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Elevated dynorphin in the hippocampal formation of aged rats: Relation to cognitive impairment on a spatial learning task

(prodynorphin mRNA/frontal cortex/[Met^5]enkephalin)

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ABSTRACT **Radioimmunoassay revealed increased dynorphin A(1–8)-like immunoreactivity [dynA(1–8)LI] in the aged rat brain. Among a number of brain regions examined, an age-related dynA(1–8)LI elevation was found only in the hippocampal formation and frontal cortex. Moreover, the increase in dynA(1–8)LI in the aged hippocampus was associated with a decline in spatial learning ability: dynA(1–8)LI distinguished aged rats that were behaviorally impaired from aged cohorts that learned the spatial task as rapidly as younger animals. Northern blot hybridization using a ^{32}P-labeled complementary RNA probe encoding rat prodynorphin indicated that the abundance of prodynorphin mRNA was also significantly increased in the hippocampal formation of aged rats with identified spatial learning impairments.**

The neurobiological status of the aging hippocampal formation includes a loss of afferent input (1, 2), a modest decline in neuron number (3–5), a diminished complement of synaptic connections (6, 7), and decreases in some measures that reflect neurochemical function (8, 9). A more severe deterioration in this system is evident in the brains of patients with Alzheimer disease (10, 11). The effects of aging and age-related pathology on the hippocampal formation are likely to contribute to the emergence of mild cognitive deficits in normal aging and the progressive cognitive decline associated with Alzheimer dementia.

In contrast to the attrition reported for many parameters in the aged hippocampus, this study describes a significant elevation of a neuropeptide system that, within the hippocampal formation of young adult rats, is largely restricted to the dentate granule cells and their mossy fiber projections to CA3 (12). We report that dynorphin A(1–8)-like immunoreactivity [dynA(1–8)LI] and prodynorphin mRNA are increased in the aged hippocampal formation relative to young adults. This neurobiological change was also examined in relation to the performance of aged rats on a spatial learning task that is sensitive to hippocampal dysfunction (13).

METHOD

Subjects. Male Long–Evans rats 4–5, 14–16, and 25–27 mo old were used as subjects. All animals were obtained as pathogen-free rats from Charles River Breeding Laboratories and were resident in the Psychology Department vivarium for at least 1 mo prior to the experiments. Middle-aged and aged rats were obtained as retired breeders at 8–9 mo of age. The animals were housed in a pathogen-free colony in single cages with food and water provided ad libitum. The vivarium is climate controlled (25°C) and maintained on a 12-hr light/12-hr dark cycle (lights on at 7 a.m.). Blood samples taken from ≈30% of the aged animals at the time of sacrifice were negative for a panel of murine viral antibodies, confirming the pathogen-free status of this colony as indicated by routine screening. Necropsies were performed at the time of sacrifice to eliminate from data analysis any aged rat with evidence of pituitary tumor. Two aged animals were excluded on this basis.

Behavioral Testing. With the exception of animals that were experimentally naive at the time of sacrifice, rats were tested for spatial learning ability in a Morris water maze. The water maze consists of a circular tank (diameter, 1.83 m; height, 0.58 m). It was filled with tepid (27°C) water opacified by the addition of powdered milk (0.9 kg). A white escape platform (height, 34.5 cm) was located 1 cm below the water surface near the center of one of the four quadrants in the maze. The maze was surrounded by white curtains with patterns affixed to provide a configuration of spatial cues. Data were analyzed by a video tracking system (HVS Image Analyzing VP-112) and IBM PC computer with software developed for the water maze by San Diego Instruments.

Rats were trained to locate the camouflaged escape platform, which remained in the same position in the maze throughout training. Animals received three trials per day for 12 consecutive days, using a 60-sec intertrial interval. On each training trial, an animal was released in the maze from one of four equally spaced starting positions around the perimeter of the tank. The starting position varied from trial to trial, thus precluding the effective use of a response strategy (e.g., always turning left from the starting location to locate the escape platform). If an animal did not locate the escape platform within 120 sec on any trial, the experimenter placed the animal on the platform, where it remained for 30 sec. Every sixth trial consisted of a 30-sec probe trial to assess the development of a spatial bias in the maze. During these trials, animals swam with the platform removed from the pool. A criterion performance was achieved on a probe trial when an animal crossed the precise position of the escape platform's location at least twice and traveled at least a third of its total search within the maze quadrant that contained the escape platform during training (Fig. 1).

Radioimmunoassay. Animals were sacrificed by decapitation and brains were rapidly dissected over ice. Brain samples were frozen at −70°C until assay. Radioimmunoassays of dynA(1–8)LI and [Met]enkephalin-like immunoreactivity were performed following published procedures (14). Briefly, tissues were homogenized in 2 M acetic acid and then heated in a boiling water bath for 5 min. After centrifugation, the supernatant was lyophilized. Aliquots of the reconstituted samples were used for radioimmunoassay. The specificity of the antiserum against [Met]enkephalin has been

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Abbreviations: dynA(1–8)LI, dynorphin A(1–8)-like immunoreactivity; cRNA, complementary RNA.

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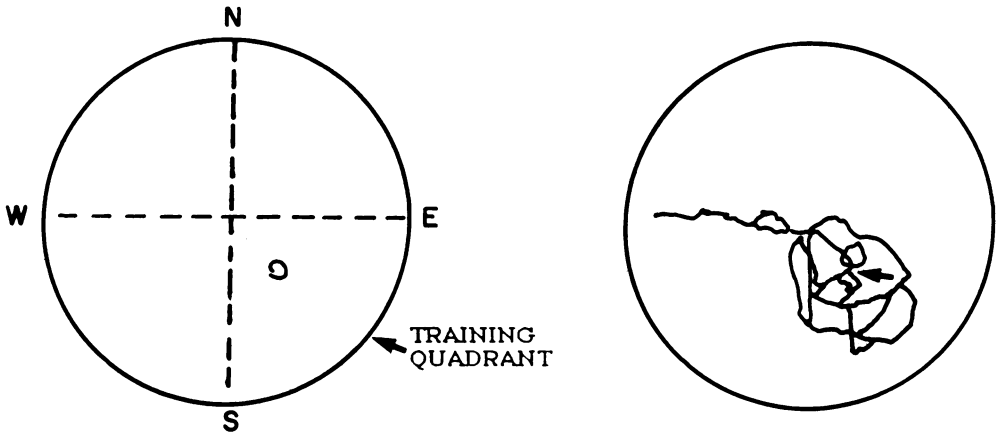

FIG. 1. (*Left*) Schematic illustration of the water maze (aerial view). North (N), south (S), east (E), and west (W) are designated starting locations for the training trials. (*Right*) Videotracking record of the path taken by a young animal on a probe trial (escape platform removed). This search pattern indicates that the animal had learned the location of the escape platform: there is a strong spatial bias for the appropriate vicinity of the maze and multiple crossings at the correct position for the platform.

described (15). The antiserum against dynA(1–8) does not cross-react with [Met]enkephalin but it does cross-react to a small degree with dynA(1–13) (0.02%) and dynA(1–17) (0.01%).

Northern Blot Hybridization. The abundance of mRNA encoding prodynorphin was determined by a Northern blot hybridization method following described procedures (16). Total RNA was extracted from the hippocampal formation. A ^{32}P-labeled complementary RNA (cRNA) probe was prepared from the plasmid p64D 1.7, which contains a 1.7-kilobase *Bgl* II/*Bam*HI fragment of a rat prodynorphin genomic clone. This fragment, encoding the entire 3′ main exon of the rat prodynorphin gene, encodes the majority of the translated region and the entire 3′ untranslated region of the rat prodynorphin mRNA. The plasmid p64D was linearized with *Eco*RI and transcribed in the presence of SP6 polymerase and [^{32}P]UTP. The resulting [^{32}P]cRNA was used for Northern blot hybridization. Quantification was by densitometry, as described (14).

RESULTS

Groups of young (n = 18), middle-aged (n = 8), and aged rats (n = 22) were trained on the spatial learning task. The results in Fig. 2 depict the rate of spatial learning for animals in each of the age groups. Analysis of variance conducted on these data (groups × blocks of trials to reach criterion) indicated an overall difference among the groups: $F(2,45) = 4.56$; $P < 0.02$. Post hoc comparisons (Newman–Keuls test) between groups revealed that the aged group differed significantly from the young group ($P < 0.02$). The young and middle-aged groups did not differ significantly from one another. Comparable results were obtained in an analysis of the latencies to locate the platform during the training trials. Analysis of variance revealed a significant group difference: $F(2,45) = 5.52$; $P < 0.005$. The aged rats were slower to locate the escape platform on all but the first block of five training trials ($P < 0.01$). The escape latencies of the middle-aged group, however, did not differ significantly from the young group (data not shown). These results are in agreement with other studies reporting age-related learning impairments in this task (17–19).

Hippocampal opioid peptides, dynA(1–8)LI and [Met]enkephalin-like immunoreactivity, were determined for all animals in the behavioral study. For a subset of these animals (n = 8–10 for each age group), both opioid peptides were also assessed in a number of other neuroanatomical regions. The results for dynA(1–8)LI are shown in Table 1. There was no significant age-related difference in dynA(1–8)LI in most brain areas (hypothalamus, striatum, basal forebrain, parietal cortex). However, a significant age-related increase was found in hippocampus and frontal cortex: $F(2,45) = 4.80$, $P < 0.01$; $F(2,25) = 5.52$, $P < 0.001$, respectively. In contrast, there was no significant age-related difference in [Met]enkephalin-like immunoreactivity in all the brain areas analyzed (data not shown).

To determine the relationship, if any, between the age-related increase in hippocampal dynA(1–8)LI and the spatial learning capacity of these same animals, the subjects were subdivided into four groups based on their behavioral performance. These consisted of a young, a middle-aged, and an aged group of animals that reached criterion within 15 training trials—i.e., by the third probe trial. A fourth group consisted of the animals that required in excess of 15 training trials to reach criterion: these animals fell outside the entire range of performance of the young group. This impaired group included 11 of the aged rats and 2 middle-aged rats. Fig. 3 represents the dynA(1–8)LI in hippocampus for these groups. Analysis of variance revealed a significant difference among the groups: $F(3,44) = 7.75$; $P < 0.0005$. Comparisons

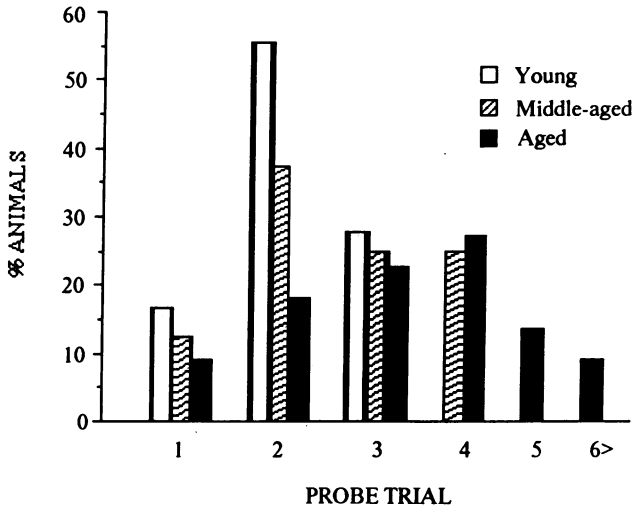

FIG. 2. Acquisition of criterion performance on the spatial learning task. Bars represent the percentage of animals in each age group that initially reached criterion at each probe trial. Comparisons between groups showed that the aged group differed significantly from the young group ($P < 0.02$) (Newman–Keuls test). There was no significant difference between the middle-aged and young groups.

Table 1. Age-related differences in dynA(1–8)LI

	DynA(1–8)LI, pmol per g of tissue		
	Young	Middle-aged	Aged
Hippocampal formation	11.14 ± 0.5	13.82 ± 1.59*	15.01 ± 0.75†
Frontal cortex	1.05 ± 0.10	1.10 ± 0.12	1.48 ± 0.12*
Parietal cortex	5.08 ± 0.88	5.41 ± 0.77	4.49 ± 0.62
Basal forebrain	32.33 ± 2.17	32.34 ± 1.86	33.76 ± 1.86
Striatum	18.48 ± 1.54	16.78 ± 2.07	20.99 ± 1.32
Hypothalamus	36.14 ± 3.49	31.37 ± 2.31	33.67 ± 3.84

*$P < 0.05$ compared to young group.
†$P < 0.01$ compared to young group.

between groups (Newman–Keuls test) indicated that the unimpaired groups (young, middle-aged, and aged) did not differ from one another. The impaired group differed significantly from the young, middle-aged ($P < 0.01$), and aged ($P < 0.05$) unimpaired groups. Thus, these data indicate that the groups of animals that readily learned the task had relatively comparable hippocampal dynA(1–8)LI, irrespective of age. DynA(1–8)LI was significantly elevated only in the behaviorally impaired animals. In contrast to these results for the hippocampal formation, dynorphin content in frontal cortex did not distinguish between aged animals that were unimpaired and those that performed outside the range of the young group [mean ± SEM = 1.47 ± 0.16 and 1.44 ± 0.10 pmol per g of tissue (wet wt), respectively].

Another series of brains was analyzed to assess whether experimentally naive young ($n = 8$; 6 mo old) and aged ($n = 9$; 28 mo old) rats have a similar pattern of dynA(1–8)LI. In these brains, dynA(1–8)LI content in the aged rats was elevated ≈43% and ≈54% in the hippocampal formation and frontal cortex, respectively, as compared with the young rats: these differences were again statistically significant ($P < 0.01$). No age differences in dynA(1–8)LI, however, were observed in the other regions that were analyzed in these brains—i.e., basal forebrain, striatum. Thus, a highly comparable age-related change in dynA(1–8)LI was found in experimentally naive and behaviorally tested rats.

To characterize further the status of dynorphin in the hippocampal formation of aged rats, prodynorphin mRNA was assessed by a Northern blot hybridization method using a [^{32}P]cRNA probe encoding rat prodynorphin. Each sample analyzed for mRNA consisted of three pooled hippocampi obtained from three animals, either young or aged. Four samples from young animals and three from aged animals were included in the experiment. Prior to sacrifice, these animals were trained on the identical spatial learning task described above. Aged hippocampi were obtained from the subpopulation of aged rats that fell outside the entire range of behavioral performance exhibited by the young rats in this experiment—i.e., in excess of 15 training trials to reach criterion. A significant elevation in prodynorphin mRNA was observed in the aged hippocampus when compared to the samples taken from young rats ($P < 0.05$; Fig. 4).

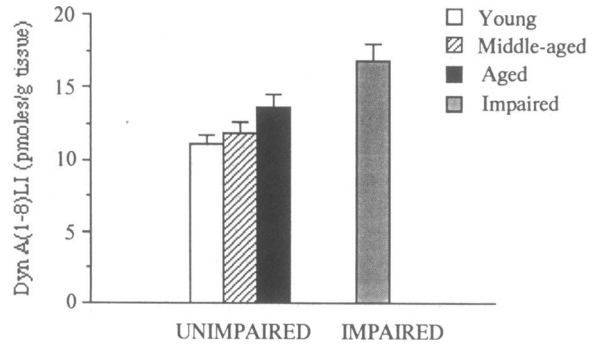

FIG. 3. Hippocampal dynA(1–8)LI according to spatial learning ability. The unimpaired groups at each age consisted of animals that reached criterion within 15 training trials. The impaired group included all animals (2 middle-aged and 11 aged rats) that required in excess of 15 trials to reach criterion. The impaired group differed significantly from the young, middle-aged ($P < 0.01$), and aged ($P < 0.05$) unimpaired groups. The unimpaired groups (young, middle-aged, and aged) did not differ from one another.

DISCUSSION

This report of an age-related elevation in a neuropeptide system is relatively unique. With few exceptions (20, 21), prior studies examining peptide content in the aged brain describe either no change or decreases (22–27). Elevations in peptide content and/or peptide gene expression can be induced experimentally by manipulations that alter neural activity. Hippocampal opioid peptides, in particular, are sensitive to a variety of treatments that induce limbic seizures. For example, repeated electroconvulsive shocks or electrically kindled convulsions cause a decrease in the levels of dynA(1–8)LI and prodynorphin mRNA (14, 28, 29). More physiological stimulation of the dentate gyrus can also regulate hippocampal dynorphin activity: brief high frequency stimulation produced a prolonged decrease in prodynorphin mRNA within the dentate granule cell/mossy fiber pathway (30). Moreover, transsynaptic regulation of granule cell dynorphin was recently demonstrated by stimulation of the perforant pathway, which produced a robust decrease in dynA(1–8)LI and prodynorphin mRNA (T. Xie, C. Mitchell, J. McGinty, and J.-S.H., unpublished observations).

The evidence that expression of the prodynorphin gene is negatively regulated by neuronal activity may have implications for the results of the present study. A loss of perforant path afferents from entorhinal cortex to the granule cells of the dentate gyrus is evident in both normal aging and in Alzheimer disease. This pathway carries major cortical inputs to the hippocampal formation. A decrease in synaptic profiles within the zone of perforant path termination in the dentate gyrus of aged rats has been reported (6, 7). Electrophysiological evidence also indicates that the dentate granule cells receive a sparser perforant innervation in the aged animal (1, 2). In Alzheimer disease, the neurons that give rise to the perforant path are laden with neurofibrillary tangles, and a marked loss of these neurons in layer II of the entorhinal cortex is found in the brains of patients with this disease as compared to age-matched controls (31). In light of this age-related loss of afferents to the dynorphin-containing dentate granule cells, it is possible that the elevated dynorphin observed in the present experiments is a consequence of diminished perforant innervation/activity.

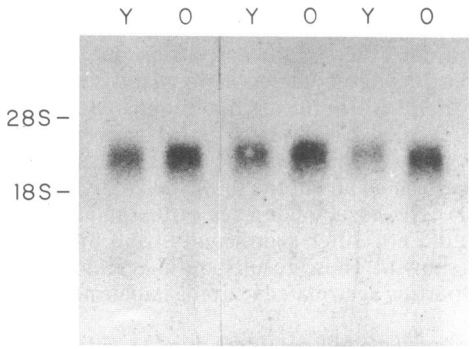

FIG. 4. Autoradiogram generated from Northern blot hybridization for prodynorphin mRNA in the hippocampal formation. Three pairs of samples from young and aged brains are shown. Each sample contained hippocampi from three young (lanes Y) or three old (lanes O) brains.

Given the evidence reported here that the capacity to learn a spatial task was related to hippocampal dynA(1–8)LI content in aged rats, is it possible that elevated dynorphin function is the cause of this behavioral impairment? Whether elevated dynA(1–8)LI translates into impaired neuronal processing that normally underlies learning in this task was not addressed by this research. In this context, however, it is notable that opiate antagonist administration has been found to improve the performance of both young and aged rats on spatial learning tasks (32–34).

Our findings are in accord with the general view that the effects of brain aging on the hippocampal formation are related to behavioral impairments in "memory-deficient" aged rats (35). More specifically, our results add to a number of other observations demonstrating a relationship between neurobiological parameters in the hippocampal formation of aged rats and age-related impairments on spatial learning tasks. In an analysis of 2-deoxyglucose uptake in 45 regions of aged rat brains, a significant correlation between the degree of spatial learning impairment and a decrease in 2-deoxyglucose uptake was limited to hippocampal/septal regions and prefrontal cortex (18). In other work, the severity of a learning deficit among aged rats on a spatial task was significantly correlated with a more rapid rate of decay for long-term potentiation at perforant/dentate synapses (1). Geinisman and colleagues (36) have reported that aged rats with a spatial learning deficit differ from aged cohorts that are relatively unimpaired in having a smaller complement of perforated synapses in the dentate gyrus. The status of glucocorticoid receptors in the hippocampus may also distinguish aged rats that are impaired on spatial tasks (37). The functional interrelationship among various hippocampal parameters that distinguish the learning ability of aged animals is poorly understood. Such an understanding is, nonetheless, important for developing either therapeutic strategies to treat age-related cognitive impairments or interventions aimed at decelerating the aging process itself.

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1. Barnes, C. A. (1979) *J. Comp. Physiol. Psychol.* **93,** 74–104.
2. Barnes, C. A. & McNaughton, B. L. (1980) *J. Physiol. (London)* **309,** 473–485.
3. Brizzee, K. R. & Ordy, J. M. (1979) *Mech. Ageing Dev.* **9,** 143–162.
4. Landfield, P. W., Baskin, R. K. & Pitler, T. A. (1981) *Science* **214,** 581–584.
5. Mani, R. B., Lohr, J. B. & Jeste, D. V. (1986) *Exp. Neurol.* **94,** 29–40.
6. Geinisman, Y. & Bondareff, W. (1976) *Mech. Ageing Dev.* **5,** 11–23.
7. Geinisman, Y., Bondareff, W. & Dodge, J. T. (1977) *Brain Res.* **134,** 541–545.
8. McGeer, E. & McGeer, P. L. (1976) in *Neurobiology of Aging*, eds. Terry, R. D. & Gershon, S. (Raven, New York), pp. 389–403.
9. Decker, M. W. (1987) *Brain Res. Rev.* **12,** 423–438.
10. Ball, M. J. (1977) *Acta Neuropathol.* **37,** 111–118.
11. Hardy, J., Adolfsson, R., Alafuzoff, I., Bucht, G., Marcusson, J., Nyberg, P., Perdahl, E., Wester, P. & Winblad, B. (1985) *Neurochem. Int.* **7,** 545–563.
12. McGinty, J. F., Henriksen, S. J., Goldstein, A., Terenius, L. & Bloom, F. E. (1982) *Life Sci.* **31,** 1797–1800.
13. Morris, R. G. M., Garrud, P., Rawlins, J. N. P. & O'Keefe, J. (1982) *Nature (London)* **297,** 681–683.
14. Kanamatsu, T., McGinty, J., Mitchell, C. & Hong, J.-S. (1986) *J. Neurosci.* **6,** 644–649.
15. Hong, J.-S., Yang, H.-Y. T., Fratta, W. & Costa, E. (1978) *J. Pharmacol. Exp. Ther.* **205,** 141–145.
16. Civelli, O., Douglas, J., Goldstein, A. & Herbert, E. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 4291–4295.
17. Gage, F. H., Dunnett, S. B. & Bjorklund, A. (1985) *Neurobiol. Aging* **5,** 43–48.
18. Gage, F. H., Kelly, P. A. T. & Bjorklund, A. (1984) *J. Neurosci.* **4,** 2837–2847.
19. Rapp, P. R., Rosenberg, R. A. & Gallagher, M. (1987) *Behav. Neurosci.* **101,** 3–12.
20. Rogers, J., Shoemaker, W. J., Morgan, D. G. & Finch, C. E. (1985) *Neurobiol. Aging* **6,** 1–9.
21. Steger, R. W., Sonntag, W. E., Van Vugt, D. A., Forman, L. J. & Meites, J. (1980) *Life Sci.* **27,** 747–753.
22. Barden, N., Dupont, A., Labrie, F., Merand, Y., Rouleau, D., Vaudry, H. & Boissier, J. R. (1981) *Brain Res.* **208,** 209–212.
23. Buck, S. H., Deshmukh, P. P., Burks, T. F. & Yamamura, H. I. (1981) *Neurobiol. Aging* **2,** 257–264.
24. De Ceballos, M. L., Boyce, S., Taylor, M., Jenner, P. & Marsden, C. D. (1987) *Neurosci. Lett.* **75,** 113–117.
25. Dupont, A., Savard, P., Merand, Y., Labrie, F. & Boissier, J. R. (1981) *Life Sci.* **29,** 2317–2322.
26. Gambert, S. R., Garthwaite, T. L., Pontzer, C. H. & Hagen, T. C. (1980) *Neuroendocrinology* **31,** 252–255.
27. Landfield, P. W., Sundberg, D. K., Smith, M. S., Eldridge, J. C. & Morris, M. (1980) *Peptides* **1,** 185–196.
28. McGinty, J. F., Kanamatsu, T., Obie, J., Dyer, R. S., Mitchell, C. L. & Hong, J.-S. (1986) *Neurosci. Lett.* **71,** 31–36.
29. Mitchell, C. L., Grimes, L., Hudson, P. M. & Hong, J.-S. (1987) *Brain Res.* **435,** 343–347.
30. Morris, B. J., Feasey, K. J., Bruggencate, G. T., Herz, A. & Höllt, V. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 3226–3230.
31. Hyman, B. T., Van Hoesen, G. W., Damasio, A. R. & Barnes, C. L. (1984) *Science* **225,** 1168–1170.
32. Collier, T. J. & Routtenberg, A. (1984) *Brain Res.* **310,** 384–387.
33. Gallagher, M., King, R. A. & Young, N. B. (1983) *Science* **221,** 975–976.
34. Gallagher, M., Bostock, E. & King, R. A. (1985) *Behav. Neural Biol.* **44,** 374–385.
35. Landfield, P. W. (1980) in *The Psychobiology of Aging: Problems and Perspectives*, ed. Stein, D. G. (Elsevier/North Holland, Amsterdam), pp. 227–252.
36. Geinisman, Y., de Toledo-Morrell, L. & Morrell, F. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 3027–3031.
37. Meany, M. J., Aitken, D. H., Berkel, C. V., Bhatnagar, S. & Sapolsky, R. M. (1988) *Science* **239,** 766–768.