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# STRESS AND THE DEVELOPING LIMBIC–HYPOTHALAMIC–PITUITARY–ADRENAL AXIS

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## SUMMARY

The postnatal limbic–hypothalamic–pituitary–adrenal (LHPA) axis in the rodent is remarkably different from the adult, both in structure and function. The first 2 weeks postnatally are characterized by a 'silent period' during which the developing animal is hyporesponsive to stress (stress hyporesponsive period—SHRP), followed by a new and unique phase of stress responsiveness when the animal fails to swiftly terminate glucocorticoid secretion. In this review, we summarize our work which focuses on the regulatory biology of the components of the LHPA system and the consequences of its disruption on the adaptive responses of the developing organism. We find that the animal during the first 2 weeks of life responds to an intermittent chronic challenge increasing anterior pituitary POMC post-translational events, while the adult increases genomic events. The result for both the mature and the developing animal is the same, an increase in corticosterone (CS) levels. In addition, we have found evidence of impaired rate sensitive feedback in the weanling animal, as well as changes in ACTH clearance. Similar to the young animal emerging from SHRP, maternally deprived pups during the first week of life exhibit a substantial and sustained ACTH and CS response to stress. In the deprived animal these changes are accompanied by decreases in mineralocorticoid receptor gene expresion in the hippocampus, suggesting that changes in mineralocorticoid to glucocorticoid receptor ratios may be important in this phenomena. What has become evident from our studies is that mechanisms underlying normal LHPA development are dynamic, age dependent and distinct to the strategies used by the mature organism to cope with stress. 

**Keywords**—Rats; Animal; Development; Feedback; Corticosteroid receptor; Hippocampus; mRNA.

## INTRODUCTION

Numerous behavioral, endocrine and clinical studies have shown that various stressors, both physical and psychological, which occur early in life, can produce profound alterations in growth and development (Barlow et al., 1978; Dachir et al., 1993; Duncan 1977; Fride et al., 1986; Fride and Winstock 1988; Henry et al., 1994; Meaney et al., 1988). The

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endocrine system which is most closely linked to stress in mammals is the limbic–hypothalamic–pituitary–adrenal axis (LHPA). A central feature of the LHPA stress response is the synthesis and the secretion of glucocorticoids from the adrenal cortex. Glucocorticoids facilitate the mobilization of substrates for energy source, potentiates the release of catecholamines, increase cardiovascular tone and suppress 'nonessential systems' for immediate survival, such as immunity, growth and reproduction. In order to maintain stress responsiveness, both rapid activation and rapid inhibition of the stress response are necessary. Failure to activate the stress response places the organism in a very fragile state. Failure to inhibit the stress response once initiated, increases the vulnerability to diseases and results in permanent effects on growth and differentiation of a number of developing systems, including the central nervous system (De Kloet et al., 1988). The normal sequence of development of the LHPA axis underscores this notion and highlights the fact that this is not a static system.

The ability to respond to stressful events develops after birth in the rodent. During the neonatal period, the rat is limited in its ability to increase its main circulating glucocorticoid, corticosterone (CS), following stressful stimuli (stress hyporesponsive period (SHRP). This relative inactivity of the adrenocortical axis can be viewed as adaptive, since it limits catabolic processes initiated by circulating glucocorticoids and favors the predominating anabolic events (De Kloet et al., 1988). The rat is weaned at about 3 weeks, just as it is fully emerging from the SHRP; at that time, it exhibits a new and unique profile of stress responsiveness: it fails to swiftly terminate glucocorticoid secretion, exhibiting high and sustained levels of circulating corticosteroids (Goldman et al., 1973) and ACTH (Vázquez and Akil, 1993a). Alterations of events that are part of this developmental program can either be adaptive leading to a swift response and inhibition of the system or maladaptive, resulting in physical consequences or in long-term changes in setpoint which sensitize the organism to stress and preclude its efficient and rapid termination of the stress response. An insight into the mechanisms underlying normal LHPA development is essential for the understanding of altered stress related abnormalities observed in early human life; conditions such as 'failure to thrive and childhood depression' (Berwick et al., 1982; Gordon and Vázquez, 1985a,b; Powell et al., 1973).

The theoretical and practical approach to studying the developing animal has to be framed in the context of what we know in the mature organism. Therefore, we will briefly summarize what is known in: (1) in the adult animal; and (2) in the developing animal. We focus on the work from our laboratory which investigates the regulatory biology of the components of this system and the consequences of its disruption on the adaptive responses of the developing organism.

## THE ADULT LHPA AXIS

The LHPA axis is a neuroendocrine circuit with complex feedback mechanisms (Fig. 1). The activating pathways whereby the brain translates stimuli into the final, integrated response at the hypothalamus is presently ill-understood. It appears to involve neuronal inputs from catecholaminergic, serotonergic and possibly cholinergic brain nuclei, including locus coeruleus, nucleus tractus solitarius, A1 cell group in the ventral lateral medulla and raphe nucleus (Gillies and Grossman, 1985). The parvocellular neurons of the paraventricular nucleus (PVN) in the hypothalamus represent the final common path

for the integration of the stress response in the brain. They express corticotropin releasing hormone (CRH) and, to a lesser extent, arginine vasopressin (AVP), both of which are secretagogues of the LHPA (AVP in the neighboring magnocellular neurons of the PVN is primarily involved in water balance). In response to physiological and psychological stressors, CRH and AVP are secreted from the median eminence into the hypophyseal portal circulation to reach the anterior pituitary, where they synergistically stimulate the proopiomelanocortin (POMC) producing cells of the anterior lobe to release ACTH, an end-product of POMC precursor molecule processing. ACTH transported in the circulation interacts with the adrenal cortex receptors causing steroidogenesis and elevation of plasma glucocorticoids. Repeated exposure to elevated glucocorticoid levels has been

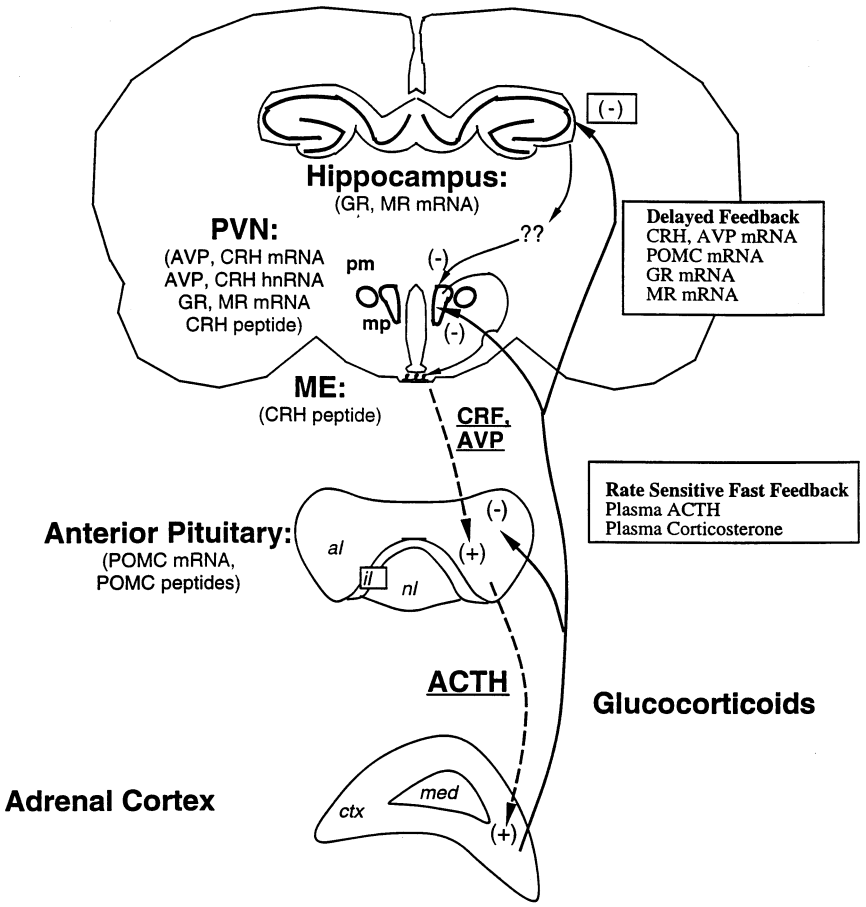


Fig. 1. The limbic–hypothalamic–pituitary–adrenal axis. The structures involved in this system include: the hippocampus, periventricular nucleus (PVN) of the hypothalamus, the anterior pituitary and the adrenal gland. Inhibition of the stress response is accomplished by three mechanisms: rate sensitive feedback, intermediate feedback and delayed feedback (see text for details). GR, glucocorticoid receptor; MR, mineralocorticoid receptor; CRH, corticotropin releasing hormone; AVP, vasopressin; POMC, proopiomelanocortin; ACTH, adrenocorticotropin; hnmRNA, heteronuclear mRNA; pm, posterior magnocellular PVN; mp, medial parvocellular PVN; ME, median eminence; al, anterior lobe; il, intermediate lobe; nl, neural lobe; ctx, cortex; and med, medulla.

implicated in neuronal death, among other deleterious effects. Therefore, inhibition of steroid secretion is also an important feature of this system. The termination of stress responsiveness appears to operate through at least three relatively independent types of mechanisms, a rate sensitive fast feedback, an intermediate feedback and a delayed feedback. Fast feedback is a rapid phenomenon (within minutes) whereby the rate sensitive rise of steroids 'turns off' CRH and ACTH secretion, and the more rapid the rate of rise, the more effective the inhibition. Intermediate feedback begins 30–90 min following steroid administration and it is proportional to the total dose of steroids administered. The mechanisms of rate dependent feedback are ill-understood, but are likely to be include neuronal mechanisms, most likely suprahypothalamic (Abdulla et al., 1995; Buijs et al., 1993a,b; Calogero et al., 1988, 1992; Dallman et al., 1991; Widmaier and Dallman, 1983a). It is evident that some aspects of fast feedback inhibition are influenced by glucocorticoid binding to specific receptors in limbic structures, primarily the hippocampus. Neural connections, from the hippocampus to the hypothalamic CRH neurons appear to play a critical role in the fast feedback inhibition of ACTH secretion, such that specific lessions or decreased glucocorticoid receptors (e.g. aging) result in a selective decrease in the termination of the stress response (Feldman and Conforti, 1980; Herman et al., 1995; Jacobson and Sapolsky, 1991; Sapolsky et al., 1984). Delayed feedback, on the other hand, works over the course of hours, whereby the translocated corticoid–receptor complex acts at the transcriptional level by suppressing gene expression, therefore decreasing the ACTH stores in the pituitary and all other key molecules in LHPA structures (e.g. hypothalamic CRH, hippocampal glucocorticoid receptors) (Fremeau et al., 1986; Young and Vázquez, 1996). The hippocampus also appears to be a key structure as hippocampectomy upregulates CRH mRNA expression, even in the face of increases in circulating glucocorticoids (Herman et al., 1989b; Jacobson and Sapolsky, 1991). Furthermore, genomic feedback at the level of the hypothalamus appears to depend on an intact hippocampus since glucocorticoids fail to inhibit the system if the animal lacks the hippocampus (Akil and Morano, 1996; Jacobson and Sapolsky, 1991).

Two types of corticoid receptors have been described in the brain based on biochemical and functional characteristics (Funder, 1986; Reul and De Kloet, 1985). Type I or mineralocorticoid receptor (MR), resembles the kidney mineralocorticoid receptor and has stringent specificity, binding selectively CS, the main glucocorticoid of the rat. In the brain, MR is most densely localized in hippocampal and septal neurons. It is often described as a 'high affinity, low capacity corticoid receptor system'. Conversely, type II or glucocorticoid receptor (GR) is known as a 'low affinity, high capacity receptor system'. The GR receptors are widely distributed in brain neurons, including hippocampus, hypothalamus, glial cells and pituitary cells. However, they bind corticosterone (CS) with a lower affinity compared with MR. Their highest affinity is for potent synthetic glucocorticoids, such as dexamethasone (Funder, 1986; Reul and De Kloet, 1985). These receptor characteristics complement each other and put both the MR and GR in a position to modulate LHPA responsivity. The MR receptors appear to be operative at low CS concentrations and may offer tonic inhibition to the axis during the nadir of the circadian rhythm (Dallman et al., 1987; Funder, 1986; Reul and De Kloet, 1985). When high concentrations are present, MR receptors saturate, and the GR receptors appear to 'take over' to ensure the return of homeostasis. It is, thus, apparent that dysfunction on any of the complex pathways within the system could severely affect the organism adaptive capacity.

## THE DEVELOPING LHPA AXIS: THE IMMEDIATE POSTNATAL PERIOD

The rodent fetal and postnatal LHPA axis is remarkably different from the adult, both in structure and function. The first 2 weeks postnatally are characterized by a 'silent period' during which the developing animal is hyporesponsive to stress (De Kloet et al., 1988). Specifically, after postnatal day 4 and until $\approx$ day 12 of life, rat pups respond weakly, to a variety of acute stressors, including ether, surgery and handling. These stressors, would otherwise cause a reliable dramatic plasma CS increase in older animals. In the neonatal rat the ACTH and CS responses are limited in magnitude, and the appearance of a response is time and stressor-specific (Walker et al., 1991). The pattern of response is that of hyporesponsiveness at all levels of the axis, namely: (1) a blunted pituitary ACTH secretion, resulting from a combination of immaturity of neural inputs to the CRH neurons, decreased pituitary peptide content or decreased sensitivity to CRH stimulus; and (2) an adrenal gland hyporesponsive to circulating ACTH levels. A unique pattern of glucocorticoid receptor in the brain and pituitary which enhances the sensitivity of the pituitary to the inhibitory feedback effects of circulating glucocorticoids has also been postulated (De Kloet et al., 1988).

Studies in the neonatal period have mainly focused on the response of individual components of the system to acute stimulus (Guillet et al., 1980; Walker et al., 1986a,b). In order to study the regulatory ability of the developing LHPA axis we have focused on cellular events consisting of genomic expression, post-translational processing, secretion during normal development and changes resulting from chronic intermittent stress. Thus, these studies were designed to investigate, the complete basal profile of the POMC cell by evaluating, simultaneously, during the first 3 weeks of life: (a) POMC mRNA levels; (b) POMC peptides (ACTH, aMSH, $\beta$E and *N*-Ac $\beta$E); and (c) plasma peptide and CS levels (Vázquez and Akil, 1992). The advantage to this approach is that the different cellular levels of the pituitary compartment, i.e. POMC mRNA, peptide content and release, is studied within the same normal developing animal. The same approach is then applied to the pup subjected to chronic intermittent stress. The tools used in these studies include: mRNA Northern gel analysis, mRNA solution hybridization technique and radioimmunoassay of the different plasma and pituitary peptides (Vázquez and Akil, 1992).

### *Anterior and Intermediate Pituitary: Peptide and POMC mRNA Ontogeny*

One key component of the adrenocortical response to stress is the proopiomelanocortin (POMC) producing cell, or corticotroph, which constitutes 5–10% of all the hormone producing cells of the mature anterior pituitary (AP) and virtually all the hormone producing cells of the intermediate lobe (IL) (Eipper and Mains, 1980). POMC is the precursor molecule of several tissue specific peptides. In the AP, POMC is cleaved to yield primarily the stress related hormone, adrenocorticotropin ($ACTH_{1-39}$) and beta-lipotropin hormone ($\beta$–LPH). $\beta$-LPH in turn is cleaved to give the opioid active beta-endorphin ($\beta E_{1-31}$). By contrast, in the IL melanotrophs, $ACTH_{1-39}$ is further cleaved to give rise to $\alpha$MSH. $\beta$-Endorphin is in turn modified by a combination of *N*-acetylation and carboxy terminal cleavage, to produce the opiate inactive forms *N*-Ac $\beta E_{1-27}$ and *N*-Ac $\beta E_{1-26}$ (Eipper and Mains, 1980). In fact, in the adult, 90% of the stored IL peptides are either MSH or $\beta$E forms (Eipper and Mains, 1980; Sachar et al., 1971; Seizinger et al., 1984).

We characterized the AP peptide content in animals during their first 3 weeks of life (Vázquez and Akil, 1992). Panel A on Fig. 2 shows that the AP peptide content increases significantly with age for each individual peptide. ACTH and $\beta$E increased 4–8-fold from

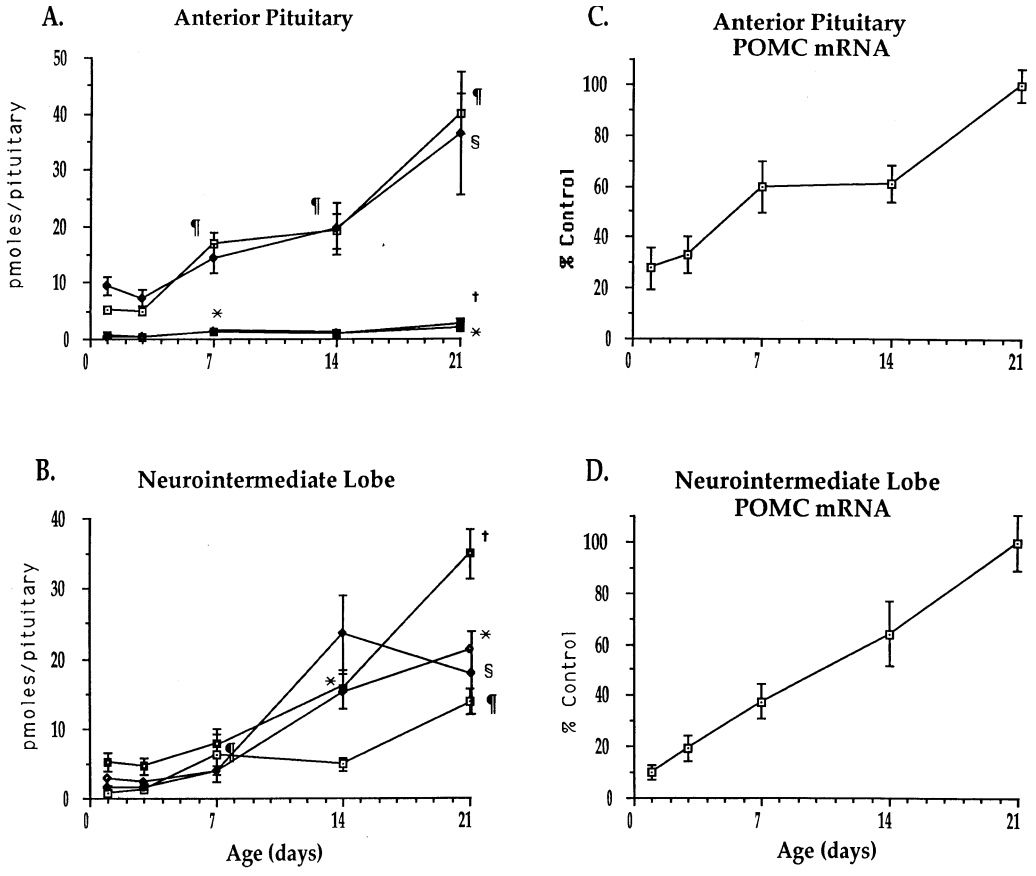


Fig. 2. Total proopiomelanocortin derived peptides stored in the pituitary of the unhandled rat pup from newborn (NB) to day 21 of life. Anterior pituitary peptide content is shown on panel A and content of the neurointermediate lobe on panel B. Two factor analysis of variance revealed a main effect of age for each peptide ($p = .001$). Significant post-hoc comparisons between NB and other ages are indicated with the following symbols: hard return symbol, ACTH; § $\beta$-LPH/$\beta$E; * MSH; † $N$-Ac $\beta$E (Fisher PLSD, $p = .05$). Number of animals per age are as follows: NB, 18; day 3, 16; day 7, 15; day 14, ten; day 21, ten. Panels C and D show the developmental progression of POMC mRNA from anterior and neurointermediate pituitary of undisturbed pups. Comparison across groups carried out by expressing densitometry units relative to the 21 day-old unhandled animal. Number of individual determinations are as follows: NB, six; day 3, seven; day 7, eight; day 14, six; day 21, eight.

newborn to day 21 of life. However, in view of the fact that an adult unstressed AP contains $\approx 100$ pmoles of $\beta$E (Shiomi et al., 1986), only 35% of the $\beta$E adult content is achieved by day 21. In agreement with other studies (Chatelain and Dupony, 1985), $\alpha$MSH and $N$-Ac $\beta$E were also detected in the AP during this period of time and both increased between birth and the third week of life. When comparing the proportions of post-translationally processed material to its immediate precursor, it became evident that there is a greater proportion of cleaved and acetylated end products in the developing anterior pituitary which are typical of the adult intermediate lobe (Table I). This suggests a relatively greater proteolytic cleavage and acetyl transferase activity during early life

which later diminishes despite the availability of greater amounts of the biosynthetic intermediates, $\beta$E and ACTH.

The neurointermediate lobe of the developing animal, presented in Fig. 2, panel B, also exhibits an increase of peptide content with increasing age. However, in this tissue, POMC peptides increased markedly and at a greater rate when compared with the rate of increase in the AP. In the 21 day-old rat, *N*-Ac $\beta$E, ACTH and $\alpha$MSH increased 7–20-fold relative to the newborn NIL. All peptides had the greatest rate of increase between day 7 and 14 of life. Interestingly, $\beta$E follows the same pattern of increase until day 14 at which time it begins to decline, a pattern which corresponds to the sharp increase of *N*-Ac $\beta$E forms. Although the amount of peptide stored, appears to be increased in the later portion of the curve, the NIL which contains $\approx$1000 pmoles of *N*-Ac $\beta$E in the adult animal (Eipper and Mains, 1980; Rudman et al., 1979) had attained only 6% of its final content by day 21.

POMC gene expression paralleled the increase of peptides in both AP and NIL and their greatest rate of increase also occurred between day 7 and 21 of life (Fig. 2, panel C and D). The appropriate mature mRNA size for POMC, 1.2 kb, was confirmed on the hybridized Northern membranes and were corrected for loading artifacts using the ubiquitous gene product, P1B15 cRNA (Danielson et al., 1988). High molecular weight mRNA bands with sizes consistent with unspliced forms can be detected in Northern Gel analysis (Fig. 3). This is consistent with work from Pintar and co-workers who reported POMC heteronuclear mRNA levels using intronic solution hybridization assays on fetal and postnatal anterior and intermediate lobe tissue (Scott et al., 1990). It is very likely that this reflects the increased level of mitosis present during early life.

*Ontogeny of Plasma Hormonal Levels*. Plasma ACTH levels changed significantly on day 21 of life (Fig. 4). The CS levels were similar to the pattern described in previous studies (Sapolsky and Meaney, 1986) showing an initial decrease in CS after birth which was followed by an increase on day 14 and 21. Two interesting observations are evident in the pattern of plasma ACTH and CS levels of the 14 and 21 day-old pups. First, on day 14 the adrenal cortex appears more sensitive to ACTH as can be seen on Table I—ACTH:CS ratio and by comparing panels A and B of Fig. 4. There is a tendency to release a greater amount of CS on day 14 though similar amounts of ACTH are

Table I. Relative molar ratios of POMC related peptides and message in the anterior and intermediate lobes of the developing pituitary

| Age (days-old) | Anterior pituitary | | |
|---|---|---|---|
| | **$\beta$E:*N*-Ac $\beta$E (unhandled)** | **ACTH:MSH (unhandled)** | **Plasma ACTH:CS (unhandled)** |
| NB | 100:10.7 $\pm$ 0.03 | 100:29.2 $\pm$ 0.1 | 100:(3.5 $\pm$ 0.9) $\times 10^3$ |
| 3 | 100:4.3 $\pm$ 0.01 | 100:65.7 $\pm$ 0.3 [a] | 100:(3.8 $\pm$ 1.6) $\times 10^3$ |
| 7 | 100:156.9 $\pm$ 1.0 [a] | 100:11.4 $\pm$ 0.02 [a] | 100:(2.6 $\pm$ 0.5) $\times 10^3$ |
| 14 | 100:15.6 $\pm$ 0.1 [a] | 100:9.9 $\pm$ 0.04 | 100:(6.0 $\pm$ 1.9) $\times 10^3$ |
| 21 | 100:19.8 $\pm$ 0.1 | 100:5.2 $\pm$ 0.02 | 100:(1.6 $\pm$ 0.2) $\times 10^3$ |
| Adult | 100:1.0* | 100:1.0* | 100:10.8 $\times 10^{3*}$ |

* Data obtained from Shiomi et al. (1986).

[a] $p < 0.05$ by ANOVA; $p < 0.05$ by Fisher PLSD compared to previous age.

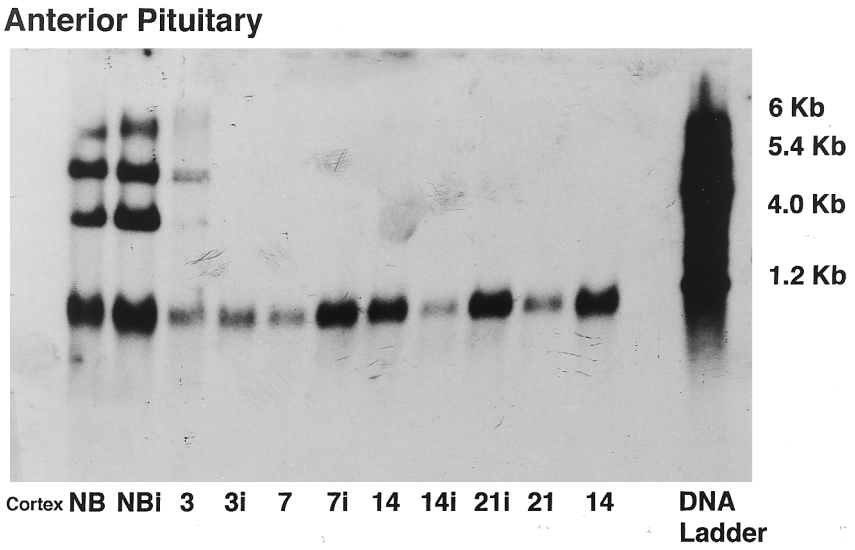


Fig. 3. Northern blot autoradiograph of anterior pituitary extracts showing multiple POMC mRNA signal bands across ages and treatment. High molecular weight mRNA bands was obtained with an exon 3 cDNA probe. The signals obtained are consistent with unspliced POMC heteronuclear mRNA forms: 6.0 kb, primary transcript; 5.4 kb, exon 1 + intron A + exon 2 + exon 3; 4.0 kb, exon 1 + 2 + intron B + exon 3. After reprobing with intron A cDNA two bands were obtained (6.0 and 5.4 kb)conforming that the bands were part of the POMC hnmRNA. Molecular markers are shown on the right. i, Isolated.

circulating on both days (Table I—ACTH:CS ratio). Second, although there are high levels of circulating ACTH on the 21 day-old animal, a corresponding CS rise is not evident. This is consistent with the decrease in adrenal sensitivity to circulating ACTH and limited adrenal stereidogenic capacity observed at this age (De Kloet et al., 1988; Henning and Genovese, 1985).

In sum, the developing animal, under basal conditions, exhibits: (1) an age related increase in pituitary POMC mRNA levels; (2) an age related increase in pituitary POMC peptide levels which parallels the increase in mRNA levels; (3) a relative insensitivity to ACTH at the level of the adrenal before day 14 of life; and (4) a decrease in plasma corticosterone levels during the first 2 weeks of life. It thus appears that the developing pituitary has a progressive maturation of the pre-translational and translational components of the POMC synthetic machinery during the first 3 weeks of life. Are there other differences present in the dynamics of the basal biosynthetic processes of the developing POMC cell? Are post-translational events also changing with age?

*De novo POMC Peptide Synthesis and Post-Translational Processing*. In a separate study, we addressed POMC post-translational events (Vázquez and Akil, 1989). *De novo* synthesis of POMC was investigated using a pulse labeling paradigm. In these series of experiments the pituitary cells were dispersed by the Mulder and Smelik method and [$^3$H]Leu radioactivity labeled amino acid was allowed to be incorporated into the newly synthesized peptide molecules. The cells are then ruptured and the peptide of interest is immunoprecipitated with a specific antibody. An antiserum was chosen that recognizes $\beta$E, $\beta$-LPH and the POMC precursor molecule. The different POMC derived molecular

forms were separated using SDS-PAGE electrophoresis. In order to validate the immunoprecipitation procedure, the following controls were carried out using both adult anterior lobe and neurointermediate lobe: (a) primary antibody added (midportion $\beta$E antibody); (b) excess of $\beta E_{1-31}$ added to inhibit primary antibody binding; and (c) no primary antibody. The results presented on Fig. 5 demonstrate that immunoprecipitation using the primary antibody specifically precipitates $\beta$E forms. The AP cells obtained from developing animals underwent the same procedure. After separating the precipitated POMC and POMC products using SDS gel electrophoresis, it became obvious that the developing

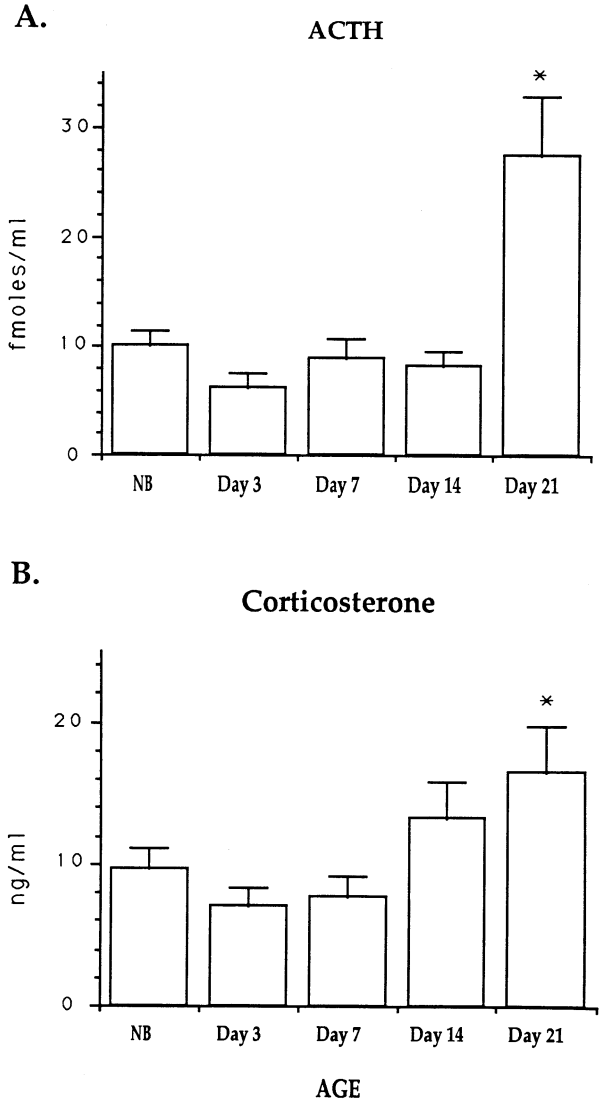


Fig. 4. Plasma corticosterone and ACTH levels from unhandled pups. The following number of samples were assayed per group: newborn, 11; day 3, eight; day 7, 12; day 14, ten; and day 21, ten. Statistical significance was achieved across ages (CS: $p = .0001$; ACTH: $p = .0001$). * $p = .05$ versus unhandled (Fisher PLSD).

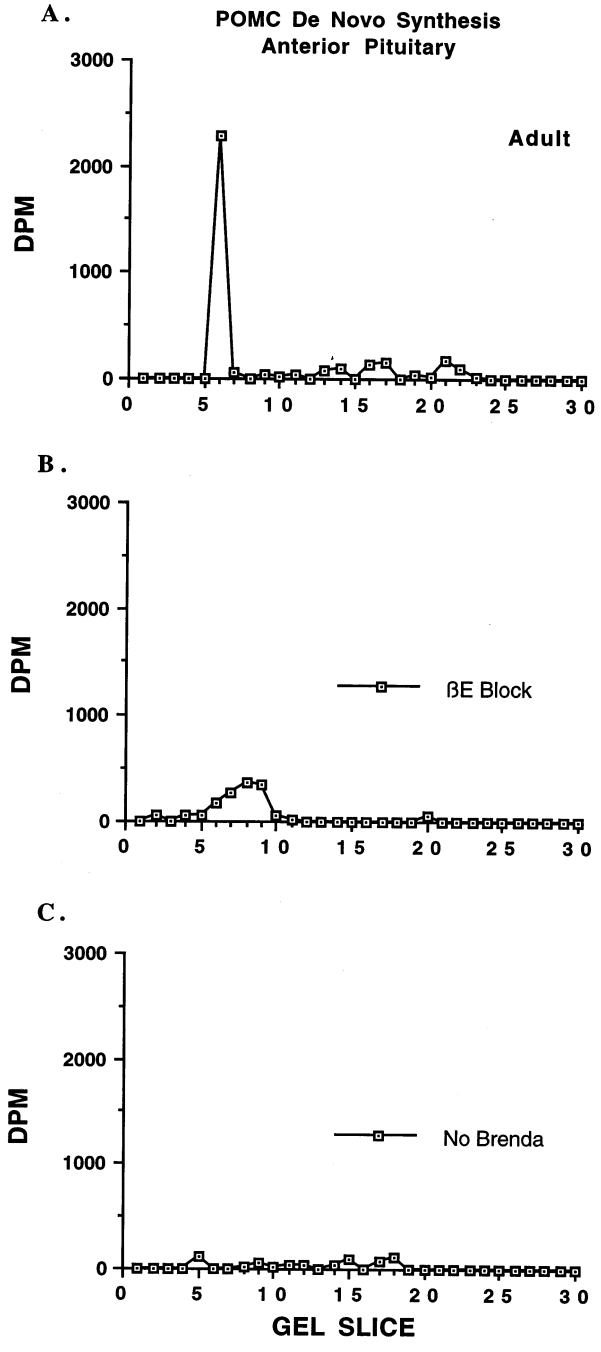


Fig. 5. Validation of the immunoprecipitation procedure used to determine *de novo* POMC synthesis. Adult anterior pituitary cells were dispersed and [$^3$H]Leu radioactivity labeled amino acid was allowed to be incorporated into the newly synthesized peptide molecules. SDS-PAGE electrophoresis was carried out on the extract obtained from the lysed cells. The following controls were carried out: panel A, primary antibody added (midportion $\beta$E antibody); panel B, excess of $\beta E_{1-31}$ added to inhibit primary antibody binding; and panel C, no primary antibody. This procedure specifically precipitates $\beta$E forms.

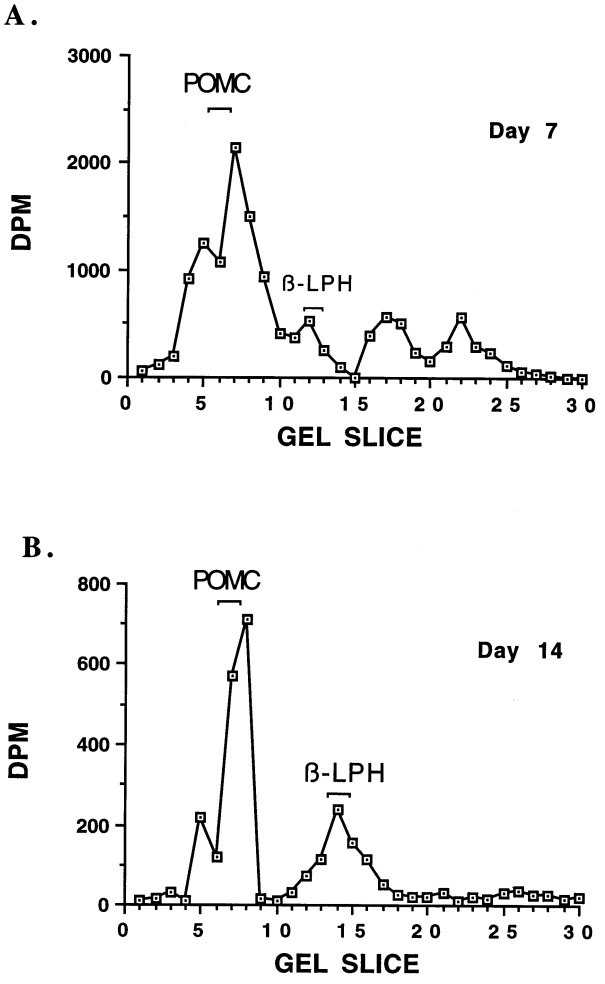


Fig. 6. *De novo* POMC synthesis in 7 (A) and 14 day-old (B) anterior pituitary cells. On Fig. 5 the adult AP showed a single peak of POMC precursor after incorporation of [$^3$H]Leu. Within the same time frame, the developing anterior pituitary processes the POMC precursor to other POMC related peptides: $\beta$-LPH and $\beta$-endorphin.

corticotroph appears to process the POMC precursor at a faster rate than the adult corticotroph (Fig. 6). Several peaks corresponding to the electrophoretic profile of POMC precursor, $\beta$-LPH and $\beta$E are seen in day 7 and 14 animals, while, as expected, a single peak corresponding to the POMC precursor molecule is obtained from the adult animals (31K:$\beta$-LPH ratio 1:0.86 in day 14 vs. 1:0.06 in the adult). Day 21 pups profile is similar to the profile obtained from adult animals.

The apparent increased rate of synthesis and the multiple POMC mRNA forms described above, are two additional differences between the developing and mature LHPA axis. What importance might these two findings have if increased demands are imposed to the POMC cell? Are these, yet, another level by which the corticotroph can regulate the demands imposed by the environment?

*Regulation: Maternal Isolation as a Chronic Intermittent Stressor*

Maternal isolation, which is a model of stress used for behavioral and pharmacological studies (Kuhn et al., 1990; Levine, 1994), causes activation of the LHPA axis in the pup postnatally. We employed a repeated stress paradigm in order to investigate if there was an altered developmental pattern of POMC cell, in terms of the dynamics of its biosynthetic processes: pre-translational events, post-translational processing and release of products, upon chronic stress. Isolation from the mother for 1 h, on 3 consecutive days, results in a small but significant increase in CS levels by the third session (Fig. 7). This is not surprising since increasing responsiveness to a change in environment is seen as the animal matures beyond 12 days of age (end of SHRP).

*Pituitary Peptide and POMC mRNA Content.* Pups challenged with isolation from the mother for 1 h, on 3 consecutive days, did not exhibit changes in stored AP peptides with the exception of the 21 day-old pups when depletion of content occurred (Vázquez and

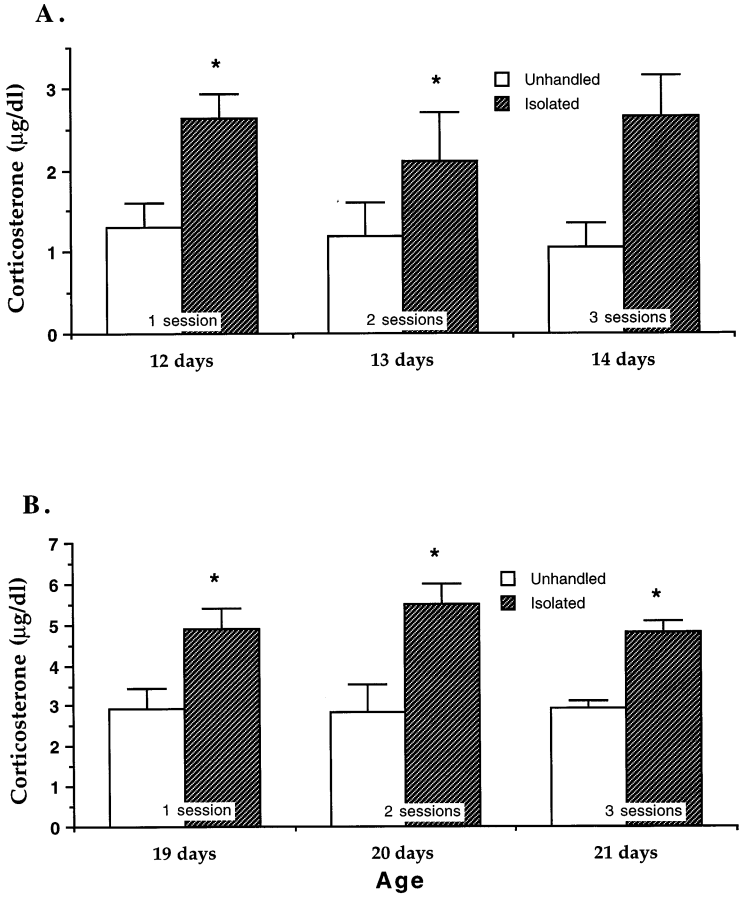


Fig. 7. Corticosterone response to consecutive one hour maternal isolation during the second and third week of life. Animals (12 and 13 days-old) have elevated levels after the end of each isolation session ($p < .05$, Fisher PLSD). However, on the third session significance is not achieved. From 19 to 21 days of age all sessions result in significantly elevated corticosterone levels ($n = 8$ per group).

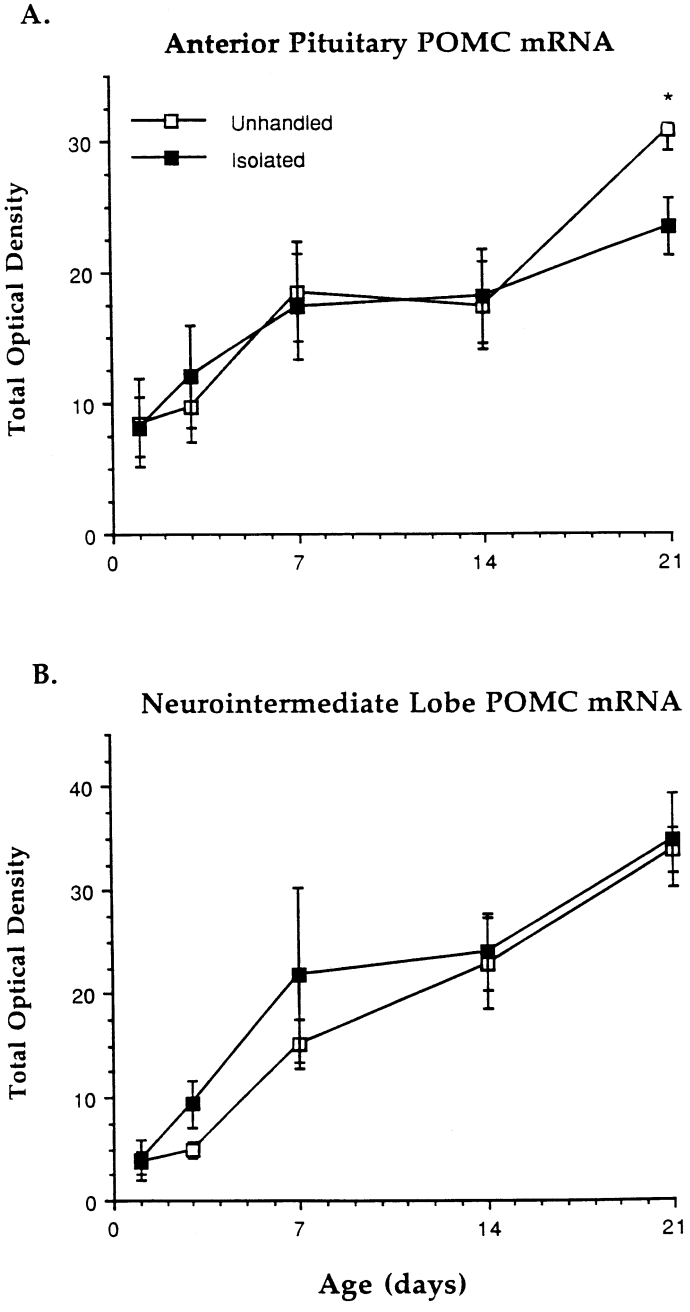


Fig. 8. Effect of maternal isolation on the content of the anterior pituitary and neurointermediate lobe POMC mRNA levels. (A) POMC mRNA extracted from the anterior pituitary of unhandled and maternally isolated animals; (B) POMC mRNA extracted from the neurointermediate lobe of unhandled and maternally isolated animals. Comparison across groups carried out by expressing densitometry units relative to the 21 day-old animal. The number of individual determinations are as follows: unhandled and isolated: age, day 1, six; day 3, seven; day 7, eight; day 14, seven; day 21, eight. * $p = .05$ by paired two tailed student $t$-test.

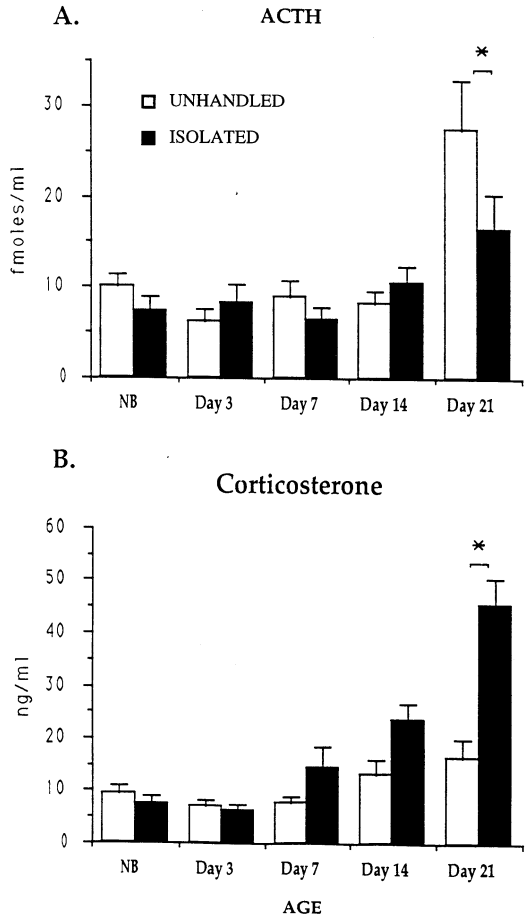


Fig. 9. Plasma ACTH and corticosterone levels from unhandled pups and pups isolated from their mothers. Unhandled pups are represented with open bars and isolated pups with closed bars. The following number of samples were assayed per group: newborn, 11; day 3, eight; day 7, 12 unhandled, six isolated; day 14, ten unhandled, 12 isolated; and day 21, ten unhandled, 16 isolated). Statistical significance was achieved across ages (CS: $p = .0001$; ACTH: $p = .004$). There was also an age to treatment interaction (CS: $p < .0001$; ACTH: $p < .04$). * $p = .05$ versus unhandled (Fisher PLSD).

Akil, 1992). At this age all peptides measured decreased significantly. Likewise, maternal isolation for 1 h for 3 consecutive days, did not cause significant changes in AP POMC mRNA levels during the first 2 weeks of life (Fig. 8). However, the 21 day-old pup responded to this stressor by decreasing its AP POMC mRNA content.

The developing NIL, like the developing AP, responded to the stressor on day 21 of life only (Vázquez and Akil, 1992). Consistently, ACTH, $\alpha$MSH and *N*-Ac $\beta$E NIL content decreased post-stress in the 21 day-old animal. In addition, the developing NIL POMC mRNA content did not change in response to the maternal isolation regimen. Therefore, these data suggest that the IL responds to repeated intermittent maternal isolation with secretion of IL peptides.

*Plasma Hormonal Levels.* The challenged animal showed an increasing CS response on day 7 of life which did not reach significance (Fig. 9). In this particular age group, the plasma ACTH levels were unaltered by stress. Nevertheless, a greater CS to ACTH ratio was observed following the maternal isolation stress, suggesting greater adrenal sensitivity to circulating ACTH levels in the 7 day-old animal which was subjected to 3 days of intermittent maternal isolation. This, in fact, is very similar to what was observed in the 14 day-old animal during development (Table I) and is consistent with reports obtained from maternally deprived pups (Rosenfeld et al., 1992). Extremely low levels of ACTH injections (in the order of magnitude of 0.001 IU of ACTH) are capable of inducing a CORT response in pups deprived from their mothers for 24 h at all ages tested (days 4, 8, 12 and 16).

A definite adrenocortical response was present in the 14 and 21 day-old animal (Fig. 9). The increase in CS levels on day 21 was associated with a failure to increase plasma ACTH levels. However, it is evident that despite similar circulating plasma ACTH levels, the 7, 14 and 21 day-old isolated animal maintain a greater level of CS when compared with the their unhandled controls.

In sum, chronic intermittent challenge in the form of maternal isolation for one h, on three consecutive days causes: (1) a tissue peptide response in both the AP and NIL of the 21 day-old animal only (a decrease); and (2) an increase in plasma corticosterone levels on days 7, 14 and 21, despite a small ACTH response, which suggests an increase in adrenal sensitivity to circulating ACTH. There are also two other features present in the 21 day-old animal: (1) a decrease in plasma ACTH levels concomitant with a high CS release; and (2) an inhibition of AP POMC gene expression in the 21 day-old pup. The combination of these observations—an increase in circulating corticosteroids, a decrease in POMC mRNA levels, a decrease in pituitary peptide stores, and a decrease in peptide plasma levels, all suggest the emergence of negative corticosteroid feedback by day 21 of life.

The pattern observed after stress in the developing animal is quite unusual, differing dramatically from what is seen in the adult animal. In the mature rat, a combination of chronic and acute stress, as used in the present study, results in an increase in POMC mRNA, a slight increase in both plasma ACTH and corticosterone and a near normal or slightly elevated pituitary content of POMC products (Shiomi et al., 1986). Thus, the adult animal differs from the 21 day-old in that: (1) it responds to increasing demands by increasing POMC expression; and (2) the increased drive which accompanies repeated stress more than counteracts the inhibitory effects of glucocorticoids.

*Effect of Chronic Intermittent Stress on de novo POMC Peptide Synthesis.* Since POMC mRNA, peptide content and secretion are not significantly altered in either lobe, this finding suggests that either maternal isolation, as applied above, is a weak stressor for the developing animal or the developing POMC cell responds to chronic demand differently than the adult animal. Thus, to further investigate the regulation of the pituitary POMC cell during the SHRP, we used a pulse paradigm to assess cellular dynamics on rats age 7 and 14 days of age. Anterior pituitary cell suspensions were prepared from pituitaries obtained from unhandled and maternally isolated pups. Radiolabled amino acid, [$^3$H]Leu, was used for incorporation and purified by immunoprecipitation using a midportion $\beta$-endorphin ($\beta$E) antibody. The different POMC derived molecular forms were separated on a SDS-PAGE. Day 7 and 14 AP cells from isolated animals processed the POMC

precursor at a faster rate when compared with the aged matched unhandled animals (Fig. 10). It, thus, appears that the developing corticotroph responds to the chronic intermittent stress of maternal separation by increasing translational events. This mechanism of response results in a minimal (if any) increase of AP peptide content and a limited increase of circulating POMC related peptides (Vázquez and Akil, 1989).

*Inhibitory Effects of Glucocorticoids.* In the mature animal the increased drive which accompanies repeated stress predominates, such that inhibitory effects of glucocorticoids are not evident (Keller-Wood and Dallman, 1984). This is not the case in the 21 day-old maternally isolated animal in which POMC mRNA levels are found to be decreased. A seemingly perplexing plasma response is also observed, with a decreased ACTH plasma

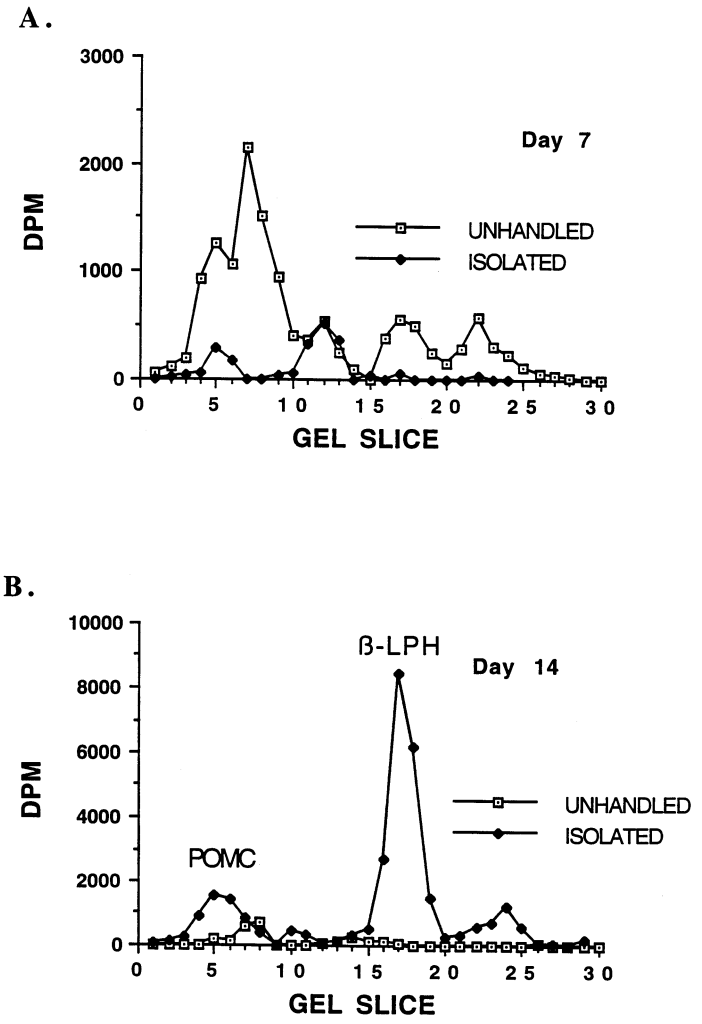


Fig. 10. *De novo* POMC synthesis in (A) 7 and (B) 14 day-old anterior pituitary cells obtained from unhandled and maternally isolated pups. An increased rate of processing of the POMC precursor is observed.

level while CS levels remain elevated (Fig. 9). How can we explain the decrease in POMC mRNA levels in the 21 day-old animal in view of the relatively mild stressor and short time of exposure to daily stress? Is it possible that fast feedback or the ability to rapidly shut-off the adrenocortical response is defective in these animals at this particular age? Such a defect in rapid 'turnoff' would result in an elevation in circulting CS, which would in turn lead to a decrease in POMC levels and ACTH release through intermediate and genomic feedback. Interestingly, Goldman et al. (1973) have shown a prolonged adrenocortical response pattern after an acute stressor in the weanling animal. Although a robust adrenocortical response was present in 25 day-old rats, the termination of the response was impaired in the 25 day-old animal such that resting CS levels were still elevated 2 h after the stressor. Therefore, similar to the aged rat (Sapolsky et al., 1983), in which failure to terminate stress has been related to a decrease in hippocampal glucocorticoid receptors, it is possible that the immature animal has an impairment of the neuroregulatory mechanisms terminating the stress response. In the case of the developing animal, the inability to rapidly 'shut-off' of the stress response may be due to the possibility that the neuronal circuitry that is needed for fast feedback has not yet matured. This may be compounded by prolonged half-life of free CS and its binding protein, CBG during the first month of life,which allows for tissues to be exposed for a longer period of time to the circulating CS (Henning, 1978; Schapiro et al., 1971). Therefore, two possibilities may explain the decrease in POMC mRNA levels in the 21 day-old animal: (1) a slow peripheral CS metabolism; and (2) a failure in fast feedback regulation due to a inhibitory circuitry which is not fully developed.

Although Walker and co-workers favor the notion of an enhanced corticosteroid inhibition during the SHRP (Walker et al., 1986b), our data suggest that this, in fact, may be occurring immediately after the second week of life when LHPA neuronal regulatory mechanisms emerge. In addition, we would suggest that this increased sensitivity to corticosteroids may be due to the presence of negative genomic feedback in the absence of an adequate acute termination of the adrenocortical response, resulting in exaggerated corticosteroid levels. These notions were tested in the following series of studies.

## THE DEVELOPING LHPA AXIS: THE POST-WEANING PERIOD

Fast feedback is the first line of defense against elevated CS levels and occurs immediately during periods of increasing CS concentration (Dallman et al., 1987); only with CS and cortisol (Jones and Hillhouse, 1976; Jones and Tiptaft, 1977); and its effect is on stimulated CRH and ACTH release (Widmaier and Dallman, 1983a,b). We strongly suspected an impaired fast feedback mechanism in view of our results in the maternally isolated 21 day-old animal. In addition, Goldman et al. have shown that, compared with the adult animal, the weanling animal has a robust CS peak but a slower return to resting CS levels after exposure to ether vapor (Goldman et al., 1973). This led to the notion that glucocorticoid suppression of the LHPA axis remains immature and continues to develop during the postweaning period (Sapolsky and Meaney, 1986). However, Goldman et al. did not measure plasma ACTH levels and did not assess the CS inhibition of pituitary ACTH directly.

In a series of studies, we sought direct evidence for an immature negative feedback inhibition in the weanling animal (Vázquez and Akil, 1993a; Vázquez et al., 1997). For this purpose, we compared the ACTH and CS response with ether vapors in the developing

Table II. Pharmacokinetic parameters: half life ($t_{1/2}$), apparent volume of distribution ($V_d$) and metabolic clearance rate (MCR) of [3-I$^{125}$]Iodotyrosyl$^{25}$ ACTH$_{1-39}$ in plasma of animals at different ages

| Age (days) | BW (g) | Half life ($t_{1/2}$) min | Apparent volume of distribution ($V_d$) | | Metabolic clearance rate (MCR) | |
|---|---|---|---|---|---|---|
| | | | ml·50 g BW | ml | ml·50g BW·min | ml·min |
| 14 (6) | 36.2 ± 0.97 | 7.47 ± 0.9* | 19.7 ± 0.5*† | 14.2 ± 0.3 | 2.05 ± 0.25* | 1.5 ± 0.19* |
| 25 (6) | 50.7 ± 0.49 | 6.48 ± 0.4* | 14.1 ± 0.1* | 14.3 ± 0.002 | 1.56 ± 0.18* | 1.6 ± 0.18* |
| Adult (13) | 339.1 ± 6.6 | 4.46 ± 0.2 | 3.1 ± 0.9 | 21.3 ± 5.8 | 0.44 ± 0.08 | 3.0 ± 0.56 |

Values are given as mean ± SE. The number in parenthesis indicate the number of animals in each group.
* Significantly different from adult, $p < .05$.
† Significantly different from 25 day-old, $p < .05$

weanling and adult animal. Specifically, we addressed the following questions: (1) does a delayed ACTH response explain the late CS peak and subsequent elevated levels in the weanling animal?; (2) are the sustained ACTH increases observed after a stressor reflecting, in part, a reduced ability to remove this peptide from circulation?; (3) is the 25 day-old animal unable to inhibit ACTH secretion once the stress response is initiated?; and (4) if the latter is true, does exogenous administration of a glucocorticoid inhibit the ACTH response to ether stress in the weanling animal?

*Hyporesponsive Adrenal versus Inadequate ACTH Activation*

Rats age 14, 18, 25 days and adults were subjected to ether vapors for 3 min in the morning. Plasma was collected for ACTH and CS determination by radioimmunoassay (RIA) at 5, 15, 30, 60 and 120 min after ether exposure. Maximum CS levels were observed at different times after exposure: 15 min in the adult animal and 30 min in the day 14, 18 and 25 animal (Fig. 11, panel A). The CS delay observed in the younger animals was not due to a delayed ACTH response since maximal ACTH values were observed 5 min after ether exposure in all ages (Fig. 11, panel B). CS levels were significantly elevated at 60 min in the day 25 and 18 when compared with the adult. In terms of magnitude, the day 25 and adult rats had a comparable ACTH and CS response. However, in the day 25 the ACTH level remained significantly elevated until 30 min after the ether vapor challenge and declined thereafter.

*Slow ACTH Metabolism versus Deficient Regulation of the Rate Sensitive Fast Feedback*

Three explanations for the delayed return to resting ACTH levels were considered: (1) a prolonged ACTH metabolic clearance; (2) an immature adrenal which leads to a delayed and slow CS rise that is not conducive to an appropriate fast feedback signal; and (3) a deficient regulation of the rate sensitive fast feedback mechanism at the pituitary and brain level. To address the first possibility, we designed a study to determine the kinetics of ACTH in 14 and 25 days-old animals and compare these with ACTH kinetics in the adult (Vázquez et al., 1997). Animals received an equivalent dose of synthetic ACTH in the form of [3-I$^{125}$]Iodotyrosyl$^{23}$ ACTH$_{1-39}$. The [3-I$^{125}$]Iodotyrosyl$^{23}$ ACTH$_{1-39}$ disappearance curve was best fit to equations derived from compartmental modeling and kinetic analysis.

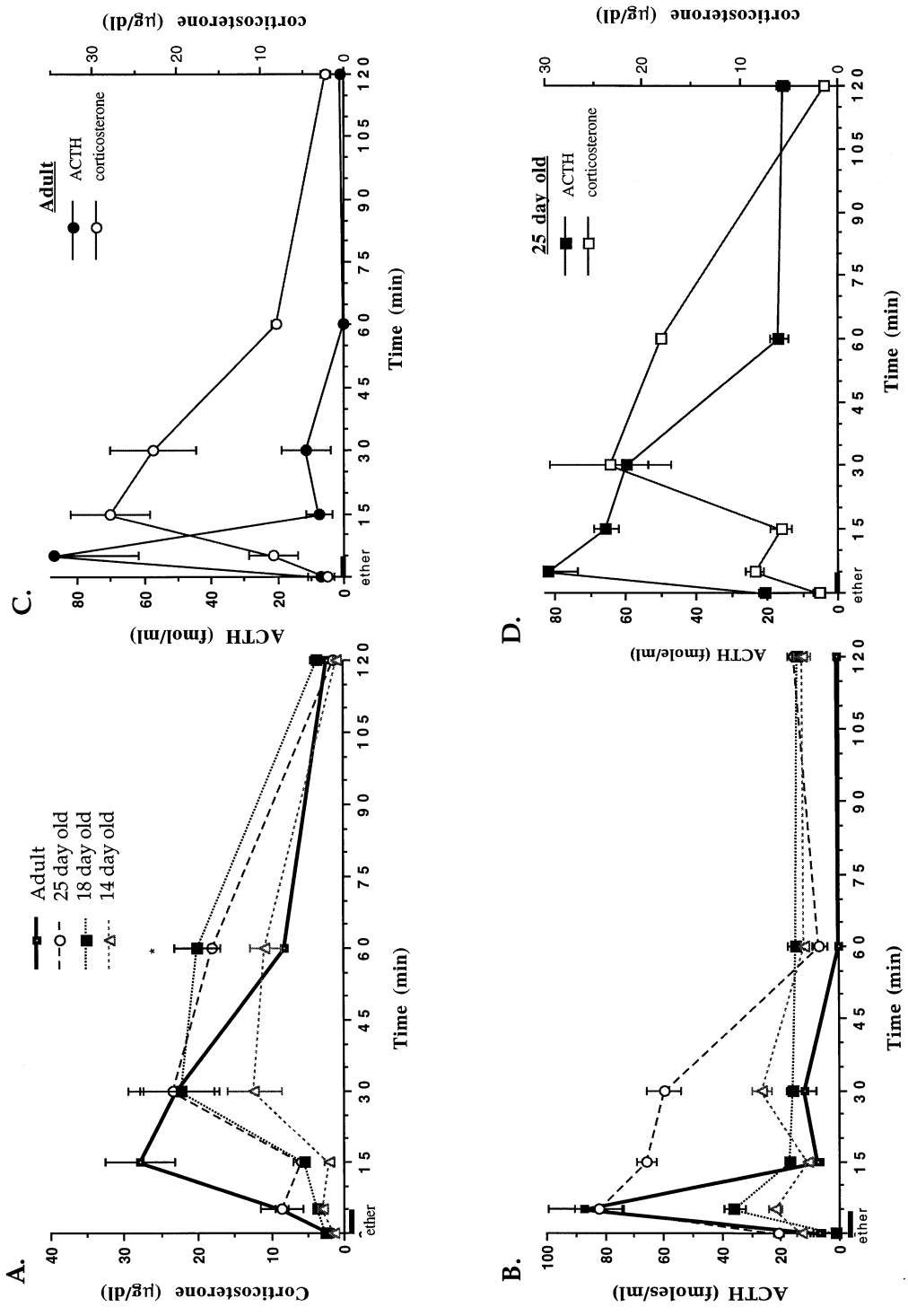


Fig. 11. Plasma ACTH and corticosterone response to ether vapors in the developing and adult animal. Panel A: corticosterone response; panel B: ACTH response; panel C: adult ACTH and corticosterone response; and panel D: 25 day-old ACTH and corticosterone response. Panels C and D are derived from the data presented on panels A and B. While the adult animal has an effective fast feedback, the developing animal has a hyporesponsive adrenal and prolonged ACTH secretion.

Table II shows that [3-I$^{125}$]Iodotyrosyl$^{23}$ ACTH$_{1-39}$ metabolism and its plasma distribution varies with age.

It takes 1.5 × longer for 50% of the [3-I$^{125}$]Iodotyrosyl$^{23}$ ACTH$_{1-39}$ to decline in the young animals when compared with the adult (Table I: $t_{1/2}$ values). This is, in part, reflecting the apparent volume of distribution ($V_d$) which in the immature animals is larger despite its smaller body size. Similarly, although calculated by a different method, the [3-I$^{125}$]Iodotyrosyl$^{23}$ ACTH$_{1-39}$ metabolic clearance in the 14 and 25 day-old animals is half of that which is seen in the adult (Table II: MCR; $p = .05$). Thus, the pharmacokinetic parameters, especially $t_{1/2}$ and MCR, suggest that ACTH concentrations are eliminated slowly in the immature animal. Would the metabolism of ACTH alone explain the prolonged ACTH profile observed in the young animal after a pituitary challenge? To answer this question, the ACTH profile obtained in the 25 day-old and adult animal after exposure to ether vapors were corrected using the half life values derived from the [3-I$^{125}$]Iodotyrosyl$^{23}$ ACTH$_{1-39}$ kinetic study. As can be seen in Fig. 12 the prolonged ACTH metabolism does not entirely explain the elevated ACTH response in the 25 day-old animal. Correction of the 25 day-old plasma response profile using the half life value for this age shows a slightly delayed response, but the delay is minor, compared to what is in fact observed in 'real life'. Thus, in the 25 day-old animal ACTH remains elevated beyond the time predicted by considering clearance alone (Fig. 12; $t_{1/2} = 6.5$).

The delayed return to resting ACTH levels observed in the weanling animal appears to be consistent with the time delay observed for the CS release from the adrenal (Fig. 11; panel D). If the delayed adrenal secretion is bypassed by the administration of cortisol, the day 25 rat suppresses ACTH release in a manner similar to the adult (Vázquez and Akil, 1993a). However, unlike the fast feedback in the adult, the cortisol induced ACTH

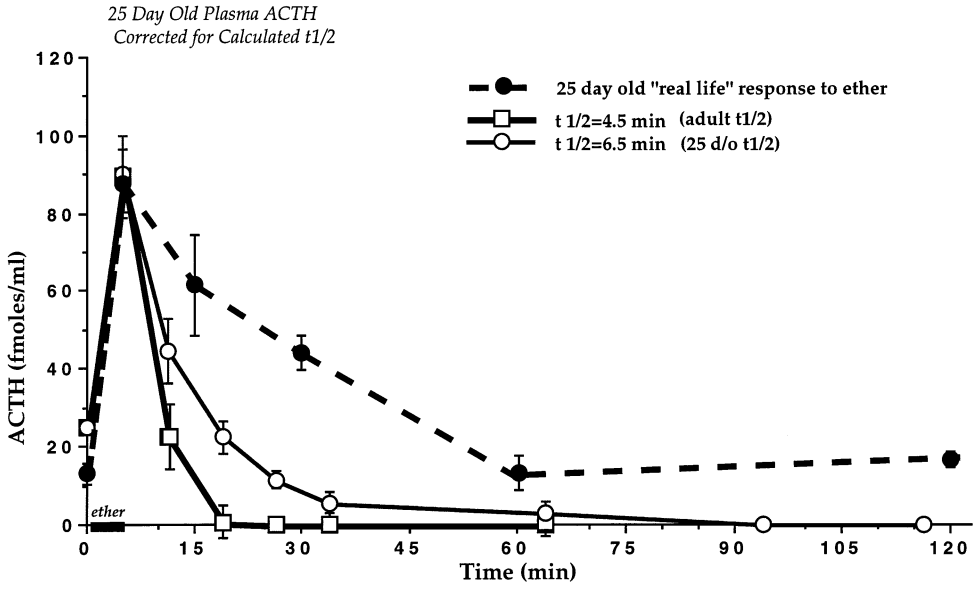


Fig. 12. ACTH plasma profile of the 25 day-old (top profile) when the ACTH half life corresponding to the adult (4.5 min) or when the ACTH half life corresponding to the 25 day-old (6.5 min) is applied to the original pattern of secretion. A slow ACTH metabolism does not entirely explain the prolonged ACTH response in the 25 day-old animal.

inhibition in the weanling animal requires a greater cortisol rate of rise and a longer time frame before basal ACTH levels are achieved. We concluded that in the weanling rat the inability to rapidly terminate the adrenocortical response following certain stressors results from a combination of adrenal developmental factors leading to a slow CS rise, and pituitary and/or brain factors which result in decreased responsiveness to negative feedback, even when the rate of glucocorticoid increase is high (Vázquez and Akil, 1993a). Thus, the weaning period represents a distinct phase of organization of the LHPA axis in the developing animal.

## THE DEVELOPING LHPA AXIS: THE HIPPOCAMPUS AND GLUCOCORTICOID INHIBITION

Late gestation is a period of time when circulating CS levels are comparable with those seen in the adult. At birth these levels decrease dramatically. A similar developmental pattern is seen for the glucocorticoid receptor system within the limbic circuit, such that [$^3$H]dexamethasone (dex) binding is higher during the fetal period (similar to adult concentrations) when compared with early postnatal life (Meaney et al., 1985a). During the first week of life receptor concentration declines to $\sim 20\%$ of levels found in the adult (Meaney et al., 1985b,c). The decline is due to a decrease in total number of receptors rather than a dilution from an increase in the number of growing neurons (Meaney et al., 1985b). Interestingly, a similar decline in GR concentrations after birth does not occur in the pituitary, where receptor numbers remain similar to adult numbers throughout the postnatal period (Olpe and McEwen, 1976). After this initial decrease in hippocampal GR concentration postnatally, GR expression increases, paralleling the increasing CS levels. About 65–80% of the adult hippocampal GR and MR concentration is reached at 3 weeks of age (Clayton et al., 1977; Meaney et al., 1985c; Rosenfeld et al., 1988a). In general, it is believed that in the adult rats, corticosteroids down-regulate their own hippocampal receptors, as evidenced by adrenalectomy studies (Tornello et al., 1982). However, based on previous studies, it appears that, in the developing rat, an increase in receptors occurs despite this steroid increase (Meaney et al., 1985c). This is a paradoxical response which may be unique of the developmental period, but we also questioned if the methodology employed to arrive at this conclusion needed to be revised (see below). In addition, because in the adult animal there is evidence that different parts of the hippocampus may be playing different physiological roles, e.g. subiculum in the termination of the stress response, BNST-PVN pathway in tonic inhibition (Cullinan et al., 1993; Herman et al., 1989b, 1992, 1995; Jacobson and Sapolsky, 1991), we were interested in regional patterns of development. In a series of studies we asked: (1) is the increase in corticoid receptors (GR and MR) seen in the developing animal specific to certain areas of the developing hippocampus (HC)?; (2) how do the patterns of gene and receptor expression in the developing rat compare with those of the adult?; (3) do these receptors exhibit adult like modulations by glucocorticoids? We were also interested on whether the developmental pattern of GR and MR correlated with LHPA function in the developing animal or more importantly if the relative balance of GR with MR may actually guide LHPA function. Therefore, the developing hippocampus was studied in terms of CS binding affinity and gene expression.

Table III. 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) | $B_{max}$ (fmole/mg/nM) | $K_d$ (nM) | $B_{max}$ (fmole/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 |

*Glucocorticoid and Mineralocorticoid Receptor Binding Capacity*

We started these set of studies describing the basal developmental pattern of GR and MR within the hippocampus as an important prelude to assessing their potential role in controlling the stress response in the developing animal. As mentioned on the previous section, as the young rat emerges from the SHRP, failure to terminate the CS rise is evident following certain stressors. Sapolsky has described a similar response to restraint stress in the aged and the chronically stressed animal (Sapolsky et al., 1983). The failure to terminate the adrenocortical response in these two models correlates with a lower GR and MR hippocampal binding. Thus, using standard in vitro biochemical methods (Eldridge et al., 1989; Landfield and Eldridge, 1989; Spencer et al., 1990), 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 500-fold excess of dexamethasone ($R^2$ ranged from 0.88 to 0.99; Table III).

Our results suggested that the failure to terminate the stress response is not a result of low GR and MR binding capacity or affinity (Vázquez et al., 1993b) as reported in the aged animal. GR and MR protein abundance, as inferred by in vitro biochemical techniques, increases from low levels on day 6 to adult levels by day 22. Furthermore, a lower affinity for CS is not likely since the affinity of GR and MR does not change with age. Thus, the greatest absolute increase for both receptors occurs between days 22 and 45, at a time when failure to terminate the adrenocortical response is described, suggesting that the receptor number and affinity is not the explanation for an impaired 'turn off' of the stress response.

*Short Term Adrenalectomy and the Developing Animal: Implications for the Receptor Binding Assays*

Short term adrenalectomy (ADX) is a well accepted procedure which is carried out in order to remove all endogenous corticosteroids which will interfere with the displacement reaction used to determine in vitro biochemical and in vivo autoradiographic steroid receptor binding techniques. Under these circumstances, adult animal studies have shown that the binding capacity of the hippocampus for CS increases in a biphasic pattern (McEwen et al., 1974; Olpe and McEwen, 1976). A rapid initial increase occurs during the first 2 h, followed by a second rise 12 h post-ADX. This second rise 12 h after ADX reaches a plateau 3–5 days after surgery. It is widely accepted that the binding capacity

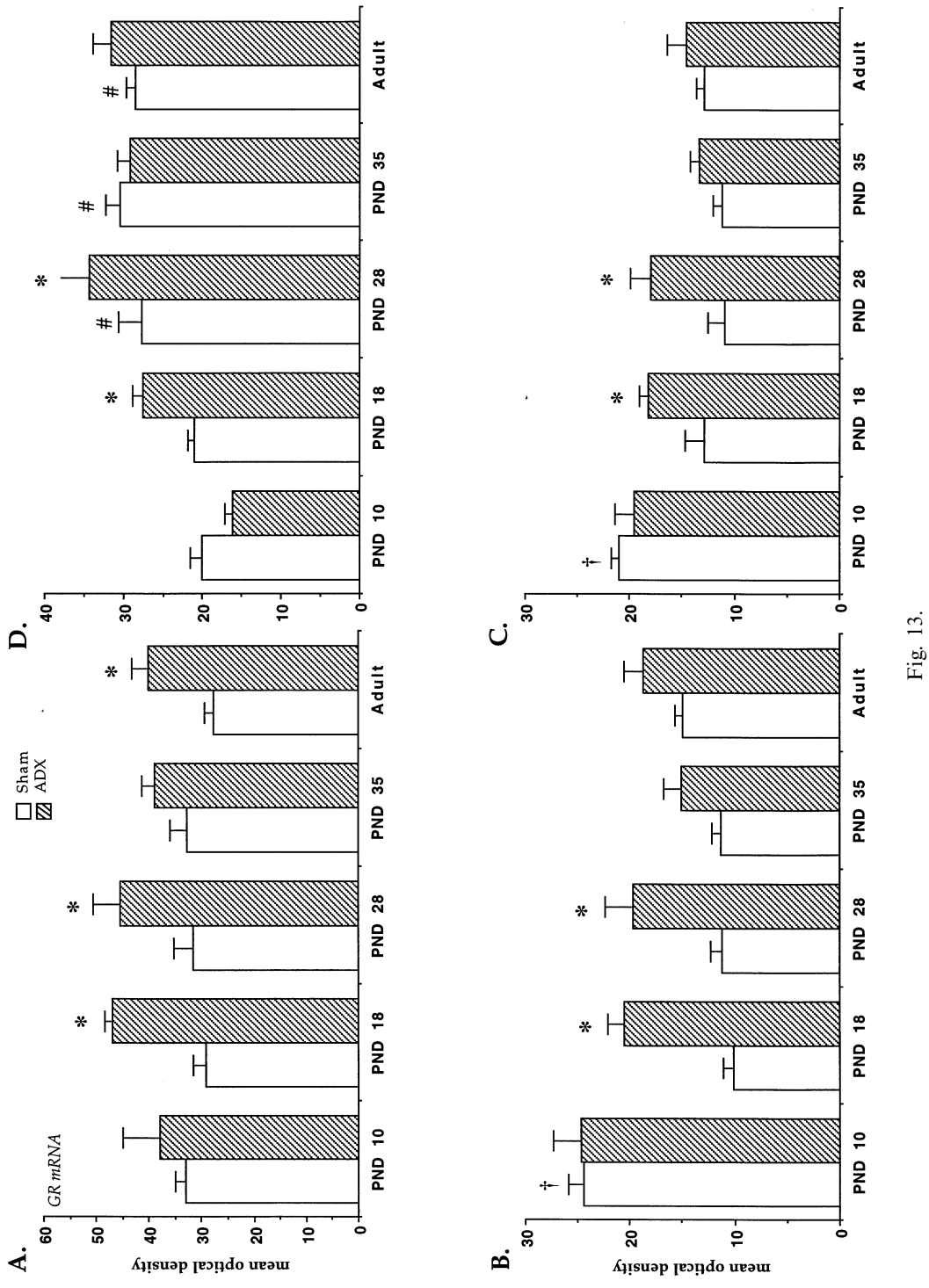
A.
B.
C.
D.
GR mRNA
Sham
ADX
mean optical density
PND 10
PND 18
PND 28
PND 35
Adult

Fig. 13.

Table IV. Plasma corticosterone levels of the group of animals from which the in situ hybridization analysis is derived

| 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) | $1.9 \pm 0.8$ | $0.1 \pm 0.1$ |

Values expressed as $\mu$g/dl $\pm$ SE.
ND, not detected; * age sham versus adult sham; $p = .05$; $n = 6$/age/condition.

measured after 2 h and up to 14 h post-ADX reflects the endogenous binding of the hippocampus in the absence of endogenous CS, while increases beyond this time reflect up-regulation and increased synthesis of the receptors (Sarrieau et al., 1988a). Although our GR and MR ontogenic progression study was consistent with previous reports (Clayton et al., 1977; Meaney et al., 1985c; Olpe and McEwen 1976; Sarrieau et al., 1988b), we wondered if our conclusion with respect to the weanling animal was accurate. Thus a study was undertaken to explore the possibility that the hippocampus of the developing animal may be more sensitive to changes of circulating CS levels and that in fact, the normal GR and MR progression obtained using standard steroid binding methods, reflects an adrenalectomy induced up-regulation of these systems in the developing hippocampus.

We chose to assess cellular modulatory and synthetic activity at the genomic level using in situ hybridization. By this method, it has been shown that the gene expression of MR and GR mRNAs are in agreement with the neuroanatomical distribution revealed by receptor binding (Herman et al., 1989a). This technique does not require the removal of endogenous CS. Moreover, it offers the unique opportunity to address the regulation of GR and MR in an anatomical context in brain structures. Thus, animals were ADX or sham operated and sacrificed 14 h after surgery, on postnatal day 10, 18, 28, 35 or 65 (adult animals). The CS levels are presented in Table IV.

The in situ hybridization results document that the GR and MR systems in the developing hippocampus are sensitive to removal of circulating CS levels, even under a short time frame. This sensitivity is age specific and site specific (Vázquez et al., 1993b). Hippocampal GR mRNA changes were evident over subfields CA1, CA2, CA3-4 and DG in the 18 and 28 day-old animals (Fig. 13) Adult animals also showed a selective GR mRNA increase over subfield CA1 only. Analysis of whole hippocampus (all subfields

Fig. 13. 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 14 h adrenalectomy on GR mRNA (ADX). Panel A: subfield CA1; panel B: subfield CA2; panel C: subfield CA3-4; and panel D: dentate gyrus (DG). The developmental distribution of GR mRNA can be appreciated by observing the progression of SHAM animals at different ages. PND 10 GR mRNA content was significantly denser in subfields CA2 and CA3-4, 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 for PND 18, 28 and adult. In addition, ADX caused a significant effect on subfields CA2 and CA3-4 in the PND18 and 28 animal. PND, postnatal day; $^{\dagger}$ $p < .05$; PND 10 SHAM versus SHAM of other ages; $^{\#}$ $p < .05$; SHAM versus SHAM PND 10; * $p < .05$; SHAM versus ADX.

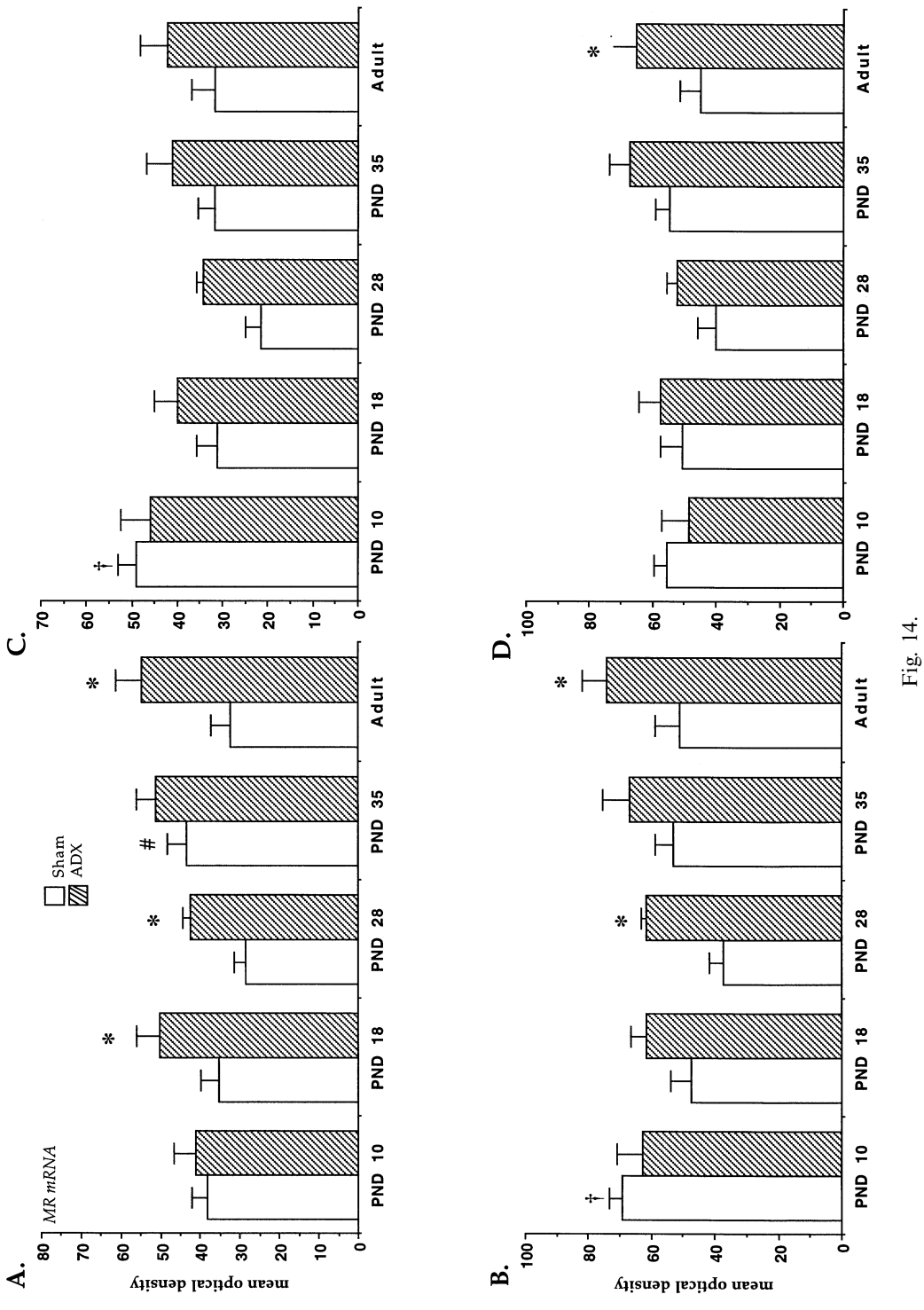
A.
MR mRNA
mean optical density
Sham
ADX
PND 10
PND 18
PND 28
PND 35
Adult
B.
mean optical density
C.
D.

Fig. 14.

included in one densitometric value) showed significant differences in the 18 and 28 day-old animal only (data not shown). The GR mRNA signal detected over the cortex was also analyzed as an internal control treatment and no significant ADX treatment effect was detected.

Adrenalectomy caused an overall tendency of MR mRNA to increase in all hippocampal subfields (Fig. 14). 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 over subfield CA1, the 28 day-old animal, in subfield CA2 and the adult, in subfields CA1, CA2 and DG. Whole hippocampus densitometric analysis demonstrated a significant increase in MR mRNA levels for the adult animal only (Vázquez et al., 1993b).

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 up-regulation 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 up regulation of these proteins in the developing animal. Moreover, the mineralocorticoid receptor system is very sensitive to corticosterone levels 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 adrenalectomized animals are also, most likely, reflecting up-regulation of the GR and MR system, rather than steady state levels of this receptor. We suggest that extreme caution is necessary when interpreting ontogenic and adult data which relies on 'short term' adrenalectomy to allow for the 'clearance' of endogenous CS. Up-regulation 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 increase synthesis of glucocorticoid and mineralocorticoid receptors which in turn will increase the GR and MR binding capacity in this structure.

The physiological significance of the site specific areas and the ages which demonstrate an ADX GR and MR mRNA effect is unclear. However, given the emergence of LHPA functions during development (activation after day 10, termination and circadian rhythms after day 21 of life), it is tempting to speculate that specific areas may contribute to different extents to the three aspects of HPA activity: stress response, circadian rhythmicity and corticosteroid feedback inhibition. In subsequent studies we explored the effect of activating the LHPA axis at a time when the system is quiescent. We focused on the

Fig. 14. 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 14 h adrenalectomy on MR mRNA (ADX). Panel A: subfield CA1; panel B: subfield CA2; panel C: subfield CA3-4; and panel D: dentate gyrus (DG). The developmental distribution of MR mRNA can be appreciated by observing the progression of SHAM animals at different ages. PND 10 MR mRNA content was significantly denser in subfields CA2 and CA3-4, while CA1 MR mRNA density was significantly denser in the PND 28 animal. There was a significant effect of treatment on subfield CA1 for PND 18 and 28. In addition, ADX caused a significant effect on subfields CA2 in the PND28 and adult animal. Adult animals showed a significant effect of treatment in CA1, CA2 and DG. PND, postnatal day; $^{\dagger}\ p < .05$; PND 10 SHAM versus SHAM of other ages; $^{\#}\ p < .05$; SHAM versus SHAM PND 10; $^{*}\ p < .05$; SHAM versus ADX.

hippocampal corticoid receptor systems in the maternally deprived animal shortly after birth.

## REGULATION OF GLUCOCORTICOID AND MINERALOCORTICOID RECEPTOR mRNAs IN THE HIPPOCAMPUS: THE IMMEDIATE POSTNATAL PERIOD

One proposed mechanism for the stress hyporesponsiveness of the infant rat is that the SHRP is due to a decreased amount of circulating corticosteroid binding protein (CBG), leading to high levels of free CS and an enhanced negative feedback (Sapolsky and Meaney, 1986). However, there are a number of findings which contradict this interpretation. For example, as discussed above, the anterior pituitary POMC molecule which gives rise to ACTH, increases steadily, both in terms of gene and peptide expression throughout postnatal life, suggesting that inhibition is minimal at the pituitary level during SHRP (Scott et al., 1990; Vázquez and Akil, 1992). In addition, the concentration of GR in hypothalamus, where CRH secreting neurons reside, is extremely low during the SHRP, making it unlikely to be a major target of direct GR-mediated glucocorticoid feedback (Rosenfeld et al., 1988a,b; Van Eekelen et al., 1987).

Maternal behavior influences several physiological processes in the developing infant, including the relative stress hyporesponsiveness unique to the SHRP. Levine and co-workers have found that following 24 h of maternal deprivation, the neonatal rat responds with significant increases on ACTH and CS when exposed to novelty, injection of isotonic or hypertonic saline, and ether vapors (Rosenfeld et al., 1992). These endocrine responses observed in the maternally deprived animals are unique and resemble the stress response seen in developing animals during their weaning period (25 days-old) (Suchecki et al., 1993; Vázquez and Akil, 1993a) and also the stress response of adult animals subjected to disruption of their hippocampal–hypothalamic connections (Herman et al., 1995). In all of these models, the animal has two salient features: (1) elevated basal CS levels; and (2) the CS levels remain elevated for a prolonged time after the stressor. In view of the proposed role of the hippocampal structure in modulating the basal tone of the LHPA and in the magnitude and duration of stress responses, we, in collaboration with Dr Levine, examined the effect of 24 h of maternal separation on the hippocampal corticoid receptors (GR and MR) gene expression (Vázquez et al., 1996). We also studied the effect of a mild stressor, saline injection, on this same parameter. In situ hybridization was the technique used for this purpose. We specifically asked: "what is the GR and MR receptor pattern in the hippocampus of maternally deprived animals (DEP) compared to unhandled animals (NDEP)?" Is there evidence that hippocampal corticoid receptors from DEP and NDEP animals may be sensitive to the rising CS levels seen after a mild stressor?

Twelve groups of animals were studied. Six groups included 24 h maternally deprived (DEP) and non-deprived rat pups (NDEP) at three ages: 6, 9, and 12 days; the other six groups included pups similarly treated, but challenged with a saline injection and sacrificed 1 h thereafter.

### *Effect of Maternal Deprivation on Plasma Hormone Levels*

Basal ACTH levels did not change with age in the NDEP animal (Fig. 15, panel A). However, CS levels increased with age (panel B). Thus, as the animal matured, the

developing adrenal became more sensitive to low ACTH levels. In contrast, both ACTH and CS increased with age in the DEP animal. Compared with the CS effect, the ACTH elevations are modest, suggesting that events triggered by maternal deprivation increase the sensitivity of the adrenal to ACTH.

We have previously reported, 24 h of maternal deprivation results in increased basal and stress (saline injection) induced CS levels in all ages tested (Fig. 15, panel B). In contrast, among the NDEP pups, only the older animals respond significantly to acute stress which is consistent with the normal HPA developmental pattern, whereby an adrenocortical stress response begins to appear by 10 days of age.

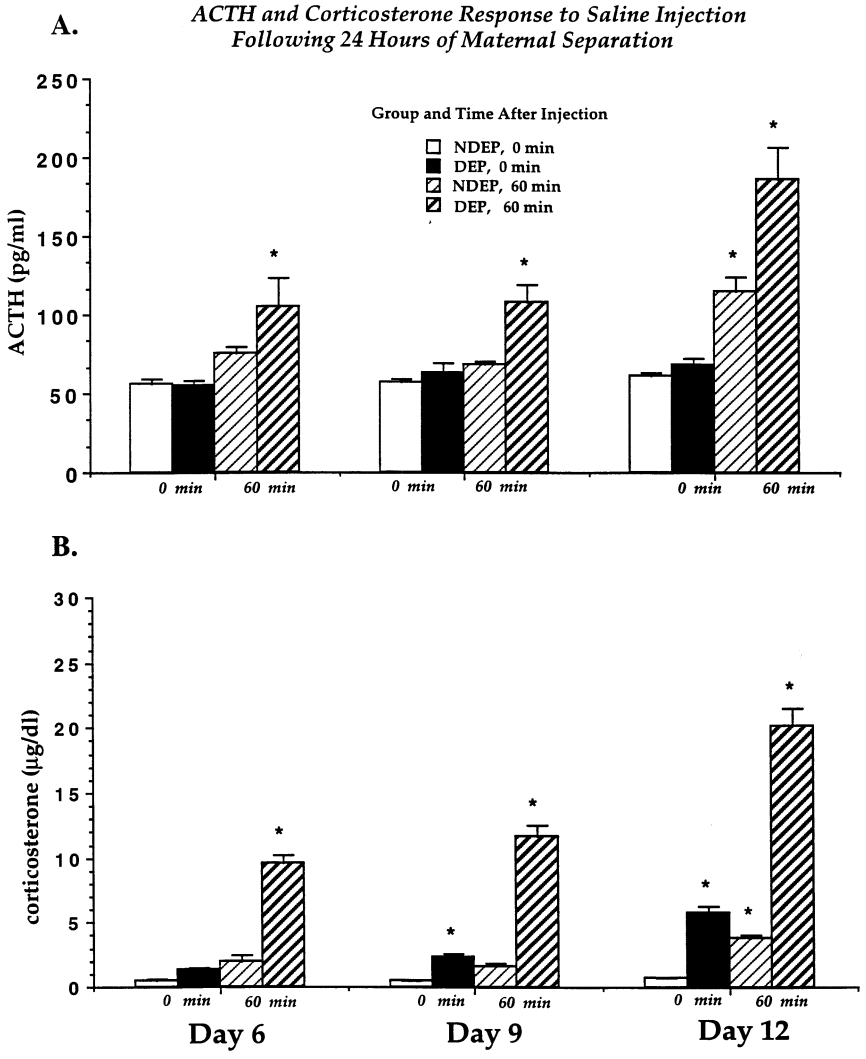


Fig. 15. Plasma ACTH (panel A) and corticosterone levels (panel B) on NDEP versus DEP animals. Hormonal values at basal (0 min) and 1 h after saline treatment (60 min) are presented on each panel for each group. Twenty four hours prior to testing, 6, 9, and 12 day-old pups were either individually-deprived or left undisturbed with their mothers. Testing was carried out at the end of the deprivation period or at the equivalent time point for the NDEP animals.

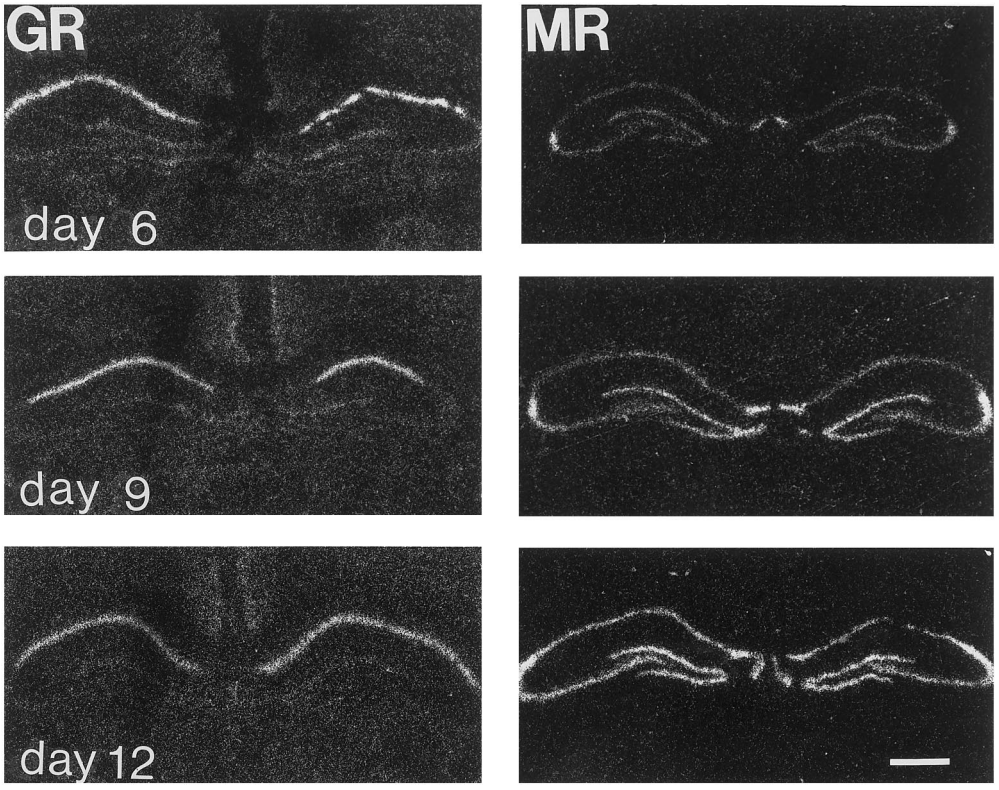


Fig. 16. A representative photomicrograph depicting the ontogenic progression of the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) mRNA. Non deprived (NDEP) and maternally deprived animals (DEP) followed a similar progression for both receptor sub-types (Vázquez et al., 1996).

The stress induced CS levels seen in DEP animals at ages corresponding to the SHRP are related to an elevation of ACTH levels (Fig. 15, panel A). Plasma ACTH were significantly elevated 1 h after saline injection in the DEP animals at all ages. Thus, consistent with previous studies (Levine et al., 1991), the pattern of ACTH and CS secretion in both the DEP and NDEP animals which respond to the acute stress differs from what we know from the adult animal in that levels remain elevated for a fairly long period of time (60 min after the stressor). How is this feature related to the hippocampal glucocorticoid and mineralocorticoid receptor pattern?

*Mineralocorticoid and Glucocorticoid Receptor mRNA within the Specific Subfields of the Hippocampus*

We first analyzed the normal developmental pattern of MR and GR receptor gene expression in the NDEP animals, followed by the analysis of the effect of the stress and maternal deprivation treatments on receptor mRNA levels. Three significant findings were detected. We found an age effect in almost every hippocampus subfield for both MR and GR mRNAs: MR increases with age, while GR decreases (Fig. 16). A significant and specific regional effect on MR gene expression was detected in the hippocampus of the

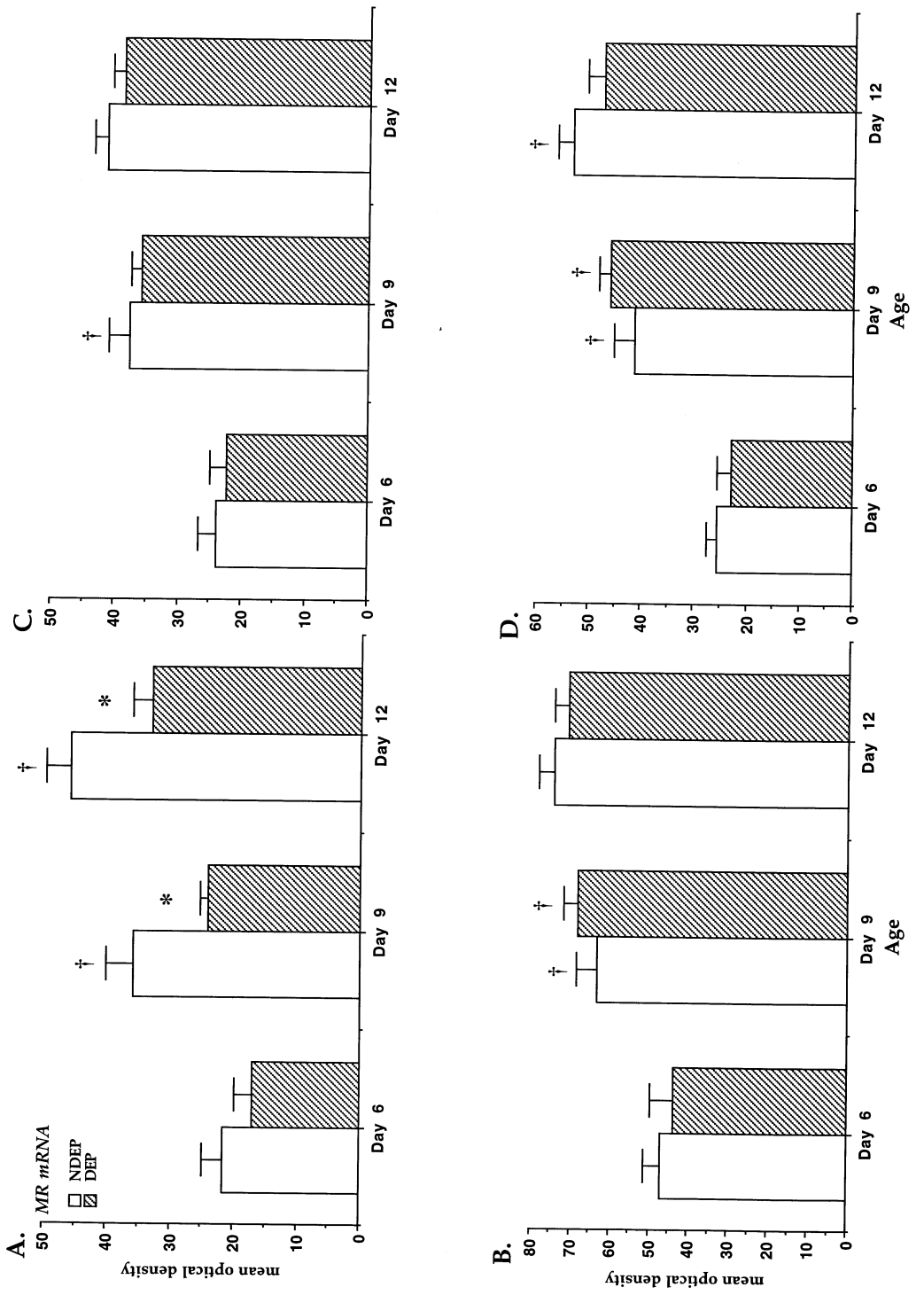
A.
MR mRNA
NDEP
DEP
mean optical density
Day 6
Day 9
Day 12
B.
Age
C.
D.

Fig. 17.

Table V. Maternal deprivation effect on the mineralocorticoid receptor gene expression in the CA1 hippocampal region

| Age (days-old DEP versus NDEP | CA1 hippocampal region | | |
|---|---|---|---|
| | MR mRNAa (%) | MR:GR mRNA ratio | |
| | | NDEP | DEP |
| 6 | $-29.6 \pm 0.7$ | $1.0 \pm 0.05$ | $1.0 \pm 0.2$ |
| 9 | $-25.3 \pm 3.0$* | $2.0 \pm 0.7$ | $1.0 \pm 0.2$* |
| 12 | $-34.9 \pm 6.5$ | $3.0 \pm 0.6$ | $2.0 \pm 0.3$* |

[a] Percent change MR mRNA calculated from data depicted on Figs. 17 and 18.
[b] MR to GR ratio calculated from densitometric measurements from control animals sacrificed at time 0.
* DEP versus NDEP, $p < .05$, Fisher PLSD; $n = 10$ per age, per group.

maternally deprived animals (Fig. 17). The effect was observed in all ages studied and it was evident that maternal deprivation caused a 25–35% decrease of hippocampal MR mRNA in the CA1 subfield, which alters the relative MR to GR ratios present in this region (Table V).

Significant glucocorticoid receptor mRNA level changes were not detected in any region as a result of the maternal deprivation treatment. However, the saline injection, which was associated with a significant plasma CS elevation at 60 min, caused an overall effect on GR receptor levels, again over the CA1 pyramidal cell region (Fig. 18).

The biochemical profile of the two different types of corticoid receptors, MR and GR, complement each other. The MR appear to be operative at low CS concentrations under which condition they may offer tonic inhibition to the axis during the nadir of the circadian rhythm (Reul and De Kloet, 1985). When high concentrations are present, MRs saturate, and the GRs are then available, acting coordinately to ensure the return of homeostasis. Thus, the dual action of these receptors in the hippocampus appear to be central for both basal modulation and stress regulation of the LHPA. The selective down regulation in the CA1 pyramidal cell region is not likely to be a random event. Chronic stress causes a decrease of MR and GR mRNA expression, exclusively, in subfields CA 1 and the DG of adult animals (Herman and Watson, 1995). A difference from the pattern observed after adrenalectomy which causes up-regulation primarily in CA1 and CA2 regions (Vázquez et al., 1993b). In the maternally deprived animal, we can see the contribution of these receptors to the endocrine profile. We observe a down-regulation of MR in the maternally deprived animal accompanied by an alteration of the tonic inhibition of the system, manifested by an elevation of basal CS levels. We believe that this is an important factor, since sustained CS elevations during the nadir of the circadian rhythm have been associated with alterations in brain MR system (Walker et al., 1992). The resulting CS elevations are postulated to release the HPA from tonic inhibition which

Fig. 17. Densitometric analysis of the mineralocorticoid receptor (MR) mRNA in DEP and NDEP animals. Panel A: subfield CA1; panel B: subfield CA2; panel C: subfield CA3-4; and panel D: dentate gyrus (DG). A three way analysis of variance did not show a saline injection effect. Therefore, subsequent analysis was collapsed across this variable and a significant 25–35% decrease on MR gene expression was detected in the CA1 hippocampal pyramidal field of the maternally deprived animals ($p = .05$; for each age, NDEP, $n = 10$; DEP, $n = 10$).

in turn may trigger facilitation of stress induced responses and decreased sensitivity of ACTH to CS feedback (Walker et al., 1992). Although in our study it is not clear if this was through a direct effect of maternal deprivation or secondary to the initial increase in CS levels, it is apparent that the small but significant decrease in MR mRNA levels may be sufficient to off-set the normal age related increase in MR mRNA levels in this region. Consequently, the MR to GR relationship (MR:GR ratio) is also altered. The combination of increased basal CS levels and decreased CA1 hippocampal MR expression may be associated with a new glucocorticoid inhibition 'set-point', and may lead to an increased sensitivity to stress in maternally deprived infant rat. However, since GR is not altered in

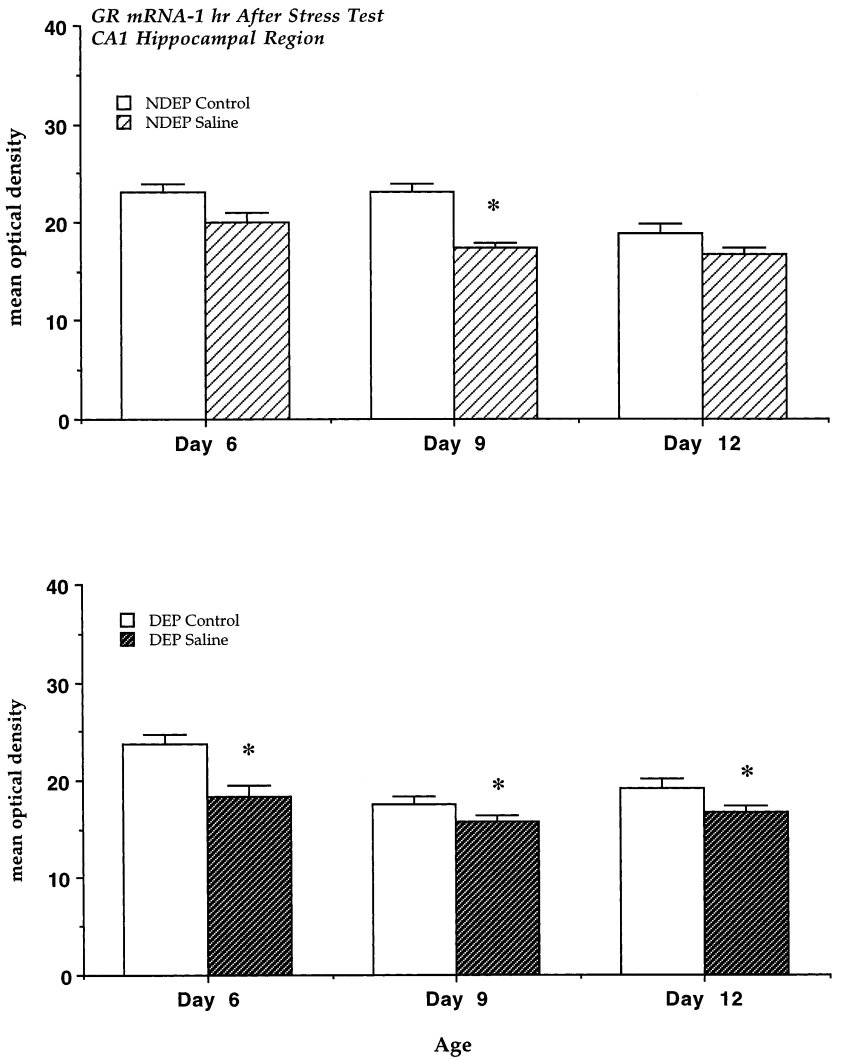


Fig. 18. Densitometric analysis of the glucocorticoid receptor mRNA digitized images through CA1 pyramidal region. Following a three factor analysis of variance, a small but significant decrease was found between the none-treated controls time 0 min and saline injected animals (time 60 min) over CA1 subfield only ($p < .05$). For each age:treatment group, five animals were analyzed.

the maternally deprived animal prior to the saline stress, we can not explain the prolonged ACTH and consequent CS response observed in the maternally deprived animal. As mentioned in the Section 1, the CRH neurons are glucocorticoid insensitive in hippocampectomized adult animals (Herman et al., 1989b; Jacobson and Sapolsky, 1991). It is, thus, possible that a greater contributor to the prolonged LHPA response is the immaturity of the hippocampal circuitry connecting into the PVN.

In sum, our results indicate that GR and MR in the developing hippocampus are sensitive to circulating CS. They also suggest that the relative ratio of GR:MR in the CA1 region may contribute to the enhanced and sustained ACTH response seen after a mild stressor in DEP animals.

## CONCLUDING REMARKS

It is clear that during development the LHPA axis is different from the adult in both structure and function. The immediate postnatal period is characterized by system that appears to be oblivious to environmental perturbations, followed by a new and unique phase of stress responsiveness when the animal fails to swiftly terminate glucocorticoid secretion. Given the delay in the development of stress related molecules and circuits, it is not surprising that there are profound differences in stress activation and in termination in the developing animal when compared with the adult. Over the last decade we have emerged from the dogma that this system is static during early life. What has become evident from our studies is that the mechanisms underlying normal LHPA development and the mechanisms of adaptation are not necessarily those which the mature system would employ under those same challenges. Thus, during the first 2 weeks of life, the animal responds to an intermittent chronic challenge increasing anterior pituitary POMC post-translational events, while the adult increases genomic events. Minimal increases of circulating ACTH peptide are present, yet an adrenocortical response is observed after the first week as the adrenal becomes more sensitive to circulating ACTH levels. In both the mature and weanling animal (21 days-old), the adrenocortical response to a chronic intermittent challenge is the same: an increase in corticosterone levels. However, the mature animal achieves this by increasing the releasable pool of ACTH, and a diminished steroid feedback at the level of the pituitary. The developing animal increases POMC molecule processing which translates to a minimal increase in ACTH circulating levels. However, a combination of an underlying decreased responsiveness to negative feedback and a more sensitive adrenal, which responds to small amounts of circulating ACTH, results in a longer exposure to circulating CS at a time when the corticoid receptor systems are being laid down and when the brain neurotransmitter systems are establishing neural contacts. The question that remains is whether early stress translates into any consequence on the long term function of the LHPA axis. There is a growing evidence that points to long term effects of early experience dictating individual differences in the stress response (Maccari et al., 1991; Meaney et al., 1993a,b; Plotsky and Meaney, 1993; Vázquez, 1997). In this aera where child neglect and abuse is on the rise and where 25 week gestation premature infants are surviving with the help of synthetic glucocorticoid treatment for respiratory conditions, we should be concerned about the impact of early experiences in creating the substrate for developmental, behavioral or mood disorders. At this stage there is evidence that links parental loss during childhood with an increased risk for major depression and generalized anxiety disorder. Epidemiological studies indicate that one of

the major factors involved in this phenomenon is 'lack of maternal care' prior to age 17 (Kendler et al., 1992). Other provocative correlations link the LHPA axis, through CS secretion, to the acceleration of behavioral sensitization and drug seeking behavior. In animal models, prolongation of CS secretion after a stressor is a predictor of individual vulnerability to acquire amphetamine self administration (Piazza et al., 1991). In humans, psychosocial stressors, such as child abuse and neglect are risk factors for subsequent drug use and dependence (Dinwiddie et al., 1992; Duncan, 1977; Gutierres et al., 1994). Thus, alterations of the hippocampal corticoid receptor system have important implications on LHPA function and the individual vulnerability to pathological conditions. At this point we are beyond 'circumstantial evidence which links early experience to HPA reactivity, personality types or ability to cope with environmental challenges' (De Kloet et al., 1988). The correlates have been identified. Now there is an urgency to understand the normal and altered mechanisms of the developing LHPA axis for its obvious relevance to the fields of medicine and psychiatry.

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