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Testis development in the opossum *Monodelphis domestica*

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ABSTRACT

Testis development in the grey short-tailed opossum, *Monodelphis domestica*, was investigated by light and electron microscopy in 180 animals. On the day of birth, half the karyotyped males were found to have histologically differentiated testes. By day (d) 1 testicular cords were clearly distinguished in all XY gonads and the tunica albuginea was fully developed. At this stage the large and pale primordial germ cells could be differentiated from dark pre-Sertoli cells. From d 3 the testis became progressively rounded and testicular cords were surrounded by peritubular cells. Leydig cells were then distinguishable by the expected ultrastructural features of steroidogenically active cells, showing abundant vesicles of SER, extensive mitochondria with tubular cristae and numerous lipid inclusions. Subsequently these cells formed clusters and were surrounded by envelope cells until wk 12. Testes were located in the abdomen, attached to the large mesonephroi, until d 24 after birth when they began their descent to the scrotal sac. From 7 wk the interstitial tissue became less cellular. At the prepubertal stage (12 wk), the seminiferous tubules lacked lumina. Leydig cell cytoplasm was electron-dense with increased amounts of SER forming parallel profiles. By 4 mo (pubertal stage), seminiferous tubules were patent and various spermatogenic stages, including spermatozoa, were seen for the first time. Leydig cells then greatly outnumbered other interstitial tissue cells and were closely-packed around blood vessels but no longer clustered by envelope cells; their SER was very highly organised into masses of parallel arrays and lipid inclusions were reduced. In the adult (1 y) Leydig cells reached their greatest size; their morphological features resembled those seen at 4 mo except that lipid inclusions were sparse. In ageing Leydig cells (2–3 y), large amounts of SER were present but disorganised.

Key words: Marsupials; Leydig cell differentiation.

INTRODUCTION

The grey short-tailed opossum, *Monodelphis domestica*, is a South American marsupial which, although only recently introduced into biomedical research (VandeBerg, 1983, 1990), is increasingly used as a laboratory species since its size and rate of reproduction are similar to those of standard laboratory rodents (Adam et al. 1988; Fadem, 1985; Fadem et al. 1992; Fadem & Rayve, 1985; Moore & Thurstan, 1990). Basic information on its reproductive biology is therefore essential.

One of the ways in which marsupials may differ from eutherians is in the mechanism of sexual differentiation. In eutherians the female phenotype will develop by default unless the testis-determining gene on the Y chromosome switches gonadal development to form a testis (for review see George & Wilson, 1994), whose hormones then control masculinisation of the reproductive tract, external genitalia and brain (Short, 1982). A master gene on the Y chromosome, SRY in the human and Sry in the mouse, is thought to act to control a hierarchy of secondary regulatory genes: triggering of differentiation of the somatic cell line along the Sertoli cell pathway is a key event (Hunter, 1995). In marsupials, however, some sexually dimorphic structures—the scrotal and mammary gland primordia—are said to develop prior to gonadal differentiation (O et al. 1988; Renfree & Short, 1988; Renfree et al. 1992; Ullmann, 1993) and, as such, appear to be independent of androgens secreted by the Leydig cells. Since sexual differentiation of the testis in *Monodelphis domestica* has variously been reported as a prenatal event (Baker et al. 1990, 1993; Maitland & Ullmann, 1993) or a postnatal one (Moore & Thurstan, 1990; Fadem et al. 1992) a systematic study is timely.

In widely-documented species such as the lab-

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Table. *Details of animals used**

Age	Number of litters	Number of animals
Day 0	4	27
Day 1	2	12
Day 2	4	23
Day 3	2	14
Day 4	3	15
Day 5	2	13
Day 8	1	8
Day 16	2	15
Day 24	1	11
4 weeks	—	4
7 weeks	—	4
8 weeks	—	3
9 weeks	—	3
12 weeks	—	4
4 months	—	9
1–3 years	—	25

* From d 0 to d 24 at least 3 animals of each sex were used. Beyond that only males were investigated.

oratory mouse, testis differentiation occurs in a stepwise manner. At about 12.5 d post coitum (dpc) the testis is first distinguishable morphologically both by its greater vascularity and by the appearance of testicular cords (Sertoli and germ cells) at its cranial end. The tunica albuginea develops by 15 dpc; 2 d later a basal lamina and peritubular cells surround each cord and Leydig cells are identifiable morphologically. The ovary remains indifferent until 14 dpc, when clusters of germ cells begin to form indistinct ovigerous cords (Mackay & Smith, 1989). A similar sequence of events is seen in the rat, although it begins about a day later reflecting the longer gestation in this species (Jost & Magre, 1988).

Fetal and postnatal populations of Leydig cell have been distinguished on ultrastructural criteria in the rat (Zirkin & Ewing, 1987). The transient fetal population is responsible for the prenatal masculinisation of the reproductive and nervous systems, while the adult population is activated at puberty. The fetal population arises from precursors which probably originate from mesonephric mesenchyme (De Kretser & Kerr, 1988). They were thought to disappear during postnatal life as a result of either cell death or dedifferentiation (Gondos et al. 1974), but Kerr & Knell (1988) have reported their persistence as a small, distinct population of cells in the adult testis where their physiological role remains enigmatic. The fetal Leydig cells initially regress during the perinatal period, but then a phase of prepubertal growth is reinitiated before a further phase of regression occurs during the 2nd wk post partum. Coincident with terminal regression of the fetal Leydig cells is the development of adult-type Leydig cells which are believed to differentiate de novo from primitive interstitial fibroblasts (or mesenchymal cells) rather than to transdifferentiate from fetal cells. In rats, therefore, there appear to be 3 consecutive waves of Leydig cell development: fetal, early juvenile and juvenile–adult; a similar developmental sequence pattern has been reported for the pig (De Kretser & Kerr, 1988).

The main objectives of this study are (1) to characterise testis development morphologically; (2) to examine early differentiation of the testis; and (3) to ascertain whether there are separately identifiable neonatal and adult populations of Leydig cells.

MATERIALS AND METHODS

Animals

The animals used in the present study were bred at Glasgow University. A total of 190 opossums of the following ages were used: 0, 1, 2, 3, 4, 5, 8, 16 and 24 d after birth (the day of birth being designated as d 0), 4 wk and 7–9 wk. Animals aged 12 wk (prepubertal) 4 mo (pubertal) and 1–3 y (adult) were also collected. Details of animals were used at each age point as shown in the Table. Young animals (d 0–24) were killed by inhalation of CO_2 or halothane gas and testes were immersion fixed. Older animals (over 4 wk of age) were anaesthetised by an intraperitoneal injection of 4% sodium pentobarbitone and fixed by perfusion with phosphate buffered-fixative (see below).

Karyotyping

Karyotyping was carried out according to the method of Evans (1987). The liver was removed, minced and cultured in Williams E medium (GIBCO) containing colchicine (0.4 mg/ml) for 1 h at 37 °C and transferred to hypotonic 0.56% KCl for 10 min. The tissue was then fixed in methanol: glacial acetic acid (3:1 v/v) and spread onto slides. Chromosomes were stained with Giemsa's solution in 0.1 M phosphate buffer at pH 6.8 for 15 min.

Tissue preparation

Testes were perfusion or immersion-fixed with a primary fixative (modification of Karnovsky, 1965), composed of 3% glutaraldehyde + 1% formaldehyde

in 0.1 M phosphate buffer (pH 7.2–7.4) for 24 h at room temperature (20 °C); then, after a buffer wash, tissue was postfixed with 1 % OsO_4 in 0.1 M phosphate buffer for 30–60 min. Subsequently, specimens were dehydrated through an ascending ethanol series before being embedded in Araldite.

Light microscopy

For light microscopy, semithin (1–2 μm) sections were cut on a Reichert–Jung Ultracut with glass knives and then stained with Mayer's haemalum and eosin. All photographs were taken on a Leitz Vario–Orthomat photomicroscope.

Transmission electron microscopy

For transmission electron microscopy, ultrathin sections were cut on a Reichert–Jung Ultracut E with a diamond knife (Diatome-MP3455) and double post-stained with saturated uranyl acetate solution and Reynolds' lead citrate (Reynolds, 1963). Specimens were examined in a Philips-CM 100 transmission electron microscope.

RESULTS

Light microscope observations

New born and 1 d old specimens were karyotyped and semithin sections of gonads examined. The diploid chromosome number for *Monodelphis domestica* is 18; the karyotypes of male and female opossums are shown in Figure 1. The sex chromosome pair is the smallest, the Y being particularly small.

At birth (d 0) the gonads in both sexes were elongate in shape, extending along the medial aspect of the mesonephroi. At this stage some male gonads remained undifferentiated (Fig. 2*a*), resembling those of females of the same age (Fig. 2*b*). In others testicular cord development appeared to be beginning (Fig. 2*c*). The prospective testis was surrounded by a tunica albuginea of flattened fibroblasts, 4–5 cells in thickness. In developing testicular cords several cell types were already distinguishable: large pale staining cells with subspherical nuclei and fibroblast-like cells. At this stage the larger pale cells were either scattered in small groups or aggregated into cord-like structures (Fig. 2*c*). The latter presumably included the pre-Sertoli cells, but at this stage it was difficult to distinguish between these and the primordial germ cells at LM level. In immersion-fixed material, small capillaries with nucleated erythrocytes were seen occasionally (Fig. 2*c*).

By d 1 the testis primordium was clearly distinguishable in all male specimens. As can be seen from Figure 3, a tunica albuginea 4–8 cells deep then completely surrounded the organ. It was possible to distinguish between the 2 cell types: primordial germ cells were large pale cells and the pre-Sertoli cells had darkly stained nuclei. Both cells types were then all gathered into testicular cords located in a broad zone below the tunica albuginea. Peritubular cells were beginning to surround the cords. The central region of the gonad was occupied by a stroma consisting of undifferentiated fibroblast-like cells.

By d 2–5 post partum, the testis was becoming rounded, in contrast to the ovary which remained elongated. In 4 animals measured on d 5, mean testis length was 0.4 ± 0.03 mm (mean ± S.E.M.). The tunica albuginea became somewhat reduced in thickness as the cords grew. The 1–2 testicular cords seen per section were located peripherally and were composed of primordial germ cells and pre-Sertoli cells surrounded by 3–4 layers of peritubular cells (Fig. 4). Mitotic figures occurred in both germ cells and pre-Sertoli cells. Spindle-shaped Leydig cells were observed between the cords in the enlarging interstitial spaces and were identifiable by their intense basophilia.

By d 16 in 5 animals measured, mean testis length had increased to 0.99 ± 0.02 mm and each section revealed cord profiles (Fig. 5). Leydig cells increased in both size and number and occupied a major proportion of the interstitial tissue; their irregular granular nuclei were very obvious (Fig. 5). Mitotic figures were present in all cell types within the testis. The testis continued to grow, mean length measuring 1.5 ± 0.02 mm by d 24 (3 animals) and by the end of wk 4, the testicular cords dominated the organ (Fig. 6).

Between 7–12 wk after birth the testis length increased from 3.7 ± 0.2 to 6.5 ± 0.8 mm (5 animals used per stage). Flattened peritubular cells were reduced to a single layer and the interstitial tissue was becoming less cellular (Fig. 7). Mitosis was still occurring both in germ cells (Fig. 7) and Sertoli cells. Blood vessels and lymphatics in the interstitial tissue were readily identifiable by the lymphoprotein content of the latter. In addition, the interstitial tissue contained macrophages which could be distinguished from Leydig cells by their inclusions and reduced basophilia. At 12 wk the testicular cords were not yet patent and mature sperm were still absent (Fig. 7).

By 4 mo, testis length was 7.4 ± 0.39 mm (9 animals) and rose to 8.5 ± 0.78 mm (18 animals) in adults (1–3 y). Each testicular cord possessed a lumen and

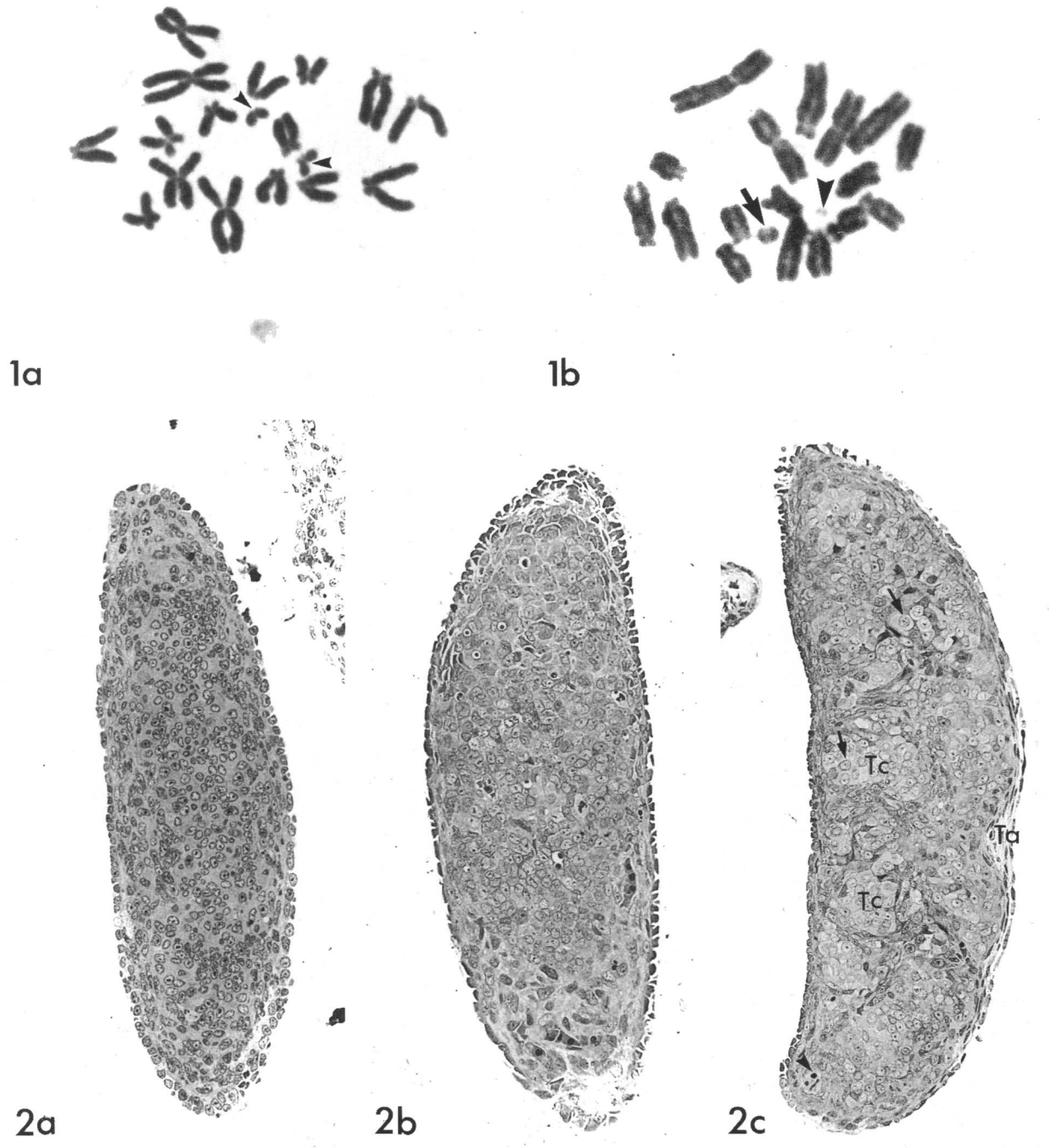
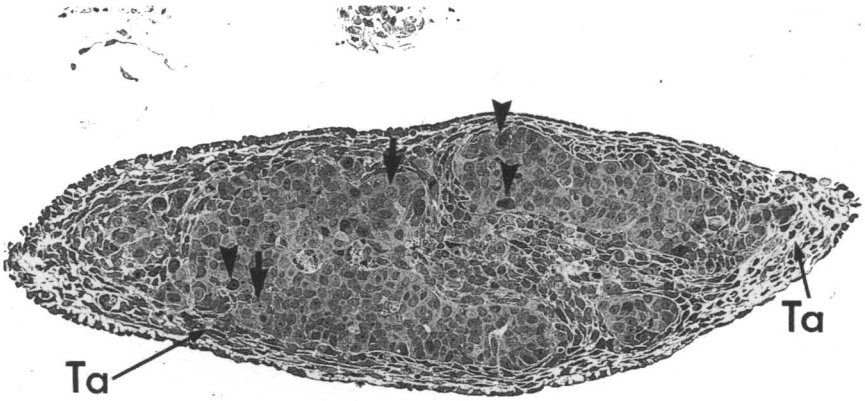


Fig. 1. Chromosome spread from new born opossums (d 0). (*a*) Chromosomes of a female opossum. The X chromosomes pair (arrowheads) is the smallest. × 1600. (*b*) Chromosomes of a male opossum. The X chromosome (arrow) is small and the Y chromosome (arrowhead) is minute. × 2600.

Fig. 2. Longitudinal sections of XY and XX gonads of neonatal opossums (d 0). (*a*) An XY gonad lacking testicular cords. × 200. (*b*) The XX gonad shows no obvious cord formation. × 200. (*c*) The XY gonad is surrounded by a tunica albuginea (Ta) 3–4 cells thick. Note initial formation of testicular cords (Tc) with large pale cells (arrows). A small capillary with nucleated cells is seen (arrowhead). × 200.

spermatogenic stages up to and including mature sperm were visible, so it was appropriate to refer to them as seminiferous tubules (Figs 8, 9). The peritubular cell layer had become further flattened (Fig. 8). Mature Sertoli cells were columnar in shape and their cytoplasmic processes were associated with late spermatids (Fig. 8). Leydig cells then greatly outnumbered other interstitial tissue cell types and in the adult could be seen closely packed around blood vessels (Fig. 9).



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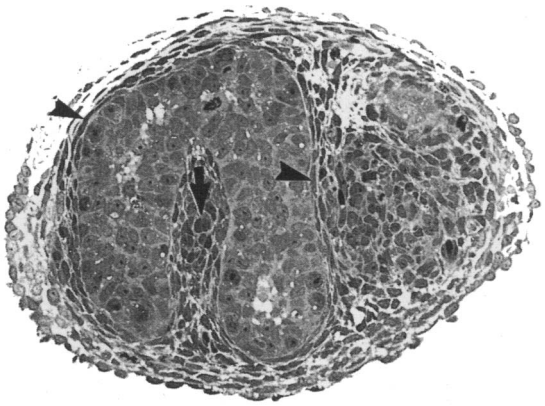

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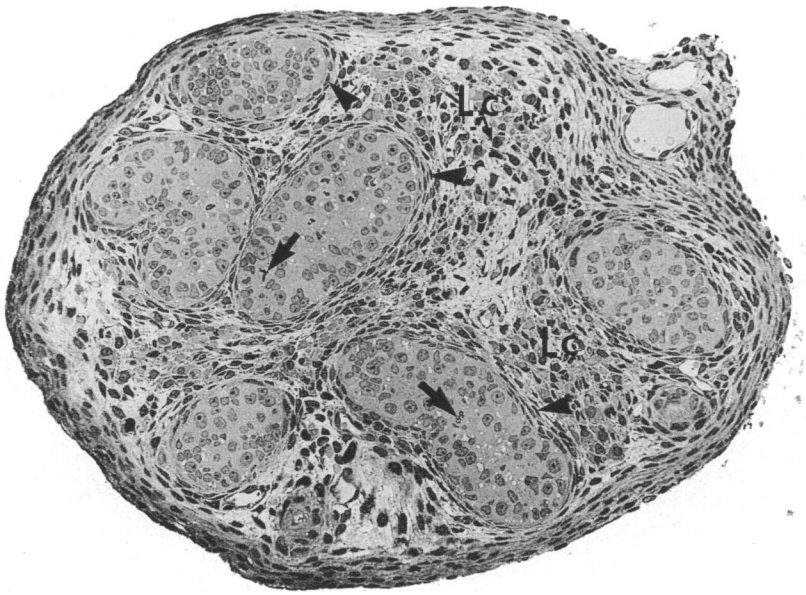

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Fig. 3. Longitudinal section through the male gonad (d 1), showing the 4–8 cells deep tunica albuginea (Ta). The sex cords are well defined and could easily be distinguished from interstitial tissue. Note primordial germ cells (arrows) and pre-Sertoli cells (arrowheads). × 200.

Fig. 4. Transverse section through the male gonad on d 3. Note Leydig cells (arrows) and peritubular cells (arrowheads). × 200.

Fig. 5. Transverse section through the male gonad (d 16), showing both germ cells and Sertoli cells. Note mitotic figures (arrows), 3–4 layers of peritubular cells (arrowheads) and well defined Leydig cells (Lc). × 200.

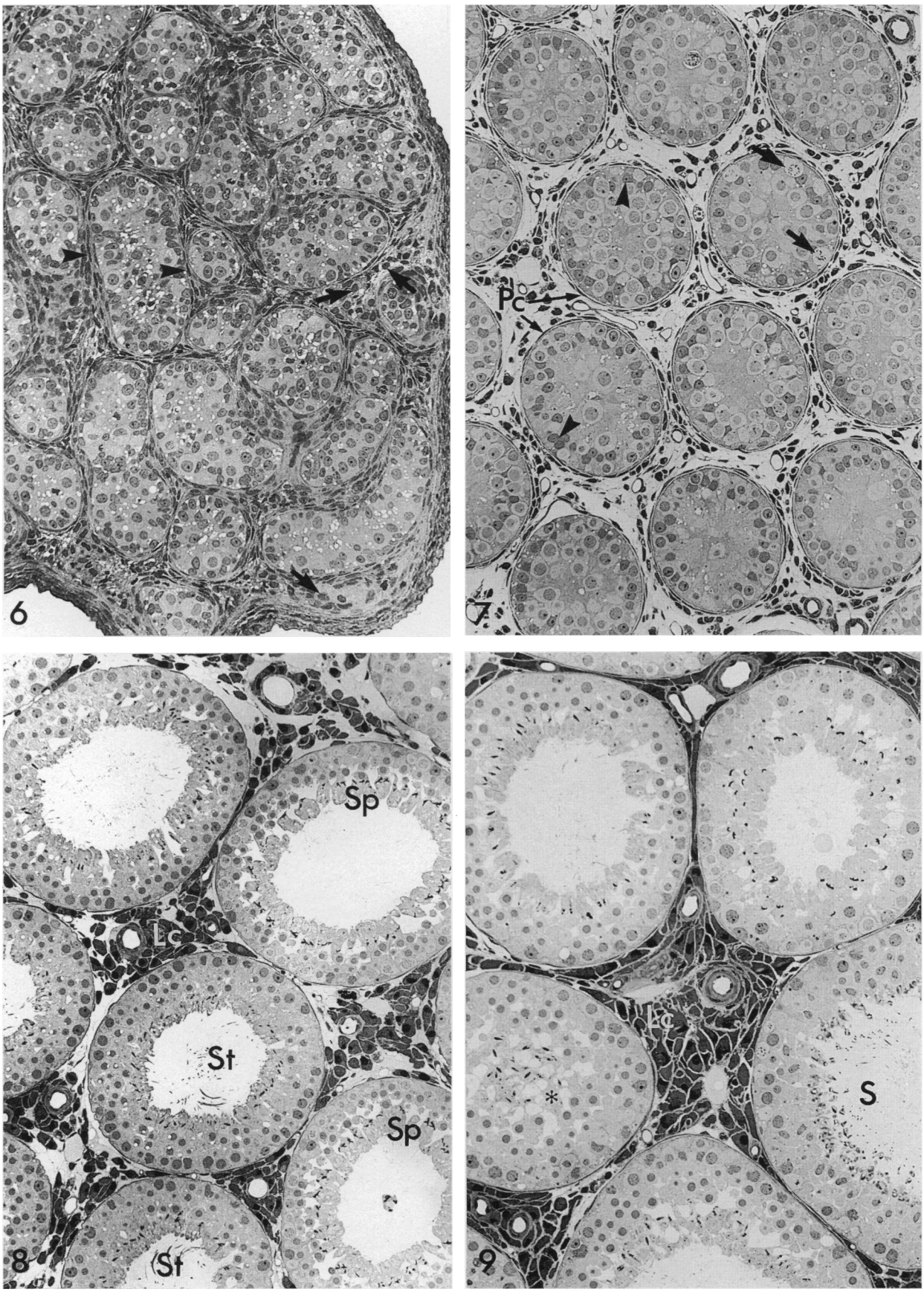

Fig. 6. Transverse section through the male gonad (d 24). The testicular cords predominate. Note Leydig cells (arrows) and 2–3 layers of peritubular cells (arrowheads). × 200.

Transmission electron microscopy

Pre-Sertoli and Sertoli cells

Pre-Sertoli cells could first be distinguished from primordial germ cells in the postnatal period (d 0–5) by the high electron density of their nuclei (Fig. 10). At both 12 wk and 4 mo, Sertoli cells proper were readily seen and had apical and lateral cytoplasmic processes which made contact with germ cell surfaces (Figs 11*a*, 12*a*). Sertoli cells were tall, irregularly columnar in shape and rested on the basal lamina of the seminiferous epithelium. Their nuclei were large and irregular in shape with numerous clumps of heterochromatin. One or two nucleoli with granular and other components of varying electron density were found in each nucleus. The Sertoli cell cytoplasm contained many tubular mitochondria and numerous electron-dense lipid inclusions. Both rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum (SER) were present, the latter being more abundant. Microtubules and microfilaments were occasionally found in the cytoplasm. By 4 mo well defined tight junctions formed between the processes of adjacent Sertoli cells with many parallel lines of fusion of the apposed membranes (Fig. 12*b*) and gap junctions were found between Sertoli cells and germ cells (Fig. 12*a*).

Germ cells

Primordial germ cells were pale, relatively large and rounded in shape with predominantly rounded nuclei. At juvenile stages (up to 8 wk), spermatogonia were common within the testicular cords and neighbouring cells were connected by intercellular bridges. Spermatogonial cytoplasm shows numerous mitochondria with tubular cristae, profiles of both SER and RER were present, as were abundant free ribosomes. By 12 wk spermatocytes with synaptonemal complexes appeared (Fig. 11*a*, *b*). From 4 mo, all stages of germ cell development could be seen in the testis (Fig. 12*a*). In general, spermatogonia lay against the basement membrane and primary spermatocytes were found towards the periphery of the tubule wall while other cell types such as spermatids and mature spermatozoa were located closer to the lumen. Primary spermatocytes were characterised by a large spherical nucleus; when present, synaptonemal complexes were

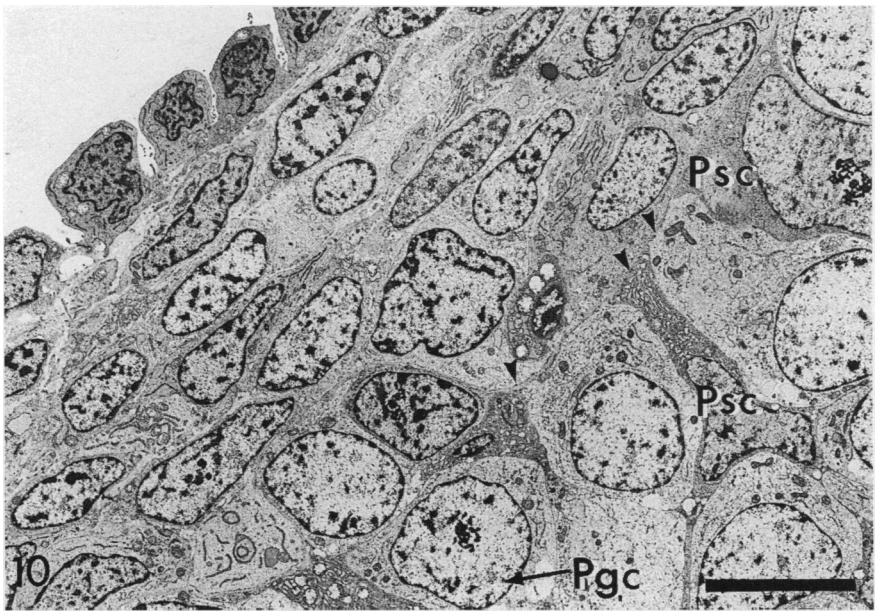

Fig. 10. Electron micrograph of a neonatal male gonad (d 0), showing pre-Sertoli cells (Psc), primordial germ cells (Pgc) and a basal lamina (arrowheads). Bar, 10 μm.

Fig. 7. Transverse section through the prepubertal testis (12 wk), showing the interstitial tissue becoming less cellular. Note a single layer of peritubular cells (Pc), germ cells in mitosis (arrows) and abundant Sertoli cells (arrowheads) with extending cytoplasm. There are extensive capillaries and lymphatics in the interstitium. × 200.

Fig. 8. Transverse section through the pubertal testis (4 months) showing various stages of germ cell development in the seminiferous tubules and closely packed Leydig cells (Lc) around blood vessels in the interstitial tissue. Note spermatids (Sp), sperm tails (St). This is the first stage at which the seminiferous tubules are patent. × 200.

Fig. 9. Transverse section through the adult testis (1 y). Note abundant Leydig cells (Lc) and numerous sperm (S). * The apparent absence of a lumen in this tubule is an effect of the plane of section. × 200.

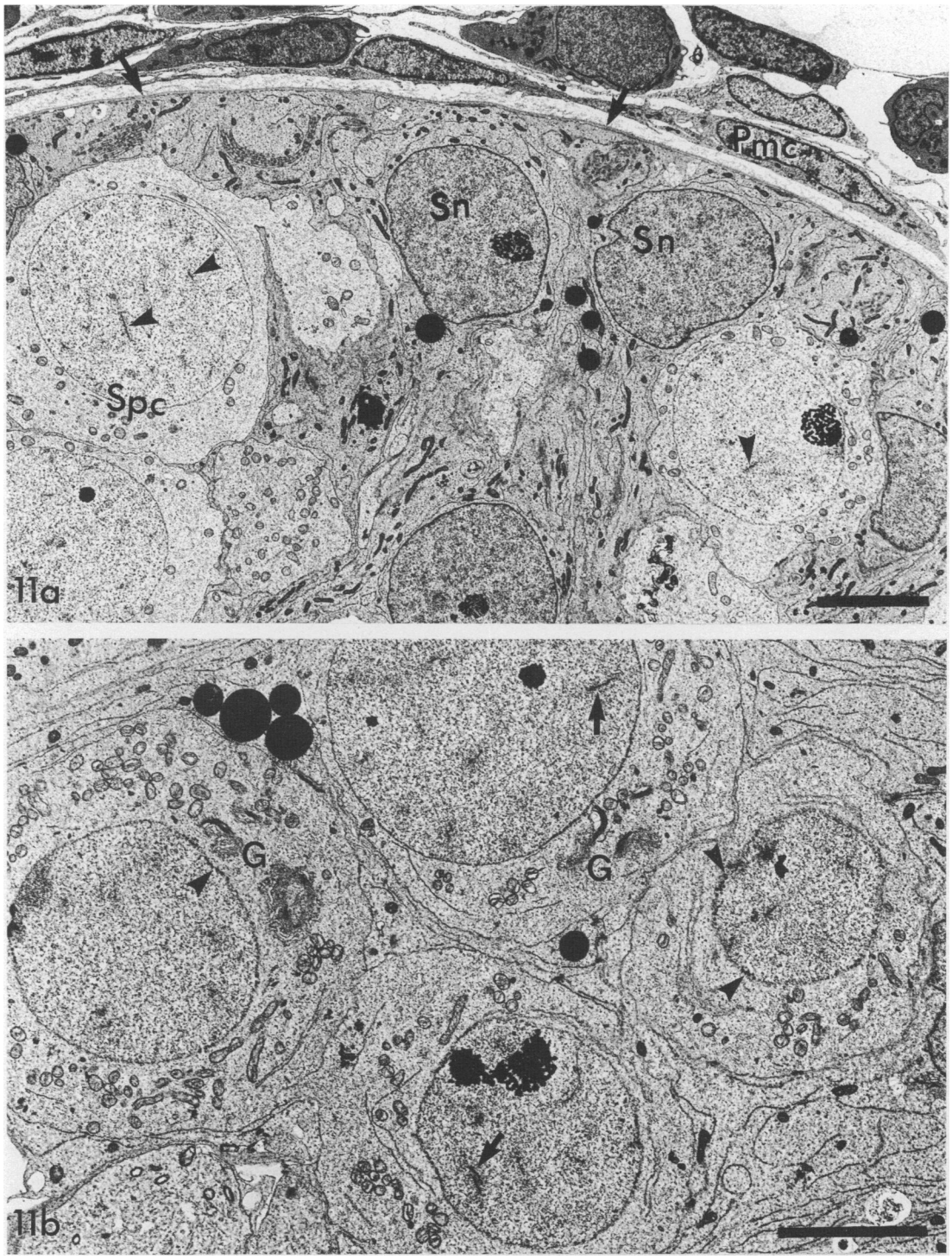

Fig. 11. Electron micrograph of prepubertal testis (12 wk). (*a*) Lower power micrograph of testicular cords showing the following features: peritubular myoid cells (Pmc), basement membrane (arrow), spermatocytes (Spc) with synaptonemal complexes (arrowheads), Sertoli cell nuclei (Sn). Bar, 5 μm. (*b*) Spermatocytes with synaptonemal complexes (arrows) and nuclear pores (arrowheads). Note well developed Golgi complex (G). Bar, 5 μm.

evidence that the pachytene stage of meiosis had been reached (Fig. 12*b*). Mitochondria were ovoid and occasionally aggregated into groups of 2 or 3. Well defined Golgi bodies appeared as large complexes of several lamellae and associated vesicles. Immature spermatids were rounded and smaller in size than spermatocytes. The spermatid had a centrally placed spherical nucleus with homogeneous chromatin, a well developed Golgi complex, and an adjacent centriole; the first signs of acrosome development were seen (Fig. 12*a*). Late spermatids were elongated in shape and their nuclei were condensed and the

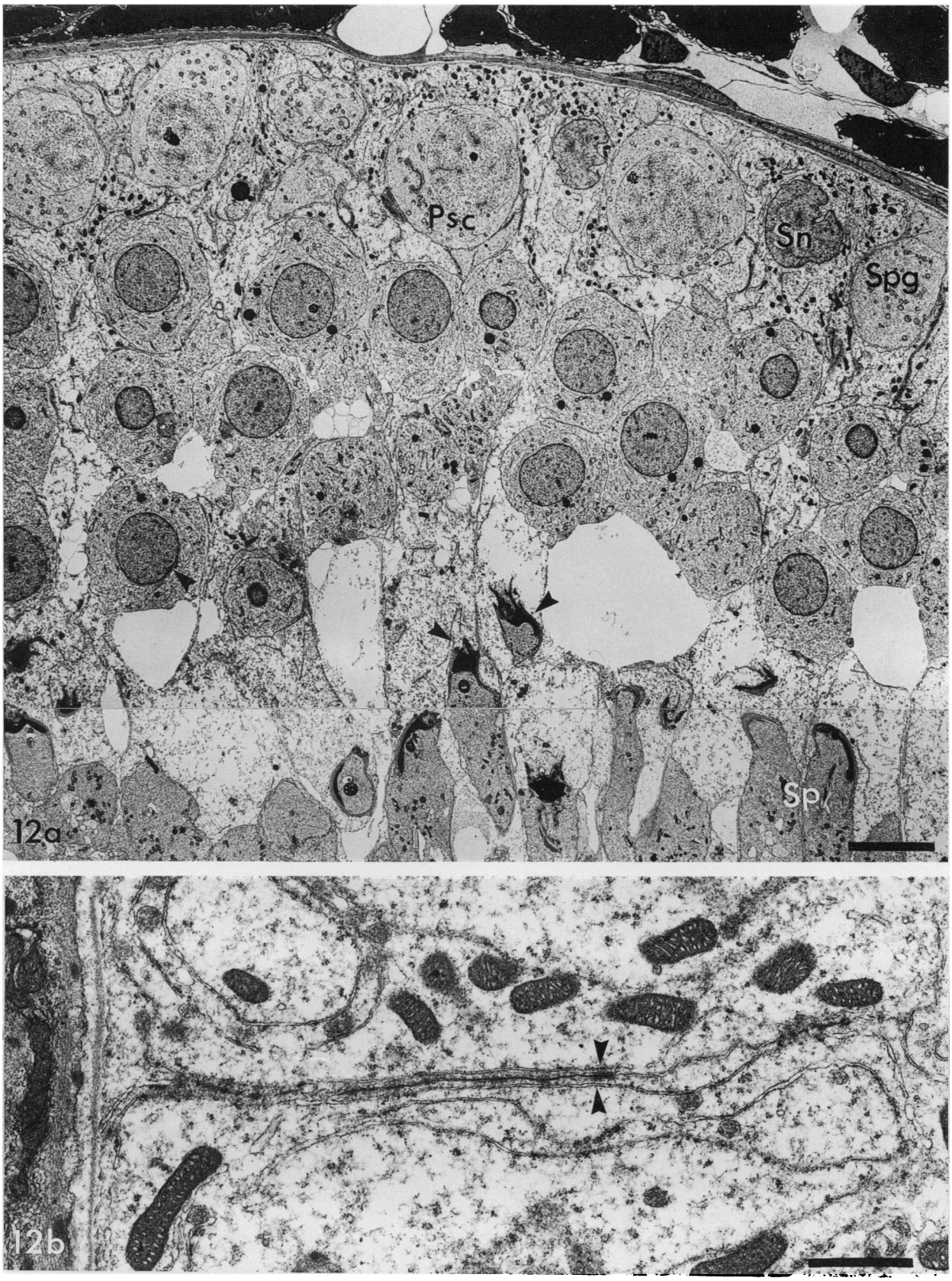

Fig. 12. Electron micrograph of pubertal testis (4 mo). (*a*) Low-power electron micrograph of seminiferous tubule (4 mo) showing the following features: spermatogonia (Spg), primary spermatocytes (Psc), spermatids (Sp), acrosomes (arrowheads) and Sertoli cell nuclei (Sn). Bar, 10 μm. (*b*) Well defined tight junction showing the parallel lines of fusion of the apposed membranes and cisternae of endoplasmic reticulum (arrowheads) Bar, 0.1 μm.

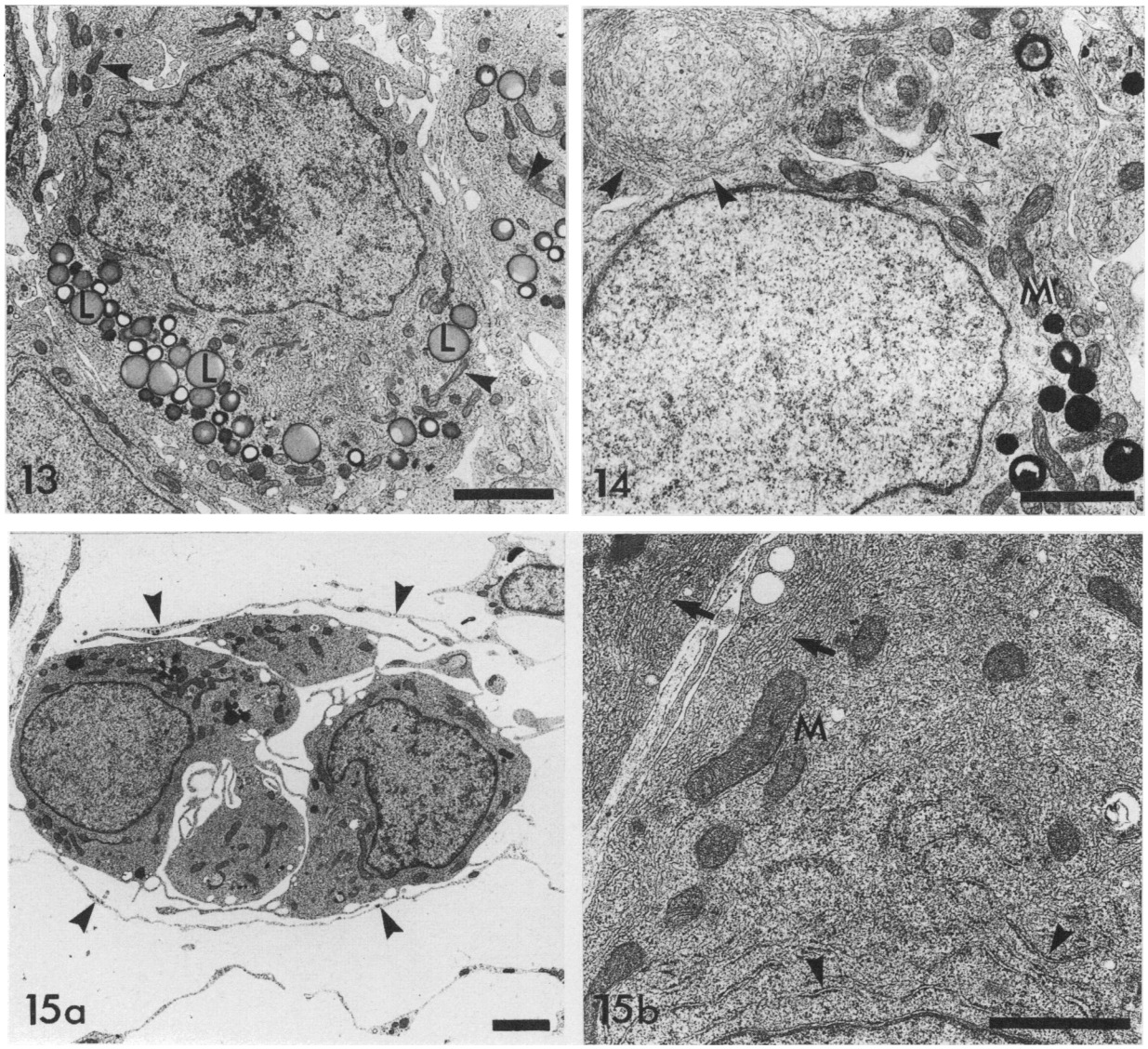

Fig. 13. Day 3 Leydig cell showing vesicles of SER; mitochondria (arrowheads) and abundant lipid inclusions (L). Bar, 2 μm.

Fig. 14. Day 8 Leydig cell showing elongate profiles and vesicles of SER (arrowheads) and mitochondria with tubular cristae (M). Bar, 2 μm.

Fig. 15. Electron micrograph of prepubertal testis (12 wk). (*a*) Leydig cells clustered by surrounding envelope cells (12 wk). Note envelope cell processes (arrowheads). Bar, 2 μm. (*b*) Portion of Leydig cell showing parallel alignment of SER profiles (arrows). Note RER (arrowheads) and mitochondria (M). Bar, 1 μm.

acrosome was more obvious. During spermiogenesis the nuclear shape changed and the mature spermatozoon nucleus became U-shaped. A fully formed acrosome was present at the head end of the mature spermatozoon and axial filaments were found in the tail.

Leydig cells

Leydig cells were not detectable at birth, but were first seen on d 3 (Fig. 13) when they could readily be distinguished from fibroblasts by their highly electron dense nuclei and cytoplasm. Over the course of the next few days they developed the expected ultrastructural features of steroidogenically active cells, namely a variable amount of elongate profiles and vesicles of SER, mitochondria with tubular cristae, and abundant lipid droplets of high electron density clustered in the cytoplasm (Figs 13, 14). By this stage SER was extensive and occasionally RER and Golgi bodies were visible; nuclei were irregular in shape and the nucleus:cytoplasm ratio was approximately 1.5:1. Subsequently Leydig cells were surrounded by envelope cells.

From 7 to 12 wk Leydig cells still formed clusters (Fig. 15*a*). SER had increased in amount and formed parallel profiles (Fig. 15*b*). At this stage lipid inclusions were still present but reduced in number

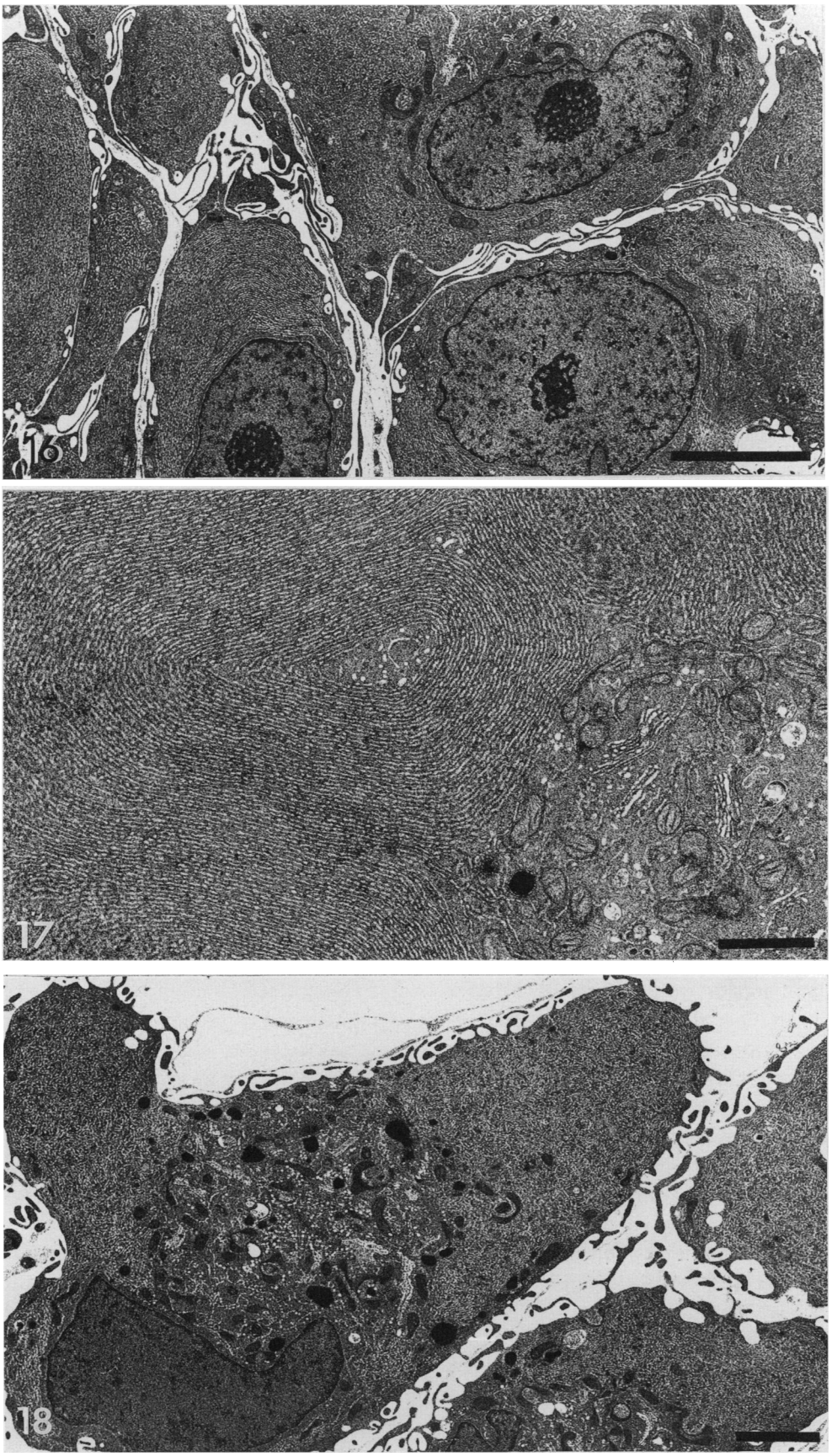

Fig. 16. Electron micrograph of pubertal testis showing closely packed Leydig cells. Bar, 10 μm.

Fig. 17. Electron micrograph of adult testis (1 y) showing part of a Leydig cell. The cytoplasm shows an abundance of SER highly organised into masses of parallel arrays. Bar, 1 μm.

Fig. 18. Electron micrograph of ageing testis (3 y) showing masses of SER, which is disorganised. Bar, 2 μm.

compared with previous stages. Leydig cell nuclei were irregular in shape and the nucleus:cytoplasm ratio was 1:1 as a result of an increase in the amount of cytoplasm.

At 4 mo Leydig cells remained closely packed but were no longer surrounded by envelope cells. Masses of SER formed highly organised parallel arrays which were not seen at earlier stages (Fig. 16) and lipid inclusions were further reduced. Nuclei were round or irregularly ovoid with finely dispersed chromatin, and the nucleus:cytoplasm ratio was 1:3 as the amount of cytoplasm had further increased.

In the adult, Leydig cells reached their greatest size and their morphological features resembled those seen at 4 mo, except that lipid inclusions were sparse (Fig. 17). With ageing (2–3 y), masses of SER were present but disordered (Fig. 18). Small amounts of RER and some secondary lysosomes were visible. Nuclei were irregular in shape and position and some heterochromatic nuclei could be seen at this stage.

DISCUSSION

While gonadal differentiation occurs in eutherians before birth (George & Wilson, 1994; Hunter, 1995), the limited observations on marsupials indicate that this process is a perinatal event. In bandicoots (Ullmann, 1981) the testis can initially be identified on the 1st day of pouch life, while in the marsupial native cat (Ullmann, 1984) and brown marsupial mouse (Taggart et al. 1993*a*) it is not distinguishable until d 3 postpartum. In the brush tail possum gonadal differentiation occurs perinatally (Ullmann, 1993). Tammar wallaby gonads were initially described as indifferent at birth (O et al. 1988) but, more recently, subtle ultrastructural differences between the sexes have allowed prenatal identification of the testis (Renfree et al. 1992).

Among American marsupials, McCrady (1938) reported testicular cord formation in the Virginia opossum as a prenatal event; but early observations on testis development in *Monodelphis* suggested that the gonads were undifferentiated at birth. Thus Fadem et al. (1992) first recorded testis development on d 4 postpartum, while Moore & Thurstan (1990) observed this on d 3. However, Baker et al. (1990, 1993) and Maitland & Ullmann (1993) described recognisable testes at birth, indicating that testis differentiation is a prenatal event in this species. In the present study about half the karyotyped males were found to have histologically differentiated testes at birth. It thus appears that in *Monodelphis* (as in the brush tail possum) testis differentiation is a truly perinatal event.

Fadem & Rayve (1985) and Adam et al. (1988) have already reported that puberty occurs at 4 mo, but it is unclear whether these authors used morphological, endocrinological or behavioural criteria to calculate the onset of puberty. Our studies provide evidence first, that, at 3 mo, the seminiferous tubules have not yet developed lumina and sperm are absent, from which we deduce that this stage is prepubertal. Secondly, by 4 mo the seminiferous tubules are patent, various spermatogenic stages, including sperm, can be seen and Leydig cells greatly outnumber other interstitial cell types. Thus the morphological features associated with the onset of puberty in *Monodelphis* appear at 4 mo.

The Sertoli cell is regarded as a principal regulator of materials delivered to the germ cells and also secretes fluid into the tubular lumen, the direction of fluid secretion being determined by the presence of the Sertoli cell barrier (George & Wilson, 1994). The latter is widely presumed to have a marked influence on spermatogenesis. Several studies have demonstrated its morphological and functional development in which inter-Sertoli junctional specialisations realign in order to provide an effective barrier (De Kretser & Kerr, 1988). Russell et al. (1989) described its postnatal development in the rat as on d 15–16 and its completion in all tubules prior to d 18. Cavicchia & Sacerdote (1991) indicated that the appearance of the blood-testis barrier takes place earlier (d 13–20) with the appearance of zygotene-pachytene spermatocytes. In *Monodelphis*, the development of the blood-testis barrier occurs before puberty with the appearance of inter-Sertoli cell tight junctions at 12 wk. In addition, synaptonemal complexes are found in some primary spermatocytes at this stage, indicating that the pachytene stage of meiosis has been reached. By 4 mo (puberty), the number of inter-Sertoli cell tight junctions (perpendicular to the basal lamina and parallel to each other) are abundant, suggesting the morphological substrate of the blood testis barrier is now fully developed.

Rod-like crystalloid inclusions occur in Sertoli cells of the koala (Harding et al. 1982) and the Virginia opossum (Duesberg, 1919), often located close to the nucleus and the basal lamina. However, such inclusions are absent in *Monodelphis*.

The spermatogenic cells of *Monodelphis* show few unusual characteristics. The present study indicates that the nucleus is oval in spermatocytes, spherical in immature spermatids and U-shaped in mature sperm. This confirms a study based on immunostaining of

suspensions of dissociated cells in *Monodelphis* by Olson & Winfrey (1991); similar findings were repeated by Rattner (1972) in *Didelphis virginiana* and the mouse opossum *Marmosa mitus*. Pairing of spermatozoa in the epididymis probably occurs in all American marsupials (Rodger, 1982; Taggart et al. 1993*b*) and our observations of this phenomenon in *Monodelphis* confirm those of Taggart et al.

Our ultrastructural study confirms the earlier tentative identification of Leydig cells in *Monodelphis* on d 3 (Moore & Thurstan, 1990), by which time they possess the machinery for steroidogenesis. In the rat, in which Leydig cells have been most intensively studied, fetal Leydig cells are characterised by numerous lipid inclusions and form clusters surrounded by envelope cells (Kerr & Knell, 1988; Kuopio et al. 1989). In the adult testis Leydig cells generally lack lipid inclusions and are not surrounded by envelope cells (Kerr & Knell, 1988). In *Monodelphis*, although the clusters of Leydig cells found in immature animals are more loosely organised than in the rat, nevertheless the delicate processes of envelope cells can be demonstrated around them at the ultrastructural level. Moreover, the lipid inclusions which occur in these cells at early stages (d 3 onwards) are rapidly reduced at the onset of puberty (4 mo).

The adult structure of Leydig cells is attained by 4 mo and is characterised by the extraordinary abundance of SER which is highly organised into masses of parallel arrays and occupies the major part of the cytoplasm, giving it great density. Christensen & Fawcett (1961) also demonstrated an unusually abundant SER in Virginia opossum Leydig cells, but this species lacks parallel arrays. Leydig cells may possess species-specific characteristics such as the crystals of Reinke in man (De Kretser & Kerr, 1988) and glycogen granules in the fetal rat (Kerr & Knell, 1988), but such inclusions are not found in *Monodelphis*.

Adult type Leydig cells in the rat are believed to differentiate from mesenchymal cells, after the disappearance of the fetal type cells (Kerr & Knell, 1988; Kuopio et al. 1989). According to these authors, some fetal Leydig cells persist in the adult rat testis. Christensen & Fawcett (1961) also reported the persistence of relatively undifferentiated mesenchymal cells in the testis of the adult Virginia opossum that are believed to be capable of developing into adult-typical Leydig cells. In *Monodelphis* we could not identify mesenchymal cells or fetal-type Leydig cells in the mature testis, nor could we recognise a period, subsequent to their appearance on d 3, when Leydig cells were absent. We therefore hypothesise that, in *Monodelphis*, the fetal-type Leydig cells give rise directly to the adult cells. Associated with this transformation is a 2–3 fold increase in the amount of cytoplasm, the remarkable development of the SER and the loss of envelope cells.

To summarise, our study suggests firstly that testicular differentiation in *Monodelphis* is a truly perinatal phenomenon since only about 50% of the animals show gonadal sex differentiation at birth. Secondly, although a morphological difference is evident in the Leydig cells of immature and mature animals (which equate to the fetal and adult-type cells described in eutherians), 2 separate and distinct populations could not be recognised, neither could a period in postnatal development be identified in which Leydig cells were absent. Since neither degenerating Leydig cells nor mesenchymal cells were evident in the testis, we hypothesise that fetal-type Leydig cells transform directly into adult-type cells by loss of lipid inclusions, loss of surrounding envelope cells and the development of highly organised arrays of SER. Thirdly, we demonstrate the appearance of patent seminiferous tubules with mature sperm by 4 mo, the period when peripheral testosterone begins to rise to adult levels (unpublished observations). On the evidence of our data we conclude that in *Monodelphis* the pubertal period is reached 4 mo after birth.

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