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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 disector method was preferred to the optical fractionator method to estimate cell number. For volume (V_{ref}) 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

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Abbreviations: asf, area sampling fraction; CE, coefficient of error; LB, lower blade; N_V, numerical density; ssf, section sampling fraction; tsf, thickness sampling fraction; UB, upper blade; V_{dis}, volume of the disector; V_{ref}, reference volume.

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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 animals of this group are defined here neonatal animals (neonatal males; neonatal females) and four males and four females 45–46 days old. In the guinea-pigs females reach puberty at 40–45 days of age and males at 45–70 days of age. The 45–46 days old animals are defined here peripubescent animals (peripubescent males; peripubescent females). Males and females were reared in the same cage up to 30 days of age. Thereafter males and females were assigned to different cages, where animals of the same sex were housed. The experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, in compliance with the Italian guidelines for care and use of laboratory animals and after receiving governmental veterinary approval. The animals utilized in the present research were treated humanely, properly fed and their surroundings kept in a sanitary condition. All efforts were made to minimize the number of animals used and their suffering.

Tissue processing

The animals were killed with an overdose (100 mg/kg, i.p.) of anesthetic (Farmotal; Pharmacia, Milano, Italy) and perfused transcardially with 4% paraformaldehyde in phosphate buffer. The brain was excised and cut along the midsagittal plane, weighed, stored in the fixative for 24 h and then kept in 20% sucrose for additional 12 h. Both hemispheres were cut in parasagittal serial sections of 30 μm thickness by a freezing microtome. Parasagittal sections were chosen because in this plane of section the dorsal and ventral hippocampus are more clearly recognizable (Fig. 1A). The sections were stored at 4 °C in phosphate-buffered saline. Every third section was mounted on slides and stained with the Nissl method.

Subregions and layers of the dentate gyrus

In rodents, the hippocampal formation (hippocampus proper plus dentate gyrus) has a longitudinal, dorso-ventral orientation in the brain. This orientation underlies its subdivision into two regions, called dorsal and ventral hippocampal formation, respectively. 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 LB, 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

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