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Synaptic correlates of memory and menopause in the hippocampal dentate gyrus in rhesus monkeys

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Abstract

Aged rhesus monkeys exhibit deficits in hippocampus-dependent memory, similar to aging humans. Here we explored the basis of cognitive decline by first testing young adult and aged monkeys on a standard recognition memory test (delayed nonmatching-to-sample test; DNMS). Next we quantified synaptic density and morphology in the hippocampal dentate gyrus (DG) outer (OML) and inner molecular layer (IML). Consistent with previous findings, aged monkeys were slow to learn DNMS initially, and they performed significantly worse than young subjects when challenged with longer retention intervals. Although OML and IML synaptic parameters failed to differ across the young and aged groups, the density of perforated synapses in the OML was coupled with recognition memory accuracy. Independent of chronological age, monkeys classified on the basis of menses data as peri/post-menopausal scored worse on DNMS, and displayed lower OML perforated synapse density, than pre-menopausal monkeys. These results suggest that naturally occurring reproductive senescence potentially influences synaptic connectivity in the DG OML, contributing to individual differences in the course of normal cognitive aging.

Keywords

delayed nonmatching-to-sample; disector method; estrogen; hippocampus; menopause; outer molecular layer; perforated synapse; post-synaptic density; recognition memory

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1. Introduction

Memory loss is a hallmark of Alzheimer's disease thought to reflect, in part, structural alterations in the perforant path (Hyman et al., 1984; Flood et al., 1987; Cabalka et al., 1992; Morrison and Hof, 1997), an excitatory projection from the entorhinal cortex to the dentate gyrus (DG) of the hippocampus (Witter et al., 1989; Lambert and Jones, 1990). Humans and nonhuman primates can also experience cognitive impairment in the absence of neurodegenerative disease, in association with normal aging and menopause-related estrogen deficiency (Sherwin, 1988; Rapp and Amaral, 1991; Peters et al., 1996; Roberts et al., 1997; Erickson and Barnes, 2003). Rhesus monkeys provide an attractive model for studying the basis of cognitive aging, because while they develop age-related hippocampal dysfunction (Presty et al., 1987; Moss et al., 1988), and undergo menopause similar to humans (Gilardi et al., 1997; Walker and Herndon, 2008), they fail to develop the neurodegenerative characteristics of Alzheimer's disease (Peters et al., 1999; Kimura et al., 2003).

Recognition memory, measured in monkeys by the delayed nonmatching-to-sample task (DNMS), requires the hippocampal formation and the perirhinal cortex (Mishkin, 1978; Squire and Zola-Morgan, 1991). DNMS performance is vulnerable to age-related decline (Presty et al., 1987; Moss et al., 1988), although some aged monkeys score as accurately as young adults (Rapp and Amaral, 1991). In rhesus monkeys, hippocampal volume remains stable during aging and fails to correlate with task acquisition or accuracy across delays in DNMS (Shamy et al., 2006). A recent magnetic resonance imaging study, however, reported that the DG is particularly vulnerable to normal aging, and that energy metabolism in this region is correlated with DNMS performance (Small et al., 2004). Given that DG granule cell number remains stable in the aged monkey (Peters et al., 1996), we targeted structural alterations in DG synapses and their potential role in cognitive aging.

The current study tested the possibility that recognition memory in the aged monkey is coupled with synaptic morphology in the DG. Additionally, we investigated the influence of reproductive senescence on DG synaptic measures and associated deficits in recognition memory. In order to explore the potential circuit specificity of observed changes, we compared the outer two thirds (OML) and inner one third (IML) of the DG molecular layer, which receive multimodal, associational input from the entorhinal cortex (Witter et al., 1989), and intrinsic projections from the DG hilus (Kondo et al., 2008), respectively. Three primary morphological measures were derived: synapse density, postsynaptic density (PSD) length, and density/percentage of perforated synapses. These measures were selected based on previous studies of the monkey prefrontal cortex, documenting an age-dependent decline in spine density that was reversed with estrogen treatment, predominantly involving plastic and motile small spines (Hao et al., 2006, 2007). Perforated synapses were of particular interest because of their association with hippocampus-dependent spatial memory in aged rats (Geinisman et al., 1986a, 1986b; Nicholson et al., 2004). Results of the present study are the first to document a morphological correlate of recognition memory in rhesus monkeys that is affected by naturally occurring reproductive senescence.

2. Methods

2.1. Animals

Subjects comprised 6 young adult (age range, 9–14 years old, mean=10.8 years old; 4 females, 2 males) and 12 aged (age range, 22–35 years old, mean=30.3 years old; 10 females, 2 males) rhesus monkeys (*Macaca mulatta*). The average life span of captive rhesus monkeys is under 25 years and human age equivalence is estimated at 1:3 (Tigges et al., 1988). By this ratio rhesus monkeys undergo menopause very late in life relative to

humans (Gilardi et al., 1997; Walker and Herndon, 2008). No subjects participated in previous invasive or pharmacological studies expected to influence the cognitive or neurobiological measures examined here. Monkeys were housed in colonies of ~ 40 individuals at the California National Primate Research Center, University of California, Davis. Visual inspection for vaginal bleeding was conducted daily for 2 years prior to perfusion. Normal cycle length (interval between the first days of menses) in young adult rhesus macaques is approximately 24–34 days (Gilardi et al., 1997). Because vaginal bleeding may have escaped detection in some cases, we adopted a more liberal cycle length criterion of 24–45 days (average cycle length in the last 12 months) to classify monkeys as eumenorrheic (regular menstruation). Average cycle lengths outside these values were classified as oligomenorrheic (irregular menstruation), or amenorrheic (absence of menstruation) in cases where monkeys had 2 days or less of vaginal bleeding in the last 12 months of observation. Eumenorrheic monkeys were classified as pre-menopausal. The transition between peri- and post-menopausal status is difficult to pinpoint with precision, and here we adopted the convention of earlier studies (Gilardi et al., 1997; Roberts et al., 1997), classifying oligomenorrheic and amenorrheic aged monkeys together as peri/post-menopausal. All experiments were conducted in compliance with the National Institutes of Health Guidelines for the Care and Use of Experimental Animals and approved by the Institutional Animal Care and Use Committee at the University of California, Davis.

2.2. Delayed nonmatching-to-sample test

Delayed nonmatching-to-sample (DNMS) testing was conducted in a manual apparatus as described in detail elsewhere (Rapp and Amaral, 1991; Rapp et al., 2003). Briefly, a sample object was placed over a food reward in the central well of the test tray, and after a response, an opaque barrier was lowered to impose the retention interval. The sample item was subsequently presented together with a novel object that covered a reward. Objects were drawn from a large pool such that a new pair was presented on each trial. Throughout the trials, a one-way mirror hid the experimenter from view when monkeys responded, and white-noise masked extraneous sounds. During the acquisition phase, monkeys learned the nonmatching rule of the task with a 10 sec retention interval, to a criterion of 90% correct or better (across 100 trials, 20 trials/day, inter-trial interval = 30 sec throughout testing). Once monkeys reached the criterion, recognition memory was challenged by imposing successively longer delays of 15, 30, 60, 120 sec (100 trials total at each delay, 20 trials/day), and 600 sec (50 trials total, 5 trials/day). Monkeys remained in the test chamber during all retention intervals.

2.3. Perfusion and tissue processing

At the conclusion of behavioral assessment, animals were deeply anesthetized with ketamine hydrochloride (25 mg/kg) and pentobarbital (20–35 mg/kg, i.v.), intubated, and mechanically ventilated. The chest was opened to expose the heart and 1.5 ml of 0.5% sodium nitrate was injected into the left ventricle. The descending aorta was clamped, and animals were perfused transcardially with cold 1% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.2) for 2 min, followed by 4% paraformaldehyde in 0.1 M PB at 250 ml/min for 10 min. Fixative flow rate was then reduced to 100 ml/min for 50 min. Following perfusion, the brain was removed from the skull and dissected, taking care to include the entire hippocampal region in a single block. The hippocampal block from the right side of the brain was postfixed for 6 hr in 4% paraformaldehyde in 0.1 M PB with 0.125% glutaraldehyde, washed in 0.1 M PB, and cut into 400 μ m thick sections on a vibratome (Leica, Nussloch, Germany). Freeze substitution and low-temperature embedding of the specimens were performed as described previously (Chaudhry et al., 1995). Briefly, slices were cryoprotected by immersion in increasing concentrations of glycerol (10, 20, and 30%) in 0.1 M PB, followed by plunging in liquid propane cooled by liquid nitrogen (-190°C) in

a Universal Cryofixation System KF80 (Reichert-Jung, Vienna, Austria). Samples were immersed in 1.5% uranyl acetate in anhydrous methanol (-90°C , 24 hr) in a cryosubstitution Automatic Freeze-Substitution System unit (Leica, Vienna, Austria). The temperature was gradually raised in steps of 4°C/hr from -90 to -45°C . The samples were washed with anhydrous methanol and infiltrated with Lowicryl HM20 resin (Electron Microscopy Sciences, Fort Washington, PA) at -45°C with a progressive increase in the ratio of resin to methanol for 1 hr each, followed by 100% Lowicryl overnight. Polymerization was performed with UV light (360 nm) at -45°C for 48 hr, followed by 24 hr at room temperature. Six or more consecutive ultrathin sections were cut into 90 nm-thick sections using a Diatome diamond knife (Electron Microscopy Sciences) and mounted on each formvar-supported slot grid (Electron Microscopy Sciences) for disector analysis. The thickness was later confirmed to be accurate, using Small's technique of minimal folds (88.9 ± 2.2 nm; Small, 1968). Images were examined at 75 kV with a Hitachi H-7000 transmission electron microscope (Hitachi High Technologies America, Inc., Pleasanton, CA). Fifteen sets of three serial sections were captured at $12,000\times$ magnification for each DG subregion (OML and IML) in each animal, using an AMT Advantage CCD camera (Advanced Microscopy Techniques, Danvers, MA). For validations of PSD length and perforated synapse density measurements, a slot grid with 16 consecutive sections was examined (described below). In order to ensure that images were taken from the correct subregion, the thickness of the entire molecular layer was measured. Images for the IML were only acquired from an area well within one third of the entire height of the molecular layer from the border of the granule cell layer (Kondo et al., 2008). OML images were only taken from the remaining two thirds of the molecular layer thickness (Witter et al., 1989). Fields containing somata and blood vessels were excluded. The electron micrographs were adjusted for brightness and sharpness using Adobe Photoshop (version 7.0.1 Adobe Systems Inc., San Jose, CA, USA), and then imported into PowerPoint, Microsoft Office, 2003 to make composite plates.

2.4. Quantitative analyses of synapse density, PSD length, and perforated synapses

Unbiased quantification of synapse density was conducted as previously described, based on a disector analysis of serial ultrathin sections (Sterio, 1984; de Groot et al., 1986; Tigges et al., 1996; Adams et al., 2001). Earlier studies (Calhoun et al., 2004; Makris et al., 2010), including an analysis of animals from the same study population examined here (Shamy et al., 2006), have independently confirmed that the volume of the hippocampus remains stable during aging in rhesus monkeys. Also, there are no differences in hippocampal volume by gender (Shamy et al., 2006) or with surgical menopause (ovariectomy) in young or aged rhesus monkeys (Hao et al., 2003). Based on these studies, it is assumed that there are no changes in the volumes of the regions in which the densities are measured and that differences in density are directly proportional to changes in total numbers. In addition, the primary focus of the present experiments was to determine the influence of age and endocrine decline on the relative representation of synaptic sub-types in the DG. On the basis of these considerations, synaptic density was selected as the appropriate measure of interest. The PSD was used as the counting unit. The physical disector approach samples objects in proportion to their number, independent of size, shape, or orientation of the PSDs. Fifteen sets of 2 serial sections from each animal and subregion were analyzed (total volume examined = $12,144 \mu\text{m}^3$). One was considered the reference and the other the look-up section (Fig. 1). Only asymmetric axospinous synapses present in the reference, but not the look-up, were counted. This procedure was repeated, switching the reference and the look-up sections. The criteria defining axospinous PSDs included the presence of synaptic vesicles in the axon terminal and a distinct asymmetric density in the postsynaptic dendritic spine. Because this approach considers only the leading edge of the PSD, it fails to distinguish

between macular (non-perforated) and perforated subtypes, and both were included in the synapse density measure.

PSD length was selected as a proxy for spine head size, since the two are strongly correlated (Harris and Stevens, 1989). Spine head size was of interest because small spines are relatively more motile and plastic (Kasai et al., 2003; Holtmaat et al., 2005), whereas larger spines have greater glutamate AMPA receptor representation and are more stable (Nusser et al., 1998). For PSD length measurements, we first examined approximately 100 axo-spinous synapses in a series of 16 consecutive sections to determine the longest PSD length within each synapse, and the average number of sections the PSDs spanned. The ruler tool in Photoshop (version 7.0.1 Adobe Systems Inc.) was used to measure all PSDs, and the proctor tool was employed for curved PSDs. We found that, on average, PSDs in the DG molecular layer spanned 3.2 sections, and that the longest length in 3 sections underestimated the “true longest PSD length”, derived from all 16 sections, by only 5.5%. Therefore, PSD measurements in the present analysis utilized 15 sets of 3 serial sections from each animal and each subregion (total volume examined = $18,216 \mu\text{m}^3$). First, all asymmetric axo-spinous synapses in the middle section (section 2) were identified. Then, for each synapse, the longest PSD in 3 sections was identified and measured (Fig. 1). Thus, for PSDs spanning all 3 sections, the longest of the 3 was measured, while for PSDs spanning 2 sections (sections 1/2 or 2/3), the longer of the 2 was measured. For the smallest class of axo-spinous synapses whose PSDs were only present in section 2, measurements were taken on section 2. Although this method of measuring PSD lengths results in a 5.5% underestimate of true PSD length, it applies across all animals and is unlikely to systematically bias the outcome of group comparisons. For perforated synapses, the lengths of all PSD segments were summed and total length was used in the statistical analyses. Approximately 400 PSDs were measured per animal and subregion (total of 6987 synapses in the OML and 6386 synapses in the IML).

Perforated synapses were defined by the presence of a discontinuity in the PSD of at least 25 nm. For perforated synapse counts, we first examined 23 axo-spinous perforated synapses in a single series of 16 consecutive sections. Perforated synapses in the DG molecular layer spanned an average of 5 sections, with the largest extending across 10. Because perforated synapses in the monkey DG molecular layer are scarce relative to other cortical regions and the DG of other species (unpublished observations), it was not practical to image the number of 10+ serial section sets necessary to derive an estimate of absolute perforated synapse density. Instead, similar to the PSD length analysis, 15 sets of 3 serial sections for each animal and each subregion were used to determine the area density and percentage of perforated synapses (total volume examined = $18,216 \mu\text{m}^3$). The area density of perforated synapses was derived by counting the number of PSDs in the middle section (section 2) that were perforated in any of the 3 sections, divided by the section 2 area. This method was superior to evaluating single sections, because while 46% of perforated synapses appeared non-perforated when one section was examined (perforation present in other sections), only 21% observed in full 16-section series escaped detection when the analysis used 3 serial sections. Although this method significantly underestimated area density, the size of perforated synapses, measured by PSD length, provided a basis for testing whether the degree of underestimation was similar across animals. The percentage of perforated synapses was calculated as the number of PSDs in the middle section (section 2) that appeared perforated in any of the 3 sections, divided by the total number of PSDs in section 2.

2.5. Statistical analyses

Statistical analyses were performed using SPSS 11.0 (SPSS Inc., Chicago, IL). The behavioral and morphological data were normally distributed ($p > 0.5$, one-sample

Kolmogorov-Smirnov test), and accordingly, parametric statistics were used to test for potential differences across groups. Preliminary analyses examined possible age or gender effects on behavioral and morphological measures using two-way analyses of variance (ANOVA). No gender differences ($p>0.05$) or age $\times$ gender interactions ($p>0.05$) were observed for any of the outcome variables. Thus adopting the approach of earlier studies that have similarly failed to find sex differences on standard tests of learning and memory (Moss et al., 1988, 1997; Herndon et al., 1997; Small et al., 2004; Shamy et al., 2006; Alexander et al., 2008), the results were collapsed across gender. Repeated measures ANOVA assessed possible age effects on DNMS performance across increasing retention intervals (15, 30, 60, 120, and 600 sec). One-way ANOVA was used to determine whether behavioral or morphological measures differed by age. The data were further explored by analysis of covariance (ANCOVA), controlling for the influence of chronological age, in order to evaluate potential effects of menses status on behavior and synaptic morphology in female monkeys. To determine if the frequencies of different-sized dendritic spines, as measured by PSD length, differed between groups, the cumulative frequency distribution of PSD length was compared using the Kolmogorov-Smirnov test. Pearson correlations evaluated the relationships between morphological indices in the OML and IML with DNMS acquisition and delay performance. Chronological age among the monkeys examined followed a bimodal distribution, and accordingly, non-parametric Spearman rho correlations were computed to examine associations between this parameter and the morphological indices. The α level for statistical significance was set at 0.05. Observed power was calculated in ANOVA to confirm that the sample size was sufficient to support the data.

3. Results

3.1. Behavioral characterization

Results from the DNMS test of visual recognition memory were similar to many previous reports (e.g., Rapp et al., 2003; Shamy et al., 2006). Aged monkeys ($n=12$; mean $\pm$ S.E.M.; 873.58 ± 125.42) required significantly more trials than young adults ($n=6$; 174.00 ± 49.64) to acquire the DNMS task initially with a 10-sec delay (Fig. 2A; one-way ANOVA; main age effect; $F_{(1, 16)}=14.567$, $p=0.002$, observed power=0.947). When memory was subsequently challenged with successively longer delays (15–600 sec), repeated measures ANOVA revealed main effects of age (Fig. 2B; main age effect; $F_{(1, 16)}=10.245$; $p=0.006$; observed power=0.852), and delay (delay effect; $F_{(4, 64)}=39.650$; $p<0.00001$; observed power=1.000), but no age by delay interaction ($p>0.05$).

In order to test whether DNMS acquisition or delay performance varied as a function of menses status, subsequent analysis compared data for pre- ($n=6$) and peri/post-menopausal ($n=8$) females. Pre-menopausal monkeys had their last observed menses within a month prior to perfusion, and they displayed an average ($\pm$ S.E.M.) of 9.33 ± 0.80 menstrual cycles during the final 12 months of the experiment, consistent with previous normative data (Roberts et al., 1997). In contrast, peri/post-menopausal monkeys exhibited only 1.50 ± 0.71 cycles during the same period, with some having no recorded menses for 2 years or more prior to euthanasia. All 4 young and 2 aged females were premenopausal, whereas the remaining 8 aged females were peri/post-menopausal.

Because menopausal status was confounded with age, we examined the influence of reproductive senescence on DNMS by ANCOVA, including age as a covariate. Controlling for age, pre- and peri/post-menopausal females learned the DNMS task at equivalent rates (Fig. 2C; ANCOVA; covariate=age; $F_{(1, 12)}=0.977$; $p>0.05$). An analysis of accuracy on the delay portion of the task, in contrast, revealed significant main effects of menses status (Fig. 2D; repeated measures ANCOVA; covariate=age; menses effect; $F_{(1, 12)}=5.278$, $p=0.042$, observed power=0.554), and delay (delay effect; $F_{(4, 48)}=4.520$, $p=0.004$, observed

power=0.916), with no menses status by delay interaction ($p>0.05$). Using accuracy averaged across delays as a summary measure, aged pre-menopausal monkeys (ages: 25 years 8 months, 32 years 9 months) scored better than any aged peri/post-menopausal subject (age range: 22 years 9 months to 34 years 8 months, $n=8$), and well within the range of young adults (average DNMS accuracy $\pm$ S.E.M.: young females, 87.73 ± 2.44 ; aged pre-menopausal, 84.0 and 84.4 ; aged peri/post-menopausal, 77.44 ± 1.24). Indeed, recognition memory accuracy among the two pre-menopausal aged monkeys was numerically superior to scores for the peri/post-menopausal group at all individual retention intervals longer than 15 s (Fig. 2D; mean $\pm$ S.E.M.: 30 s, aged pre-menopausal= 88.00 , 88.00 , aged peri/post-menopausal= 81.59 ± 1.39 ; 60 s, aged pre-menopausal= 86.00 , 92.00 , aged peri/post-menopausal= 79.75 ± 1.98 ; 120 s, aged pre-menopausal= 85.00 , 85.00 , aged peri/post-menopausal= 77.80 ± 2.46 ; 600 s, aged pre-menopausal= 70.00 , 72.00 , aged peri/post-menopausal= 63.00 ± 2.42). Together the results suggest that the effects of aging on recognition memory are more strongly linked to reproductive senescence than chronological age, per se.

3.2. Synaptic morphology in the aged DG

There was a high density of axo-spinous synapses on small dendritic spines in the DG molecular layer in all monkeys, independent of age, sex, and menses status. We first evaluated whether morphological indices in the OML and IML differed between young and aged monkeys. A one-way ANOVA failed to reveal an effect of age on synapse density ($F_{(1, 16)}=3.20$), PSD length ($F_{(1, 16)}=0.52$) and perforated synapse percentage ($F_{(1, 16)}=0.384$) and area density ($F_{(1, 16)}=1.523$) in the OML (Fig. 3; $p>0.05$ for all analyses). Similarly, a one-way ANOVA showed a lack of an age effect on synapse density ($F_{(1, 16)}=0.00$), PSD length ($F_{(1, 16)}=4.068$) and perforated synapse percentage ($F_{(1, 16)}=0.277$) and area density ($F_{(1, 16)}=0.019$) in the IML ($p>0.05$ for all analyses; Supp. Fig. 1). We also tested for potential age-dependent change in the frequency of different-sized dendritic spines, as reflected by PSD length. Results of a Kolmogorov-Smirnov test indicated that the cumulative frequency distributions of PSD length were equivalent across groups ($p>0.05$), suggesting that the relative proportions of small and large dendritic spines are similar in young and aged monkeys.

3.3. Menses status effect on DG morphology

Prompted by the observation that recognition memory declines in association with menopause, next we tested for corresponding effects on DG synaptic morphology. A one-way ANCOVA indicated that, after controlling for chronological age, the density of perforated synapses in the OML was significantly higher in pre- (covariate-corrected mean $\pm$ S.E.: 15.78 ± 1.94) than peri/post-menopausal (7.04 ± 1.60) monkeys (Fig. 4A; ANCOVA; covariate=age; menses effect; $F_{(1, 12)}=8.974$, $p=0.012$, observed power=0.778). Consistent with the conclusion that this effect occurred independent of aging, per se, chronological age failed to correlate with perforated synapse density in the OML (Fig. 5; Spearman rho correlation; $p>0.05$). Indeed, one of the aged pre-menopausal monkeys (age: 25 years 8 months) displayed the highest OML perforated synapse density of all subjects examined, young or old, and the other (age: 32 years 9 months) had the third highest density in the aged group ($n=12$). By contrast, two of the youngest peri/post-menopausal subjects (ages: 22 years 9 months, 26 years 3 months) displayed the lowest OML perforated synapse density of any monkey examined (Fig. 5). Although the number of males available for analysis was limited (2 young adult and 2 aged), average values among these subjects most closely approximated results for the peri/post-menopausal group (mean number of perforated synapses per $1000\text{ }\mu\text{m}^2\pm$ S.D.: pre-menopausal= 14.32 ± 4.04 ; peri/post-menopausal= 8.14 ± 3.60 ; males= 9.07 ± 2.22). A one-way ANCOVA also showed that the corresponding percentage of the total synapse population in the OML that was perforated was significantly

higher in pre- (covariate-corrected mean $\pm$ S.E.: 8.27 $\pm$ 1.09) versus peri/post-menopausal (3.21 $\pm$ 0.90) monkeys (Fig. 4B; ANCOVA; covariate=age; main menses effect; $F_{(1, 12)}=9.589$, $p=0.010$, observed power=0.805). There were no age, sex, or menses status effects on the size distribution of OML perforated synapses, as reflected in PSD length (ANOVA; $p>0.05$, data not shown). Overall synapse density (Fig. 4C; $F_{(1, 12)}=0.366$) and PSD length (Fig. 4D; $F_{(1, 12)}=3.00$) in the OML failed to differ between pre- and peri/post-menopausal females (ANCOVA; $p>0.05$). None of the morphological measures (synapse density, PSD length, and density/percentage of perforated synapses) in the IML differed significantly across the pre- and peri/post-menopausal groups (Supp. Fig. 2; ANCOVA; $p>0.05$ for all analyses).

3.4. Relationships between DG morphology and recognition memory

Potential relationships between DG synaptic indices and recognition memory were evaluated by a bivariate correlation approach. We first examined the relationship between DNMS acquisition and OML morphological measures. None of these parameters significantly correlated with the number of trials required to learn the task with a 10 sec delay (Pearson correlation, $p>0.05$, data not shown). There was, however, a trend toward an inverse correlation between DNMS acquisition and OML synapse density (Pearson correlation; $r=-0.434$, $p=0.072$), suggesting a potential association between increasing OML synapse density and rapid DNMS acquisition (data not shown). None of the IML morphological measures correlated with DNMS acquisition (Pearson correlation; $p>0.05$, data not shown).

Next, we examined potential synaptic relationships with behavior by evaluating the morphological measures in comparison with recognition accuracy, averaged across successively more challenging retention intervals (15 – 600 sec). This analysis revealed a significant positive correlation between the density of OML perforated synapses per 1000 μm^2 and DNMS accuracy (Fig. 6A; Pearson correlation; $r=0.489$, $p=0.039$). None of the other correlations, computed for either the OML (Fig. 6B–D) or IML (data not shown) for all subjects considered together, were statistically significant (Pearson correlation; $p>0.05$). Because perforated synapses are generally large, with long combined PSD lengths, we tested whether DNMS performance might be correlated with the proportion of all large synapses, rather than the perforated subset specifically. No correlation, however, was found between the percentage of OML large synapses (% of synapses with PSD length over 400 nm) and DNMS accuracy averaged across delays (Pearson correlation; $p>0.05$, data not shown).

4. Discussion

The present report provides novel evidence that, beyond the influence of chronological age, menopause in rhesus monkeys is coupled with recognition memory impairment and an associated shift in synaptic representation in DG OML. In particular, our findings suggest that the decrease in estrogen that accompanies menopause may impact memory at least in part by promoting the selective loss of perforated synapses that support enhanced synaptic efficacy (Geinisman et al., 1993). The behavioral and morphological findings we report have important implications for the development of novel hormone replacement therapies aimed at supporting cognitive health in postmenopausal women.

4.1. Methodological considerations

Menses status was determined by recording vaginal bleeding daily for two years until the day of the perfusion. Since monkeys can have break-through bleeding without ovulation, or experience light menstruation that escapes detection, our method for tracking reproductive senescence was not as precise as assaying ovarian hormones in daily urine or serum

samples. Earlier evidence from the same animal population, however, indicates that menstrual bleeding patterns accurately reflect ovarian activity; most eumenorrheic monkeys are pre-menopausal, and most oligomenorrheic and amenorrheic monkeys are peri/post-menopausal when confirmed by hormonal data (Gilardi et al., 1997). Pre- and peri/post-menopausal monkeys in the present study displayed average numbers of menstrual cycles per year consistent with an earlier analysis in rhesus monkeys that also included direct urinary endocrine measurements (Roberts et al., 1997). This background provides substantial confidence that the criteria applied here accurately classified the pre- or peri/post-menopausal groups.

Perforated synapses are morphologically characterized by a discontinuity in the PSD, and substantial evidence points to their important role in memory-related plasticity (Geinisman et al., 1991; Toni et al., 2001). They can be further divided on the basis of morphology into three subtypes: fenestrated (hole or holes in the PSD), horseshoe-shaped (PSD shaped like a horseshoe), and segmented (PSD consisting of 2–8 separate plates) (Geinisman et al., 1992a). Although the approach used here did not permit a comprehensive sub-type classification, it proved substantially superior to single-section methods for estimating the area density of perforated synapses. While underestimating this parameter, because there was no difference in perforated synapse size by age, sex, or menses status, the degree of underestimation was equivalent across groups. Using 3 serial sections, fenestrated perforated synapses were the most likely to escape detection, since often they are perforated only in one or two sections. In contrast, because segmented perforated synapses have multiple, completely separate PSD plates (Geinisman et al., 1993), all segmented synapses were detected by the approach used here. Our sensitivity for detecting this synaptic subtype is noteworthy in light of previous evidence documenting a selective increase in the number of segmented synapses in rat DG after induction of long-term potentiation (Geinisman et al., 1993).

4.2. Lack of age effect on synaptic measures in the OML and IML

The monkeys examined in this study displayed age-dependent impairments in DNMS acquisition and accuracy across delays, similar to previous reports (Rapp et al., 2003; Shamy et al., 2006). In contrast to our recent study in monkey dorsolateral prefrontal cortex that revealed a significant age-related decrease in synapse density and a shift in the distribution of dendritic spine size (Dumitriu et al., 2010), axo-spinous synapse density, PSD length or percentage/density of perforated synapses in the DG OML and IML failed to differ between young and aged groups. However, these observations are consistent with earlier reports that also employed the unbiased disector approach in monkey DG, and showed no correlation between OML or IML morphological measures and chronological age (Tigges et al., 1995, 1996). The number of subjects available for analysis is typically limited in nonhuman primate studies, and Tigges et al., for example, examined 10 subjects spanning the entire adult life span of this species (4–35 years old). In rodents, there is no age-related loss of DG granule cell number (Calhoun et al., 1998; Gallagher et al., 2003; Rapp and Gallagher, 1996; Rasmussen et al., 1996) or synaptic proteins, even in cognitively-impaired subjects (Nicolle et al., 1999). However, an unbiased double-disector study showed age-dependent decreases in both perforated and non-perforated axo-spinous synapse numbers per granule cell in the rat DG molecular layer (Geinisman et al., 1992b; Geinisman et al., 1995). There is precedent for differences between rats and monkeys in the propensity for morphological plasticity. The capacity for estrogen-induced increase in CA1 dendritic spine number is retained during aging in rhesus monkeys, for example, but markedly attenuated in aged rats (Adams et al., 2001; Hao et al., 2003).

4.3. Correlations between OML synaptic measures and DNMS

This study provides the first set of morphological correlates of recognition memory in rhesus monkeys. Significant correlations were found between the representation of perforated synapses in the OML and DNMS accuracy. These results are consistent with rodent studies, reporting that the number of perforated synapses is decreased in aged rats with impaired hippocampus-dependent spatial memory, and that this loss correlates with the degree of memory impairment (Geinisman et al., 1986a, 1986b). Although perforated synapses are generally found on large mushroom-shaped dendritic spines, we did not find a correlation between DNMS accuracy and the proportion of large synapses, as measured by PSD length. These findings suggest that perforated synapses comprise a distinctive synaptic population important for recognition memory dependent on the hippocampal region. It is also clear, however, that a complete account of age-related decline in recognition memory will involve neurobiological parameters and circuits beyond those considered here.

Overall OML synapse density, including both macular and perforated synapses, tended to vary inversely with DNMS acquisition such that monkeys with higher values learned DNMS more quickly. In an independent study of the same monkeys examined here, synapse density in area 46 of the prefrontal cortex showed a significant inverse correlation with DNMS acquisition (Dumitriu et al., 2010), suggesting that both area 46 and the hippocampus may be importantly engaged under conditions that emphasize the acquisition of novel task rules.

None of the synaptic measures collected from the IML correlated with DNMS acquisition or delay performance. This pattern of selectivity is consistent with other studies suggesting that morphological effects of aging observed in the DG preferentially target perforant path inputs from the entorhinal cortex, largely sparing intrinsic projections to the IML (Smith et al., 2000). Related evidence from people similarly points to the likely involvement of perforant path disruption in normal cognitive aging (Yassa et al., 2010).

4.4. Menses status effect on recognition memory and OML perforated synapse density

Average DNMS scores were significantly higher in pre-menopausal than peri/post-menopausal monkeys, even after controlling for the influence of age. Although only two aged pre-menopausal monkeys were available, both scored more accurately than any of the aged peri/post-menopausal monkeys. Together, our findings suggest that the influence of aging on recognition memory is more closely associated with reproductive senescence than chronological age, per se. Additionally, aged individuals that continue to have menstrual cycles may be more resistant to memory decline. These observations are consistent with our previous work showing that cyclic estrogen administration moderately improves DNMS delay performance in ovariectomized aged monkeys (Rapp et al., 2003). Longitudinal studies in larger numbers of age-matched pre- and peri/post-menopausal monkeys will be needed to document the degree to which menopause accounts for the recognition memory impairment frequently reported in aged monkeys (Presty et al., 1987; Moss et al., 1988, 1997; Herndon et al., 1997; Small et al., 2004; Shamy et al., 2006; Alexander et al., 2008; Dumitriu et al., 2010). Nonetheless, given the striking parallels in reproductive physiology and patterns of endocrine senescence across humans and nonhuman primates (Matt et al., 1998; Gill et al., 2002; Woller et al., 2002; Nichols et al., 2005; Walker and Herndon, 2008), our initial observations have potentially important implications for research on menopause-associated cognitive decline in women.

Consistent with the proposal that ovarian hormone decline might regulate the trajectory of normal cognitive aging, our results are among the first to demonstrate that synaptic morphology in the aged monkey hippocampus varies in relation to reproductive senescence. Specifically, here we found that the density and proportion of OML perforated synapses is

significantly higher in pre- compared to peri/post-menopausal monkeys. Notably, the difference observed as a function of menses status remained significant even when the influence of age was statistically controlled. In the present investigation, all young adult and two aged females were pre-menopausal. Future studies in a larger number of age-matched pre- and peri/post-menopausal monkeys will help determine the interactive influence of aging and ovarian senescence on perforated synapse density in the OML. Interestingly, the two young and two aged male monkeys examined in the present analysis displayed OML perforated synapse densities equivalent to those of the peri/post-menopausal group. Taken together, these findings suggest a significant role for estrogen in maintaining OML perforated synapses that contribute to recognition memory. Although the underlying mechanisms are yet to be determined, substantial interest centers on glutamate NMDA receptors. Estradiol-mediated increases in CA1 dendritic spine density require NMDA receptor activation (Woolley and McEwen, 1994; Woolley et al., 1997), and estradiol treatment in ovariectomized rats up-regulates NMDA receptor NR1 subunit protein concentration in DG granule cells (Gazzaley et al., 1996a). Furthermore, compared to young adults, aged monkeys exhibit a significant decrease in NR1 expression, selectively in the OML (Gazzaley et al., 1996b). Estrogen may also help maintain OML perforated synapses by upregulating glutamate AMPA receptors (Liu et al., 2008; Zadran et al., 2009), which are preferentially expressed in these synapses compared to non-perforated ones (Ganeshina et al., 2004). A comprehensive accounting will valuably inform the development of novel hormone replacement strategies aimed at supporting cognitive health in postmenopausal women.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Electron micrographs illustrating the disector method for synapse density, measurements of postsynaptic density (PSD) length, and criteria for perforated synapse counts in the dentate gyrus molecular layer. (Top) For synapse density measurements, two serial sections were used. One section was considered the reference (1) and the other the look-up (2). Only asymmetric axo-spinous synapses that are present in the reference (shaded in yellow), but not the look-up were counted and divided by the disector volume. This procedure was repeated by switching the reference and the look-up. Synapses present in both the reference and the look-up are shaded in green. (Middle) For PSD length measurements, three serial sections were used. All asymmetric axo-spinous synapses in the middle section (2) were identified (shaded in different colors). For each synapse, the longest PSD in 3 sections was identified and measured. (Bottom) For perforated synapse counts, of PSDs present in section 2, ones that are perforated in any of the 3 sections were counted. In this example, the dendritic spine shaded in yellow has a PSD that is macular in sections 1 and 2, but perforated in section 3 (red arrow). Thus this PSD was counted as a perforated synapse. The percentage of perforated synapses was determined by the number of perforated synapses (as calculated above) divided by the total number of PSDs present in section 2. Scale bar, 500 nm.

Figure 2.

Acquisition and delay performance on delayed nonmatching-to-sample test (DNMS) by age and menses status. (A) Aged monkeys took significantly more trials to learn the task to a 90% correct criterion with a 10 sec delay interval than young adults. (B) Across longer DNMS retention intervals (15, 30, 60, 120 and 600 sec), accuracy in the aged group was significantly impaired compared to young. (C) DNMS acquisition did not differ significantly between pre- and peri/post-menopausal female monkeys when the influence of age was controlled. (D) Pre-menopausal monkeys scored significantly better across DNMS delays than peri/post-menopausal monkeys. Symbols indicate the scores for individual aged pre-menopausal monkeys. Otherwise, group results are expressed as mean $\pm$ standard error of mean. A, B. young, $n=6$ (4 females, 2 males); aged, $n=12$ (10 females, 2 males); C, D (females only). young pre-menopausal, $n=4$; aged pre-menopausal, $n=2$; aged peri/post-menopausal, $n=8$.

Figure 3. Morphological measures in the DG OML by age. Perforated synapse area density (A), percentage of perforated synapses (B), total axospinous synapse density (C), and postsynaptic density length (D) in the OML are expressed as mean $\pm$ standard error of mean. young, n=6 (4 females, 2 males); aged, n=12 (10 females, 2 males).

Figure 4. Morphological measures in the DG OML by menses status. Perforated synapse area density (A), percentage of perforated synapses (B), total axospinous synapse density (C), and postsynaptic density length (D) in the OML are expressed as covariate-corrected mean $\pm$ standard error. *p<0.05. pre-menopausal females, n=6; peri/post-menopausal females, n=8.

Figure 5. Scatter plot of the relationship between chronological age for individual monkeys and DG OML perforated synapse density (per 1000 μm^2). n=18.

Figure 6.

Morphological measures for individual subjects in the DG OML in relation to delayed nonmatching-to-sample (DNMS) delay performance. Perforated synapse area density (A), percentage of perforated synapses (B), density of all axospinous synapses (C), and postsynaptic density length (D) in the OML are plotted against memory scores (accuracy averaged across all retention intervals) on DNMS. A significant correlation was seen between the density of OML perforated synapses and DNMS performance. $n=18$.