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Androgen action during male sex differentiation includes suppression of cranial suspensory ligament development

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The cranial suspensory ligament is located on the border of the cranial (mesonephric) mesentery in adult female mammals, which runs between the cranial pole of the internal genitalia and the dorsal abdominal wall. Absence of the cranial suspensory ligament in male mammals depends upon exposure of its primordium to fetal testicular androgens and is a prerequisite for testis descent. Female rats were exposed to 5α-dihydrotestosterone propionate at different stages of genital development, and cranial suspensory ligament development was studied in neonatal and in adult animals. Androgens suppressed cranial suspensory ligament development when exposure started during the early stages of genital development, until day 19 postconception (pc). Androgen receptor expression was immunohistochemically detected in the cranial mesentery of both sexes from day 16 pc onwards. A decrease of androgen receptor expression in female fetuses from day 18 pc onwards coincided with the appearance of a differentiated cranial suspensory ligament, as evidenced by the expression of two cell differentiation markers: α-smooth muscle (α-SM) actin and desmin. α-SM actin was located on the outer border of the cranial mesentery of both sexes at day 17 pc, and expression increased only in female fetuses. On day 19 pc, desmin expression was also detectable in the α-SM actin-positive cells. Proliferation and apoptosis indices of cells in the cranial mesentery, as analysed by 5′-bromodeoxyuridine incorporation and by detection of DNA strand breaks (TUNEL method) respectively, did not show any difference between the sexes, neither on day 17 nor on day 18 pc. Since primordial cells of the cranial suspensory ligament highly express the androgen receptor during the period of gestation when androgens can suppress cranial suspensory development, altered morphogenesis of these cells may be a direct consequence of androgen action.
Key words: **androgen/genital system/sex differentiation/ testis descent**

Introduction

The male-specific process of testis descent, as it occurs in many mammalian species, has been indicated as a partially androgen-dependent process, and searches have been made for the target structures of this androgen action (Hutson *et al*., 1994; Heyns and Hutson, 1995; Husmann and Levy, 1995). Almost exclusively attention has been paid to structures caudal to the testis, with the gubernaculum as the main target structure. However, in contrast to other specific structures of the male genital system such as the epididymis, vas deferens, seminal vesicles, and prostate, it is still a matter of debate whether androgens are involved in male-specific gubernaculum development (Heyns and Pape, 1991; Van der Schoot and Elger, 1993; Husmann and Levy, 1995). An under-exposed phenomenon in the discussion of possible targets is the absence of the cranial suspensory ligament (CSL) in adult males of most mammalian species (Van der Schoot and Emmen, 1996). The CSL is a muscular cord-like structure which borders the cranial part of the mesonephric mesentery, attaching the ovary and genital duct to the craniolateral surface of the dorsal abdominal wall, near the ventral aspect of the last rib. Besides keeping the uterus in position during pregnancy, the CSL is supposed to play a role in the autonomic innervation of the ovary (Mohsin and Pennefather, 1979). During early genital development, the primordium of the CSL is present in both sexes of, among others, the rat, human and pig (Van der Schoot and Emmen, 1996). In rats, the sexual dimorphism in CSL development occurs during the last days of fetal life and correlates with the noticeable sex-specific position of the gonads (Van der Schoot and Elger, 1992). Lack of outgrowth of the CSL probably is a prerequisite for testis descent, although this does not imply that absence of the CSL results in testis descent. The complex process of testis descent involves an interplay of different structures and factors (Hutson *et al*., 1997).

The prevention of outgrowth of the fetal CSL in male rodents is an androgen-dependent process: prenatal exposure of females to androgens prevents development of the CSL, whereas males prenatally exposed to anti-androgens show CSL development in a female-like fashion (Van der Schoot and Elger, 1992; Shono *et al*., 1994; Barthold *et al*., 1994; Cain *et al*., 1995). These exposed male rats may demonstrate cryptorchidism in adulthood (Van der Schoot and Elger, 1992). Furthermore, androgen-insensitive mice and rats provide strong evidence for the essential role of androgens in CSL development; these animals have a non-functional androgen receptor and show persistence of the CSL, combined with cryptorchidism (Hutson, 1986; Barthold *et al*., 1994). Studies on testis descent in experimental animals may contribute to our understanding of the pathophysiology of testis descent in the human, which is of much interest in view of the high incidence of cryptorchidism in newborn boys (Frey and Rajfer, 1982).

The aims of the present study were: (i) to examine whether

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androgen action on CSL development is limited to a critical period of genital development; (ii) to gain further support for androgen action by demonstrating the local and/or time-specific presence of the androgen receptor in the primordium of this structure; (iii) to understand in more detail the cellular and molecular mechanism underlying the specific suppressive androgen action.

Materials and methods

Animals and tissue preparation

Wistar rats (R-Amsterdam strain) were obtained from TNO (Rijswijk, The Netherlands). Animals were housed in the departmental animal quarters on a fixed lighting regime (lights on at 05.00 h and off at 19.00 h), and were provided with tap water and standard laboratory pelleted food *ad libitum* (Hope Farms Standard Laboratory Diet, Woerden, The Netherlands). Rats were mated overnight, and the day when copulatory plugs were found in the morning was considered the day of conception (day 0 of pregnancy).

Neonatal females were injected s.c. on postnatal days 1 (= day of birth), 3 and 5 with 2 mg 5α-dihydrotestosterone propionate (DHTP; Steraloids, Hamilton, NH, USA) in 0.1 ml olive oil ($n = 10$), or with oil only ($n = 10$). They were killed at day 7 or at 3 months of age. Furthermore, pregnant female rats were daily injected s.c. with 10 mg DHTP in 0.2 ml oil, or with oil only, from day 11, 15, 17 or 19 postconception (pc) up to and including day 21 pc. They were killed on the expected day of parturition (day 22 pc). Fetuses were removed from the uterus, placed on ice, decapitated and fixed in 10% neutral buffered formalin. After preliminary fixation for 4–6 h, the abdomen was opened and intestines were removed. Fetuses were embedded in paraffin, sectioned parasagitally at 7 μm, mounted on slides (1:10) and stained with haematoxylin and eosin. Other fetuses were kept alive and reared by foster mothers. The animals were killed at the age of 3 months by cervical dislocation and the internal genitalia were visualized through macroscopical dissection and photographed *in situ*. In each treatment group, at least five animals were studied at the day of birth and five animals in adulthood.

5′-Bromodeoxyuridine (BrdU) is a thymidine analogue which can cross the placenta and is incorporated into DNA during the S-phase of the cell cycle (Packard *et al*., 1973). Pregnant rats were injected i.p. with a single pulse of BrdU (Boehringer Mannheim, Germany) dissolved in saline (100 mg/kg body weight) on day 17 or 18 pc. Two hours after injection, fetuses were removed from the uterus, fixed in 10% neutral buffered formalin and further processed for immunohistochemical detection of incorporated BrdU. Maternal intestine was included as control tissue.

For immunohistochemistry, fetuses were removed from the uterus by Caesarean section on days 16–22 pc and further prepared as described above. Tissue sections were cut at 5 μm and sections were selected for immunostaining. At each time point, one unilateral part of the urogenital tract was studied from at least four male and four female fetuses, which had been obtained from two different mothers.

Scanning electron microscopy

Day 16, 17, 18, 20, and 22 pc rat fetuses were processed for scanning electron microscopy (SEM) as follows. After preliminary fixation with Bouin's fixative for 6 h, the abdomen was opened and intestines were removed. After 48 h of fixation, tissue was dehydrated through a graded ethanol series to hexamethyldisilazane (Electron Microscopy Sciences, Fort Washington, PA, USA), mounted onto specimen stubs, coated with gold, and examined in a JOAL JSM 25-CF scanning electron microscope at 15 kV.

Immunohistochemistry

Sections were mounted on slides coated with 3-aminopropyl triethoxysilane (Sigma, St Louis, MO, USA). Negative controls were always included. After deparaffinization, sections were treated with 3% H_2O_2/methanol solution to block endogenous peroxidase activity and transferred to phosphate-buffered saline (PBS).

Immunohistochemical detection of the androgen receptor (AR) was performed on paraffin-embedded sections as described by Janssen *et al*. (1994). Sections were microwaved for 3×5 min at 700 W in 0.01 M citric acid monohydrate buffer, pH 6.0 (Merck, Darmstadt, Germany). The sections were then preincubated with normal goat serum, followed by incubation at 4°C overnight with primary polyclonal antibodies SP197 (raised against a synthetic peptide corresponding to the first 20 amino acid residues of the N-terminal domain of the human and rat AR), diluted 1:7000 in 5% bovine serum albumin (BSA)/phosphate-buffered saline (PBS) (Bentvelsen *et al*., 1995). After rinsing in PBS, the sections were treated with biotinylated goat anti-rabbit antibody (dilution 1:400; Dako, Glostrup, Denmark) followed by treatment with streptavidin–biotin–peroxidase complex (ABC; diluted 1:1:200; Dako) for 30 min at room temperature. Peroxidase activity was developed with 0.07% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Fluka, Basel, Switzerland) for 7 min. Negative controls included replacement of SP197 antibody by preimmune serum (1:7000) or peptide-blocked SP197 (1:7000), prepared by incubation of the antibodies with an excess (1 mg/ml) of the free synthetic peptide that was used to raise the antibodies. Haematoxylin was used for counterstaining.

Detection of apoptosis was performed by the TUNEL method (Gavrieli *et al*., 1992). Briefly, tissue sections were incubated with proteinase K (7.5 μg/ml) for 15 min, before treatment with 3% H_2O_2 in PBS to block endogenous peroxidase activity. Sections were pretreated with terminal deoxynucleotidyl transferase (TDT) buffer (30 nM Trizma base, 140 nM sodium cacodylate, 4 mM cobalt chloride, 0.1 mM DTT) for 5 min and then incubated with biotinylated deoxyuridine triphosphate (dUTP; 10 μM biotin–16-dUTP; Boehringer Mannheim) and TDT (0.3 U/μl; Promega, Madison, WI, USA) in TDT buffer for 2 h at 37°C. The reaction was terminated by transferring the slides into TB buffer (salt sodium citrate buffer) for 15 min. After washing for 10 min in 2% BSA in PBS, slides were incubated with ABC complex (diluted 1:200) and a solution of 0.07% DAB.

For BrdU immunostaining, monoclonal anti-BrdU was used as primary antibody (diluted 1:25; Sigma). The sections were digested with pronase 0.1% (Boehringer Mannheim) for 30 min at 37°C, treated with 2 N HCl for 30 min at 37°C, and neutralized with 0.1 M borate buffer (pH 8.5). After preincubation with 10% normal goat serum (Dako) to reduce non-specific binding, sections were subsequently incubated with anti-BrdU for 1 h at 37°C, then peroxidase-conjugated goat-anti-mouse (dilution 1:100; Sigma) for 30 min at room temperature, and 0.07% DAB for 7 min.

To study smooth muscle cell differentiation, monoclonal anti-α-smooth muscle (α-SM) actin (diluted 1:200; Biogenex anti-αsm-1 clone 1A4, San Ramon, CA, USA) and monoclonal anti-desmin (diluted 1:25; Sanbio mon-3001, Uden, The Netherlands) were used. Sections were treated as for BrdU staining except that pretreatment with pronase and HCl was not performed and the second antibodies were biotin-conjugated goat anti-mouse (Dako) diluted 1:50.

Evaluation of immunohistochemical results

Cells with nuclear (AR) or cytoplasmic (α-SM actin and desmin) immunostaining were interpreted as being positive. The intensity of positive staining was expressed in terms of three relative intensities: strong (+++), moderate (++) and low (+). Prostate and intestine

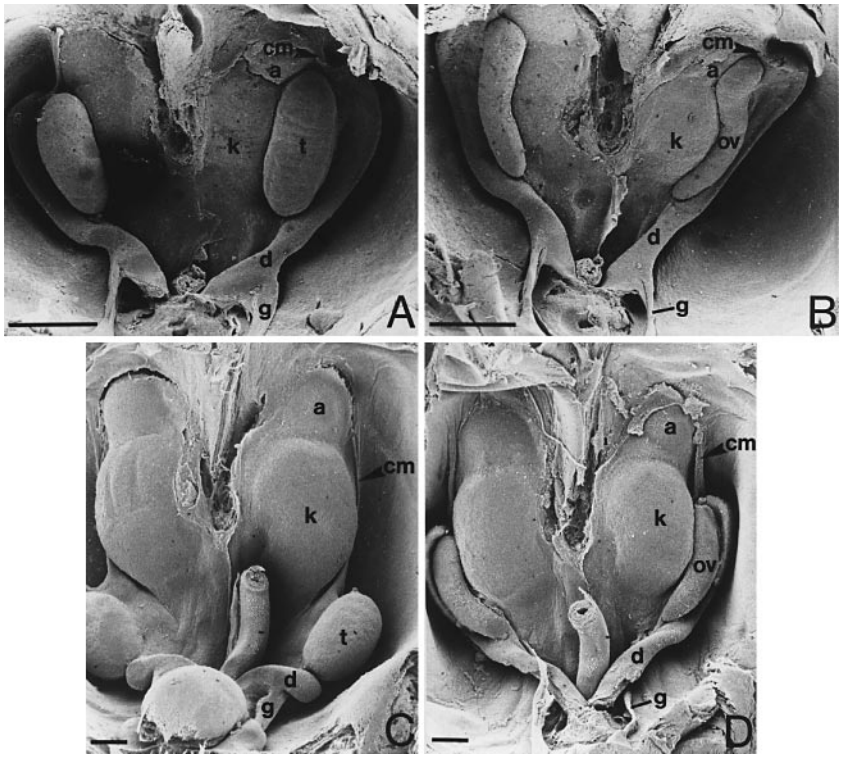

Figure 1. Scanning electron microscopy images of the urogenital system of male and female rat fetuses. (**A**, **C**) Male, and (**B**, **D**) female rat fetuses on day 16 postconception (pc) (top) and day 17 pc (bottom). Except for the gonadal form and size, the genital system of both sexes on day 16 pc is very similar; e.g. position of the gonads, and appearance of cranial mesentery (cm), gubernacular cord (g), and duct (d). On day 17 pc, the testis (t) has gained a lower position compared to the ovary (ov). There is a marked difference in the shape of cranial mesentery and gubernacular cord between the sexes (gubernacular cord of the female broke during preparation). a, adrenal; k, kidney. Scale bar = 400 μm.

of adult rat were used as a positive control for AR and actin/desmin staining respectively and were graded as +++.

Proliferation and degeneration indices of the cranial mesentery were determined by counting a minimum of 300 cells and a maximum of 1500 cells per specimen ($n = 4$) and subsequent division of the number of positive staining nuclei by the total number of counted cells. The cranial mesentery was considered to be the area between the efferent tubules of the gonad and cranial point of attachment to the abdominal wall. The cranial mesentery was not divided in different cellular compartments. Statistical significance was evaluated using two-factor analysis of variance.

Results

Normal development of CSL

On day 16 pc, the gonads of both male and female fetuses were found to be positioned on the ventrolateral aspect of the developing kidneys (Figure 1A,B). Differences in gonadal size and shape could be recognized: the ovary was more elongated and smaller compared to the testis. In both sexes, the gonads and adjacent ducts were connected to the abdominal body wall by two strands: one strand running from the gonad towards the diaphragm, which is the cranial (mesonephric) mesentery containing the CSL primordium, and a second strand connecting the duct with the inguinal abdominal wall, the gubernacular cord.

On day 17 pc, the testis showed a lower abdominal position than the ovary (Figure 1C,D). The ovaries of female rats were positioned lateral from the caudal pole of the kidneys, whereas the testes of male rats of the same age were situated well below the kidneys. The cranial mesentery also showed sexual dimorphism, appearing like a thickened cord in the female fetuses in contrast to the elongated tissue strand in male fetuses. Furthermore, a difference in gubernacular cord appearance between the sexes was noticed: a thicker cord in the male compared to the female fetus.

From day 18 pc onwards, the ovaries remained positioned lateral to the kidneys, and connected to the middle area of the last rib via a thick mesentery. In contrast, the testes gained a position well below the kidneys, and were connected to the abdominal wall at the level of the caudomedial tip of the kidney by a flattened mesenteric fold that sometimes was hardly distinguishable. At the light microscopical level, the cranial mesentery of female rats was bordered by a differentiated ligament, the CSL, which appeared as condensed mesenchyme (Figure 2A) and later was identified as smooth muscle by immunohistochemistry. The male counterpart was a small mesenteric fold without specifically differentiated cells in its free border (Figure 2B).

Androgen exposure and CSL development

Rat fetuses were exposed to DHTP at different stages of development to determine the time period of inhibitory androgen action upon the CSL primordia. DHTP was used rather than testosterone propionate (TP), since DHTP virilizes the

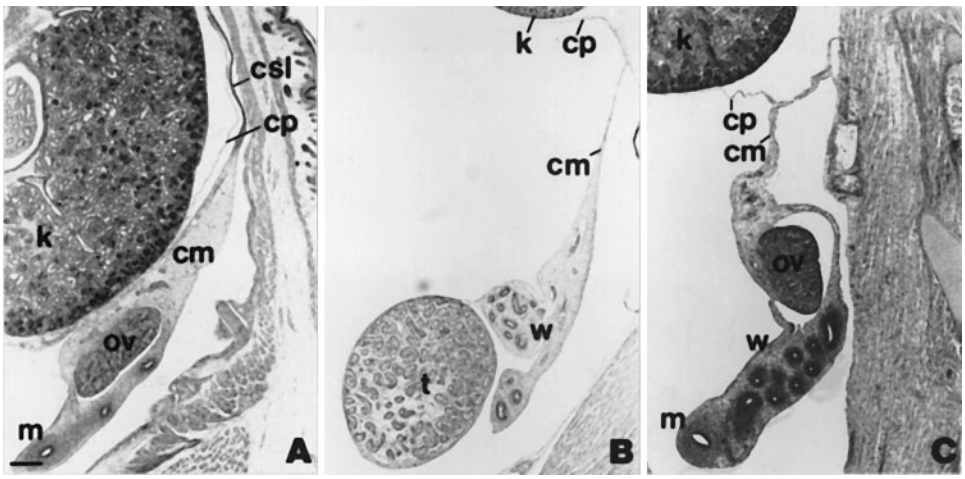

Figure 2. Sagittal histological section of the urogenital system of male and female rat fetuses on day 22 postconception (pc) (day of birth). (**A**) Control female, (**B**) control male and (**C**) female rat fetus exposed to 5α-dihydrotestosterone propionate (DHTP) from days 15 pc to day 21 pc. In the female fetus (**A**), the ovary (ov) is lying at the caudal pole of the kidney (k) and is connected to the cranial part of the dorsal abdominal wall via the cranial mesentery (cm) which is bordered by the cranial suspensory ligament (csl). The testis (t), surrounded by Wolffian duct derivatives (w), of the male fetus (**B**) has gained a position below the kidney and is connected to the abdominal wall via a thin fold of the cranial mesentery, without a specific structure. In the DHTP-exposed female (**C**), the ovary is located caudal from the kidney and surrounded by both Müllerian duct (m) and Wolffian duct derivatives. Only a thin mesenteric fold was observed between the genital system, the abdominal wall and the kidney capsule (cp). Scale bar = 200 μm.

Table I. The presence of cranial suspensory ligament (CSL) in female rats after prenatal exposure to dihydrotestosterone propionate (DHTP)

Treatment group (day pc)	At birth	In adulthood
11–21	0/8[a]	0/16
15–21	0/8	0/20
17–21	0/10	0/10
19–21	10/10	18/18
Control	10/10	26/26

The fetuses were exposed to DHTP (10 mg/day), starting on different days postconception (pc) up to and including day 21 pc. Examination was performed at day of birth (day 22 pc) by histology, and in adulthood by dissection.
[a]Number of ovaries with CSL/total number of ovaries.

genital system of the female rat fetus to a similar degree as TP but avoids the frequent disruption of pregnancy observed after TP injection (Schultz and Wilson, 1974).

Prenatal exposure of male fetuses to DHTP had no visible effect on the cranial part of the genital system. All male animals, control and DHTP-treated, displayed no development of a CSL at birth and had normally descended testes in adulthood (results not shown).

In female rats the CSL was absent from three out of four different treatment groups (Table I). Histological analysis of the internal genitalia of day 22 pc female fetuses exposed to DHTP before day 19 pc showed no distinct ligament in the free border of the cranial mesentery (Figure 2C). The ovaries were positioned below the caudal pole of the kidney. However, the ovaries were closer to the kidney than the testes and the cranial mesentery was united with the kidney capsule whereas in males the cranial mesentery was separated from the kidney capsule (Figure 2B,C). In female fetuses exposed to DHTP from day 19 pc onwards, as well as control female fetuses, the ovaries were located lateral to the caudal pole of the kidneys and were connected via a well-developed ligament to the cranial part of the dorsal abdominal wall (Figure 2A). The degree of internal genital virilization varied between the different treatment groups, with the strongest degree of virilization in female fetuses exposed to DHTP from day 11 pc onwards.

Autopsy of adult female rats from different treatment groups confirmed the microscopical observations at the time of birth (Table I). Dissection of adult female rats, exposed to DHTP before day 19 pc, demonstrated absence of the CSL in all animals (Figure 3C). However, unlike the situation in normal males, the cranial mesentery had remained connected to the kidney capsule. In all exposed female rats, the morphological pattern of the ovarian–uterine vascular system was similar to that in control females, and no structure similar to a male-specific plexus pampiniformis was found. Virilization of exposed females from the different treatment groups was confirmed by presence of a penis with the urethra ending on its tip, enlargement of the anogenital distance, and absence of nipples.

Female rats neonatally exposed to DHTP showed unimpaired development of the CSL, visible microscopically on postnatal day 7 and macroscopically during adulthood when compared to control females (Figure 3A,B).

Developmental pattern of AR expresssion within the cranial part of the developing genital system

AR expression was examined in untreated fetuses of both sexes between day 16 pc and day 22 pc. The AR was continuously expressed in the cranial part of the male urogenital system during genital differentiation, from day 16 pc up to and including day 20 pc (Table II). On day 16 pc, positive cells were found in the mesenchymal and epithelial cells of the mesonephric tubules, and in the cranial mesentery between the gonads and the diaphragm. This cranial part of the genital

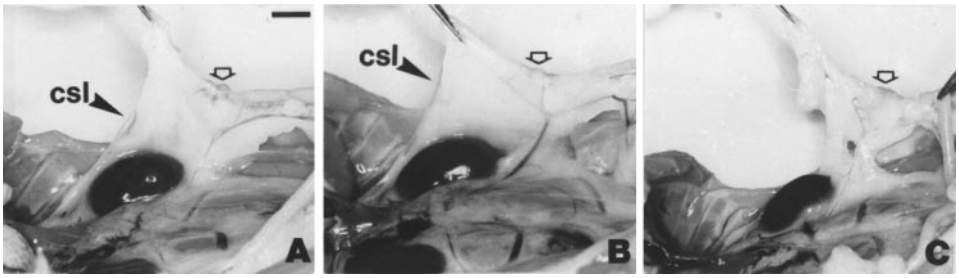

Figure 3. Ventral macroscopic view on the left kidney and cranial part of the urogenital system of adult female rats. (**A**) control treatment, (**B**) postnatal exposure to 5α-dihydrotestosterone propionate (DHTP) and (**C**) prenatal exposure to DHTP. By gently pulling at the left ovary (open arrow) presence of the cranial suspensory ligament (csl, black arrowhead) can be confirmed in the control (**A**) and postnatally treated (**B**) female rats, whereas in the prenatally treated (**C**) female rat only the cranial mesentery without a specific border structure is present. Scale bar = 2 cm.

Table II. Expression of different markers in the cranial part of the genital system of male and female rats at different stages of development

Marker	Days postconception						
	16	17	18	19	20	22	Day 7 postnatal
Androgen receptor							
Male	++	+++	+++	+++	+++	+++	nd
Female	++	+++	++/+	++/+	+	+/–	+/–
α-Smooth muscle actin							
Male	–	+	+	+	+	+	nd
Female	–	+	++/+	++	+++/++	+++	+++
Desmin							
Male	–	–	–	–	–	–	nd
Female	–	–	–	+	+	++	++

Intensity of positive immunostaining was graded as strong (+++), moderate (++), weak (+). nd = not determined; – = no staining. Use of two symbol groups (+++/++, ++/+, +/–) indicates that intensity of positive staining varied among different specimens within one group.
At each time point, one unilateral part of the urogenital tract was studied in at least four male and four female fetuses.

system had a more intense AR immunostaining than the caudal part, containing the Wolffian duct with AR-positive mesenchymal cells surrounding the duct. On day 17 pc, the pattern of AR expression was similar to day 16 pc but the staining was more intense. AR was detected in virtually all cells of the cranial mesentery (Figure 4A). From day 18 pc on, the AR was highly expressed throughout the male genital system and differential expression between the cranial part and the caudal part of the genital system was no longer detectable.

In female fetuses, the level of expression of the AR was more dependent on the different stages of development than in male fetuses (Table II). Highest expression was found on day 17 pc. A subsequent decrease in expression was observed, until a low level was reached at day 20 pc and later days. The epithelium and surrounding mesenchyme of the efferent tubules showed moderate AR expression up to day 22 pc. As in male fetuses of days 16 pc and 17 pc, differential AR expression within the genital system could be observed; the cranial part of the female genital system stained more intensely than its caudal part, which contains the degenerating Wolffian duct, with a low level of AR in the surrounding mesenchymal cells. The AR expression pattern in the genital system of newborn female rats, prenatally exposed to DHTP, was similar to that in newborn males (results not shown).

AR expression was also studied in postnatal day 7 female rats, both in control and DHTP-treated rats. AR expression was hardly detectable in the genital system of control females (Table II). In contrast, the cranial mesentery including the CSL contained a high level of AR expression in DHTP-exposed neonatal female rats (Figure 4C).

Smooth muscle differentiation in CSL during development

The immunolocalization of differentiating smooth muscle cells in the cranial part of the genital system was determined from day 16 pc up to and including postnatal day 7, using the smooth muscle markers α-SM actin and desmin (Table II).

In the day 16 pc fetus, α-SM actin was not detectable in cells of the CSL primordia. From day 17 pc onwards, α-SM actin was found to be located in the border of the cranial mesentery of both sexes, the primordial cells of the CSL (Figure 4B). Expression of α-SM actin continued to increase in the outer border of cranial mesentery in female rat fetuses. At postnatal day 7, the α-SM actin-containing cells form a distinct muscular ligament, the CSL, as anterior border of the cranial mesentery (Figure 4D). Differences in α-SM actin expression in the developing CSL between sexes were demonstrated from day 19 pc onwards (Table II). In male fetuses, the outer border of the cranial mesentery remained weakly positive for α-SM actin and no formation of a muscular outer layer could be observed at the end of fetal development. Extensive α-SM actin labelling was detected within the growing tunica albuginea (Figure 4B) and peritubular myoid cells of the testis at all different stages, and in vascular cells

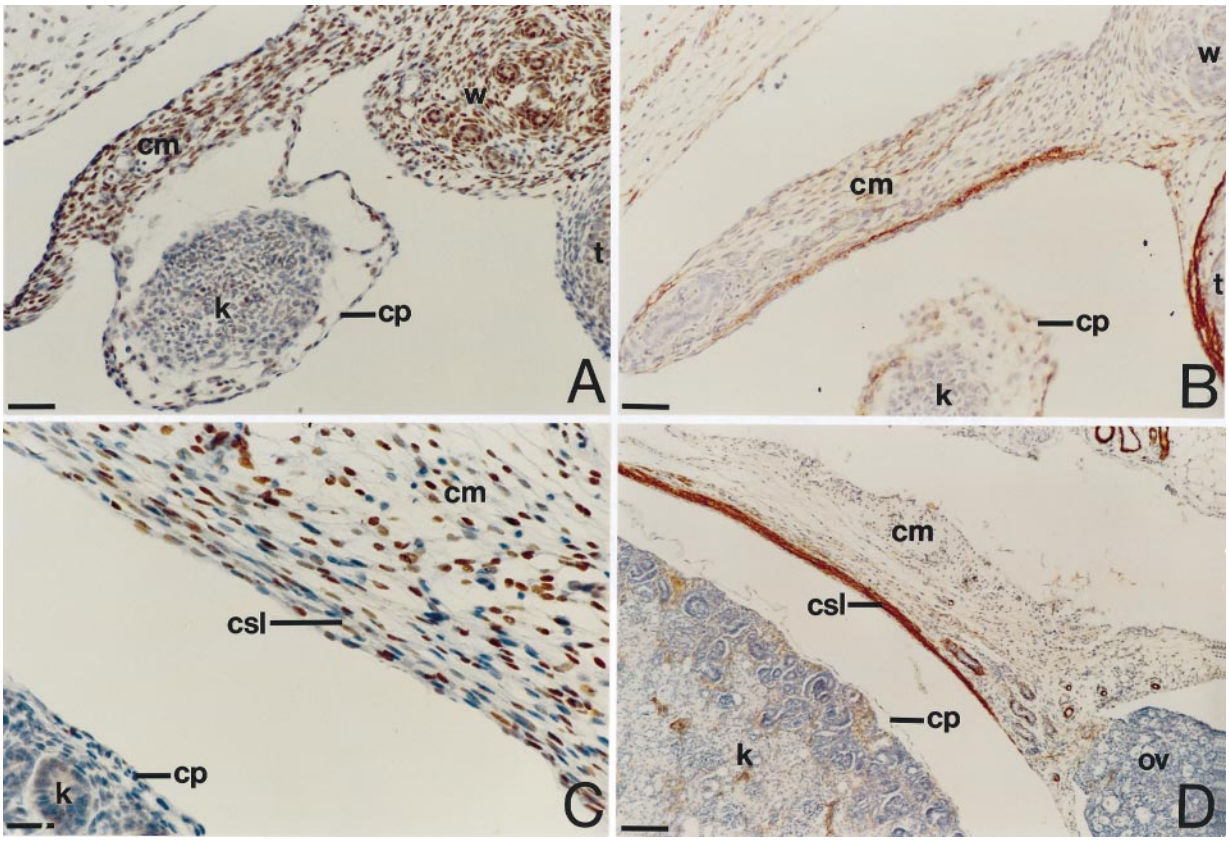

Figure 4. Immunohistochemical localization of α-smooth muscle (α-SM) actin and androgen receptor (AR) in the cranial part of the urogenital system of male rat fetuses and female neonates. (**A**, **C**) AR immunostaining and (**B**, **D**) α-SM immunostaining in male fetuses of day 17 postconception (pc) (top) and female neonates on postnatal day 7 (bottom). In a male fetus of day 17 pc, AR (**A**) is expressed in virtually all cells of the cranial mesentery (cm), whereas α-SM actin-positive cells (**B**) are mainly found in the outer border of the mesentery, the primordial cells of the cranial suspensory ligament (CSL). Caudally, the Wolffian duct derivatives (w) is surrounded by AR-positive cells (**A**). The tunica albuginea demonstrates a strong anti-actin immunoreaction (**B**). Detail of CSL (csl) of a neonatal female after postnatal exposure to androgen (**C**) shows that many cells in differentiated CSL and surrounding mesenchyme have high expression of AR. In overview (**D**), the CSL on postnatal day 7 is present as the outer border of the cranial mesentery, containing strands of actin-positive cells. The ovary (ov) is located at the caudal tip of kidney (k). t, testis; cp, kidney capsule. Scale bar = (**A**, **B**) 50 μm, (**C**) 25 μm, (**D**) 200 μm.

of both sexes. From day 17 pc onwards, the mesenchyme surrounding the mesonephric tubules and differentiating Müllerian and Wolffian ducts gave a positive reaction.

Desmin expression was absent from the CSL primordium at days 16, 17 and 18 pc in both sexes (Table II). At day 19 pc, desmin-positive cells were first observed in the developing CSL of female fetuses, which were also positive for α-SM actin. At later developmental stages, desmin staining was increasing in the actin-containing cells. In male fetuses, no desmin positive cells were detected in the CSL primordia. In contrast, desmin was detected in the testis at all different stages.

Proliferation and apoptosis in CSL during development

Cells in S-phase of the mitotic cycle were labelled using BrdU followed by anti-BrdU immunohistochemistry. Many positive nuclei were found in all the different fetal tissues, including the cranial mesentery. Positive staining cells did not appear to be restricted to particular parts of the mesentery. Although there was large variability in cell proliferation rates within and between specimens, particular parts of the genital system showed consistently different proliferation rates. In day 18 pc fetuses, proliferation rates of areas containing genital ducts were higher than those of the cranial mesentery.

The occurrence of DNA strand breaks, indicative of apoptotic cell death, was visualized *in situ* by the TUNEL method. Although very few cells stained positive for dUTP, the nuclear staining could be found in the cranial mesentery. In female fetuses, the epithelium of the degenerating Wolffian duct and gonadal germ cells at day 18 pc displayed a slightly higher incidence of positively stained nuclei compared with other structures of the genital system (not shown). Proliferation and apoptosis indices of the cranial mesentery determined at days 17 and 18 pc are presented in Table III and were not different between the sexes.

Discussion

The results presented in this study address earlier observations that prevention of development of the CSL, during male sex differentiation in fetal rats, is an androgen-dependent process (Van der Schoot and Elger, 1992; Barthold *et al*., 1994). Fetal treatment, but not postnatal treatment, of female rats with

Table III. Percentages of cells undergoing proliferation and degeneration in the cranial mesentery at days 17 and 18 postconception (pc) in male and female rat fetuses

	Day 17 pc	Day 18 pc
Proliferation		
Male	12.5 ± 3.5	9.9 ± 1.7
Female	9.2 ± 3.3	11.1 ± 2.0
Degeneration		
Male	1.0 ± 0.9	0.5 ± 0.2
Female	1.5 ± 0.9	0.9 ± 0.4

Values are mean ± SEM. No statistically significant differences were found. Minimum 300 cells and maximum 1500 cells per specimen were counted.

androgens did interfere with CSL development. Androgens prevented CSL differentiation in the anterior free border of the cranial mesentery in female fetuses, provided that the exposure had started at day 17 pc or earlier. Thus, the critical period during which CSL development can be abolished by androgens ends around day 17 or 18 pc. Since the rat testis starts to produce testosterone on days 15.5–16.5 pc (Warren *et al*., 1972), this androgen effect is an early phenomenon during male sex differentiation, with an onset well before fetal testosterone production reaches its maximum on day 18.5 pc (Habert and Picon, 1984).

SEM images demonstrated a possible correlation between the sex dimorphic development of the CSL and the development of the sex-specific position of the gonads during the last days of rat fetal life. If suppression of CSL development in male rats is an important prerequisite for testis descent, the critical period for androgen action on the CSL primordia should correlate with the precise time period of androgen action on testis descent. Indeed, earlier studies demonstrated that inhibition of testis descent in male rats by anti-androgens was most effective when these compounds were applied during the early phases of genital differentiation (Husmann and McPhaul, 1991; Spencer *et al*., 1991). Exposure of male rat fetuses to the anti-androgen flutamide during different time intervals showed maximal disturbance of testis descent when this compound was administered during days 16–17 pc (Husmann and McPhaul, 1991) or days 15.5–17 pc (Spencer *et al*., 1991). Cain *et al*. (1995) measured CSL length in male fetuses exposed to flutamide, DHT or oil alone during days 15–17 pc, the time period of maximal androgen action during testis descent. No difference in CSL length was observed at day 18 pc, but on day 20 pc the flutamide-treated animals had a significantly shorter CSL compared with both other treatment groups (Cain *et al*., 1995). It should be noted that Cain *et al*. (1995) have used the term cranial ligament to point to the complete mesentery present cranial to the gonads, whereas we have applied this term to the ligament in the border of the mesentery. Recent reports on case studies on bilateral cryptorchidism in a dog and a pig also suggested a correlation between disturbed androgen action, persistence of CSL at both sides, and cryptorchidism (Kersten *et al*., 1996; Van der Schoot and Emmen, 1996).

In the present study, no difference was observed between the cranial mesentery of prenatally androgen-treated and control females, except for the anterior border of this mesentery; in control females the muscular CSL is present at this border, which is not identifiable in females exposed to androgens before day 19 pc. The absence of the CSL could explain the more caudal position of the ovaries compared to the normal ovarian position. In general, the ovaries, oviducts, and uterus are embedded in mesenteric folds, which attach the female genital system to the kidney capsule and posterior abdominal wall. This anatomical relationship was not abolished in the androgen-exposed female rats, in contrast to male rats, and could be of importance in determining the final position of the gonads. No ovarian descent was observed in the DHTP-exposed females, as was also observed in earlier studies (Elger *et al*., 1970; Van der Schoot and Elger, 1992). Possibly, exogenous androgen does not reach the developing genital system of the fetus in a sufficiently high concentration to cause complete virilization of that system (Schultz and Wilson, 1974; Bentvelsen *et al*., 1995). Although complete stabilization and differentiation of the Wolffian ducts (Elger *et al*., 1970; Schultz and Wilson, 1974), and marked virilization of external genitalia were observed after fetal exposure of females to androgen, the development of the ovarian vasculature into a male-like plexus pampiniformis, and male-specific gubernaculum development, had not taken place in these androgen-treated females. The observed anatomical differences between male and androgen-exposed female rats cannot be explained by the absence of anti-Müllerian hormone (AMH) in the female fetuses. AMH-deficient (gene knock-out) male mice show normal testis descent, despite presence of Müllerian duct derivatives (Behringer *et al*., 1994). Concerning gubernaculum development, involvement of an unknown third testicular factor has been suggested (Fentener van Vlissingen *et al*., 1988; Van der Schoot *et al*., 1995; Visser and Heyns, 1995). Lack of such a factor in androgen-exposed females cannot be ruled out as a possible explanation for the observed anatomical differences between androgen-exposed females and normal males.

Since local expression of the AR is a prerequisite for direct androgen action on the cranial part of the urogenital system, the distribution of nuclear AR within this part was analysed. Immunolocalization of AR in the fetal urogenital system of the rat has been studied before (Bentvelsen *et al*., 1995; Madjic *et al*., 1995), but not in relation to the cranial part of this system. Madjic *et al*. (1995) reported a heterogeneous distribution of AR among the mesenchymal cells of the mesonephric area in male rat fetuses on day 16 pc. The present study confirmed this observation, but also showed AR-positive cells in the cranial mesentery. From day 18 pc onwards, male fetuses showed intense nuclear AR staining in the cranial mesentery, whereas expression in female fetuses decreased. This sex-dependent difference in expression has also been observed in other structures of the fetal genital system, and it was suggested that the AR level in the fetal urogenital system is initially hormone independent, whereas continued AR expression is dependent upon testicular androgen production (Bentvelsen *et al*., 1994). The present study demonstrated the presence of AR in the cranial mesentery, including primordial cells of the CSL, during the period when androgens are effective in suppressing CSL development (up to and including

day 17 pc). Thus, the CSL primordium can be considered a direct target tissue for fetal testicular androgens. Furthermore, the present observations indicate that the primordium of the CSL has lost its androgen responsiveness by day 17 pc, although AR expression in the differentiated CSL continues after day 17 pc, but obviously without a morphogenic effect upon androgen exposure. Such a phenomenon was also observed in the nipple primordia of mice (Wasner *et al*., 1983).

The cellular and molecular mechanisms of the suppressive androgen action on CSL development are of interest, in view of many other effects of androgens on growth and differentiation of fetal male tissues (Wilson *et al*., 1981; Cunha *et al*., 1992). Another sex dimorphic process in which androgens suppress development is differentiation of the nipples of the mammary glands in rats and mice. Nipple development is abolished in female rats and mice after exposure to androgens during an early phase of sex differentiation (Goldman *et al*., 1976; Kratochwil, 1977). A major difference between nipple and CSL development is that the fetal nipple primordium consists of an epithelial bud surrounded by mesenchymal cells, whereas the CSL primordium only consists of mesenchymal cells. During destruction of the nipple primordium, androgens act on the AR-positive mesenchyme, rather than on the epithelial cells, and mesenchymal–epithelial interactions eventually cause degeneration of the epithelial bud (Dürnberger and Kratochwil, 1980). In the CSL primordium, suppression of development involves direct action of androgens on the mesenchymal cells.

The CSL of adult female rats is a ligament at the anterior border of the cranial mesentery which contains smooth muscle cells (Mohsin and Pennefather, 1979). Since smooth muscle cells are characterized by expression of both α-SM actin and desmin (Lazarides and Hubbard, 1976), the expression patterns of these two markers in the developing CSL were analysed. From day 17 pc, α-SM actin was found in the CSL primordia of both sexes, but this expression started to show a distinct sex-specific pattern between days 18 and 19 pc. From day 19 pc onwards, the α-SM actin-positive cells of the female CSL started to express desmin, which could not be demonstrated in the CSL of male fetuses. Such a sequence of first expression of smooth muscle markers is a common observation in smooth muscle differentiation, and is also demonstrated in the prostate and seminal vesicles (Hayward *et al*., 1996). Since desmin expression was first detected in prostate at postnatal day 5 and in seminal vesicles at postnatal day 10, smooth muscle differentiation in the CSL of female rats is an early event compared to muscle differentiation in prostate and seminal vesicles. The relative absence of smooth muscle cells in the CSL of male fetuses, as compared to the female, indicates that androgens suppress smooth muscle cell differentiation in the CSL. Mitotic activity and apoptosis of the cells in the cranial mesentery was studied to see whether the difference in CSL development between the sexes was accompanied by a difference in cell kinetic characteristics of the cranial mesentery. However, no such sex difference was found. Gonadal germ cells in both sexes demonstrated cell degeneration and proliferation at day 17 and 18 pc, as reported by others (Hilscher *et al*., 1974; Coucouvanis *et al*., 1993). Although no sex difference in cell kinetics was detected, the cranial mesenteric tissue of the male fetus at day 18 pc appeared more loosely structured compared to the female cranial mesentery with differentiating CSL. Furthermore, SEM images already showed a sex difference in cranial mesentery appearance on day 17 pc, when the sex difference in CSL differentiation could not yet be detected by immunohistochemistry. This could indicate changes in cell density, cell size, and/or extracellular matrix (ECM) between the male and female cranial mesentery. In general, the ECM plays an important role in morphogenesis (Hay, 1991). The ECM consists of different types of macromolecules including collagen, fibronectin and laminin which can bind specifically to receptors at the cell surface. Cell growth and differentiation can by affected by cell–ECM interactions because the ECM can bind or activate peptide growth/differentiation factors, such as TGFβ, which in turn react with the cell. The ECM controls cell differentiation and morphogenesis, and can exert a direct effect on gene expression (Streuli *et al*., 1991). Complex interaction between ECM, androgens and growth factors is involved in the control of prostate and mammary gland differentiation (Streuli *et al*., 1991; Kooistra *et al*., 1995). A similar complex interaction may play a role in the control of the development of the cranial mesentery, containing the CSL primordium.

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