Low CA1 Spine Synapse Density is Further Reduced by Castration in Male Non-human Primates

The hippocampus plays a major role in learning and memory and its morphology and function are readily affected by gonadal hormones in female non-human primates. We sought to determine whether the gonads also affect pyramidal cell spine synapse density in the CA1 hippocampal area of male primates. Unbiased electron microscopic stereological calculations were performed to determine the volumetric density of pyramidal cell spine synapses and semi-quantitative analyses on the surface density of glial fibrillary acidic protein-containing glia processes and the diameter of pyramidal cell apical dendrites in the CA1 area of intact and orchidectomized (1 month) St Kitts vervet monkeys (Chlorocebus aesthiops sabaeus). The volumetric density (number of spine synapse/µm³) of spine synapses was significantly lower (40%) in the orchidectomized animals than in control monkeys; conversely, the density of glia processes was significantly higher (15%) and the diameter of dendritic shafts located in this area was also larger (30%) in the orchidectomized animals than in the controls. Strikingly, when compared to female values, intact male primates had lower spine synapse densities than either intact or ovariectomized females. Since the primate hippocampus is very similar to that of a human’s, the present observations suggest that physiological levels of circulating androgen hormones are necessary to support normal spine synapse density in the CA1 stratum radiatum of human male hippocampus.

Keywords: electron microscopic unbiased stereological calculation, glial fibrillary acidic protein, orchidectomy, pyramidal cell dendrite, sexual differences, vervet monkey

Introduction

Recent data have demonstrated that experimental manipulations and physiological changes in the levels of circulating female gonadal hormones greatly influence the density of pyramidal cell spine synapses in the CA1 hippocampal subfield of both female rats (Gould et al., 1990; Woolley et al., 1990, 1997; Woolley and McEwen, 1992, 1993; Leranth and Shanabrough, 2001; Leranth et al., 2000) and monkeys (Leranth et al., 2002). Interestingly, the integrity of the male hippocampus also requires normal levels of circulating male hormones. For example, it has been shown that testosterone (T), but not estrogen (E) has a synaptoplastic effect on the hippocampus of gonadectomized male rats (Leranth et al., 2003). In addition, it is well established that a large population of neurons in the monkey hippocampus, similar to the rat hippocampus (Simery et al., 1990; Brown et al., 1995) contains androgen receptors (Pomerantz and Sholl, 1987; Choate et al., 1998; Abdelgadir et al., 1999; Roselli et al., 2001). In the rat hippocampus, androgen receptors appear to be primarily located in pyramidal neurons (Clancy et al., 1992; Kerr et al., 1995). Because both the rat and monkey hippocampus contain low levels of aromatase (MacLusky et al., 1994; Roselli et al., 2001), effects of T could be mediated by either actions on androgen receptors or conversion to E. Although major differences have been shown in the morphology and functional connections of the hippocampus between monkeys and rats, great similarities exist in this respect between monkeys and humans (e.g. Rosene and Van Hoesen, 1987), so the findings presented here may have significant implications for humans. Therefore, this study aimed to define whether hormonal manipulations influence synaptic plasticity in the hippocampus of male subhuman primates. Furthermore, we wished to address the question of whether the same hormonal manipulation influences the density of astroglia processes in the same brain region, as is seen in rats (Lam and Leranth, 2003).

Materials and Methods

Young adult (5–6 years; 5.6–6.3 kg) male (n = 6) St Kitts vervet monkeys (Chlorocebus aesthiops sabaeus) were used. The animals were housed in individual cages (water and regular monkey chow were provided in excess of nutritional needs) at the St Kitts Biomedical Research Foundation, St Kitts (West Indies). The facility is in full compliance with all applicable US regulations, and the treatment and care of these monkeys were in compliance with the Guide for the Care and Use of Laboratory Animals (1996) US Public Health Service, Washington, DC, National Academy Press. Experimental protocols were approved by the Institutional Animal Care and Use Committee of Yale University Medical School and Axion Research Foundation. All efforts were made to minimize discomfort and pain and the number of animals used. Half of the monkeys were anesthetized (ketamine sedation, 10 mg/ kg i.m., followed by 20 mg/kg pentobarbital, i.v.) and orchidectomized (GDX), under sterile conditions. One month later, before killing the animals (ketamine sedation 10 mg/kg i.m., followed by an overdose, 100 mg/kg of pentobarbital, i.v.), blood was collected and centrifuged; each sample was divided into three aliquots, placed into liquid nitrogen and shipped to Yale. To minimize measuring errors, serum T level was measured in each aliquot using radioimmunoassay; the sensitivity of the radioimmunoassay used is <0.2 ng/ml.

Thereafter, the chest was opened, the descending aorta clamped, the right atrium opened and after rinsing the vascular system via transcardial perfusion using a motorized peristaltic pump (Masterflex® L/S; Cole Palmer, San Francisco, CA) with 1000 ml 0.9% heparinized (1 unit/ml) saline, animals were further perfused for 10 min with 1500 ml of fixative containing 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.35; PB). The temperature of both solutions was 20°C. Brains were postfixed at 4°C for 2 h in the same fixative and stored in PB at 4°C. The hypothalamus and the nigrostriatal systems of these animals were used in other studies. The hippocampi were dissected out and divided into three equal pieces along its longitudinal axis. Thereafter, small 2–3 mm long blocks were cut (perpendicular to the longitudinal axis) out from the middle portion of these tissue samples and 50 and 200 µm thick sections were cut perpendicular to the longitudinal axis of the hippocampal formation on the vibratome for light microscopic immunostaining and electron microscopic statistical analyses, respectively.
Immunostaining for Glial Fibrillary Acidic Protein (GFAP)

In order to eliminate unbound aldehydes, the 50 μm vibratome sections were pretreated with 1% sodium borohydride in PBS for 30 min. After this, the sections were rinsed in PB (3 times 10 min), and glutaraldehyde (1% glutaraldehyde in 40 ml PB) for 10 min (Sigma, St Louis, MO) and washed 3 times in PB (10 min each). Thereafter, sections were incubated in the primary antibody – rabbit anti-GFAP (Dako, Carpinteria, CA), 1:1000 in PB containing 2% normal goat serum (ICN Biomedicals, Aurora, OH) and 0.5% Triton X-100 (Acros Organics, Fairlawn, NJ) – overnight at room temperature. Sections were washed 3 × 10 min in PB and further incubated in the secondary antibody – biotinylated goat anti-rabbit IgG (Vector Laboratories Burlingame, CA) 1:500 in PB for 2 h at room temperature – followed by another rinse in PB (3 × 10 min) and an incubation in the ABC Elite reagents (1:500; Vector Laboratories Burlingame, CA). After washing in PB (3 × 10 min), the tissue bound peroxidase was visualized using nickel–diaminobenzidine (Ni–DAB: 15 mg DAB, 12 mg ammonium chloride, 0.12 mg glucose oxidase, 600 μl 0.05 M nickel ammonium sulphate and 600 μl 10% H2O2) in 40 ml PB for 8 min. Sections were mounted on gelatin-coated slides, dehydrated and mounted in Permount. To ensure that sections taken from the gonadectomized and control animals were treated under identical conditions, they were incubated together throughout the procedures.

Light Microscopic Analysis

The stratum radiatum of the hippocampal CA1 subfield was examined. Data analysis was performed as described recently (Lam and Leranth, 2003). Briefly, from each GFAP-immunostained section (25/animal), 100 fields (including those from area located between the upper third of the CA1 stratum radiatum (300–500 μm from the pyramidal cell layer) with the observer blinded to the experimental treatment. As in the light microscopic analysis, the location of the first photograph was randomly selected. Thereafter, non-overlapping photographs were taken from the left and right-hand sides of the first photographed area. Potentially interfering structures, such as blood vessels, large dendrites or glial cells, were intentionally avoided. In order to obtain a comparable measure of synaptic numbers, unbiased for possible changes in synaptic size, the disector technique (Braendgaard and Gundersen, 1986) was used. Thus, the density of spine synapses (which were identified by the lack of mitochondria, but the presence of spine apparatus and dark post synaptic bar adjacent to the synaptic contact in the head of the spines (Gray type 2 synapses) and vesicle accumulation at the synaptic contact in the presynaptic bouton) of pyramidal cell apical dendrites was calculated with the help of a reference grid superimposed on the EM photographs. The disector volume (volume of reference) was the unit area of the reference grid multiplied by the distance between the upper faces of the reference and look-up sections. Section thickness (average 0.075 μm) was determined by using the electron scattering technique (Small, 1968). Only those spine synapses were counted that were present on the reference section, but absent in the corresponding look-up section and vice versa (Fig. 1). The measured synaptic density values were divided by the volume correction factor kv. This correction provided a synaptic density estimate normalized with respect to the density of pyramidal cells and also accounted for possible changes in hippocampal volume.

At least five neuropil fields were photographed on each electron microscopic grid. With at least eight grids from each vibratome section (containing a minimum of two pairs of consecutive, serial ultrathin sections), each animal provided 5 × 8 × 2 = 80 neuropil fields. Each pair (photos of reference and look-up sections) of photographs represented an 8.9 × 8.9 × 0.075 μm = 5.94 μm3 volume. As mentioned, from each monkey 80 photographs were analyzed that represented a 475 μm3 volume.

In addition to the spine synapse density calculation, a semiquantitative analysis was performed on the diameter of dendrites located in this area. On all prints used for this statistical analysis, the diameter of dendritic profiles (30 dendrites in randomly selected photos taken from each animal) were measured and compared.

Statistical Analyses

For all dependent variables (glia process density, dendritic diameter and spine synapse density), the means determined for each animal were used for the statistical analysis. The non-parametric F-test was used to test the difference in the ratios of means between groups and group means were compared using pairwise t-tests (two-tailed proba-
Figure 1. Electron micrographs demonstrate two consecutive serial sections: a reference (a) and look-up section (b). Only those spine synapses (arrows) were counted that were present in just one section. Scale bar = 1 μm.
A level of confidence of $P < 0.05$ in two-tailed tests was adopted. $P$ and $t$ values as well as the degrees of freedom (d.f.) are given in the figure legends. In the text, the mean ± SEM values are given.

**Results**

**Serum T Levels**

There was a major difference between the levels of circulating T in the GDX and control monkeys. In GDX monkeys, the mean T level was <0.2 ng/ml (the minimum sensitivity of radioimmunoassay is <0.2 ng/ml). In control monkeys, the average serum T level was 1.134 ± 0.016 ng/ml (Table 1), which is the same as we measured previously in the serum of same-age St Kitts male monkeys and very similar to the lower end of T levels measured in human men.

**Astroglia Process Density**

Astroglia processes could easily be detected due to the specific GFAP staining. Light microscopic observations already suggested a difference in the density of astroglial processes between the control and GDX monkeys. In the CA1 of GDX animals, the fiber density was increased compared to the control (Fig. 2). The semiquantitative analysis confirmed this optical impression (Fig. 3). In control animals, the mean density of glia processes was 108.8 ± 2.952/8000 µm², whereas in the castrated animals it was 124.8 ± 3.926/8000 µm² (Table 1). This relative increase (15%) in the density of glia processes was statistically significant ($P < 0.031$). In contrast, there was no significant difference in the density of GFAP-containing cell bodies in the CA1 regions of castrated and control animals (data not shown).

**Spine Synapse Density**

No significant differences were observed in the density of pyramidal cell nuclei between the control (840 ± 73.901/mm²) and GDX animals (832 ± 64.663/mm²; Table 1). Furthermore, in the electron microscope, no obvious qualitative differences could be observed by comparing the ultrastructure of CA1 stratum radiatum of control and GDX monkeys. However, surveying all of the electron micrographs, it appeared that the density of small glia processes is higher in the material depicted from GDX monkeys than in the control animals. Because of technical difficulties and the extremely time-consuming nature of a meaningful electron microscopic statis-
tical analysis on the density of these processes and having the data of the light microscopic surface density calculation of glia processes (see below), an unbiased electron microscopic stereological calculation was not performed.

The result of the unbiased electron microscopic stereological calculation on the spine synapse density demonstrated a major, significant ($P < 0.001$) difference in the density values of CA1 area pyramidal cell spine synapse density of the two groups. The total numbers of spine synapses/µm$^3$ volume of control monkeys were $259,825, 287,802,$ and $276,45$ (0.343 ± 0.00674/µm$^3$). Whereas, in GDX animals, these numbers were $176,675, 156,75$ and $163,875$ (0.578 ± 0.0171/µm$^3$), in the same volume (Table 1 and Fig. 4), which represents a 40% decrease.

**Diameter of Dendritic Shafts**

The semiquantitative analysis on the diameter of dendritic shafts in the outer third of the stratum radiatum of the CA1 subfield indicated that the average diameter of these dendrites is significantly ($P < 0.001$) larger in the GDX animals (1.322 ± 0.03 µm) than those (1.025 ± 0.018 µm) of control monkeys (Leranth et al., 2002). The enlargement of dendritic processes seen here in male monkeys may be due to a similar spine retraction.

**Technical Notes**

In spite of the 40% loss of spine synapses in the CA1 area of GDX monkeys, there was no apparent difference in the density of pyramidal and glia cells. Thus, a change in the volume of the hippocampus could be excluded. Two compensatory factors may be responsible for this phenomenon: the significant sprouting of glia processes and the significant, 30% enlargement of the diameter of pyramidal cell apical dendritic shafts. Since the increase in glia process density (15%) was much less than the decrease in the spine synapse density (40%), the remaining vacant space may have been filled up by the increased mass of dendrites. Enhancement of dendritic processes has been observed in female monkeys following OVX and this has been assumed to result from incorporation of the cytoplasm of spines that are retracted into the dendrites (Leranth et al., 2002). The enlargement of dendritic processes seen here in male monkeys may be due to a similar spine retraction.

**Discussion**

**Functional Considerations**

The major observation of this study is that, similar to gonadal hormone action on female rats (Gould et al., 1990; Woolley et al., 1990, 1997; Woolley and McEwen, 1992, 1993; Leranth and Shanabrough, 2001; Leranth et al., 2000), female monkeys (Leranth et al., 2002) and male rats (Leranth et al., 2003), experimental manipulation of gonadal hormone levels greatly influences the density of pyramidal cell spine synapses in the CA1 hippocampal subfield of male subhuman primates. Thus, it can be assumed that the male primate hippocampus, in addition to other gonadal hormone-targeted structures that have major roles in mnemonic processes, e.g. the mesencephalic...
dopamine system in conjunction with the prefrontal cortex (McCarthy et al., 1996; Goldman-Rakic, 1998), is a major participant in the system by which gonadal hormones influence mnemonic functions. However, the specific mnemonic consequences of the observed spine synapse density changes in male monkeys are unclear at the present time. In conjunction with the estrogenic facilitation of hippocampal LTP and neurogenesis seen in female rats (e.g. Wong and Moss, 1992; Warren et al., 1995; Tanapat et al., 1999), it has been assumed that the increased spine density reported during proestrus in cycling rats or in ovariectomized rats after exogenous estrogen should lead to improvements in learning and memory. Indeed, increased spine density in the CA1 region of the hippocampus has been associated with spatial learning (Moser et al., 1994, 1997). However, studies in intact cycling rats have failed to find fluctuations in spatial memory that mirror changes in spine density (Frye, 1995; Berry et al., 1997; Stackman et al., 1997; Warren and Juraska, 1997). Furthermore in male rats, exogenous testosterone has been shown to impair the acquisition of a spatial water maze task (Goudsmit et al., 1990; Naghdhi et al., 2001). Nevertheless, several studies in humans have demonstrated a positive relationship between testosterone levels and several types of memory, particularly in older men (Gouchie and Kimura, 1991; Janowsky et al., 1994, 2001; Barrett-Connor et al., 1999; Silverman et al., 1999; Cherrier et al., 2001; Moffat et al., 2002). In several cases, men with lower T levels exhibited impaired memory relative to those with higher T (Barrett-Connor et al., 1999; Moffat et al., 2002). Because the cytoarchitecture is similar between humans and monkeys (e.g. Rosene and Van Hoesen, 1987), it is possible that humans with low testosterone may have lower spine densities.

Sexual Differences

Data from previous experiments regarding the response of the pyramidal cell spine synapse density to the manipulation of gonadal hormone levels of male (Leranth et al., 2003) and female (Leranth et al., 2000) rats, as well as female (Leranth et al., 2002) and male monkeys can be directly compared. In rats, similar to monkeys, experimental manipulations of circulating gonadal hormone levels have no effect on pyramidal cell density in either female or male animals. In contrast, gonadectomy has a significant effect on the density of the dendritic spine synapses of pyramidal cells in both female and male rats, as well as female and male monkeys (Table 2).

From this table, it appears that, in percentage terms, the magnitude of the synaptoplastic effect of female and male gonadal hormones is similar in rats (50%) and monkeys (40%) of both genders. The absolute spine synapse density values of control female rats (1.08/µm²) and monkeys (1.15/µm²) are also very similar. However, there are marked differences between the absolute values of spine synapse densities of control male rats (0.9/µm²) and monkeys (0.58/µm²). Consequently, there are major differences between the spine synapse density values of female and male monkeys regardless of the levels of circulating gonadal hormones. Both the control (0.58/µm²) and GDX males (0.34/µm²) have ∼48% fewer spine synapses/µm² than control (1.15/µm²) and OVX (0.65/µm²) female monkeys, respectively. Furthermore, the spine synapse density in the intact, control male monkey hippocampus (0.58/µm²) is >10% lower than in the OVX female monkey (0.65/µm²).

In light of these data, assumptions regarding spine density and memory may need to be reevaluated or reconsidered. It is generally accepted that hippocampal long-term potentiation (LTP) is associated with synaptic plasticity and memory (Cohen and Eichenbaum, 1991) and electrophysiological experiments have demonstrated that E-induced spine synapse density in OVX rats is associated with augmented CA1 long-term potentiation (LTP). Cordoba Montoya and Carrer, 1997; Fay et al., 1984; Good et al., 1999; Ito et al., 1999). In contrast, T administration to castrated male rats has been reported to have an opposite, negative effect on CA1 LTP (Harley et al., 2000), in spite of the fact that T administration dramatically increases the spine synapse density in both male rats (50%; Leranth et al., 2003) and monkeys (40%; recent study). However, sex differences favoring males have been observed in some hippocampal-dependent memory tasks (Williams et al., 1990; Williams and Meck, 1991; Roof and Havens, 1992; Roof, 1993; Markowska, 1999). Exogenous T or E during early postnatal development can reverse this sex difference and promote the development of a male-like hippocampus in females (Williams et al., 1990; Williams and Meck, 1991; Roof and Havens, 1992; Roof, 1993). Because males excel at some hippocampal-dependent memory tasks, despite having fewer CA1 spine synapses than females, it is tempting to speculate that either spine density has little to do with hippocampal-dependent memory processing, or that both LTP and gonadal hormones induce synaptic sprouting without causal relationships. On the other hand, the organizational effects of steroid hormones on the brain may necessitate that females have a greater density of CA1 spine synapses to compensate for spine reductions or other alterations elsewhere in the brain. Alternatively, high numbers of spine synapses may not be beneficial to memory (e.g. by producing excess disruptive input to the dendrite) and the impairments shown by females in some spatial memory tasks may be the result of too many spine synapses. Correlations between spine synapse density and memory measurements performed in the same animals will be necessary to clearly elucidate this issue.

Table 2

Summary of spine density values in the CA1 hippocampal subfield of gonadectomized and control female and male rats and monkeys

<table>
<thead>
<tr>
<th>Animals</th>
<th>Gonadectomized</th>
<th>Control</th>
<th>Difference [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female rat</td>
<td>0.71/µm²</td>
<td>1.00/µm²</td>
<td>34</td>
</tr>
<tr>
<td>Male rat</td>
<td>0.45/µm²</td>
<td>0.90/µm²</td>
<td>50</td>
</tr>
<tr>
<td>Female monkey</td>
<td>0.65/µm²</td>
<td>1.15/µm²</td>
<td>40</td>
</tr>
<tr>
<td>Male monkey</td>
<td>0.34/µm²</td>
<td>0.56/µm²</td>
<td>40</td>
</tr>
</tbody>
</table>

Notes

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