CaMKIV/Gr is dispensable for spermatogenesis and CREM-regulated transcription in male germ cells

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CaMKIV/Gr is dispensable for spermatogenesis and CREM-regulated transcription in male germ cells. Am J Physiol Endocrinol Metab 281: E931–E937, 2001.—The calcium/calmodulin-dependent protein kinase type IV/Gr (CaMKIV/Gr) is expressed in male germ cells and spermatids and has been implicated in controlling the differentiation of germ cells into mature spermatozoa. The function of CaMKIV/Gr in spermatogenesis was investigated using CaMKIV/Gr-deficient mice generated by targeted gene disruption. CaMKIV/Gr-deficient males exhibited normal spermatogenesis, and their fertility was similar to that of wild-type littermates. Notwithstanding the function of CaMKIV/Gr as an activator of cAMP response element (CRE)-dependent transcription, mRNA levels of several testis-specific CRE modulator (CREM)-regulated genes were unaltered. These results indicate that CaMKIV/Gr is not essential for spermatogenesis or for CRE-regulated gene transcription in the testis.

calcium signaling; calmodulin-dependent protein kinase; spermiogenesis; cyclic adenosine monophosphate response element modulator

SPERMATOCYTES, the differentiation of progenitor male germ cells or spermatogonia into spermatooza, is a complex process that occurs in seminiferous tubules and proceeds through several ordered steps (20). It is initiated upon the commitment to differentiation of spermatagonia present at the periphery of the seminiferous tubule. Committed spermatagonia undergo mitotic division to give rise to spermatocytes. These in turn undergo meiosis to give rise to postmeiotic spermatids. Spermiogenesis, the differentiation of spermatids into spermatozoa, involves complex morphological differentiation including acrosomal formation and nuclear condensation.

A number of molecular mechanisms have been implicated in regulating the progression of male germ cell differentiation. Of particular interest has been the role of a calcium-signaling pathway involving the calcium/calmodulin-dependent protein kinase type IV/Gr (CaMKIV/Gr) (13, 15, 16). Several attributes of CaMKIV/Gr have marked it as a candidate regulator of germ cell differentiation. In the mouse testis, CaMKIV/Gr is expressed in male germ cells in a stage-specific manner. It locates in spermatogonia and spermatids (but not spermatocytes) and associates with chromatin and the nuclear matrix of elongating spermatids (25). The CaMKIV/Gr gene encodes two different CaMKIV/Gr isoforms, a shorter α- and a longer β- that extends an extra 28 amino acids at the NH2-terminal (12, 18, 23). CaMKIV/Gr gene expression is under distinct, tissue-specific regulation in the three tissues in which it is enriched; only the α-isofrom is expressed in the testis, whereas both α- and β-isoforms are expressed in neurons and T lymphocytes (8, 9, 16, 25). Moreover, the CaMKIV/Gr gene encodes a second protein, calserpin, which is expressed exclusively in spermatids. Calserpin is composed of 164 amino acids that correspond to the calmodulin-binding and the carboxyl-terminal-associative domains of CaMKIV/Gr, and it is transcribed from a separate internal promoter in the 10th intron of the CaMKIV/Gr gene (15, 17, 22, 23).

Although the function of CaMKIV/Gr in male germ cell differentiation has remained undefined, it has been suspected of serving both transcriptional and posttranscriptional regulatory functions. CaMKIV/Gr has been demonstrated to activate members of the cAMP response element (CRE) binding protein (CREB) family of transcription factors, including the testis-specific CRE modulator-γ (CREMγ) (11, 21, 22, 24). This factor, which is activated by both cAMP- and calcium-dependent signaling pathways, has been revealed as a master molecular switch that is indispensable to the initiation of spermiogenesis (19). CREMγ regulates the expression of several haploid germ cell-specific genes, including those encoding protamine-1 and -2 and transitional protein-1 and -2 as well as calserpin itself. Mice deficient in CREMγ suffer from postmeiotic arrest in the first step of spermiogenesis, leading to complete lack of spermatozoa and resultant infertility (2, 14).

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Expression of CaMKIV/Gr and calispermin in spermatids may also serve posttranscriptional regulatory functions at later stages in spermatogenesis (25).

The present report makes use of CaMKIV/Gr-deficient mice generated by targeted gene disruption to analyze the function of this kinase in male germ cell development. We find that CaMKIV/Gr is dispensable for spermatogenesis.

MATERIALS AND METHODS

Mice. CaMKIV/Gr-deficient mice were derived by targeted disruption of the 79-bp-long exon III of CaMKIV/Gr gene, corresponding to bp 195–273 of murine CaMKIV/Gr cDNA (5'-GGGTGCTACATCGGTGAGTGGAGAGAAGGGGACAGAGGCTATGCTCAAAGTTTAAAGAA-AAAAC-3'). Details of the targeting strategy have been reported elsewhere (9). Germline transmission of the mutant CaMKIV/Gr gene was carried out by breeding male chimeric mice with both inbred C57BL/6 and outbred Black Swiss mice. Mice were back-crossed for at least five generations on both backgrounds. Wild-type (WT), heterozygous (HET), and homozygous deficient (KO) littermate mice were derived by mating heterozygous animals. The mice used in these studies were housed on a 12:12-h light-dark cycle with ad libitum access to rodent chow. All mouse protocols were in accordance with National Institutes of Health guidelines and were approved by the Animal Care and Use Committee of Washington University School of Medicine.

Genotyping was initially performed by Southern blot analysis, as described (9). Subsequent genotyping was performed by PCR analysis of mouse tail genomic DNA with the use of three oligonucleotides in the amplification mix. The first is a sense oligonucleotide common to both WT and mutant alleles that corresponds to a CaMKIV/Gr intron II sequence downstream of the neo cassette (5'-AAATTACATCCATACACCTAAC-3'). A WT allele-specific sense primer corresponds to an intron II sequence located downstream of the neo cassette insertion site (5'-TGATAAGGGAGGACGCTCACA-3'). This sequence is deleted in the targeted gene. A mutant allele-specific antisense primer corresponds to a sequence in the inserted neo gene (5'-TACTCCATTTGTGACCT-3'). PCR amplification was carried out as follows: denaturation at 95°C for 15 s, annealing at 50°C for 30 s, and elongation at 72°C for 30 s for a total of 35 cycles. The resultant amplification products include a 464-bp-long WT allele-specific fragment and a 326-bp-long mutant allele-specific fragment.

Histopathology and TdT-mediated nick end labeling. Testes were fixed in Bouin's fixative and embedded in paraffin. Sections were cut at 5 μm and stained with periodic acid Schiff-hematoxylin. TdT-mediated nick end labeling (TUNEL) staining was performed as described (27). Briefly, after dehydration, the sections were incubated in 2× SSC at 80°C for 20 min followed by washing twice with water and once with proteinase K buffer (20 mM Tris-HCl, pH 7.4, 2 mM CaCl₂) for 5 min each. The slides were then treated with proteinase K (10 μg/ml, Roche Molecular Biochemicals, Indianapolis, IN) in proteinase K buffer at 37°C for 30 min. An aliquot of 3'-end labeling reaction mixture containing 4 μl of 5× terminal deoxynucleotidyl transferase (TdT) buffer (Promega), 0.1 μl of digoxigenin-11-ddUTP (10 nmol/μl Roche Molecular Biochemicals), 0.2 μl of ddATP (5 mM; Promega), 1 μl of TdT (Roche Molecular Biochemicals), and 14.7 μl nuclease-free water (Promega) was applied to one section. The slides were kept in a humidified box, incubated at 37°C for 1 h, and then washed three times with TBST buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 0.1% Tween-20) for 10 min each. An anti-digoxigenin-horseradish peroxidase monoclonal antibody (DAKO, 1:200 dilution in TBST containing 1% BSA) was applied, and the slides were incubated in the humidified box at room temperature for 1 h and then washed three times with TBST for 5 min each. Finally, the labeled cells were visualized by 3,3'-diaminobenzidine tetrahydrochloride (Sigma) for 0.5–2 min.

Immunoblotting. Homogenates of testes of adult WT, HET, and KO littermate mice were cleared by high-speed centrifugation, and 50-μg protein samples were resolved by SDS-PAGE and then transferred to nitrocellulose membranes for immunoblotting. Membranes were blocked in fat-free milk and then probed with one or more of the following antibodies as indicated: mouse monoclonal anti-CaMKIV/Gr catalytic domain antibody, anti-CaMKIIα antibody, and anti-protein kinase A (PKA) catalytic subunit (PKAc) antibody (Transduction Laboratories); goat polyclonal anti-CaMKIV/Gr COOH-terminal peptide antibody, rabbit polyclonal anti-ERK or anti-CREB antibodies, and mouse monoclonal anti-Rsk-2 or anti-CREB antibodies (Santa Cruz Biotechnology). The blots were developed using horseradish peroxidase-conjugated secondary antibodies and enzyme-linked enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

Northern blot analysis. Total RNA was extracted from mouse testis using RNAzol. RNA (10 μg/lane) was loaded onto a formaldehyde gel, resolved by electrophoresis, and transferred to Hybond-XL membranes (Amersham Pharma- cia Biotech). Membranes were hybridized with the indicated probes overnight at 42°C in ULTRAhyb solution (Ambion). The membranes were washed, exposed, and then stripped and reprobed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts to confirm equal loading. Probes for the following transcripts were obtained as expressed sequence tag clones from Human Genome Systems (St. Louis, MO): protamine-1 (GenBank accession no. AA064036), protamine-2 (AI528784), transitional protein-1 (AA144629). GAPDH and CaMKIV/Gr full-length cDNA were derived by PCR from mouse brain cDNA. A DNA fragment corresponding to CaMKIV/Gr cDNA bp 39–791 (5'-end cDNA) was derived by EcoRI digestion (10). An exon III-specific single-stranded oligonucleotide probe was synthesized that spans bp 195–243 of murine CaMKIV/Gr cDNA 5'-GGGTGCTACATCGGTGAGTGGAGAGAAGGGGACAGAGGCTATGCTCAAAGTTTAAAGAA-AAAAC-3'. A WT allele-specific anti-sense primer corresponds to an intron II sequence located downstream of the neo cassette insertion site (5'-TGATAAGGGAGGAGCTCACACAC-3'). PCR amplification was performed as follows: denaturation at 95°C for 15 s, annealing at 50°C for 30 s, and elongation at 72°C for 30 s for a total of 35 cycles. The resultant amplification products include a 464-bp-long WT allele-specific fragment and a 326-bp-long mutant allele-specific fragment.

RC-PCR analysis and sequencing. cDNA was derived from total testis RNA of WT, HET, and KO mice by reverse transcription using an oligo dT primer and then subjected to RT-PCR analysis with the use of the following exon III sequence flanking primers: 5'-TCTCTGGGGGCGATTCTTCGCCCTG-3' (sense primer; bp 153–172 of murine CaMKIV/Gr cDNA) and 5'-CTGGAGCTTCGCTCAAGAAGGGACCACCATAGCTACACAC-3' (antisense primer; bp 1957–2057 of murine CaMKIV/Gr cDNA) (10). PCR conditions were 95°C for 1 min followed by 35 cycles at the following settings: 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s. Amplified products were sequenced using an ABI 377 sequencer with dye-labeled dideoxy terminators.

Sperm count. The cauda epididymides of WT and KO mice were dissected, and their sperm content was released into phosphate-buffered saline medium. Sperm number and motility (measured as percent sperm with active flagella) were determined using a hemocytometer.

Fertility assessment. In this study, 6-wk-old WT and KO littermate males were derived by mating heterozygous F₅-F₇ Black Swiss parents. These were mated with WT F₅-F₇ Black Swiss females to generate WT and KO males.

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Swiss females. One female was placed with each male, and the number of days leading to the first two deliveries was counted to a maximal follow-up period of 11-wk. Litter sizes were counted at birth.

Testosterone assay. Fifty microliters of serum were collected from males by retroorbital bleeding. Serum testosterone levels were determined using an RIA kit (Diagnostic Products, Los Angeles, CA) following the manufacturer’s recommendations.

RESULTS

CaMKIV/Gr-deficient mice were generated by targeted disruption of the 79-bp-long exon III of CaMKIV/Gr gene (9). The targeting strategy aimed to disrupt expression of CaMKIV/Gr α- and β-isofoms while sparing that of calspermin by avoiding targeting of CaMKIV/Gr or calspermin promoter sequences or of calspermin coding sequences. Replacement of CaMKIV/Gr exon III with a neomycin resistance gene was predicted to result in the generation of out-of-frame, alternatively spliced CaMKIV/Gr transcripts that lacked the deleted exon III sequence. The mutant transcripts were predicted to encode a catalytically inactive 55-amino acid peptide before terminating at a premature stop codon. These predictions were verified by several criteria. First, Northern blot analysis with a full-length CaMKIV/Gr cDNA probe (bp 39–791) revealed normal levels of CaMKIV/Gr and calspermin transcripts in testes of mutant mice (Fig. 1A). Levels of CaMKIV/Gr transcripts were similarly unaffected in brains and thymi of mutant mice (data not shown). However, both Northern blot analysis and RT-PCR confirmed the lack of exon III sequence from CaMKIV/Gr transcripts of KO mice. Testicular CaMKIV/Gr transcripts of KO mice failed to hybridize to an oligonucleotide probe specific for exon III sequence, whereas transcripts of HET mice hybridized at 50% of the level noted for those of WT littermates. Furthermore, RT-PCR analysis with flanking primers confirmed the absence of targeted exon III sequence in transcripts of KO mice, whereas HET mice manifested both WT and exon III-deficient transcripts (Fig. 1B).

The lack of exon III sequence in CaMKIV/Gr transcripts of KO animals was further verified by direct sequencing of exon III-flanking RT-PCR products. Figure 1C demonstrates that, although WT transcripts showed the predicted transition from exon II to exon III sequence at codon 50, mutant KO transcripts skipped directly from exon II to exon IV sequence. The loss of exon III sequence rendered the KO transcripts out of frame. This resulted in a garbled 5-codon sequence downstream of codon 50 followed by a premature stop codon. On the basis of our previous studies on NH2-terminal, catalytically inactive CaMKIV/Gr fragments, such a peptide would fail to express in targeted cells due to instability/degradation (4).

Expression of CaMKIV/Gr protein in gene-targeted animals was evaluated by immunoblotting with the use of antibodies directed at different domains of CaMKIV/Gr, including the catalytic and the COOH-terminal associative domains. CaMKIV/Gr protein expression was found to be totally absent in KO mice and was reduced by 50% in HET mice compared with WT littermates (Fig. 1D) (9). In contrast, expression of the testis-specific calspermin protein, as detected by an anti-CaMKIV/Gr COOH-terminal peptide antibody, was normal (9). Similarly, there was normal expression in CaMKIV/Gr KO mice of other protein kinases, such as CAMKIIα, extracellular signal-regulated kinase-1 and -2, receptor-activated signal kinase-2, PKA, and the CaMKIV/Gr substrates CREB and CREM (Fig. 1D).

Transmission of WT and mutant alleles was analyzed in weaned mice derived by mating of heterozygous parents. One thousand eighteen mice were examined in this way, pooled from matings of parents on predominantly outbred (F4–F5 Black Swiss), inbred (F6–F8 C57BL/6J), or mixed backgrounds (F2–F5 129 Black Swiss or 129× B57BL/6J). Of those, 245 (24%) carried only the WT allele, 529 (52%) had one copy of the mutant allele, and 244 (24%) carried two copies of the mutant allele. The ratio of the three groups of mice is consistent with Mendelian transmission of both alleles. Analysis of mutant gene transmission in subgroups of out- and inbred populations yielded similar results. These findings indicated that the presence of the mutant CaMKIV/Gr allele did not impair fetal development or early postnatal survival.

Measurement of testis weights revealed no significant difference between WT and KO mice (Table 1). Testosterone levels were also found similar in the two groups (Table 1). Sperm count and motility of CaMKIV/Gr KO mice were examined and compared with those of WT littermates; results revealed no significant differences between WT and KO mice in the total sperm counts or in the proportion of motile sperm. To examine the fecundity of CaMKIV/Gr KO male mice, WT and KO males derived from matings of F5–F7 Black Swiss HET parents were mated with WT and HET females of similar background. The results, shown in Table 2, revealed no significant difference between WT and KO groups in the proportion of females attaining pregnancy, the number of days it took to deliver the first and second litters, or the litter sizes (with the exception of modestly larger 2nd litter sizes in the KO group). The progeny appeared normal.

Examination of periodic acid Schiff-hematoxylin-stained, paraffin-embedded sections of testes from 3-mo-old WT and KO mice on both outbred (Black Swiss) and inbred (C57BL/6J) backgrounds showed normal histological features in the KO group (Fig. 2A). The seminiferous tubules of KO mice appeared normal in diameter and lumen size. They also appeared to contain normal numbers of Sertoli cells, spermatogonia, spermatocytes, and round and condensed spermatids. Spermatogenesis proceeded normally until step 16 spermatids, and the lumen contained mature sperm. To determine whether CaMKIV/Gr deficiency was associated with enhanced apoptosis of male germ cells, in situ TUNEL was performed to detect DNA-free ends, a cardinal feature of apoptosis. Figure 2B demonstrates that similar numbers of TUNEL-stained
cells were detected in WT and KO testes, indicating that CaMKIV/Gr deficiency was not associated with enhanced male germ cell apoptosis. The results demonstrating the normal histology, spermatogenesis, and apoptosis in the KO testes are consistent with the adequate sperm count observed in these animals.

Because CaMKIV/Gr has been implicated as an activator of CREM$^\gamma$, we examined the expression in KO testes of CREM$^\gamma$-regulated gene transcripts including those encoding protamine-1 and transitional protein-1 and -2. No difference in the expression of these transcripts was observed between WT and KO mice (Fig. 3). These results, together with the normal levels of calispermin in KO mice, are consistent with the lack of an effect of CaMKIV/Gr deficiency on CREM$^\gamma$-mediated transcription.
Table 1. Testis weights, sperm counts and motility, and testosterone levels of WT and CaMKIV/Gr KO males

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<thead>
<tr>
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<th>WT</th>
<th>KO</th>
<th>P Values</th>
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<tr>
<td>Testis weight, mg</td>
<td>139.3 ± 8.59</td>
<td>145.6 ± 10.55</td>
<td>0.656</td>
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<tr>
<td>Epididymis weight, mg</td>
<td>38.5 ± 1.57</td>
<td>42.2 ± 1.79</td>
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<tr>
<td>Seminal vesicle weight, mg</td>
<td>23.4 ± 1.25</td>
<td>20.8 ± 0.49</td>
<td>0.089</td>
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<tr>
<td>Testosterone level, ng/ml</td>
<td>3.03 ± 1.128</td>
<td>2.02 ± 0.69</td>
<td>0.493</td>
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<tr>
<td>Sperm count, 10^6/ml</td>
<td>23.50 ± 5.54</td>
<td>16.35 ± 2.85</td>
<td>0.241</td>
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<tr>
<td>Sperm motility, %</td>
<td>43.22 ± 7.32</td>
<td>46.50 ± 6.66</td>
<td>0.744</td>
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Results represent means ± SE. Tests were carried out on F2 WT and KO mice derived from matings of HET parents. Sample sizes were 5 mice (age 12 wk) per group for organ weights, 15 mice (average age 10 wk) per group for serum testosterone levels, and 8 WT and 10 KO mice for sperm counts and 9 WT and 10 KO for sperm motility (12–15 wk). Statistical analysis was carried out using two-tailed t-test. WT, wild type; KO, knockout; CaMKIV/Gr, calcium/calmodulin-dependent protein kinase type IV/Gr; HET, heterozygous.

DISCUSSION

Products of the CaMKIV/Gr gene, including CaMKIV/Gr and calsermin, are expressed in male germ cell in a developmentally regulated manner, raising the possibility of a role for these products in Ca^2+–mediated regulatory events during spermatogenesis. In this study, we have analyzed mice in which CaMKIV/Gr expression was specifically abrogated while that of calsermin was maintained. We found that CaMKIV/Gr deficiency did not compromise fetal development or early postnatal survival. Also, CaMKIV/Gr-deficient males were fertile and had normal testis weight and serum testosterone levels and normal number and motility of sperm. Testis histology and the progression of spermatogenesis appeared normal, and apoptosis of the germ cells was not increased in KO mice relative to WT controls. It thus appeared that CaMKIV/Gr deficiency did not affect spermatogenesis or male fertility.

CaMKIV/Gr has been suspected of playing a role in spermatogenesis due in part to its capacity to activate CREM-dependent transcription (23, 24). This activation may involve phosphorylation by CaMKIV/Gr of a regulatory serine residue that is conserved among CREB family members (Ser^133 of CREB, Ser^117 of CREM^1 and CREM^2) and is targeted by CaMKIV/Gr phosphorylation (6, 11, 21). It may additionally involve activation by CaMKIV/Gr of co-activator proteins such as p300/CBP binding protein (CBP) (5). Consistent with the role of CaMKIV/Gr as a CREB activator, neuronal Ser^133 CREB phosphorylation and CREB-dependent gene expression were markedly depressed in CaMKIV/Gr KO mice (9). In addition, several forms of CREB-dependent neuronal plasticity models were also impaired, including a late phase of cerebellar long-term depression and hippocampal CA1 long-term potentiation (1, 3, 9). However, we found no evidence of impaired CREM^1 function in the testis. Transcription of CREM^1-regulated genes including calsermin itself, protamine-1 and -2, and transitional protein-1 was normal. Sparing of testicular CREM^1 activation was also inferred by the finding of normal spermiogenesis in CaMKIV/Gr KO, whereas spermiogenesis is arrested in CREM^1 KO mice.

It is possible that CREM^1 function in CaMKIV/Gr KO mice is rescued by activation of other signaling pathways, including cAMP-dependent PKA and mitogen-activated protein kinases (6). Alternatively, testicular CREM^1 activation may proceed by phosphorylation-independent mechanisms involving the testis-specific CREM^1 co-activator ACT (activator of CREM in testis) (7). Unlike p300/CBP, the capacity of ACT to associate with and transactivate CREM^1 is not regulated by phosphorylation but is constitutive (7). Both CREM^1 and ACT accumulate synchronously in the adult testis and co-localize in spermatids. Hence, the sparing of CREM^1 activity in testes of CaMKIV/Gr KO mice would follow from the regulation of testicular CREM^1 function at the level of CREM^1 and ACT expression rather than by phosphorylation-induced activation.

Table 2. Fecundity of CaMKIV/Gr KO males

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<th>WT</th>
<th>KO</th>
<th>P Values</th>
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<tr>
<td>Fraction bearing 1st litter</td>
<td>6/8</td>
<td>7/8</td>
<td>1.0</td>
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<tr>
<td>Days to 1st litter</td>
<td>32.0 ± 8.67</td>
<td>35.8 ± 8.49</td>
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<tr>
<td>First litter size</td>
<td>8.00 ± 0.92</td>
<td>9.00 ± 1.21</td>
<td>0.523</td>
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<tr>
<td>Fraction bearing 2nd litter</td>
<td>6/8</td>
<td>5/8</td>
<td>1.0</td>
</tr>
<tr>
<td>Days to second litter</td>
<td>24.6 ± 1.21</td>
<td>28.4 ± 3.49</td>
<td>0.296</td>
</tr>
<tr>
<td>Second litter size</td>
<td>9.00 ± 0.84</td>
<td>12.2 ± 0.73</td>
<td>0.020</td>
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Values other than fractions are means ± SE. Matings of WT and KO males were followed up over 2 successive litters for a maximal period of 11 wk. Statistical analysis included χ²-test for fraction of females bearing litters and two-tailed t-test for other endpoints.
Recently, Wu et al. (26) have reported the generation by targeted gene disruption of CaMKIV/Gr-deficient mice. Similar to the mice reported herein, those of Wu et al. exhibited no abnormality in CREM$t^{-}$-dependent transcription; however, they suffered from impaired spermiogenesis and male sterility. The reason for the discrepant phenotypes of the CaMKIV/Gr-deficient mice of Wu et al. and those described in this report is not clear. The difference cannot be ascribed to residual CaMKIV/Gr expression in the mice utilized in this report, which is totally lacking not only in the testis but in all other tissues examined, including the brain and T-lymphocytes. The normal testicular phenotype is in contrast with the manifestation by the same mutant mice of both neuronal and lymphoid abnormalities that are consistent with CaMKIV/Gr deficiency. These include the aforementioned deficits in synaptic plasticity, CREB activation, and Ca$^{2+}$-dependent gene transcription (9) as well as impaired positive selection and Ca$^{2+}$-dependent gene transcription in developing T-cells (V. Raman and T. Chatila, unpublished results). Potential explanations for the discrepancy between the results of Wu et al. and those reported herein include differences in the embryonal stem cell clones utilized in the respective study, the gene-targeting strategies, or the mouse strains on which studies were carried out. Of note, the gene-targeting strategy of Wu et al. resulted in the deletion of the promoter sequences of both $\alpha$- and $\beta$-CaMKIV/Gr together with exons I and II. These sequences were spared in our mice. It remains to be determined whether these or other factors may have contributed to the different outcomes in the two studies.

Fig. 2. Normal progression of spermatogenesis in CaMKIV/Gr-deficient mice. The testicular histology and progression of spermatogenesis in KO mice (A) are comparable to that in WT animals (B); spermatocytes (Sp), round spermatids (rSd), and elongated spermatids (eSd) are indicated. TdT-mediated nick end labeling (TUNEL) analysis demonstrates occasional apoptotic spermatogonia (arrowheads) in KO (C) and WT (D) mice. Different stages of the seminiferous epithelial cycle are indicated by Roman numerals. Bar, 50 $\mu$m.

Fig. 3. Analysis of transcripts of CREM$t^{-}$-regulated genes in testes of WT and KO mice. Total testicular RNA of either WT (+/+; lane 1), HET (+/-; lane 2), or KO (-/-; lane 3) mice was hybridized with cDNA probes specific for transcripts of the following CREM$t^{-}$-regulated genes: protamine-1, protamine-2, and transitional protein-1. Control hybridization was performed with a cDNA probe specific for GAPDH transcripts.
Finally, our study does not rule out a latent role for CaMKIV/Gr in spermatogenesis that may be uncovered under special conditions including deficiency of other regulatory pathways implicated in spermatogenesis. Also presently unclear is the function in spermatogenesis of calsperrin, whose expression was left unaffected by our targeting strategy. Derivation of mice that suffer from combined deficiencies of CaMKIV/Gr and other regulatory pathways as well as the selective ablation of calsperrin expression by gene-targeting approaches may further clarify the function of products of the CaMKIV/Gr gene in male germ cell differentiation.

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REFERENCES