side compartments, or whether neurons with this capability retained their responsiveness to NGF. Although reintroduction of NGF into a side compartment after 29 days of deprivation resulted in resumption of neurite advance (Fig. 2b), the neurites of a subpopulation of neurons capable of surviving but not elongating during NGF deprivation could have been responsible for this. Neurites of the hypothetical subpopulation could have been present just out of sight under the barrier in the side compartment, where exposure to reintroduced NGF could have caused their growth to resume. The 1- to 2-day lag between addition of NGF and resumption of neurite advance would have been sufficient for these neurites to overtake and pass the previous growth. Thus, neurons capable of regenerating neurites during NGF deprivation may not, in fact, be responsive to NGF.

Alternatively, neurites of mature sympathetic neurons may be capable of limited regeneration in the absence of NGF, but full regeneration may require reestablishment of contact with an NGF supply. Postganglionic axotomy results in irreversible atrophy of the superior cervical ganglia when performed in rats before the 12th postnatal day (*11*). This atrophy was preventable by a period of intravenous administration of NGF (there was functional recovery of the innervation), but axotomy carried out after 3 weeks of age was followed by reinnervation without the aid of exogenous NGF. The timing of the appearance of the unaided, regenerative capability is well correlated with my observation of regeneration in NGF-deprived neurons maintained 1 month or more in culture. Taken together, the results suggest that NGF-independent neurite regeneration may be a first, crucial stage in the reestablishment of sympathetic innervation after peripheral nerve damage in the adult rat. This capability may develop independent of any direct postnatal influences by nonneuronal cells.

A second stage of regeneration, as well as long-term maintenance of the neurons, may require reestablishment of contact with an NGF source—perhaps the tissues to be reinnervated. Interestingly, NGF production by the iris is stimulated by denervation (*12*), and truly long-term survival of sympathetic neurons may require NGF, even in the intact, adult sympathetic nervous system (*4*).

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Brain Aging Correlates: Retardation by Hormonal-Pharmacological Treatments

Abstract. *Mid-aged rats were either adrenalectomized and chronically maintained, or left intact and treated daily for a 9- to 10-month period with a potent analog of the peptide adrenocorticotropin (residues 4 to 9), which has some stimulant properties, or with the neural stimulant pentylenetetrazole. All three treatments reduced hippocampal morphologic correlates of brain aging (neuronal loss, glial reactivity). The pentylenetetrazole and peptide treatments also improved reversal learning. These results suggest that certain endogenous peptides, with stimulant properties, may also exert long-term, trophic effects on brain structure and function.*

Little is known about the etiological factors that influence the rate of brain aging, although research on possible causes of senile dementia or normal brain aging (or both) is currently focused on cerebrovascular processes, slow viruses, immune reactions, toxic metals, and genetic mechanisms (*1*). Additionally, we have been pursuing the hypothesis that endocrine factors, particularly glucocorticoids, may normally accelerate some aspects of brain aging (*2*). Our prior studies have so far yielded data consistent with predictions of that hypothesis (*2*), and other studies have implicated elevated corticoids in age-like cardiovascular decline (*3*). However, clear evidence of retarded brain aging also seems needed to test a hypothesis of causal factors in normal brain aging. We recently reported, in preliminary form, that long-term adrenalectomy does reduce some morphologic correlates of aging in the rat hippocampus (*4*) [which is rich in corticosterone receptors (*5*)].

Adrenalectomy not only lowers corticoids, however, but also results in widespread changes in endocrine-metabolic measurements. Among the major consequences of adrenalectomy, of course, is an elevation in adrenocorticotropic hormone (ACTH), which exerts direct behavioral and biochemical actions on the brain (*6*); moreover, ACTH and its non-steroidogenic, brain-active fragments (for example, ACTH residues 4 to 10) induce electrophysiological patterns similar to those of neural stimulants (*7*). It therefore seemed feasible that the prolonged neural stimulation resulting from elevated ACTH, rather than the reduction in steroids per se, could be largely responsible for the retardant action of adrenalectomy on brain aging (possibly by maintaining neuronal metabolism). In this study, we examined these possibilities.

We now report that long-term treatment of rats with a neural stimulant, pentylenetetrazole (PTZ), or with a potent and behaviorally active analog of the ACTH[4–9] molecule (ORG 2766), which does not affect glucocorticoid release (*8*), can retard the development of both some neuromorphologic and some behavioral correlates of brain aging in rats. We also report that the long-term absence of the adrenal glands has effects on brain aging correlates different from those of ORG 2766 and PTZ. These studies, then, suggest that peptides, as well as steroids, can influence brain aging and that stimulation may be an important element in these effects.

We used a number of established morphometric correlates to quantify the degree of hippocampal aging. Neuronal loss, lipofuscin, and glial reactivity have

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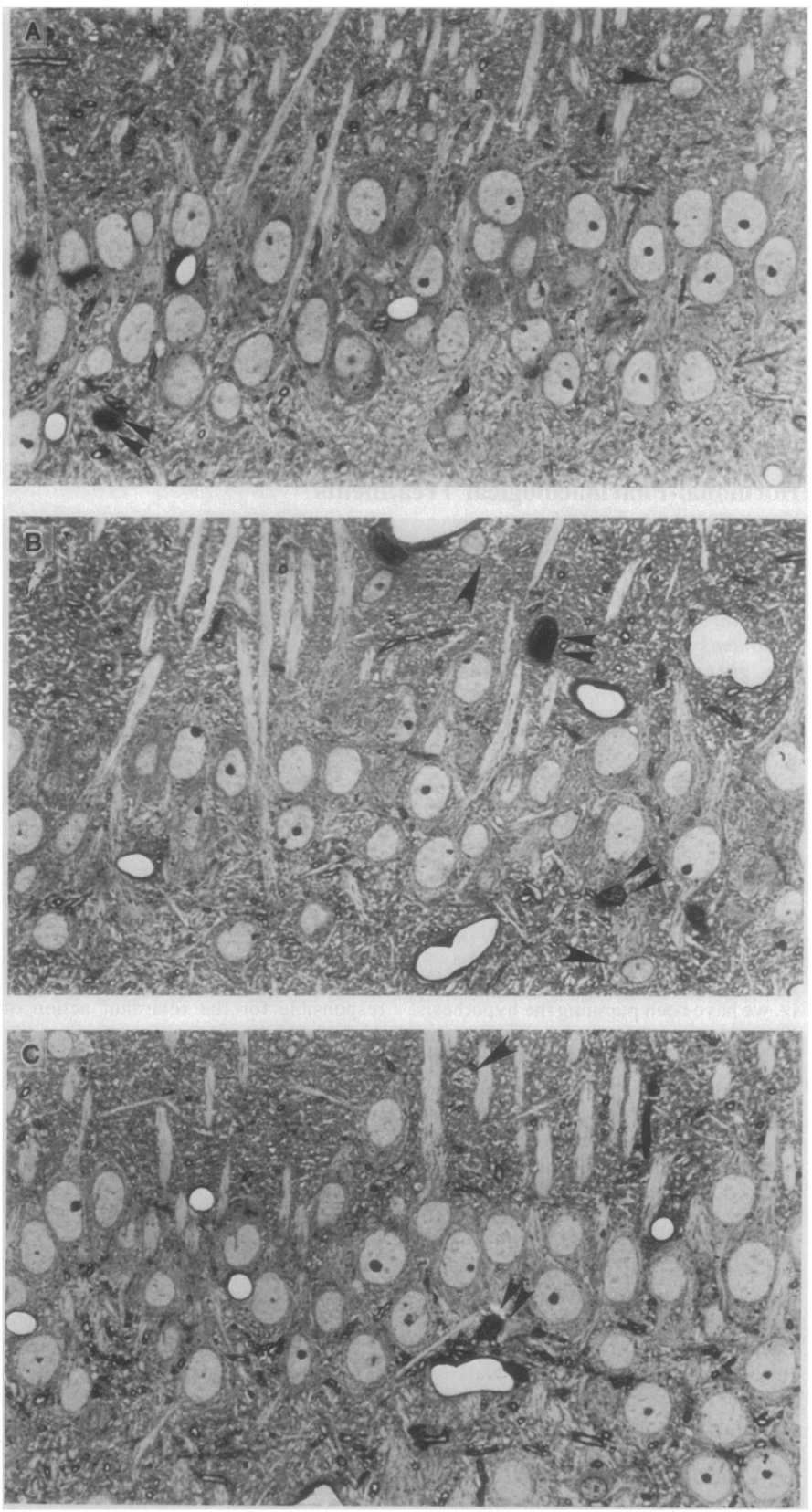

Fig. 1. Examples of CA1 pyramidal cells in the soma layer in semithin sections from young rats (A), aged controls (B), and aged rats adrenalectomized 9 months earlier (C). All sections are cut perpendicular to the somal layer, from the CA1 region just dorsal to the tip of the dorsal limb of the dentate gyrus granule cells. Neuronal nuclei and major glial species can be recognized—astrocytes with lucent cytoplasm (arrowheads) and the darker microglia and oligodendrocytes, with chromatin clumps in the nucleus (double arrowheads) [compare with (*17*)].

been reported to be consistent correlates of brain aging in humans, monkeys, dogs, and rodents (*9*). Morphologic correlates were assessed in semithin sections. We recently described the appearance and quantification of these variables in semithin sections of aged rat hippocampus in some detail (*10*). In addition, we noted that neuronal nuclei subjectively appear "rounder" in younger rats, and we therefore also assessed nuclear roundness as a means of obtaining another neuronal (as opposed to glial) measure (*11*). Since many behavioral changes, including apparent memory decline, develop in aging humans, monkeys, and rodents (*12*), we also incorporated behavioral measures into our assessment of brain aging. Rats exhibit age-related deficits in complex maze tasks, particularly when trials are temporally spaced or when reversal paradigms are included (*12*). We used a task incorporating these features to assess performance in our animals (*13*).

Male inbred Fischer rats 16 months old (cesarean-derived, barrier-reared, from the Charles River colony of the National Institute on Aging) were divided into three groups, matched for mean weights and open-field activity. The groups received subcutaneous injections of ORG 2766 (N = 12), PTZ (N = 12), or appropriate vehicle (N = 14), once each day, five times a week, for 8.5 months (*14*). Drugs were then withheld beginning 1 week before the onset of behavioral tests, and for their duration (lasting ~ 2.5 weeks); drugs were thereafter continued for an additional 1.5 months, at which point all animals were killed. An additional (untreated) aged control group (N = 14) with the same birth date was obtained 5 months after the experiment began and was then concurrently maintained until the end of the study. Since the vehicle and untreated aged control groups were essentially identical on every variable measured, they were combined into one aged control group for statistical purposes. Nineteen other animals with the same birth date were adrenalectomized at 18 months of age and were maintained concurrently for 9 months on a 1 percent NaCl drinking solution, which included 5 μg of cortisol per milliliter as replacement glucocorticoid (*14*). A young-mature control group (N = 11) was also obtained 5 months after the experiment began. Precautions against infection were maintained throughout the study (*15*). All aged animals were 27 months old, and the young-mature control group was 8 months old, when the study ended.

Due to attrition from natural causes, from inadequate perfusion, or from a sudden death syndrome (possibly hypoglycemia) in adrenalectomized animals that otherwise appeared healthy, the final group sizes during the behavioral tests were: aged controls, 20 animals; ORG 2766, 10; PTZ, 10; adrenalectomized 11; young-mature, 11. For morphologic analyses the final group sizes were: aged controls, 17 (vehicle, 7; untreated, 10); ORG 2766, 8; PTZ, 8; adrenalectomized, 8; and young, 11. At the end of the 11-month study, rats were anesthetized with ether; beginning 5 minutes after the start of anesthesia, 3 to 5 ml of blood was withdrawn from the abdominal aorta of each rat for hormonal and electrolyte analysis (*16*). Animals were then quickly perfused through the heart with mixed aldehyde solutions, and brains were prepared for semithin sections. Quantitative light microscopic analyses were performed blind and checked by a second investigator (*10, 11*).

Most adrenalectomized animals resembled the young animals on morphologic variables (Fig. 1), but two adrenalectomized animals exhibited considerable evidence of brain aging (for example, they scored above the median for aged controls on the brain aging index). The appearance of drug-treated animals was intermediate in pattern between young and aged controls. As shown in Fig. 2, on every morphologic measure except lipofuscin (Fig. 2D) the three experimental groups were more similar to the young-mature groups than aged controls were. Because of variance, differences on some variables were not significant ($.05 < P < .10$). Nevertheless, when combined in a composite index these variables also contribute to the degree of difference from aged controls (Fig. 2G). Young animals and the drug groups performed significantly better than aged controls did on the maze reversal task, as measured by latency values (which are increased by incorrect or uncertain responses) (Fig. 2A). Analysis of data on correct choices yielded similar results (*13*). Adrenalectomized animals did not perform better than aged controls despite morphologic evidence of reduced brain age. However, plasma analyses revealed a wide spectrum of endocrine-physiologic disturbances in the adrenalectomized animals (*16*), and one of these side effects may acutely decrease maze performance and thereby counteract any long-term effects of adrenalectomy on neurobiologic structure. Since group differences in behavior were found only on the reversal phase, the age and drug effects cannot be ascribed to obvious performance variables (such as sensory-motor functions or pain threshold) and may reflect changes in information processing or storage systems or in perseveration (*12*). Drug treatments were withheld beginning 1 week before the behavioral trials; because the metabolism of these agents is complete by 24 hours (*8, 14*), the drugs could not have affected maze performance by an acute action. Therefore, long-term drug treatment seems to have induced a prolonged change in the structure or physiology of the brain (or both), allowing aged rats to perform more like the young.

Our findings suggest that the feedback-dependent increase of ACTH (*16*) may account for some of the effects of adrenalectomy on brain correlates of age, since administration of the ORG 2766 peptide retarded brain aging indices in the absence of any alteration of steroids (*16*). However, the profile of the ORG 2766 effect was not the same as that of adrenalectomy; that is, some adrenalectomized animals exhibited substantially less evidence of brain aging than any of the ORG 2766–treated animals. Therefore, reduction of steroids seems to have contributed to the retardation of brain aging correlates, and steroids may exert actions partially opposing those of peptides. The similarity of the effects of ORG 2766 and PTZ on brain morphology and behavior suggests that these drug treatments may both have acted by the common mechanism of neural stimulation, particularly since ACTH and its fragments have stimulant properties (*7*). These data also raise the possibility that certain endogenous peptides with stimulant properties may exert trophic, long-

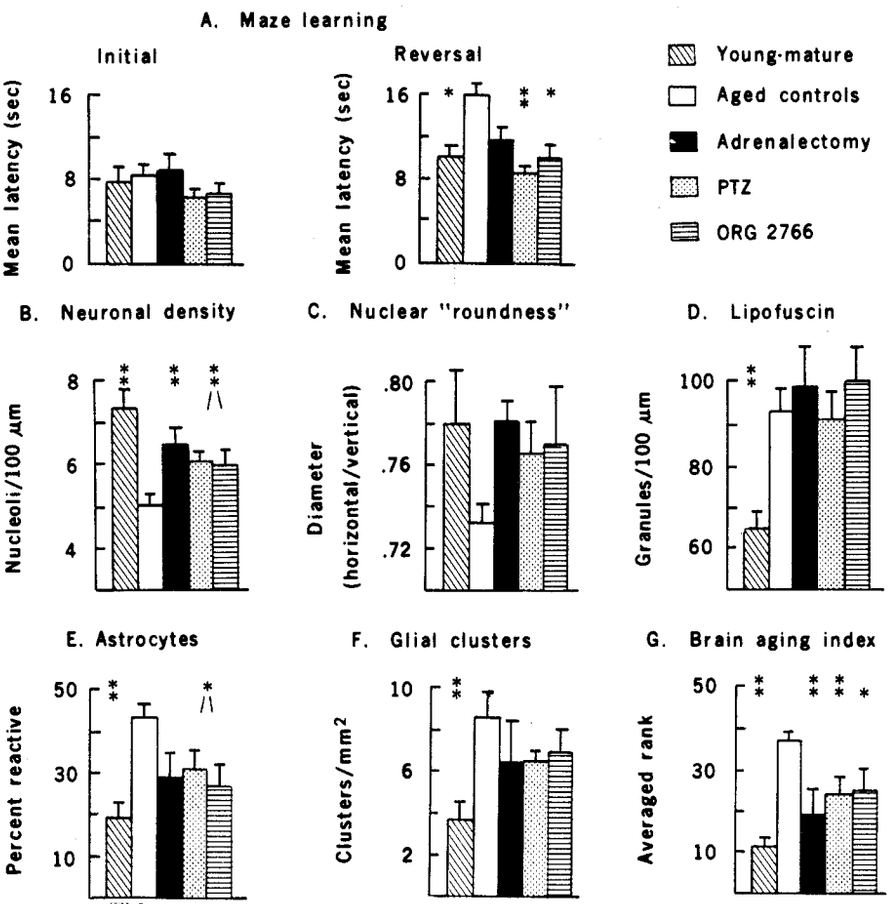

Fig. 2. Means (± standard errors of the means) of behavioral and morphologic variables. (A) The age-dependent behavioral impairment, indicated by elevated latency, is confined to the reversal phase. Both drug groups, but not the adrenalectomized animals, performed significantly better than aged controls. (G) Composite index incorporating variables shown individually in (B, C, E, and F) (*11*). Because the PTZ and ORG 2766 groups exhibited similar profiles and were not statistically different on any variable, and because the two drugs are proposed to act by a similar mechanism, these two groups were combined for statistical analyses on the individual morphologic variables (but not on behavioral measures or on the composite index). Each group is still significantly different from aged controls when groups are not combined, at the next level of significance below that shown. Significance level of difference from aged controls: $^{**}P < .01$, $^{*}P < .05$; (two-tailed).

term effects on brain structure and function. The relative balance between long-term peptide and steroid hormonal effects could be important influences on the rate of vertebrate brain aging.

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11. Semithin sections (1.5 μm thick) were prepared according to standard methods (*10*). Neuronal packing density was determined by counting all pyramidal cell nuclei with distinct nucleoli in the stratum pyramidale. Mean diameter of nucleoli did not differ among groups and therefore correction factors for split-nucleoli error were not applied (*10*). Mean nuclear roundness (ratio of horizontal diameter to vertical diameter of the nucleus) was calculated for each animal from the ten largest pyramidal cell nuclei from one section. Astrocyte reactivity was taken as the percentage of all astrocytes with both (i) extensive cytoplasm around the nucleus and (ii) numerous inclusion granules in the cytoplasm. Glial clusters were defined as any instance of membrane apposition between at least one astrocyte and at least one microglial or oligodendroglial cell (*10*). Glial counts were confined to the CA1 regions between the alveus and the perforant path. Lipofuscin granules were counted throughout the pyramidal cell layer. If aged and young controls differed significantly according to a *t*-test on a variable, that variable was considered sensitive to age differences; all aged groups were then examined on that variable with an analysis of variance and post hoc (Scheffe) comparisons. A brain aging index was also calculated for each animal by averaging its rank score across the four variables that most clearly exhibited the largest experimental effects (neuronal density, roundness, astrocyte reactivity, and glial clusters); this average was used to re-rank all animals on a composite brain aging index. Kruskal-Wallis one-way analyses of variance and Mann-Whitney *U* tests were used to assess this variable.
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13. Animals were trained in a cross-shaped maze (three arms plus one start box) to choose one arm (left) to avoid or escape footshock; after 1 week, they were then reversed and trained to the right arm. A footshock trial consisted of placing a rat in the start box and after 5 seconds delivering shock (0.35 mA) to the grids of the start alley and to the two incorrect maze arms until a correct choice was made. On nonfootshock trials, rats were placed in the maze until a choice was made, or until 2 minutes had elapsed, but no shock was given. At the beginning of initial training, three acquisition footshock trials were given, and animals were then tested for retention performance by giving them one nonfootshock trial, and one footshock trial on each of days 1, 4, 5, and 6 after the acquisition session. Two (reversal) acquisition footshock trials were then given for the reversal phase, and animals were subsequently tested in the same manner and sequence as after the initial phase acquisition trials. Mean latencies (time from start to correct choice) on footshock trials were subjected to analyses of variance. Acquisition footshock trials were not included in this analysis since the study was aimed at assessing retention performance (although reversal data are also signficant if acquisition trials are included).
14. Initial drug dosages were set at levels above those known to exert behavioral effects [(*6*, *8*); J. L. McGaugh, *Science* **153**, 1351 (1966)], but were lowered if abnormal patterns of weight loss, food intake, or pathology appeared (all of which were monitored). The initial dose of ORG 2766 was 70 μg per day per rat, which was reduced to 30 μg per day after 3 months because of pathology in one rat, then raised to 40 μg per day 2 months later. The initial dose of PTZ was 5 mg per rat per day, which elicits electroencephalographic effects but is below seizure threshold [P. Landfield, *Brain Res. Bull* **1**, 9 (1976)]. After 7 months this was raised to 9 mg per rat for 1.5 months, but was then returned to 5 mg per rat after several animals exhibited pathology or weight loss. Administration of corticoids in drinking water is well characterized [J. Ramaley, *Biol. Reprod.* **14**, 151 (1976)]. A concentration of 5 μg/ml is far below that needed (> 160 μg/ml) to raise plasma corticosterone to minimal resting levels. We use cortisol, rather than corticosterone, because it is more potent in most rat assays (thereby avoiding higher concentrations and precipitate formation in water), and since cortisol substantially cross-reacts with brain corticosterone receptors (*5*).
15. Our long-term colony was maintained behind a high-efficiency particulate air filter barrier to prevent airborne infections. No respiratory illness was found in any animal. All injections were given by an investigator with clean laboratory coat, surgical mask and gloves, and sterile needle. Animals were killed if major overt pathology appeared. The 50 percent survival age among our aged controls was comparable to that in the parent colony, despite added behavioral stress and handling. A veterinary pathologist assessed cause of death in most cases.
16. Six plasma hormones were analyzed by radioimmunoassay for each animal and a comprehensive blood analysis was also performed. Drug treatments had no effect on these values, but in adrenalectomized animals, ACTH was greatly elevated (> 500 percent), insulin and cholesterol were increased, and thyroxine and glucose were decreased. Only minimal levels of corticosterone were found. A complete report of these data is being prepared.
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