Effect of Neonatal Handling on Age-Related Impairments Associated with the Hippocampus

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In rats, an environmental manipulation occurring early in life resulted in changes in the adrenocortical axis that persisted throughout the entire life of the animals and attenuated certain deficits associated with aging. Rats handled during infancy had a permanent increase in concentrations of receptors for glucocorticoids in the hippocampus, a critical region in the negative-feedback inhibition of adrenocortical activity. Increased receptor concentrations led to greater hippocampal sensitivity to glucocorticoids and enhanced negative-feedback efficacy in the handled rats. Thus, at all ages tested, rats that were not handled secreted more glucocorticoids in response to stress than did handled rats. At later ages, nonhandled rats also showed elevated basal glucocorticoid levels, with the result that there was a greater cumulative exposure to glucocorticoids in nonhandled rats. Increased exposure to adrenal glucocorticoids can accelerate hippocampal neuron loss and cognitive impairments in aging. Hippocampal cell loss and pronounced spatial memory deficits emerged with age in the nonhandled rats, but were almost absent in the handled rats. Previous work showed that glucocorticoid hypersecretion, hippocampal neuron death, and cognitive impairments form a complex degenerative cascade of aging in the rat. The present study shows that a subtle manipulation early in life can retard the emergence of this cascade.

FUNCTIONAL DEFICITS EMERGE AS most physiological systems age (*1*). However, considerable individual variation in the severity of such degeneration occurs, reflecting individual differences in biological and environmental life histories. We demonstrated that an environmental manipulation restricted to the first 3 weeks of life in the rat will, 2 years later, attenuate neuroendocrine, anatomical, and behavioral impairments related to hippocampal dysfunction during aging.

The environmental manipulation used was the handling of neonatal rats, which has been shown to alter the adrenocortical response to stress (*2*). Infant, male Long-Evans rats (*3*) were handled daily from the day of birth until the day of weaning (day 22). The mothers of handled (H) litters were removed from their cages. The pups were then removed and placed in a plastic container lined with paper towel. After 15 minutes, the pups and then the mother were returned to their cages. Nonhandled (NH) litters were left undisturbed.

We found earlier that postnatal handling is associated with increased concentrations of glucocorticoid receptors (GC-Rs) in the hippocampus in animals tested at 5 months of age (*4*). We first examined the possibility that this effect might persist throughout the animal's life-span. With aging, GC-R concentrations decline in hippocampal neurons, with no change elsewhere in the brain, except the amygdala (*5, 6*). Receptor affinity does not change, and the loss does not occur in the glial population of GC-Rs (*6*). Using an in vitro binding assay (*7*), we found that, although the H rats showed a loss of GC-Rs by 24 months of age, the effect of the early handling persisted throughout the life-span, with the result that H rats had significantly higher hippocampal GC-R concentrations than did NH rats at all ages tested (Fig. 1A). In agreement with our previous findings (*4, 6*), there was no effect of either age or handling on GC-R concentrations in the hypothalamus or pituitary.

Such elevated GC-R concentrations in H rats appeared to have important neuroendocrine consequences. The adrenocortical axis is a classical, closed-loop endocrine system in which elevated concentrations of the end product, the glucocorticoids (GCs), inhibit subsequent secretion of corticotropin from the hypothalamus and adrenocorticotropin from the pituitary (*8*). This negative-feedback regulation is mediated by the pituitary, hypothalamus, and a number of suprahypothalamic sites, including the hippocampus. As evidence for the role of the hippocampus, lesions of this structure produce GC hypersecretion and negative-feedback insensitivity (*9*). The inhibitory effects of GCs at the hippocampus appear to be mediated by the binding of GCs to GC-Rs. The hippocampus has high concentrations of GC-Rs (*10*). A loss of GC-Rs (without damaging hippocampal neurons or altering GC-Rs elsewhere in the brain) desensitizes the system to feedback regulation and produces GC hypersecretion under both basal and stress conditions (*11*). Similarly, as aging rats lose GC-Rs, they become insensitive to GC feedback regulation and hypersecrete corticosterone (the predominant GC in the rat) both basally and after the end of stress (*12*). We examined corticosterone secretion before and at various times after the termination of a 20-minute restraint stress in H and NH animals, 6, 12, or 24 months of age. Blood samples were taken from the tail vein of each animal before and at 0, 30, 60, 90, 120, and 180 minutes after the termination of the stressor. Plasma corticosterone levels were determined by means of a radioimmunoassay. The differences between H and NH animals were apparent at each age (at 6 months, $F = 5.35$, df = 5,105, $P < 0.001$; at 12 months, $F = 3.10$, df = 5,135, $P < 0.01$; at 24 months, $F = 3.29$, df = 5, 80, $P < 0.01$). At each age tested, the H animals secreted significantly less corticosterone during restraint stress and terminated corticosterone secretion after stress sooner than did the NH rats (Fig. 1B). As expected on the basis of the GC-R data, age-related deficits were far less pronounced in the H rats. At later ages, both H and NH animals showed prolonged post-stress elevations in corticosterone levels; however, this effect of age was greater in the NH animals (Fig. 1B). Moreover, the age-related rise in basal GC levels, often seen in aged rats (*9*), was observed in the NH but not in the H animals (Fig. 1C).

These findings suggest that cumulative GC exposure over the life-span was greater in NH than in the H animals. We next examined an important neuropathological consequence of this difference. The hippocampus loses neurons with age, particularly in the CA1 and CA3 cell fields (*13*). The degree of exposure to GCs, which inhibit glucose utilization in the hippocampus, appears to act as a major determinant of hippocampal neuron loss after neurological insult and during the normal aging process (*13, 15*). Neuron loss after hypoxia-ischemia, seizure, or exposure to antimetabolites is exacerbated by high GC concentrations and decreased by adrenalectomy (*14*). Senescent loss of hippocampal neurons is prevented by adrenalectomy at mid-age (*15*) and accelerated by chronic exposure to corticosterone in concentrations in the upper physiological range [equivalent to those occurring during major stressors (*16*)]. Thus, the reduced cumulative exposure to GCs in the H rats suggested that hippocampal degeneration would be less pronounced in these animals, and this was observed (*17*): among NH animals, but not H animals, there was a significant loss of neurons with

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age in both CA1 and CA3 hippocampal cell fields (Fig. 1D). The number of hippocampal neurons in H and NH rats did not differ at 6 months of age, but was greater in the H rats at later ages (Fig. 1D).

The difference in the number of hippocampal cells between the H and NH animals at the later ages is of functional significance. In addition to its neuroendocrine role, the hippocampus is important in learning and memory, and hippocampal injury profoundly disrupts cognition (*18*). The aged rat develops cognitive impairments similar to those seen after hippocampal damage (*19*). Adrenalectomy at 12 months of age not only prevents hippocampal neuron loss, but also reduces cognitive impairments due to senescence (*15*). These findings suggested that the older H rats, with attenuated cell loss in the hippocampus, should show less evidence of age-related cognitive impairments than older NH rats.

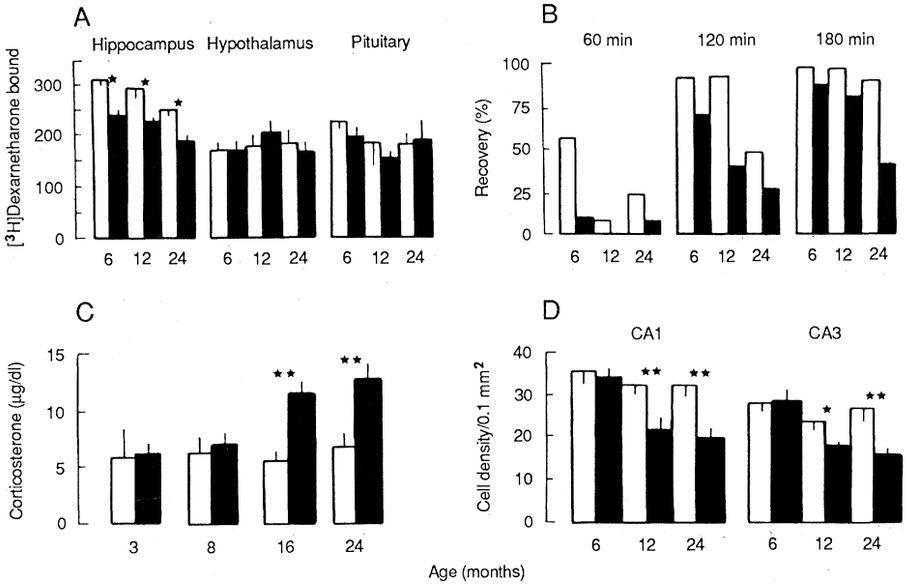

Fig. 1. (**A**) Mean (±SEM) of [^{3}H]dexamethasone binding (measured as femtomoles per milligram of protein in hippocampus, hypothalamus, and pituitary in H (□) and NH (■) animals at various ages. Assays on hypothalamic and pituitary tissues were performed on pools of tissue from two or three animals (n = 3 or 4 per group), while the assays on hippocampal tissue were performed with tissue from a single animal (n = 7). Age (F = 4.78; df = 2,19; $P \leq 0.05$) and treatment group (F = 9.33; df = 2,19; $P \leq 0.01$) differences are statistically significant for hippocampus (differences significant at $^*P \leq 0.05$). (**B**) Cumulative percentage of animals in each age by treatment group exhibiting recovery to basal corticosterone levels (recovery defined as a value ≤ prestress level + 20%) at various intervals following a 20-minute restraint stress (n = 10 to 17, with the variation due to the exclusion of certain assay points and the loss of animals at the older ages). (**C**) Basal corticosterone values (measured as micrograms per deciliter) at 1000 hours in the 24-month-old H (□) and NH (■) animals sampled at 3, 8, 16, and 24 months of age (n = 12 per group). The treatment effect (F = 15.01; df = 3,93; $P \leq 0.001$) is statistically significant (differences significant at $^{**}P \leq 0.01$). (**D**) Neuron density per 0.1 mm^2 in the CA1 and CA3 pyramidal cell fields of the hippocampus in H (□) and NH (■) animals of varous ages. Significant treatment effects were found for both CA1 (F = 17.66; df = 1,26; $P \leq 0.001$) and CA3 (F = 12.11; df = 1,26; $P \leq 0.01$; group differences significant at $^*P \leq 0.05$ and $^{**}P \leq 0.01$; n = 4 or 5 per group). There were no consistent differences observed in the CA2 or CA4 cell fields.

We used the Morris swim maze to test spatial memory (*19, 20*). Spatial memory deficits emerged with age in the NH rats (Fig. 2), with the result that the 24-month-old NH animals differed significantly from the 6-month-old animals in all but the first 3 of 18 trials. In contrast, among the H rats there were no statistically significant age differences in spatial memory. In order to control for possible differences in swimming speeds, we also analyzed the data for the last eight trials by scoring the distance the rats swam before locating the submerged platform. Again, aged NH rats showed a clear deficit not shown by the aged H rats (Fig. 2). In subsequent tests, H and NH rats of all ages performed similarly when the platform was made visible by raising it above the water level, indicating that the differences between the H and NH rats were due to spatial rather than motor skills. These spatial memory deficits in the older NH animals may be related to the hippocampal damage seen in these animals, since similar deficits are observed after lesioning of the dorsal hippocampus (*20*).

The aged rat shows GC hypersecretion and negative-feedback insensitivity, as well as the loss of hippocampal neurons and GC-Rs (*9*). These deficits form a complex and self-perpetuating cascade; that is, a consequence of GC hypersecretion is accelerated loss of neurons in the aging hippocampus, including the loss of corticosterone-concentrating neurons, and a consequence of hippocampal damage is adrenocortical negative-feedback insensitivity and GC hypersecretion. Handling produced lower stress GC concentrations throughout the life-span, the capability to terminate GC secretion more rapidly after stress, as well as lower basal GC levels later in life. These changes appear to prevent, or at least delay, the degenerative glucocorticoid cascade that characterizes aging in the rodent.

In view of the importance of hippocampal GC-Rs in the negative-feedback regulation of the adrenocortical axis (*9, 11*), increased GC-R number appears to be critical for the handling effect. We have recently demonstrated that H animals show greater feedback sensitivity to both corticosterone and dexamethasone (*21*). Also, selective down-regulation of hippocampal GC-Rs (with chronic corticosterone treatment) in H animals virtually eliminates the differences between young, adult H, and NH animals in post-stress GC secretion. The handling effect on hippocampal GC-Rs appears to be mediated, at least in part, by the pituitary-

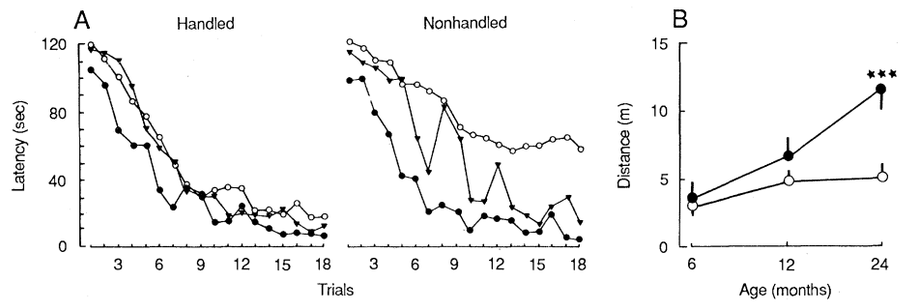

Fig. 2. (**A**) Mean latency (seconds) to find the platform for H and NH animals of various ages: (●) 6 months old; (▼) 12 months old; (○) 24 months old; n = 9 to 12 animals per group. The data for the 18 trials were obtained while the platform was submerged below the water level (spatial learning condition). Statistical analysis of the results revealed a significant age by treatment effect (F = 3.92; df = 2,66; $P \leq 0.05$; the difference between the 24-month-old H and NH animals is significant, $P \leq 0.01$). (**B**) Mean (±SEM) swimming distance to the platform over the last eight trials for H (○) and NH (●) animals of various ages. Statistical analysis of the results revealed a significant age by treatment interaction (F = 6.52; df = 2,66; $P \leq 0.01$; differences are significant $^{***}P \leq 0.001$).

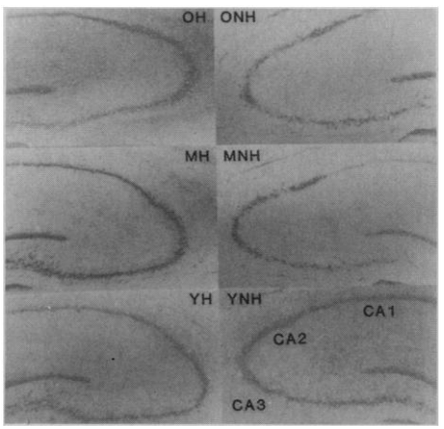

Fig. 3. Photomicrographs of 10-μm cresyl-violet–stained sections of dorsal hippocampus from H and NH animals at ages (Y) 6 months, (M) 12 months, and (O) 24 months. The neuron loss in CA1 and CA3 in the 12- and 24-month-old NH animals can be discerned by comparing these regions to the unaffected CA2 region.

thyroid axis, since administration of thyroid hormones during the first week of life mimics the handling effect on GC-R concentrations, whereas propylthiouracil, an inhibitor of thyroid hormone synthesis, blocks the effect of handling on GC-R development (*22*). In contrast, handling has little effect on corticosterone secretion in neonatal rats (*23*) and therefore cannot be readily interpreted as being a classical neuroendocrine stressor.

The diminished rate of hippocampal neuron loss (Fig. 3) in the aging H rats probably reflects the lower cumulative lifetime exposure to GCs. The GCs appear to damage hippocampal neurons indirectly, by compromising the ability of the neurons to survive metabolic challenges, through a disruption of energy metabolism (*9, 14*). Regardless of the mechanism, the decreased cumulative exposure to GCs over the lifespan was associated with the same result as was reported for rats adrenalectomized at mid-age (*15*)—that is, attenuated senescent loss of hippocampal neurons. It should be noted that this outcome is the product of two apparently opposing trends. Although increased concentrations of hippocampal GC-Rs is related to the enhanced negative-feedback sensitivity and decreased GC secretion in H rats, the same high GC-R concentrations could conceivably sensitize the hippocampus to the endangering effects of GCs. In this instance, the decreased secretion apparently outweighs the risk of increased target sensitivity, perhaps by ensuring that the prolonged GC exposure necessary for the endangering effects does not occur.

Thus, relatively subtle individual differences in early experience can alter profoundly the quality of aging years later (in female as well as in male rats). This not only underscores the importance of developmental critical periods and explains a possible source of variability of aging studies, but also suggests strategies early in life to attenuate some of the dysfunctions of aging. It should be emphasized that only some of these handling-induced changes represent legitimate deceleration of the aging process. For example, even though hippocampal GC-R concentrations were always higher in the H than in the NH rats, the H rats still lost GC-Rs. In contrast, the number of hippocampal neurons and spatial memory ability did not differ between the H and NH rats at 6 months of age. Thus, the difference between H and NH rats in these measures at later ages represents a true retardation of the aging process in the H rats.

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7. GC-R concentrations in pituitary and selected brain regions were analyzed by means of an in vitro binding assay with [^{3}H]dexamethasone, a synthetic GC that labels GC but not mineralocorticoid binding sites, as described earlier (*4, 6*). The results were analyzed by the method of Scatchard [G. Scatchard, *Ann. N.Y. Acad. Sci.* **51**, 660 (1949)] and expressed as femtomoles of [^{3}H]dexamethasone binding per milligram of protein. Statistical analysis was performed with a two-way ANOVA (age by treatment) with Tukey post-hoc tests.
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17. Cell counting was performed on 10-μm, cresyl-violet–stained sections as described (*16*). Briefly, raw cell counts were obtained using a 250 by 250 μm grid over a magnified (×40) section with a light microscope. Three to five sections of dorsal hippocampus (corresponding to plates 21 or 22 of the rat brain atlas of Paxinos and Watson [G. Paxinos and D. Watson, *The Rat Brain in Stereotaxic Coordinates* (Academic Press, New York, 1982)] were analyzed for each animal. Raw cell counts of the pyramidal cell fields were transformed into measures of neuron density per 0.1 mm^2 by the method of Abercrombie [M. Abercrombie, *Anat. Rec.* **94**, 239 (1946)] to correct for split cell artifacts. In addition, the area of the cell fields was estimated with camera lucida tracings onto a graphics tablet providing a measure of the length of each cell field. We found that the length of each cell field remained stable for the various age versus treatment groups. Thus, differences in neuron density could not be attributed to changes in the length of the cell fields. Statistical analysis was performed using a two-way ANOVA (age by treatment) with Tukey post hoc tests.
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20. The Morris swim maze [R. Morris, in *Neurobiology of the Hippocampus*, W. Seifert, Ed. (Academic Press, New York, 1985), p. 185] is a behavioral test in which animals are required to find a platform submerged (2 cm) in a pool (1.6-m diameter) of opaque water using only distal, spatial cues available within the testing room. The animals were given 18 trials over 5 days with the platform submerged, followed by 5 trials in which the platform was elevated (2 cm) above the water level so that the animals could use the obvious visual cues of the platform. The critical measures are the time elapsed (latency) and the swimming distance traveled before the animal locates and climbs onto the platform. The latency data were analyzed with a three-way ANOVA (age by treatment by trials) and the distance data were analyzed with a two-way ANOVA (age by treatment). Post hoc analysis was performed using Tukey post hoc tests.
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