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Apparent Age-Related Resistance of Type II Hippocampal Corticosteroid Receptors to Down-Regulation During Chronic Escape Training

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Corticosteroids appear to modulate neuronal loss in the hippocampus during aging. However, there is a seeming paradox in the literature in that age-related neuronal loss develops more prominently during the later phases of the lifespan, whereas brain corticosterone receptors have been reported to decline with aging, an effect that might be anticipated to reduce the impact of corticosteroids on cell loss. In order to study the regulatory sensitivity of hippocampal corticosteroid receptors (HCSR) during aging, which could play a role in this apparent paradox, rats of 3 ages (4, 12, and 18 months old at the start of training) were given 6 months of chronic escape training using a mild footshock in a 2-way shuttle-escape task (4 hr/d, 5 d/week). Animals were killed either 1 d or 3 weeks following the 6 month training paradigm. Nontrained home cage controls also were maintained in parallel with each age group. Although previous studies have measured receptors in rats adrenalectomized 12 hr or more prior to death, rats intact at death were used in the present studies to avoid possible confounding effects from age differences in receptor up-regulation or response to surgery. Receptor capacity was analyzed with a saturation assay able to measure available type II HCSR in intact rats.

Results showed that, in intact young-mature rats (10 months old at death), type II HCSR were down-regulated at 1 d, but not at 3 weeks, after the end of the 6 months of training. However, significant decreases in HCSR were not observed in late mid-aged (18-month-old) or aged (24-month-old) rats at the 1 d point, indicating apparent resistance to down-regulatory stimuli. Moreover, levels of type II binding were unchanged or higher with aging, even in nontrained controls. This result contrasts with prior findings in rats adrenalectomized before death, suggesting age-related resistance even to tonic, down-regulatory influences from endogenous steroids. In a second experiment, the time course of down-regulation over the 6 month escape training period was examined in late mid-aged rats only (12 months old at the start of training). Down-regulation was not found after 2 months but was present after 4 months of training. By 6 months of training some recovery from the down-regulation had appeared.

Although detailed studies of the occupied (nuclear) receptor populations must still be conducted, the present data suggest that, during aging, type II HCSR develop resistance to down-regulatory influences. This effect could help resolve the question of why corticosterone-dependent hippocampal neuronal decline develops more prominently during the later phases of the lifespan.

Adrenal corticosteroid binding sites in the hippocampus are known to be highly labile and sensitive to the concentrations of circulating glucocorticoids. Hippocampal corticosteroid receptors (HCSR) up-regulate substantially in response to adrenalectomy (ADX) (McEwen et al., 1974; Tornello et al., 1982; Reul et al., 1987a) and, conversely, down-regulate in response to administration of corticosterone (CORT) or chronic exposure to stress (Tornello et al., 1982; Sapolsky et al., 1984; Reul et al., 1987b; Brinton and McEwen, 1988). These receptors have been viewed as important in a variety of brain functions, including the negative-feedback regulation of adrenocorticotropin (ACTH) and numerous biochemical and behavioral processes (Angelucci et al., 1980; McEwen et al., 1986; Sapolsky et al., 1986; de Kloet and Reul, 1987; Masters et al., 1987; Schlatter and Dokas, 1987).

In addition, glucocorticoids appear to accelerate the development of age-related changes in neurons and glial cells in rat hippocampus (Landfield, 1978, 1987; Landfield et al., 1978, 1980, 1981a, 1988; Sapolsky et al., 1986; Kerr et al., 1986), and this effect seems to be mediated by specific steroid receptors (Sapolsky et al., 1985, 1986). Nevertheless, an important and unresolved paradox appears to be associated with studies on the glucocorticoid hypothesis of brain aging (cf. Landfield, 1978, 1987; Sapolsky et al., 1986) in that, although the development of hippocampal aging changes is most pronounced in the second half of the lifespan (Lindsey et al., 1979; Landfield et al., 1980, 1981b), a number of studies have found that the density of brain corticosteroid receptors decreases in aged rats (Roth, 1974, 1976; Sapolsky et al., 1983; de Kloet et al., 1987; Reul et al., 1988). This decrease might be expected to protect against deleterious corticosteroid actions and to slow the loss of neurons.

Although there are a number of possible explanations of this seeming paradox (see Discussion), one clue to a possible reso-

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lution may lie in recent findings that HCSR of aged rats exhibited a reduced response to up-regulatory stimuli (e.g., to ADX) (Eldridge et al., 1987, 1989). That is, if HCSR also developed resistance to down-regulatory stimuli with aging, this might result in an increasing impact of corticosteroids on brain cells. The present experiments examined down-regulatory influences on one type of HCSR as a function of age.

Because age differences in ADX-stimulated up-regulation (Eldridge et al., 1987, 1989) or in the response to surgery could affect estimates of HCSR in ADX animals, animals intact at death were used in these studies. The use of intact animals also facilitated simultaneous analyses of the large numbers of animals used in the present experiments. However, there are considerable problems associated with measures of HCSR binding in intact animals (see McEwen et al., 1974), and we therefore conducted extensive pilot studies to develop suitable assay methods. The recent availability of highly specific synthetic steroids (Teutsch et al., 1981; Raynaud et al., 1984) has revealed the presence of at least 2 types of specific HCSR in hippocampus (see Reul and de Kloet, 1985; de Kloet and Reul, 1987). The subpopulation referred to as type I has higher affinity for CORT than the type II subpopulation. Because of this higher affinity, type I sites are almost fully occupied at normal plasma CORT levels, whereas type II are occupied only at more elevated (e.g., stress-induced) concentrations of CORT (Reul et al., 1987a). Thus, it has been proposed that type II HCSR is the subtype that plays the important role in hippocampal contributions to negative-feedback control of stress-induced ACTH release (de Kloet and Reul, 1987).

Using dexamethasone as the tracer (for which type II has higher affinity), we have been able to measure essentially the full type II HCSR population [as determined by comparison with values from our laboratory for early post-ADX periods (see Materials and Methods and Eldridge et al., 1989)] but almost none of the type I population. This difference in type I and type II availability may occur because type II sites are essentially unoccupied during the diurnal trough of endogenous corticosteroid secretion (Reul et al., 1987a, b) or because type II is more accessible for exchange by tracer. However, the latter possibility seems less likely because it appears that transformed corticosteroid receptors of either type are unable to rebind steroid following initial ligand association (McEwen et al., 1974; Brinton and McEwen, 1988; Chou and Luttge, 1988). Therefore, the present binding studies in intact animals were limited to a consideration of the unbound type II HCSR population.

Materials and Methods

Animals. A total of 135 male Fischer-344 rats were obtained from the National Institute on Aging colony (Harlan Industries) and were housed in a barrier facility, 2 per cage, paired by age and experimental grouping. Subjects were monitored frequently by veterinary staff for indications of pathology, and any animals exhibiting overt pathology were excluded from the study. Food and water were provided *ad libitum*. Room lighting was on from 8:00 a.m. to 10:00 p.m. daily. Death occurred by rapid decapitation after removal from the housing area, between 8:00 a.m. and 10:00 a.m. Animals were killed in cage-mate pairs, alternating across different age and experimental groups.

Chronic stress training. The chronic escape training procedure has been described elsewhere in some detail (O'Steen, et al., 1987; Odio and Brodish, 1988). Briefly, groups of 6–8 animals were placed in one of several adjacent Plexiglas boxes containing a metal grid floor and were trained simultaneously for 4 hr/d, 5 d/week, on a 2-way shuttle-escape task. Half of the floor area was electrified on alternate trials, using a current of just sufficient intensity (0.75 mA, constant-current source) to ensure shuttling to the nonelectrified side. The electrified half was alternated at random intervals (15 sec–5 min, mean interval = 105 sec ± 55 SD), and each alternation was preceded by a loud, 2 sec auditory cue. The animals learned quickly to escape immediately from the foot-shock area, which minimized the amount of shock received over the 6 month period. However, the rats were motionless and appeared tense during the interval between tone signals, indicating continuing stress during the period in the box.

Age-related differences in electric shock sensitivity threshold have been reported, but only with very low currents [e.g., below 0.4 mA (Gordon, 1978)]. At our current level of 0.75 mA, all groups appeared to act similarly. In addition, age-related differences in learning were not a factor, since age has little effect on 2-way shuttle tasks or on simple tasks with extensive overtraining (see Goodrick, 1972; Sprott and Stavnes, 1975; Arenberg and Robertson-Tchabo, 1977; Ingram, 1988). No physical injuries or trauma were seen during the 6 month training, and no animals died during the study. All procedures were approved by our institutional committee on animal care.

Experimental design. In the first experiment, animals at 4 months old (young), 12 months old (mid-aged) and 18 months old (aged) were exposed to the training paradigm for 6 months. Subjects were killed at either 1 d or 3 weeks after the last training session of the 6 month period. Unstressed home cage controls for each age group were also maintained in parallel and killed with the trained cohorts. Each trained group (2 per age) and each control group (1 per age) contained 8 animals (72 animals total).

The second study examined the time course of stress effects on HCSR density, using only mid-aged animals (begun at 12 months of age). Animals were trained for 2, 4, or 6 months, and killed on the day following the final session. Unstressed home cage controls were also maintained in parallel and killed at each time point. Each of the 6 groups contained 8–10 rats (53 animals total). This study was conducted to determine whether adaptation of down-regulation might play a role in the reduced effect of stress (see Results) on receptors in aging rats.

Reagents. Receptor assays were carried out in a hypotonic buffer (TEG) containing 5 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, 10 mM molybdate, and 10% glycerol, pH 7.40. The radioligand was ^{3}H-dexamethasone (DEX), obtained from Amersham Corp. Specific type II receptors were identified by displacement of tracer with RU-28362 (RU), a synthetic type II agonist (Teutsch et al., 1981; Raynaud et al., 1984; Reul and de Kloet, 1985). The radioimmunoassay buffer (GBS) was composed of 0.15 M NaCl, 0.01 M PO_4, 0.1% azide, 0.1% gelatin, pH 7.40. Radioactive corticosterone (^{125}I) was purchased from Cambridge Medical Diagnostics, Inc., as was an anti-CORT antiserum. The second antibody (anti-rabbit gamma globulin) was acquired from Antibodies, Inc. All other reagents were obtained from Sigma Chemical Co.

Receptor analysis. The quantity of type II corticosteroid binding was determined with a single-dose saturation assay described and validated elsewhere (Eldridge et al., 1989). Briefly, both hippocampi from each animal were rapidly dissected at autopsy following decapitation and immersed in ice-cold TEG. The tissue was homogenized and centrifuged at 105,000 × *g* to prepare cytosol. A set of incubation tubes was prepared for each animal, containing 20 nM ^{3}H-DEX alone, to assess total binding, tracer plus 100-fold excess nonradioactive RU-28362 (RU), to displace type II sites, or tracer plus 500-fold excess nonlabeled DEX, to displace total specific binding. Cytosol was added to all tubes and incubated overnight at 4°C. Separation of bound and free steroid was accomplished with dextran–charcoal. Type II binding (fmol/mg protein) was calculated from the difference between total binding of ^{3}H-DEX and tracer remaining bound in the presence of excess RU. In intact animals, the additional displacement by unlabeled DEX over that by RU (which estimates type I binding) is essentially undetectable, indicating that our assay measures only type II sites. Cytosol protein was determined by the Lowry method.

In a separate study in ADX animals from this laboratory, using ^{3}H-CORT (Eldridge et al., 1989), we found hippocampal type II capacity (B_{max}) in young animals to be 133.2 ± 17.0 (SE) fmol/mg protein at 24 hr post-ADX. After 48 hr ADX, the mean B_{max} was 171.3 ± 16.5 fmol/mg and exhibited further up-regulation by 7–10 d (311.2 ± 31.7 fmol/mg). In aged rat hippocampus, type II binding at 2 d post-ADX was 145.3 ± 22.4 fmol/mg, and by 7–10 d, 172.2 ± 28.5 fmol/mg. These 24–48 hr post-ADX figures are comparable to the estimates obtained in the present study. This indicates that, in unstressed intact rats, the population of type II hippocampal CSR remains essentially unoccupied and available for analysis by radioligand binding.

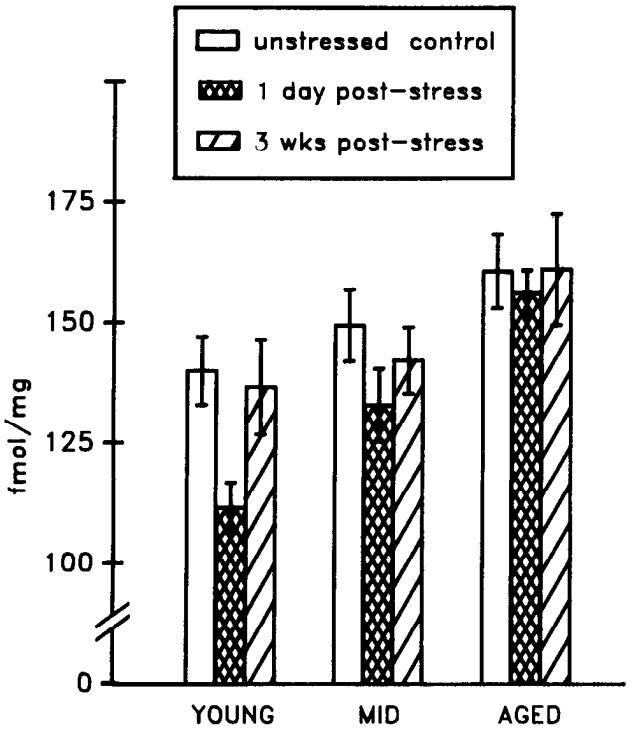

Figure 1. Type II corticosteroid receptor content in hippocampal cytosol after 6 months daily training in footshock-escape stress. Each bar represents the mean ± SEM of 8 animals. Ages at beginning of stress: young = 6 months, mid-aged = 12 months, aged = 18 months. Death at 1 d or 3 weeks following final training session. Main effects: age, $F(2,71) = 9.963$, $p < 0.001$; stress, $F(2,71) = 3.700$, $p < 0.05$.

Corticosterone RIA. As noted, an extensive literature (see review by de Kloet and Reul, 1987) indicates that the low plasma CORT levels existing in rats during the first hour of light are not sufficient to cause significant occupation of type II receptor sites. However, as a further check that small differences in CORT secretion at this diurnal period would not substantially affect measures of type II HCSR, plasma was obtained from control anmals at death in Experiment 1 and stored frozen for subsequent analysis of CORT by radioimmunoassay. Techniques were similar to those we have published elsewhere (Sonntag et al., 1987), with substitution of an ^{125}I-labeled tracer and a double-antibody separation. This method correlates extremely well with the former one (slope = 0.95, y-intercept = 0.038, correlation coefficient = 0.98, $n = 26$). The between-assay precision, including extraction, was 13.0% (20 runs), and the within-assay variation was 5.6% (75 duplicates).

Quantitative and statistical analyses. Group mean data were examined by analysis of variance and individual group contrasts were assessed by Bonferroni post hoc tests (Crunch Software Corp.). Correlational analyses were also assessed with Crunch programs.

Results

Experiment 1: effects of age and training on down-regulation

Analysis of variance showed significant main effects on type II HCSR of both age ($p < 0.001$) and chronic stress training ($p < 0.05$). The effects of age were accounted for mainly, but not solely, by the age difference in response to stress training at the 1 d poststress point (Fig. 1). Training significantly reduced HCSR only in the youngest animals (10 months old at death); a significant fall of 21% was seen at 1 d poststress, in comparison with unstressed young controls ($p < 0.05$). By 3 weeks poststress, HCSR binding returned to control levels in the young group (control vs 3 weeks, NS) and recovered significantly above the down-regulated 1 d poststress level (3 weeks vs 1 d, $p < 0.05$). Neither the mid-aged group (18 months old at death) nor the aged group (24 months old) exhibited a significant reduction of HCSR at 1 d or 3 weeks poststress, although, in mid-aged animals, there was a nonsignificant trend to down-regulation at 1 d. This trend was absent in aged animals. Although the significant effect of age was due mainly to differences at the 1 d point, all aged groups tended to show higher HCSR values than did comparable young-mature groups and contributed to the strong overall main effect of age ($p < 0.001$). This result is opposite to that previously reported in most studies of ADX animals (see Discussion).

Table 1. Plasma corticosterone at death and correlation with type II corticosteroid receptor capacity in hippocampal cytosol[a]

Age at death (months)	Plasma CORT (ng/ml)[b]	Correlation (r) with type II HCSR[c]
10 (Y)	44.2 ± 13.7	0.124 (NS)
18 (M)	52.8 ± 6.6	0.246 (NS)
24 (A)	42.4 ± 18.2	−0.225 (NS)

[a] Death between 8:00 and 10:00 a.m., unstressed controls.
[b] Mean ± SEM, 8 animals per group; $F(2,27) = 0.159$ (NS).
[c] Correlation of plasma CORT and HCSR in individual animals.

As anticipated for this time of the diurnal period, plasma CORT levels were consistently low in the control animals (Table 1) and were not different across the 3 ages. In addition, the analysis of possible correlations between receptor binding and plasma CORT levels at death across individual animals indicated that no correlation existed for any of the 3 age groups. A significant inverse correlation would have been predicted if resting endogenous CORT concentrations were high enough to occupy a sufficient number of type II sites to influence availability for tracer binding. While we did not examine CORT in trained animals, plasma CORT concentrations in rats chronically trained in this apparatus, or in similar tasks, have been found to be even lower than in unstressed controls (presumably because of adaptation) (see Pollard et al., 1976; Odio and Brodish, 1989), and therefore would also not be expected to reduce type II receptor availability in the trained groups.

Experiment 2: time course of development of chronic training effects

In order to evaluate the time course of the appearance of HCSR down-regulation with stress training, and to determine whether down-regulation in older animals might be more pronounced earlier in the 6 month training period, we examined a large group of mid-aged rats (12 months old at the outset) in the same chronic stress paradigm. Subgroups of 8–10 animals each were removed after 2, 4, or 6 months exposure and killed on the day following the last session. Parallel groups of home-cage unstressed controls were also killed at those points.

A significant ($p < 0.01$) overall effect of training exposure was seen, which was shown by individual group contrasts to be due largely to differences between the 4 months stress group and its age-matched control ($p < 0.02$) (Fig. 2). Individual group contrasts also showed a significant difference between the 2 and 4 month groups, indicating that the full effect of this paradigm did not develop in mid-aged animals until after 2 months of moderate stress. Further, the 6 months stress group did not differ significantly from its matched unstressed control, suggesting that

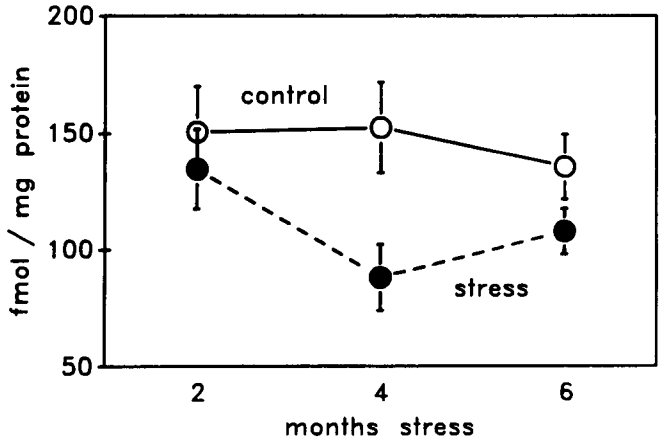

Figure 2. Type II corticosteroid receptor content in hippocampal cytosol after chronic escape training of mid-aged rats. Each point represents the mean ± SEM of 8–10 animals. Stress was begun at 12 months of age and death occurred on the day after the final session. Main effect of stress, $F(1,50) = 6.885$, $p = 0.01$.

HCSR down-regulation in mid-aged animals may exhibit some recovery with prolonged stress (Fig. 2).

Discussion

The results of this study indicate that type II HCSR appear less sensitive in aged than in young rats to the down-regulatory effects of 6 months of chronic escape training. In addition, the age-related decrease in HCSR density seen previously in ADX rats (Roth, 1974, 1976; Sapolsky et al., 1983; de Kloet et al., 1987; Reul et al., 1988) was not observed in intact control rats (Fig. 1). In fact, a significant overall elevation of type II HCSR was present in intact aged animals, in comparison to the young animals, and both control and trained groups contributed to this main effect of age. The most direct interpretation of these 2 main results seems to be that the type II HCSR population becomes less labile with aging and is therefore more resistant both to the down-regulatory influences of chronic stress training and to tonic down-regulatory influences that may normally operate in intact animals.

It seems possible that recent studies indicating that post-ADX up-regulation of type II HCSR is impaired in aged animals (Eldridge et al., 1989) might help to reconcile the present results, as well as studies that found no age effect on HCSR, with other apparently contradictory studies that found age-related decreases in HCSR. That is, most other studies on HCSR and aging have been conducted in rats adrenalectomized from 12 to 72 hr prior to receptor analysis. However, because of age differences in up-regulation, age differences in HCSR might develop or increase with time following ADX and might not be representative of conditions in intact animals. Nelson et al. (1976) found no age differences in brain CSR of mice adrenalectomized 12 hr earlier, whereas Roth (1976) found a substantial age difference in brain CSR of rats adrenalectomized 4–7 d earlier. Studies by Sapolsky et al. (1983), de Kloet et al. (1987), and Eldridge et al. (1989), but not Rachamin et al. (1987), also found age-related differences in brain CSR (aged animals lower), after at least 12–16 hr post-ADX. If the comparatively greater up-regulation of type II HCSR in younger animals begins within a few hours after ADX, older ADX animals might appear to have lower brain CSR as soon as 12–16 hr post-ADX. Nevertheless, this explanation may not apply to reports of age-related declines in type I HCSR (Reul et al., 1988) or in CSR of peripheral tissues (see reviews by Kalimi, 1984; Roth, 1985) because age differences have not yet been observed in post-ADX up-regulation of those receptor populations.

An age-related impairment of up-regulation has also been seen for adrenergic receptors in brain and other systems (Randall et al., 1981; Weiss et al., 1984; Roth et al., 1986; Scarpace and Abrass, 1988) and, as noted, for type II HCSR (Eldrige et al., 1989). In conjunction with those data, the present observations of apparent age-related resistance to down-regulation seems to raise the possibility of a generalized alteration in brain receptor plasticity with advancing age. However, extensive studies in other systems will be needed to test this possibility.

Although the simplest and most direct interpretation of the present data seems to be a reduced plasticity and greater population of type II HCSR with aging, an alternative interpretation might be that the results are due to increased availability of unbound type II HCSR for the tracer, arising from differences in either pituitary-adrenal activity or receptor affinity. However, as shown in Table 1, there were no age differences in resting CORT levels under the conditions of the present study. Other studies have found no age differences (Sonntag et al., 1987) or age-related increases in plasma CORT under various conditions (Lewis and Wexler, 1974; Landfield et al., 1978, 1980; Angelucci et al., 1980; Sapolsky et al., 1986). This suggests that there should be at least as much occupation of HCSR by endogenous steroid in aged as in young rats. Nevertheless, it is clear that the present studies do not address the total receptor population, and that bound endogenous steroids in intact animals can alter the brain binding patterns for other steroids (cf. Yongue and Roy, 1987). Therefore, studies of occupied receptors and bound hormone, including the population bound to chromatin, will be needed before the issue of whether these measures of higher type II populations in intact, aged rats reflect a greater functional capacity for CORT activation can be resolved definitively. As noted earlier, however, occupied and transformed corticoid receptors do not seem accessible to measurement by conventional exchange techniques (McEwen et al., 1974; Chou and Luttge, 1988), and studies of the total receptor population and of bound hormone will likely require a combination of approaches, including receptor immunocytochemistry (e.g., van Eckelen et al., 1987), receptor mRNA analysis (Chao and McEwen, 1988), and measures of radioactive ligand uptake *in vivo,* or RIA's of brain tissues in intact animals (McEwen et al., 1976; Yongue and Roy, 1987).

Because a single-point assay was used, the results also could arise from age-related increases of receptor affinity rather than capacity. If type II receptors in aged animals exhibited relatively greater affinity for the DEX tracer, then an apparent age-related increase in CSR binding might be observed. Although previous studies have found few effects of age or stress on CSR affinity (Kalimi, 1984; Roth, 1985; Sapolsky et al., 1984; de Kloet et al., 1987), this possibility cannot be excluded fully on the basis of available data. However, if altered affinity rather than density is found to account for aspects of the results presented in Figure 1, such data would still indicate that a major alteration occurs during aging that increases the impact of corticosteroids on brain cells.

The data in Figure 2 indicate that HCSR in mid-aged animals exhibit down-regulation by the fourth month of training but that the HCSR recover somewhat from down-regulation by 6

months. Therefore, it may be that differences in the rate at which down-regulation develops or recovers could account in part for the age differences in HCSR observed after 6 months of stress training (Fig. 1). However, additional studies will be needed to determine whether the rate of onset or the time course of down-regulation differs as a function of age. In addition, it should also be noted that the down-regulatory effects of chronic stress on HCSR may not necessarily be a result of elevated plasma CORT, and future studies will be needed to examine other physiologic responses to stress which might indirectly modulate corticosteroid receptor binding.

In summary, the present data are consistent with the view that hippocampal type II corticosteroid receptors in aged rats are more resistant to down-regulation by chronically elevated CORT levels (e.g., under conditions of stress), and perhaps to tonic down-regulation by normal CORT levels as well. These observations could have important implications for the seeming paradox of why CORT-dependent brain morphological deterioration develops more prominently with advancing age, whereas HCSR density has been found to decrease with aging. That is, the present data suggest that there may be no paradox because, under physiological (intact) conditions, type II receptor density (and, therefore, the capacity for CORT activation of the type II system) may not decrease with aging, or may even increase. There could, of course, be alternative explanations of this paradox. For example, levels of plasma CORT elevations, or the frequency or duration of corticosteroid activation, rather than the number of available receptor sites, could be important variables in corticosteroid-dependent brain aging. In addition, nonreceptor cellular defense mechanisms against deleterious aspects of steroid activation might decline with age. However, the present data on the apparent resistance to down-regulation and the generally higher levels of type II HCSR binding in intact aged animals, suggest that this receptor system might play a role in the mechanisms of brain aging. Moreover, the reduced plasticity of receptors observed here could be a factor in a variety of other reported age-dependent changes in behavioral and pituitary-adrenal responses (e.g., Landfield et al., 1978; Angelucci et al., 1980; McEwen et al., 1986; de Kloet and Reul, 1987).

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