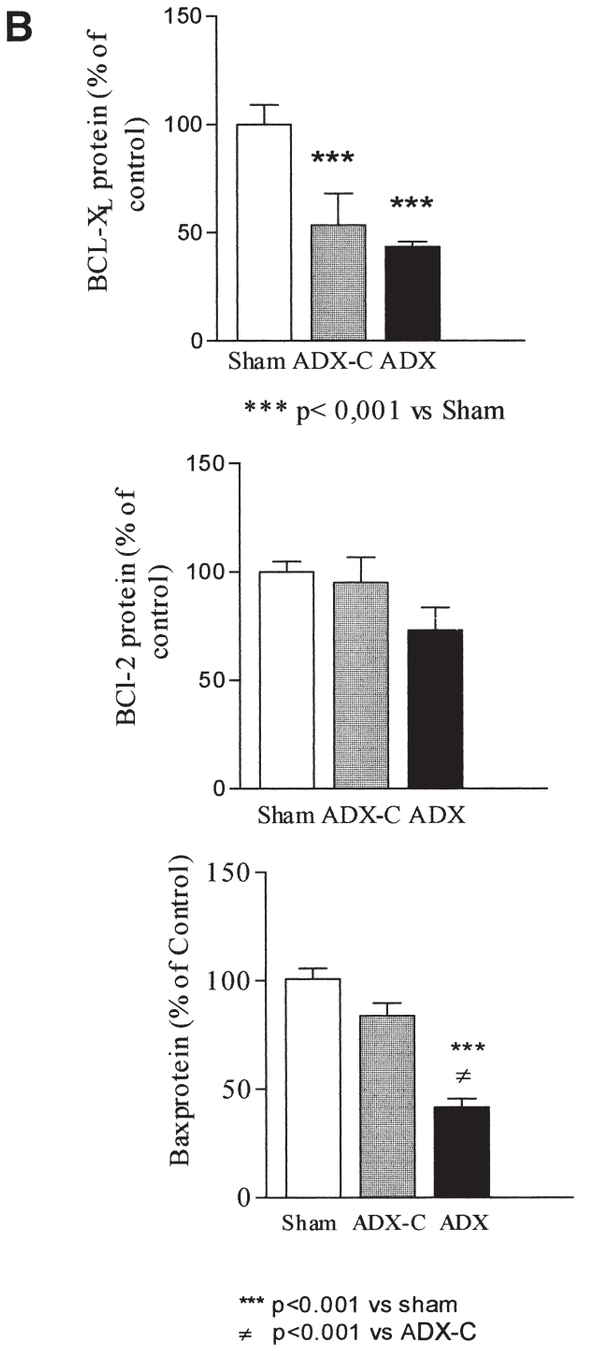

Fig. 4. (**B**) Protein bands from the immunoblots were scanned and the relative intensity of the bands with respect to Sham (100%) was calculated. The data are shown as the mean ± SEM (n = 4–7). ***$p < 0.001$ vs Sham $^{\neq\neq\neq}p < 0.001$ vs ADX-C.

(14,39,40), suggesting its participation in the control of programmed cell death during early development and in the adult CNS *(41)*. Furthermore, Bax is normally expressed in the CNS *(15,42)* and in several models of neuronal delayed cell death, this protein is induced, acting as an apoptosis inducer *(15–19)*. The cell death-inducing actions of this molecule can be antagonized by the presence of Bcl-2 or Bcl-X_L *(20,21)*, indicating that the ratio between Bcl-X_L + Bcl-2 and Bax determines whether an individual cell will survive or succumb by apoptosis.

We found that ADX results in a reduction in the steady-state level of *bax* mRNA, which is restored by CORT replacement. This data strongly suggests that CORT upregulates the gene expression of this apoptotic protein in hippocampus, probably indirectly via activation of MR. However, it has recently been demonstrated by *in situ* hybridization that bax levels are not modified in any hippocampal subfield studied after MR blockade *(43)*. However this technique does not have the high sensitivity of RT-PCR, which was used in our work to observe the reduction in mRNA levels in whole hippocampus. Moreover, our findings indicate that a reduction in both *bax* and *bcl*-2 levels by ADX, with no change in the ratio between pro- and antiapoptotic mRNA, could protect the CA3-CA1 region from apoptosis. Considering also that the hippocampal tissue obtained from ADX rats is comprised of normal neurons and a small population of degenerating cells, it is plausible that ADX triggers apoptosis through a mechanism that involves a reduction in *bcl*-2 and/or an induction of *bax* levels only in cells which are already undergoing apoptosis in the GD. Several investigators have pointed out that p53 induction promotes a delayed cell death in different models of apoptosis *(17,19,44,45)*. This protein binds to a 10-mer DNA consensus sequence located in the *bax* gene, stimulating its expression *(44)*; in contrast p53 negatively regulates bcl-2 gene expression, inducing apoptosis *(46)*. Induction of p53 mRNA and injured hippocampal granule cells are observed after ADX and both events were prevented by CORT administration *(47)*. After ADX we also found an induction of *bax* only in the GD using *in situ* hybridization, an effect prevented by CORT (Fiedler J. L., personal communication), so the possibility exists that the increase in bax and p53 mRNA could be causally linked.

A decrease in *bcl*-2 mRNA levels in hippocampus of ADX rats was also determined, an effect is prevented by CORT administration, suggesting a positive regulation in its gene expression via MR activation. Accordingly, a recent work shows that spironolactone, a MR antagonist, reduces *bcl*-2 mRNA content in the CA1 and CA3 regions of the hippocampus and to a lesser extent in the GD, indicating that the *bcl*-2 gene is positively regulated by the MR *(43)*. Overall, these data suggest that MR modulates *bcl*-2 expression in the hippocampus and may therefore play a role in pyramidal and granular cell survival.

We also demonstrated that *bcl-*X_L levels are independent of CORT treatment of ADX rats as it did not alter *bcl-*X_L mRNA level. These results agree with a previous report which demonstrated that two daily ip administrations of CORT to normal young rats, at doses which activate only MR, did not change the expression of this transcript *(48)*.

The mechanisms by which changes in CORT levels regulate *bcl*-2 and *bax* remain unknown. A GenBanK data-

base search failed to reveal potential MR or GR responsive elements, so it is likely that the observed effects on their mRNA levels are indirect and are probably mediated by other transcription factors which are modified by adrenalectomy.

ADX Reduces the Content of Pro-apoptotic Bax and Anti-apoptotic Bcl-X_L

After adrenalectomy we observed a reduction in Bax level which was reversed by CORT administration, with the same effect on its mRNA level. In contrast, the observed reduction in *bcl*-2 mRNA levels by ADX was not related to Bcl-2 protein content, perhaps indicating that the turnover of this protein is not affected by the hormonal status of the animals. Another plausible explanation is that a reduction in CORT activates some process which increases the half-life of Bcl-2. The other antiapoptotic protein studied, Bcl-X_L, had significantly reduced levels under ADX; this change was not blocked by hormone administration. This implies that the steady-state level of Bcl-X_L is altered by ADX, possibly due to the alteration of a posttranslational process. It remains to be established whether this effect is dependent on other hormones altered by ADX. A recent publication reports the effect of CORT or dexamethasone administration to normal Wistar rats on *bcl-X_L* transcripts, as discussed above *(48)*. The authors also showed a representative immunowestern blot for this protein; they did not however discuss the fact that *Bcl-X_L* is reduced by CORT and that a further reduction is observed by dexamethasone, both agonists having been administrated in two daily doses for eight days *(48)*. Other studies have put forward the notion that MR and GR concentrations depend on the hormonal state of the animal *(49,50)*. Administration of CORT downregulates GR, leaving MR unaffected. Dexamethasone also downregulates GR, but upregulates MR *(50)*. These results could be interpreted as MR and GR agonists inducing downregulation of GR, explaining the decrease of Bcl-X_L under these conditions. In other words, GR activation might upregulate this protein by a mechanism altering the posttranslational process.

In the hippocampus, Bcl-2 and Bcl-X_L proteins are localized in pyramidal cells of the CA3 region, with lower expression in neurons of the CA1 field *(15)*. In contrast, Bcl-2 immunostaining is undetectable in granule cells of the GD *(15)*, in spite of the fact that its mRNA is expressed in this area *(42,51)*. Furthermore, basal Bax immunoreactivity is present in pyramidal neurons in the CA3-CA1 regions of the hippocampus and in granule cells of the GD *(16)*. These findings imply that the reduction observed in Bax and Bcl-X_L under ADX is probably restricted to whole hippocampus.

Thus, an interesting implication of our study is that CORT plays a dual function—acting as a neuroprotective factor which induces an increase in *bcl*-2 or act as neuronal injurer inducing *bax*, and may therefore play a role in hippocampus cell survival. ADX also induces changes in Bcl-X_L levels which probably determine cell survival in the adult hippocampus. However further investigation, probably *in situ* hybridization experiments, are required to establish whether ADX induces regional alterations in bcl-2, bax, and bcl-X_L gene expression or in their gene products and, finally, if these effects are related to the activation of MR and/or GR.

Conclusion

In this study we demonstrated that CORT levels regulate *bcl*-2 and *bax* mRNA levels. We also found that ADX was accompanied by a reduction in Bax and Bcl-X_L levels, the former being restored to control values under adrenal hormone administration. Probably, differential sensitivity to ADX among neurons from the hippocampus suggest that one potential contributor of apoptosis, in at least the GD, could be the ratio between apoptosis repressors (Bcl-X_L and Bcl-2) and apoptosis inducers (Bax). These findings may be of considerable importance, given that the organism is frequently exposed to changing levels of CORT due to variations in adrenocortical activity or sustained high levels of this hormone as in stress situations, either of which could modulate cell survival in the hippocampus.

Materials and Methods

Animals

Adult male Sprague Dawley rats (320–350g), derived from a stock maintained at the University of Chile, were used. They were allowed free access to pelleted food, maintained with controlled temperature (22ºC) and photoperiod (lights on from 0700 to 1900h) and all animals received 0.9% saline drinking water. All animals procedures were performed using protocols approved by the Institutional ethical committee of Faculty Chemical and Pharmaceutical Sciences, University of Chile.

Animal Treatment and Tissue Preparation

Animals were divided into three groups: Bilaterally adrenalectomized (ADX) under anesthesia, ADX animals with hormone replacement (ADX-C), which received drinking water supplemented with 20 µg/mL of CORT (Sigma, St. Louis, MO) and Sham-operated control rats. Five days after ADX, the animals were sacrificed and hippocampi were dissected and rapidly frozen in liquid nitrogen. At the time of sacrifice, trunk blood was collected for the determination of serum CORT levels.

Tissue Fixation

Animals were anesthetized with ether and a sample of blood was taken from the heart. Then they were transcardiacally perfused with 100 mL of 0.9% NaCl followed by 500 mL of 3.7% paraformaldehyde in 0.1 *M* phosphate buffer pH 7.4 (PB). The brain was dissected from the skull and post-fixed overnight and the next day were immersed

Table 2
Primer Sequences used in RT-PCR Analysis

Gene	Primer sequence[a]	cDNA[b] position	RT-PCR product	Reference[c]
Bcl-X	5'-GTCTCAGAGCAACCGGGAGCT-3'	74–94	765 bp	22
	5'-GTGTCTGGTCACTTCCGACTG-3'	780–760		
Bax-α	5'-GGTTTCATCCAGGATCGAGCA-3'	85–105	324 bp	23
	5'-TGATGGTTCTGATCAGCTCGG-3'	408–388		
bcl-2	5'-TGACTTCTCTCGTCGCTACCG-3'	531–541	350 bp	24
	5'-GTCTTCAGAGACAGCCAGGAG-3'	881–861		
β-*actin*	5'-GCATTGTAACCAACTGGGACG-3'	1551–1571	351 bp	25
	5'-CATGAGGTAGTCTGTCAGGTC-3'	2364–2344		

[a]The upper and the lower primers of each amplification pair represent sense and antisense primers, respectively.
[b]Nucleotides correspond to the cDNA published sequence, except β-actin which corresponds to the gene sequence.
[c]Reference from which the primer sequence was derived.

in 30% sucrose in 0.1 *M* phosphate buffered saline (PBS), then kept at 4ºC for two days. Fixed brains were mounted in (optimal cutting temperature, O.C.T. Miles, Elkhart, IN) cryostat embedding medium, frozen in cold isopentane and stored at -80ºC. For the TUNEL-based assay, 15-µm-thick coronal sections, spanning the entire hippocampus, were cut from each brain on a cryostat and placed on glass slides pretreated for electrostatic adherence and dried thoroughly overnight.

In Situ DNA Fragmentation

Coronal cryostat sections were processed according to the TUNEL-based detection kit NeuroTACS™ (R&D Systems, Inc., MN). Briefly, coronal slices of each animal were washed in PBS for 10 min at room temperature, incubated with Neurospore solution for 25 min at room temperature and then washed two times in DNAse-free water for 2 min or in the presence of DNAase as a positive control. The samples were then immersed in an H_2O_2 quenching solution for 5 min, washed in PBS and incubated in 1xTdT Labeling Buffer for 5 min at room temperature. Afterwards, a reaction buffer containing biotinylated-dNTP as well as TdT was applied for 90 min at 37ºC in a humidity chamber. As a negative control, a set of sections was incubated in the absence of TdT. The reaction was stopped with TdT Stop buffer and the slices were washed twice with PBS and incubated with Streptavidin-HRP solution for 10 min. After PBS rinsing, slides were immersed in DAB solution for 4–10 min, washed with PBS and counterstained with Neuro TACS Blue counterstain, dehydrated in a graded alcohol series and mounted with Entellan (Merck). The number of dark brown DAB stains in each nucleus was counted in five brain sections per animal. These sections were selected from rostral to middle dentate gyrus at the region where the suprapyramidal and infrapyramidal blades are joined (at the crest and where the dentate gyrus is oriented horizontally beneath the corpus callosum). In the same section we counted smaller degenerating cells, which showed morphologic breaking up of the condensed nucleus and cytoplasm—characteristics of apoptotic cells.

Corticosterone Radioimmunoassay

Serum corticosterone levels were determined by RIA following the manufacturer's instructions (Sigma, St. Louis, MO). Intra- and interassays variations were less than 5%; the minimal detectable value of CORT was 0.3 µg.

RNA Preparation and Semiquantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from hippocampi using Trizol Reagent® (Gibco BRL, Gaithersburg, MD) and pretreated for 30 min at 37ºC with RNAase-Free DNAase RQ1 (Promega) to eliminate genomic DNA contamination. Reverse transcription (RT) was performed using 5 µg of total RNA from each sample. The reaction was carried out at 22ºC for 10 min and then at 42ºC for 45 min using 1.6 m*M* dNTPs, 10 m*M* dithiothreitol (DTT), 176 n*M* random hexamers (Gibco BRL), 25 U RNAsin (Promega, Madison, WI), 125 U reverse transcriptase (Gibco BRL) and first strand buffer in a final volume of 30 µL. Dilutions of the RT reaction were incubated with 1 U of DNA *Taq* polymerase (Promega), 0.8 m*M* dNTPs, 25 pmol of each primer in a final volume of 50 µL. For each sample, the same RT reaction was used to measure the level of specific mRNA by PCR but in different tubes to avoid competition between primers. The PCR was conducted for 35 cycles (*bcl*-2), 28 cycles (*bcl*-X_L), 26 cycles (*bax*) or 21 cycles (β-actin) and consisted of denaturation at 94ºC for 1 min, followed by annealing at 60ºC for 60 s and extension at 72ºC for 120 s using a DNA thermal cycler (MJ Research Inc, Watertown, MA). Oligonucleotide primers for PCR were designed using published cDNA sequences and analyzed with BLASTA program to avoid nonspecificity (Table 2). To verify that the mRNA samples were not contaminated with genomic DNA, β-actin

primers were designed from a codogenic sequence of the β-actin gene which spans an intronic sequence and generates either a 351 bp fragment or a 809 bp fragment for cDNA or genomic DNA respectively. All RT-PCR and PCR reactions included the use of water instead of template as a negative control. The PCR products were initially sequenced using the ss-dideoxy method (Gibco, BRL) and all corresponded to the predicted sequences. RT-PCR products were electrophoresed in 1.0% agarose gels, stained with ethidium bromide and photographed. The band intensity was measured with the UN-SCAN-IT program. Data were normalized according to the intensity of the β-actin bands.

Western Blot Analysis

Hippocampi were homogenized with a glass-glass homogeneizer in 5 volumes of 10 m*M* HEPES pH 7.9, 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 0.1 m*M* EGTA, 0.1 m*M* EDTA. 0.5 m*M* DTT, 0.1 m*M* Na_3VO_4, 100 µg/mL PMSF, 2 µg/mL leupeptin, 2 µg/mL aprotinin, and 0.05% Triton X-100. Following centrifugation at 10,000*g* for 15 min, the supernatant was collected and the protein content was determined according to Bradford *(26)*. A total of 100 µg of protein extract was resolved on a 15% SDS-polyacrylamide gel (100 V) and blotted onto a 0.45 µm nitrocellulose membrane (1 h, 95 V). The equality of protein in all wells was confirmed by Ponceau Red (Sigma) staining. Membranes were blocked at room temperature for 1 h in PBS containing 0.05% Tween-20 (PBST) with 5% (w/v) nonfat dry milk for Bcl-X_L and Bcl2 determinations or in Tris-buffered saline containing 0.05% Tween-20 (TBST) including 2% gelatin (BioRad) for Bax-α determination. Membranes were incubated 1 h at room temperature with PBST-1% milk containing a rabbit polyclonal anti-rat Bcl-X_L (3 µg/mL)$_L$raised against amino acids 126–188 of the carboxy terminal of human Bcl-X_L (Santa Cruz Biotechnology) or with rabbit polyclonal anti-rat Bcl-2 (3 µg/mL) that recognizes amino acids 20–34 of the protein (Calbiochem, Cambridge, MA). For Bax-α determinations, membranes were incubated in TBST containing 1 µg/mL of rabbit anti-Bax protein Ab-1 (Calbiochem, Cambridge, MA) corresponding to residues 150–165 of the human Bax. After washing, membranes were incubated with peroxidase-conjugated anti-rabbit IgG (Calbiochem) at a 1:5,000 dilution in PBST for Bcl-X_L and Bcl-2 or in TBST for Bax-α determinations. After additional washes, membranes were incubated with Super-Signal Substrate (Pierce Chemical), an enhanced chemiluminescent substrate, according to the manufacturer's instructions and exposed to X-ray film (MR-1 Eastman-Kodak, Rochester, NY). The intensity of the bands was determined and analyzed using the UNSCAN-IT program.

Statistical Analysis

To determine the effect of ADX on serum CORT levels, morphological determinations, gene expression and changes in protein levels, all data were analyzed by one-way ANOVA followed by the Bonferroni post-hoc test. Comparisons were made with respect to age-matched controls and treatment. The level of significance was set at $p \leq 0.05$.

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