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The Development of the Glucocorticoid Receptor System in the Rat Limbic Brain. III. Negative-Feedback Regulation

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In the neonatal rat, there are parallel increases with age in the concentrations of glucocorticoid receptors in the limbic system, and in the sensitivity of the hypothalamic-pituitary-adrenal (HPA) axis to negative-feedback inhibition by circulating glucocorticoids. We speculated that the increasing receptor concentrations may mediate this increasing sensitivity of the HPA axis to the suppressive effects of glucocorticoids. To examine this idea we treated rats with exogenous corticosterone from days 29 to 34, resulting in a down-regulation of glucocorticoid receptors in the brain at Day 35 to levels similar to those of younger animals. Subjects whose maturational increase in receptors was reversed in this manner were less sensitive to feedback inhibition of glucocorticoids. Specifically, compared to controls they continued to secrete corticosterone after the end of stress, and were relatively insensitive to the suppressive effects of the synthetic glucocorticoid, dexamethasone, on corticosterone titers. Our data specifically implicate the hippocampus in modulating feedback sensitivity, as down-regulation was extensive in this structure, and did not occur in the septum, amygdala, hypothalamus or pituitary.

INTRODUCTION

As reviewed in the first paper in this series[4], a number of functions of the hypothalamic-pituitary-adrenal (HPA) axis mature slowly in the neonatal rat. Among these functions is negative-feedback regulation of the axis by circulating glucocorticoids. Goldman et al.[1] have shown that immature animals are relatively insensitive to the suppressive effects of dexamethasone upon subsequent stress-induced corticosterone secretion. Further, it was shown that this immature negative-feedback regulation manifested itself as a delay in terminating the secretion of corticosterone at the end of a stressor.

Negative-feedback regulation of the HPA axis by circulating glucocorticoids presumably is mediated by glucocorticoid receptors (G_{Rec}) in the brain and pituitary[3,6]. Our previous two papers demonstrated that receptors occur in low concentrations in the rat limbic system perinatally and increase steadily thereafter[4,5]. This suggests that sensitivity of the HPA axis to negative-feedback regulation might increase ontogenetically as a result of the increasing concentrations of G_{Rec} during development. The present paper offers data supporting this idea, in that an experimental manipulation which reverses the maturational increase in receptor concentration also leads to a reversal of the maturation of sensitivity to negative-feedback regulation.

MATERIALS AND METHODS

Female Long–Evans rats (Charles River Breeding Farms, Kingston, NY) were obtained at 23 days of age. Animals were supplied with food and water ad libitum and were maintained on a 14:10 h light:dark cycle (lights on 05.00–19.00 h). Subjects used in stress tests were rapidly bled through the tail vein (within 2 min of handling) and placed for 1 h in a cage on a shaker. At the end of the hour, another blood sample was taken, and subjects were returned to their home cage to recover quietly. An additional

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sample, at varying points during the recovery period, was then taken. Dexamethasone suppression tests were administered by injecting 10 μg/100 g b.wt. dexamethasone (Sigma, St. Louis, MO, dissolved 250 μg/ml in sesame oil) s.c. at 09.00 h. The rats were killed by decapitation within 2 min of handling, either at the time of injection, 4 or 8 h later. Trunk blood was collected in chilled, heparinized containers, and immediately centrifuged. Plasma was separated and frozen until assay. Corticosterone was measured by radioimmunoassay[2] using an antiserum generated against cortisol 21-succinate bovine serum albumin (Antisera F21-53, Endocrine Sciences, Tarzana, CA). This antiserum cross reacts with corticosterone (approximately 60%) but less than 1% with dexamethasone or progesterone. [1,2,6,7-^{3}H]cortisol was used as the tracer and corticosterone as the standard. The sensitivity of the assay was 10 pg; sufficiently large volumes of plasma were used so that the level of detection in plasma was 0.25 μg/100 ml. Coefficients of variation within and between assays were 8 and 11%, respectively (n = 8).

Subjects chronically administered corticosterone were injected s.c. daily with 1 mg/100 g b.wt. of corticosterone (Sigma, 10 mg dissolved/ml sesame oil). In pilot studies, we found that with this dose corticosterone titers were elevated approximately 30 μg/100 ml for 12 h afterward and returned to baseline within 24 h of administration.

Quantitative autoradiography of corticosterone receptors in the brain was conducted as previously described[7] using tritium-sensitive, sheet film (LKB Ultrofilm, LKB, Inc). Subjects were adrenalectomized and injected 12 h later s.c. with a 100 μCi/100 g b.wt. dose of [1,2,6,7-^{3}H]corticosterone (82.1 Ci/mmol, Amersham) and were decapitated 2 h later. This dose of [^{3}H]corticosterone saturates brain glucocorticoid receptors[10]. Non-specific binding was assessed in one control animal injected with 5 mg unlabeled corticosterone concurrently with the labeled steroid. Frozen, 32 μm thick sections of brain were cut with a cryostat at −15° and thaw-mounted onto subbed, glass slides. After overnight storage at −40°, the slides were dried on a hot plate and apposed against LKB Ultrofilm in cardboard X-ray film cassettes. After a 60-day exposure at room temperature, the film was developed in Kodak GB/X X-ray film developer and optical densities of the autoradiograms were quantified by projecting the autoradiogram onto a moveable photocell connected to a digital voltmeter. The level of resolution is 50 μm with this technique. Optical densities of autoradiograms were converted into molar amounts of bound corticosterone with the tritium brain-mash standards.

Cytosolic G_{Rec} assays were conducted as described in the first paper of this series[4].

Endocrine data were analyzed by analysis of variance followed by Newman–Keuls tests, and autoradiographic data were analyzed by independent *t*-test.

TABLE I

Fmol [^{3}H]corticosterone bound/mg protein in control subjects and subjects treated chronically with corticosterone, as analyzed by quantitative autoradiography

	Controls (n = 4)	*Experimental (n = 4)*
Hippocampus		
Subiculum	320 ± 100	152 ± 12
CA1		
Stratum oriens	40 ± 14	62 ± 40
Stratum pyramidale	320 ± 90*	160 ± 12
Stratum moleculare	60 ± 26	50 ± 8
CA2		
Stratum oriens	50 ± 24	52 ± 4
Stratum pyramidale	268 ± 84	170 ± 24
Stratum moleculare	44 ± 14	14 ± 14
CA3		
Stratum oriens	32 ± 18	40 ± 20
Stratum pyramidale	150 ± 40	100 ± 12
Stratum moleculare	50 ± 28	50 ± 16
CA4		
Stratum pyramidale	150 ± 36*	52 ± 16
Stratum moleculare	80 ± 22	40 ± 20
Dentate gyrus	420 ± 80**	170 ± 10
Hilus	100 ± 36	52 ± 18
Septum		
Lateral	80 ± 60	68 ± 8
Medial	0 ± 0	0 ± 0
Amygdala		
Cortical	76 ± 14	78 ± 42
Medial	10 ± 4	12 ± 4
Hypothalamus		
Ventral medial/PVN	38 ± 5	36 ± 11

Mean (± S.E.M.); *,** indicate experimentals differed significantly from controls at the 0.05, 0.01 level, respectively (two-tailed *t*-test). Three slices were analyzed per animal per structure, and were then pooled into a single determination, and that determination was then the basis for grouped values.

RESULTS

We determined whether decreasing G_{Rec} concentrations in the brain or pituitary would decrease the capacity of subjects to terminate their corticosterone stress-response at the end of stress. Neural G_{Rec} levels were reduced in Day 35 subjects by daily injections on Days 29–34 with 1 mg/100 g b.wt. of corticosterone. We have previously shown[4] that this treatment reduces hippocampal corticosterone receptors by about 40% in pubertal animals.

Table I demonstrates the down-regulation of G_{Rec} in Day 35 subjects following 5 days of corticosterone treatment, as analyzed by quantitative autoradiography. Chronic corticosterone treatment led to little or no down-regulation in the septum, amygdala or hypothalamus. Further, in a single-point assay with a 75 nM saturating dose (i.e., 10 times K_d) of [^{3}H]dexamethasone, there was no indication of down-regulation of pituitary G_{Rec} number (controls n = 5), 424 ± 31 fmol/bound mg cytosolic protein; chronic corticosterone-treated rats (n = 5), 393 ± 53 fmol bound/mg protein; n.s., paired *t*-test). In agreement with previous biochemical studies[4,9], substantial declines in G_{Rec} levels occurred within the hippocampus. At least 50% declines in binding were observed in the subiculum, dentate gyrus and in the pyramidal layers of CA1 and CA4. Lesser, but consistent declines were also observed in pyramidal layers of CA2 and CA3, while no consistent changes occurred throughout the stratum oriens or moleculare of corticosterone-treated subjects. Anterior–posterior gradients of binding were not observed.

We then examined the capacity of control and down-regulated Day 35 subjects to terminate their corticosterone secretory response to vibratory stress. Adult females have a significant elevation in corticosterone titer after 60 min of stress, and have recovered to basal levels within 1 h of recovery (Fig. 1). Day 35 control subjects, in contrast, were relatively slower in recovering from the effects of stress, continuing to have corticosterone titers significantly elevated above baseline at 1 h into the recovery period. Day 35 subjects whose G_{Rec} concentrations had been down-regulated by corticosterone treatment were even more delayed in their termination of the stress-response, displaying elevated titers 2 h into the recovery period. Thus, down-regulation of G_{Rec} caused an exaggeration of the pattern of Day 35 subjects to hypersecrete corticosterone at the end of stress. Further, down-regulated subjects were relatively less sensitive to the suppressive effects of dexamethasone on basal titers of corticosterone than were controls (Fig. 2).

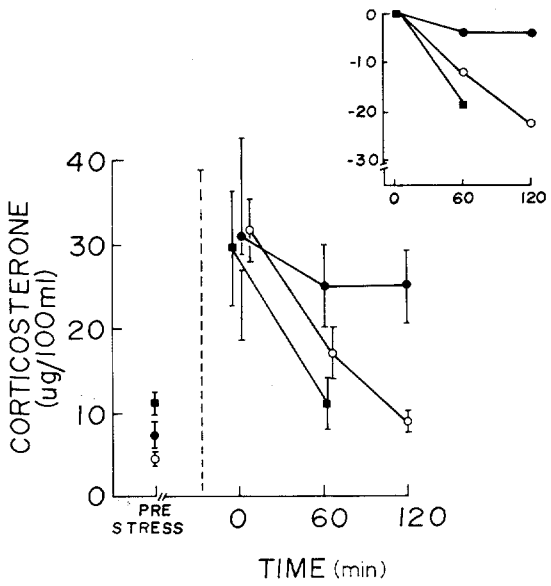

Fig. 1. Mean (± S.E.M.) corticosterone levels (μg/100 ml) in adult (■), down-regulated Day 35 (●), and untreated Day 35 (○) animals prior to stress (baseline levels) and at 0, 60 and 120 min following the termination of stress. The inset represents recovery from stress-induced corticosterone levels (i.e. decrease in corticosterone levels from maximum stress levels over time). Corticosterone levels at time 0, 60 and 120 were statistically analyzed for each group using a one-way analysis of variance to analyze the degree of recovery. Corticosterone levels in down-regulated, Day 35 animals were statistically indistinguishable over time ($F = 0.2$; df = 2,11; n.s.) in contrast to effect of time seen in untreated, Day 35 animals ($F = 9.44$; df = 2,18; $P < 0.001$) and in adult animals ($F = 5.62$, df = 1,10; $P < 0.04$). In the untreated, Day 35 animals the significant effect was due to the decrease in corticosterone levels by 120 min post-stress.

DISCUSSION

Goldman et al.[1] have demonstrated that the immature HPA axis is not fully sensitive to negative-feedback regulation. Although weanlings and adults show equivalent corticosterone responses to ether stress, dexamethasone treatment is more effective in suppressing ether-induced corticosterone secretion in adults than in weanlings. This insensitivity to feedback inhibition is further demonstrated with the impaired capacity of weanlings to terminate a cortico-

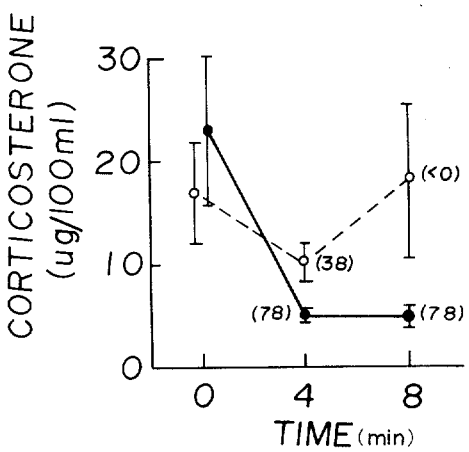

Fig. 2. Mean (± S.E.M.) corticosterone levels (μg/100 ml) 0, 4 and 8 h after dexamethasone administration in down-regulated, Day 35 animals (○) and untreated, Day 35 animals (●) (n = 8 for each group). Numbers in parentheses represent the percent of decline from baseline levels. Statistical analysis revealed a significant time × treatment effect ($F = 22.2$; df = 2,18; $P < 0.01$) that was due to the differential reduction in corticosterone levels 4 and 8 h following dexamethasone between the groups.

sterone stress-response. Adult animals, exposed to 1 min of ether- or shock-stress, show elevated corticosterone titers that return to basal levels within 60 min, where weanlings still have significantly elevated titers of corticosterone at that time.

Glucocorticoid feedback inhibition depends on receptors for the steroid, and we reasoned that the limited sensitivity of weanlings to such inhibition was related to the gradual maturation of the G_{Rec} system in the limbic brain. In testing this, we have demonstrated a correlation between G_{Rec} concentration and ability to terminate the stress-response. By chronically treating subjects with exogenous corticosterone, we have down-regulated specific limbic populations of G_{Rec} to less mature levels[4,5]. When receptor concentrations are reduced to less mature levels functional aspects of the HPA axis are also less mature. Down-regulated animals are delayed in their termination of the stress-response at the end of vibratory-stress, and are less sensitive to the inhibitory effects of dexamethasone on basal corticosterone titer.

Goldman et al.[1] show that weanlings and adults do not differ in their sensitivity to feedback inhibition of the HPA axis when glucocorticoids were implanted in the hypothalamus. They concluded that the feedback immaturity detected in their prior experiments was anatomically based in a suprahypothalamic locus. Our data implicate the hippocampus as one possible site in regulating the sensitivity of the HPA axis to negative-feedback inhibition. Chronic corticosterone treatment led to receptor declines only in the hippocampus, with no change in the septum, amygdala, hypothalamus or pituitary. Study of G_{Rec} down-regulation in the adult rat has demonstrated the identical anatomical profile of receptor reductions[9], suggesting that the hippocampus, which has the highest concentration of G_{Rec} in the brain, is particularly sensitive to down-regulation.

A number of other studies support the relationship between a reduction in hippocampal G_{Rec} and limited sensitivity to feedback inhibition. In the aged rat, where there is corticosterone hypersecretion at the end of stress[8] and insensitivity to glucocorticoid feedback inhibition[11] receptor losses occur in the hippocampus and amygdala, with no changes elsewhere in the brain or pituitary[10]. In the Brattleboro rat, congenitally deficient in vasopressin, corticosterone hypersecretion is observed during recovery from stress[12] and a deficit in corticosterone receptor concentrations occurs in the hippocampus and pituitary, but in no other brain region[14]. Thus, loss of hippocampal G_{Rec} appears to be the common anatomical locus in each of the syndromes of corticosterone hypersecretion. In support of this, destruction of the hippocampus leads to elevated corticosterone titers under a variety of conditions, as well as attenuated sensitivity to negative-feedback regulation (reviewed in ref. 3).

In agreement with previous studies[1] we have found that untreated Day 35 animals hypersecreted corticosterone at the end of stress, as compared with adults (although they did so less than did down-regulation Day 35 subjects). However, untreated Day 35 subjects have concentrations of G_{Rec} in the hippocampus equivalent to adult concentrations, when determined biochemically[1]. Our autoradiographic study[5] showed, however, the G_{Rec} concentrations did not mature at an equal rate in all cell fields in the hippocapus. Similarly, the autoradiographic analysis in this paper showed that down-regulation did not occur to the same extent in all cell fields. Finally, autoradiographic analysis of the receptor losses in aged and Brattleboro rats demonstrated anatomically unequal extents of depletion in different hippocampal cell fields[11]. Taken together, these data suggest that the hippocampal G_{Rec} involved in this particular form of

feedback inhibition of the HPA axis might be localized rather discretely within the hippocampus, most probably in pyramidal layers. Further studies are in progress to better define which receptor populations are most critical to this regulation.

In conclusion, our data suggest that increasing concentrations of hippocampal G_{Rec} play a role in the neonatal maturation of feedback inhibition of the HPA axis. Presumably, with age, glucocorticoids have an increasing impact upon hippocampal function, via its higher G_{Rec} concentrations. The hippocampus, in turn, could influence HPA activity either by direct control of release of CRF-like neurohormones, or by modulation of hypothalamic sensitivity to stimulatory or inhibitory inputs.

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