Serotoninergic system blockage in the prepubertal rat inhibits spermatogenesis development

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Abstract

The stimulatory and inhibitory role of serotonin in gonadotropin secretion and in the onset of puberty in the male rat has been previously described, but its role in the establishment of spermatogenesis is not known. The aim of this study was to investigate the effects of serotoninergic inhibition by p-chloroamphetamine (pCA) on the prepubertal-to-adult stage of the rat reproductive system. Hypothalamic serotonin, gonadotropins and sex steroid hormone concentrations were measured, and a histopathological analysis of seminiferous epithelium was carried out on animals treated with pCA from day 30 and killed at 45 or 65 days of age. The pCA treatment significantly reduced the hypothalamic levels of serotonin and its metabolite (5-hydroxyindole-3-acetic acid). This inhibition did not affect the sex steroid hormone or LH concentrations, but rather it induced an increase in FSH concentration in animals of both ages. Spermatogenesis was impaired by pCA treatment. Disruption of seminiferous epithelium and the death of numerous germ cells were observed. Sperm produced by pCA-treated animals was of poor quality and appeared in small quantities. Apparently, serotonin depletion did not affect communication between the hypothalamus and the pituitary, but the FSH increase could have been related to alterations in the seminiferous epithelium effects. The seminiferous epithelium cycle was altered in rats killed at both 45 and 65 days of age, because at each age of killing the distribution of spermatogenesis stages was different. Germ cell apoptosis did not appear to be related to changes in the FSH concentrations, but other factors produced during spermatogenesis could have been involved in this induction. This study showed that serotonin was necessary for the development of normal spermatogenesis in prepubertal rats.

Introduction

Puberty is the transitional stage of development during which reproductive competence is achieved. The onset of puberty in the male rat is characterized by completion of the first wave of spermatogenesis, i.e. when it is possible to find testicular sperm. In the male rat, testicular sperm appears at ~45–50 days of age (Russell et al. 1987, Malkov et al. 1998) and at ~65 days of age it is possible to observe sperm in the vas deferens (Russell et al. 1987). The regulation of mammalian spermatogenesis includes chemical communication between the hypothalamus–hypophysis axis and the gonad itself. The production of spermatozoa is regulated by luteinizing hormone (LH) and follicle-stimulating hormone (FSH) which act in the gonad on the Leydig and Sertoli cells respectively (Weinbauer & Nieshlag 1993, Sharpe 1994). Both hormones are produced in response to gonadotropin-releasing hormone (GnRH). Anatomical studies support the existence of interrelationships between the serotoninergic system and GnRH-secreting neurons (Justo et al. 1989). Serotonin, among other neurotransmitters, plays a role in the modulation of gonadotropin secretion and in the onset of sexual maturation in the male rat (Mogulevsky et al. 1985, Justo et al. 1989, Pinilla et al. 1994, Shiskina & Dygalo 2000). Several reports have indicated that serotonin has both an inhibitory and a facilitatory role in the modulation of gonadotropin secretion, depending on the age and sex of the animal (Mogulevsky et al. 1985, Justo et al. 1989, Shiskina & Dygalo 2000). There is evidence for the existence of such a differential role of serotonin during the development of the male rat. High levels of endogenous serotonin, induced by the administration of 5-hydroxytryptophan, increase FSH release in male rats at 26, 30 and 60 days of age; however, these effects are not observed at 18 and 20 days (Justo et al. 1989). Furthermore, the inhibition of serotonin synthesis with p-chlorophenylalanine (PCPA) in male rats between 40 and 44 days of age...
decreases the endocrine function of the testis in 60-day-old animals; the same treatment in male rats between 40 and 44 days of age inhibits steroidogenesis and spermatogenesis (Shishkina & Borodin 1989). Justo et al. (1989) suggested that the differential effect of serotonin on gonadotropin secretion during prepubertal development of male rats is related to the prevailing steroid milieu; other authors have made reference to this in the female rat as well.

In addition, a number of reports indicate that serotonin is present and acts in the testis (Aguilar et al. 1995), epididymis (Leung et al. 1999) and vas deferens (Celuch & Soley 1988). Still others have introduced the idea that serotonin modulates testosterone production (Tinajero et al. 1993) and blood flow (Collin et al. 1996) in the testis. However, the role of serotonin, if any, in the control of testis functions remains unknown.

Our decision to analyze the effects of the continuous blockade of serotonin synthesis at the onset of puberty and at the adult stage of the rat was based on the following: (i) the fact that the role of the serotoninergic system in regulating the onset of puberty in male rats and the functions of the testis is still not clear; (ii) the fact that several differential effects of the serotoninergic system during prepubertal development have been observed (Justo et al. 1989, Shishkina & Dygalo 2000). In the present study, we measured the hypothalamic changes in serotonin concentrations, circulating gonadotropins and steroid sex hormones. A histopathological evaluation was made of the seminiferous epithelium and the sperm.

Material and Methods

Animals and treatments

Thirty-day-old male rats, CII-ZV strain (weighing 75–90 g), from our own stock were used. They were kept in individual cages under conditions of controlled lighting (lights on from 0500 to 1900 h) with free access to water and food (Purina chow). All experiments were performed following the parameters of the US National Institutes of Health (NIH) guide for live animal experiments. p-Chloroamphetamine (pCA; Sigma) was dissolved in saline, and each rat was treated at day 30 and 44 days of age inhibits steroidogenesis and spermatozoa (Shishkina & Borodin 1989). Justo et al. (1989) and at the adult stage of the rat was based on the following: (i) the fact that several differential effects of the serotoninergic system in regulating the onset of puberty in male rats and the functions of the testis is still not clear; (ii) the fact that several differential effects of the serotoninergic system during prepubertal development have been observed (Justo et al. 1989, Shishkina & Dygalo 2000). In the present study, we measured the hypothalamic changes in serotonin concentrations, circulating gonadotropins and steroid sex hormones. A histopathological evaluation was made of the seminiferous epithelium and the sperm.

Measurement of serotonin and 5-hydroxyindole-3-acetic acid (5-HIAA)

After decapitation, the brain of each rat was quickly removed and placed in a chilled solution and frozen on liquid nitrogen. After careful removal of the nerves and optic chiasm, the anterior and median hypothalamus were dissected following the parameters described in the stereotaxic atlas of Paxinos & Watson (1982). The anterior hypothalamus (Bregma −0.8 to Bregma −1.8) included the removal of the lateral and median pre-optic nuclei, the suprachiasmatic, the paraventricular, the periventricular, and the stria terminal pre-optic area. The medial hypothalamus (Bregma −2.3 to Bregma −3.2) included the median eminence and arcuate nucleus. Both hypothalamic regions were also stored at −70°C, until the concentrations of serotonin, and its metabolite 5-HIAA, were measured using HPLC.

The concentrations of serotonin, noradrenaline, dopamine (and their metabolites 5-HIAA, MHPG (4-hydroxy-3-methoxyphenyl glycol) and DOPAC (3,4-dihydroxyphenylacetic acid) respectively) were measured following the methodology described previously (Aylal et al. 1998). Samples of hypothalamus were weighed in a precision balance, homogenized in 300 μl of 0.1 M perchloric acid and centrifuged at 12 000 g, at 4°C for 30 min. The supernatant was filtered using 0.2 μm regenerated cellulose filters; 20 μl of this extract were injected to a chromatography column via a Rheodyne valve.

Calibration was performed by producing a standard curve over a range of 0.1–2 ng/ml. Serotonin and its metabolites were identified by relative retention times compared with standards. Results are expressed as nanograms of neurotransmitter per milligram of wet tissue. The sensitivity was 0.01 ng. The serotoninergic activity was estimated as previously described, following the suggestions of Kerdelhué et al. (1989). Serotoninergic activity is the ratio of the concentrations of 5-HIAA and serotonin ( = [5-HIAA] /[5-HT]).

Gonadotropins and sex steroid hormones

After decapitation, trunk blood from each animal was collected, allowed to clot at room temperature and centrifuged at 1000 g for 15 min. The serum was separated and stored at −20°C until assayed. FSH and LH concentrations were measured by RIA, using the double-antibody technique, with reagents and protocol supplied by the National Hormone and Pituitary Program (Baltimore, MD, USA). The results were expressed in ng/ml as the international references standard rLH-RP-2 and rFSH-RP-2. Intra- and interassay coefficients were, respectively, 5.74 and 7.91% for FSH and 6.82 and 9.32% for LH. Serum concentrations of progesterone, testosterone, and 17β-estradiol were measured by RIA, using kits purchased from Diagnostic Products (Los Angeles, CA, USA). Hormone concentrations were expressed for progesterone and testosterone in ng/ml and for 17β-estradiol in pg/ml.
Intra- and interassay coefficients of variations were, respectively, 4.3 and 7.8% for progesterone, 3.9 and 7.6% for testosterone and 7.2 and 8.5% for 17β-estradiol.

**Seminiferous epithelium cycle**

After killing the animals, the testes, epididymis, prostate, coagulant glands and seminal vesicle were dissected and weighed on a precision balance. The left testis was fixed by immersion in Bouin solution, dehydrated and embedded in paraffin wax. Serial sections, 8 μm thick, were taken every 100 μm and stained with periodic acid-Schiff (PAS)–hematoxillin. Spermatogenesis stages were identified using the criteria of Russell et al. (1990). From each animal, 50 seminiferous tubules were taken randomly and the spermatogenesis stage was determined in each. The percentage of each spermatogenesis stage was determined; the distribution (percentage per stage of the total) is presented here. In cases where the tissue had been altered by the treatment and the stage could not be identified, we used morphological criteria other than spermiogenesis steps for staging.

**TUNEL**

The right testis was fixed by immersion in paraformaldehyde (4% in phosphate buffer, pH 7.4), dehydrated and embedded in paraffin wax. Serial sections of a thickness of 8 μm were taken every 100 μm. DNA end labeling was performed with the aid of an apoptosis kit according to the manufacturer’s instructions (Roche); briefly, sections were mounted on slides coated with poly-l-lysine, deparaflinized, hydrated and treated with 20 μg/ml proteinase K (Roche) for 30 min at 37°C, in a humidifier chamber. The endogenous peroxidase was blocked for 20 min with block solution (Roche). The tissue was treated with permeabilization solution with Triton (0.1% Triton in 0.1% sodium citrate) for 2 min. Tissue sections were treated with label solution (terminal deoxynucleotidyl transferase (TdT) enzyme and biotinylated 16-dUTP) for 60 min. After each step the tissue was rinsed twice, for 5 min in each. The tissue sections were treated with converter-POD (anti-fluorescein conjugated with horse-radish peroxidase; 30 min at 37°C) and counterstained with Mayer-hematoxillin, dehydrated and mounted for light microscopy. In the negative control slides, either the TdT enzyme or biotinylated 16-dUTP was omitted in labeling the reaction.

**Sperm analysis**

The vas deferens and its contents were obtained and placed in 2 ml Tyrode's solution (Sigma) at 37°C and mixed to homogenize the suspension. A drop of the sperm suspension was placed on a slide for observation. For each animal 200 spermatozoa were analyzed (Linder et al. 1992, Seed et al. 1996) at ×400 using a phase-contrast microscope (Nikon). The percentage of motile sperm was defined as the ratio of cells showing movement to the total number of cells ×100 (Seed et al. 1996).

Sperm viability was determined using the 1% trypan blue technique (Sigma) and 200 cells were analyzed for each animal. Sperm concentration was determined in a Neubauer chamber (PROPER Lumycite 1/100 mm depth).

**Statistics**

The following data were checked for normality using the Kolmogorov–Smirnov test before ANOVA and Tukey’s test processing: weight of organs, hormone serum levels, serotonin, 5-HIAA concentration and [5-HIAA]/[5-HT] ratio. Sperm motility and viability data were analyzed by the Mann–Whitney U-test. Differences were accepted if P < 0.05. All analyses were performed on a PC with SPSS 9 software.

**Results**

**Organ weights**

After the animals were killed, the testes, epididymis, prostate, seminal vesicle and coagulant glands were dissected, and their weights registered. No changes were observed in the weight of organs obtained from the saline group compared with that of the absolute control group (Table 1). Rats treated with pCA and killed at 45 days of age showed a significant reduction in body weight and testis weight, whereas animals that had been pCA treated and were killed at 65 days of age exhibited a reduction in body weight and testis weight and their weights registered. No changes were observed in the weights of organs obtained from control male rats and treated with pCA from 30 day and sacrificed at 45 or 65 days of age.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight</th>
<th>Testisa</th>
<th>Epididymisa</th>
<th>Prostate</th>
<th>Seminal vesicle</th>
<th>Coagulant gland</th>
</tr>
</thead>
<tbody>
<tr>
<td>Killed at 45</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute control</td>
<td>168.8 ± 3.1</td>
<td>0.93 ± 0.03</td>
<td>0.11 ± 0.00</td>
<td>0.15 ± 0.02</td>
<td>0.08 ± 0.01</td>
<td>0.03 ± 0.00</td>
</tr>
<tr>
<td>Saline</td>
<td>160.3 ± 3.6</td>
<td>0.77 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.14 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.03 ± 0.00</td>
</tr>
<tr>
<td>pCA</td>
<td>149.6 ± 4.4a</td>
<td>0.61 ± 0.04b</td>
<td>0.10 ± 0.00</td>
<td>0.14 ± 0.01</td>
<td>0.06 ± 0.01</td>
<td>0.03 ± 0.00</td>
</tr>
<tr>
<td>Killed at 65</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute control</td>
<td>275.9 ± 5.8</td>
<td>1.40 ± 0.07</td>
<td>0.29 ± 0.01</td>
<td>0.44 ± 0.06</td>
<td>0.29 ± 0.02</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>Saline</td>
<td>295.4 ± 9.1</td>
<td>1.54 ± 0.08</td>
<td>0.30 ± 0.02</td>
<td>0.55 ± 0.04</td>
<td>0.33 ± 0.03</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>pCA</td>
<td>236.7 ± 5.9a, b</td>
<td>1.00 ± 0.10ab</td>
<td>0.22 ± 0.01ab, b</td>
<td>0.34 ± 0.01b</td>
<td>0.25 ± 0.01b</td>
<td>0.12 ± 0.01</td>
</tr>
</tbody>
</table>

* Testsis and epididymis are average weight of organs.

aP < 0.05 vs Absolute control; bP < 0.05 vs Saline.

Table 1 Body and reproductive organs weight (g) of control male rats and treated with pCA from 30 day and sacrificed at 45 or 65 days of age.
Serotonin and 5-HIAA

There were no differences in the serotonin and 5-HIAA concentrations, nor in the [5-HIAA]/[5-HT] ratio of the hypothalamus, between animals treated with saline and the absolute control. The administration of pCA induced a significant decrease in the serotonin and 5-HIAA concentrations in the anterior and medium hypothalamus when animals were killed at 45 or 65 days of age in comparison with the saline group (Figs 1 and 2).

The [5-HIAA]/[5-HT] ratio (serotonergic activity) in the anterior hypothalamus was not modified in animals treated with pCA (Fig. 1), but a significant increase in this ratio was observed in the medium hypothalamus in those animals killed at 65 days of age (Figs 1 and 2).

pCA treatment induced no changes in the noradrenaline and dopamine concentrations in rats killed at 45 or 65 days of age.

**Figure 1** Serotonin and 5-HIAA concentrations in the anterior hypothalamus of control male rats and of those treated with pCA from day 30 to 45 or 65 days of age. Serotonergic activity ([5-HIAA]/[5-HT]) was calculated from serotonin and 5-HIAA values. AC, absolute control; Vh, vehicle (saline). *p < 0.05 vs absolute control; **p < 0.05 vs vehicle.

**Figure 2** Serotonin and 5-HIAA concentrations in the medium hypothalamus of control male rats and of those treated with pCA from day 30 to 45 or 65 days of age. Serotonergic activity ([5-HIAA]/[5-HT]) was calculated from serotonin and 5-HIAA values. AC, absolute control; Vh, vehicle (saline). *p < 0.05 vs absolute control; **p < 0.05 vs vehicle.
Gonadotropins and sex steroid hormones

LH concentrations were found to be unmodified in animals treated with pCA, when compared with control groups. pCA treatment induced a significant increase in the FSH concentration when animals were killed at 45 or 65 days of age (Fig. 3).

pCA treatment did not induce changes in progesterone, testosterone or estradiol concentrations in animals killed at the two different ages (Fig. 4). Testosterone and estradiol concentrations in animals killed at 65 days of age showed an increase with respect to those in animals killed at 45 days of age, independent of treatment.

Seminiferous epithelium

Absolute control and saline animals of both ages exhibited normal testicular tissue, with typical seminiferous epithelium (Fig. 5a and c). Scattered seminiferous tubules with disruption of spermatogenesis were observed in animals treated with pCA and killed at 45 or 65 days of age. Shrunken tubules with giant multinucleate cells and aphaic seminiferous tubules were observed (Fig. 5b and d). Some tubules showed a total loss of spermatocytes and spermatids, although spermatogonia remained (Fig. 5b and d).

Rats in the control groups that were killed at 45 days of age revealed all the spermatogenesis stages, including those where testicular sperm were present (stages I to VIII). A similar distribution of spermatogenesis stages was observed in the absolute control and vehicle groups. No tissue alterations were observed in control groups (Fig. 6a). Animals pCA treated and killed at 45 days of age presented 53.81% of seminiferous tubules with different degrees of degeneration; the remaining tubules showed dramatic changes in the distribution of spermatogenesis stages. Nearly 80% of the total seminiferous tubules observed were in stages I, XII and XIV. Spermatogenesis stages II to XI were either less than 2% or were not present in the pCA group (Fig. 6a).

In the absolute control animals killed at 65 days of age, the distribution of spermatogenesis stages was similar to that of the saline group (Fig. 6b). This distribution in pCA-treated animals was disrupted. In the pCA group, 80% of spermatogenesis stages were grouped in stages IV to VIII. Like the animals treated with pCA and killed at 45 days of age, the distribution of spermatogenesis stages was similar to that of the saline group (Fig. 6b). This distribution in pCA-treated animals was disrupted. In the pCA group, 80% of spermatogenesis stages were grouped in stages IV to VIII. Like the animals treated with pCA and killed at 45 days of age, the distribution of spermatogenesis stages was similar to that of the saline group (Fig. 6b). This distribution in pCA-treated animals was disrupted. In the pCA group, 80% of spermatogenesis stages were grouped in stages IV to VIII.
days old, the pCA-treated rats killed at 65 days of age showed 51.34% of seminiferous tubules with different degrees of degeneration.

Stages VII to IX were the most advanced spermatogenesis stages observed in the pCA-treated group at 65 days of age. Calculation of the duration of spermatogenesis stages (based on Russell et al. 1990) together with the age at killing showed that the most advanced spermatogenesis stages of rats pCA treated and killed at 65 days of age (VIII to IX) were approximately in stage I 20 days earlier. This elapsed time was one and a half times the seminiferous epithelial cycle and corresponded exactly with the most obvious spermatogenic stage observed in animals pCA treated and killed at 45 days of age (Fig. 7).

**TUNEL assay**

Numerous shrunken seminiferous tubules with TUNEL-positive germ cells were observed in animals treated with pCA (Fig. 8a–d). Neither Leydig nor Sertoli cells positive to TUNEL were observed in pCA-treated animals. Most of the seminiferous tubules showed TUNEL-positive germ cells in the region of round spermatids, although massive germ cell staining was also noted.

**Sperm**

No sperm were obtained from deferens ducts of the control animals or from those of the pCA-treated male rats killed at 45 days of age. This finding indicated that although spermatogenesis was complete in the testis, the sperm still needed to pass through the epididymis to reach the deferens ducts. Sperm were obtained from all control and pCA-treated animals killed at 65 days of age. Although spermatogenesis was not suppressed in pCA-treated animals, sperm viability, motility and production decreased significantly (Table 2).

**Discussion**

The aim of this study was to identify the effects of continuous chemical serotonin depletion on the seminiferous epithelium of rats from the prepubertal stage to the adult stage. The present results suggest that the serotonergic system had a stimulatory role in the neuroendocrine system regulating the onset of puberty and spermatogenesis in the rat, since depletion of serotonin was accompanied by the loss of germ cells in the testis and then by a decrease in sperm production.
According to Soghomonian et al. (1998), the different hypothalamic nuclei contain serotoninergic fibers that arise in the dorsal and medial raphe nuclei. Also, other authors have suggested the existence of serotoninergic cells in the dorsomedial hypothalamic nucleus (Frankfurt et al. 1981). Our results showed that the administration of pCA resulted in a diminution in the serotonin concentration in the anterior and medium hypothalamus, without any change in the catecholamine concentrations. Serotonin depletion in the central nervous system is attributed to a specific modification in tryptophan hydroxylation, a reaction catalyzed by the enzyme tryptophan hydroxylase (Sanders-Bush & Sulser 1970). The effects of pCA are evident 2 days after treatment and last up to 10 days (Sanders-Bush et al. 1972). Therefore, it seems that the i.p. dose of pCA and the administration schedule were effective in inducing a decrease in the concentration of serotonin in the hypothalamus.

Previous studies in prepubertal rats have shown that stimulation of the serotoninergic system by the

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**Figure 6** Distribution of stages of the cycle of the seminiferous epithelium in controls and pCA-treated rats. AC, absolute control; Vh, vehicle (saline). (A) Groups killed at 45 days of age. (B) Groups killed at 65 days of age.

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**Figure 7** Schematic representation of stages of the cycle of seminiferous epithelium and age at which animals were killed. (A) The two horizontal lines show the ages at the beginning of pCA treatment and when animals were killed. (B) The double, horizontal segmented line with roman numbers represents a seminiferous tubule with stages of the cycle, each one of different duration (Russell et al. 1990); not all spermatogenesis stages are indicated. Each division in the solid line indicates a spermatogenesis wave. (C) The line indicates the coincident duration in days, as the time elapsed from the most advanced spermatogenesis stages in animals killed at 65 days of age (VIII to IX) to the most obvious stage (I) observed at 45 days of age, which corresponds to one and a half spermatogenesis waves.
serotonin precursor 5-hydroxytryptophan (5-HTP), as well as the systemic chemical inhibition of serotonin synthesis, produce changes in gonadotropins and sex steroid hormone concentrations during sexual maturation (Becu-Villalobos et al. 1989, Justo et al. 1989, Shishkina & Dygalo 2000). An increase in FSH concentration has been observed when prepubertal and adult male rats are treated with a concentration greater than 50 $\mu$g/kg of 5-HTP (Becu-Villalobos et al. 1989, Justo et al. 1989). Our results differed in this respect because serotonergic inhibition by the pCA treatment increased the FSH concentration significantly. The reason for administering the serotonin precursor 5-HTP is to increase serotonin production, but the above authors do not show the effect of 5-HTP on serotonin concentration. The data of Shishkina & Dygalo (2000) on serotonin concentration after 5-HTP treatment of prepubertal male rats do not show changes in the serotonin levels. It is possible then, that factors other than stimulus of serotonin synthesis could have caused the increase in the FSH level that we observed.

FSH synthesis is not completely regulated by GnRH; other factors such as inhibin, activin, and follistatin also regulate it (Winters & Moore 2004). Inhibin is a hormone produced in the testis by Sertoli cells. This hormone has an effect on the pituitary and its levels correlate inversely with FSH concentrations (Anawalt et al. 1996, Woodruff et al. 1996). The immuno-neutralization of inhibin has been shown to induce a rise in the circulating FSH in juvenile male rats (Culler & Negro-Vilar 1988, Rivier et al. 1988). Such information leads us to think that the increase in the FSH level in animals with serotonin depletion could be due to an alteration in the production of inhibin by Sertoli cells. This could occur as a result of germ cell loss in the seminiferous epithelium, then the pituitary without inhibin restriction could increase FSH synthesis.

Testosterone and progesterone biosynthesis in Leydig cells is regulated by LH, which is synthesized and secreted by the hypophysis. Testosterone and progesterone then regulate the secretion of LH by means of the interaction with their receptors in the hypothalamus, which increases or diminishes the concentration of GnRH (Turner et al. 2001). The fact that progesterone, testosterone and estradiol concentrations did not decrease with the pCA administration to the animals killed at 45 or 65 days old, and the fact that concentrations of the same steroid hor-

Table 2 Characteristics of sperm obtained from control male rats and rats treated with pCA from day 30 and killed at 65 days of age.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Motile sperm (%)</th>
<th>Viable sperm (%)</th>
<th>Sperm concentration ($\times 10^6$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute control</td>
<td>67.66 ± 1.93</td>
<td>82.39 ± 2.48</td>
<td>235.48 ± 13.03</td>
</tr>
<tr>
<td>Saline</td>
<td>53.99 ± 3.16</td>
<td>76.33 ± 4.04</td>
<td>275.60 ± 9.11</td>
</tr>
<tr>
<td>pCA</td>
<td>3.09 ± 4.08ab</td>
<td>15.55 ± 4.70ab</td>
<td>64.49 ± 15.97ab</td>
</tr>
</tbody>
</table>

*a* $P < 0.05$ vs absolute control; *b* $P < 0.05$ vs saline.

Figure 8 TUNEL-stained testis sections from saline- and pCA-treated rats. (a and b) Saline control rats killed at 45 and 65 days of age respectively. (c and d) pCA-treated animals killed at 45 and 63 days of age respectively. Arrows indicate germ cell dying. Magnification, $\times$ 400.
Serotonin depletion and spermatogenesis

Serotonin was shown to be essential for normal spermatogenesis, but its deficiency did not totally prevent the development of spermatogenesis, as demonstrated by analysis of seminiferous epithelium cycle stages. However, a differential disruption in the seminiferous epithelium cycle was observed in animals with serotonin depletion, depending on the age at which they were killed.

The effects of serotonin and other catecholamines, and neurotransmitters such as GABA, on the hypothalamus change during pubertal development (Mogullevsky et al. 1990, 1991). Distinct and well-defined populations of germ cells interact with Sertoli cells in a cyclic pattern. Sertoli cells secrete a large number of proteins and regulatory factors that are crucial for germ cell development (Griswold 1998). Germ cells are needed to maintain the cyclicity of Sertoli cells (Jegou & Pineau 1995, Weinbauer & Wessels 1999). Then perhaps the different distribution of the spermatogenesis stages in the pCA-treated animals at 45 or 65 days of age was related to intra-testicular chemical communication; on the one hand, as a result of changes in the action of hypothalamic factors related with age, and on the other hand because of a depletion of specific germ cells. In order to elucidate this, information is needed about the first damaged germ cells resulting from serotonin depletion.

Shishkina & Dygalo (2000) reported that adult-stage male rats treated with pCPA on days 30 and 40 and 44 show a decrease in testis weight and sperm

mones were greater in 65-day-old animals than in those that were 45 days old indicate two things: (i) the reduction observed in the serotonin hypothalamic concentration as a result of pCA administration apparently had no effect on the synthesis of GnRH because the LH concentration did not show changes in either the 45-day-old or the 65-day-old rats; (ii) in the testis, the Leydig cells increased their functional capacity with the animal's age independently of pCA treatment.

This study revealed that the establishment of spermatogenesis was disrupted by the inhibitor of serotonin synthesis, pCA. Serotonin depletion during the prepubertal-to-pubertal stage in the adult male induced a reduction in sperm production. This reduction was related to germ cell loss in the seminiferous epithelium. FSH and testosterone are related to apoptosis induction in the seminiferous epithelium of the pCA-treated rats and these factors could be related to the type of germ cell dying. Analysis of the seminiferous epithelium by TUNEL staining exhibited many tubules with TUNEL-positive germ cells in the region of round spermatids. This fact suggest that factors involved in round-spermatid differentiation were related to germ cell depletion by apoptosis, but the mechanisms remain to be identified.

In the testis, there are several potential serotonin sources: nerve endings in the testicular capsule, mast cells and Leydig cells (Gaytan et al. 1989, Campos et al. 1990, Tinajero et al. 1993). However, the contribution of the different potential sources to the total amount of serotonin in the testis is not known (Aguilar et al. 1995). Serotonin has some effects known to be relevant to testis physiology. As mentioned above, serotonin inhibits testosterone synthesis (Tinajero et al. 1993), functions as a potent vasoconstrictor of the testicular subcapsular artery at physiological and elevated transmural pressures (Davis 1992) and decreases the blood flow and vasomotion (Collin et al. 1996). The effects of an elevated testicular blood pressure include focal apoptosis of spermatogonia and primary spermatocytes (Bergh et al. 2001); whereas, a reduction in testicular blood flow could induce testicular hyperemia and edema (Davis 1992). In the present study we did not observe testicular edema and apoptosis occurring in round spermatids. None of these effects were observed in the morphology of testis from pCA-treated animals in the present study. This indicates that apparently the regulation of blood flow was not modified by pCA treatment. It is not known whether other factor(s), such as catecholamines, that function as weak vasoconstrictors were modified, but hypothalamic concentrations of these neurotransmitters were not modified in our work.

The present study revealed that serotonin was essential for normal spermatogenesis, but that its deficiency did not totally prevent the development of spermatogenesis, as demonstrated by analysis of seminiferous epithelium cycle stages. However, a differential disruption in the seminiferous epithelium cycle was observed in animals with serotonin depletion, depending on the age at which they were killed.

The effects of serotonin and other catecholamines, and neurotransmitters such as GABA, on the hypothalamus change during pubertal development (Mogullevsky et al. 1990, 1991). Distinct and well-defined populations of germ cells interact with Sertoli cells in a cyclic pattern. Sertoli cells secrete a large number of proteins and regulatory factors that are crucial for germ cell development (Griswold 1998). Germ cells are needed to maintain the cyclicity of Sertoli cells (Jegou & Pineau 1995, Weinbauer & Wessels 1999). Then perhaps the different distribution of the spermatogenesis stages in the pCA-treated animals at 45 or 65 days of age was related to intra-testicular chemical communication; on the one hand, as a result of changes in the action of hypothalamic factors related with age, and on the other hand because of a depletion of specific germ cells. In order to elucidate this, information is needed about the first damaged germ cells resulting from serotonin depletion.

Shishkina & Dygalo (2000) reported that adult-stage male rats treated with pCPA on days 30 and 44 show a decrease in testis weight and sperm
count. Our results on the sperm count of animals with serotonin depletion agreed with the data obtained by Shishkina and Dygalo, and shed light on the quality of sperm produced under conditions of serotonin depletion. It is now clear that low testis weight and sperm count in adult animals with serotonin depletion are due to a loss of germ cells by apoptosis.

Sperm were not present in the vas deferens of the control or of the pCA-treated animals killed at 45 days of age. This was because testicular sperm require time to move through the epididymis and vas deferens. Sperm were observed in all the groups of 65-day-old rats, but their quality and quantity were low in the pCA-treated animals. The low sperm motility and viability induced by the pCA treatment was perhaps related to effects on the epididymis. This suggestion is supported by evidence that physiological phenomena occur in the epididymis and that serotonin is present in the reproductive accessory ducts. In the epididymis, sperm achieve the capacity to move (Robaire & Hermo 1988) and undergo changes in the cytoplasmic membrane (Shalgi et al. 1989). Serotonin is present in the epididymis (Leung et al. 1999) and vas deferens (Celuch & Sloley 1988), but its function is not known.

In summary, the present study showed that serotonin is necessary for the development of normal spermatogenesis in prepubertal rats. The inhibition of serotonin synthesis by pCA disrupts the establishment of normal spermatogenesis through induction of germ cell apoptosis. In animals with induced serotonin depletion, gonadotropin and sex steroid hormones apparently were not related to germ cell apoptosis in the tests. Although severe spermatogenesis disruption was induced by pCA, mature sperm were formed, but their population was poor in quality and quantity.

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