

Short-Term Adrenalectomy Increases Glucocorticoid and Mineralocorticoid Receptor mRNA in Selective Areas of the Developing Hippocampus¹

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In the central nervous system, adrenal steroids bind to two different types of corticosteroid receptors: glucocorticoid (GR) or mineralocorticoid (MR). *In vitro* biochemical and autoradiographic techniques have been used to infer GR and MR protein abundance in the hippocampus. Adrenalectomy (ADX) is routinely performed to measure the normal receptor number in absence of corticosterone (B), which would otherwise interfere with the binding reaction. The developing rodent has low basal B levels until the third week of life. We were interested in whether removal of circulating B may have a greater impact in the developing hippocampus than in the adult animal. In this study we examined the effect of a 14-h ADX on hippocampal GR and MR binding capacity (B_{max}) by standard binding techniques and on gene expression by *in situ* hybridization. ADX was performed on Day 6, 10, 14, 18, 22, 28, 35, and 45 and on adult animals. GR B_{max} increased from Day 6 to adult levels by Day 22 ($d6 = 159.0 \pm 27$; $d22 = 369.1 \pm 43$; $a = 344.8 \pm 23$, fmol/mg protein \pm SE). In contrast, MR B_{max} had adult levels on Day 6 and increased above these until Day 45, when it decreased and approached adult concentrations ($d6 = 83.2 \pm 22$; $d45 = 123 \pm 23$; $a = 76.9 \pm 13$, fmol/mg protein \pm SE). The greatest absolute increase for both receptors occurred between Days 22 and 45 and correlated with increases in GR and MR gene expression. Moreover, age- and region-specific changes were evident in the developing hippocampus. In addition, the adult animal also exhibited an MR mRNA upregulation after 14 h of adrenalectomy. We propose extreme caution when interpreting GR and MR B_{max} values obtained after short-term adrenalectomy in both adult and developing animals since upregulation of these genes is evident in this short time frame. © 1993 Academic Press, Inc.

INTRODUCTION

Corticosteroid suppression of the hypothalamic-pituitary-adrenal axis (HPA) is of vital importance for restoring homeostasis in an organism. High circulating corticosterone levels cause neuronal loss (1, 2), immunosuppression, and metabolic alterations (3) in the adult animal. During development, high levels of corticosteroids have greater impact in brain, where cell proliferation, myelination, synaptogenesis, and axonal growth are inhibited (4, 5). There is an adaptive and protective period during the early postnatal life which is characterized by low stable circulating levels of corticosterone, undetectable corticosterone circadian rhythmicity, and reduced capacity to secrete corticotropin hormone (ACTH) and corticosterone in response to stressful stimuli (6). By the end of the second week of life the HPA shows signs of maturation, with gradual increases in basal corticosterone levels, greater adrenocortical response to stress, and gradual emergence of the corticosterone circadian rhythm. However, the ability to rapidly terminate the secretion of corticosterone at the end of a stressor appears to be delayed beyond the weaning of the pup from the mother, a process which normally occurs by 21 days of age (7).

The ability of corticosterone to terminate a stress response is presumably mediated by glucocorticoid receptors in the brain and pituitary (8). In the central nervous system, adrenal steroids bind to two different types of corticosteroid receptors: glucocorticoid (GR or Type 2) or mineralocorticoid (MR or Type 1). The two receptor subtypes differ in their affinity for corticosterone, their neuroanatomical distribution, their regulation, and their function (9-11). The classical GR binds corticosterone and dexamethasone, while, of these two ligands, MR binds corticosterone only. In addition, MR binds corticosterone with greater affinity than does GR. Original studies by McEwen *et al.* showed the greatest concentration of corticosterone binding in brain was in the hippocampal formation (12). Subsequently, autoradiographic studies using tracer amounts of [³H]corticosterone have shown labeling

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of MR primarily to cell nuclei of neurons in the hippocampus and lateral septum (13, 14). [³H]Dexamethasone used to label GR shows a strikingly different pattern, being retained widely in brain by both neurons and glia, but being especially prominent in the paraventricular nucleus of the hypothalamus and the pituitary (15–17). The fact that MR binds corticosterone with a higher affinity than GR explains the extensive occupation of this hippocampal receptor at physiological levels of corticosterone. This has led to the initial notion that MR is key to circadian rhythmicity, while GR, which requires high corticosteroid levels for occupation, may be involved in inhibition of the stress response (11). There is now evidence suggesting that both hippocampal GR and MR are involved in stress response, circadian rhythm, and feedback inhibition (18, 19). Partial or complete hippocampal lesions have a marked effect on HPA activity (20–24). Hippocampal removal elicits marked increase of CRH and vasopressin mRNAs in paraventricular neurons of the hypothalamus (25), pointing to a potential inhibitory role of the hippocampus on HPA function. Studies performed to distinguish between the importance of each of the receptor systems in feedback have shown that the administration of MR antagonists induces prolonged adrenocortical secretion following stress (26), suggesting that MR has a role in mediating feedback control. However, occupancy of both hippocampal receptor types appears to be important for the feedback suppression of CRH secretion during stress (27).

Studies addressing the specific regulation of hippocampal MR and GR protein have proven critical for our understanding of the modulation of the HPA. To this effect, *in vitro* biochemical and *in vivo* autoradiographic techniques use specific ligands which allow for the discrimination between the binding capacity of the two receptor subtypes (28). However, adrenalectomy (ADX) is routinely performed in these experiments in order to measure the normal receptor number in absence of endogenous hormone. Under these circumstances, adult animal studies have shown that the binding capacity of the hippocampus for corticosterone increases in a biphasic pattern (29, 30). A rapid initial increase occurs during the first 2 h, followed by a second rise after 12 h post-ADX. This second rise after 12 h post-ADX reaches a plateau 3–5 days after surgery. It is widely accepted that the binding capacity measured after 2 h and up to 14 h post-ADX reflects the endogenous binding of the hippocampus in the absence of endogenous corticosterone, while increases beyond this time reflect upregulation and increased synthesis of the receptors (31).

A valuable method for assessing cellular modulatory and synthetic activity at the genomic level is *in situ* hybridization. By this method it has been shown that the gene expression of MR and GR mRNAs is in agreement with the neuroanatomical distribution revealed by receptor binding (32, 33). This technique does not require the

removal of endogenous corticosterone. Moreover, the measurement of specific mRNA levels which code for GR and MR receptor can be used to infer the state of *activation* of the cells synthesizing these receptors. It also offers the unique opportunity of addressing the regulation of GR and MR in an anatomical context in brain structures.

Several anatomical and biochemical studies have been performed to characterize the hippocampal glucocorticoid receptor system in the developing rat based on the receptor binding capacity methodology (34–36). As mentioned above, most of these studies relied on the removal of the adrenals 12 to 14 h prior to the determination of the receptor binding capacity. Based on these studies, one would conclude that the developing animal's failure to terminate the adrenocortical response to stress is not due to a decrease in glucocorticoid or mineralocorticoid receptor number. Given the relatively stable corticosteroid levels which "bathe" all organs during early postnatal life, and given that adrenalectomy will not significantly reduce these circulating corticosterone levels at very early ages, one would expect that the determination of the receptor binding capacity is indeed accurate during the first 2 weeks of life. However, we reasoned that beyond 2 weeks of age, the developing hippocampal glucocorticoid system may be more sensitive to changes in corticosterone levels than the same treatment in the adult animal. Therefore, ADX, even for the limited time required for the determination of hippocampal GR and MR binding capacity, may indeed be of greater consequence than that for the adult animal. In this study we examined the effect of 14 h of adrenalectomy on hippocampal GR and MR binding capacity and on gene expression. We used standard binding techniques to evaluate GR and MR binding capacity and *in situ* hybridization to assess the state of activation of the hippocampal cells synthesizing these receptors. We specifically asked, "is the increase in receptor number seen in the developing animal reflecting normal developmental progression or is the increase due at least in part to the removal of glucocorticoids?" We find that the developing hippocampus appears exquisitely sensitive to changes in its corticosterone milieu.

MATERIALS AND METHODS

Animals

Sprague-Dawley female rats (Charles Rivers, Wilmington, MA) were mated in our facilities. Pregnant rats had free access to water and food and were kept on a 14-h light, 10-h dark cycle. On the day after delivery, which was considered Day 1, the litters were removed from the mother, randomly mixed, and 12 pups, half females and half males, were returned to a lactating mother. Pups were randomly selected from 25 litters. The litters were undisturbed except for a brief period for the adrenalectomy.

Weaning from the mother was performed when the pups were 21 days old. Animals were sacrificed in the morning (0900 to 1100) at 10, 18, 28, and 35 days of age. Sham and adrenalectomized animals came from the same litter. Adult male rats (60 days old, 300–400 g body wt) were used as reference.

Adrenalectomy was performed on six animals from each litter (3 females, 3 males). The remaining six animals (3 females, 3 males) were sham-operated (SHM). ADX was performed via a dorsal approach under Metafane vapor anesthesia (Pitman-Moore, Mundelein, IL). SHM animals had the same surgical procedure except that a small amount of suprarenal adipose tissue was removed. After recovery from the anesthesia, the animals were returned to their home cage and left undisturbed until 14 h later when they were sacrificed by decapitation. Brains were rapidly removed, frozen in liquid isopentane (-42°C), and stored at -80°C . Subsequently, they were sectioned in the coronal plane at $10\ \mu\text{m}$ on a Bright-Hacker cryostat (Hacker Instruments, Fairfield, NJ), which was maintained at -20°C , and thaw mounted onto polylysine-coated microscope slides. Brain sections were stored at -80°C until processed for *in situ* hybridization. In a separate experiment, ages Postnatal Day (PND) 6, 10, 14, 18, 22, 28, 35, and 45 and adults were included. Hippocampi were dissected on ice and frozen immediately on dry ice for later estimates of B_{max} by single near-saturation dose binding assay. In addition, certain ages were selected for Scatchard analysis to estimate both K_d and B_{max} for GR and MR.

Plasma Corticosterone Determination

Trunk blood was collected in tubes containing EDTA and spun at 2000 rpm for 7 min to obtain plasma. Corticosterone was assayed to confirm the removal of the adrenal using a radioimmunoassay as previously described (37). The antibody cross reacts 2.2% with cortisol and less than 1% with other endogenous steroids. The detection limit is 1 pg per milliliter and the intra- and inter-coefficient of variation is 2 and 3%, respectively.

Receptor Binding Capacity

A rough estimate of the capacity of the receptor (B_{max}) is possible by utilizing single-point binding assays for individual animals from the hippocampus (38–40). A saturating concentration of ligand, i.e., $10\ \text{nM}$ [^3H]-dexamethasone, which gives the x -intercept in the Scatchard plot, is used for this purpose. This method has been described before (38–40). In the test tube, dexamethasone binds to both GR and MR in the cytosolic hippocampi homogenate, allowing for the determination of both receptors as explained below (38–40). Briefly, frozen hippocampi were homogenized in a hand-held glass homogenizer in $10\ \text{mM}$ ice-cold Tris buffer, pH 7.4, with $1\ \text{mM}$ EDTA, 10% glycerol, $40\ \text{mM}$ molybdate, and $1\ \text{mM}$

dithiothreitol. The homogenate was centrifuged in an ultracentrifuge at 40,000 rpm for 30 min, and then the cytosol (supernatant) was transferred to another tube which remained on ice. A $50\text{-}\mu\text{l}$ aliquot of the cytosol was incubated with a saturating concentration of [^3H]-dexamethasone ($10\ \text{nM}$, New England Nuclear, Boston, MA), with and without $500\ \text{nM}$ of the GR agonist RU 26988 (Roussel-Uclaf, Romainville, France) and excess ($2.5\ \text{mM}$) dexamethasone (Sigma, St. Louis, MO). Thus, individual total binding (cytosol + [^3H]-dexamethasone), MR binding (cytosol + [^3H]-dexamethasone + $500\ \text{nM}$ RU 26988), and nonspecific binding (cytosol + [^3H]-dexamethasone + excess $2.5\ \text{mM}$ dexamethasone) were determined. In addition, an aliquot representing one-fourth of a hippocampus was obtained from animals of the same age (PND 6, 14, 22, 28, and 35 and adult) and pooled to construct a Scatchard plot. For this purpose, increasing concentrations of [^3H]-dexamethasone (0.1 – $10\ \text{nM}$) were used along with the above mentioned reagent combinations. The samples were incubated overnight (a minimum of 16 h) at 0° . The bound ^3H ligand was separated from free using charcoal suspension [2% (Norit A, JT Baker, Phillipsburg, NJ)], 0.2% Dextran, MW = 7000 (Sigma). A small sample of the original cytosol was assayed for protein content with Bio-Rad protein assay (Bio-Rad Corp., Wilmington, DE), and the values are expressed as fmol bound/mg protein. Scatchard analysis is performed using the LIGAND receptor binding program.

Riboprobe Preparation

GR and MR probes were prepared as previously described (33), to match equivalent specific activities and "stickiness" (GC content). The GR probe was a 544-nucleotide fragment of a cDNA clone directed against the protein binding region and the 3' untranslated region of the GR mRNA (cDNA clone provided by Dr. Keith Yamamoto, U.C.S.F.). The MR probe was a 347-nucleotide fragment of our MR clone, directed against the 3' untranslated region of the MR mRNA (41). We have previously shown that the probes do not contain any regions of high homology, which obviates any potential for cross-hybridization. Antisense riboprobes were prepared using the appropriate restriction endonucleases and transcribed using either T7 or SP6 promoter sites in the presence of [^{35}S]UTP ($1000\ \text{Ci/mmol}$). The reaction yielded a specific activity of 1.37×10^5 and $1.42 \times 10^5\ \text{Ci/mmol}$ for GR and MR probes, respectively.

In Situ Hybridization

Tissue sections were removed from the -80°C freezer and incubated with a $1\ \mu\text{g/ml}$ solution of proteinase-K to permeabilize the tissue and inactivate endogenous RNase. After this treatment, sections were incubated in succession in water (1 min), $0.1\ \text{M}$ triethanolamine (pH 8.0; 1 min), and 0.25% acetic anhydride in $0.1\ \text{M}$ triethanol-

amine (10 min). The tissue was then washed in $2 \times \text{SSC}$ (0.3 mM NaCl, 0.03 mM Na citrate, pH 7.2; 5 min) and dehydrated through graded concentrations of ethanol. Sections were then hybridized with 1.5×10^6 dpm of [^{35}S]UTP-labeled GR or MR cRNA probes in 20 μl of a hybridization buffer containing 75% formamide, 10% dextran sulfate, $3 \times \text{SSC}$, 50 mM sodium phosphate buffer (pH 7.4), $1 \times \text{Denhart's}$ solution, 0.1 mg/ml yeast tRNA, and 0.1 mg/ml sheared salmon sperm DNA. Tissue sections were covered with coverslips which were sealed with rubber cement. The slides were incubated overnight at 50°C. On the following day the rubber cement was removed, the coverslips were soaked off with $2 \times \text{SSC}$, and the tissue sections were washed for 10 min in fresh $2 \times \text{SSC}$ solution. Single-stranded probe not hybridized with endogenous mRNAs was removed by incubating the sections for 30 min in 200 $\mu\text{g}/\text{ml}$ solution of RNase-A at 37°C. The tissue was then washed in increasingly stringent SSC solutions (2, 1, and $0.5 \times \text{SSC}$; 10 min each), followed by a 1-h wash in $0.5 \times \text{SSC}$ at 50°C. After this final wash, the tissue sections were dehydrated using graded concentrations of ethanol, air dried, and prepared for detection by X-ray autoradiography on Kodak XAR-5 film.

Controls for Hybridization

Controls for verifying the specificity of GR and MR hybridization have been previously described (33). Controls included the use of sense strand RNA probes of the same length and specific activity as the GR and MR probes. In addition, GR and MR cRNA-mRNA hybrids were melted by incubation of hybridized tissue sections under increasingly stringent conditions. True hybrids show dissociation at a predictable level of stringency.

Microdensitometric Analysis

Autoradiograms generated were analyzed using an automated image analysis system (Dage camera, MAC II/IMAGE program). The person analyzing the images was not aware of the treatment conditions under analysis. Hippocampal subfields were defined with reference to Nissl-stained sections and the stereotaxic atlas of Paxinos and Watson (42). Accordingly, four hippocampal areas, corresponding to (1) subfield CA1-2, (2) subfield CA3 proximal, (3) subfield CA3 distal, and (4) dentate gyrus (see Fig. 5), were digitized from a given dorsal hippocampal section. The mean optical density of the region of interest was measured at 100 \times magnification. This measurement corrects for the size of the structure analyzed (mean optical density/area). Background labeling was measured from the corresponding internal molecular layer of the hippocampus and subtracted from each individual subfield value obtained. Eight sections per animal were analyzed. These sections spanned the dorsal hippocampus. The mean of these was used as the individual value for a particular area in an animal.

Statistical Analysis

A two-factor ANOVA was used for the analysis of age and treatment interaction. Post hoc comparisons were made by using the Fisher protected least-significant difference.

RESULTS

Glucocorticoid and Mineralocorticoid Receptor Binding Capacity in the Developing Hippocampus

Receptor binding capacity of GR and MR was measured in young animals 14 h after bilateral adrenalectomy, a time period that allows for the clearance of circulating endogenous corticosterone that would otherwise interfere in the binding assay. Plasma corticosterone levels were undetectable or at the detection limit of the RIA in all ages studied (data not shown). The binding assay used was one developed as a GR and MR microassay by Landfield and co-workers (40). We have also validated this method in our laboratory (data not shown) and the extrapolated B_{max} estimates using this method correlate closely with large Scatchard plots ($r = 0.80$ for MR; $r = 0.98$ for GR). Figure 1 shows the developmental progression of GR and MR expressed as amount of receptor per milligram of protein using this microassay method. Whereas GR binding capacity had a steady increase during the first 2 weeks of life and remained steady (d6 = 159.0 ± 27 ; d10 = 286 ± 35 ; d22 = 369.1 ± 43 ; d45 = 391.2 ± 40 ; $a = 344.8 \pm 23$, fmol/mg protein \pm SE), the MR B_{max} for younger animals was significantly greater than that for adults from Day 10 until Day 45 (d10 = 132.5 ± 28 ; d22 = 175.4 ± 23 ; d35 = 144 ± 10 ; d45 = 89.0 ± 17 ; $a = 76.9 \pm 13$, fmol/mg protein \pm SE), when it decreased and approached adult concentrations. Analysis of variance showed that both receptor populations varied significantly with age (GR, $P = 0.03$; MR, $P = 0.03$). Table 1 shows the apparent affinity constants and capacity obtained from the Scatchard analyses. Note that since the Scatchard analysis allows for the determination of the B_{max} using a linear regression, the resulting B_{max} by Scatchard plot (Table 1) is generally slightly higher than the B_{max} which is derived from single near-saturation doses of radioactive ligand (Fig. 1) (39, 40, and our own observations). Despite these discrepancies, the ontogenic progression is in general agreement with others. Furthermore, the receptor binding constants (K_d) obtained by Scatchard plot are also in general agreement with other investigators (31, 34, 40).

In Situ Hybridization Study: Plasma Corticosterone Levels of Sham and ADX Animals

Table 2 presents corticosterone values of the SHM and ADX animals used for the hippocampal GR and MR *in situ* analysis. ADX animals had corticosterone levels at

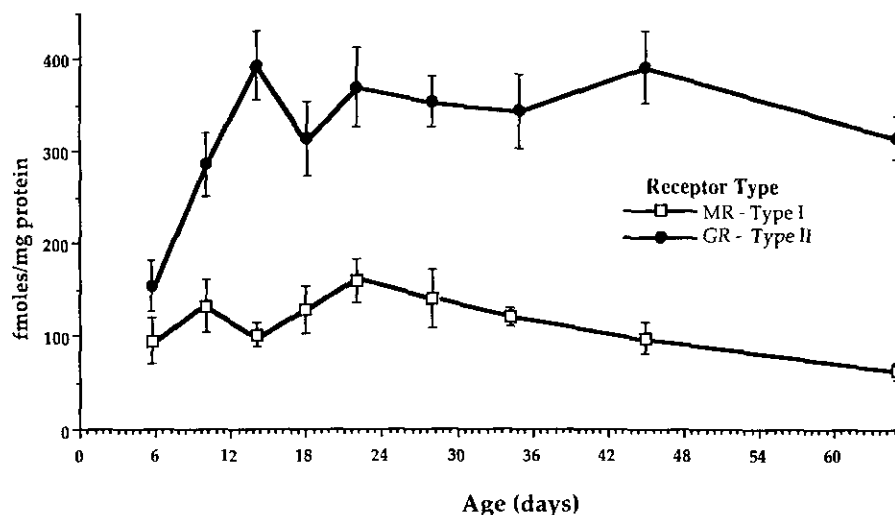


FIG. 1. Glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) binding capacity in the developing hippocampus. Animals were sacrificed 14 h after bilateral adrenalectomy, a time period that allows for the clearance of circulating endogenous corticosterone that would otherwise interfere in the binding assay. Hippocampal GR and MR binding capacity (B_{max}) analyses were performed utilizing a single-point binding assay from a hippocampus for individual animals. Saturating amounts of [3 H]dexamethasone, RU26938, a GR agonist, and dexamethasone were used as ligands to calculate specific GR or MR B_{max} (as described under Materials and Methods). Values are the mean \pm SE B_{max} (fmol/mg protein \pm SE) of 6–8 animals per time point.

the detection limit of the RIA. SHM 28- and 35-day-old animals had a significantly higher corticosterone level when compared to adult SHM animals ($P \leq 0.05$).

Ontogenic Progression of Glucocorticoid and Mineralocorticoid Receptor mRNA within the Specific Subfields in the Developing Hippocampus

An ontogenic analysis by hippocampal subfield is possible since all brains from all ages were processed identically throughout the *in situ* hybridization procedure. GR

mRNA was localized to the pyramidal cell field of the hippocampal formation (CA1–2, CA3p, and CA3d) and the granular cell layer of the dentate gyrus (DG) (see Fig. 2). The signal intensity was greater over the pyramidal cell subfield CA1–2, of intermediate intensity over the DG, and lowest over the CA3/CA4 area (CA1–2 > DG > CA3/CA4). The 10-day-old animal exhibited a different pattern in which the intermediate intensity of the signal was seen equally over subfield CA3 and the DG (CA1–2 > DG = CA3). Quantification of the signal revealed a significant effect of subfield on hybridization intensity across the hippocampus by two-way ANOVA ($P = 0.001$). A preponderance of GR mRNA appears to be present over specific subfields in the 10-day-old animal. Specifically, analysis of the GR mRNA levels reveals that the 10-day-old animal has significantly higher GR mRNA levels over subfields CA1–2 and CA3d when compared to

TABLE 1

Rat Hippocampal Binding Constants of [3 H]Dexamethasone to Cytosolic GR and MR Receptors across Several Ages

Age (days)	Glucocorticoid receptor		Mineralocorticoid receptor	
	K_d (nM)	B_{max} (fmol/mg/nM)	K_d (nM)	B_{max} (fmol/mg/nM)
6	2.55	253.9	2.83	56.7
14	3.08	420.5	3.40	164.0
22	2.50	332.9	1.18	150.6
28	3.37	300.6	1.20	145.5
35	2.48	493.3	2.88	109.3
60 (adult)	2.57	311.6	2.80	86.6

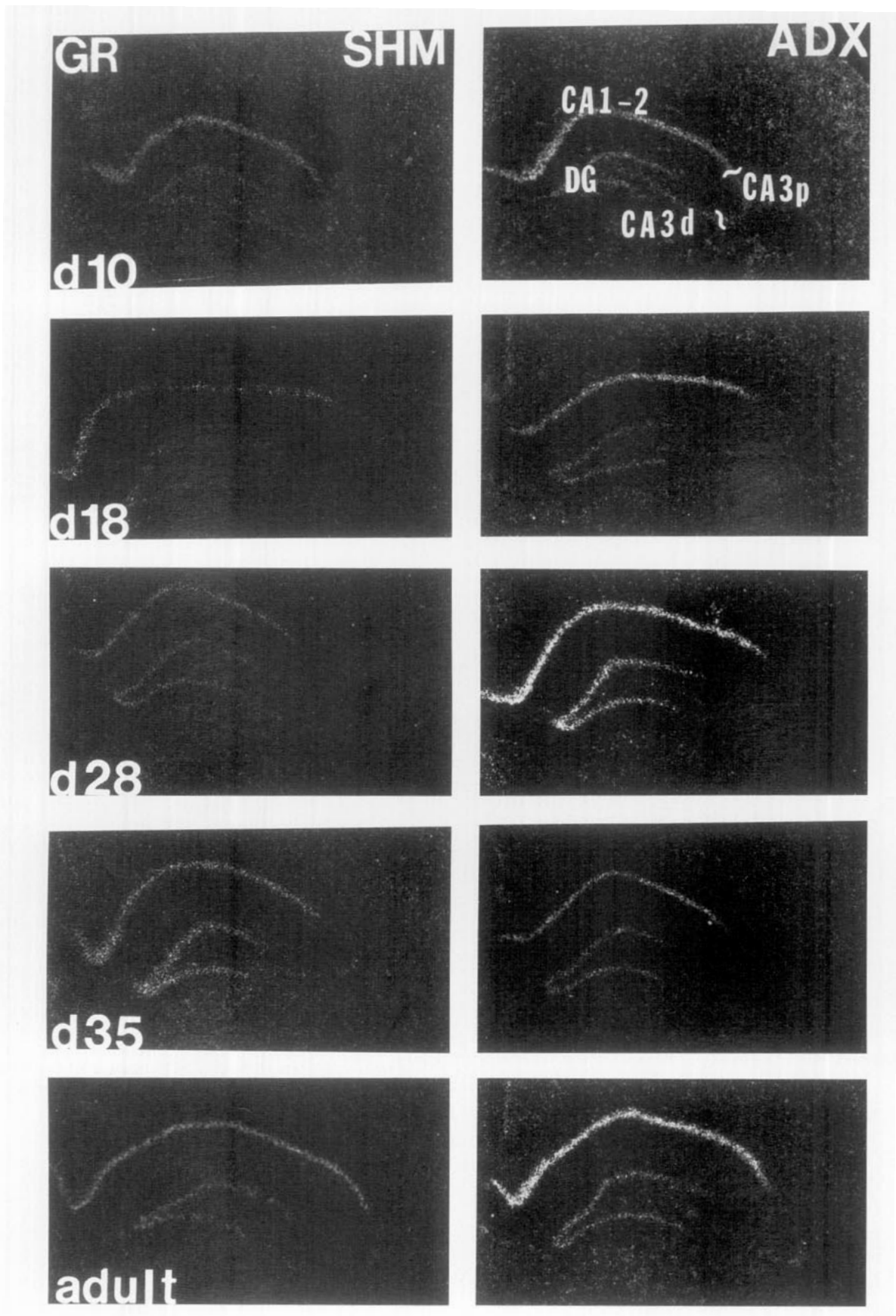
Note. The apparent affinity constants (K_d) and binding capacities (B_{max}) were calculated from Scatchard plots derived from the binding data of [3 H]dexamethasone in the presence of 500 nM of the glucocorticoid agonist RU 26988 and a 500-fold excess of dexamethasone. R^2 ranged from 0.88 to 0.99.

TABLE 2

Plasma Corticosterone Levels of the Group of Animals from which the *in Situ* Hybridization Analysis Is Derived (Values Expressed as μ g/dl \pm SE)

Age (days)	Sham	ADX
10	1.9 \pm 0.3	ND
18	3.1 \pm 0.8	ND
28	5.4 \pm 2.1*	0.3 \pm 0.1
35	6.4 \pm 1.6*	0.2 \pm 0.1
60 (adult)	1.9 \pm 0.8	0.1 \pm 0.1

Note. ND, not detected; *age sham vs adult sham, $P \leq 0.05$.



the other ages studied ($P \leq 0.05$; see Figs. 3B and 3C). The DG, however, shows an increasing signal pattern with age. Animals older than 18 days had significantly higher GR mRNA levels when compared to the 10-day-old animal in this particular region (all comparisons, $P \leq 0.05$; Fig. 3D). When the hippocampus is analyzed as a whole structure (see Fig. 6A), the 10-day-old SHM animal exhibited the highest GR mRNA level, which subsequently decreased with age ($P \leq 0.05$).

MR mRNA was also readily detected in the hippocampal formation of the developing animals (see Fig. 4). In contrast to the GR mRNA, MR mRNA signal was stronger over the CA3 region, particularly in the area closest to the CA2 area (CA3p). An intermediate signal was present over the DG and CA1-2 regions. This pattern was evident in the adult and 35- and 10-day-old animals (CA3p > DG \geq CA1-2). However, in the 18- and 28-day-old animals, the DG exhibited the heaviest hybridization, followed by CA3p and the CA1-2 region (DG > CA3p > CA1-2). Quantification of the signal revealed a significant effect of subfield on hybridization intensity across the hippocampus by two-way ANOVA ($P = 0.04$). The MR mRNA analysis in SHM animals also revealed significantly higher MR levels in the 10-day-old animal over subfields CA3p and CA3d ($P \leq 0.05$; see Figs. 5B and 5C). Analysis of the whole hippocampus shows that the MR mRNA levels are also significantly higher in the 10-day-old SHM animal compared to all other ages ($P \leq 0.05$; see Fig. 6B).

Effect of a 14-h Adrenalectomy on Glucocorticoid and Mineralocorticoid Receptor mRNA Levels in the Developing Hippocampus

Hippocampal GR mRNA appears to be altered by the absence of steroids in a site-specific manner in the developing animal (ANOVA, $P = 0.0001$; see Figs. 2 and 3). The effect was evident over subfields CA1-2, CA3p, CA3d, and the DG (all SHM vs ADX comparisons by Fisher, $P \leq 0.05$) in the 18- and 28-day-old animals. Adult animals also showed a selective GR mRNA increase over subfield CA1-2 only ($P \leq 0.05$). Analysis of the whole hippocampus showed significant differences in the 18- and 28-day-old animals only (see Fig. 6; SHM vs ADX by Fisher, $P \leq 0.05$). The GR mRNA signal detected over the cortex was also analyzed as an internal control for ADX treatment (see Fig. 3F) and no significant treatment effect was detected.

Adrenalectomy caused an overall tendency of MR mRNA to increase in all hippocampal subfields (see Figs. 4 and 5). This was a pattern observed in all ages except for the 10-day-old animal, which maintained steady MR mRNA levels. Significant treatment effects were observed in the 18- and 28-day-old animals over subfield CA1-2 (ANOVA, $P = 0.0004$; SHM vs ADX by Fisher, $P \leq 0.05$), in the 28-day-old animal in subfield CA3p (ANOVA, $P = 0.002$; SHM vs ADX by Fisher, $P \leq 0.05$), and in the adult in subfields CA1-2, CA3p, and DG (ANOVA, $P = 0.05$; SHM vs ADX by Fisher, $P \leq 0.05$). Whole hippocampus densitometric analysis demonstrated a significant increase in MR mRNA levels for the adult animal only (see Fig. 6; SHM vs ADX by Fisher, $P \leq 0.05$). Two-factor ANOVA did not reveal age-treatment interaction.

DISCUSSION

Short-term adrenalectomy is a well-accepted procedure which is performed in order to remove all endogenous corticosteroids which will interfere with the displacement reaction used to determine *in vitro* steroid receptor binding capacity. The present study was undertaken to explore the possibility that the hippocampus of the developing animal may be more sensitive to changes of circulating corticosterone levels and that in fact the normal GR and MR progression obtained using standard steroid binding methods reflect upregulation of these systems in the developing hippocampus. Our study demonstrates an ontogenic increase of GR and MR binding capacity in the developing hippocampus which is in general agreement with other investigators. However, this increase in receptor protein is accompanied by an upregulation of hippocampal GR mRNA levels in specific hippocampal subfields at specific ages and an overall tendency for an increase of hippocampal MR gene expression in the developing animal. The increase in mRNA levels for both of these receptor systems 14 h after adrenalectomy suggests that receptor binding measurements which relay in short-term adrenalectomy may, in fact, reflect upregulation of these proteins in the developing animal. Moreover, the mineralocorticoid receptor system is very sensitive to corticosterone level changes even in the adult animal, in which significant MR mRNA changes are detected. Thus, previous hippocampal measurements of basal glucocorticoid and mineralocorticoid receptor capacity which were made by using hippocampi obtained from developing and adult

FIG. 2. Photomicrograph of the glucocorticoid receptor mRNA expression in the developing hippocampus and the effects of a 14-h adrenalectomy. Hippocampi from adrenalectomy (ADX) and sham-ADX (SHM) animals that best represent the mean effect observed by subfields are presented here. Animals were sacrificed in the morning, 14 h after surgery, on animals 10, 18, 28, 35, and 60 days of age. *In situ* hybridization was performed as described under Materials and Methods using a 544-nucleotide fragment of a cDNA clone directed against the protein binding region and the 3' untranslated region of the GR mRNA. The probe does not contain any regions of high homology with MR which obviates any potential for cross-hybridization. The four hippocampal areas analyzed are shown and correspond to (1) subfield CA1-2, (2) subfield CA3 proximal (CA3p), (3) subfield CA3 distal (CA3d), and (4) the dentate gyrus (DG). Background labeling was measured from the corresponding internal area from each section. PND, postnatal day.

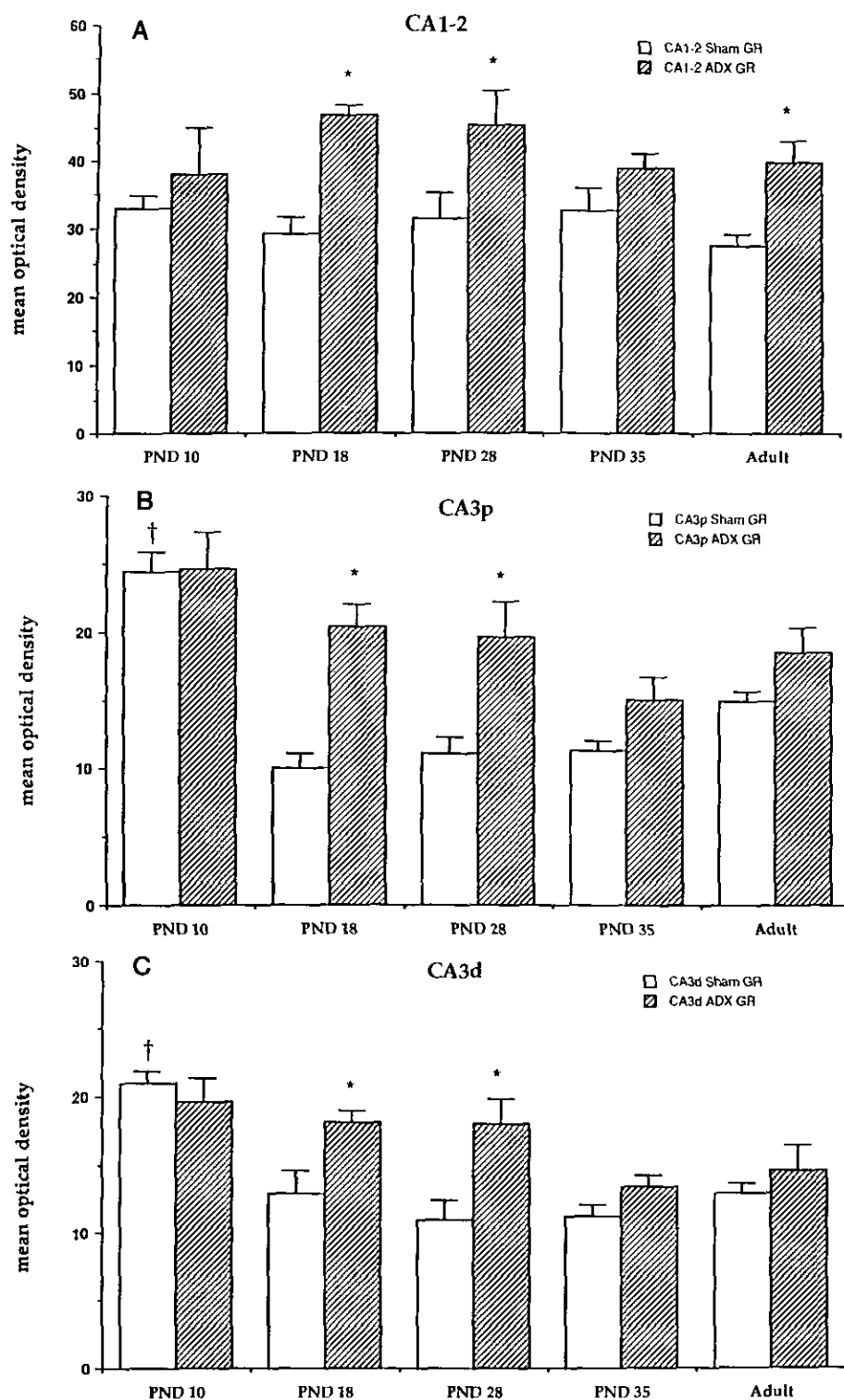


FIG. 3. Densitometric analyses of digitized images through the different subfields of the hippocampal formation of the developing animal illustrating the developmental GR mRNA expression (Sham) and the effect of a 14-h adrenalectomy on GR mRNA (ADX). (A) Subfield CA1-2, (B) subfield CA3 proximal (CA3p), (C) subfield CA3 distal (CA3d), (D) dentate gyrus (DG), and (E) cortex. The developmental distribution of GR mRNA can be appreciated by observing the progression of SHM animals at different ages. PND 10 GR mRNA content was significantly denser in subfields CA3p and CA3d, while DG GR mRNA density was significantly denser in the older animals (PND 28-adult). There was a significant effect of treatment on subfield CA1-2 for PND 18 and 28 and adult animals. In addition, ADX had a significant effect on subfields CA3p and CA3d in the PND18 and 28 animals. PND, postnatal day. Symbols are as follows: †, $P < 0.05$, PND 10 SHM vs SHM of other ages; #, $P < 0.05$, SHM of other ages vs SHM PND 10 and PND 18; *, $P < 0.05$, SHM vs ADX.

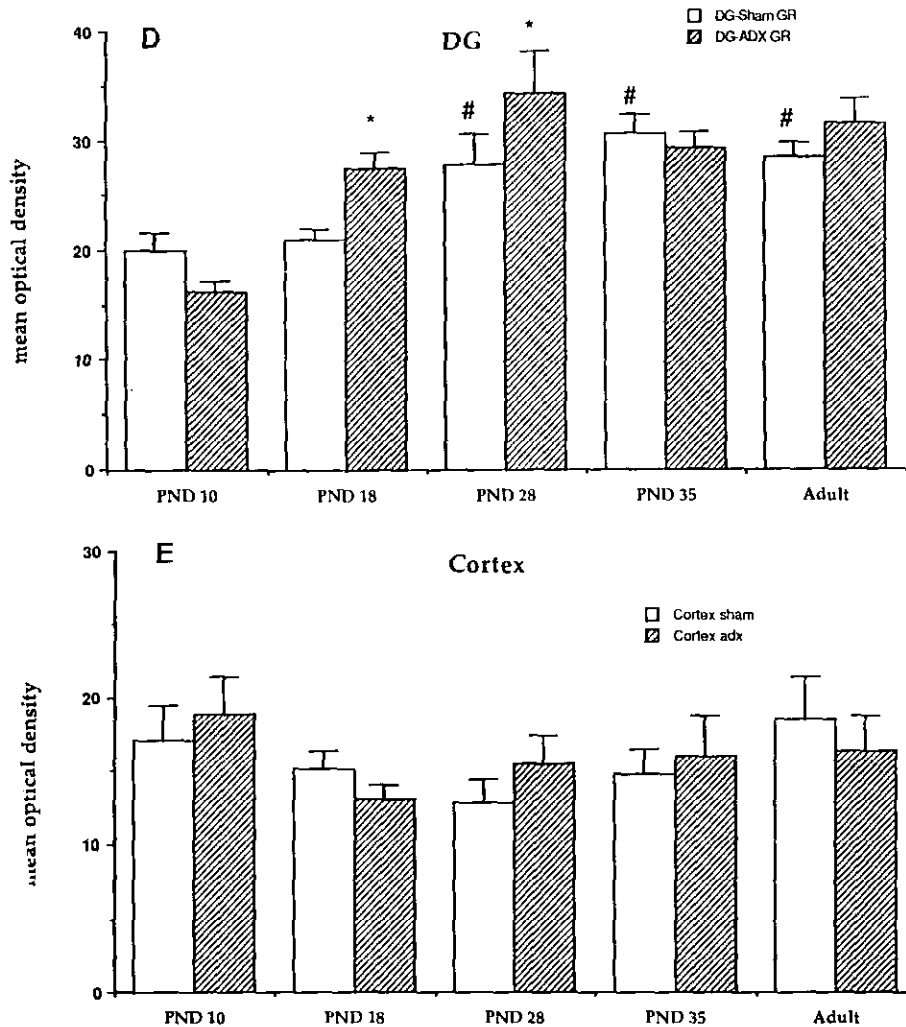


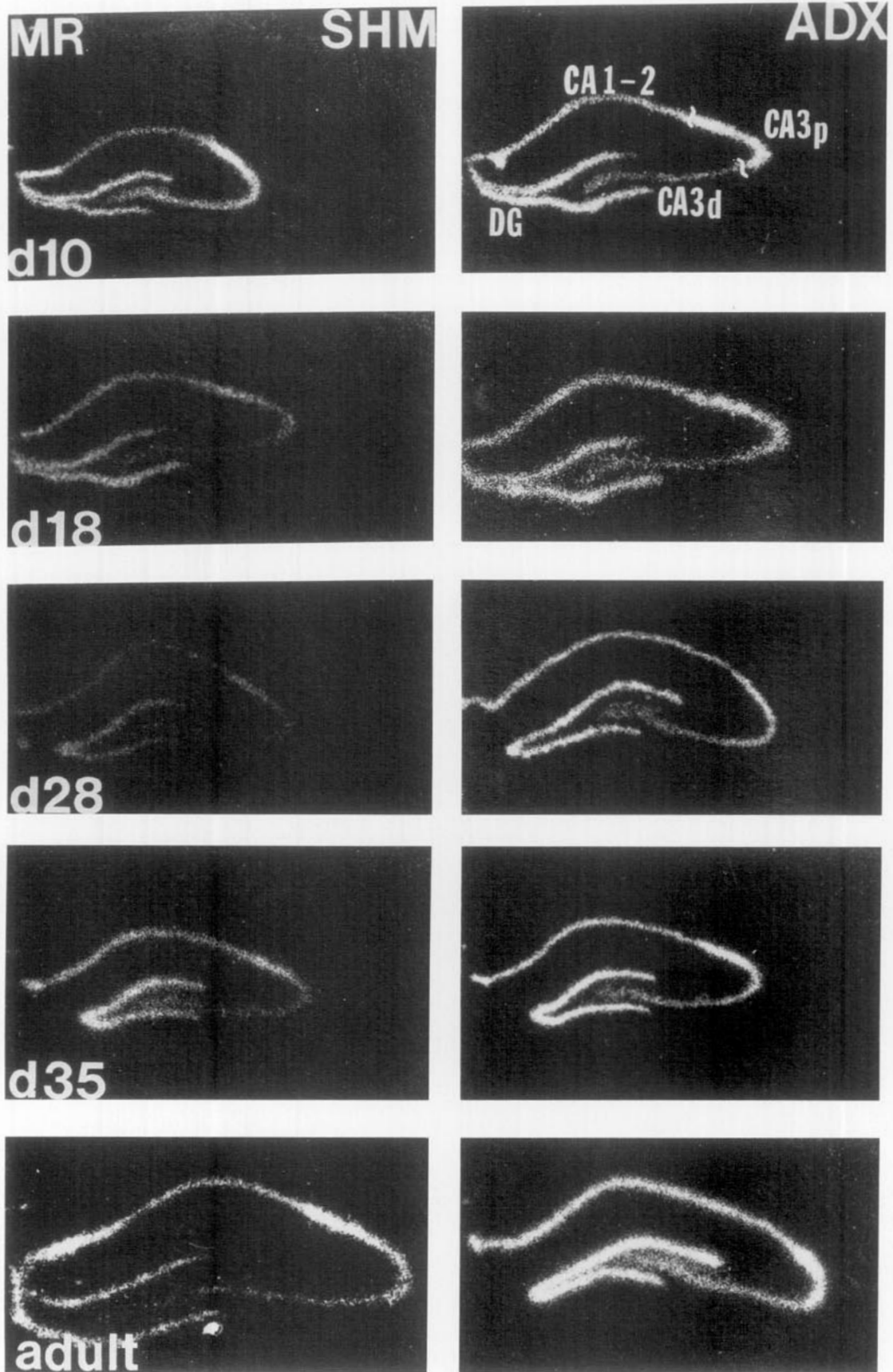
FIG. 3—Continued

adrenalectomized animals are also, most likely, reflecting upregulation of the GR and MR system, rather than steady-state levels of this receptor.

Glucocorticoid and Mineralocorticoid Receptor Binding Capacity in the Developing Hippocampus

The developing hippocampus has an increase in GR binding capacity which reaches adult levels by the second week of life. In contrast, less dramatic changes are observed in the developing hippocampal MR system, which reaches relatively stable levels early in ontogeny. The [³H]dexamethasone binding data reported here is similar to those obtained in previous developmental studies which used [³H]dexamethasone or the GR agonist [³H]RU28362 as radioligand to react with inactive cytosolic glucocorticoid receptors obtained from homogenates of hippocampal tissue (30, 34, 35, 43). In these studies fewer ages than those reported here were used (PND 5, 7, 14, 21, and 35). Much like our study, these studies report GR

adult-like levels by 15 days of age, followed by an increase above adult levels between 15 and 35 days. Although the MR binding capacity data in the present study were derived from the difference between total binding and specific GR binding, the relationships from age to age are similar to those obtained with the selective MR agonist [³H]aldosterone (35, 36, 46). This is not surprising since, *in vitro*, [³H]dexamethasone binds to hippocampal MR, in the presence of 0.5 μ M RU26988, with an affinity constant which is twofold higher than that obtained with [³H]aldosterone (38–40). This also explains the relatively similar GR and MR affinity constants derived from our Scatchard plots. Despite differences in methodologies, which are primarily related to the radioligands used to measure GR and MR binding capacity, our data are consistent with others in showing that steroid binding capacity measured in the developing hippocampus after short-term adrenalectomy results in a glucocorticoid receptor pattern which differs from the mineralocorticoid



receptor in the hippocampal system. Our data adds more ages to actually demonstrate the ontogenic progression beyond 3 weeks of life. How do these ontogenic increases in binding capacity relate to GR and MR mRNA expression in the different hippocampal regions of the developing hippocampus? Does short-term ADX, which is used to obtain an accurate binding capacity, impact on the cell GR and MR synthetic activation? We first looked at the GR and MR gene expression in a precise anatomical context in the animal with intact adrenals. We then compared these data to that data obtained from the animals that underwent adrenalectomy.

Ontogenic Progression of Glucocorticoid and Mineralocorticoid Receptor mRNA within the Specific Subfields of the Developing Hippocampus

The abundance and distribution of the glucocorticoid and mineralocorticoid message follows *individually distinct patterns* in the developing hippocampal formation and dentate gyrus. It is evident that both glucocorticoid and mineralocorticoid receptors are detected in every subfield of the hippocampus. Their expression, as in the adult animal, appears to differ across the pyramidal cell subfields (CA1-4) and the granular cells of the dentate gyrus. The general agreement is that in the adult rat GR receptors and mRNA levels are concentrated in the CA1-2 region, while the mineralocorticoid receptors and mRNA levels predominate in the CA3 region (46-49). The developing hippocampus follows a different pattern of intensity and distribution when compared to the adult animal. Our data reveal that animals at the earliest age studied, 10 days old, exhibit a signal intensity which is greatest in CA1-2 and DG for GR mRNA, while the MR message is greatest over DG and CA3p. The GR mRNA organization seen beyond this age resembles the adult distribution reported by Herman *et al.* (CA1-2 > DG > CA3) (33). This progression of the GR gene expression very much agrees with the ontogenic progression of the protein reported independently by Van Eekelen *et al.* and Rosenfeld *et al.* (49, 54). In their immunocytochemical studies performed in hippocampi obtained from intact animals, the 8-day-old animal showed intense GR signals over the CA1-2 fields and the DG, but markedly reduced staining over CA3-4. This reduced staining over CA3-4 becomes indistinguishable from background by 12 days of age, a pattern that is well recognized in the adult (33, 49, 50).

The developing hippocampus does not acquire an adult-like MR mRNA distribution until after Postnatal Day

28. Prior to this age there is a relative abundance of MR message in the DG region. The adult pattern of greatest abundance over CA3p is evident on Postnatal Day 35, beyond which the adult pattern of CA3p > DG > CA1-2 is present (33). To date, there is not an adequate description of hippocampal mineralocorticoid receptor protein distribution during development or in the adult. The ontogenic progression of the MR protein has been described using autoradiographical methods which depend on short-term adrenalectomy prior to the *in vivo* administration of tracer doses of [³H]corticosterone (54 and 44 for review). This approach, we find, may result in receptor up-regulation due to the operative procedure. In addition, binding distribution assessed by an *in vivo* administration of [³H]corticosterone not only reflects variation in binding sites due to developmental maturity but may also reflect other age-related factors such as differential tissue distribution, differences in steroid metabolism, and changes in mechanisms whereby the radioligand reaches neuronal tissue (54).

A postnatal ontogeny of mineralocorticoid and glucocorticoid receptor gene expression has been previously reported (51). However, in this latter study, depending on the age of the animal, different tissue preparation and prehybridization protocols were performed to obtain the optimal hybridization signal. This precluded the analysis of the GR and MR mRNA signal as a function of age (age to age comparison), while comparisons of different structures within a given age were possible. All the animals in the present study were prepared in the same manner, using identical prehybridization and hybridization procedures without compromising the optimal signal and such that age to age comparisons could be made. A clear MR mRNA decrease after PND 10 is seen in the CA3 region (both CA3p and CA3d). The developmental progression of the GR gene presented in this study appears to follow a distinctive decrease from Day 10 to adult within the pyramidal cells of the CA1-4 region and an increase from PND 10 to adult in the granular cell layer of the DG. The latter DG pattern follows the neurogenesis and neuronal migration pattern described in studies of hippocampal development (53-58). Considerable neurogenesis and neuronal migration is evident in the DG with more than 70% of the dentate neurons populating this area during the first 2 weeks of life. Neurogenesis of the pyramidal cell layer, on the other hand, occurs during prenatal life (54, 55, 58). This suggests that the high density of GR mRNA in areas CA1-2 and MR mRNA in CA3 in the 10-day-old animal is due to an increased transcription of

FIG. 4. Photomicrograph of the MR mRNA expression in the developing hippocampus and the effect of a 14-h adrenalectomy. Hippocampi from ADX and SHM animals that best represent the mean MR mRNA effect observed by subfields are presented here. Animals were sacrificed between 0900 and 1100, 14 h after the surgeries. *In situ* hybridization was performed as described under Materials and Methods using a 347-nucleotide fragment of a cDNA MR clone, directed against the 3' untranslated region of the MR mRNA. The probe does not contain any regions of high homology with GR which obviates any potential for cross-hybridization. PND, postnatal day.

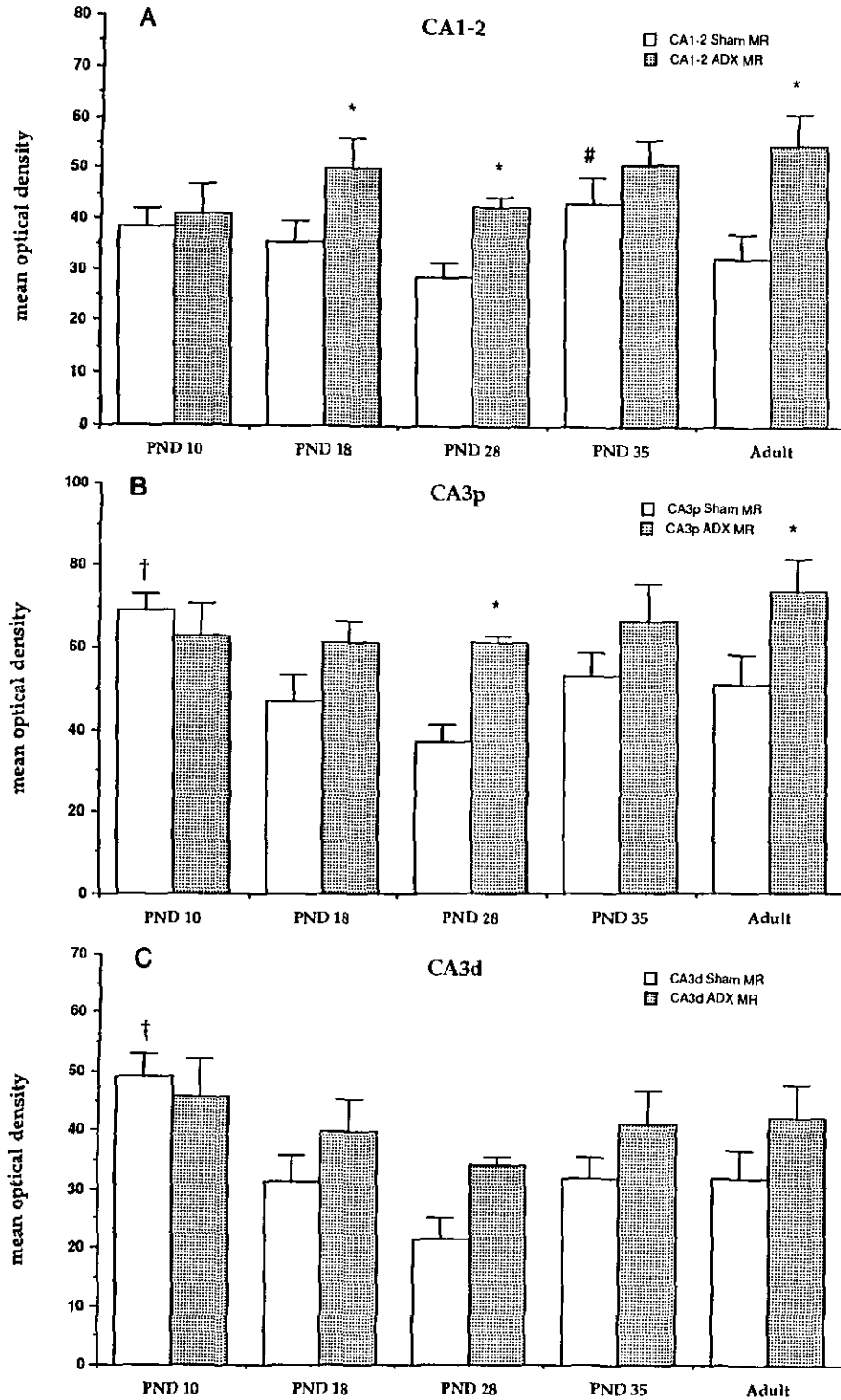


FIG. 5. Densitometric analyses of the MR mRNA expression through the different subfields of the hippocampal formation illustrating the developmental progression of MR mRNA (Sham) and the effect of a 14-h adrenalectomy on MR mRNA (ADX). (A) Subfield CA1-2, (B) subfield CA3 proximal (CA3p), (C) subfield CA3 distal (CA3d), and (D) dentate gyrus (DG). The developmental distribution of MR mRNA can be appreciated by observing the progression of SHM animals at different ages. PND 10 MR mRNA content was significantly denser in subfields CA3p and CA3d, while CA1-2 MR mRNA density was significantly denser in the PND 28 animal. There was a significant effect of treatment on subfield CA1-2 for PND 18 and 28 animals. In addition, ADX had a significant effect on subfield CA3p in the PND 28 and adult animal. Adult animals showed a significant effect of treatment in the CA1-2, CA3p, and DG areas. PND, postnatal day. Symbols are as follows: †, $P < 0.05$, PND 10 SHM vs SHM of other ages; #, $P < 0.05$, SHM of other ages vs SHM PND 10 and PND 18; *, $P < 0.05$, SHM vs ADX.

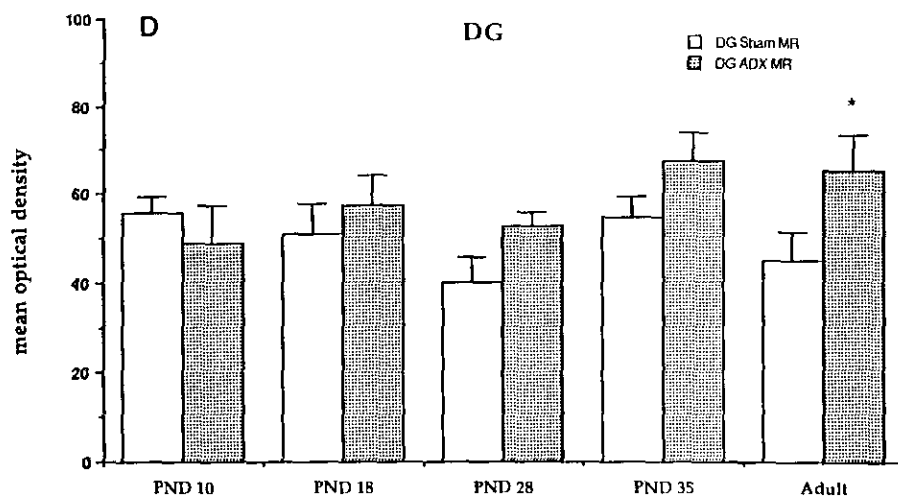


FIG. 5—Continued

these genes within these cell clusters. Ontogenic expression of GR and MR as assessed by receptor autoradiography (34, 52) or immunoreactive staining (36) also shows that early on, in the hippocampus of 9-day-old animals, there is an apparent thickness of the pyramidal cell layers which decreases by the end of the second week of life. This apparent thickness reflects both actual increase in receptor concentration and increased neuronal density (34, 52).

In general, the pattern of GR and MR expression is an image of the changes in circulating corticosterone which are present in the developing animal. Thus, the expression of GR and MR is high in the 10-day-old animal when corticosterone levels are low, decreasing when corticosterone levels are high, i.e., PND 18–28. Following the post-natal period, there is still a slight increase in both GR and MR expression to adult levels, which may represent the final “fine tuning” as corticosterone metabolism and circulating corticosterone binding globulin (CBG) levels, which act as a buffer to the system, are fully in place. Is this expression altered by short-term adrenalectomy?

Effect of a 14-h Adrenalectomy on Glucocorticoid and Mineralocorticoid Receptor mRNA Levels in the Developing Hippocampus

The present study documents that the developing hippocampus GR and MR systems are sensitive to removal of circulating corticosterone levels even under a short time frame. As suggested above, it is important to keep in perspective that during the first 2 weeks of life the developing rat maintains very low basal levels of corticosterone. While key elements of the HPA axis are immature, the corticosterone molecule itself has particular qualities that contribute to a stable steroid environment. The biological half-life of corticosterone is prolonged about three times that of the adult (6, 44). Corticosterone binding globulin

(CBG), which limits the amount of corticosterone available to tissues, is markedly reduced (6, 44). This combination results in a constant steroid environment which apparently is necessary for normal development. Within the hippocampus proper, it is known that elevations of the corticosterone environment by hydrocorticosterone administration during the first 4 days of life causes a decrease of [³H]thymidine-labeled granule cells throughout the dentate hilus (where these cells originate) and also in the stratum granulosum (where they migrate) (56, 57). This suggests a decrease in granular cell neurogenesis and migration as a result of high corticosterone levels, at a time when low constant levels are maintained. The pyramidal cell layer also appears to be very sensitive to its corticosterone environment during this time, as reflected by long-lasting reductions of glucocorticoid receptors in the CA1 region of animals treated with corticosterone during the first week of life (59). Though developmental factors other than corticosterone environment cannot be underestimated, these findings point to the vulnerability of the hippocampal cells during this period, particularly for the DG area where neurogenesis and cell migration are active processes during the first weeks of life. Thus, it is interesting to note that the 10-day-old animal has a relatively low basal and constant corticosterone level which although reduced to undetectable levels by ADX does not constitute a significant change in steroid milieu. Then it follows that at this age we do not observe a great impact on either GR or MR mRNA hippocampal levels. However, hippocampal GR and MR mRNA appears to be regulated by corticosterone in a site- and age-specific manner at later ages. Fourteen hours after adrenalectomy GR mRNA was significantly increased over the CA1–2, CA3p, CA3d, and DG regions in the 18- and 28-day-old animals. This is unlike the adult animal which shows an increase in the CA1–2 and CA2/CA3 junction only. As

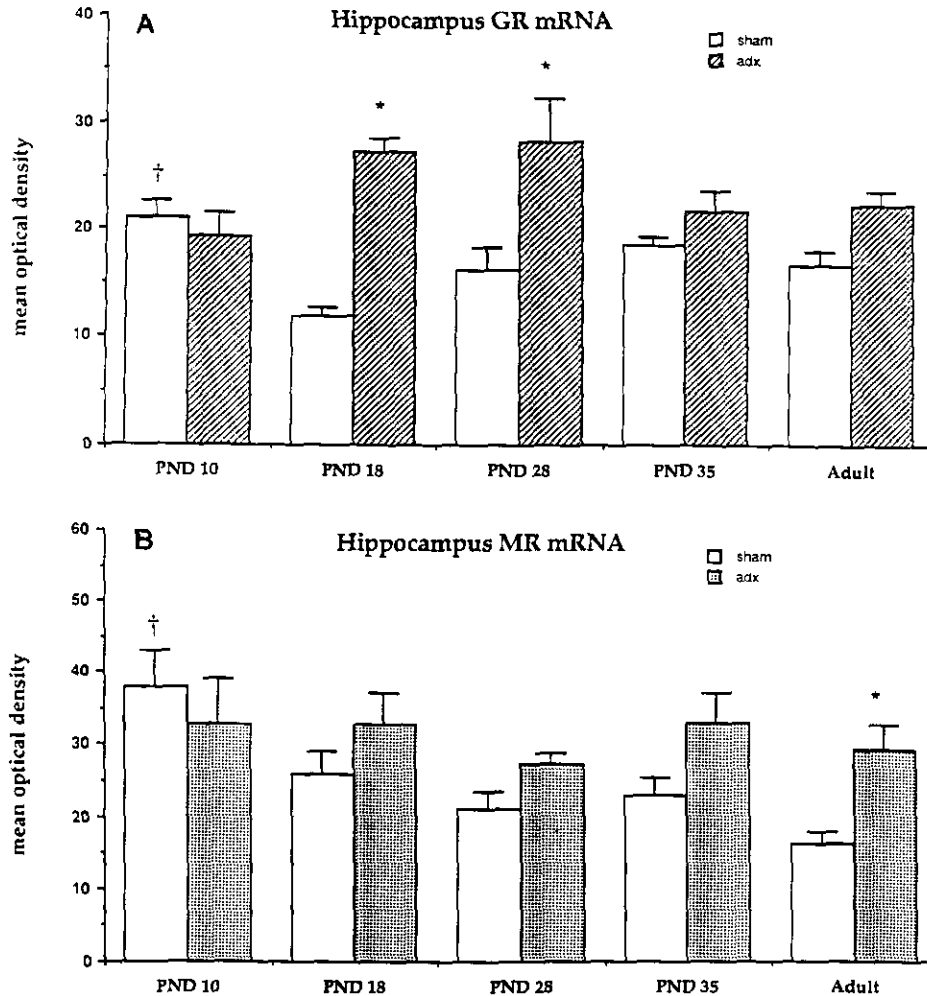


FIG. 6. Densitometric analyses of digitized images throughout the hippocampal formation of the developing animal illustrating the effect of a 14-h adrenalectomy on GR mRNA and MR mRNA in the whole hippocampus. The complete pyramidal cell and granular cell layer of the hippocampus was included in this analysis. Background labeling was measured from the corresponding internal area from each section. Overall digitized analysis shows: (1) the developmental progression of the expression of these genes and (2) the effect of ADX on whole hippocampus GR and MR mRNA expression. GR mRNA and MR mRNA expression decreases with increasing age (†, $P < 0.05$, PND 10 SHM vs SHM of other ages). The ADX treatment effect on GR mRNA is observed in the PND18 and 28 animals, while an MR mRNA effect is seen in the adult only (*, $P < 0.05$, SHM vs ADX).

predicted by the fact that MR has a high affinity for corticosterone, MR mRNA had an overall trend of increasing in all subfield at all ages (except Day 10). Statistical significance was limited to the CA1-2 area in the 18-day-old animal, the CA1-2 and CA3p areas in the 28-day-old animal, and the CA1-2, CA3p, and DG areas in the adult. Thus, the pyramidal and granular cells of the developing hippocampus have the ability to detect alterations in their steroid environment and this capacity is more prominent during the transition time when the different organ systems are experiencing normal increases in circulating corticosterone.

Adult animal studies describing corticosterone and dexamethasone binding to the hippocampus by biochemical methods (using hippocampal homogenates) have

shown a biphasic increase of the corticosterone receptor population (29). A rapid initial increase occurs during the first 2 h, followed by a second rise which begins after 12-h post-ADX and reaches a plateau 3-5 days after the surgery. Because of the short time span present in the first phase in relation to the disappearance of the endogenous corticosterone, this first increase was interpreted as an elevation of the binding protein probably due to unmasking of preexisting binding sites (29). Due to the long delay of the second phase, *de novo* synthesis, stabilization of receptor protein, and/or further unmasking of preexisting binding sites were likely explanations for the second peak in this phenomenon. Chao and co-workers pursued this question of *de novo* synthesis in a later study where GR and MR mRNA expression was assessed using total hip-

hippocampal RNA preparations obtained from adult animals 3 days after ADX (60). Their results point to a stable GR and MR mRNA level which is unaffected by a 3-day adrenalectomy. Reul and co-workers described a different time course of GR and MR mRNA expression after adrenalectomy. In this study animals were tested several days after adrenalectomy (1 day post-ADX was the first time point) and a single animal per day was used for mRNA determination. Increases in GR mRNA receptor were present 2 days post-ADX, while MR mRNA was elevated within 24 h after ADX (45). Both of these groups use the same RNase protection methodology, which masks changes that may be present in specific hippocampal regions and may also dilute a significant finding. Our study focuses on an early time point and shows two significant findings for both development and normal adult physiology. The first one is that ADX, after a shorter time frame than examined by others, causes significant changes in both GR and MR gene expression in both developing and adult animals. The second is that *in situ* hybridization has in fact unmasked region-specific phenomena and "pin-pointed" the particular areas that are sensitive to absence of circulating corticosterone—areas that most likely would have been missed using a homogenate method such as RNase protection assay. Specifically, in the adult animal, the area of greater GR mRNA abundance, CA1-2, increases its GR expression as a result of short-term adrenalectomy. MR mRNA has a broader response involving areas of lower abundance (CA1-2 and DG) as well as areas of greater MR gene expression (CA3p). This effect of short-term adrenalectomy in the adult animal persists even as the analysis is performed to include whole hippocampus measurements (see Fig. 6B). Thus, in the adult animal: (a) the hippocampal formation's GR and MR systems are indeed sensitive to circulating corticosterone levels in a region specific manner, and (b) this effect is an early one, occurring within 14 h after adrenalectomy.

Overall, there seems to be a direct relationship between circulating basal corticosterone levels and the degree of effect of short-term adrenalectomy in the developing animal. The effects are in general seen in the 18- and 28-day-old animals. The fact that the 18- and 28-day-old animals appear to be more sensitive to the absence of corticosterone than at later ages is consistent with the changes in the hypothalamic-pituitary-adrenal axis during this age. Soon after Day 14, corticosterone target tissues are exposed to a substantial change in basal corticosterone levels. Corticosterone levels have increased from $<1 \mu\text{g/dl}$ to $3\text{--}5 \mu\text{g/dl}$ levels while CBG concentrations remain low (6, 37). Thus, at this age, the hippocampus (and other regions) may be exposed to particularly high effective concentrations of glucocorticoids, which may restrain the expression of GR and MR. Once circulating levels are decreased, these particular ages appear to be more sensitive to the change in corticoid environment and now reflect the capacity of the system to respond to

changes in its corticosterone environment. Thus, the finding described here points to cellular activation of pyramidal and granular cells in the developing hippocampus in an age-specific and site-specific manner. This cellular activation, that is, increased GR gene expression in the 18- and 28-day-old animals and the MR mRNA trend of increasing in all ages, correlates with an overall high GR and MR binding capacity in these ages (see Fig. 1 and Table 1).

The physiological significance of the site-specific areas and the ages which demonstrate ontogenic and an ADX GR and MR mRNA effect is unclear. Due to the high concentration of both GR and MR in the hippocampus, a great deal of effort has been channeled toward the understanding of the role of this structure in HPA regulation. All three aspects of HPA activity have been found to be linked to this structure: stress response, circadian rhythmicity, and corticosteroid feedback inhibition (19). It is also evident that the two "interlocking C's" which constitute the pyramidal (CA1-4) and granular cell layer (DG) intercommunicate with each other via parallel series of coronal lamellae and considerable transverse septo-temporal connections (19, 58). Given the complexity of these connections and the conflicting data regarding the effect of stimulating certain hippocampal subfields (61-63), it has been proposed that the different HPA responses are due to different combinations of neurons recruited at different activation thresholds (19). Since organization of hippocampal cell layers is a postnatal event (53-55), it is tempting to speculate that perhaps suppression of these hippocampal regions may occur with the establishment of synaptic contacts from other hippocampal areas. Other tissues, such as the intermediate lobe of the pituitary (IL), show that GR expression is sensitive to presynaptic activity. For example, GR is induced with denervation of the IL and is also present in the developing IL from newborn to Postnatal Day 3, prior to catecholaminergic innervation of this structure (64, 65). It is also possible that more subfield areas respond to the absence of corticosterone in the developing animal due to the relatively low levels of plasma CBG or membrane-bound CBG molecules which allows for a greater corticosterone "starving."

In summary, the developing hippocampus is exquisitely sensitive to its corticosterone environment in an age- and site-specific manner. This is evident at a time when the developing animal has increasing basal corticosterone levels and is becoming stress responsive. We propose that extreme caution is necessary when interpreting ontogenic and adult data which relies on "short-term" adrenalectomy to allow for the "clearance" of endogenous corticosterone. Upregulation of the GR and MR genes is evident in specific areas of the developing and adult hippocampus after 14 h of adrenalectomy and this may be reflected in an increased synthesis of glucocorticoid and mineralocorticoid receptors which in turn will increase the GR and MR binding capacity in this structure.

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