Corticosterone-induced negative feedback mechanisms within the hypothalamo–pituitary–adrenal axis of the chicken

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Abstract

This paper reports the results of in vivo and in vitro experiments on the feedback effects of corticosterone on the hypothalamo–pituitary–adrenal axis in embryos at day 18 of incubation and in 9-day-old chickens. In vivo, a significant negative feedback was detected on the levels of corticotropin–releasing factor (CRF) precursor (proCRF) mRNA and on the plasma concentration of corticosterone, two hours after a single intravenous injection with 40 µg corticosterone. In contrast, the levels of CRF peptide in the hypothalamic area, the CRF receptor type 1 (CRF-R1) mRNA and pro-opiomelanocortin (POMC) mRNA levels in the pituitary were not affected by the in vivo administration of corticosterone. In vitro, incubation with 1 µM corticosterone did not affect the CRF-R1 mRNA levels in the pituitary, but significant feedback inhibition was observed on the POMC mRNA levels. These in vitro effects were the same at the two ages studied. The in vitro feedback effect on the proCRF gene expression, however, differed with age. In 9-day-old animals a decrease in gene expression was observed which was not detectable in embryonic tissue at day 18 of the ontogeny.

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Introduction

It has been known for decades that stressful situations cause an increase in the activity of the hypothalamo–pituitary–adrenal (HPA) axis, and this has been studied in great detail in mammals (Vazquez 1998, Makino et al. 2002, Tsigos & Chrousos 2002) as well as in birds (Harvey & Hall 1990). The normal function of the HPA axis is the result of different feedback mechanisms which take place at various levels of the neuroendocrine system (Keller-Wood & Dallman 1984, Dallman et al. 1994). Being the end product of the action of the HPA axis, glucocorticoids act at several loci to exert a negative feedback, which results in an inhibition of adrenocorticotropic (ACTH) secretion. Many years ago, these feedback effects were first demonstrated in mammals at the pituitary level and at the hypothalamic site of the brain (Sayers & Portanova 1974, Sato et al. 1975). In addition, glucocorticoid feedback effects were detected in other regions of the central nervous system, such as the prefrontal cortex and the hippocampus (Feldman & Conforti 1980, Diario et al. 1993, Mizoguchi et al. 2003). At the hypothalamic level, a major feedback target is the corticotropin–releasing factor (CRF) gene expression via the glucocorticoid receptor (GR) (Kretz et al. 1999, Feldman & Weidenfeld 2002). Glucocorticoids have been found to regulate CRF mRNA at two sites, by inhibiting gene transcription and by decreasing the mRNA stability (Ma et al. 2001). In addition, glucocorticoids were also shown to influence the stimulus-evoked secretion of CRF from the median eminence (ME) (Spinedi et al. 1991). At the level of the pituitary, glucocorticoids have been shown to have an effect on ACTH secretion (Dallman et al. 1987) and a direct inhibitory effect via the GR on the pro-opiomelanocortin (POMC) mRNA level (Eberwine & Roberts 1984). Besides the direct effects on POMC and ACTH, glucocorticoids have also been shown to cause significant reductions in the pituitary content of CRF receptors (CRF-R) (Hauger et al. 1987, Pozzoli et al. 1996, Aguiler et al. 2001). In addition, these steroid hormones also reduce the levels of CRF-R mRNA (Luo et al. 1995, Makino et al. 1995).

In contrast to the enormous number of studies of this feedback topic in mammals, the knowledge of this subject in birds is disappointing even though this mechanism is of great importance in the response to stress. The HPA axis of pigeons is stated to be more sensitive to suppression by glucocorticoids than that of mammals (Westerhof et al. 1994). These observations indicate that some of the negative effects of an excess of glucocorticoids are likely to be at least as frequent and severe in birds as in mammals. Even more than pigeons, the chicken and its reaction to stress has its importance in science and industry (Barnett & Hemsworth 2003).
In this paper we report results of in vivo and in vitro experiments on the feedback effects of cortisol on the HPA axis in chickens. Since the literature showed that in mammals the feedback effects of glucocorticoids differ between animals of different ages (Scaccianoce et al. 1990, Baram & Schultz 1992), we conducted the in vitro experiment with embryos at day 18 of incubation (E18) and with 9-day-old chicks (C9). In mammals, the total feedback mechanism of corticosterone consists of both a fast and a delayed feedback effect at the level of the hypothalamus and the pituitary gland (Keller-Wood & Dallman 1984). The fast feedback mechanism, evident within 15 min and independent of de novo mRNA synthesis (Hinz & Hirschelmann 2000), acts by inhibition of release whilst the delayed feedback inhibits both synthesis and release (Jones et al. 1977). Since we were interested in the delayed, genomic effects of corticosteroid feedback together with the effects on the release of CRF and corticosterone, the first samples were taken 30 min after corticosterone administration (in vivo) or after 30 min of incubation (in vitro).

Materials and Methods

Animals

All studies were conducted on Ross broiler chickens (Avibel, Halle-Zoersel, Belgium) purchased as fertilized eggs or as one-day-old chicks of both sexes. Eggs were incubated in a forced-draft incubator at a temperature of 37.8 °C with increasing humidity and ventilation from day 14 onwards, with continuous lighting and a 45° rotation every hour. The start of incubation was designated embryonic day 1 (E1). Hatched chicks were kept under a 14 h light/10 h darkness photoperiod and fed a commercial mixed diet (Hendrix, Merksem, Belgium). Water and food were available ad libitum. The K U Leuven Ethical Committee for Animal Experiments approved all experimental protocols.

In vivo feedback experiment

Nine-day-old Ross broiler chicks (C9) were given a 100 µl intravenous injection (in the leg vein) of either vehicle or 40 µg corticosterone (0.2 µg/g). This concentration has been demonstrated to be effective in feedback experiments in posthatch chicks (Geris et al. 1999). Corticosterone was first dissolved in a small volume of ethanol (0.5% of the total volume) and then further diluted to the appropriate concentration with saline (0.9% NaCl). Vehicle consisted of saline with the same percentage of ethanol as used in the test conditions. Thirty-five chicks were killed by decapitation without receiving any injection (condition 0 h). Other animals were decapitated 0.5, 1, 2, 4 and 6 h after injection (n=35 animals per condition). Blood was collected from 15 animals/group in heparinized tubes and plasma was stored at −20 °C until assayed for corticosterone. From 3 animals the total diencephalon was dissected and kept intact; from the other 32 animals the diencephalon was dissected and the ME (8 pools of 4) was separated from the rest of the tissue. Pituitaries (3 pools of 5 pituitaries) were collected from 15 animals/group. All of these tissues were snap frozen in liquid N2 and stored at −80 °C until total RNA isolation and CRF extraction. The obtained RNA was assayed for levels of chicken CRF precursor (proCRF) (Vandenborne et al. 2005), POMC (Gerets et al. 2000) and CRF-receptor type 1 (CRF-R1) (Yu et al. 1996) by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Extracts of diencephalon and ME were assayed for CRF protein levels by radioimmunoassay (RIA).

In vitro feedback experiments

In vitro experiments were conducted using a static incubation system with 6-well plates (Nalge Nunc Int., Roskilde, Denmark) at 37 °C. For every condition, 20 diencephalons (pooled per 2) and 30 pituitaries (pooled per 5) from 18-day-old embryos (E18) and 9-day-old chicks (C9), were dissected and placed in 2 ml ice-cold medium M199 ( Invitrogen, USA). All tissues were equilibrated in the medium for 1 h at 37 °C, followed by a stimulation period of varying length (0, 0.5, 1, 2, 4 and 6 h) at the same temperature. During this stimulation period half of the samples were incubated in pure medium, the other half were incubated in 1 µM solution of corticosterone in medium M199 (see Geris et al. 1999). Corticosterone was first dissolved in a small volume of ethanol (0.5% of the total volume) and then further diluted to the appropriate concentration with medium. Vehicle consisted of medium with the same percentage of ethanol as used in the test conditions. After the stimulation period, the tissues were removed from the wells, snap frozen in liquid N2, and stored at −80 °C until total RNA isolation. The RNA obtained was subsequently assayed for levels of chicken proCRF, POMC and CRF-R1 mRNA by semi-quantitative RT-PCR. Secreted hormones were not measured because a quantitative technique sensitive enough to measure chicken CRF and ACTH secreted into the incubation media from these in vitro experiments is not yet available.

Semi-quantitative RT-PCR analysis

Semi-quantitative RT-PCR was used to measure pro-CRF mRNA levels in the diencephalon and CRF-R1 and POMC mRNA levels in pituitary tissue. Total RNA was extracted from tissue using the Total RNA Isolation System (Promega, The Netherlands). The integrity of the RNAs was verified by 1·5% denaturing agarose gel electrophoresis followed by ethidium bromide staining. To
rule out the possibility that PCR products would result from the amplification of genomic DNA contaminating the RNA sample. RNA samples were treated with DNase I using the DNA-free kit (Ambion, Austin, TX, USA). As an extra control, a PCR was performed on total RNA samples that did not undergo a reverse transcription, showing that this indeed did not result in an amplification of cDNA. For RT-PCR, 1 µg total RNA was denatured at 75 °C for 5 min in the presence of an oligo(dT)-primer (Roche, Germany) followed by reverse transcription with AMV reverse transcriptase (2·5 U) (Roche) at 42 °C for 60 min. The subsequent PCR amplification was carried out on 5 µl of the RT reaction mix. The PCR reaction (20 µl) contained PCR buffer (10 mM Tris-HCl pH 9·0, 1·5 mM MgCl₂, 50 mM KCl, 0·1% Triton X-100 and 0·01% gelatin), 1 mM of each dNTP (Roche), 1 µM of each primer, and 1 U SuperTaq DNA polymerase (HT Biotechnology, Cambridge, Cambridgeshire, UK). After initial denaturation by incubating at 94 °C for 5 min, a number of cycles (35 for proCRF and CRF-R1 and 25 for POMC) including denaturation for 30 s at 94 °C, annealing of the primers for 1 min at 55 °C (proCRF and CRF-R1) or at 70 °C (POMC) and extension at 72 °C were performed, followed by a final elongation step at 72 °C for 5 min. The primers (Table 1) designed for the semi-quantitative RT-PCR analyses were based on the known sequences of chicken proCRF (accession # AJ621492) (Vandenborne et al. 2005), chicken CRF-R1 (accession # L41563) (Yu et al. 1996) and chicken POMC (accession # AB019555) (Gerets et al. 2000). The number of cycles used for each gene was empirically determined using a linear amplification range beginning at 20 cycles and increasing to 50 cycles in 5-cycle increments. The midpoint of the linear part of the amplification curve was chosen as the optimal number of cycles for each gene. PCR products were visualized on a 1% agarose gel containing ethidium bromide (0·5 µg/ml).

Radioimmunoassay for CRF

The CRF content in diencephalon and ME was measured using an adaptation of the method of Mastorakos et al. (1995) as described by Boorse and Denver (2004). This RIA was validated for use in chicken tissue extracts (Vandenborne et al. 2005).

CRF extraction from tissue

Tissue samples (diencephalon and ME) were dissected and weighed. Ten volumes of boiling acetic acid (2 M) were added (1 ml per 0·1 g tissue weight) and samples were boiled for 10 min. This was followed by addition of β-mercaptoethanol to a final concentration of 5% (v/v) and incubation of the samples for 10 min at room temperature. Samples were then placed on ice and homogenized. The homogenate was centrifuged for 30 min at 15 000 × g (4 °C) and the supernatant was transferred to a new microcentrifuge tube. Three volumes of acetone were added (0·3 ml per 0·1 g tissue) and the sample was mixed thoroughly. The mixture was then centrifuged (15 min, 15 000 × g, 4 °C) and the supernatant was dried under vacuum. The residue was dissolved in 500 µl CRF assay buffer (0·1 M Na₂HPO₄·2H₂O, 0·05 M NaCl, 0·01% (m/v) NaN₃, 0·1% (m/v) BSA, 0·1% (v/v) Triton X-100, pH 7·4).

RIA

In this technique an antibody against Xenopus laevis CRF (xCRF) (Boorse & Denver 2004) was used. The xCRF differs from the mutually identical chicken (c), rat (r) and human (h) CRF (c/r/hCRF) in only 3 of 41 residues (Stenzel-Poore et al. 1992, Vandenborne et al. 2005) and the r/hCRF exhibits identical potency and parallelism with the xCRF standard curve in the xCRF RIA (Boorse & Denver 2004). The tracer, [¹²⁵I]Tyr⁴-r/hCRF, was purchased from PerkinElmer (Wellesley, MA, USA). Synthetic r/hCRF (Neosystem, Strasbourg, France) was used to produce a standard curve (0·05 ng/ml to

Table 1. Primers used for semi-quantitative RT-PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense</th>
<th>Antisense</th>
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<tbody>
<tr>
<td>proCRF</td>
<td>5'-TCTCCCCGACCTGACTTGC-3'</td>
<td>5'-GAGGTGACATCAAGACAGCA-3'</td>
</tr>
<tr>
<td>CRF-R1</td>
<td>5'-CCATCATGCTGGCTCGCCGCTGG-3'</td>
<td>5'-ACGCTCTCCGACACAGCGAA-3'</td>
</tr>
<tr>
<td>POMC</td>
<td>5'-CAGGCGGTGTCAGGAACAGC-3'</td>
<td>5'-CTCCATGCGGTAAACTCTTCAG-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-CAAAGGCAAACAGAGAAGA-3'</td>
<td>5'-TCAAGCAAAATTTCCTTCT-3'</td>
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6.25 ng/ml, diluted in CRF assay buffer). Standard and sample solutions (100 µl) were incubated with 200 µl CRF assay buffer and 100 µl anti-xCRF (diluted 1/1500 in CRF assay buffer) in polystyrene tubes for 24 h at 4 ºC. Subsequently, radioiodinated r/hCRF (100 µl; 15000 c.p.m. per tube) was added and the tubes, with a final volume of 500 µl, were incubated for another 24 h at 4 ºC. Separation of free and bound label was achieved by immunoprecipitation using 100 µl Sac-cell anti-rabbit γ-globulins (IDS, Boldon, Tyne and Wear, UK). After 1 h of incubation at room temperature and then centrifugation (1500 × g, 15 min, 4 ºC), the radioactivity of the precipitate was measured in a γ-counter. Intra-assay and inter-assay coefficients of variation were 1.6% and 4.1% (Vandenborne et al. 2005).

Corticosterone

Plasma concentrations of corticosterone were determined using a commercial RIA kit (ICN Biomedicals, Irvine, CA, USA), which has been adapted and validated for use in chicken plasma (Darras et al. 1996). Intra-assay and interassay coefficients of variation of the kit were 7.3% and 6.9% respectively.

Validation of the in vitro system

In order to validate the in vitro system used, the viability of the tissues was tested. For the diencephalon, this was carried out by pre-incubating them in medium for different periods of time (1, 2, 4 and 6 h), and testing if a 1-h KCl (56 mM) treatment was still able to stimulate somatostatin release from these diencephalons (Wren et al. 2002). Similarly, the viability of the pituitaries after 1, 2, 4 and 6 h was tested by examining their ability to release thyrotropin (alpha-subunit) upon a 30-min stimulation with thyrotropin-releasing hormone (TRH) (100 nM). Both somatostatin and alpha-subunit were measured using RIAs, as described by Geris et al. (1998) and Berghman et al. (1993) respectively. These experiments showed that both tissues at both ages (C9 and E18) were viable and able to react to stimuli during the entire 6-h study period (data not shown).

Statistics and data analysis

Statistical analysis of the data obtained with the RIAs (corticosterone and CRF peptide) was conducted using the Graphpad Prism software (version 4.0, GraphPad Software, San Diego CA, USA). Statistical significance was assessed by two-way ANOVA (effect of both time and treatment), followed by Bonferroni post tests. Data from the RT-PCR assays were analyzed for the effect of treatment by a non-parametric test (Kruskal-Wallis) using the SAS program (SAS Institute, Cary, NC, USA).

Results

In vivo feedback experiment

Figures 1 and 2 show the results of the in vivo experiment. Two-way ANOVA showed that there is a significant time × treatment interaction (P<0.0001) for the plasma corticosterone concentrations. Thirty minutes after injection, the circulating corticosterone concentration was increased (Fig. 1A), compared with the control group (P<0.001). The highest level of corticosterone, caused by the injection together with the stress of handling, was probably already reached before this time. Two hours after the injections the corticosterone concentrations in the plasma of the corticosterone-injected animals were significantly lower (P<0.01) compared with the control animals that received a single injection with saline. In contrast, we could not detect any significant treatment effects on the content of CRF peptide in the diencephalon (Fig. 1B) or in the ME (Fig. 1C). However, the CRF peptide content in the diencephalon showed significant changes in the course of time, which could be due to the stress of handling.

Two hours after the injection the level of proCRF mRNA (Fig. 2A) was significantly lower in the test animals compared with the control animals (P<0.05). However, the levels of POMC mRNA (Fig. 2B) and CRF-R1 mRNA (Fig. 2C) remained unaffected by the administration of corticosterone.

In vitro feedback experiments

The results of the in vitro experiments using C9 diencephalons and pituitaries are shown in Fig. 3, while Fig. 4 shows the effects in E18 embryos. In the diencephalon of C9 chicks, the levels of proCRF mRNA were significantly lower (P<0.05) after 2 h of incubation with the corticosterone-supplemented medium compared with those incubated with the control medium (Fig. 3A), whereas this effect was not observed in E18 animals (Fig. 4A). The POMC mRNA levels were also significantly inhibited by corticosterone after 2 h (P<0.05) in the C9 pituitaries (Fig. 3B), while this effect was even more pronounced in the E18 animals (Fig. 4B), where the inhibition was still significant after 4 h of incubation (P<0.05). The levels of CRF-R1 mRNA were not affected by the corticosterone treatment in either of the age groups tested (Figs 3C and 4C).

Discussion

Glucocorticoids are well recognized for their regulatory actions on the HPA axis in mammals (Dallman et al. 1994).
Because of the lack of information on this topic in birds, the aim of this study was to investigate the glucocorticoid feedback mechanism in vivo as well as in vitro in chicken brain and pituitary.

In the plasma of the chicken, the effect of an exogenous administration of corticosterone was directly detectable.

Figure 1 The effect of an in vivo injection with saline (solid bars) or with 40 μg corticosterone/animal (open bars) on the plasma corticosterone concentration (n=15) (A), the amount of CRF in the diencephalon (n=8) (B) and in the ME (n=8 pools of 4) (C) of 9-day-old chickens. Data shown represent means ± S.E.M. Statistically significant differences between treatments are indicated as follows: **P<0.01, ***P<0.001.

Figure 2 The effect of an in vivo injection with saline (solid bars) or with 40 μg corticosterone/animal (open bars) on the mRNA levels of proCRF (A) in the diencephalon (n=3) and of POMC (B) and CRF-R1 (C) in the pituitary (n=3 pools of 5) of 9-day-old chickens. mRNA levels are expressed as relative units (expression after normalization with β-actin expression). Data shown represent the median ± range. Significant statistical differences between treatment groups as determined by the Kruskal-Wallis test are indicated as follows: *P<0.05.
in the corticosterone concentration. After a steep rise caused by the injection, the concentration of this steroid decreased to levels lower than the corticosterone
concentration in control animals. It should be stated here that this endogenous decrease could even be underestimated because of the administration of exogenous corticosterone.

At the hypothalamic level, the major target of the feedback mechanism by glucocorticoids in mammals seems to be CRF gene expression, which is affected via the GR (Jingami et al. 1985, Herman et al. 1990, Kretz et al. 1999). In our experiments, the levels of proCRF mRNA in C9 chicks were significantly decreased in vivo 2 h after corticosterone administration. The negative feedback was also observed on the proCRF mRNA levels in diencephalons from C9 chicks in vitro. In contrast, this in vitro feedback on proCRF gene expression was not observed in the embryonic diencephalons. This observation is in agreement with earlier studies in mammals where the general activity of the HPA axis, especially at the hypothalamic level, seems to increase with age (Scaccianoce et al. 1990, Baram & Schultz 1992), an observation that was linked to the absence of a glucocorticoid negative feedback effect on CRH gene expression. However, in chickens it has been shown that the glucocorticoid feedback already becomes functional between E15 and E18 (Bordone et al. 1997), although at this stage in development the binding of corticosterone to its receptor is significantly decreased compared with the situation in posthatch chicks. The latter observation might also explain why, in our current study, corticosterone had no effect on proCRF mRNA levels in E18 chicken embryos, whereas it decreased proCRF mRNA levels in posthatch chicks.

In contrast, the amount of CRF protein in the total diencephalon and in the ME was not affected. The reduced amount of proCRF mRNA suggests a decreased synthesis. It is possible that the amount of CRF peptide is lowered in the paraventricular nucleus (PVN), but that this effect is masked by the presence of other pools of CRF peptide in the total diencephalon (Richard et al. 2004). In addition, the lack of feedback effects at the CRF peptide level in the ME suggests that, in chickens, glucocorticoids do not influence the secretion of CRF from the ME. The latter is in contrast to the mammalian situation, where the ME seems to be an important site of glucocorticoid action (Jones et al. 1977, Keller-Wood & Dallman 1984, Spinedi et al. 1991, Feldman et al. 1992, Feldman & Weidenfeld 2002). The decrease in CRF content seen in the diencephalon during the first hour after in vivo treatment with corticosterone could be caused by the stress of handling. When chickens receive an injection they are likely to show stress effects, a steep increase in corticosterone concentration being the most important consequence. However, this increase was not detected in our experiment since this effect is known to occur immediately (within 10 min after handling) and to have disappeared 30 min after the stressful action (Harvey & Hall 1990). This short increase in corticosterone immediately after the injection may have caused its own negative feedback, resulting in the decrease in CRF content in the diencephalon during the first hour of the experiment.

At the level of the pituitary, we were interested in the effect of corticosterone administration on the level of CRF-R1 mRNA since this is the CRF-R subtype that is involved in regulation of ACTH secretion from the corticotropes (Yu et al. 1996). In both the in vivo and in vitro experiments the levels of CRF-R1 mRNA remained constant after corticosterone treatment. This suggests that, in chickens, glucocorticoids do not exert any feedback regulation on the pituitary CRF-R1 mRNA levels. This is in contrast to what has been found in mammals, where it was demonstrated, both in vivo and in vitro, that glucocorticoids have an inhibitory effect on the CRF-R1 gene expression (Luo et al. 1995, Makino et al. 1995, Pozzoli et al. 1996). In addition, the amount of CRF-R1 is also decreased by glucocorticoids in rats (Childs et al. 1986, Hauger et al. 1987). Since we did not measure CRF-R1 protein levels, we cannot exclude the possibility that in chickens also the amount of CRF-R1 in the pituitary is affected by glucocorticoid treatment.

At the pituitary level, we also measured the levels of POMC mRNA, since POMC is the precursor of ACTH which stimulates the secretion of glucocorticoids from the adrenals. In mammals, glucocorticoids are known to cause a decrease in POMC gene transcription (Eberwine & Roberts 1984, Jingami et al. 1985). In chickens, in vitro incubation of chicken pituitaries with corticosterone had a direct inhibitory effect on the POMC mRNA levels. In pituitary cultures from C9 chickens this inhibition was only detected after 2 h, whereas in the E18 pituitaries, the duration of this effect was longer and remained significant until after 4 h of incubation. On the other hand, in the in vivo experiment, POMC mRNA levels were not significantly affected by the corticosterone injection. The effect seen in vitro is possibly masked in the in vivo experiment. A possible explanation can be found in the stress caused by handling during the in vivo experiment. Due to stress, the levels of β-endorphin rise in both mammals and birds (Johnston & Negro-Vilar 1986, Barna et al. 1998). Since POMC is also the precursor for β-endorphin, a higher need for this peptide during stress could account for the inability of corticosterone to induce an in vivo negative feedback effect on the expression levels of POMC mRNA.

In summary, a single injection of corticosterone caused a significant decrease in plasma corticosterone levels after 2 h. This low concentration is the result of a cascade of feedback effects beginning in the PVN of the hypothalamus, passing through the pituitary and ending in the inhibition of corticosterone production by the adrenal cortex. In vivo, administration of corticosterone caused a negative feedback at the proCRF mRNA levels in the hypothalamic area. In contrast, neither POMC nor CRF-R1 mRNA levels were affected by corticosterone.
administration. In vitro, there is no direct effect noticeable on CRF-R1 mRNA levels, but a significant feedback inhibition occurs on POMC mRNA levels. These observations are the same at the two ages studied. However, the in vitro negative feedback on the proCRF gene expression differs with age. A decrease is observed in C9 animals which is not detectable in E18 embryonic tissue, possibly due to a lowered degree of binding to the GR at this embryonic stage.

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