No loss of synaptic proteins in the hippocampus of aged, behaviorally impaired rats

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Abstract

The levels of three different synaptic proteins in the hippocampus of young (6 months of age) and aged (26–27 months of age) Long Evans rats were examined using quantitative Western blotting. An important feature to this study is that prior to the neurobiological analysis, hippocampal function was determined by assessing spatial learning ability in the Morris water maze. A subset of the aged rats was impaired on the learning task while the remaining aged cohort performed within the range of young rats. The amount of immunoreactivity for synaptophysin, synaptotagmin, and synaptosomal associated protein-25 did not differ between the young and aged rats. In addition, the aged rats with severe cognitive impairment had levels of these synaptic proteins that were similar to those of the aged rats with preserved cognitive function. This finding of no change in the levels of synaptic proteins suggests that substantial synapse loss in the hippocampal formation does not underlie cognitive decline in normal aging. © 1999 Elsevier Science Inc. All rights reserved.

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1. Introduction

In normal aging, the structural integrity of the hippocampus may be preserved to a greater extent than was formerly appreciated. For example, recent anatomical studies using stereology have shown no loss of principle neurons in the aged hippocampus in rodent and human brains [17,19,28]. Against that background, the extent to which synaptic connections are altered in this system is of interest as a potential basis for age-related decline in cognitive functions that depend on the hippocampus. Connectional alterations, rather than frank neuron degeneration, may be related to the activation of certain markers in this structure during aging. For example, glial fibrillary acidic protein (GFAP) mRNA, a marker of glial reactivity, is elevated in the hippocampus during aging (e.g., [14,22]). This increase could indicate a glial response to age-associated structural alterations or synaptic reorganization within the hippocampus. Indeed, removing the synaptic innervation of the hippocampal dentate gyrus in young rats causes an increase in GFAP immunoreactivity and concomitant decreases in synaptic proteins, including synaptophysin and synaptosomal-associated protein 25 (SNAP-25), in the denervated area [5,7,26]. If synapse loss or synaptic reorganization contributes to cognitive decline in aging, parallel changes in levels of proteins known to be associated with synapses would be predicted. In the present study we quantified three proteins known to be localized to the synapse: synaptophysin, synaptotagmin, and SNAP-25.

Synaptophysin is the most abundant integral membrane protein associated with small neurotransmitter-containing synaptic vesicles [29]. It is localized to the principle neurons of the hippocampus and has been used as a marker of synaptic reorganization following lesions of afferent input [9,13]. Synaptotagmin I is an integral membrane protein of synaptic vesicles and it functions as a calcium sensor in the calcium-triggered release of neurotransmitter in the hippocampus [27]. Although there are several isoforms of synaptotagmin with varying functions, synaptotagmin I is the most abundant in the hippocampus [27]. SNAP-25 is a presynaptic plasma membrane protein [16]. It is a constit-
ulent of the soluble NSF attachment protein receptor (SNARE) complex, a combination of synaptic proteins that mediate fusion between the neurotransmitter vesicle and the plasma membrane [21]. Immunocytochemistry has localized this protein in the presynaptic terminals of the hippocampal granule cell mossy fibers and in the inner molecular layer of the dentate gyrus [16].

Prior to quantification of the proteins in hippocampus from young and aged rats, the subjects were evaluated for performance in a hippocampal-dependent task. The spatial learning assessment in the Morris water maze used in this study reveals an impairment in aged rats that is attributed to hippocampal dysfunction (e.g., [1]). We used this behavioral assessment in the current research to determine if age-related cognitive decline is associated with generalized synaptic loss. Our quantification of synaptophysin, synaptotagmin, and SNAP-25 immunoreactivity in the hippocampus with Western blotting revealed no alterations in the levels of these markers either as a function of age or the severity of age-related cognitive decline.

2. Methods

2.1. Subjects

Eight young (6 months of age) and sixteen old (26–27 months of age) male Long-Evans rats served as subjects. All rats were obtained pathogen-free from Charles River Laboratories (Raleigh, N.C.) and resided in the University of North Carolina Psychology Department vivarium for a minimum of one month prior to behavioral testing. Rats were singly housed in an environment that was climate controlled at 25°C and maintained on a 12 h light/dark cycle (lights on at 7 a.m.). Food and water were provided ad libitum. Routine exams throughout the experiment, as well as necropsies at the time of killing, were performed to assess the health of the subjects. Approximately one week after behavioral testing, subjects were sacrificed by decapitation and the hippocampus was dissected on ice then quickly frozen and stored at −80°C. Animal housing and experimental procedures followed the National Institute of Health guide for the care and use of laboratory animals.

2.2. Behavioral testing

The water maze is a circular tank (1.83 m diameter and 0.58 m height) with a removable escape platform centered in one of the four maze quadrants. During testing, the tank was filled to a depth of 35.5 cm with 27°C water clouded by the addition of non-toxic white tempura paint (150 mL). The top of the escape platform was submerged 2 cm below the water surface. The maze was surrounded by white muslin curtains affixed with black felt patterns for providing spatial cues. Sensory-motor ability was determined by cue training to a visible black platform extending 2 cm above the water surface.

Data were analyzed using a video tracking system (HVS Image Analyzing VP-112) and software developed for the water maze by Richard Baker, HVS Imaging (Hampton, U.K.). During an eight-day period, in sessions consisting of three trials a day, the rats were trained to locate the camouflaged platform that remained stationary throughout training. During a training trial, the animal was placed in the water at the perimeter of the pool and allowed 90 s to locate the escape platform. If at the end of this interval the rat had failed to escape, it was placed onto the platform and allowed to remain there for 30 s. The position of entry for the animal was varied at each trial. There was a 60 s intertrial interval. Every five training trials made up a trial block. Every sixth trial consisted of a free swim ("probe trial") which served to assess the development of a spatial bias in locating the escape platform. During such probe trials, the animal was allowed to swim a total of 30 s without the escape platform available for escape. A "learning index," which was generated from the proximity of the rat to the escape platform during probe trials (described in detail in [6]), was used in correlations with the neurobiological data.

Cue training was conducted on the last day of place training. Cue training consisted of one session of 6 trials. During these trials, a visible platform was moved to different locations in the pool between trials to test for sensorimotor and motivational factors that may influence spatial learning. Each rat was given 30 s to reach the platform and it remained on the platform briefly. Trials were separated by a 30 s intertrial interval.

2.3. Western blotting

Protein was extracted from previously frozen hippocampal tissue by homogenization in cold 10 mM Tris, pH 7.4, containing 1 mM ethylenediaminetetraacetic acid (EDTA) with the use of a Tekmar Tissuemizer (Cincinnati, OH). Homogenates were centrifuged at 37,000 × g for 10 min, and the pellets (membrane fraction) were resuspended in 0.7% sodium dodecyl sulfate (SDS) and boiled for 5 min. Aliquots of the SDS-solubilized proteins were frozen at −80°C until use.

One μg of protein extract from each subject was loaded into each well of a 10% Bis-Tris gel. For quantification of these proteins, each gel also contained a standard curve of 0.5, 1.0, and 2.0 μg hippocampal protein from a Long Evans rat. An additional lane of the lowest standard concentration was run on the opposite side of the gel to confirm uniformity in the protein transfer from the gel to the PVDF membrane. In repeated runs, the samples were counterbalanced for lane position, age, and learning index score, thereby eliminating any potential confound due to loading sequence or position on the gel. A minimum of 3 experiments was performed for each antibody.

Electrophoresis, using the NuPage system (Novex), was at 200 volts for 60 min in 3-(N-morpholino) propane sulfonic acid (MOPS)-0.5% SDS buffer. The protein was
transferred to PVDF membrane in NuPage transfer buffer plus 10% methanol for 2 h at 24 volts. For synaptotagmin and SNAP-25, membranes were cut horizontally at about 40 kDa, and the top was blotted for synaptotagmin and the bottom was blotted for SNAP-25. Synaptophysin was blotted independently. A general procedure was followed for immunoblotting: Membranes were blocked for one hour in 10 mM PBS, pH 7.4, containing 3% nonfat dry milk (Sigma). After a 3 × 5 minute wash in 10 mM PBS + 1% bovine serum albumin (BSA) (PBS + BSA), pH 7.4, the membranes were exposed to 1:1000 dilutions of antisynaptotagmin (Transduction Laboratories), anti-SNAP-25 (Chemicon), or antisynaptophysin (Chemicon) in PBS + BSA, pH 7.4, for one hour. After washing 3 × 5 minutes in PBS + BSA, blots were incubated for 30 min with HRP-conjugated antimouse IgG (Santa Cruz Biotechnology, Inc.) at a 1:5000 dilution (synaptophysin) or a 1:10,000 dilution (synaptotagmin and SNAP-25) in PBS + BSA plus 2% goat serum (Vector). Three 5 min washes with PBS + BSA were followed by two 5 min washes with ddH₂O. The signal was visualized with chemiluminescent substrate (Pierce Ultra SuperSignal) and captured on Amersham ECL film.

2.3.1. Quantification of Western blots

Autoradiograms of the chemiluminescent Western blots were quantified using NIH Image. The optical densities obtained from the subjects were in the linear range of the film and of the standard curve. The optical density of the subjects were converted to “hippocampal equivalents” based upon the standard curve. Data was from at least three experiments for SNAP-25 and synaptotagmin and two experiments for synaptophysin.

3. Results

The aged rats performed more poorly on the spatial learning task than the young rats based on training trial and probe trial data. As shown in Fig. 1a, aged rats were less proficient in locating the escape platform than the young rats. This was evident in a two-way repeated measure ANOVA (age × trial block) with a significant main effect of Age [F(1,22) = 19.20, p < 0.0002]. Fig. 1b shows that the aged rats were deficient in learning the task, evidenced by significantly greater learning index scores in the aged rats (young = 179.8 ± 3.0 and aged = 230.6 ± 2.87; F(1,22) = 8.5, p < 0.01). The distribution of these scores, however, shows that some of the aged rats performed within the range of young rats and others performed outside the range of young performance and are poor spatial learners. All rats in this study had proficient sensorimotor skills, evidenced by equivalent latency scores to escape during cue training to the visible platform (young = 7.99 ± 1.95 s and aged = 6.34 ± 0.59 s, F(1,22) = 1.70, p = 0.21).

Examples of typical Western blots for SNAP-25, synaptotagmin, and synaptophysin are shown in Fig. 2. Incorporation of a range of “standard” proteins on each blot allowed for quantification of the relative level of immunoreactive material in the hippocampal extracts. The blotting experiment was replicated 3 times for SNAP-25 and synaptotagmin and 2 times for synaptophysin. The data were averaged together across experiments. Sample loading order was counterbalanced for age and learning index to eliminate any systematic errors. One-way analysis of variance (ANOVA) showed that aged rats were not different from young rats in levels of SNAP-25, synaptotagmin, or synaptophysin immunoreactivity (Table 1). Fig. 3 shows the distribution of immunoreactivity levels across the range of learning index scores for the young and aged rats, clearly indicating no loss of synaptic markers in the aged rats and no correlation with behavioral performance (r values ranging from .03 to .09).
4. Discussion

The current study measured three proteins that are markers of synapses in the hippocampus of young and aged rats. The quantitative methods used provide good sensitivity for alterations in protein immunoreactivity. Given the variability observed and the sample sizes employed in this study, relatively modest changes (10–15%) would have been statistically significant. Nonetheless, no age difference was found for any of the three proteins that were examined.

The lack of an age-effect on hippocampal synaptic markers in the current study is consistent with another report indicating no change in SNAP-25 mRNA level in any subregion of the hippocampus in a comparison of 3 and 24 month old Sprague Dawley rats [12]. Other studies have reported no change in synaptic density in CA1 [20] or the dentate gyrus [3] in aged rodent hippocampus. In addition, non-human primates do not exhibit synaptic loss in the dentate gyrus supragranular region [25]. In such studies, it is possible that a loss of synapses might be obscured by simply grouping subjects based on chronological age, because significant synapse loss might only occur in those aged animals with cognitive impairment. Our study addressed that issue by evaluating the behavioral status of aged subjects in a hippocampal dependent task. Our study, however, showed no relationship between immunoreactivity for three synaptic proteins in the hippocampus and cognitive decline; synaptic markers are maintained even in aged rats with cognitive deficits in a hippocampal-dependent task. This stability of synaptic markers in normal aged rodents and primates contrasts with a 50% loss of synaptophysin immunoreactivity in the hippocampus of Alzheimer’s disease patients [23].

It is important to note that the current findings are compatible with evidence for structural reorganization of synaptic connections within the hippocampal formation. The connections made by the entorhinal cortex/perforant path projection to the dentate gyrus become more sparse both in normal aging and, to a greater degree, in Alzheimer’s disease [8,10,18]. In a study of aged rat brain using the dissector method, Geinisman et al. [8] reported that the alteration is type-specific; only perforated synapses in the middle and outer molecular layer are decreased. In our previous work with aged Long Evans rats, evidence was obtained for restructuring within this same region [15,18]. Importantly, decreases in the connectional zone for entorhinal input defined by Timm stain were accompanied by increases in the connectional zone defining the inner molecular layer. A similar pattern of sprouting of intrinsic hippocampal connections from the inner molecular layer is also seen in young animals after entorhinal lesions and in AD, indicating that a decrease in synapses from one source may be offset by an increase from another source. Such alterations might result in no net loss of synapse numbers, consistent with our observation of no overall change in synaptic proteins on immunoblots.

Reorganization of synapses could conceivably be the cause of increased glial activation commonly reported in the hippocampus of normally aged rodents and humans [11,14, 22] and humans [4]. Nonetheless, the present study, along with previous research in the same study population, indicates that glial activation in the aged hippocampus is not a result of a widespread net loss of synapses or neurons.
Moreover, the spatial learning decline associated with normal aging in the Long Evans rat does not appear to be caused by frank neurodegeneration or a major decrease in the total complement of hippocampal synaptic connections. It appears that disruption of information processing in the hippocampus in normal aging [2,24] may be attributable to functional alterations in a system where the underlying structure remains largely intact.

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References


Fig. 3. Plots show the lack of relationship between immunoreactivity level of SNAP-25, synaptotagmin, and synaptophysin with spatial learning in the water maze. Higher scores indicate poorer learning.


