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Apoptosis is involved in the anti-proliferative effect of corticosterone in non-tumoral ACTH pituitary cells

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SUMMARY

Glucocorticoids are the physiological negative feedback on ACTH secretion and may be involved in regulating the maintenance of the population of hypophyseal ACTH-cells, because following surgical ablation, adrenalectomy induces an increase in the number of these cells in the early stages. Glucocorticoids have been implicated in the induction of apoptosis in several tissues; but they have been little considered as hormonal inducers of apoptosis in the pituitary gland. By means of a double immunohistochemical study for PCNA and ACTH and a double assay by ISEL and immunohistochemistry for ACTH, the aim of the present study was to analyse whether corticosterone induces apoptosis and inhibits cellular proliferation to control non-tumoral ACTH-cells in the pituitary gland. For this purpose untreated, sham-operated and adrenalectomized rats, treated or not with corticosterone, were compared. The results of the present study demonstrate a very important decrease in proliferation and an increase in apoptosis of pituitary ACTH-positive cells induced by corticosterone, suggesting that the number of pituitary ACTH-producing cells is mainly controlled by glucocorticoids by means of cellular proliferation and apoptosis.

Key words: Hypophysis – Apoptosis – Cellular proliferation – Glucocorticoids

INTRODUCTION

Glucocorticoids are the physiological negative feedback on ACTH secretion and may be involved in regulating the maintenance of the population of hypophyseal ACTH-cells. However other factors as well as vasopressin have also been implicated (McNicol et al., 1990).

Glucocorticoids have been implicated in the induction of apoptosis in several tissues. However, they have been little considered as hormonal inducers of apoptosis in the anterior hypophysis (Nolan et al., 1998).

Apoptosis is considered to be a physiological event aimed at regulating different cell populations in endocrine glands, including the anterior pituitary (Aoki et al., 1998). Apoptotic regulation of ACTH-tumoral cells, AtT-20 cells, has been reported in response to the inhibitory effect of bromocriptine (Yin et al., 1994), in a similar way to the responses observed for other pituitary cells (Drewett et al., 1993; Yin et al., 1993; Aoki et al., 1998).

However, the role of glucocorticoides in controlling both the proliferation and apoptosis of pituitary ACTH-producing cells is not

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well defined, and the main mechanism —proliferation or apoptosis— controlling the population of these cells has not yet been established.

Using a double immunohistochemical study for PCNA and ACTH and a double assay by ISEL and immunohistochemistry for ACTH, the aim of the present study is analyse, if corticosterone induces apoptosis and inhibits cellular proliferation to control the non-tumoral ACTH-cells in the anterior hypophysis. Thus, untreated, sham-operated, and bilaterally adrenalectomized rats, treated or not with corticosterone, were compared.

Materials and methods

Animals and treatments

Fifty male Wistar adult rats (b.w.: 200g) were used, divided into 5 groups (10 animals per group): Ten animals were untreated animals. Twenty animals were sham-operated animals in which the surgical procedure for adrenalectomy was simulated. The animals were anaesthesized by intraperitoneal administration of 10 mg/Kg body weight of Ketolar ®. Ten of the sham-operated animals were treated with two daily doses of 150 mg/Kg body weight of corticosterone (Sigma®) over 7 days. Twenty animals were adrenalectomized rats. After ketolar anaesthesia, adrenalectomy was carried out by dorsal extraperitoneal surgical intervention. The drinking water of these animals was supplemented with 0.9% NaCl and 5% glucose (Silverman et al., 1980). Ten of the adrenalectomized animals were treated with corticosterone in a similar way what was previously described for the sham-operated animals. All animals were kept under standard stable conditions (temperature: 20±2ºC, relative humidity: 50±5%, lights on 8.00 to 20.00h, a Panlab balanced diet and water ad libitum). The animals were handled according to guidelines of the European Communities Council directive (86/609/EEC) and current Spanish legislation for the use and care of laboratory animals (BOE 67/8509-12, 1998).

The rats were sacrificed by decapitation under ether anaesthesia and their pituitaries were carefully removed and fixed in 4% paraformaldehyde in phosphate buffer (0.1M, pH 7.4). The glands were embedded in paraffin and 5 μm coronal serial sections were obtained and processed for study.

Determination of cellular proliferation by double immunocytochemical labelling for ACTH and PCNA

To determine PCNA-positive cells, the biotinylated-avidin-peroxidase immunohistochemical method was implemented in a similar fashion to what has been reported previously (Carretero et al., 1995a, 1995b, 1997). Endogenous peroxidase was blocked with H_2O_2 in methanol and non-specific reactions of the secondary antibody were blocked by incubation in normal goat serum (Dako®, diluted 1:30). Cultures were incubated overnight at 4ºC with the mouse PC10 (PCNA) mAb (Dako®, diluted 1:3000 in TBS). Biotinylated goat anti-mouse IgG (Dako®, diluted 1:100) and Avidin-Biotinylated horseradish peroxidase complex (ABC kit Dako®, diluted 1:100) were successively applied at room temperature for 40 min and 30 min, respectively. Reactions were developed in freshly prepared 3-3'DAB (0.025% in TRIS buffer containing 0.03% of H_2O_2).

To determine ACTH-positive cells, the biotinylated-avidin-peroxidase immunocytochemical method was implemented. Following immunocytochemical detection of PCNA, slides were incubated overnight at 4ºC with the rabbit anti-ACTH polyclonal antibody (Dako®, diluted 1:800 in TBS). Swine anti-rabbit IgG (Dako®, diluted 1:100) and Peroxidase-antiPeroxidase soluble complex (Dako®, diluted 1:100) were successively applied at room temperature for 40 min and 30 min, respectively. Reactions were developed in freshly prepared 4-1Cl-Naphtol (0.03% in TRIS buffer containing 0.03% of H_2O_2).

Controls included substitution of the primary antibody by normal rabbit serum or TBS, as well as omission of the secondary antibody; after both tests, no immunoreactivity was detected. The cross-reaction of goat anti-rabbit antiserum IgG with mouse or rat immunoglobulins was determined by ELISA and was also very low (less than 1%).

Determination of cellular apoptosis by ISEL and immunocytochemistry labelling for ACTH

BrdU in-situ tailing of fragmented DNA. Apoptotic cells were labelled by the in situ DNA end-labelling bromodeoxyuridine (BrdU) method (ISEL), according to the in vivo method of Aschoff et al. (1996). After careful rinsing in 66 mM PBS, slices were incubated with TdT-reaction mixture

(Boehringer, Mannheim®) consisting of 8 µl (200 units) of TdT (Terminal d-Transferase), 8 µl of TdT-reaction buffer, 3 µl of $CoCl_2$ stock solution (25 mM), and 1 µl of BrdU (2.5 mg/ml) in 400 µl of 66 mM TBS (8.8 g NaCl, 6.06 g Tris/1000 ml). Slices were incubated with the reaction mixture at 37ºC for 1 hour. Incorporated BrdU was visualised by immunocytochemistry. After in situ hybridization of BrdU, to study the percentage of BrdU-positive cells the Biot-Stav-Pox immunohistochemical method was implemented. Endogenous peroxidase was blocked with H_2O_2 in methanol and non-specific reactions of the secondary antibody were blocked by incubation in normal goat serum (Dako®, diluted 1:30). Slides were incubated overnight at 4ºC with the mouse anti-BrdU monoclonal antibody (Dako®, diluted 1:250 in TBS). Biotinylated goat anti-mouse IgG (Dako®, diluted 1:100) and Avidin-Biotinylated horseradish peroxidase complex (ABC kit Dako®, diluted 1:100) were successively applied at room temperature for 40 min and 30 min, respectively. The reaction was developed in freshly prepared 3-3'DAB (0.025% in TRIS buffer containing 0.03% of H_2O_2). Controls included replacement of the primary antibody by normal mouse serum or TBS, as well as omission of the secondary antibody or ABC and PAP complex; after both tests, no immunoreactivity was detected. The cross-reaction of swine anti-rabbit antiserum IgG with mouse or rat immunoglobulins was determined by ELISA and was also very low (less than 1%). After ISEL labelling, ACTH immunocytochemical detection was carried out as was previously explained for double immunocytochemical labelling.

Quantification of ACTH-positive and ACTH- and PCNA- or ISEL-positive cells

Ten thousand cells per dish and study were evaluated using an Axioplan Zeiss® microscope equipped with an ocular grid at a final magnification of 400x. The cells were randomly selected from different areas of the gland (1000 cells per pituitary section; the sections were separated by 125 µm from one to another). Only cells with visible nuclear and cellular profiles in the plane of section were considered. ACTH-positive, ACTH- and PCNA-positive or ACTH- and ISEL-positive cells were determined and the percentages of immunoreactive cells were calculated by the double blind method.

Statistical analysis

The results obtained were processed statistically using GraphPad Prism 4, and are expressed as arithmetic means ± standard error of the mean. The differences observed were compared using analysis of variance, accepting $p<0.05$ as significant for the Bonferroni test.

Results

Percentage of ACTH-positive cells (Figures 1 and 2)

The typically stellate ACTH-positive cells of untreated animals were widely distributed in the gland and only 7.59±0.27% of the hypophyseal cells were ACTH-positive cells. The percentage of these cells increased significantly in the sham-operated animals ($p<0.01$), 2 (17.83±1.03) and 4 (19.08±1.01) days after simulation. However, similar values to those of the untreated animals were found after 6 days of simulation (8.66±0.31). Two days after adrenalectomy, no changes in the percentage of ACTH-positive cells with respect to with the sham-operated animals were found (18.43±0.79). However, 4 and 6 days after adrenalectomy, significant increases ($p<0.01$) were found (27.05±2.01 and 31.30±1.19, respectively). When the animals were treated with corticosterone the percentages of ACTH-positive cells in the sham-operated rats were similar to those found in the untreated animals. The percentage of ACTH-positive cells in the corticosterone-treated adrenalectomized animals decreased significantly ($p<0.01$ with respect to adrenalectomized animals). The differences observed with the sham animals were not statistically significant.

Percentage of PCNA- and ACTH-positive cells (Figures 1 and 3)

ACTH- and PCNA-positive cells were not numerous in the pituitary gland of the untreated animals (0.3±0.08%). PCNA- and ACTH-positive cells increased 2 days after simulation in the sham operated animals (7.57±0.26, $p<0.01$ with respect to the untreated animals). This percentage decreased after 4 days (5.02±0.18, $p<0.05$) and was similar to the values found in the untreated animals after 6 days. Two days after adrenalectomy, a significant increase in ACTH- and PCNA-positive cells was found (18.08±0.63, $p<0.01$ with respect to 2-day

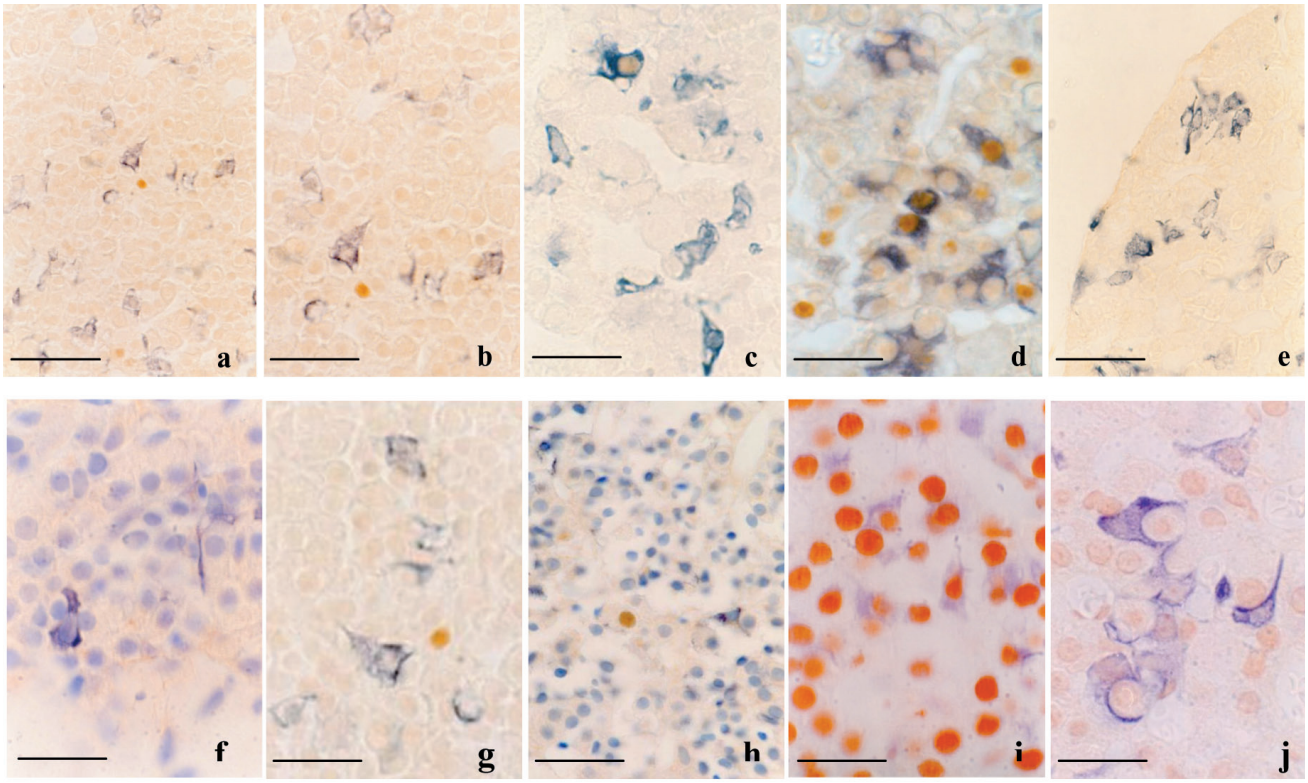

Figure 1. Micrographs showing the morphology and reaction for ACTH-positive cells in untreated, sham, adrenalectomized or adrenalectomized animals and treated with corticosterone. **a-e**: double labelling for ACTH and PCNA: untreated (a,b), sham-operated (c), adrenalectomized over 4 days (d), adrenalectomized and treated with corticosterone over 6 days (e). **f-j:** double-labelling for ISEL and ACTH: untreated (f), sham-operated (g), adrenalectomized (h), adrenalectomized and treated with corticosterone for 2 days (i) and 4 days (j). Scale bars: a,e,h: 50 μm; b,c,d,f,g: 20 μm; i,j: 10 μm.

sham operated animals). This increase was higher 4 days after adrenalectomy (20.98±0.73, $p<0.01$ with respect to 4 days in the sham operated animals). However, although ACTH- and PCNA-positive cells were found 6 days after adrenalectomy (4.47±0.16, $p<0.05$ with respect to 6-day sham operated animals), a significant decrease with respect to the values found on the 2nd and 4th days was observed ($p<0.01$). Treatment with corticosterone abolished the stimulation of cellular proliferation in ACTH-positive cells observed in the sham and adrenalectomized animals.

Percentage of ACTH apoptotic cells (Figures 1 and 4)

ACTH- and ISEL-positive cells were not found in the pituitary gland of the untreated and sham animals. Following adrenalectomy, a slight increase in the percentage of ACTH-apoptotic cells appeared at 4 (1.61±0.03%) and 6 (1.21±0.01%) days post-intervention.

Corticosterone treatment induced an increase in ACTH-apoptotic cells in the sham and adrenalectomized animals. Following treatment with corticosterone, the sham-operated animals showed increased percentages

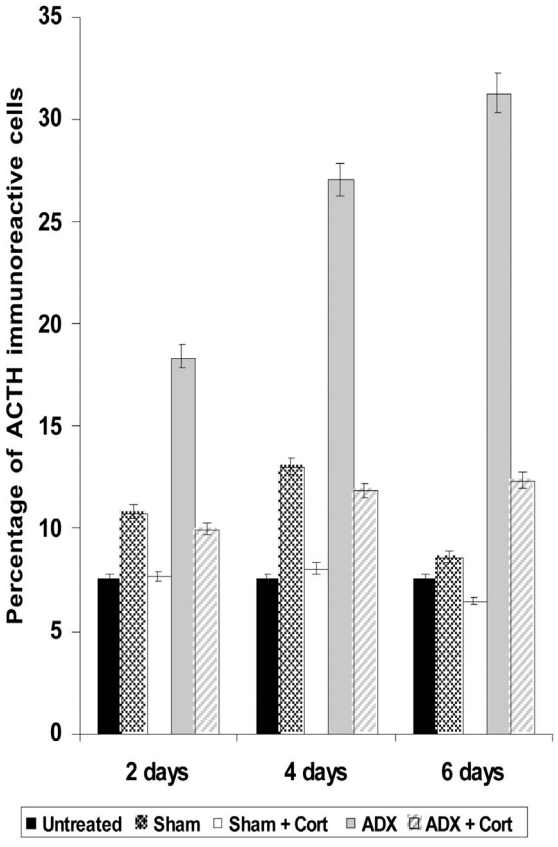

Figure 2. Plot showing variations in the percentage of ACTH-positive cells after adrenalectomy or adrenalectomy and treatment with corticosterone.

with respect to the sham-operated animals after 2, 4 and 6 days of treatment ($p<0.01$).

At two days, about 59% of ACTH-positive cells were apoptotic cells in the pituitary glands of the adrenalectomized and corticosterone-treated animals ($p<0.001$ with respect to the 2-day adrenalectomized rats). The percentage of ACTH-apoptotic cells decreased after 4 and 6 days of treatment with corticosterone in the adrenalectomized rats with respect to the percentages observed at 2 days ($p<0.01$). However, these percentages were about 30%, significantly higher than in the adrenalectomized or sham-operated animals ($p<0.01$).

Discussion

In the literature, low percentages of pituitary ACTH-positive cells have been reported. However, very important differences can be found depending on the methodology used and animal species analyzed. By means of histochemical studies, Siperstein (1963) defined the population of ACTH cells in 0.13% of total pituitary cells. Percentages ranging from 2% to 9.2% have been described using electron microscopy (Surks and DeFesi, 1977; Takahashi and Kawashima, 1982). By means of immunocytochemistry, the percentages ranged from 2.93% to 8.10% (Childs et al., 1982; Dada et al., 1984). The findings of the present study are validated, because the percentage of ACTH-positive cells found in the untreated animals lies within the ranges described by other authors.

The irregular and stellate shape of ACTH-producing cells (Yoshimura y Nogami, 1981; Häusler et al., 1984; Dacheux, 1984; Shirasawa et al., 1985; Asa et al., 1986; Montero et al., 1990) is lost after adrenalectomy, and, as expected, their cytoplasms show a granular pattern of reaction, very similar to those described by other authors in similar treatment (Siperstein y Allison, 1965; Kurosumi y Kobayashi, 1966; Pelletier, 1970; Baker y Dummond, 1972; Kraicer et al., 1973; Bowie et al., 1973; Sánchez et al., 1988).

The inhibitory effects of glucocorticoides on ACTH secretion is well known, and inhibitory feedback is re-established in adrenalectomized animals following treatment with corticoids (Hodges et al., 1962; Jobin et al., 1975; Nicholson et al., 1984).

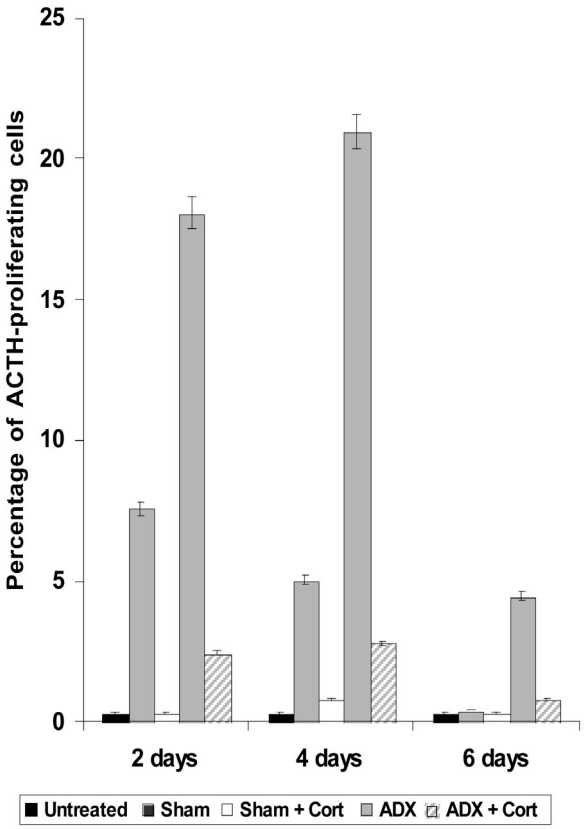

Figure 3. Plot showing variations in the percentage of proliferating ACTH-positive cells after adrenalectomy or adrenalectomy and treatment with corticosterone.

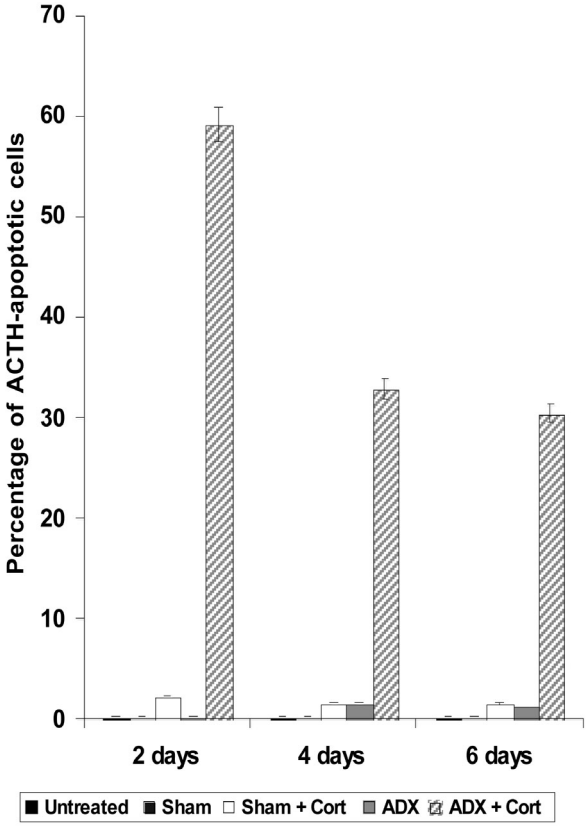

Figure 4. Plot showing variations in the percentage of apoptotic ACTH-positive cells after adrenalectomy or adrenalectomy and treatment with corticosterone.

Together with these regulatory effects of corticoids on ACTH secretion described by other authors, our results demonstrate that the increase in the percentages of ACTH-producing cells induced by adrenalectomy disappears after treatment with corticosterone and that this is accompanied by decrease in ACTH-proliferating cells and increases in ACTH-apoptotic cells. This suggests that proliferation and apoptosis are involved in the maintenance of the physiological percentages of ACTH-positive cells in the pituitary gland and that both proliferation and apoptosis are regulated by glucocorticoid levels.

Regulation of the proliferation and apoptosis of ACTH-positive cells by corticosterone could be carried out in the pituitary gland because it is well known that corticosterone is able to inhibit *in vitro* stimulated ACTH release (Abou-Samra et al., 1986; Lim et al., 2002). However, its regulatory effects could be developed indirectly through other ACTH-regulators that modify the secretion of ACTH and the proliferation of ACTH-producing cells, such as vasopressin, CRF, or interlukin 1β (IL-1β).

IL-1β stimulates the secretion of ACTH and corticosterone (Xiao et al., 2001; Toftegaard et al., 2002-2003; Goshem et al., 2003) and corticosterone inhibits the IL1-β-induced release of ACTH (Philip et al., 2002) and the inflammation-stimulated secretion of IL-1β (Wahl et al., 1975; Besedovsky et al., 1986). Moreover, corticosterone inhibits the stress-stimulated hypothalamic and pituitary release of IL-1β (Nguyen et al., 2000) and blocks the IL-1β-estimulated secretion of ACTH (Holland et al., 2002). Arg-vasopressin and CRF stimulate the secretion of pituitary ACTH (Prickett et al., 2000), and vasopressin stimulates the proliferation of ACTH-producing cells (McNicol et al., 1990). However, Nolan et al. (2004) described that anterior pituitary trophic responses following bilateral adrenalectomy are more likely to be mediated through direct glucocorticoid withdrawal at the level of the pituitary rather than via changes in exposure to hypothalamo-hypophyseal-releasing factors.

In sum, our results, demonstrate that adrenalectomy induces an evident increase in ACTH cells by induction of cellular proliferation; this it is abolished when the animals are treated with corticosterone. Moreover, corticosterone induces apoptosis of ACTH-producing cells mainly when the proliferation of these cells is previously stimulated, suggesting that the maintenance of the pituitary ACTH-cells population is regulated by glucocorticoids.

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