SEX DIFFERENCES IN THE STEREOLOGICAL PARAMETERS OF THE HIPPOCAMPAL DENTATE GYRUS OF THE GUINEA-PIG BEFORE PUBERTY

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Abstract—Studies in rats and mice have shown several sex-dependent functional and structural differences in the hippocampal region, a brain structure playing a key role in learning and memory. The aim of the present study was to establish whether sex differences exist prior to puberty in the stereological parameters of the dentate gyrus in the guinea-pig, a long-gestation rodent, whose brain is at a more advanced stage of maturation at birth than the rat and mouse. The number of granule cells and volumes of the granule cell layer, molecular layer and hilus were evaluated in Nissl-stained brains of neonatal (15–16 days old) and peripubescent (45–46 days old) guinea-pigs. Based on a pilot study, the optical dissector method was preferred to the optical fractionator method to estimate cell number. For volume (Vref) estimation with the Cavalieri principle, contour tracing was preferred to the point counting method, as the latter appeared to underestimate volumes. The results showed that neonatal males had more granule cells than females in both the dorsal and ventral dentate gyrus and a larger volume in all layers. Peripubescent males had a larger volume of the granule cell layer than females in both the dorsal and ventral dentate gyrus, more granule cells in the ventral dentate gyrus, a larger volume of the hilus in both the dorsal and ventral dentate gyrus and a larger volume of the molecular layer in the ventral dentate gyrus. The results show that sex differences are present in the guinea-pig dentate gyrus prior to puberty and go in the same direction at both investigated ages, with males exhibiting more granule cells and larger volumes than females. The widespread distribution of these sex differences suggests that in the guinea-pig, similarly to other rodents, hippocampus-dependent functions may be sexually dimorphic. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: sexual dimorphism, hippocampal formation, development.

Numerous studies have reported widespread sex-dependent differences in the morphology and physiology of the hippocampal formation. The sexual dimorphism in the anatomy of this brain region includes dimorphisms in the overall volume of the hippocampus (Diamond et al., 1983), morphology and number of hippocampal neurons (Madeira et al., 1988; Juraska et al., 1989; Gould et al., 1990; Madeira et al., 1992; Madeira and Paula Barbosa, 1993; Lavenex et al., 2000), volume of the mossy fiber system (Madeira et al., 1991b), morphology of the granule cells (Juraska et al., 1985), size of the dentate granule cell layer and number and density of granule cells (Wimer and Wimer, 1985, 1989; Madeira et al., 1988, 1991a; Wimer et al., 1988; Roof and Havens, 1992; Roof, 1993a; Tabibnia et al., 1999). In general, all these studies report sex differences favoring males, which have a larger volume, more cells and a more developed dendritic tree than females. These differences in a fundamental center of learning and memory, such as the hippocampus, suggest that there must be some relationship between structural differences in hippocampal anatomy and memory. Indeed, there are several reports concerning sex-dependent differences in spatial learning, such as the different behaviors of laboratory rodents, favoring males, in the radial arm maze and the Morris water maze (Juraska et al., 1984; Roof and Havens, 1992; Roof, 1993b; Galea et al., 1995, 1996; Kavaliers et al., 1996).

A significant deficiency in the existing literature is that almost all the data on the sex differences in the hippocampal region are derived from rats and mice and the degree to which these sex differences are species-specific or reflect general features across mammals is still a matter not fully elucidated. In previous studies we obtained evidence for a strong sex dimorphism in the dendritic tree of the hippocampal pyramidal neurons and dentate granule cells of the guinea-pig (Bartesaghi and Serrai, 2001; Bartesaghi and Severi, 2002; Bartesaghi et al., 2003a), but in this rodent the sexually dimorphic pattern of the pyramidal neurons and granule cells had a direction in most cases opposite to that demonstrated in the rat (Juraska et al., 1985, 1989; Juraska, 1990). Unlike rats and mice, that are short-gestation rodents, the guinea-pig is a long-gestation rodent. In short-gestation species the brain is very immature at birth and the perinatal period represents a critical time window during which gonadal steroids operate the sexual differentiation of the brain (Toran-Allerand, 1980, 1984; Toran-Allerand et al., 1983). In long-gestation species such as the guinea-pig, the brain has a high degree of neurological maturity at birth and the sexual differentiation of the brain is largely organized by gonadal steroids before birth (Resko and Roselli, 1997). It is thus possible that the differences in the sexual dimorphism of the granule cells and hippocampal neurons (and, possibly, of other brain structures) of rats and guinea-pigs are related to differences in their brain developmental pattern. Whatever the
explanation, the differential sex effects on pyramidal neuron and granule cell morphology in rats (Juraska et al., 1985, 1989; Juraska, 1990) and guinea-pigs (Bartesaghi and Serrai, 2001; Bartesaghi and Severi, 2002; Bartesaghi et al., 2003a,b) demonstrate that not all species exhibit the same sex differences. Hence, it may not be valid to simply extrapolate work in rats and mice, because it may not be paradigmatic for all mammals.

Wimer and Wimer (1985) demonstrated that in some inbred strains of mice the granule cell layer of the dentate gyrus has a higher neuron density in males compared with females. This difference, however, occurred only in the strains that had a high number of granule cells, whereas in the strains with a low number of cells no sexual dimorphism was present in the number of granule cells. Analysis across development (Wimer et al., 1988) revealed that the number of granule cells underwent a reduction between postnatal days 20–27 in both sexes, but that the reduction was larger in females and, consequently, a sex dimorphism appeared at this age. The presence of sex differences in the granule cell layer has been demonstrated also in the adult rat, a species in which, similarly to the mouse, males have more granule neurons and a larger volume of the granule cell layer compared with females (Madeira et al., 1988, 1991a). However, these sex differences were not confirmed by another report, in adult animals of younger age (Tanapat et al., 1999). All these data suggest that the developmental stage of the animals may be an important variable to take into account when analyzing sex differences and comparing different species.

The work presented here and in a previous study (Bartesaghi et al., 2003a) was performed to address the issue of sex differences in the dentate gyrus of the guinea-pig, a relatively unstudied rodent, during different stages of development. In a previous report we investigated sex differences in the dendritic architecture of the granule cells at two ages before puberty, and obtained evidence that these differences changed with age (Bartesaghi et al., 2003a). The aim of the present study was to establish whether at these same ages sex differences exist in the number of granule cells and volume of the dentate gyrus and whether possible differences change during development.

### EXPERIMENTAL PROCEDURES

#### Animals and housing conditions

Albino guinea-pigs (Brescia strain; Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia, Brescia, Italy) of either sex were used. Animals were maintained under standard laboratory conditions: 12-h light/dark cycle; temperature of 20 °C; ad libitum availability of pellet food and drinking solutions. The experimental groups were four males and four females 15–16 days old. The portions of the dentate gyrus located in the dorsal and ventral hippocampal formation will be called here dorsal and ventral dentate gyrus, respectively (Fig. 1A). In parasagittal sections the dorsal and ventral dentate gyrus are easily recognizable, because they appear as distinct structures (Fig. 1A). Only in lateralmost parasagittal sections the dorsal and ventral dentate gyrus merge into a single dentate gyrus. In these sections we have divided the dentate gyrus into a dorsal and ventral portion by drawing a horizontal line midway its longitudinal extent. The cellular layer of the dentate gyrus is the granule cell layer (Fig. 1B). This layer is C-shaped and its two arms are classically named upper blade (UB) and lower blade (LB), respectively (Fig. 1B). The UB, which faces field CA1, is alternatively called suprapyramidal blade. The LB, which faces the telodiencephalic fissure and is opposite to the UB, is alternatively called infrapyramidal blade (Amaral and Witter, 1995). The molecular layer, which is a relatively thick layer situated above the granule cell layer (Fig. 1B), contains the dendrites of the granule cells and several interneuron types. The hilus is the region of the dentate gyrus enclosed by the granule cell layer (Fig. 1B). Its outer portion, adjacent to the granule cell layer, contains the progenitor of the granule cells (Altman and Das, 1967; Altman and Bayer, 1975; Schlessinger et al., 1975; Bayer, 1980) and its inner portion contains a variety of interneurons (Amaral and Witter, 1995). The border between the hilus and the hippocampal pyramidal cell layer is difficult to determine in Nissl-stained sections. This border was here arbitrarily defined by drawing a straight line between the tips of the UB and LB. For analogy with the subdivision of the granule cell layer into two blades, we have subdivided the molecular layer and the hilus into two portions, corresponding to the UB and LB of the granule cell layer, respectively (Fig. 1C).

#### Equipment

The following stereology system was used: i) light microscope (Leitz, Germany) equipped with a motorized stage and focus control system; ii) color digital video camera attached to the microscope; iii) Image Pro Plus (Media Cybernetics, Silver Spring, MD, USA) with the StagePro module for controlling the motorized stage in the x, y and z directions, as primary software. A macro based on the BASIC
programming language developed by King et al. (2002) was used for the automatic conducting of the optical fractionator process.

Pilot study

Stereological methods use geometric probes to estimate geometrical parameters of structures, such as area, volume, and population size. The granule cell layer of the dentate gyrus is a thin (thickness: 70–100 μm, in rodents) and elongated structure, with a complex orientation (see Fig. 1A). We were concerned with the possibility that for such a structure, probes of inadequate size may affect the precision of the estimates of the volume and total cell number. Thus before undertaking the present investigation we sought to obtain preliminary information on this issue.

Point counting versus tracing for evaluation of the volume of the different layers of the dentate gyrus. The method of choice to estimate the volume of an object from sections is based on Cavalieri’s principle (Gundersen and Jensen, 1987; West, 1990; West and Gundersen, 1990). Parallel sections with thickness t are cut through the object. Every nth section is chosen from the series. The volume ($V_{ref}$) estimate is obtained from the section area ($A$) estimates (see below) in the chosen sections and the spacing $T$ ($T=t\times n$) between sections

$$V_{ref}=T\times(A_1+A_2+\ldots+A_n)$$

Section areas can be estimated by either i) point grid overlays or ii) boundary contour tracings. Point counting is very commonly used in stereology for unbiased area and volume estimation (Gundersen and Jensen, 1987), where counts are made of randomly overlaid lattice points that fall within a closed area. A convenient test system is a square grid of side s (hereafter called the “grid size”). An unbiased estimator of $A_i$ is

$$A_i=P_i\times a_p$$

Fig. 1. Methods. (A) Nissl-stained parasagittal sections, at different distances from the midline, across the hippocampal formation. The dorsal and ventral portions of the dentate gyrus (DG) are indicated. (B) Photomicrograph showing the three layers of the DG (Gr, granule cell layer; H, hilus; Mol, molecular layer). The Gr is subdivided into an UB and a LB. (C) The molecular layer (MOL; middle section) and hilus (H; section on the right) have been subdivided into an upper and lower portion, corresponding to the UB and LB of the Gr (section on the left). The white line in each section shows how the area of each layer was measured by contour tracing. (D) Photomicrograph of the DG with a superimposed counting grid formed by optical dissectors used to estimate the $N_v$ of granule cells with the stereological method. Grid distance: 150 μm. (E) Detailed view of the optical dissector. The sides of the dissector have a 30 μm length. The dots indicate the cells corresponding to the inclusion rules that have been counted. Scale bar = 1000 μm in A; in B and C = 500 μm. hf, hippocampal fissure; tdf, telodiencephalic fissure.
where \( a_i \) is the area associated with one point in the grid and \( P_i \) is the number of test points hitting a section of area \( A_i \). Thanks to the development of new software, the area of a structure of interest can now be directly measured by tracing its contour on video images displayed on computer. Contour tracing obviously appears the safest way to determine the area, because it provides a direct measurement of the area, while point counting provides an estimate of the area. The advantage of the point counting method is that it does not require much time. In order to establish the grid size giving the best approximation of the area (and, hence, volume) of the granule cell layer, we compared the volume estimates (Eq. 1) obtained with grids of different sizes with the value obtained by careful contour tracing. The same analysis was carried out also for the molecular layer and hilus. For this pilot study the right and left hemispheres of one animal from the group of neonatal males were used. One out of 12 serial parasagittal sections was sampled, with a total of 13 sections per hemisphere. Tracing and point counting were carried out at a final magnification of 160×. The same operator carried out the complete analysis. Contours were traced with the aid of the software Image Pro Plus. To validate the intraoperator consistency, in one section tracing of the granule cell layer was repeated 10 times by the same operator. The results (data not shown) were similar across sessions (±3% of the mean value), which validated the intraoperator reliability. To validate the interoperator reliability of contour tracing. For point counting, a square grid was randomly positioned over each section by the Image Pro Plus software. Grids with size of 60 \( \mu m \), 120 \( \mu m \), 150 \( \mu m \) and 200 \( \mu m \) were used to estimate the area of the granule cell layer and grids with size of 120 \( \mu m \), 150 \( \mu m \) and 200 \( \mu m \) to estimate the area of the molecular layer and hilus. The results showed that estimation of the volume of the granule cell layer with any of the four grids gave values significantly smaller compared with contour tracing (Fig. 2B). The estimates with the grids of 60 \( \mu m \), 120 \( \mu m \), 150 \( \mu m \) and 200 \( \mu m \) had values that were 85%, 75%, 65% and 68%, respectively, compared with that obtained by contour tracing (Fig. 2Bb1). Estimation of the volume of the molecular layer with the grids of 120 \( \mu m \), 150 \( \mu m \) and 200 \( \mu m \) yielded values that were 80%, 81% and 75%, respectively, compared with that obtained by contour tracing (Fig. 2Bb2). Estimation of the volume of the hilus with any grid yielded values not significantly different from contour tracing (Fig. 2Bb3). These findings indicate that the volume estimate of the granule cell layer even with grids of small size differed significantly from that obtained by contour tracing. Similar considerations hold for the molecular layer, though the difference was smaller, very likely due to its greater thickness. The hilus was the only layer where point counting gave values similar to contour tracing. It has been shown that the greater the shape coefficient (ratio between the perimeter and the square root of the surface area) is, the greater is the variation of volume estimates (Gundersen and Jensen, 1987). The hilus and the molecular layer have a similar area but the molecular layer, due to its C shape, has a larger perimeter. Calculation of the shape coefficients gave values of 9–10 for the molecular layer and 4–5 for the hilus. The lower shape coefficient of the hilus may explain why point counting yielded a better area/volume estimate compared with the molecular layer. Calculation of the coefficient of error (CE; West et al., 1990) for estimation of the area with each grid gave values <0.1. For instance, the total number of points counted in the granule cell layer of one hemisphere with the grids of 60 \( \mu m \), 120 \( \mu m \), 150 \( \mu m \) and 200 \( \mu m \) was 1504, 343, 167, and 104, respectively, and the CE values were 0.032, 0.038, 0.039 and 0.044. Though these values are considered acceptable by most studies, volume estimation by point counting significantly differed from that obtained by tracing up to 35%. For all layers, the estimate improved by reducing the grid size. The best estimate for the granule cell layer was obtained with the grid of 60 \( \mu m \). With such a grid, the number of points hitting the layer in each section ranged from 73 to 192, with a total of 1504 points in 13 sections. The time required to count such a large number of points greatly outweighed the time required to trace the contours of the granule cell layer. Considering that even with that grid the estimated volume was 15% smaller than the actual volume, we concluded that the more precise and easier way to evaluate the volume of the granule cell layer was to trace its contour.

Optical fractionator method versus optical disector method for evaluation of total number of granule cells. Total number of cells in a structure can be estimated with either the optical fractionator method or the optical disector method (Gundersen and Jensen, 1987; West, 1990; West and Gundersen, 1990; Dorph Petersen et al., 2001; Keuker et al., 2001). The optical fractionator method systematically partitions the tissue contained in a uniformly spaced series of sections into equal volumes of equal size (disectors). In each of the sections, the disectors are spaced in rows and columns at constant distances (Fig. 1D, E) and neurons are counted in all disectors that hit the region of interest. For evaluation of population size three sampling fractions are used: i) the section sampling fraction (ssf) is the proportion of the sections across the entire structure that is sampled for evaluation; ii) the area sampling fraction (asf) is the proportion of the sectional area that is investigated within the sampled sections; iii) thickness sampling fraction (tsf) is the part of the section thickness that is investigated. The estimated total number of particles (\( N \)) is obtained by multiplying the reciprocals of the fractions with the total particle count (\( \sum Q \)), obtained with the optical disectors

\[
N = \sum Q \times 1/\text{ssf} \times 1/\text{asf} \times 1/\text{tsf}
\]  

(3)

The optical disector method estimates the total number of particles (\( N \)) from the product of the volume (\( V_{\text{vol}} \)) and numerical density (\( N_p \))

\[
N = V_{\text{vol}} \times N_p
\]  

(4)

\( V_{\text{vol}} \) is typically estimated by use of Cavalieri's principle. The \( N_p \) of particles is estimated from systematic disector sampling:

\[
N_p = \frac{\sum Q \sum \text{dis}}{V_{\text{dis}}}
\]  

(5)

where \( \sum Q \) is the summed number of particles counted in the disectors hitting the region of interest, \( \sum \text{dis} \) is the total number of disectors and \( V_{\text{dis}} \) is the volume of the disector (sampling volume). To estimate total granule cell number, in the present study we have sampled granule cells using the optical fractionator sampling scheme described below. This scheme allowed us to estimate total cell number according to Eq. 3 (optical fractionator method). Since from this scheme cell \( N_c \) can be calculated (Eq. 5) and we had measured \( V_{\text{vol}} \) of the granule cell layer (by contour tracing), we could estimate total cell number also with Eq. 4 (optical disector method). To establish whether these two methods yielded similar results, in a group of animals we evaluated granule cell number with both methods. The comparison showed that the two methods did not yield the same results and that total cell number estimated with Eq. 3 was significantly smaller (20%) than that obtained with Eq. 4 (Fig. 2C). With our sampling scheme, the number of disector hitting the granule cell layer in 12–14 sampled sections was 178–234 and the number of counted cells 1058–1268. Calculation of CE of \( N \) (Keuker et al., 2001) gave values between 0.035 and 0.041. Though these values are considered acceptable (Gundersen and Jensen, 1987; Keuker et al., 2001), the estimated cell number was smaller than with the optical disector method. With the optical fractionator method there is no need to measure the volume of the region of interest, because the
The latter is implicitly determined by the combination of the fractions in Eq. 3 (Keuker et al., 2001). The data reported above showed that for the granule cell layer the precision of volume estimate was critically related to the number of points hitting the layer. This suggests that if the number of disectors hitting the granule cell layer is not sufficiently high, Eq. 3 may underestimate total cell number. This problem will not be encountered by evaluating cell number with Eq. 4, provided that $V_{ref}$ has been estimated with

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**Fig. 2.** Estimation of the volume of the dentate gyrus and granule cell number with different methods. (A) Volumes of the different layers of the dentate gyrus estimated based on Cavalieri’s principle from contour tracing of the areas. Contour tracing was performed by two different operators (operator 1 and operator 2). Note the interoperator consistency. (B) Volume of the granule cell layer (b1), molecular layer (b2) and hilus (b3) estimated by contour tracing (T) and point counting with square grids of the indicated sizes (60 μm, 120 μm, 150 μm and 200 μm). The upper panel in (b1), (b2) and (b3) shows the estimated volume in mm$^3$; in the lower panel volumes are expressed as percent of the volume obtained by contour tracing. All measurements were carried out by the same operator (operator 1). Data are the mean $\pm$ S.D. of the two sides. The volume estimated with each grid was compared (two-tailed t-test) with that obtained by contour tracings. (*) $P<0.06$; * $P<0.05$; ** $P<0.01$. (C) Total number of granule cells estimated with the optical disector method ($N = V_{ref} \times N_V$) and with the optical fractionator method ($N = \sum Q \times 1/ssf \times 1/asf \times 1/tsf$). See text for explanation. Data are the mean $\pm$ S.D. from the group of neonatal males ($n=4$). Two-tailed t-test: * $P<0.05$. 
precision by contour tracing. While it is theoretically feasible to increase the number of disectors to improve the estimate, it should be noted that with our sampling scheme, where the distance between disectors (150 μm) was smaller than in many studies, 2–3 h were required to sample each section and a further increase in the sampling frequency would render cell count time-consuming. We concluded that the optical disector method is more convenient than the optical fractionator method to estimate granule cell number, because it provides a better estimate with a relatively low sampling frequency.

**Detailed methods**

Two stereological parameters were evaluated: $V_{\text{tot}}$ of each layer and $N_v$ of the granule cell layer. From these parameters, the total number of granule cells was then evaluated. One guinea-pig performed separately for each blade in both the dorsal or ventral dentate gyrus was calculated by adding the areas of their respective blades. The total area of each layer was obtained by summing the areas of the dorsal and ventral dentate gyrus. Volumes were determined based on Cavalieri’s method (Eq. 1) by multiplying the area measured in the series of sections and the distance ($T$) between sections. The latter was obtained by multiplying the number of sections interposed between the analyzed sections (12) and the section thickness (30 μm); $T=12 \times 30=360 \mu m$.

**Granule cell density.** The granule cell density was determined in the selected sections using systematic disectors samples. Counting frames with 30 μm side length and 6 μm height (i.e. depth) spaced in a 150 μm square grid were systematically used (Fig. 1D, E). Cells were counted in all disectors hitting the granule cell layer. The 3 μm border at the top of the tissue section was omitted. The cell nucleus of the granule cells was used as counting criterion (West et al., 1990; Keuker et al., 2001). Granule cell nuclei were counted with a 100× (Leitz) oil objective (1.4 NA). Granule cell nuclei intersecting the uppermost focal plane and the exclusion lines of the count frame were not counted. When the counting frame did not fall entirely within the layer, the number of frame corners within the layer was noted and the frame considered as a fractional frame, the fraction being that of the corners of the frame hitting the layer (West et al., 1990). The location of the disectors in different subregions of the dentate gyrus was noted during neuron sampling, in order to separately estimate cell density in each subregion. The neuron density ($N_v$) was calculated from Eq. 5. For calculation of the $V_{\text{tot}}$ the disector height was corrected for section shrinkage in the z-plane (Dorph Petersen et al., 2001) according to the formula: $h=(\text{counting thickness} \times (\text{original thickness/measured thickness})$. The section thickness was measured during neuron counting at different random locations. In the analyzed sections, the mean section thickness was 14.8 μm (range: 12.5–16.2 μm). Granule cell density was calculated separately for each blade in both the dorsal and ventral dentate gyrus. Mean cell density in either the dorsal or ventral dentate gyrus was obtained by averaging the values of the dorsal and ventral dentate gyrus.

**Granule cell number.** The number ($N$) of granule cells in each subregion was calculated by Eq. 4. Cell number in either the dorsal or ventral dentate gyrus was calculated by adding the values of the respective UB and LBs. Total granule cell number was evaluated by adding the values of the dorsal and ventral dentate gyrus.

**Statistical comparisons**

All data are presented as mean±S.D. Volume of each layer, granule cell density and granule cell number were used as dependent variables and sex, brain side and age were the independent variables. Values were examined by three- or two-way ANOVA, taking as factors sex, age and brain side. The effects of sex and age were determined with Tukey-HSD comparisons (significance set to 0.05). The effects of side in each group were determined with individual two-tailed t-tests. A probability level of $P<0.05$ was considered to be statistically significant.

**RESULTS**

**Body and brain weight**

In neonatal animals the body and total brain weight were significantly larger in males than in females. Analysis of each hemisphere showed that the sex difference in brain weight was due to the left hemisphere (Table 1). In peripubescent animals no sex differences were observed in body and total brain weight (Table 1). Analysis of the sex differences in either hemisphere, however, showed that the right hemisphere was significantly larger in males than in females (Table 1). The comparison of the weights of the two hemispheres showed a side difference only in neonatal males, with the weight of the right hemisphere significantly larger than the left. No side-related differences were observed in neonatal females and in peripubescent animals of either sex (Table 1).

**General results**

The three-way ANOVA, taking as factors sex, age and brain side showed a few interactions (Table 2). Values were then examined by two-way ANOVA, taking as factors i) sex and brain side; ii) age and brain side; iii) sex and age. No interactions were observed between either sex and side or between age and side, but numerous interactions were observed between sex and age (Table 2). While numerous main effects of sex and age were observed, very few main effects of side emerged from the analysis. The absence of interactions between either sex and side or age and side suggested that the effects of sex and age were not lateralized. This was confirmed by post hoc comparison of the effects of either sex or age carried out separately in each side. For each age group, this analysis provided similar sex (and age) effects in either hemisphere. Hence, the analysis of the sex and age effect was performed on the pooled data from the two sides. The sex and age effects presented in the following sections refer to the analysis of pooled data.

**Effect of sex on the stereological parameters of the dentate gyrus in neonatal animals**

**Granule cell layer.** The total volume of the granule cell layer was larger in males than in females; the percentage value in males was 22% higher than in females (Fig. 1).
Table 1. Sex and age effects on the body and brain weights of the guinea-pig before puberty

<table>
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<th>Sex/age/side</th>
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<td>Weight (grams)</td>
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Table 2. Statistical analysis: interactions between sex, age and side

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<td>ns</td>
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<td>0.035</td>
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*Statistical analysis with three-way ANOVA taking as factors sex, age and brain side and two-way ANOVA taking as factors sex and age. P<0.05 was considered to represent statistical significance. Significant P values from ANOVA are reported. ns, not significant. The experimental groups were eight neonatal (15–16 days old) males (n=4) and females (n=4) and eight peripubescent (45–46 days old) males (n=4) and females (n=4). dDG, dorsal dentate gyrus; dLB, dorsal lower blade; dUB, dorsal upper blade; TOT, total dentate gyrus; vLB, ventral lower blade; vUB, ventral upper blade. See Experimental Procedures for definition of the different regions of the dentate gyrus.
Fig. 3. Effects of sex on the stereological parameters of the dentate gyrus. The graphs show for each region of the dentate gyrus: volume of the granule cell layer (A), granule cell density (B), granule cell number (C), volume of the molecular layer (D), volume of the hilus (E) in neonatal animals and volume of the granule cell layer (F), granule cell density (G), granule cell number (H), volume of the molecular layer (I), volume of the hilus (J) in peripubescent animals. Data (mean ± S.D.) refer to one dentate gyrus (average of measurements from both sides). Neonatal males (n=4) and neonatal females (n=4) were 15–16 days old. Peripubescent males (n=4) and peripubescent females (n=4) were 45–46 days old. The asterisk indicates a sex effect in each age group; the symbol # indicates an age effect with respect to animals of the same sex: (*) P<0.06; * P<0.05; # P<0.05 (Tukey-HSD comparisons). dDG, dorsal dentate gyrus; dLB, dorsal lower blade; dUB, dorsal upper blade; TOT, total dentate gyrus; vDG, ventral dentate gyrus; vLB, ventral lower blade; vUB, ventral upper blade. See Experimental Procedures for definition of the different regions of the dentate gyrus.
ventral dentate gyrus was sexually dimorphic, with males having more cells than females (+26%, in the dorsal UB and +20%, in the ventral UB).

**Molecular layer.** The total volume of the molecular layer was 8% larger in males than in females (Fig. 3D). Analysis at the level of the dorsal and ventral dentate gyrus showed that males had a larger volume in both the dorsal (+7%) and ventral (+10%) dentate gyrus, though in the dorsal dentate gyrus this difference was not statistically significant (Fig. 3D). Analysis at the level of each blade showed that the UB had a larger volume in males than in females. The value in males was 18% larger than in females in the dorsal dentate gyrus and 22% in ventral dentate gyrus. In contrast, the LB of males had a volume that was 88% of that of females both in the dorsal and ventral dentate gyrus. However, this difference was significant only in the dorsal dentate gyrus (Fig. 3D).

**Hilus.** The total volume of the hilus of males was 22% larger than that of females. The volume of the hilus evaluated in the dorsal and ventral dentate gyrus was larger in males than in females (+24%, in the dorsal dentate gyrus and +16%, in the ventral dentate gyrus; Fig. 3E). Analysis of each blade showed that in the dorsal dentate gyrus both blades had a larger volume (+32%, for the UB and +13%, for the LB) in males than in females. In the ventral dentate gyrus, only the UB was sexually dimorphic, being larger (+20%) in males than in females (Fig. 3E).

**Effect of sex on the stereological parameters of the dentate gyrus in peripubescent animals**

**Granule cell layer.** The total volume of the granule cell layer was larger in males than in females, with males having a volume 20% larger than females (Fig. 3F). The sex dimorphism in total volume was due to both the dorsal and ventral dentate gyrus; the volume of the granule cell layer in males was 14% larger than in females, in the dorsal dentate gyrus, and 40% larger, in the ventral dentate gyrus (Fig. 3F). Analysis of each blade showed that males had a larger LB than females in both the dorsal (+16%) and ventral (+46%) dentate gyrus (Fig. 3F). No sex differences were present in the volume of the UB. Unlike in neonatal animals, in peripubescent animals no sex differences were present in granule cell density (Fig. 3G). Similarly to neonatal animals, in peripubescent animals the total number of granule cells was larger (+20%) in males than in females, but this difference only approached significance (Fig. 3H). Analysis of the sex effects on total cell number in the dorsal and ventral dentate gyrus showed a significant sex difference only in the ventral dentate gyrus, where the number of granule cells of males was 18% larger than that of females (Fig. 3H). The sex difference in the ventral dentate gyrus was due to the LB, where the number of granule cells of males was 47% larger than that of females. Likewise, in the dorsal dentate gyrus the LB had more cells (+14%) in males than in females, whereas the number of cells in the UB was not sexually dimorphic (Fig. 3H).

**Molecular layer.** In peripubescent animals the volume of the molecular layer was similar in the two sexes, save for the volume of the UB in the ventral dentate gyrus and total volume of the ventral dentate gyrus (Fig. 3I), where males had a volume 26% and 20% larger, respectively, than females.

**Hilus.** Males had a larger total volume (+23%) of the hilus than females and a larger total volume of the dorsal (+18%) and ventral (+40%) dentate gyrus. Analysis of each blade revealed that these differences were due to the LB only, whereas the volume of the UB was not sexually dimorphic. The volume of the LB was 25% larger in males than in females, in the dorsal dentate gyrus, and 83% larger, in the ventral dentate gyrus (Fig. 3J).

**Effect of age on the stereological parameters of the dentate gyrus**

**Granule cell layer.** In either sex the volume of the granule cell layer, granule cell density and granule cell number underwent an overall increase with age. The total volume of the granule cell layer was 24% larger in peripubescent than in neonatal males and 25% larger in peripubescent than in neonatal females (Fig. 3A, F). The mean granule cell density across the dentate gyrus increased with age in both sexes. The percentage increase was 23% in males and 18% in females (Fig. 3B, G). The total number of granule cells significantly increased with age, with a percentage increase of 57% in males and 47% in females (Fig. 3C, H). Analysis of individual regions of the dentate gyrus revealed that both blades in the dorsal dentate gyrus contributed to the observed age-related increase in the volume of the granule cell layer. In contrast, the UB and LB in the ventral dentate gyrus of both males and females did not exhibit an age-related volume increase (Fig. 3A, F). Consequently, while the volume of the dorsal dentate gyrus increased with age, the ventral dentate gyrus had a similar volume in neonatal and peripubescent animals (Fig. 3A, F). Granule cell density increased in all regions of the dentate gyrus in both sexes (Fig. 3B, G), and consequently, the number of granule cells increased also in the regions that did not exhibit a volume increase, with the exception of the UB in the ventral dentate gyrus of males (Fig. 3C, H).

**Molecular layer.** In either sex the total volume of the molecular layer underwent an overall increase with age (Fig. 3D, I). The volume of the molecular layer was 8% larger in peripubescent than in neonatal males and 10% larger in peripubescent than in neonatal females. Analysis of individual regions of the dentate gyrus revealed a significant volume increase only in the dorsal dentate gyrus. In contrast, the UB in the ventral dentate gyrus of males, and the UB and LB in the ventral dentate gyrus of females did not exhibit an age-related volume increase and, consequently, the total volume of the ventral dentate gyrus did not undergo and age-related volume increase (Fig. 3D, I).

**Hilus.** In either sex the hilus underwent an age-related volume increase. The volume of the hilus was 22%...
larger in peripubescent than in neonatal males and 23% larger in peripubescent than in neonatal females (Fig. 3E, J). Analysis of individual regions of the dentate gyrus revealed a significant increase in the volume of both the dorsal and ventral dentate gyrus, in males, but only of the dorsal dentate gyrus, in females (Fig. 3E, J).

Effect of side on the stereological parameters of the dentate gyrus

Very few side differences were observed in neonatal animals and no side differences in peripubescent animals. If present, the side differences went in the same direction in males and females, with the values in the right hemisphere being greater than in the left hemisphere. Both neonatal males and females had a larger volume in the right than in the left hemisphere in the following regions: i) granule cell layer in the dorsal dentate gyrus (males: 1.76±0.09 mm³ versus 1.52±0.03 mm³, P<0.01; females: 1.39±0.05 mm³ versus 1.28±0.03 mm³, P<0.04, two-tailed t-test); ii) total granule cell layer (males: 2.47±0.16 mm³ versus 2.20±0.01 mm³; females: 2.02±0.05 mm³ versus 1.82±0.04 mm³, P<0.01, two-tailed t-test); iii) hilus (males: 7.98±1.11 mm³ versus 7.39±0.12 mm³, P<0.01; females: 7.71±0.39 mm³ versus 6.66±0.23 mm³, P<0.01, two-tailed t-test); iv) molecular layer in the LB of the ventral dentate gyrus (males: 0.66±0.01 mm³ versus 0.63±0.01 mm³, P<0.04; females: 0.85±0.05 mm³ versus 0.64±0.03 mm³, P<0.01, two-tailed t-test). In addition, neonatal males and females had a larger number of granule cells in the right than in the left hemisphere in the dorsal dentate gyrus (males: 434±15×10³ versus 376±11×10³, P<0.01; females: 409±35×10³ versus 362±14×10³, P<0.05, two-tailed t-test) and a total number of granule cells larger in the right than in the left dentate gyrus (males: 605±31×10³ versus 535±21×10³, P<0.03; females: 557±30×10³ versus 496±16×10³, P<0.04; two-tailed t-test).

DISCUSSION

The stereological parameters of the dentate gyrus are sexually dimorphic prior to puberty

In this study we have examined sex and developmental differences in layer volumes, granule cell density and granule cell number in the dentate gyrus of the guinea-pig. Two ages were analyzed, corresponding to the early postnatal and peripuberal periods. The results show that at both ages sex differences are present in the volume of the dentate gyrus and number of granule cell, with males having a larger volume and more neurons than females. The current results are in agreement with the presence of sex differences in granule cell dendritic architecture in animals of the same ages as those used here (Bartesaghi et al., 2003a).

The presence of sex differences in the granule cell layer before puberty has been observed in the rat (Roof, 1993a) and several strains of the mice (Wimer and Wimer, 1989). In mice, sex differences in granule cell number appear at 20–27 days of age. Since this is the time at which male mice exhibit an increase in plasma levels of androgens, this increase has been thought to underlie the establishment of the sexually dimorphic pattern of the dentate gyrus (Wimer and Wimer, 1989). There are no follow-up studies of plasma levels of gonadal steroid in the guinea-pig from birth to adulthood. In our previous study (Bartesaghi et al., 2003a), we obtained evidence that in both males and females plasma levels of gonadal steroids are very low in 15 days old animals and begin to increase in 45 days old animals. In view of the low levels of testosterone in 15 days old males, it seems unlikely that the sexually dimorphic pattern of their dentate gyrus is directly mediated by testosterone. Since in the guinea-pig, similarly to the rhesus monkey, the critical period for brain masculinization occurs early during gestation (Resko and Roselli, 1997), it seems likely that the sexually dimorphic pattern of the dentate gyrus in neonate animals is related to prenatal events. In 45 days old guinea-pigs, the sex differences in the volume of the dentate gyrus and total cell number went in the same directions as in 15 days old animals. Though in females estrogens appear to induce a larger production of new granule neurons compared with males, this increase is transient and no sex differences are present in the number of surviving cells after 15 days (Tanapat et al., 1999). This may explain why in peripubescent females the increase in plasma levels of estrogens (Bartesaghi et al., 2003a) does not revert the direction of the sex differences in total granule cell number.

Only the sex differences in granule cell density changed with age, though at either age no sex differences are present in granule cell size (Bartesaghi et al., 2003a). While in neonatal animals females had a larger cell density than males, this difference disappeared in 45 days old animals. As granule cell density increased with age in both sexes, these data indicate that the density increase was proportionally larger in males. This effect may be related to the fact that from the age of 15 days to the age of 45 days males underwent an increase in granule cell number that was larger (+57%) than that exhibited by females (+47%). The larger net cell production exhibited by males may be related to the rise in plasma androgen concentration taking place from 15 to 45 days of age (Bartesaghi et al., 2003a).

Distribution of the sex differences in the dentate gyrus

In the present study we have investigated the distribution of sex differences in the layers and different subregions of the dentate gyrus. The results showed that sex differences were present in all layers. The sex differences in the volume of the molecular layer and hilus were altogether similar to those in the granule cell layer, with males having a larger volume than females. This analogy is not surprising, considering that the molecular layer contains the dendrites of the granule cells and the hilus contains the mossy cells, a population of excitatory interneurons that are innervated by the granule cells and form a powerful associational connections in the dentate gyrus (Amaral and Witter, 1995).

Analysis of different subregions of the dentate gyrus showed that sex differences were present in both blades.
and in both the dorsal and ventral dentate gyrus. Numerous data suggest that the hippocampus is functionally differentiated along its dorso-ventral (septo-temporal) axis, with the dorsal hippocampus being specifically involved in spatial learning and the ventral hippocampus being very likely involved in autonomic and neuroendocrine functions (see Moser and Moser, 1998). The widespread distribution of the sex differences across the dentate gyrus suggests that functions subserved by both the dorsal and ventral hippocampus may be sexually dimorphic.

The stereological parameters of the dentate gyrus increase with age

Unlike most neuronal populations that are produced during the prenatal period, the granule cells in the dentate gyrus possess the unusual property to continue to be generated during postnatal and adult life (Angevine, 1965; Altman and Das, 1967; Schlessinger et al., 1975; Rakic and Nowakowski, 1981; Gueneau et al., 1982; Wyss and Sripandkulchai, 1985; Gould et al., 1997, 1998). The present data demonstrate that in the guinea-pig the granule cell number increases during development in a significant manner. This is in agreement with a previous demonstration that a substantial neurogenesis takes place in the guinea-pig dentate gyrus during the first postnatal month (Guidi et al., in press). From the increase in granule cell number observed here from the age of 15 days to the age of 45 days, it can be calculated that 8600 and 7600 new neurons are added each day to the male and female guinea-pig dentate gyrus, respectively. These values are consistent with our previous study, though their magnitude is larger than previously demonstrated. In addition, the total number of granule cells found here in 15 days old animals is larger than the value previously observed in 30 days old animals (Guidi et al., 2005). This difference may be due to the use of different methods to evaluate total cell number, because in our previous study total cell number was evaluated with the optical fractionator method which, as described above, appears to underestimate total granule cell number. In addition, in the present investigation we have used guinea-pigs of a different strain. In the mouse, notable differences exist among strains in the rate of granule cell proliferation and survival (Kempermann and Gage, 2002). Hence, it is also possible that the larger number of granule cells found here is a feature of the used strain of guinea-pigs.

In both males and females the increase in granule cell number was associated with an increase in granule cell density. The granule cell layer contains the cell bodies of the granule cells, arranged in several rows, and the proximal part of their dendritic arbor. A previous study showed that the granule cell soma has a circumference of 54–55 μm in 15 days old animals and 57–61 μm in 45 days old animals (Bartesaghi et al., 2003a). This small (4–10%) age-related increase in cell size was accompanied by a large reduction (15–35%) in the number of first and second order dendritic branches and an increase in the number of more distal branches. The reduction in the number of proximal branches implies an increase the space available within the layer. This may account for the age-related density increase of the granule cells, in the presence of a slight increase in soma size.

The age-related increase in the volume of the granule cell layer was accompanied by a parallel increase in the volume of the molecular layer and hilus. The volume increase of the molecular layer is consistent with the size increase of the distal dendritic tree of the granule cells (Bartesaghi et al., 2003a). For analogy, it seems very likely that the volume increase of the hilus is due to an increase in the size of the dendritic arbor of the interneurons present in this layer.

The dentate gyrus of the guinea-pig is scarcely lateralized

Very few side asymmetries were present in the young guinea-pig and no asymmetries in the peripubescent guinea-pig, indicating that the guinea-pig dentate gyrus, unlike that of the rat and mouse (Diamond et al., 1983; Roof and Havens, 1992; Roof, 1993a; Tabibnia et al., 1999) is scarcely lateralized. The pattern of laterality, if present, favored the right dentate gyrus, which corresponds to what occurs in the dentate gyrus of other rodents (Diamond et al., 1983; Roof and Havens, 1992; Roof, 1993a; Tabibnia et al., 1999). However, unlike other rodents, the few side differences observed in the neonatal guinea-pig were present in both sexes. In addition, the magnitude of the sex effects was similar in the two sides, whereas in the rat the sex effects are more pronounced in the right hemisphere (Roof and Havens, 1992; Roof, 1993a). From the present data it appears that the degree of laterality in the structure of the hippocampal formation may be differentially pronounced according to species and, possibly, age.

CONCLUSIONS

The present data demonstrate that sex differences in the volume of the dentate gyrus and granule cell number are present in the guinea-pig before puberty. The sex differences in the granule cell layer go altogether in the same direction as those demonstrated in the rat and mouse. The analysis of the sex dimorphism has been carried out separately in different layers and subregions of the dentate gyrus. This allowed us to demonstrate that sex differences i) are present in all layers; ii) are present at both dorsal and ventral levels of the dentate gyrus; iii) go in the same directions throughout the dentate gyrus. The use of animals of different ages revealed that the direction of the sex differences did not change during development, though the sex differences were less widespread in peripubescent animals. The presence of the sex differences across the dentate gyrus suggests that hippocampus-dependent functions may be sexually dimorphic prior to puberty. It remains to be ascertained whether the sex differences existing prior to puberty in the guinea-pig dentate gyrus are retained in adulthood.

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