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Loss of perforated synapses in the dentate gyrus: Morphological substrate of memory deficit in aged rats

(aging/spatial memory/synapse quantitation)

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ABSTRACT **Most, but not all, aged rats exhibit a profound deficit in spatial memory when tested in a radial maze—a task known to depend on the integrity of the hippocampal formation. In this study, animals were divided into three groups based on their spatial memory capacity: young adult rats with good memory, aged rats with impaired memory, and aged rats with good memory. Memory-impaired aged animals showed a loss of perforated axospinous synapses in the dentate gyrus of the hippocampal formation in comparison with either young adults or aged rats with good memory. This finding suggests that the loss of perforated axospinous synapses in the hippocampal formation underlies the age-related deficit in spatial memory.**

One of the cardinal signs of aging in man is an impairment of memory for recent events. This selective failure to retain new information in the face of preserved retrieval for older experiences renders the age-associated memory loss qualitatively similar to the impairments seen following bilateral temporal lobe damage involving the hippocampal formation in humans (1–3). Memory deficits similar in nature to the kinds of amnestic syndromes outlined above have also been demonstrated in rats following bilateral damage to the hippocampal system (4). A task often used to assess mnemonic function in rats is the eight-arm spatial maze (5–7). In one version of this task, every arm of the maze is baited with food. Since once consumed food is not replaced, the optimal strategy for a hungry animal is to remember where he has been and not return to an arm that has already been visited. Accurate performance in this situation, which may be viewed as equivalent to remembering an eight-item list in humans, depends crucially on the integrity of the hippocampal formation and its afferent connections and is relatively unaffected by neocortical or amygdala lesions (4, 6, 8).

Numerous experiments have shown the hippocampal formation to be especially vulnerable to the aging process (9–17). The fact that behavior in the spatial maze is critically dependent on hippocampal integrity makes the task especially advantageous for examining the relationship between age-dependent deficits in memory and changes in the morphology or electrophysiology of the anatomical system involved. In fact, results from our laboratory and those of others have shown such age-associated deficits in spatial memory to be tightly related to impaired synaptic efficacy in the entorhino–dentate pathway, suggesting that the memory deficit may depend on altered synaptic transmission in the hippocampal formation (18–20). Interestingly, however, most, but not all, aged rats exhibit the spatial memory deficit and the accompanying electrophysiological change in hippocampal synaptic plasticity.

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In the present experiment, we took advantage of such individual differences in spatial memory among aged rats in order to assess a possible morphological substrate of the age-related spatial memory deficit. Previous morphological studies concerned with the effects of aging on the hippocampal formation did not relate anatomical findings to memory impairments (10–12, 21–23). Chronological age *per se* may not be adequate to separate functionally different groups. To deal with the intrinsic variability in memory during aging, all animals were examined for memory function before morphometric analyses of synapses were carried out. We report here that aged rats with impaired spatial memory exhibit a loss of perforated axospinous synapses in the dentate gyrus in comparison with young adults or with animals of the same age with intact memory.

MATERIALS AND METHODS

The apparatus used to test spatial memory was an eight-arm radial maze similar to that described by Olton and Samuelson (5). Four-month-old (n = 6) and 26-month-old (n = 12) F344 strain male rats were first deprived of food to 80% of their body weight. After adaptation to the apparatus, they were given one trial a day in the maze and trained to a stringent criterion of three consecutive trials with no errors (i.e., no repeated entries into a given alley) or to a maximum of 30 trials. A trial consisted of placing the animal in the center of the maze and allowing 10 choices (8 if all were correct) or a maximum of 15 min, whichever came first.

For electron microscopy, animals were coded so that their age and behavioral history remained unknown during morphological analyses. The day after the last trial in the maze, rats were perfused intracardially, first with a dilute fixative (1% paraformaldehyde/1.25% glutaraldehyde/0.002% $CaCl_2$ in 0.12 M phosphate buffer, pH 7.3) and then with the same fixative at twice the aldehyde concentration. The brain was removed 4 hr later and postfixed overnight in the concentrated fixative. The right hippocampal formation was then dissected free and cut perpendicular to its long axis into blocks ≈1 mm thick. Two blocks with rostral faces 1.5 or 3.5 mm caudal to the septal pole of the hippocampal formation were osmicated, dehydrated, and embedded in Araldite using standard procedures. All blocks were randomly assigned code numbers to be decoded after completion of the morphological work. From the rostral face of each block, complete series of 25–42 ultrathin sections were prepared. The sections were obtained from the central (in the mediolateral direction) segment of the hidden blade of the dentate gyrus and included the total width of the molecular layer, from the hippocampal fissure to the granule cell layer. After staining with uranyl acetate and lead citrate, a section series was mounted on a slot grid coated with collodion-carbon and examined in a JEOL-100CX electron microscope.

Abbreviation: PSD, postsynaptic density.

Synapses were studied in the middle of the molecular layer (in relation to the hippocampal fissure and granule cell layer). The same neuropil area was photographed in consecutive serial sections. The final magnification of electron micrographs was ×20,000. At this magnification, all axodendritic synapses could be reliably identified (as compared to the same neuropil area examined at a final magnification of ×35,000) and counted in a relatively large test area (100 μm^2). Axodendritic synapses were identified in micrographs of serial sections by the presence of a postsynaptic density (PSD) and synaptic vesicles. A synaptic contact was classified as an axodendritic synapse involving a dendritic shaft if its postsynaptic element contained mitochondria and arrays of parallel microtubules; or as an axospinous synapse if its postsynaptic element was attached to a parent dendrite, contained a spine apparatus, or was lacking mitochondria and microtubules.

Synaptic counts were performed in a test area of a central serial section with the aid of the unbiased test frame of Gundersen (24), PSD being used as a counting unit. Synapses were counted if their PSD was completely within the test frame or was intersected by inclusion edges of the frame, but not by extended exclusion edges. The numerical density of synapses per unit volume of neuropil (N_V) was estimated by the method of Cruz-Orive (25), which does not depend on assumptions concerning the synaptic shape. Each synapse counted in a micrograph of a central serial section was subsequently traced in micrographs of adjacent serial sections to establish the number of sections in which its PSD could be observed. The sum total of the thickness of these sections gave a value for the projected height of a synapse. Synaptic N_V was calculated by the formula (26)

$$N_V = N_A/E(H + T),$$

where N_A is the number of synapses per unit test area, E designates the harmonic mean (i.e., the mean of reciprocal values), H is the synaptic projected height, and T is the section thickness.

The thickness of ultrathin sections was estimated by means of the "small-fold" technique (27). Minimal folds, which are perpendicular to the section plane and have a width that equals twice the section thickness, were identified and photographed at a magnification of ×100,000, together with a magnification standard. The width of minimal folds was measured on negatives. It was not possible to estimate the thickness of all serial sections studied, since minimal folds were typically found only in a third or fewer sections in each series. Because tissue blocks obtained from all animals studied were sectioned in random order, the variation in section thickness is most likely to be randomly distributed among the groups of rats under comparison. Therefore, the average thickness of sections containing minimal folds (n = 417) was assessed, and all the sections studied were assigned the mean thickness (0.082 μm), assuming that no systematic bias was introduced in this way.

All group comparisons reported with respect to the morphological or behavioral data are based on two-tailed Mann–Whitney U tests.

RESULTS

When tested in the spatial maze, all young adult rats reached the behavioral criterion. On the other hand, older animals could be divided into two groups depending on their performance in the maze—those that reached the criterion and those that did not. Although young rats reached criterion slightly faster than older rats with good memory (mean number of trials to criterion, 12.2 for the young and 18.7 for the aged), this difference was not statistically significant. Thus, the morphometric analyses were based on examination of three groups of rats: six young adult animals (5 months old at this time) that reached criterion; six aged animals (27 months old) that reached criterion (i.e., aged rats with "good" or intact spatial memory); and six aged animals (27 months old) that failed criterion (i.e., aged rats with "poor" or impaired spatial memory).

In a preliminary morphological study, the minimal sample size required for obtaining reproducible values of synaptic N_V was assessed by the technique of progressive means (28). A tissue block taken from the dentate gyrus of a 27-month-old rat was used to obtain 15 sets of electron micrographs of serial sections. N_V was estimated for all axodendritic synapses in each micrograph set separately. These sets were randomly assigned numbers from 1 to 15, and synaptic N_V estimates were progressively averaged: first for sets 1 and 2, then for sets 1, 2, and 3, etc. It was found that the number of micrograph sets necessary to bring the progressive means permanently within ± 5% confidence limits of the final value for all sets was six. Therefore, six micrograph sets of serial sections obtained from each tissue block were examined to derive synaptic N_V, and the mean of these values gave an estimate per tissue block. The final synaptic N_V estimate per animal was assessed by averaging the synaptic N_V of two blocks.

Examination of all axodendritic synapses (involving both dendritic shafts and dendritic spines) and axospinous synapses separately gave essentially the same results (Table 1). No statistically significant differences were noted among the three groups of animals, although memory-impaired aged rats showed an 11% decrease in synaptic N_V relative to young adult rats.

To answer the question of whether N_V of certain synaptic types would be differentially altered as a function of aging and memory loss, axospinous synapses were divided into nonperforated and perforated ones (29, 30). The first type included synapses in which PSD did not exhibit a discontinuity in any consecutive serial section (Fig. 1, closed arrows). The second type was identified by the presence of perforated or discontinuous PSD in at least one serial section (open arrows). An unequivocal identification of these synaptic types was only possible in serial sections. Nonperforated synapses were diffi-

Table 1. Numerical density of axodendritic and axospinous synapses per unit volume of neuropil in the molecular layer of the rat dentate gyrus

	1. Young adults		2. Aged rats, memory intact			3. Aged rats, memory impaired			
Rat	n*	N_V†	n*	N_V†	1–2‡	n*	N_V†	1–3‡	2–3‡
				Axodendritic synapses					
1	463	1.284	510	1.322		493	1.326		
2	500	1.264	422	1.171		372	1.011		
3	434	1.217	448	1.148		384	1.011		
4	449	1.164	449	1.140		376	0.994		
5	417	1.063	394	1.008		391	0.963		
6	385	1.059	369	0.911		365	0.955		
Group mean		1.175		1.117	−4.9%		1.043	−11.2%	−6.6%
				Axospinous synapses					
1	437	1.220	457	1.200		465	1.258		
2	478	1.204	404	1.121		357	0.976		
3	410	1.153	428	1.099		363	0.954		
4	418	1.088	438	1.113		352	0.935		
5	399	1.017	370	0.948		362	0.902		
6	355	0.986	344	0.852		332	0.880		
Group mean		1.111		1.056	−5.0%		0.984	−11.4%	−6.8%

*Number of synapses counted in a total test area of 1200 μm^2.
†Synaptic numerical density in μm^{-3}.
‡Difference between group N_V means.

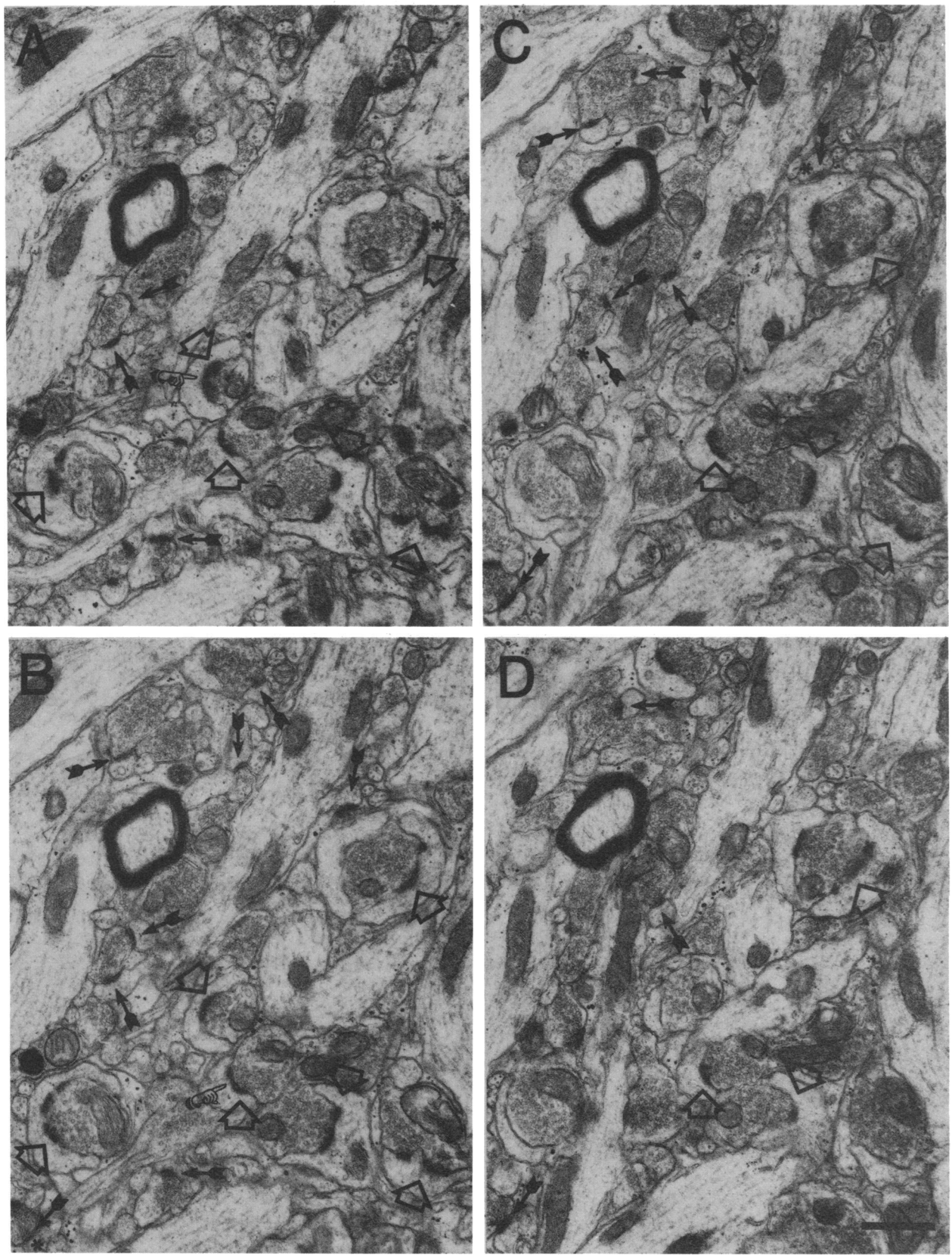

FIG. 1. Electron micrographs of consecutive serial sections demonstrating the neuropil in the dentate gyrus molecular layer of a 5-month-old rat. Perforated (open arrows) and nonperforated (closed arrows) axospinous synapses can be observed. (Bar = 0.8 μm.)

cult to recognize in a random section when they were tangentially cut (Fig. 1 *B* and *C*, asterisks). Perforated synapses could be classified mistakenly as nonperforated ones if studied in random sections (Fig. 1*A*, asterisk). Because the size of PSD perforations appeared to be relatively small in some synapses (Fig. 1 *A* and *B*, pointing fingers), the presence of PSD perforations was verified by examining micrographs of axospinous synapses with a ×10 magnifier.

Analysis of nonperforated axospinous synapses did not reveal statistically significant differences in N_V estimates among the three groups of rats under comparison, although a trend toward a decrease (10.4%) was observed in memory-impaired aged animals relative to young adults (Table 2). On the other hand, there was a substantial and highly significant decrease in N_V of perforated axospinous synapses for the group of memory-impaired aged rats as compared to either young adult rats or to aged rats without memory deficits (Table 2). The latter two groups did not differ from each other with respect to N_V of perforated synapses (Table 2).

Further examination of the behavioral data for all 18 animals in relation to the morphological results showed a significant correlation between the number of trials necessary to reach the behavioral criterion and N_V of perforated synapses (Pearson's $r = -0.725$, $P < 0.001$; for this correlation, old rats that failed the behavioral criterion were assigned a score of 33, since at least three more trials would have been required to reach criterion). Thus, whether young or old, rats exhibiting good spatial memory had a larger number of perforated axospinous synapses in a volume of the dentate gyrus. Aged rats were further rank ordered on the basis of number of trials to criterion, as well as a choice accuracy score (5) for the last three days of training. The choice accuracy score takes into account the number of errors made and where in the sequence of choices these errors occurred. A rank order correlation revealed a striking relationship between degree of memory loss and extent of decrease in N_V of perforated synapses (Spearman's $\rho = 0.937$, df = 11, $P < 0.001$). Pearson's and Spearman's correlations between the behavioral measures (as described above) and N_V of nonperforated synapses, however, did not show a significant relationship between the two ($r = -0.320$, $P > 0.05$; $\rho = 0.248$; $P > 0.05$).

DISCUSSION

The most important result of this study is that the deficit in spatial memory observed in the majority of aged rats appears to be critically dependent on a loss of perforated axospinous synapses in the hippocampal dentate gyrus. Chronological age alone is not a determinant of the morphological change observed. Only by separating old animals of the same chronological age according to their spatial memory capacity was it possible to demonstrate that the synaptic loss was related to an impairment of spatial memory.

Table 2. Numerical density of nonperforated and perforated axospinous synapses per unit volume of neuropil in the molecular layer of the rat dentate gyrus

	1. Young adults		2. Aged rats, memory intact			3. Aged rats, memory impaired			
Rat	*n*	N_V	*n*	N_V	1–2	*n*	N_V	1–3	2–3
Nonperforated axospinous synapses									
1	364	1.096	365	1.059		387	1.138		
2	395	1.079	326	0.995		290	0.866		
3	332	1.019	336	0.958		291	0.840		
4	327	0.940	346	0.977		291	0.845		
5	311	0.883	277	0.808		296	0.808		
6	279	0.853	251	0.716		257	0.762		
Group mean		0.978		0.919	−6.0%		0.876	−10.4%	−4.7%
Perforated axospinous synapses									
1	73	0.124	92	0.141		78	0.120		
2	83	0.125	78	0.126		67	0.110		
3	78	0.134	92	0.141		72	0.114		
4	91	0.148	92	0.136		61	0.090		
5	88	0.134	93	0.140		66	0.094		
6	76	0.133	93	0.136		75	0.118		
Group mean		0.133		0.137	+3.0%		0.108	−18.8%*	−21.2%*

Designations are the same as in Table 1.
*Statistically significant differences between groups of rats ($P = 0.002$; two-tailed Mann–Whitney U test).

The decrease in N_V of perforated synapses found in memory-impaired aged rats cannot be attributed to an increase in the neuropil volume. If this were the case, an equal decrease in N_V of different synaptic types would have occurred. However, the decrease in N_V of perforated axospinous synapses was much more pronounced than that of nonperforated ones (Table 2). This differential magnitude of changes in N_V of the two synaptic types is indicative of an absolute loss of perforated axospinous synapses in memory-impaired aged animals.

Earlier work had demonstrated that the spatial memory deficit seen in most aged rats was associated with impaired kindling via perforant path stimulation and with rapid decay of long-term potentiation at the terminal synaptic field of this pathway in the dentate gyrus (18–20). Both electrophysiological observations are indicative of reduced synaptic efficacy. The quantitative morphological findings described here suggest that the age-dependent decline in spatial memory and in the concomitant electrophysiological indicators of synaptic efficacy may be a direct consequence of the loss of perforated axospinous synapses in the hippocampal formation.

These findings complement the observations of Desmond and Levy (31), who examined the axodendritic synapses in the dentate gyrus of young rats after inducing long-term potentiation by high frequency electrical stimulation of the perforant path. By stimulating only one side, they were able to use a single animal as its own control and demonstrated a 32% increase in what they call concave-shaped synapses (many of which are perforated) in the potentiated region of the dentate gyrus molecular layer. Similarly, environmental stimulation as provided by rearing in a complex environment (32) or visual discriminative training (33) has also been shown to increase the proportion of perforated synapses in the occipital cortex of rats and rabbits, respectively. Thus, it appears that both electrically induced potentiation of a synaptic system and behavioral learning are associated with an increase, in a functionally relevant brain area, of just those synaptic elements that we find to be significantly diminished in the hippocampal formation of aged memory-impaired rats.

The functionally relevant brain area for the spatial memory task used in this work is the hippocampal formation. Furthermore, the input from entorhinal cortex to the hippocampal formation appears to be necessary for the registration of new experience (34, 35). For example, lesions of the entorhinal cortex produce memory loss by depriving the hippocampal formation of neocortical sensory input (4, 35). Discovery of a loss of perforated synapses in the hippocampal formation and the striking correlation between the extent of synaptic loss and the degree of age-related deficit in spatial memory suggests that this particular type of hippocampal synapse may be crucial for normal memory function.

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