Male reproductive performance is composed of two principal elements, copulation and spermatogenesis. A wealth of literature has described the intricate web of endocrine events underlying these biological processes. In the present study we show that puromycin-sensitive aminopeptidase (Psa)-deficient mice are infertile, lack copulatory behavior, and have impaired spermatogenesis. The reproductive deficits of the mutants are not restored by androgen administration, although no aberrant localization of the sex steroid receptors was detectable in their brains and testes. Considering the strong expression of the Psa gene in the brain and Sertoli cells and the degenerative morphology of Sertoli cells in Psa-deficient mice, Psa may participate in testosterone-mediated reproductive signal pathways in the brain and testes. (Molecular Endocrinology 15: 960–971, 2001)

INTRODUCTION

Male reproduction requires the coordinated regulation of reproductive hormones comprising the hypothalamic-pituitary-gonadal axis. The axis is crucial for germ cell development, reproductive organ function, and sexual behavior. A number of molecules have been investigated to determine their involvement in the appearance of reproductive performance in males (for review, see Refs. 1 and 2). However, their intricate regulatory machinery is still not fully understood.

Attempts to understand the neural regulation of sexual behavior have been based on the results of removing areas of the brain. Many lesion studies have revealed several confined brain areas that play important roles in the neural control of copulation (for review, see Ref. 1). Among these brain areas, the medial preoptic area (MPOA) appears to be an integral component of this system (3) (for review, see Ref. 1). Chemical stimuli, such as pheromones and endogenous testosterone, which stimulate neural activity in the MPOA via the olfactory neural network and testosterone-responsive pathways, are integrated in the MPOA and lead to the expression of masculine mating behavior. A number of steroid signaling pathways and neurochemical systems exist in the MPOA and project into other brain areas involved in sexual behavior in males (for review, see Ref. 1). Among these molecules, testosterone acts as a key regulator of copulation (4) through several receptors in vivo, including the androgen receptor (AR) and estrogen receptor-α (ERα) and β (ERβ) (for review, see Refs. 5 and 6).

Spermatogenesis requires the physical and tropic support of Sertoli cells, although the intrinsic regulation of spermatogenic cells is required as well (7–9). This tropic support depends on a network of reciprocal signalings among Leydig cells, Sertoli cells, peritubular cells, and germ cells within the testis, which is regulated upstream by the hypophyseal hormones (for review, see Ref. 2). These cell-cell interactions among intratesticular cells have been investigated at the cellular level. Testosterone is a key molecule also in spermatogenesis, is secreted from Leydig cells, stimulates Sertoli cell activity, and regulates spermatogenesis (for review, see Ref. 2).
In the present study, we demonstrate that puromycin-sensitive aminopeptidase (Psa)-deficient mice are infertile, lack copulatory behavior, and have impaired spermatogenesis. Psa-deficient mice (designated as \( \text{Psa}^{\text{goku/goku}} \)) produced by a gene-trap method have shown increased anxiety and impaired pain response (10). Psa has been characterized and purified as a putative extracellular enkephalinase in vivo (11). The intracellular localization of Psa (12, 13) as well as our previous study using Psa mutant mice (10), however, imply intracellular roles for Psa.

We have also found in the present study that abnormal reproductive phenotypes are insensitive to testosterone administration, and that the function or survival of Sertoli cells, in which Psa is expressed strongly, is mainly affected. Together with the comparable levels of ER\( \alpha \) in the brain and AR in testes of \( \text{Psa}^{\text{goku/goku}} \) mice to those in normal animals, Psa may participate in AR/ER responsive signalings.

**RESULTS**

**Sterility and Lack of Copulatory Behavior in \( \text{Psa}^{\text{goku/goku}} \) Male Mice**

Male \( \text{Psa}^{\text{goku/goku}} \) mice did not sire when housed with \( \text{Psa}^{+/+} \) females over 1 month. The testes and seminal vesicles of \( \text{Psa}^{\text{goku/goku}} \) mice (Fig. 1B) were significantly reduced in weight compared with \( \text{Psa}^{+/+} \) mice (seminal vesicle: \( \text{Psa}^{+/+} \) mice, 175.2 ± 14.7 mg, \( n = 5 \); \( \text{Psa}^{\text{goku/goku}} \) mice, 96.6 ± 11.8 mg, \( n = 7 \); \( P < 0.001 \)); testes: \( \text{Psa}^{+/+} \) mice, 86.0 ± 5.9 mg, \( n = 4 \); \( \text{Psa}^{\text{goku/goku}} \) mice, 45.3 ± 3.3 mg, \( n = 6 \); \( P < 0.001 \)). Adult male \( \text{Psa}^{\text{goku/goku}} \) mice, however, showed no morphological deficiency in the external genitalia. These observations imply that Psa is not sex determined.

To assess sexual behavior, we observed the copulatory behavior of 8- to 15-week-old males of each genotype when housed with a 5- to 6-week-old estrous female for 2 h (Table 1). Male sexual behavior is composed mainly of courtship and precopulatory behavior, mounting, intromission, and ejaculation (1, 14). We assessed the expression of ejaculation by confirming the presence of vaginal plugs on the day after the experiments, because it is difficult to distinguish clearly between intromission and ejaculation by our method.

During the testing periods, \( \text{Psa}^{+/+} \) males exhibited overall masculine sexual behavior within 1 h. Female mice housed with \( \text{Psa}^{+/+} \) males had a high frequency of vaginal plugs (Table 1). In contrast, \( \text{Psa}^{\text{goku/goku}} \) males exhibited adequate precopulatory behavior (allo-grooming and anogenital contact, followed by sniffing), but did not display the onset of either mounting behavior or intromission. Furthermore, vaginal plugs in females housed with \( \text{Psa}^{\text{goku/goku}} \) males were not detected the following morning (Table 1). This indicates that \( \text{Psa}^{\text{goku/goku}} \) males did not ejaculate. Although erectile dysfunction in \( \text{Psa}^{\text{goku/goku}} \) males cannot be ruled out, these observations suggest that the infertility in \( \text{Psa}^{\text{goku/goku}} \) males is due to a lack of copulatory behavior.

**Impaired Spermatogenesis in \( \text{Psa}^{\text{goku/goku}} \) Males**

Spermatogenesis is a major component of male reproduction. We found a reduction in the size of the testes of \( \text{Psa}^{\text{goku/goku}} \) males compared with \( \text{Psa}^{+/+} \) mice (Fig. 1, A and B). Therefore, spermatogenesis in \( \text{Psa}^{\text{goku/goku}} \) males was also examined. The number of sperm flushed from the caudal epididymides of \( \text{Psa}^{\text{goku/goku}} \) males at 8–12 weeks of age was significantly decreased compared with \( \text{Psa}^{+/+} \) animals \( (P < 0.001) \). Furthermore, most of the sperm collected from \( \text{Psa}^{\text{goku/goku}} \) males exhibited poor movement and some lacked any movement. We next examined fertilization by \( \text{Psa}^{\text{goku/goku}} \) sperm in vitro (15). Sperm collected from the caudal epididymides of \( \text{Psa}^{\text{goku/goku}} \) males lacked the ability to fertilize intact oocytes in vitro (Table 2). These observations suggest that spermatogenesis in \( \text{Psa}^{\text{goku/goku}} \) males is impaired by Psa deficiency.

The spermatogenic status of \( \text{Psa}^{\text{goku/goku}} \) males was assessed morphologically with cross-sections of semiferous tubules in 11-week-old mice of both genotypes. Fifty sectioned tubules of each genotype were randomly selected. The cell type and stage definitions used herein are those of Russell et al. (16). The cross-sections of \( \text{Psa}^{+/+} \) animals revealed an organized spermatogenic cycle (Fig. 1C). In contrast, the stages of most tubules from \( \text{Psa}^{\text{goku/goku}} \) males were difficult to categorize because concentric organization of the germ cells was not detected and there were only a few late elongated spermatids (steps 15–16) (Fig. 1D). Moreover, vacuolar structures were frequently observed in the tubules of \( \text{Psa}^{\text{goku/goku}} \) males (Fig. 1D), suggesting germ cell degeneration. After DNA staining of the testicular cells, the stained cells can be sorted by the cell sorter according to the intensity of the fluorescence emission, which corresponds to DNA content. Among the karyotype 1N cells of \( \text{Psa}^{+/+} \) mice, two predominant peaks of fluorescence with wide ranges were apparent (Fig. 1E), one representing round spermatids and the other elongated spermatids and spermatozoa (17). In contrast, testicular cells of \( \text{Psa}^{\text{goku/goku}} \) males showed an absence of the peaks representing elongated spermatids and spermatozoa (Fig. 1E). These results are consistent with a reduction in spermatozoa accumulated in the epididymis and the observation that elongated spermatids in \( \text{Psa}^{\text{goku/goku}} \) males are most severely affected in histological analyses (Fig. 1D).
We assessed the development of spermatogenesis in Psagoku/goku testes. Histological studies of each genotype from postnatal day 16 to 1 yr of age revealed that the spermatogenic stage from spermatogonia to spermatids at step 9 (16) appears normal (Fig. 2, A–D). Together with the decreased number of late elongated spermatids (Fig. 1, D and E), these data suggest that spermiogenesis after step 9 is affected. The observed abnormalities in the seminiferous tubules of Psagoku/goku males were progressively affected depending on the age of the mouse examined, none of which could be categorized according to the normal spermatogenic cycle by 24 weeks of age (Fig. 2, E and F). In addition, frequency and area of the vacuolar structures increased (Fig. 2, F and H). By 1 yr of age, most seminiferous tubules in Psagoku/goku males lacked most germ cells (Fig. 2H). These observations indi-
cate that the survival of germ cells is affected in addition to the defects in spermiogenesis.

Hormonal Levels and an Attempt to Restore the Phenotype of \( Psa^{goku/goku} \) Male Mice by Androgen Administration

The appearance of copulatory behavior and the induction of spermatogenesis are regulated by androgens (4). We examined the plasma levels of testosterone and also the hypophyseal hormones (LH, FSH, and PRL) that regulate male reproduction cooperatively. The mean plasma levels of testosterone, LH, FSH, and PRL in \( Psa^{goku/goku} \) males were approximately 57%, 64%, 86%, and 84% of those in \( Psa^{+/+} \) males, respectively (Table 3). Statistical analysis (Student’s \( t \) test) revealed significant differences in the levels of LH and FSH between the genotypes while the differences in testosterone and PRL were not significant (Table 3).

Next, we examined whether the disruption of male reproduction in \( Psa^{goku/goku} \) mice could be restored by androgen administration. Perinatal androgens are known to be crucial for masculine sexual behavior in rodents (18). Therefore, we prepared two types of treated \( Psa^{goku/goku} \) male mice: 1) those that received neonatal injections of testosterone propionate (TP) in olive oil (for control mice, only olive oil was injected) and implantation of a tube containing TP in the adult, and 2) those that underwent implantation of a TP tube in the adult without prenatal treatment. In both treatments, tubes containing Dulbecco’s PBS were used as controls. The effects of administration of TP were assessed after 4 weeks of implantation by measuring the plasma testosterone levels of the type 2 treated mice (\( Psa^{+/+} \), 17.10 ± 1.59 ng/ml, \( n = 3 \); \( Psa^{goku/goku} \) males, 21.73 ± 5.39 ng/ml, \( n = 6 \), respectively). The levels of testosterone in \( Psa^{goku/goku} \) males showed a significant increase compared with those of non-treated mice (Table 3, \( P < 0.01 \)). Both treatments resulted in restoration of the weight of the seminal vesicles of the \( Psa^{goku/goku} \) males (Fig. 3, A–C). The treated \( Psa^{+/+} \) males displayed intact fertility. Conversely, three type 1, and six type 2 treated \( Psa^{goku/goku} \) mice displayed neither copulation nor fertility. Furthermore, the reduction in the number of sperm accumulated in the epididymides was not restored (Fig. 3D). Impaired spermatogenesis in the testes of \( Psa^{goku/goku} \) mice was also not restored after the administration of TP, which is the same as shown in Fig. 1 (Fig. 3, E–G).

These data indicate that the impairments in copulation and spermatogenesis in \( Psa^{goku/goku} \) males are insensitive to testosterone.

Immunolocalization of Steroid Receptors and Other Related Molecules in the Brain and Testis

To examine whether the failure to repair the reproductive defects in \( Psa^{goku/goku} \) males derives from deficiencies in AR, ER, or both, we investigated the immunoreactivity of AR and ER in the brain and testes of \( Psa^{+/+} \) and \( Psa^{goku/goku} \) males.

The ER-mediated pathway is required, in part, for the appearance of sexual behavior in male mice (for review, see Ref. 1). We examined the immunolocalization of ER\( \alpha \) positive cells in the MPOA. The MPOA has been well established as a center for masculine sexual behavior in rodents. No apparent differences between genotypes in localization or intensity were detectable for cells positive for ER\( \alpha \) in the MPOA (Fig. 4, A and B). Next, we examined the immunoreactivity of other molecules associated with reproductive performance in the male: GnRH-associated peptide (GAP; characterized as a marker of the GnRH neurons), CRH, \( \beta \)-endorphin, and enkephalins in the MPOA and medial basal hypothalamus including the paraventricular nucleus, arcuate nucleus, and the median eminence (for review, see Ref. 19). Comparable staining intensities

| Table 1. Copulatory behavior is missing in \( Psa^{goku/goku} \) male mice |
|---|---|---|
| Genotype | No. of Mice Observed | Nos. of Males Exhibiting |
| | | Mounting | Intromission\(^a\) | Plug formation\(^b\) |
| \( Psa^{+/+} \) | 8 | 8.8\(^c\) | 6.8\(^c\) | 6.8\(^c\) |
| \( Psa^{goku/goku} \) | 10 | 0.0\(^c\) | 0.0\(^c\) | 0.0\(^c\) |

\(^a\) Intromission defined as the behavior with reduced speed of thrusting.

\(^b\) Female depositing a vaginal plug after housing with the tested males overnight were counted as showing positive plug formation.

\(^c\) Number in the first trial, number in the second trial.

| Table 2. In Vitro Fertilization Using Intact Oocytes |
|---|---|---|
| Genotype | No. of Oocytes Examined | No. of Oocytes Developing to |
| | | 2-cells (%) | Morulae (%) | Blastocysts (%) |
| \( Psa^{+/+} \) | 73 | 38 (52.1) | 24 (32.9) | 15 (20.5) |
| \( Psa^{goku/goku} \) | 116 | 1 (0.9) | 0 (0) | 0 (0) |
and localizations for these molecules were detected for both genotypes (GAP; Fig. 4, C and D).

AR immunoreactivity was present in the nuclei of spermatogonia, Sertoli cells, Leydig cells, and elongated spermatids in the seminiferous epithelia in the testes of \( \text{Psa}^{+/+} \) and \( \text{Psa}^{\text{goku/goku}} \) mice. This observation is consistent with previous studies (20, 21). The staining intensity of most Sertoli cells was comparable between genotypes (Fig. 4, E and F). Next, we examined the expression of inhibin \( \alpha \), a subunit of inhibin that is expressed in Sertoli cells and has important roles in spermatogenesis. The immunoreactivity of inhibin \( \alpha \) in most Sertoli cells from \( \text{Psa}^{\text{goku/goku}} \) mice revealed a reduction in intensity compared with

![Diagram of testicular development](https://example.com/testicular-development.png)
**Table 3. Plasma Levels of Reproductive Hormones from Psa<sup>+/−</sup> and Psa<sup>goku/goku</sup> Male Mice**

<table>
<thead>
<tr>
<th>Hormone (ng/ml)</th>
<th>Psa&lt;sup&gt;+/−&lt;/sup&gt;</th>
<th>Psa&lt;sup&gt;goku/goku&lt;/sup&gt;</th>
<th>p-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testosterone</td>
<td>8.20 ± 3.54 (8)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.64 ± 1.48 (5)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.464</td>
</tr>
<tr>
<td>LH</td>
<td>56.26 ± 5.46 (7)</td>
<td>35.95 ± 4.48 (7)</td>
<td>0.014</td>
</tr>
<tr>
<td>FSH</td>
<td>10.75 ± 0.39 (8)</td>
<td>9.20 ± 0.59 (7)</td>
<td>0.044</td>
</tr>
<tr>
<td>PRL</td>
<td>16.56 ± 1.75 (11)</td>
<td>13.83 ± 1.11 (11)</td>
<td>0.140</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± SEM are provided. The number of mice examined is shown in parentheses. <sup>b</sup> Student’s t test.

**Psagoku/goku** (Fig. 4, G and H), while the immunoreactivity of WT-1, which is also expressed in Sertoli cells and is a differentiation marker, did not show apparent differences, suggesting that Sertoli cells in Psa<sup>goku/goku</sup> mice differentiate developmentally (data not shown). The observation was coincident with the results of decreased levels of inhibin α in Western blot analysis (Fig. 5). We measured the intensity of the bands and compared them between genotypes. We found that those for Psa<sup>goku/goku</sup> mice were significantly reduced (Psa<sup>+/−</sup> mice, 222.2 ± 3.0, n = 3; Psa<sup>goku/goku</sup> mice, 151.1 ± 12.6, n = 3; P < 0.01), while the intensity of the bands against anti WT-1 did not exhibit significant differences (Psa<sup>+/−</sup> mice, 222.8 ± 20.8, n = 3; Psa<sup>goku/goku</sup> mice, 246.9 ± 2.8, n = 3; P = 0.31). We also detected decreased levels of inhibin β (Fig. 5) (intensity, Psa<sup>+/−</sup> mice, 228.0 ± 9.3, n = 3; Psa<sup>goku/goku</sup> mice, 121.0 ± 33.7, n = 3; P < 0.05). These results suggest that the expression of AR is normal, while functions including the expression of inhibin are impaired in Sertoli cells in Psa<sup>goku/goku</sup> mice.

**Electron Microscopic Analysis and Expression of the Psa Gene in Testes**

Transmission electron microscopic analysis (Fig. 6, A–D) was performed to characterize the aberrant status of the testes of Psa<sup>goku/goku</sup> animals. In 11-week-old Psa<sup>goku/goku</sup> animals, the morphology of the Leydig cells was not severely affected (data not shown). In contrast, some Sertoli cells were found to have degenerated morphologically compared with Psaguku<sup>+/−</sup> animals (Fig. 6, A and B). Sertoli cells from Psa<sup>+/−</sup> mice adhere tightly to germ cells and provide physical and tropic support for the developing spermatogenic cells (22). The abnormal Sertoli cell morphology in Psa<sup>goku/goku</sup> animals is likely to lead to impaired physical interactions with encompassing spermatogenic, spermatocytes, and spermatids (Fig. 6, A and B).

This observation is consistent with the appearance of multinuclear cells that may result from impaired Sertoli cell function and reflect the decreased number of sperm in the epididymides of Psa<sup>goku/goku</sup> mice, and their inability to fertilize eggs in vitro.

Next, expression of the Psa gene in the testes was examined. Because the *Escherichia coli* β-galactosidase gene (lacZ) was inserted into the Psa gene, we can monitor the expression of the Psa gene using β-galactosidase activity (10). X-gal staining was performed to examine β-galactosidase activity, and the activity was detected predominantly in Sertoli cells and Leydig cells (Fig. 6, E and F). Very weak activity was observed in spermatagonia and spermatocytes, but not in spermatids.

Together with Psa gene expression in Sertoli cells, Psa may play critical roles in either Sertoli cell survival or functions that support germ cells.

**DISCUSSION**

**The Basis of Suppression of Copulatory Behavior in Psa<sup>goku/goku</sup> Mice**

Masculine sexual behavior is regulated by androgens (Ref. 4; for review, see Ref. 1). Replacement of testosterone to castrated male animals can restore copulatory behavior (for review, see Ref. 1). However, in the present study the administration of androgen in neonates and adults failed to restore copulatory behavior in Psa<sup>goku/goku</sup> males. Psa gene is expressed strongly in almost all neurons in the brain (10). These facts strongly suggest that the lack of copulatory behavior in Psa<sup>goku/goku</sup> males results from defects in the brain, and that the defects are independent of those in the testes.

Immunohistochemical analyses revealed no significant alteration in the MPOA region of Psa<sup>goku/goku</sup> mice with respect to the distribution and staining intensity of ER and other molecules. Based on these observations, we hypothesize that Psa may be a component of the AR/ER-responsive pathways. The reproductive phenotypes of Psa<sup>goku/goku</sup> mice resemble those described for ER α gene-deficient mice. ERα deficiency leads to greatly reduced copulatory behavior (23, 24). Furthermore, previous studies suggest that AR activation is necessary to express sexual behavior as well as ERα-mediated signaling (25). Based on the observations in this manuscript, Psa may assemble and modulate both the AR and ER-mediated signaling that give rise to masculine copulatory behavior. Previous studies have re-
ported that testosterone stimulates growth in female rats and that testosterone deficiency leads to behavioral depression in mice due to castration (26, 27). These physiological effects of testosterone (including estrogens)-responsive pathways possibly support our hypothesis because \( Psa \) mice exhibit dwarfism and behavioral impairment-associated anxiety (10).

**The Basis of Spermatogenetic Disruption in \( Psa^{+/-} \) Mice**

In this study, the \( Psa \) gene was found to be expressed strongly in Leydig and Sertoli cells in the testes. \( Psa \) gene expression was not detected in elongated spermatids and spermatozoa that had advanced beyond step 10, the stage that is severely affected in \( Psa^{+/-} \) mice. Moreover, decreased levels of inhibin were observed in Sertoli cells of \( Psa^{+/-} \) mice. Together with morphological degeneration in Sertoli cells of \( Psa^{+/-} \) mice, \( Psa \) deficiency is likely to affect not only inhibin expression but also other Sertoli cell-secreting molecules. These observations further suggest that \( Psa \) in Sertoli cells is required for Sertoli cell function and survival.

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**Fig. 3. Effects of Testosterone Administration in \( Psa^{+/-} \) Animals**

A, 12W seminal vesicle with coagulating gland of \( Psa^{+/-} \) (top) and \( Psa^{+/-} \) (bottom) animals. B, Seminal vesicles from the same animals as shown in panel A collected 4 weeks after implantation of the TP-containing tube. C and D, Wet weight of seminal vesicle (C) and number of sperm accumulating in the epididymis (D) after TP treatment. Gray, orange, and green bars represent mean values from mice receiving control treatment, TP implants as adults, and TP injection neonatally followed by TP implants as adults, respectively. The number of mice examined is shown in parentheses. The error bars indicate SEM. E–G, Cross-sections of seminiferous tubules of \( Psa^{+/-} \) (E) and \( Psa^{+/-} \) animals receiving TP implants as adults (F), and a \( Psa^{+/-} \) animal receiving a TP injection neonatally in addition to a TP implant as an adult (G). Note the disorganized spermatogenesis regardless of TP treatment (F and G). Bars represent 1 mm (A and B) and 100 \( \mu m \) (E–G).
The same explanation given for the impaired copulatory behavior described above can explain the deficits in spermatogenesis of *Psagoku/goku* males. Sertoli cells express AR and ERβ in mice (28). Moreover, morphological changes in testicular cells by pharmacological destruction of the Leydig cells can be prevented by testosterone administration (29). These studies indicate that AR/ER signaling plays important roles in functions of Sertoli cells for spermatogenesis. According to this hypothesis, deficits in spermatogenesis in *Psagoku/goku* males are likely to arise from hampered AR/ER signaling within Sertoli cells.

**Putative Role of Psa in Vivo**

A possible role of Psa in steroid signal pathways can be hypothesized. The efficient signal transduction of the steroid hormones requires a number of accessory proteins (30). Upon 17β-estradiol binding, ER dissociates from the complex and works as a transcriptional factor (31). The mechanisms of signal transduction by steroid receptors are complex and not fully understood. The degenerative pathways for the heterocomplex and receptor-hormone complex are still unclear. A previous study suggested that the ubiquitin-proteas-
some pathway may contribute to part of the chaperone machinery (32). Psa protein contains two motifs showing significant similarity to a sequence in the 26S proteasome subunits (13). This suggests that Psa participates in either the proteolysis of the chaperone complex, posttranscriptional ER-ligand complexes, or both. In fact, proteasome appears to be responsible for the degradation of key regulatory proteins, such as transcriptional factors (33). Thus, ER may fail to interact with hormones because of the excess amount of chaperone complex in Psagoku/goku animals. Otherwise, a lack of degradation of the receptor-steroid complex may result in an excess flow of the transcriptional stimulation of AR/ER, which leads to a disruption in the normal regulation of reproductive performance in Psagoku/goku males.

Further analyses of Psagoku/goku mice will resolve the nature of the testosterone-insensitive deficits and Sertoli cell degeneration resulting from Psa deficiency. Psagoku/goku mice provide a tool with which to investigate the role of Psa in the molecular machinery underlying male reproductive performance.

**MATERIALS AND METHODS**

**Mice**

Psagoku/goku mice were generated by intercrossing goku heterozygous mice. F20–21 heterozygotes obtained by backcrossing with BALB/cA strain mice were used as parents. All animals were maintained one to three mice per cage with a 12-h light/12-h dark cycle. Food and water were given ad libitum. All experiments involving animals were performed in accordance with standard ethical guidelines for the care and use of laboratory animals (NIH Standards for Treatment of Laboratory Animals, 1985) and approved by the Ethical Committee of our institute.

**Copulatory Behavior and Fertilization Analyses**

Five to 6-week-old female BALB/cA mice (Clea Japan, Tokyo, Japan) were treated by ip injection with PMSG (5 IU; Serotropin, Teikoku-Zouki Co. Ltd., Tokyo, Japan) 2 days before the observation of copulatory behavior. On the day of an experiment, estrus was induced in the females by the
The Roles of the Psa Gene in Male Reproduction

Injection of human CG (hCG; 5 IU; Gonatropin, Telkoku-Zouki Co., Ltd.) eight hours before the experiment. At 2200 h, a female was placed in a separate cage with an 8- to 15-week-old virgin male mouse. The cages were videotaped for 2 h under dim light. The observed behaviors were assessed as described by McGill (14) (for review, see Ref. 1). The females were checked for vaginal plug formation the following morning. Duplicate observations were carried out weekly.

The sperm collected from the caudal epididymides of each genotype at 8–12 weeks of age were prepared for in vitro fertilization as previously described (15). Eggs were collected from BALB/cA females in which superovulation was induced (same procedure as the induction of estrus in females). Sixteen hours after the injection of hCG, insemination in vitro at a concentration of 250 sperm/µl was carried out, and the inseminated eggs were incubated in 5% CO₂ at 37 C. Six hours after insemination, eggs containing polar bodies and two pronuclei were used for further analysis. On the following day, eggs that had developed to the two-cell stage were designated as fertilized eggs.

Histological and Immunohistochemical Analysis

The testes were removed and fixed overnight in Bouin’s solution (Sigma, St. Louis, MO) at 4 C, dehydrated in ethanol, cleared with xylene, and then embedded in paraplast. The specimens were cut into 7-µm sections and stained with hematoxylin and eosin (Sigma), or with hematoxylin and periodic acid Schiff (PAS). For X-gal staining or immunohistochemistry, the testes and brains derived from 10–20-week-old mice were fixed for 5 h at 4 C in 4% paraformaldehyde in PBS (pH 7.6) and embedded in Tissue-Tech O.C.T. compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan). The testes and brains were cut into 7-µm and 40-µm sections, respectively. X-gal staining was performed to monitor Psa expression using β-galactosidase activity (10). The sections were stained overnight at 37 C as previously described (34). Paraaffin sections were used for immunohistochemistry using antinhibin α antibody without X-gal staining. Immunohistochemistry was performed with antiporcine inhibin α (diluted at 1:8,000, Ref. 35), anti-β-endorphin (diluted at 1:5,000, PG21; a gift from G. Greene, University of Chicago), anti-GAP (diluted at 1:8,000, Ref. 35), anti-AR (diluted at 1:5,000, PG21; a gift from G. Greene, University of Chicago), anti-CRH (diluted at 1:1,000, YANAIHARA Institute), anti-ERα (diluted at 1:5,000, ER21; a gift from G. Greene, University of Chicago), anti-ERβ (diluted at 1:5,000, PG21; a gift from G. Greene, University of Chicago), anti-β-endorphin (diluted at 1:5,000, Sigma), or anti-trenkaphalins (diluted at 1:5,000, Chemicon International, Inc., Temecula, CA). The sections were probed two to three overnights with first antibody for brain sections and one overnight for testicular sections at 4 C. A Vectastain ABC Elite kit (Vector Laboratories, Inc., Burlingame, CA) was used for the subsequent immunodetection.

Western Blot Analysis

Testes removed from 8-month-old mice were suspended in 2 × sample buffer (100 mM Tris-HCl, pH 6.4, 4% SDS, 0.2% bromophenol blue, and 20% glycerol), sonicated, and boiled for 5 min. The protein concentrations of the cell lysates were measured with a protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA). Lysates containing protein (50 µg) were resolved in 7.5% SDS-polyacrylamide gels and electrobotted to a nitrocellulose membrane. The filters were probed with first antibodies at 4 C overnight and incubated with HRP-conjugated antibody to rabbit-IgG at room temperature for 1 h. The immune complexes were detected by ECL detection (Amersham Pharmacia Biotech, Arlington Heights, IL). As first antibodies, antibodies against porcine inhibin α and β (diluted at 1:1,000, Ref. 35) and rat WT-1 (C-19, diluted at 1:1,000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used for the analysis. The results of blotting were scanned with a scanner, and the intensities of the bands were analyzed by the software NIH image.

Flow Cytometric Analysis

Cytological analysis of testicular cells by FACSscan was performed as described previously (17). Four 8- to 12-week-old mice of each genotype were prepared for assay. For FACSscan analysis, testicular cells were added to a concentration of 1 × 10^6 cells/ml in separation medium and stained with propidium iodide. The cells were analyzed with a FACSscan apparatus (Becton Dickinson and Co., Rutherford, NJ).

Electron Microscopy

Analysis by transmission electron microscopy was performed as described previously (36). Testes from 11- and 17-week-old mice of each genotype were removed and fixed in 2% glutaraldehyde in PBS (pH 7.6). For quick and solid fixation, a 27G needle was inserted through the tunica albuginea of the testes and a small volume of glutaraldehyde was injected before immersion fixation.

RIA

Male mice caged in groups of one to three sexually identical mice were used for the hormonal assay. The mice were decapitated without anesthetic and blood samples rapidly collected (within 30 sec). RIAAs of FSH, LH, and PRL in mouse plasma were performed in duplicate using reagents distributed by the NIDDK National Hormone and Pituitary Program. Results are expressed in terms of rat FSH-RP-2, rat LH-RP-2, and PRL (AFP-6476C). Plasma levels of testosterone were determined with a double-antibody RIA system using ^125^I-labeled radioligand as described by Taya et al. (37). Anti-serum against testosterone (GDN250; Ref. 38) kindly provided by G. D. Niswender (Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO) was used. In assays for testosterone, PRL, LH, and FSH detection, the interassay coefficients of variation (CVs) were 15.5%, 18.7%, 13.5%, and 9.9%, respectively, and the intraassay CV values were 6.0%, 12.6%, 5%, and 9.2%, respectively.

Administration of Androgen

Neonatal mice were injected with androgen as described previously (18). Male and female mice heterozygous for the goku mutation were housed for breeding and the pups on postnatal days 0–3 were injected sc with 100 µg of TP in 0.02 ml of olive oil. As control experiments, only olive oil was injected. For the administration of androgen in adults, an sc implant of 2-cm Silastic tube (outer diameter, 3.18 mm; inner diameter, 1.57 mm, Silastic medical grade tubing, Dow Corning Corp.; Midland, MI) containing either TP or PBS as a control was undertaken in 6- to 8-week-old mice.

Duplicate observations (weekly observations) of copulatory behavior were conducted 2 weeks after TP implantation as described in Copulatory Behavior and Fertilization Analysis above, and then the morphology of the testes and plasma testosterone levels were analyzed 4 weeks after TP implantation as described in Histological and Immunohistological Analysis and RIA, respectively.

Statistical Analysis

The experimental data were analyzed by Student’s t test. Values of P < 0.05 were considered as statistically significant. All values in the text are expressed as mean ± SEM.
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