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Long-term continuous, but not daily, environmental enrichment reduces spatial memory decline in aged male mice

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Abstract

Although environmental enrichment improves spatial memory and alters synaptic plasticity in aged rodents, it is unclear whether all types of enrichment treatments yield similar benefits. The present study examined the effects in aged male mice of three types of enrichment on spatial memory in Morris water maze and radial arm maze tasks, and on levels of the presynaptic protein synaptophysin in several brain regions. Non-enriched young and aged males were compared with males exposed to one of the following treatments for 10 weeks: 5 min of daily handling, 3 h of daily basic enrichment, or 24 h of continuous complex enrichment. Young controls outperformed aged controls in both tasks. Neither daily handling nor daily enrichment affected spatial memory or synaptophysin levels. In contrast, continuous enrichment significantly reduced age-related spatial memory decline in both tasks, such that this group was statistically indistinguishable from young controls in most measures of performance. Continuously enriched mice were also significantly better than other aged mice in several spatial memory measures. Despite these improvements, synaptophysin levels in the continuous enrichment group were significantly lower than those of young and aged controls in the frontoparietal cortex, hippocampus, and striatum, suggesting a negative relationship between synaptophysin levels and spatial memory in aged males. These data demonstrate that different types of enrichment in aged male mice have disparate effects on spatial memory, and that the relationship between enrichment-induced changes in synaptophysin levels and spatial memory in aged males differs from that we have previously reported in aged female mice.

Keywords: Aging; Reference memory; Working memory; Synaptophysin; Hippocampus; Neocortex

1. Introduction

One of the most common approaches to reducing age-related memory decline in humans and rodents has been to develop drugs that augment the function of specific neurotransmitter systems (Barnes, 1998; Bartus, 2000). However, in recent years, growing attention has been given to the use of behavioral treatments, such as environmental enrichment, as methods of alleviating age-related memory impairments. Environmental enrichment generally refers to any treatment that provides cognitive and/or physical stimulation beyond that which would be received in standard housing conditions (Rosenzweig & Bennett, 1996). Control animals in these standard housing conditions are either housed individually (i.e., isolated controls) or housed in small groups (i.e., social controls). Enriched mice are typically housed socially and are also provided with an array of stimulating objects and running wheels for various periods of time.

Among aging rodents, environmental enrichment alleviates age-related impairments in several types of memory and reduces neural dysfunction in related areas of the brain such as the hippocampus and neocortex. For example, in middle-aged rats and mice, enrichment significantly improves spatial reference memory in the Morris water maze (Frick, Stearns, Pan, & Berger-Sweeney, 2003; Kempermann, Kuhn, & Gage, 1998; Pham et al., 1999) and learning in the Hebb-Williams maze

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(Cummins, Walsh, Budtz-Olsen, Konstantinos, & Horsfall, 1973). It also increases forebrain weight (Cummins et al., 1973), neocortical dendritic branching (Green, Greenough, & Schlumpf, 1983), hippocampal neurogenesis (Kempermann et al., 1998), and neocortical and hippocampal neurotrophin levels (Ickes et al., 2000; Pham et al., 1999). Similarly, in aged rats and mice, enrichment improves spatial reference memory in the Morris water maze (Frick & Fernandez, 2003), reverses short-term memory deficits (Soffié, Hahn, Terao, & Eclancher, 1999), and increases spontaneous alternation (Van Waas & Soffié, 1996), incidental learning, and food-seeking behaviors (Warren, Zerweck, & Anthony, 1982). Furthermore, enrichment in aged rats and mice reduces age-induced hippocampal gliosis (Soffié et al., 1999), and increases cortical thickness (Diamond, Johnson, Protti, Ott, & Kajisa, 1985) and presynaptic vesicle number (Nakamura, Kobayashi, Ohashi, & Ando, 1999).

In aged female mice, enrichment-induced improvements in spatial reference memory in the water maze have been associated with increased hippocampal levels of the presynaptic protein synaptophysin (Frick & Fernandez, 2003), a calcium-binding glycoprotein located in the membranes of presynaptic vesicles containing neurotransmitters (Jahn, Schiebler, Ouimet, & Greengard, 1985; Wiedenmann & Franke, 1985). Reductions in synaptophysin have been associated with age-related cognitive decline in normal and demented humans (Liu, Erikson, & Brun, 1996; Sze et al., 1997) and aged rodents (Smith, Adams, Gallagher, Morrison, & Rapp, 2000). Although changes in synaptophysin levels have been most commonly interpreted as reflecting alterations in synapse number, enrichment in aged rats appears to increase the number of vesicles per synapse rather than the number of synapses (Nakamura et al., 1999). An increase in synaptic vesicles may result in increased neurotransmission, which could lead to the mnemonic improvements seen in aging rodents. Although enrichment-induced increases in synaptophysin levels are associated with improved spatial memory in aged female mice (Frick & Fernandez, 2003), it is unclear whether this relationship extends to aged males. This information is important to understanding the neurobiological mechanisms of enrichment-induced mnemonic improvements in aged rodents because similar relationships between increased synaptophysin levels and improved spatial memory have not been observed in middle-aged male and female mice (Frick et al., 2003) or in young female mice (Lambert, Fernandez, & Frick, 2005). The present study is the first to examine this relationship in aged males.

One difficulty in evaluating the effects of environmental enrichment on memory and neurobiology is the wide disparity among enrichment paradigms used by different laboratories. Enriched housing varies in cage size, composition, duration, social complexity, stimulus object complexity, and frequency of object changing. For example, studies have employed very large home cages containing various objects that were moved or replaced daily (Green et al., 1983), every other day (Green et al., 1983; Soffié et al., 1999; Van Waas & Soffié, 1996), twice a week (Ickes et al., 2000; Pham et al., 1999), or when objects deteriorated (Winocur, 1998). Others used smaller home cages with fewer objects that were changed on a daily basis (Frick et al., 2003). Still others did not house rodents with enriching stimuli, but exposed them to enrichment for 3 h/day (Frick & Fernandez, 2003; Rampon et al., 2000). Other parameters that differ among enrichment studies include the amount of experimenter handling (Diamond et al., 1985), inclusion of supplemental food treats (Kempermann et al., 1998), and exposure to sexually receptive females (Warren et al., 1982). The fact that all of these treatments improved memory or neural function in some way seems to suggest that almost any form of enrichment can improve memory in aging rodents. However, this conclusion is complicated by the fact that all of these studies evaluated different types of memory using different tasks. To date, no study has compared the effects of different types of enrichment on memory in aging rodents using the same methods.

Thus, the present study was designed to simultaneously assess the efficacy of three types of environmental enrichment in reducing age-related spatial memory deficits in aged male mice. Aged mice were divided into four groups (all of which were socially housed): controls, daily handling (5 min of daily handling and exploration of a new cage), daily enriched (enriched in a large cage for 3 h/day), and continuously enriched (housed in a very large home cage with 24 h access to numerous toys and running wheels). Young male non-enriched controls were also included to evaluate the extent to which the three enrichment conditions alleviated age-related spatial memory deficits. Spatial reference memory was first assessed in the Morris water maze, and then spatial reference and working memory were simultaneously measured in a water-escape motivated radial arm maze. To examine the relationship between enrichment-induced changes in spatial memory and synaptophysin levels, synaptophysin levels were measured in the hippocampus, neocortex, striatum, and cerebellum. We expected that aged controls would be impaired relative to young controls in both tasks (Bimonte, Nelson, & Granholm, 2003; Frick, Burlingame, Arters, & Berger-Sweeney, 2000). Although daily handling may reduce the stress associated with handling during behavioral testing, this very mild form of enrichment was not expected to improve memory or affect synaptophysin levels. In contrast, because we have previously shown in aged female mice that the daily enrichment treatment enhances spatial reference memory and synaptophysin levels in the hippocampus and neocortex (Frick & Fernandez, 2003), this treatment was expected to attenuate age-related memory decline in both tasks and augment synaptophysin levels in several brain regions. Finally, because continuous enrichment provides a more intense enrichment experience than daily enrichment, improvements produced by this treatment were expected to

be as great, or greater, than those produced by daily enrichment.

2. Methods

2.1. Subjects

Subjects were 12 young and 30 aged male C57BL/6 mice obtained from Hilltop Lab Animals (Scottdale, PA). Mice were initially quarantined for 3 weeks at the Yale School of Medicine animal facility before being transferred to a colony room in the Department of Psychology where they remained for the duration of the study. Upon arrival, mice were handled for 5 days to habituate them to the experimenter. They were then divided into experimental groups (see below) and housed in enriched or control conditions for 6 weeks prior to behavioral testing. At the beginning of behavioral testing, mice were 5 or 23 months of age. During the quarantine and handling phases, all mice were housed up to 5 in standard shoebox cages in a colony room with a 12:12 light/dark cycle (lights on at 07:00 h). Water and food (Purina LabDiet 5P00 ProLab RMH 3000) were available ad libitum throughout the study. During the environmental enrichment and behavioral testing phases, young controls, aged controls, aged daily handling, and aged daily enriched mice remained in shoebox cage housing, whereas aged continuously enriched mice were housed in larger cages containing complex enriched environments (see below). Colony room conditions remained constant throughout the study and all behavioral testing took place during the light phase of the cycle. All procedures were conducted in accordance with the standards set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and followed an approved protocol by Yale University's Institutional Animal Care and Use Committee.

2.2. Environmental enrichment

Mice were divided into five treatment groups: young controls ($n = 12$), aged controls ($n = 6$), aged daily handling ($n = 8$), aged daily enriched ($n = 8$), and aged continuously enriched ($n = 8$). Mice were enriched daily for six weeks prior to behavioral testing and during all four weeks of behavioral testing (a total of 10 weeks). On testing days, aged daily handling and aged daily enriched mice were enriched after the completion of testing, whereas aged continuously enriched mice were returned to their home cage.

Young and aged controls were group housed in shoebox cages as described above (see also Fig. 1, right), and had no exposure to extra handling or enriching objects. Aged daily handling mice were similarly housed and were also not exposed to enriching objects. However, this group was exposed to brief daily handling and exploration of a novel environment (a clean cage) to control for any effects that these aspects of the daily enrichment protocol may have on memory. Daily handling lasted for 5 min/day, during which mice were allowed to move freely about the experimenter's hands and to explore a new shoebox cage together with their cage-mates before being returned to their home cage.

Aged daily enriched mice were housed similarly to the young control, aged control, and aged daily handling groups, but were removed from their home cages for 3 h each day and placed in large, translucent, Rubbermaid bins (HiTop Storage Box, 56.5 cm long × 41.5 cm wide × 22 cm high) fitted with white ventilated lids (with approximately 75 small holes for ventilation) as in Frick and Fernandez (2003). These daily enrichment bins always contained fresh bedding, a running wheel, a plastic rodent dwelling, a plastic tube configuration requiring vertical climbing, and 2 or 3 other toys (see Fig. 1, center). Bins contained new objects in new configurations each day and care was taken not to

Fig. 1. Photo of the various enrichment conditions. Continuous enrichment is shown at left, a bin used in the daily enrichment condition is shown in the middle, and standard housing used for all groups but the aged continuously enriched group is shown at right.

include any individual wheel or toy more than once per week. Aged daily enriched cage-mates were moved together into the same enrichment bin, and bins were placed side-by-side on a table in a well-lit room that also contained a variety of visual cues (Frick & Fernandez, 2003).

Aged continuously enriched mice were all housed together in a large transparent plastic bin (Ancare, Bellmore, NY; 66 cm long × 46 cm wide × 38 cm high) fitted with a transparent snap-on lid. The lid was modified for ventilation by cutting 2 rectangular holes (30 cm long × 18.5 cm wide) 6 cm apart into the lid and fitting them with wire feeding racks covered by ventilation lids from standard shoebox cages. The large bin allowed more mice to be housed together (more social enrichment), and for larger and more complex objects to be placed in the cage (more cognitive and physical enrichment). Water access was provided by drilling a small hole (10 mm × 25 mm) 7 cm above the base of the bin on one end and inserting the spout from an exteriorly mounted stainless steel water bottle holder (Ancare, Bellmore, NY). Food pellets were placed in the overhead feeding racks (to encourage climbing behavior) and in a stainless steel food bowl on the floor of the bin. The bin was placed on a stainless steel cage rack in the same colony room as the standard shoebox cages. The bin was changed twice per week (a clean bin, bedding, and objects were provided at each change) and contained a large assortment of enrichment objects which always included: 2 running wheels, 2 rodent dwellings, a large plastic tube configuration with vertical climbing aspects (watchtowers, spiral loops, bridges, etc.), and 4 or 5 other objects used to enrich the aged daily enriched group (see Fig. 1, left). New objects were presented in new configurations each time the bins were changed.

2.3. Morris water maze

Water maze testing was conducted as in Frick, Fernandez and Bulinski (2002) and Frick and Fernandez (2003). A white circular tank (97 cm in diameter) was filled with water (24 ± 2 °C) made opaque with white non-toxic tempera paint (Dick Blick Art Materials, Galesburg, IL). The tank was divided into four quadrants and four start positions were located at the intersections of the quadrants. Extra-maze cues (abstract black/white designs, approximately 30 cm^2) were placed around the tank at each of the start positions (north, south, east, and west). The tank was located in a well-lit room that contained additional extra-maze cues attached to the walls and an overhead camera connected to an HVS 2020 automated digital tracking system (HVS Image, Hampton, England) used to record data. A smaller circular ring (55 cm) was inserted into the center of the tank (to decrease swimming area) and used to habituate the mice prior to testing. In a four trial shaping procedure, mice were first allowed to sit on a visible red platform (10 cm × 10 cm) for 10 s, and then placed into the water at three progressively further distances from the platform and given 30 s to locate the escape platform. This procedure allowed the mice to become accustomed to swimming and taught them that the platform affords escape from the water. No data were recorded during habituation. Spatial and cued versions of the Morris water maze were conducted as described below.

2.3.1. Spatial water maze

This task tests spatial reference memory, which refers to memory for spatial locations (e.g., an escape platform location) that do not change over time (Olton, 1977). In this hippocampal-dependent version of the task (Morris, Garrud, Rawlins, & O'Keefe, 1982), a transparent Lucite platform (10 cm × 10 cm) was submerged 0.5 cm under water and placed in the southwest quadrant of the tank where it remained for all trials. Five sessions were conducted on five consecutive days, and each session consisted of six trials in which the start position varied for each trial. For the first five trials, the mouse was given 120 s to locate the escape platform, upon which it was allowed to remain for another 10–15 s before being removed, dried off, and returned to the home cage for an inter-trial interval of approximately 20 min. Swim time (s), swim distance (cm), and swim speed (cm/s) were recorded during these trials. Lower numbers indicate better performance for swim time and swim distance, and indicate slower swim speeds.

The sixth trial of each session was a variable-interval probe trial (Markowska, Long, Johnson, & Olton, 1993) in which the platform was collapsed for a variable amount of time (either 20, 30, or 40 s), then raised and made available for escape. The amount of time allowed to find the raised platform varied with the duration of the variable-interval such that the total trial time was 60 s (Frick et al., 2000). While the platform was lowered and unavailable for escape, quadrant time (the percent of time the mouse spent in each quadrant of the tank) and platform crossings were recorded. For platform crossings, the number of times the mouse crossed the submerged platform location/10 s was used as the dependent variable in order to account for the varying duration of the intervals. Thus, the numbers of crossings were divided by 2, 3, or 4 for the 20, 30, and 40-s intervals, respectively. For both quadrant time and platform crossings, higher numbers indicate better performance.

2.3.2. Cued water maze

Cued testing began the day after completion of spatial testing. For this non-spatial, non-hippocampal-dependent version of the task, the platform was made visible by covering the surface in red tape, raising it 0.5 cm above the surface of the water, and attaching a flat circular disk (8 cm in diameter) perpendicularly to one side. Also, the extra-maze cues attached to the tank were removed. Three test sessions were conducted on consecutive days, with each session consisting of 6 trials in which the start position (north, south, east, and west) and quadrant containing the platform varied for each trial. Mice were given 120 s to locate the escape platform and the inter-trial interval was approximately 20 min. No probe trials were conducted in this task. Swim

time, swim distance, and swim speed data were recorded as above.

2.4. Water-escape motivated radial arm maze

Water-escape motivated radial arm maze (WRAM) testing began three days after completion of the cued task. This task assessed spatial working and reference memory simultaneously and was conducted as described in Gresack and Frick (2003). In contrast to spatial reference memory, spatial working memory refers to memory for spatial locations that change over time (Olton, 1977). The 8-arm maze was constructed of an opaque round center (44 cm in diameter) with eight clear Plexiglas arms (38 cm long × 12 cm wide × 10 cm high) radiating equidistantly from the center. The maze was placed in the center of the white circular tank used for Morris water maze testing. The tank was filled with opaque water (24 ± 2 °C). Hidden escape platforms (10 × 10 cm) were submerged just below the surface of the water at the ends of four of the arms. One arm was designated as a start arm and never contained a platform. The location of the four platforms remained constant for each mouse throughout testing but varied between mice. Platforms were placed in no more than two adjacent arms. Testing took place in the same room as Morris water maze testing.

Mice were habituated to the task using a five trial shaping procedure in which they learned to swim down the length of an arm to find a hidden escape platform. During shaping, only one arm contained a platform, which was made visible by covering it with red tape and lowering the water level to approximately 0.3 cm below the platform surface. For the first four trials, all eight arms were blocked off and the mouse was confined to the shaping arm. During trial 1, the mouse was placed on the platform for 15 s. With each successive trial, the mouse was placed at further distances from the platform such that for the fourth trial, the mouse was placed at the entrance to the shaping arm. During the final trial, the shaping arm was opened to allow access to the center of the maze (the other seven arms remained blocked). The mouse was then placed in the center and allowed to climb on the platform in the shaping arm. If, on any trial, the platform was not found in 30 s, then the mouse was gently guided to it. No data were collected during shaping.

Testing began the day after shaping. Fifteen consecutive daily sessions were conducted, each consisting of four trials. At the start of Trial 1, all four hidden escape platforms were submerged in their designated arms. The mouse was released from the end of the start arm and given 120 s to climb onto any platform. If it did not locate a platform within this time, it was gently guided to the nearest platform and allowed to remain there for 15 s. The mouse was then removed, dried off with a towel, and placed in a holding cage for a 45 s inter-trial interval. During this time, the located platform was removed from the tank, leaving three platforms in the maze. Trials 2–4 were conducted in a similar manner with the removal of an additional platform at the completion of each trial. At the end of Trial 4, the mouse was removed from the maze, dried off, and returned to the home cage. All four platforms were resubmerged in a new arrangement before the next mouse began testing.

Errors were recorded during each trial of the daily sessions as in Gresack and Frick (2003). An entry was recorded when the mouse's entire body (excluding the tail) crossed an arm opening. Three types of errors were recorded: working memory errors (entries into arms from which a platform had been removed during a daily session), initial reference memory errors (first entries into arms that never contained platforms), and repeated reference memory errors (repeated entries into arms that never contained platforms). In addition to total working memory errors committed during a session, the number of working memory errors committed during Trials 2–4 of each session were recorded (it is not possible to commit a working memory error in Trial 1) in order to assess the working memory information as the number of items to be remembered (i.e., the working memory load) increased. Values analyzed for Trials 2–4 were the average working memory errors made in each individual trial in the sessions of interest (see below).

2.5. Synaptophysin assay

Tissue extraction, homogenization, and processing for synaptophysin were conducted as previously described (Frick & Fernandez, 2003; Frick et al., 2003). Mice were briefly sedated with CO_2 and decapitated. Brains were immediately removed and the hippocampus, frontoparietal cortex, striatum, and cerebellum were dissected bilaterally on ice. Enrichment was expected to affect synaptophysin levels in brain regions associated with spatial learning, such as the hippocampus and frontoparietal cortex. The striatum and cerebellum were also collected to assess the effects of enrichment on brain regions that are associated with motor learning (which is inherent to the enrichment treatment). All tissue was weighed and immediately stored at −70 °C until the day of homogenization. Samples were resuspended in a 0.02% Triton X-100 in 0.1 mM Tris solution, pH 7.4, sonicated with a probe sonicator, and centrifuged for 10 min at 10,000*g*. The supernatant was diluted 1:5 and designated as the crude extract. This crude extract was further diluted as described below. The protein content of the samples was measured using a Bradford protein assay (Bradford, 1976).

Synaptophysin was measured using an enzyme-linked immunosorbent assay (ELISA) as previously described (Frick & Fernandez, 2003; Frick et al., 2003). Because purified synaptophysin was not available for use as a standard, synaptophysin levels in the samples are expressed as "equivalents" relative to synaptophysin immunoreactivity from whole mouse brain homogenate (termed "mouse brain standard" or "MBS"). An antibody sandwich ELISA using two different anti-synaptophysin antibodies (monoclonal

anti-synaptophysin Clone SY 38 and polyclonal rabbit anti-synaptophysin, DAKO, Carpinteria, CA) was used to determine the relative amounts of synaptophysin in the samples. Samples were diluted to 1:32,000 from the crude extract and were assayed in triplicate. Optical density was measured at a wavelength of 405 nm using a Labsystems Multiskan Plus microplate reader. The average absorbance of three wells containing no MBS was subtracted from each reading.

The relative amount of synaptophysin in the samples was calculated by plotting the absorbance of four different MBS concentrations versus the log of the total protein concentration. The equation of the straight line that resulted and the absorbance of each sample was used to determine the concentration of MBS which would have the absorbance exhibited by the sample. This apparent MBS concentration of the sample was divided by the total protein concentration of the sample (obtained from the protein assay described in the previous section) to yield the relative amount of synaptophysin in the sample versus the amount of synaptophysin in the MBS homogenate (termed "MBS synaptophysin equivalent").

2.6. Data analysis

For the spatial and cued water maze, all measures were averaged within a group for each session and were analyzed using a one-way repeated-measures analysis of variance (ANOVA) with Group as the independent variable and Session as the repeated measure (SuperANOVA, Abacus Concepts, Berkeley, CA). Errors in the WRAM were analyzed separately using one-way repeated-measures ANOVAs with Group as the independent variable and testing Block as the repeated measure. Analyzing these data by test block, rather than by test session, was necessary because a few aged animals were unable to complete some test sessions, although they were able to resume testing the following session. Blocks were as follows: Block 1 = sessions 2–5, Block 2 = sessions 6–8, Block 3 = sessions 9–12, and Block 4 = sessions 13–15. Data from Session 1 of the WRAM task were not included in any analysis because this session is the first time the mice are introduced to the entire maze, the platform locations, hidden platforms, and the concept that platforms disappear once found, and thus does not accurately measure spatial working or reference memory (Gresack & Frick, 2003). Therefore, working and reference memory are measured starting in Session 2, at which point the mice have been fully exposed to the apparatus and rules of the task. Working memory errors for the working memory load analyses were averaged for each trial and analyzed using a one-way repeated-measures ANOVA with Group as the independent variable and Trial as the repeated measure. One-way ANOVAs without repeated measures assessed group differences in individual trials. Synaptophysin levels in each brain region were analyzed using separate one-way ANOVAs. Fisher's protected least significant difference (PLSD) post hoc tests were conducted a priori to determine between-group differences for all behavioral and synaptophysin measures. These tests were conducted even when the main effect of Group was not significant to avoid the possibility that enrichment effects obscured age differences between the young and aged control groups.

3. Results

3.1. Subjects

All mice appeared in good health at the start of the experiment, although alopecia was present in most aged mice. Because the aged control and aged daily handling groups did not differ statistically in any behavioral measure, these groups were combined into one aged control group (labeled AC in the figures). All mice completed Morris water maze testing, resulting in the following sample sizes: young control (YC) = 12, aged control (AC) = 14, aged daily enriched (ADE) = 8, and aged continuously enriched (ACE) = 8. Following the completion of Morris water maze testing, two aged control mice were euthanized due to health concerns. During the course of WRAM testing, several aged mice (6 from the aged control group, and 2 each from the other aged groups) were removed from the study due to fatigue or age-related physical ailments (e.g., ulcerative dermatitis). These mice were not included in any WRAM or synaptophysin data analyses. This resulted in the following sample sizes for the WRAM: young control = 12, aged control = 6, aged daily enriched = 6, and aged continuously enriched = 6. Additionally, due to damaged tissue samples, two mice (one young control and one aged control) were eliminated from synaptophysin analyses.

3.2. Morris water maze

3.2.1. Spatial water maze

All groups learned to find the hidden platform, as suggested by significant main effects of Session for swim time ($F(4,152) = 28.6$, $p < .0001$) and swim distance ($F(4,152) = 35.9$, $p < .0001$). However, differences among the groups were evident only in swim time (main effect of Group: $F(3,38) = 4.5$, $p < .01$; Fig. 2A), but not swim distance ($F(3,38) = 0.5$, $p > 0.05$; Fig. 2B). Post hoc tests revealed that young controls exhibited significantly shorter swim times than all aged groups except the aged continuously enriched group ($ps < .05$). In addition, aged continuously enriched mice exhibited significantly shorter swim times than aged daily enriched mice ($p < .05$). Group × Session interactions were not significant for either swim time or swim distance ($Fs(12,152) = 1.3$ and 1.4, respectively, $ps > 0.05$). For swim speeds (Fig. 2C), the main effects of Group ($F(3,38) = 13.3$, $p < .0001$) and Session ($F(4,152) = 5.4$, $p < .001$) were significant, but the Group × Session interaction was not ($F(12,152) = 1.4$, $p > .05$). Post hoc tests indicated that swim speeds in all groups differed significantly ($ps < .05$) from each other in

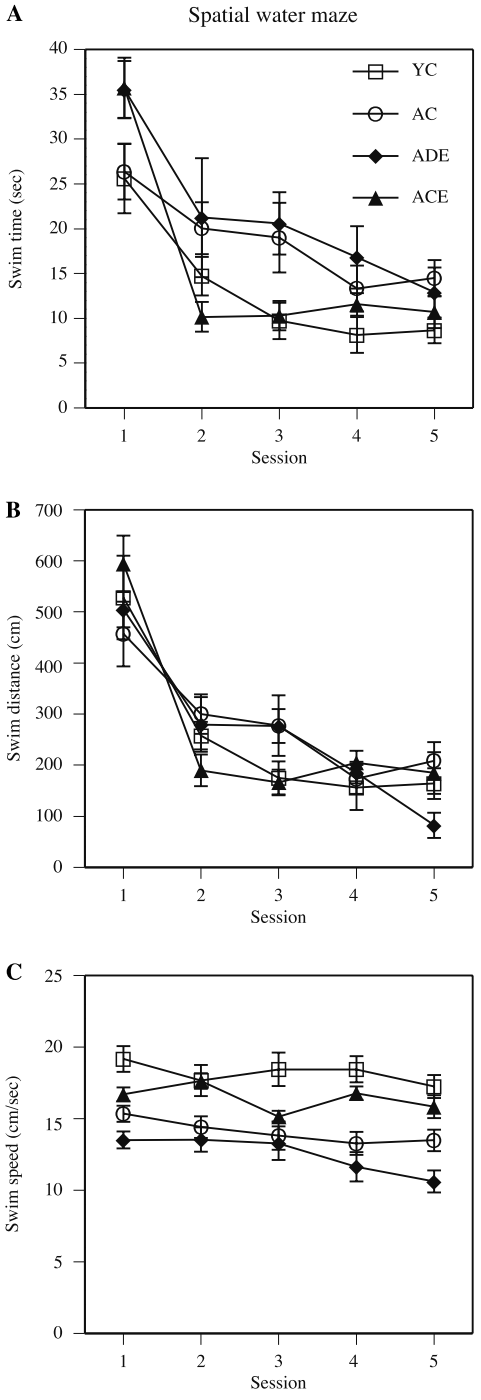

Fig. 2. Performance in the spatial water maze task as illustrated by swim time (A), swim distance (B), and swim speed (C). Each point represents the mean ± the standard error of the mean (*SEM*) of each group during one test session. Aged controls and aged daily enriched mice were impaired relative to young controls in swim time and swim speed. Aged daily enriched mice also exhibited slower swim times relative to aged continuously enriched mice; both aged controls and aged daily enriched mice had slower swim speeds than aged continuously enriched mice. Aged continuously enriched mice only differed from young controls in swim speed. No age- or enrichment-related differences were observed in swim distance.

the following order (from fastest to slowest): young control, aged continuously enriched, aged control, and aged daily enriched.

In the probe trials (Fig. 3), significant Session effects for both quadrant time ($F(4,152) = 4.1, p < 0.001$) and platform crossings ($F(4,152) = 9.5$, $p < .0001$) indicated that the groups as a whole learned to search more accurately for the submerged platform as training progressed. Although the main effect of Group was not significant for quadrant time ($F(3,38) = 1.8, p > .05$; Fig. 3A), post hoc tests indicated that young controls spent more time in the correct quadrant than aged controls ($p < .05$). Neither control group differed from the aged enriched groups. However, a differential effect of enrichment emerged in the platform crossings measure (Fig. 3B). This measure is more challenging than the quadrant time measure because it requires precise knowledge of the platform location. The main effect of Group ($F(3,38) = 3.4$, $p < .03$) and the Group × Session interaction were significant for platform crossings ($F(12,152) = 2.6$, $p < .01$), indicating differential numbers of platform crossings among the groups. Post hoc tests indicated that young controls made significantly more platform crossings than aged control and aged daily enriched mice ($ps < .05$).

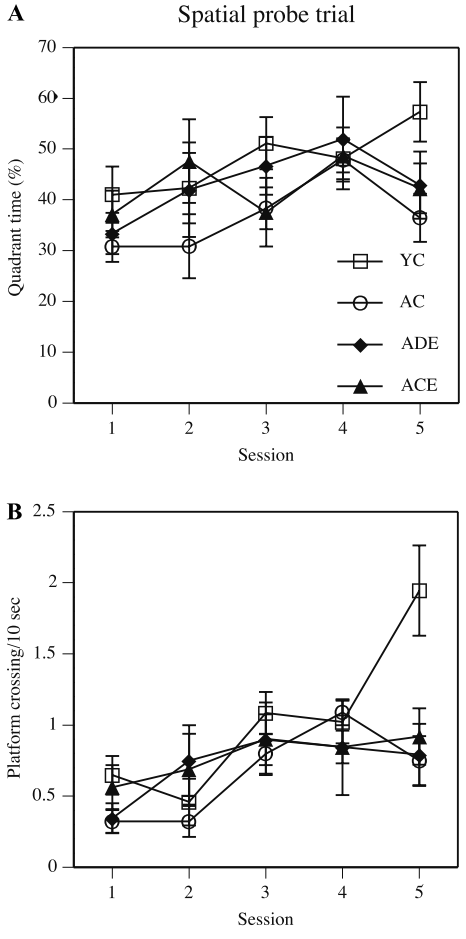

Fig. 3. Performance in the spatial probe trials as illustrated by quadrant time (A) and platform crossings (B). Each point represents the mean ± *SEM* of each group during one test session. Aged controls were impaired relative to young controls in both measures, whereas the aged daily enriched group was impaired only in platform crossings. The aged continuously enriched group did not differ from young controls in either measure.

In contrast, the young control and aged continuously enriched groups did not differ.

3.2.2. Cued water maze

Significant main effects of Session for swim time ($F(2,76) = 6.2$, $p < .01$), swim distance ($F(2,76) = 3.2$, $p < .05$), and swim speed ($F(2,76) = 3.2$, $p < .05$) indicated a general improvement in cued water maze performance over the course of testing (Fig. 4). In addition, main effects of Group were significant for all three measures (swim time: $F(3,38) = 6.0$, $p < .01$; swim distance: $F(3,38) = 3.2$, $p < .05$; swim speed: $F(3,38) = 10.8$, $p < .0001$), although no Group × Session interactions were significant ($Fs(6,76) = 0.8$–2.0, $ps > .05$). In both swim time and swim distance (Figs. 4A and B), post hoc tests revealed that young controls performed significantly better than all aged groups except the aged continuously enriched group ($ps < .01$). Additionally, aged daily enriched mice had significantly slower swim times than the other aged groups ($ps < .01$). As in the spatial task, swim speeds in all groups differed significantly ($ps < .05$; Fig. 4C) from those of the other groups such that young controls were the fastest, followed in order by the aged continuously enriched, aged control, and aged daily enriched groups.

3.3. WRAM

For working memory errors (Fig. 5A), the main effect of Treatment was significant ($F(3,26) = 6.3$, $p < .01$), but the Block effect ($F(3,78) = 0.4$, $p > .05$) and Block × Treatment interaction ($F(9,78) = 1.4$, $p > .05$) were not, indicating the presence of differences among the groups despite a lack of improvement across test blocks. Post hoc tests revealed that young controls made fewer working memory errors than all other groups ($ps < .03$); no differences were observed among the aged groups. In the analysis of working memory load (Fig. 5B), the number of working memory errors made by the groups increased significantly with each successive trial (main effect of Trial, $F(2,52) = 241.6$, $p < .0001$), although a significant Treatment main effect ($F(3,26) = 6.7$, $p < .01$) and Trial × Treatment interaction ($F(6,52) = 2.9$, $p < .02$) suggested different rates of change among the groups. Although post hoc tests on the Treatment main effect indicated that young controls committed significantly fewer working memory errors than all other groups ($ps < .02$), analysis of individual trials revealed differences among the aged groups that varied based on working memory load. Treatment main effects were significant for Trials 2, 3, and 4 ($Fs(3,26) = 3.0$, 4.1, and 5.2, respectively, $ps < .05$); post hocs demonstrated that young controls made significantly fewer errors than aged controls in each trial ($ps < .04$) and than aged daily enriched mice in trials 3 and 4 ($ps < .04$). However, young controls and aged continuously enriched mice differed only when the working memory load was the highest, in trial 4 ($p < .01$).

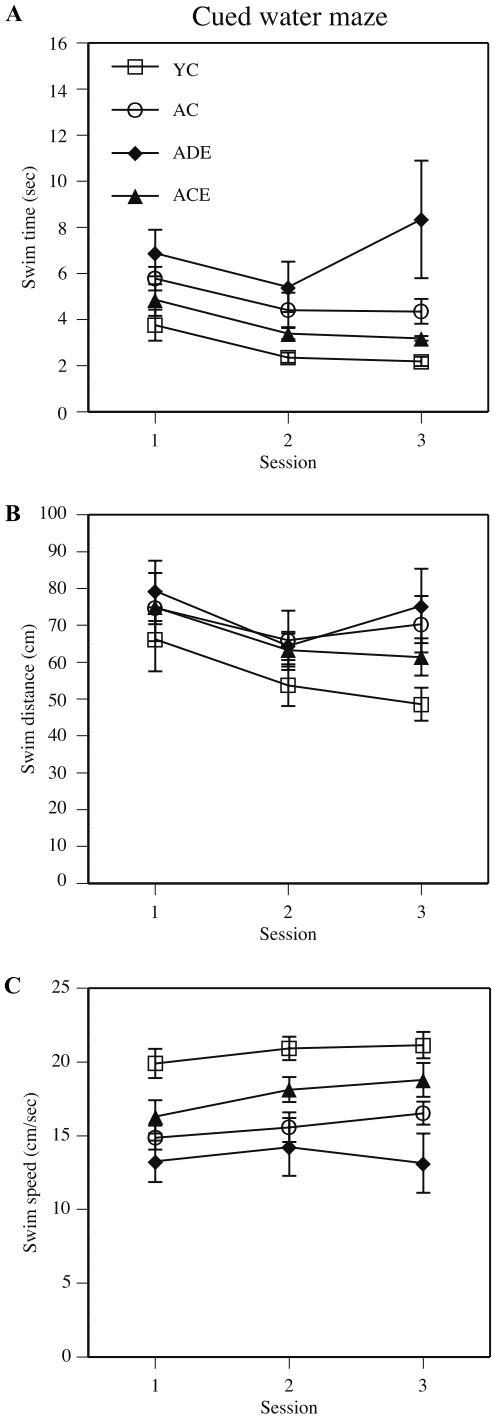

Fig. 4. Performance in the cued task as illustrated by swim time (A), swim distance (B), and swim speed (C). Each point represents the mean ± *SEM* of each group during one test session. The aged control and aged daily enriched groups were impaired relative to young controls in each measure. Both groups also had slower swim speeds than the aged continuously enriched group, and the aged daily enriched group had slower swim times than the continuously enriched group. The aged continuously enriched group only differed from young controls in swim speed.

The main effect of Treatment was significant for both initial reference memory ($F(3,26) = 13.1$, $p < .01$; Fig. 6A) and repeated reference memory ($F(3,26) = 6.3$, $p < .0001$;

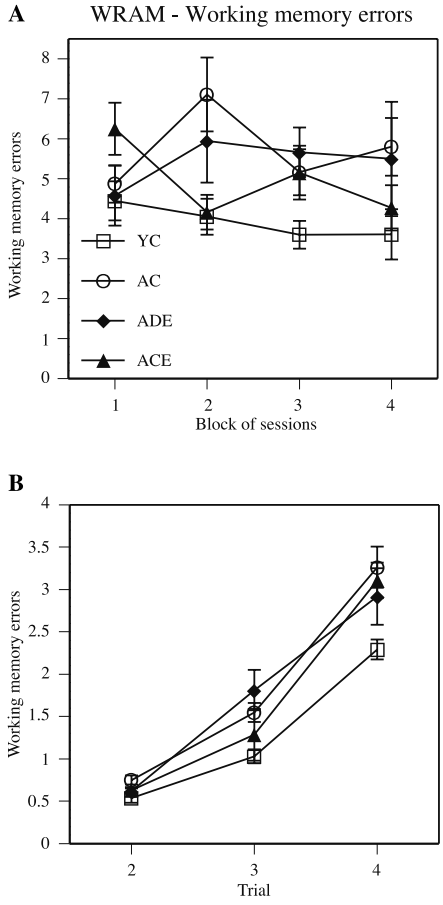

Fig. 5. Working memory errors in the WRAM made in the four testing blocks (A) and in Trials 2–4 of the sessions (B). Each point in (A) represents the mean ± *SEM* of each group during one test block, and each point in (B) represents the mean ± *SEM* of each group during a single trial of testing. All aged groups were impaired relative to young controls in terms of working memory errors examined across test blocks (A). However, only aged controls made significantly more working memory errors than young controls as working memory load increased from trial to trial (B). Neither enriched group differed from young controls at a low working memory load (trial 2). The aged daily enriched group made more working memory errors than young controls at medium (trial 3) and high (trial 4) working memory loads, whereas the aged continuously enriched group only made more errors than young controls at the high load (trial 4).

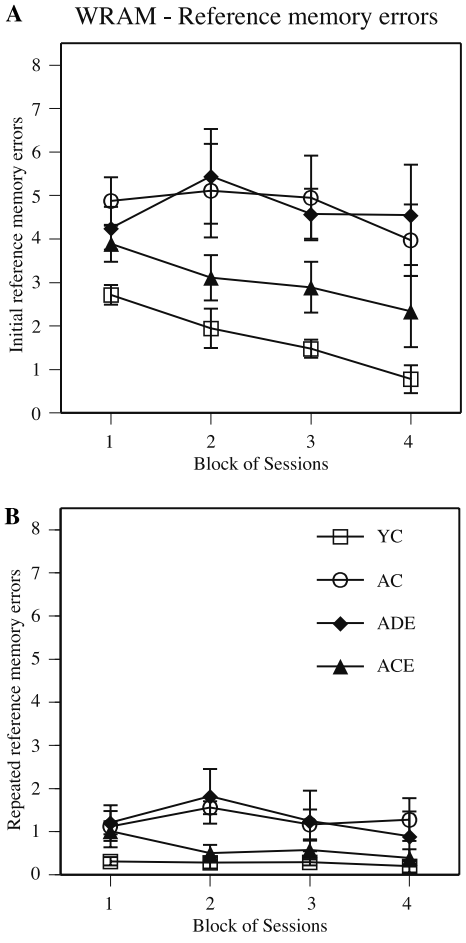

Fig. 6. Initial (A) and repeated (B) reference memory errors in the WRAM. Each point represents the mean ± *SEM* of each group during one test block. The aged control and aged daily enriched groups made significantly more initial and repeated reference memory errors than both the young control and aged continuously enriched groups. Aged continuously enriched mice made more initial reference memory errors than young controls, but did not differ from young controls in repeated reference memory errors.

Fig. 6B) errors. For initial reference memory errors, post hoc tests revealed that young controls made fewer errors than all other groups ($ps < .002$) and aged continuously enriched mice made fewer errors than the other aged groups ($ps < .001$). For repeated reference memory errors, both the young control and aged continuously enriched groups made fewer errors than the other aged groups ($ps < .01$). Unlike for initial reference memory errors, the young controls and aged continuously enriched groups did not differ in the number of repeated reference memory errors made. Across all groups, a reduction in the number of errors made during the course of testing was observed for initial reference memory errors (main effect of Block, $F(3,78) = 3.5$, $p < .02$), but not repeated reference memory errors (main effect of Block, $F(3,78) = 1.2$, $p > .05$). The Block × Treatment interactions were not significant for either reference memory error type ($Fs(9,78) < 1.0$, $ps > .05$), suggesting no changes in the effects of enrichment over the course of testing.

3.4. Synaptophysin assay

Group synaptophysin means for each brain region are listed in Table 1. Synaptophysin levels did not differ among the groups in the cerebellum ($F(3,28) = 0.4$, $p > .05$). However, the main effects of Group were significant for the neocortex ($F(3,27) = 3.6$, $p < .03$), hippocampus ($F(3,27) = 4.2$, $p < .02$), and striatum ($F(3,28) = 7.0$, $p < .01$). Post hoc tests revealed that synaptophysin levels in aged continuously enriched mice were significantly decreased in all three brain regions compared to young and aged controls ($ps < .03$), and compared to the aged daily enriched group in the neocortex ($p < .01$) and striatum ($p < .02$), but not hippocampus.

Table 1
Synaptophysin[a] data for each treatment group

Brain region	Young control	Aged control	Aged daily enriched	Aged continuously enriched
Hippocampus	0.58 ± 0.10	0.71 ± 0.07	0.45 ± 0.04	0.26 ± 0.02*
Neocortex	0.34 ± 0.04	0.39 ± 0.05	0.37 ± 0.02	0.21 ± 0.01†
Striatum	0.45 ± 0.02	0.40 ± 0.02	0.40 ± 0.01	0.32 ± 0.01†
Cerebellum	0.23 ± 0.01	0.23 ± 0.01	0.22 ± 0.02	0.21 ± 0.01

[a] Values represent means ± *SEM*. 'MBS synaptophysin equivalents' expressed as sample immunoreactivity relative to that of an equal amount of MBS.
* $p < .01$ relative to young and aged controls.
† $p < .03$ relative to all other groups.

4. Discussion

The results of this study demonstrate that different types of enrichment treatment can have vastly disparate effects on spatial memory in aged male mice. A summary of these effects is provided in Table 2. Daily handling did not affect any measure of spatial reference or working memory. Daily enrichment had little beneficial effect on spatial reference and working memory, as illustrated by the fact that this group was impaired relative to young controls in most measures of performance. In fact, in several water maze measures, daily enriched mice performed significantly worse than aged controls. In contrast, aged mice receiving continuous enrichment were statistically indistinguishable from young controls in measures from both spatial tasks, and performed significantly better than the other aged groups in several measures of spatial reference memory. Interestingly, synaptophysin levels in the aged continuously enriched group were significantly lower than those of the other groups in the hippocampus, neocortex, and striatum, perhaps suggesting a negative correlation between levels of this synaptic protein and spatial memory in aged males, which is contrary to the positive relationship we previously reported in aged female mice (Frick & Fernandez, 2003).

Table 2
Summary of impairments relative to the young control and aged continuously enriched groups

Task	Measure	Aged control	Aged daily enriched	Aged continuously enriched
Spatial water maze	Swim time	X	XX	—
	Swim distance	—	—	—
	Swim speed	XX	XX	X
	Quadrant time	X	—	—
	Platform crossings	X	X	—
Cued water maze	Swim time	X	XX	—
	Swim distance	X	X	—
	Swim speed	XX	XX	X
WRAM	WM errors	X	X	X
	WM load overall	X	X	X
	Trial 2	X	—	—
	Trial 3	X	X	—
	Trial 4	X	X	X
	IRM errors	XX	XX	X
	RRM errors	XX	XX	—

"X" indicates a significant impairment ($p < .05$) relative to the young control group only; "XX" indicates a significant impairment ($p < .05$) relative to the young control and aged continuously enriched groups; "—" indicates no difference from the young control group. WM, working memory; IRM, initial reference memory; RRM, repeated reference memory.

Our findings indicate that 10 weeks of continuous enrichment produced numerous improvements in aged males; mice in this group were unimpaired relative to young controls in all mnemonic measures (everything but swim speed) from the spatial and cued water maze tasks, in committing repeated reference memory errors in the WRAM, and in committing working memory errors at low and medium working memory loads. Furthermore, continuously enriched mice were significantly better than aged controls and aged daily enriched mice in several measures from the water maze (notably, swim speed and/or swim time), and they made fewer initial and repeated reference memory errors in the WRAM than both groups. This reduction of age-related spatial memory decline is similar to previous effects of enrichment in the water maze reported in aged female mice (Frick & Fernandez, 2003), middle-aged male and female mice (Frick et al., 2003; Kempermann et al., 1998), and middle-aged male rats (Pham et al., 1999). It is also consistent with previous enrichment-induced improvements in spontaneous alternation, incidental learning, and learning of the Hebb-Williams maze in aged and middle-aged rats (Cummins et al., 1973; Van Waas & Soffié, 1996; Warren et al., 1982).

In comparing the effects of continuous enrichment on the two spatial memory tasks, it appears as if this treatment was more consistently beneficial to memory tested in the water maze than in the WRAM; continuously enriched mice did not differ from young mice in any cognitive measure from the water maze, yet were impaired relative to young mice in several measures from the WRAM. One potential reason for this is that the water maze requires subjects to learn only a single platform location. On the other hand, this task does not provide any guidance towards the platform, unlike that provided by the arms of the WRAM, so mice need to have an exceptionally precise knowledge of the platform location in order to find it. Another explanation for the apparent difference in effects on the two tasks involves the types of memory tested. The fact that the aged continuously enriched group was unimpaired relative to young controls in the water maze and in making repeated reference memory errors in the WRAM may suggest that continuous enrichment was more

beneficial for reference memory than for working memory. This idea could be interpreted in two ways: (1) that the effects of continuous enrichment are specific to reference memory, or (2) that reference memory was more generally affected because reference memory information (e.g., remembering the location of arms that never contain platforms) is easier to remember than working memory information (e.g., remembering which platforms one has already visited today). The first interpretation is less likely, given that continuous enrichment did not eliminate the age-related increase in the number of initial reference memory errors committed. Thus, an improvement in reference memory was not observed in every reference memory measure. This finding, combined with the pattern of working memory improvements, makes the second interpretation, of a relationship with task difficulty, more appealing. First, with regard to the two types of reference memory errors, initial reference memory errors are more challenging to learn to avoid than repeated reference memory errors because they require the mice to remember information between sessions. However, once the mice have made an initial reference memory error and, thus, have been reminded that an arm does not contain a platform, enrichment may help them remember to avoid re-entering that arm again during the test session. Second, the working memory load analysis indicates that enrichment is most effective at preventing working memory errors when the task is easier (i.e., at lower working memory loads). Thus, at the lowest load (trial 2), both the daily and continuous enrichment groups were unimpaired relative to young controls. At the medium load (trial 3), only the continuous enrichment group remained unimpaired relative to young controls. Finally, when required to remember three platform locations, both enrichment groups were impaired relative to young controls. As such, the findings suggest that the extent to which continuous enrichment improves memory in aged males may depend more on task difficulty than on the type of memory being tested.

In contrast to continuous enrichment, 10 weeks of daily enrichment was of very little benefit to spatial memory in aged male mice. Furthermore, in both water maze tasks, daily enriched mice exhibited slower swim times (cued task) and speeds (both tasks) than aged controls. This is surprising, given that two weeks of the same treatment significantly improved spatial reference memory in the water maze in aged female mice (Frick & Fernandez, 2003). One potential reason for the current lack of effect of the daily enrichment treatment is the amount of time the mice spent with the enrichment stimuli. Females tend to be more active than males (Beatty, 1979), and thus, it is possible that the aged females in our previous report spent more time interacting with the objects than the aged males in the present study. Three hours may not have been sufficient for males to adequately interact with the objects and reap the benefits of treatment. The fact that spatial memory in aged male mice (this study) and middle-aged male mice (Frick et al., 2003) was improved by continuous exposure to objects may suggest that males need more time to interact with the stimuli than females. In addition to the amount of time spent with the stimuli, the time of day of the enrichment treatment may be important for males. Daily enrichment took place during the light phase of the light/dark cycle, and aged males may have been less likely to interact with the objects during the portion of the cycle in which they typically sleep. In contrast, continuously enriched mice were allowed to interact with the objects during the dark phase of the cycle (when mice are most active), which may have led to more enriching stimulation in this group than in the daily enriched group. Finally, male mice are more aggressive than females and the daily re-establishment of the dominance hierarchy upon being returned to the home cage after enrichment may have been more stressful and disruptive for males.

One potential consequence of differential exposure to the objects is discrepant levels of physical fitness between the daily and continuous enrichment groups. Although any differences in physical fitness do not appear to have influenced mortality in this study, continuously enriched mice consistently swam faster than the other aged groups in the water maze, and outperformed the daily enriched group on the swim time measure in both water maze tasks. These data may indicate that increased exercise, due to more exposure to enrichment stimuli, led to increased physical fitness and better performance among continuously enriched mice in the physically demanding swimming tasks. Indeed, the fact that the aged control and aged daily enriched groups were impaired relative to young controls in both the spatial and non-spatial water maze tasks might suggest that the benefits of continuous enrichment were due primarily to improvements in swimming ability, rather than memory. However, two pieces of evidence argue against this interpretation. First, continuous enrichment improved measures of performance in the spatial task that are not influenced by swim speed, namely quadrant time and platform crossings. Second, in the WRAM, continuously enriched mice made significantly fewer spatial reference memory errors and spatial working memory errors in trials 2 and 3 of each session than aged control and daily enriched mice. The WRAM measures are minimally affected by swim speed, and in fact, we might expect mice that swim faster (young controls and aged continuously enriched) to make *more* errors than slower swimmers because they are physically able to make more arm entries during the allotted trial time. Because this was clearly not the case, the data argue against the interpretation that an enrichment-induced increase in swimming ability, per se, is responsible for improved task performance in the continuously enriched group. Nevertheless, an increase in physical fitness could contribute to improved spatial memory in other ways. For example, in young rodents, exercise alone has been shown to significantly enhance hippocampal function (Fordyce & Wehner, 1993; van Praag, Christie, Sejnowski, & Gage, 1999a; van Praag, Kempermann, & Gage, 1999b), hippocampal and neocortical synaptophysin levels

(Lambert et al., 2005), spatial reference memory in the water maze (Fordyce & Wehner, 1993; van Praag et al., 1999a), and spatial working memory in the WRAM (Lambert et al., 2005). Thus, rather than just improving swimming ability, increased exercise in the continuously enriched group may have had direct effects on the hippocampus and neocortex that led to the observed memory improvement.

Finally, another possible reason why continuous enrichment was more beneficial than daily enrichment involves differences in stress due to experimenter handling. During the 10-week enrichment period, daily enriched mice were briefly handled by the experimenters each day while they were being transferred to and from the enrichment bins, whereas continuously enriched mice were handled only twice a week (when their cages were changed). Chronic stress leads to hippocampal dysfunction and impaired memory (Kim & Diamond, 2002), thus potentially accounting for the inability of the daily enrichment treatment to improve memory in this study. Interestingly, acute tailshock stress enhances memory in males, but impairs memory in females (Shors, 2001; Wood & Shors, 1998). As such, we find the stress explanation somewhat unlikely because our previous work showed that this daily enrichment treatment improves memory in aged females (Frick & Fernandez, 2003). However, it remains possible that aged males and females respond differently to the stress involved in the daily enrichment treatment. In fact, daily enriched males in this study performed worse than aged control males in several water maze measures (e.g., swim time, swim speed), suggesting that the daily enrichment treatment was somewhat detrimental to males despite the fact that some controls also received daily handling. The performance of this group may, thus, have been affected by other stressors endemic to the daily enrichment treatment, including dominance and territoriality squabbles over the toys or neophobia arising from the constant introduction of new toys. These issues would seem to be unique to males, as we have not previously found that daily enriched aged females performed worse than aged control females (Frick & Fernandez, 2003). This issue will need to be addressed in future work.

Daily handling did not affect any behavioral measure. Although previous work has shown that neonatal handling can significantly reduce stress and improve memory in aging male and female rats (Meaney, Aitken, Bhatnagar, & Sapolsky, 1991; Meaney, Aitken, van Berkel, Bhatnagar, & Sapolsky, 1988), we thought it unlikely that handling initiated in old age would significantly affect spatial memory. Rather, this group was included as a control for the daily handling received by the daily enrichment group. Although the fact that the daily handling group (which was collapsed into the aged control group for data analyses) and the daily enrichment group differed from the continuous enrichment group may suggest a detrimental effect of daily handling on memory, a more likely explanation is that the stimulation received by the continuous enrichment group was a more effective method of improving memory in aged males.

Synaptophysin levels in all brain regions were not reduced in aged controls relative to young controls. Although some studies have reported age-related reductions in this protein in the neocortex and hippocampus (Chen, Masliah, Mallory, & Gage, 1995; Saito et al., 1994), several others have not (Calhoun et al., 1998; Nicolle, Gallagher, & McKinney, 1999), or have reported reductions in specific hippocampal subregions (Smith et al., 2000). Nevertheless, the current data are consistent with our previous findings in aged female mice illustrating no age-related reduction in synaptophysin levels in the neocortex, hippocampus, and striatum (Frick & Fernandez, 2003). However, the effects of enrichment on synaptophysin levels in aged males are considerably different from those which we have previously reported in aged females. Whereas in aged females, two weeks of daily enrichment significantly increased neocortical and hippocampal synaptophysin levels and improved memory (Frick & Fernandez, 2003), 10 weeks of the same treatment had minimal effects on synaptophysin levels and memory in aged males. Furthermore, the continuous enrichment treatment, which successfully reduced age-related memory decline in both tasks, actually *decreased* synaptophysin levels in the neocortex, hippocampus, and striatum relative to young and aged controls. The reasons why a reduction in synaptophysin levels may be related to improved memory in aged males are unclear, given that previous studies have associated increases in synaptophysin with reduced age-related memory decline in aging mice (Calhoun et al., 1998; Frick & Fernandez, 2003). As stated previously, increases in synaptophysin have most often been interpreted as reflecting an increase in presynaptic terminals (Chen et al., 1995; Eastwood, Burnet, McDonald, Clinton, & Harrison, 1994). However, one electron micrography study indicates that it is the number of vesicles per terminal, rather than the number of terminals, that increases as a result of enrichment in aged male rats (Nakamura et al., 1999). This increase in vesicles should result in an increase in neurotransmitter secretion (Alder, Kanki, Valtorta, Greengard, & Poo, 1995). Thus, the reduction in synaptophysin levels observed in the present study may represent a decrease in vesicle number and, hence, in neurotransmission. There are two ways in which this reduction may result in a functional improvement in memory. The first is if the affected synapses are primarily inhibitory, which could release excitatory neurons (e.g., hippocampal pyramidal neurons) from inhibition. The second is if the observed age-related memory deficits are the result of interference with postsynaptic function by excess neurotransmitter release. In this case, a reduction in vesicles, and thus the amount of neurotransmitters released, would reduce interference caused by excess neurotransmitter. These clearly speculative possibilities will need to be further investigated.

The fact that reductions in synaptophysin levels in this study were associated with improved spatial memory further complicates the relationship between synaptophysin levels and memory in mice. In addition to the previously mentioned relationship between improved memory and increased synaptophysin levels in aged daily enriched female mice (Frick & Fernandez, 2003), we have recently reported that increases in synaptophysin levels induced by daily enrichment in young females do not seem to be closely associated with improvements in spatial memory (Lambert et al., 2005). In this latter study, both daily exercise and daily cognitive stimulation significantly increased synaptophysin levels in the neocortex and hippocampus, although only exercise improved spatial memory in the WRAM (Lambert et al., 2005). These data would seem to suggest that a different neural mechanism was responsible for exercise-induced improvements in spatial memory. Perhaps this is true in aged males as well. Together, these studies suggest that synaptophysin may not be the best marker for assessing enrichment-induced changes in synaptic plasticity. Certainly, many other aspects of neocortical and hippocampal function may be affected by enrichment in aging rodents; for example, neurotrophin levels (Ickes et al., 2000; Pham et al., 1999), neurogenesis (Kempermann et al., 1998), and gliosis (Soffié et al., 1999). Thus, changes in these variables, rather than in synaptophysin levels, may have been responsible for the mnemonic improvements induced by continuous enrichment. This possibility remains to be examined in future studies.

In conclusion, the results of the present study demonstrate for the first time that different types of enrichment treatment have widely discrepant effects on spatial memory and synaptophysin levels in aged male mice. These data suggest that only continuous enrichment can effectively reduce age-related spatial memory deficits and affect synaptophysin levels in aged males, which contradicts our previous report illustrating that daily enrichment benefited both spatial memory and synaptophysin levels in aged female mice. Future work directly comparing the effects of enrichment on aged males and females will help resolve this inconsistency. Nevertheless, this study illustrates that not all enrichment treatments can reduce age-related memory impairments, and that the type of enrichment should be considered carefully when planning such studies in aging rodents.

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