Cannabinoid Receptor 1 Influences Chromatin Remodeling in Mouse Spermatids by Affecting Content of Transition Protein 2 mRNA and Histone Displacement


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Cannabinoid Receptor 1 Influences Chromatin Remodeling in Mouse Spermatids by Affecting Content of Transition Protein 2 mRNA and Histone Displacement

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Marijuana smokers and animals treated with Δ9-tetrahydrocannabinol, the principal component of marijuana, show alterations of sperm morphology suggesting a role for cannabinoids in sperm differentiation and/or maturation. Because the cannabinoid receptor 1 (CNR1) activation appears to play a pivotal role in spermiogenesis, the developmental stage where DNA is remodeled, we hypothesized that CNR1 receptors might also influence chromatin quality in sperm. We used Cnr1 null mutant (Cnr1−/−) mice to study the possible role of endocannabinoids on sperm chromatin during spermiogenesis. We demonstrated that CNR1 activation regulated chromatin remodeling of spermatids by either increasing Tnp2 levels or enhancing histone displacement. Comparative analysis of wild-type, Cnr1+/−, and Cnr1−/− animals suggested the possible occurrence of haploinsufficiency for Tnp2 turnover control by CNR1, whereas histone displacement was disrupted to a lesser extent. Furthermore, flow cytometry analysis demonstrated that the genetic loss of Cnr1 decreased sperm chromatin quality and was associated with sperm DNA fragmentation. This damage increased during epididymal transit, from caput to cauda. Collectively, our results show that the expression/activity of CNR1 controls the physiological alterations of DNA packaging during spermiogenesis and epididymal transit. Given the deleterious effects of sperm DNA damage on male fertility, we suggest that the reproductive function of marijuana users may also be impaired by deregulation of the endogenous endocannabinoid system. (Endocrinology 151: 5017–5029, 2010)

Endocannabinoids are lipid mediators acting as endocrine (1) and/or local signals (2) whose effects are mimicked by those of exo- or recreational cannabinoids (3). Indeed, the latter bind to extracellular sites of cannabinoid receptors 1 and 2 (CNR1 and CNR2). Endocannabinoids were first isolated from brain (4) and later from peripheral tissues as well as reproductive fluids (5). The main endocannabinoids are: arachidonoylthanolamide (AEA or anandamide) and 2-arachidonyl-glycerol (2-AG). The effects of AEA and 2-AG, via CNR1 and CNR2, depend on...
their concentration in the extracellular space. This is controlled by a putative endocannabinoid membrane transporter and by the balance between synthetic and degrading enzymes. Endocannabinoids, receptors, transporters, and synthesis and degradation enzymes constitute the “endocannabinoid system” (6), recently characterized in isolated spermatozoa (sperm) (7) and testicular cells (8, 9). In sperm, it influences motility, acrosome reaction, and capacitation (7, 10–12); furthermore, it regulates Leydig cells, either development (9) or function (13), and Sertoli cell activity (14), whereas its role in male germ cell spermiogenesis and maturation is still unknown. Mouse germ cells possess the endocannabinoid system, which is modulated during meiosis. In particular, 2-AG exerts a prodifferentiative effect in isolated spermatogonial cells by inducing meiosis entrance through CNR2 receptor activation (15).

Spermiogenesis drives the differentiation of round spermatids into sperm. Round cell elongation and chromatin condensation lead to the development of elongating (stages 9–11 of spermiogenesis) and condensing spermatids (stages 12–13 of spermiogenesis), respectively. Finally, mature elongated cells carrying tightly packaged spermatids (stages 14–16 of spermiogenesis) are formed (16). In mammals, chromatin condensation is a double-step event (17–19): testis-specific histones (20) are transiently replaced first by transition proteins (TNPs; TNP1 and TNP2 encoded by Tnp1 and Tnp2 genes, respectively) and then by protamines (PRMs; PRM1 and PRM2, encoded by Prm1 and Prm2 genes, respectively). PRMs are sperm-specific, small nuclear proteins that are highly basic, being characterized by an arginine-rich core and cysteine residues. Arginine is used during spermiogenesis to induce chromatin condensation, whereas cysteine is used in the epididymis to make disulfide bonds between adjacent PRMs to condense chromatin further (17). These packaging events preserve DNA integrity indirectly by preventing stress agents from reaching the molecule (21). A direct role in maintaining DNA integrity has also been proposed for TNPs and PRMs (17). Indeed, during early spermiogenesis, a transient increase in DNA strand breaks has been reported (22, 23). These lesions are no longer detected at later stages and are probably related to nucleosome withdrawal. Because TNP1 and TNP2 stimulate DNA nick repairing activity in vitro (24, 25), a possible role for TNP1 and TNP2 in DNA repair after DNA strand breakage is hypothesized. Either TNPs or PRMs may act as “alignment factors” allowing specific enzymes to repair DNA. Therefore, inefficient expression and/or activity of TNPs/PRMs may facilitate DNA damage (26–29).

Sperm DNA damage has been closely associated with poorer outcomes of numerous indicators of reproductive health, including lower fertilization, embryo quality, and implantation, and also with spontaneous abortion (30). Not only is poor sperm DNA quality associated with each of the early fertility outcomes but, in turn, it can impact adversely on the short- and long-term health of children born by assisted reproductive technology. Children conceived by assisted reproductive technology, particularly by intracytoplasmic sperm injection, have a higher incidence of disease than children spontaneously conceived (31).

The recent introduction of lifestyle pastimes such as marijuana use has been reported to have negative effects on male fertility (32, 33), although the mechanisms are not yet elucidated. It may be through a deregulation of the endocannabinoid system, which we now know is pivotal for male reproductive efficiency. Recently, we have analyzed the expression and localization of CNR1 and fatty acid amide hydrolase (FAAH) in germ cells of frog (11) and rat (6). Both proteins are present in round and elongating/elongated spermatids, suggesting the involvement of the endocannabinoid system in spermiogenesis and maturation. The presence of CNR1 and FAAH in the same cell type suggests that the presence of FAAH may be necessary to establish the optimal endocannabinoid tone to accurately drive cellular morphogenesis of spermatids. Both marijuana smokers and animals treated with Δ9-tetrahydrocannabinol show alterations of sperm morphology (34, 35) supporting this hypothesis. Therefore, we took advantage of Cnr1-null mutant male mice to study spermiogenesis and to assess the possible role of endocannabinoids in chromatin remodeling.

Materials and Methods

**Drugs for in vivo and in vitro treatments**  
Anandamide (AEA); AM281 [1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamid], a selective CNR1 antagonist; and AM630 (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl] (4-methoxyphenyl) methanone), a selective CNR2 antagonist, were all obtained from Sigma-Aldrich (Milan, Italy) and used for in vivo treatments. Calphostin C, a protein kinase C (PKC) inhibitor, and H89, a protein kinase A (PKA) inhibitor, were purchased from Sigma-Aldrich and used for in vitro treatments. All drugs were of the purest analytical grade and were dissolved in ethanol or dimethylsulfoxide (DMSO) according to the manufacturer’s instructions.

**Experimental animals**  
Wild-type (WT; Cnr1+/+) male mice or males carrying a Cnr1-null mutation (36), either in heterozygous (Cnr1+/-) or homozygous (Cnr1-/-) condition, were used in this study. Heterozygous mice were bred on a CD1 background (Charles River Laboratories, Lecco, Italy) before generating male mice
(Cnr1<sup>+/+</sup>, Cnr1<sup>−/−</sup>, and Cnr1<sup>−/+</sup>). All animals were maintained on a standard pellet diet with free access to water.

Adult males (4–8 months) were killed by CO₂ asphyxia, and testes or epididymis were processed depending on experimental procedure. Each experimental analysis or treatment included at least three or four different animals for each genotype, and each animal was separately analyzed.

Experiments were approved by the Italian Ministry of Education and the Italian Ministry of Health. Procedures involving animal care were carried out in accordance with National Research Council’s publication Guide for Care and Use of Laboratory Animals (National Institutes of Health Guide).

**Total RNA preparation**

Total RNA was extracted from mice testes (three testes for each genotype were separately processed) using TRIZOL Reagent (Invitrogen Life Technologies, Milan, Italy) according with manufacturer’s instructions. RNA samples (10 µg) were treated with 1 µl Deoxyribonuclease (DNase, 10 U/µl) at 37 C for 10 min and further processed according to manufacturer’s instructions (GE Healthcare, Milan, Italy). Total RNA purity and integrity were determined by spectrophotometry at 260/280 nm and by electrophoresis.

**Reverse transcriptase-PCR analysis**

**cDNA synthesis**

Total RNA was reverse transcribed to prepare cDNA (three series of cDNA for each genotype). The reverse transcription was carried out using 2 µg total RNA in a final volume of 20 µl, following the manufacturer’s instructions (Invitrogen Life Technologies, Milan, Italy). As a negative control, 2 µg of total RNA was incubated in 20 µl of previous mix without reverse transcriptase enzyme (RT-cDNA sample).

**Semiquantitative PCR**

PCR was carried out as previously described (9). Each cDNA was amplified in triplicate. Primer sequences and PCR program are shown in Table 1. Finally, 25 µl PCR amplification mixtures were analyzed by agarose gel. Densitometry analysis of PCR signals was carried out by using the GELDOC1,00-UV system (Bio-Rad, Milan, Italy). The relative amount of Tnp2 mRNA was calculated by ratio Tnp2/Actin values and graphed as OD units.

**Quantitative real time-PCR (qRT-PCR)**

qRT-PCR analysis was performed according to the manufacturer’s instructions (CFX-96; Bio-Rad) in a 15-µl reaction mixture (SS0 Fast EvaGreen supermix; Bio-Rad) containing diluted cDNA (1:3). Assays were performed in triplicate, and a standard curve from consecutive 5-fold dilutions (2 µg–31 ng) of a cDNA pool representative of all samples was included for PCR efficiency determination. Relative gene expression analysis, corrected for PCR efficiency and normalized toward reference gene (ribosomal protein S27A, Rps27a, [GenBank no. BC002108]) was performed by CFX Manager software (Bio-Rad).

**Protein extraction and Western blot analysis**

**Protein extraction**

Protein extracts were prepared from mouse testis according to a method previously described (11), which was opportunely modified to swell spermatid chromatid. Mouse testis was homogenized on ice in PBS (pH 7.4) in the presence of protease inhibitors (1 g/ml of leupeptin, apro tin, pepstatin A, and chymostatin; and 5 g/ml of tosyl phenylalanyl chloromethyl ketone). The homogenate was supplemented with 10 mM dithiothreitol, incubated on ice for 15 min, further supplemented (0.02% sodium azide, 0.1% sodium dodecyl sulfate, 1% Nonidet P-40, 0.5% sodium deoxycholate), and then sonicated three times for 30-sec bursts, each at 60 mW.

**Western blot analysis**

Proteins (100 µg) were separated using 18% SDS-PAGE and transferred to polyvinylidene difluoride membrane (GE Healthcare) at 280 mA for 2.5 h at 4 C. The membrane was horizontally cut around 25 kDa, and the obtained filters were separately used to evaluate TNP2 and MAPK2 immunoreactivity. Filters were treated for 3 h with blocking solution [5% nonfat powdered milk, 0.25% Tween-20 in Tris-buffered saline (pH 7.6)] and then incubated with the primary antibody (both anti-TNP2 and -MAPK2 antibodies, diluted 1:500 or 1:1000, respectively) in PBS 3% nonfat powdered milk solution overnight at 4 C on an orbital shaker. After washing in Tris-buffered saline-0.25% Tween20, filters were incubated with 1:1000 horseradish peroxidase-conjugated IgG (Dako Corp., Milan, Italy) in PBS-1% normal swine serum (Dako Corp.) and then washed again. The immune complexes were detected using the enhanced chemiluminescence-Western blotting detection system (GE Healthcare) following the manufacturer’s instructions.

<table>
<thead>
<tr>
<th>Sequences (5’–3’)</th>
<th>Tm (C)</th>
<th>No. of cycles</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tnp1 S</td>
<td>accagcgcgaagctaaagactcat</td>
<td>56</td>
<td>30</td>
</tr>
<tr>
<td>Tnp1 AS</td>
<td>tccagcttgccatattacccactct</td>
<td>58</td>
<td>30</td>
</tr>
<tr>
<td>Tnp2 S</td>
<td>agggaaggcagcagcaagaaa</td>
<td>56</td>
<td>28</td>
</tr>
<tr>
<td>Tnp2 AS</td>
<td>tccctcagctgattgctactct</td>
<td>66</td>
<td>28</td>
</tr>
<tr>
<td>Prm1 S</td>
<td>ccaatattccacctctgctcaca</td>
<td>56</td>
<td>28</td>
</tr>
<tr>
<td>Prm1 AS</td>
<td>acggccagggttttgattgcag</td>
<td>66</td>
<td>28</td>
</tr>
<tr>
<td>Prm2 S</td>
<td>ccaggctgctgcagcaccag</td>
<td>56</td>
<td>25</td>
</tr>
<tr>
<td>Prm2 AS</td>
<td>ttcggcactttctggactgccc</td>
<td>56</td>
<td>25</td>
</tr>
<tr>
<td>Actin S</td>
<td>ctctccccagctctctcctctct</td>
<td>56</td>
<td>25</td>
</tr>
<tr>
<td>Actin AS</td>
<td>cttctctgctctgctctgctct</td>
<td>56</td>
<td>25</td>
</tr>
</tbody>
</table>

Product size indicates base pairs of the amplified fragments. S, Sense; AS, antisense; Tm, annealing temperature.
Both anti-TNP2 (sc-21106) and anti-MAPK2 (sc-154) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Milan, Italy). The latter was used to quantify protein content. Densitometry analysis of signals was carried out by using the GELDOC1,00-UV system (Bio-Rad, Hercules, CA). The relative amount of TNP2 was calculated by ratio of TNP2/MAPK2 values and graphed as OD units.

**Immunolocalization of TNP2**

Testes of Cnr1\(^{+/+}\), Cnr1\(^{+/-}\), and Cnr1\(^{-/-}\) animals were fixed in Bouin solution for 12 h and paraffin embedded. Serial sections (5 μm) were cut and processed for immunocytochemistry or hematoxylin-eosin staining.

**Immunocytochemistry**

For TNP2 immunolocalization, sections were subjected to antigen retrieval in 10 mM sodium citrate buffer (pH 6.0) and then incubated in PBS containing 0.3% Triton X-100 and 10% goat serum (PBS-Triton/serum, PBS-TS) at room temperature. Three hours later, slides were incubated with TNP2 antiserum diluted 1:100 (sc-21106; Santa Cruz Biotechnology, Inc.) in PBS-TS overnight at 4 C. Another section on the same slide was incubated only with PBS-TS as an immunohistochemical control. After three washes in PBS, sections were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Milan, Italy), diluted 1:100 (sc-21106; Santa Cruz Biotechnology, Inc.) in PBS-TS for 1 h at room temperature. Subsequently, the sections were incubated with Vectastain ABC reagents (Vector Laboratories) for 30 min at room temperature, and TNP2 was detected with 3,3‘-diaminobenzidine substrate according to the manufacturer’s protocol. Immunostained sections were used to analyze TNP2 distribution in seminiferous epithelium of Cnr1\(^{+/+}\), Cnr1\(^{+/-}\), and Cnr1\(^{-/-}\) animals. For each genotype, we analyzed three testes (six sections for testis, approximately 70 tubules for section) from three different animals, and for each section we counted: 1) number of tubules; 2) number of tubules at each of 12 stages; 3) number of TNP2 immunopositive tubules; and 4) number of TNP2 immunopositive tubules at each of 12 stages. The counts were used to analyze: 1) percentage of tubules at each of 12 stages (number of tubules at each stage/total tubules); 2) percentage of TNP2 immunopositive tubules at each of 12 stages (number of TNP2 immunopositive tubules at each stage/total tubules); and 3) frequency of TNP2 immunopositive tubules within each stage (number of TNP2 immunopositive tubules at each stage/total tubules at each stage).

**Hematoxylin eosin**

Sections were stained with hematoxylin eosin and used to identify stages of spermatogenesis. The identification was based on acrosomal development, spermatid nuclear shape, and meiotic cells (37). Stages IX, XI, and XII were identified by the shape of step 9, 10, and 11 spermatids, respectively. Stage XII was identified by the presence of meiotic cells. Stage I–VIII tubules were identified by the presence of round spermatids and acrosomal development. In particular, stage VII and VIII tubules were distinguished by the presence of condensed spermatids lining the luminal surface of the seminiferous epithelium, whereas in stages I–VI, the spermatids were not aligned but were embedded in the epithelium.

**In vivo experiments**

WT males (n = 18) of 21 days postpartum (dpp) were injected with the following: vehicle (0.5% ethanol plus 1.25% DMSO); AEA (2 mg/kg, dose for each injection); AEA (2 mg/kg, dose for each injection) with AM281 (1 mg/kg, dose for each injection); AM281 (1 mg/kg, dose for each injection) (38); AEA (2 mg/kg, dose for each injection) with AM630 (0.5 mg/kg, dose for each injection); and AM630 (0.5 mg/kg, dose for each injection) (38). Cnr1\(^{+/+}\) adult males (4–8 months; n = 12) were injected with the following: vehicle (0.5% ethanol plus 0.63% DMSO); AEA (2 mg/kg, dose for each injection); AEA (2 mg/kg, dose for each injection) with AM281 (1 mg/kg, dose for each injection); and AEA (2 mg/kg, dose for each injection) with AM630 (0.5 mg/kg, dose for each injection).

Because AEA and AM281 or AM630 were dissolved in different vehicles (ethanol and DMSO, respectively), all animals for each group received both vehicles diluted in physiological solutions to a final concentration of 0.5% ethanol plus 1.25% or 0.63% DMSO (for AM281 or AM630, respectively) to preserve control quality and avoid any effect due to the vehicle.

Intraperitoneal injections were carried out for 1 wk on alternate days. At the end of this period, animals were killed by asphyxia with CO₂. Testes were immediately dissected and used for total RNA preparation or histological analysis. Hematoxylin eosin staining was used to analyze testicular sections (30 sections/testis) of WT animals.

**In vitro experiments**

Testes of WT animals (n = 3) of 25 dpp were rapidly removed, washed, and then incubated with vehicle (0.1% ethanol plus 0.1% DMSO) with AEA alone (1 μM for 45 min). AEA (1 μM for 45 min) was also given in combination with Calphostin C (0.3 μM) or H89 (0.5 μM) for another 30 min. Each treatment was carried out at room temperature. After treatments, testes were stored at −80 C and then used for total RNA preparation.

**Sperm collection**

For the Comet assay, sperm samples from caput and cauda of epididymis (n = 3 for each genotype) were analyzed separately so each epididymal segment was isolated for sperm collection. Each segment was immersed in PBS (pH 7.6) and cut into a few pieces to let the sperm flow out from the ducts. The sperm were then filtered and processed. For aniline blue staining or cytometry analysis, sperm samples from caput (n = 3 for each genotype) of epididymis were analyzed. As previously reported (39), the genotype did not affect sperm viability.

**Aniline blue staining**

Aniline blue dye shows selective high affinity for lysine and is able to discriminate sperm characterized by lysine-rich histones from those possessing arginine/cysteine-rich protamines (40). We exploited this property to analyze the presence of histones in sperm nuclei of WT, Cnr1\(^{+/+}\), and Cnr1\(^{-/-}\) mice.

Caput sperm were dried on polylysine slides, fixed in 4% paraformaldehyde for 30 min at 4 C and stained with 5% aqueous aniline blue (Sigma-Aldrich) mixed with 4% acetic acid (pH 3.5) for 5 min (40). After washing, sperm were analyzed under light microscope (Leica CTR500; Leica Microsystems Inc., Milan, Italy) at ×100 magnification. A total of 100–200 sperm cells were evaluated. Results were reported according to staining intensities and graphed as percentage of unstained/stained on total sperm. Each sperm sample was analyzed in triplicate.
Acridine orange (AO) staining analysis

The fluorochrome AO intercalates into double-strand DNA as a monomer and fluoresces green. Conversely, when it binds to single-strand DNA, as an aggregate, a red fluorescence is observed. We took advantage of this metachromatic shift of AO fluorescence, from green (FL1-H) to red (FL3-H), to measure WT, Cnr1+/−, and Cnr1−/− spermatid chromatin quality under acid conditions (41). In particular, by cytofluorimetry we analyzed the high DNA stainability (HDS) and DNA denaturability (DD). The HDS value represents the percentage of intensely green (FL1-H > 103) fluorescing DNA/total green fluorescing DNA. Besides containing double-strand DNA, this parameter also determines the chromatin that is in an uncondensed state. In fact, uncondensed chromatin is highly accessible by the dye. DD value [red fluorescing DNA/total fluorescence (red plus green)] is an index of DNA susceptibility to acid denaturation because it reveals the percentage of single-strand DNA.

Aliquots of sperm (1 × 10⁶/100 μl) collected from caput of epididymides were suspended in 1 ml of ice-cold PBS (pH 7.4) buffer and centrifuged at 600 × g for 5 min. The pellet was resuspended in ice-cold TNE [0.01 M Tris-HCl, 0.15 M NaCl, and 1 mM EDTA (pH 7.4)] buffer and again centrifuged at 600 × g for 5 min. The pellet was then resuspended in ice-cold TNE-10% glycerol buffer (200 μl) and immediately fixed in ethanol (70% vol/vol) at 4°C for 24 h. Cytofluorimetry analysis was simultaneously carried out on WT, Cnr1+/−, and Cnr1−/− sperm. The samples were treated for 30 sec with 400 μl of a solution of 0.1% Triton X-100, 0.15 M NaCl, and 0.08 M HCl (pH 1.2). After 30 sec, 1.2 ml of staining buffer [6 μg/ml AO, 37 mM citric acid, 126 mM Na2HPO4, 1 mM disodium EDTA, 0.15 M NaCl (pH 6.0)] was admixed to the test tube and analyzed by flow cytometry. After excitation by a 488-nm wavelength light source, AO bound to a single-stranded DNA fluoresces green (515–530 nm), and AO bound to a double-stranded DNA fluoresces red (630 nm or greater). A minimum of 10,000 cells were analyzed by fluorescent activated cell sorting (FACSCalibur; BD BioScience, Milan, Italy).

Comet assay

The Comet assay (42) is a highly reproducible method (43). It is the only technique that allows the measurement of DNA damage in individual cells. Therefore, it is particularly useful in a heterogeneous population like sperm.

Ten microliters of sperm samples (three different sperm samples for each genotype) were mixed with 75 μl of 0.5% low melting point agarose at 37°C and spread on slides (6 × 104 cells) coated with agarose gel. The slides were immersed in fresh lysatesolution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris (pH 10)] plus 1% Triton X-100 for 1 h at 4°C. Subsequently, 10 mM dithiothreitol was added for 30 min at 4°C. Afterward, the slides were washed, drained, and treated with fresh alkaline-photoreactivation solution (300 mM NaOH, 1 mM EDTA) for 20 min, allowing the exposed DNA to unwind. Photoreactivation was carried out for 10 min at 25°C, with the current adjusted to 300 mA. Then, slides were removed from the tank, drained, and flooded with three changes of neutralization buffer [0.4 M Tris (pH 7.5)], removing any residual alkali or detergents. Slides were stained with 50 μl of 20 mg/ml ethidium bromide and immediately analyzed using a Nikon E600 epifluorescence microscope (Nikon, Tokyo, Japan) equipped with an excitation filter of 515–560 nm from a 100-W mercury lamp and a barrier filter of 390 nm.

In the Comet assay, on electrophoresis of each spermatozoa, fragmented DNA migrates out of the sperm nucleus to form a tail in the direction of the anode giving the damaged spermatozoa the appearance of a comet. The head of the comet is formed by the unfragmented DNA remaining in the nucleus, whereas the tail is composed of fragmented (damaged) DNA.

DNA fragmentation was determined for each spermatozoa using the percentage DNA in the Comet tail as calculated using an image analysis system (Komet 5.5; Kinetic Imaging, London, UK). Values were then reported as the mean percentage of DNA damage in 100 spermatozoa per slide, performed in triplicate.

Statistical analysis

Student’s t test or ANOVA followed by Duncan’s test for multigroup comparison was performed where appropriate. Data were expressed as the mean ± SEM from at least three or four independent experimental procedures.

Results

Gene expression analysis of Tnp1/2 and Prm1/2

The Tnp1/2 and Prm1/2 gene expression was evaluated in the adult testis of WT, Cnr1+/−, and Cnr1−/− animals through semiquantitative PCR analysis. Figure 1A shows the presence of Tnp1/2 and Prm1/2 in all samples. Quantitative densitometry analysis of signals did not reveal measurable differences except for Tnp2 transcript (Fig. 1B), which decreased by approximately 50% in both Cnr1−/− and Cnr1+/− testes (P < 0.01) as compared with WT.

Analysis of TNP2

TNP2 was analyzed in the adult testis of WT, Cnr1+/−, and Cnr1−/− animals. Western blot analysis revealed the protein in all samples (Fig. 2A). Quantitative densitometry analysis of signals revealed low levels of TNP2 in both Cnr1+/− and Cnr1−/− (P < 0.01), as compared with WT animals.

Immunocytochemistry showed TNP2 in seminiferous epithelium of WT, Cnr1+/−, and Cnr1−/− testis at stages X–XII and I–IV (Fig. 2B). Indeed, independently of the genotype, the protein first appeared in the nuclei of step 10 spermatids (stage X), was retained between steps 11 and 15 (stages XI–XII and I–IV), and then completely disappeared from late step 15 (stage V) onward. The number of total and TNP2-stained tubules at each stage was assessed, and results were summarized in Table 2. The percentage of tubules at each of 12 stages did not show any significant variation among animal groups (Table 2, column A). The percentage of TNP2-stained tubules/total tubules significantly (P < 0.01) decreased in Cnr1−/− (34.6%) and Cnr1+/− (32.6%), as compared with WT (44.4%). In particular, a low percentage (P < 0.01) of stained tubules at steps 14 (stages II–III) and 15 (stage IV) spermatids (Table 2, column B) was observed. Furthermore, within step 14...
and 15 spermatids, the frequency of TNP2-stained tubules/total tubules at step 14 or 15 strongly (P < 0.01) decreased (Table 2, column C).

In vivo and in vitro AEA treatments

In vivo treatments

We stimulated CNR activity by treating WT animals with AEA alone or in combination with the selective CNR1 and CNR2 antagonists, AM281 and AM630, respectively. The pharmacological treatment was performed on 21 dpp mice according to the presence of round spermatids (44). Treatment was halted 7 d later (28 dpp) because spermatid elongation was completed in the first wave in most tubules. Specifically, we used 21–28 dpp treated animals because transcription of Tnp2 gene starts at stage 7 round spermatids at 21 dpp (29); afterward, as spermiogenesis progresses, Tnp2 mRNA is stored for some days, in translationally inert ribonucleoprotein particles. Its translation, beginning as early as stage 9, around 23 dpp, is completed by d 29 (29, 45). Tnp2 mRNA degradation occurs later, after translation. Thus, this precise window is necessary to analyze the regulation of TNP2 expression at the mRNA level accurately.

Results demonstrated Tnp2 presence in all experimental groups (Fig. 3A). Interestingly, AEA treatment significantly increased Tnp2 mRNA levels (P < 0.01) in comparison with other groups. The effect was completely counteracted by AM281 but not by AM630, thus indicating the exclusive CNR1 involvement in Tnp2 mRNA regulation. Moreover, AM281 and AM630, when used alone, did not affect Tnp2 levels (Fig. 3A).

Due to the above results, testes of control and AEA ± AM281 treated animals were examined by hematoxylin-eosin staining. Histological analysis was carried out to check whether the timing was properly chosen. As expected, in 28-dpp testis (Fig. 3B), round, elongating, and condensing spermatids appeared in control animals. Furthermore, the same picture appeared in the testis of all treated animals, indicating that the treatment did not induce any alteration of gametogenesis progression (44).

We stimulated CNR1 and CNR2 activity by treating Cnr1+/−/− animals with AEA alone or in combination with the selective CNR1 and CNR2 antagonists, AM281 and AM630, respectively.

Moreover, AEA treatment in Cnr1+/−/− significantly increased Tnp2 mRNA levels (P < 0.01) to WT levels. This effect was completely counteracted by AM281 but not by AM630, thus confirming the exclusive CNR1 involvement in Tnp2 mRNA regulation (Fig. 3C). Noteworthy, qRT-PCR data demonstrated that Tnp2 mRNA levels in Cnr1+/−/− mice were significantly (P < 0.01) lower in comparison with WT animals, validating the semiquantitative data.

In vitro treatments

qRT-PCR analysis, carried out on 25-dpp WT testis, treated with AEA ± PKA/PKC inhibitors, showed that AEA significantly increased Tnp2 mRNA levels (P < 0.01) in comparison with control group. This effect was efficiently counteracted by Calphostin C and H89, PKC and PKA inhibitors, respectively. In particular, these last treatments abolished AEA effect carrying back Tnp2 mRNA levels to control levels (Fig. 3D).

Histone retention analysis

Aniline blue staining was used to analyze histone presence in sperm nuclei of WT, Cnr1+/−/−, and Cnr1−/− mice. Three classes of stained heads were observed: white (unstained), light blue (poorly stained), and dark blue
(strongly stained). We reasoned that light and dark staining were an index of the amount of retained histone.

Figure 4A shows that the percentage of white sperm was significantly higher in WT than in Cnr1\(^{+/−}\) and Cnr1\(^{−/−}\) mice (\(P < 0.01\)). Conversely, the percentage of dark blue sperm (cells with high histone retention) was higher (\(P < 0.01\)) in Cnr1\(^{−/−}\) (51.66 ± 1.47) compared with Cnr1\(^{+/−}\) (14 ± 0.71) and WT (8.66 ± 0.41) animals. Additionally, the percentage of light blue sperm (cells with low histone retention) was similar in Cnr1\(^{+/−}\) (30.33 ± 1.47) and Cnr1\(^{−/−}\) (31.33 ± 0.82) mice, but lower values were detected in WT animals (19 ± 0.71; \(P < 0.01\)).

**Evaluation of sperm chromatin quality**

We used metachromatic shift of AO fluorescence, from green to red, to measure chromatin quality of WT, Cnr1\(^{+/−}\), and Cnr1\(^{−/−}\) sperm by flow cytometry. In particular, we analyzed HDS and DD values as indices of

**TABLE 2. Percentages of tubules for each stage of seminiferous epithelium**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Step</th>
<th>Cnr1(^{+/−})</th>
<th>Cnr1(^{+/−})</th>
<th>Cnr1(^{−/−})</th>
<th>Cnr1(^{+/−})</th>
<th>Cnr1(^{+/−})</th>
<th>Cnr1(^{−/−})</th>
<th>Cnr1(^{+/−})</th>
<th>Cnr1(^{+/−})</th>
<th>Cnr1(^{−/−})</th>
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<tbody>
<tr>
<td>IX</td>
<td>9</td>
<td>8</td>
<td>8.2</td>
<td>8.5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>X</td>
<td>10</td>
<td>3.8</td>
<td>4.6</td>
<td>4.5</td>
<td>1.8</td>
<td>2.1</td>
<td>2</td>
<td>7.5</td>
<td>6.7</td>
<td>7.1</td>
</tr>
<tr>
<td>XI</td>
<td>11</td>
<td>9.5</td>
<td>8.8</td>
<td>9.6</td>
<td>7.5</td>
<td>6.7</td>
<td>7.1</td>
<td>11</td>
<td>10.3</td>
<td>10.2</td>
</tr>
<tr>
<td>XII</td>
<td>12</td>
<td>11.1</td>
<td>10.4</td>
<td>10.2</td>
<td>12.2</td>
<td>11.6</td>
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</tr>
<tr>
<td>I</td>
<td>13</td>
<td>12.1</td>
<td>11.6</td>
<td>11.3</td>
<td>12.2</td>
<td>11.6</td>
<td>9.4</td>
<td>11</td>
<td>10.3</td>
<td>10.2</td>
</tr>
<tr>
<td>II–III</td>
<td>14</td>
<td>7.8</td>
<td>8.2</td>
<td>7.6</td>
<td>7.8</td>
<td>2.4(^a)</td>
<td>2.5(^a)</td>
<td>4.3</td>
<td>1.5(^a)</td>
<td>1.4(^a)</td>
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<td>9.3</td>
<td>9.5</td>
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<td>4.3</td>
<td>1.5(^a)</td>
<td>1.4(^a)</td>
<td>46</td>
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<td>0</td>
<td>79.3</td>
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<tr>
<td>VII</td>
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<td>0</td>
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<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>VIII</td>
<td>16</td>
<td>6.8</td>
<td>6.7</td>
<td>6.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Total</td>
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<td>34.6</td>
<td>32.6</td>
<td></td>
<td></td>
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</tbody>
</table>

Column A, Percentage of tubules at each of 12 stages (number of tubules at each stage/total tubules). Column B, Percentage of TNP2 immunopositive tubules at each of 12 stages (number of TNP2 immunopositive tubules at each stage/total tubules). Column C, Frequency of TNP2 immunopositive tubules within each stage (number of TNP2 immunopositive tubules at each stage/total tubules at each stage).

\(^a\) Significance as compared with WT (\(P < 0.01\)).
uncondensed chromatin and DNA susceptibility to acid denaturation, respectively.

Figure 4B shows histograms of green (FL1-H) and red (FL3-H) stained sperm in the gated area (M1). HDS (Fig. 4C) and DD (Fig. 4D) values were significantly higher in \textit{Cnr1}\(^+/−\)/\textit{H11002}/\textit{H11002}\((P < 0.01) as compared with either WT or \textit{Cnr1}\(^+/−\)/\textit{H11001}/\textit{H11002}. Surprisingly, chromatin quality of \textit{Cnr1}\(^+/−\)/\textit{H11001}/\textit{H11001} and \textit{Cnr1}\(^+/−\)/\textit{H11001}/\textit{H11002} sperm did not show any significant difference.

Because DNA susceptibility to acid denaturation indirectly suggested possible breaks in the DNA helix, we analyzed sperm DNA integrity by the Comet assay. In particular, using an alkaline pH method (46, 47), we measured both single and double DNA strand breaks. The assay was carried out on sperm collected from \textit{caput} and \textit{cauda} epididymis of \textit{Cnr1}\(^+/−\)/\textit{H11001}/\textit{H11001}, \textit{Cnr1}\(^+/−\)/\textit{H11001}/\textit{H11002}, and \textit{Cnr1}\(^+/−\)/\textit{H11002} mice (Table 3). In the \textit{caput}, the percentage of damaged DNA was significantly higher in \textit{Cnr1}\(^+/−\)/\textit{H11002}\((5.56 ± 0.95\%) as compared with \textit{Cnr1}\(^+/−\)/\textit{H11001}/\textit{H11001}(4.6 ± 0.84\%; P < 0.01). Conversely, the percentage of damaged DNA in \textit{Cnr1}\(^+/−\)/\textit{H11002}\(4.76 ± 1.12\% was not different from WT sperm. Interestingly, the Comet assay (42) is recognized to be more sensitive than other DNA damage tests (48, 49), so baselines of damage in control sperm were higher than those reported by studies employing these other assays. Similarly, in the \textit{cauda}, a significant difference \((P < 0.001) was once again detected in \textit{Cnr1}\(^+/−\)/\textit{H11002}\(27.80 ± 4.01\%) in comparison with \textit{Cnr1}\(^+/−\)/\textit{H11001}\((17.12 ± 4.02\%) was higher but not significantly different from that of WT sperm \((15.16 ± 4.0\%). Furthermore, DNA damage increased markedly as sperm from all groups traveled through the epididymis from \textit{caput} to \textit{cauda}.

**Evaluation of \textit{Cnr1}\(^+/−\) male mice fertility**

Fertility of \textit{Cnr1}\(^+/−\) males \((n = 12) was evaluated by inducing mating with WT females \((n = 13). Control mating was carried out by crossing WT males \((n = 11) with females \((n = 14). Vaginal plug formation was checked the
next morning as an indication of successful mating. Pregnancies indicated that Cnr1/- males were fertile (Table 4). Statistical analysis of litter size indicated no substantial differences in the ability of WT (13 ± 1.54) and Cnr1/- (13 ± 1.57) males to generate offspring.

**Discussion**

CNR1 and CNR2 both immunolocalize in round spermatids during differentiation into mature elongated cells (7, 9, 11, 15). Here we show that Cnr1 gene null mutation interfered with spermiogenesis.

We used WT, Cnr1+/−, and Cnr1−/− mice to analyze the main events reported to be upstream and downstream of chromatin remodeling (Tnp1/2 and Prm1/2 mRNA expression, histone displacement, DNA protection) occurring in spermatids (18). Gene expression analysis revealed no differences of Tnp1, Prm1, and Prm2 in whole testis; whereas Tnp2 mRNA levels were 50% lower in Cnr1+/− and Cnr1−/− compared with WT animals. Accordingly, TNP2 protein levels, analyzed by Western blot, decreased. In addition, the percentage of TNP2-immunopositive tubules/total tubules as well the frequency of TNP2-immunopositive tubules within II–IV stages decreased in both Cnr1+/− and Cnr1−/− mice. This suggested an early disappearance of the protein. Therefore, in both homozygote and heterozygote, Cnr1 mutation affected Tnp2 levels, either mRNA or protein, hinting CNR1 as important fac-

**FIG. 4.** Analysis of Cnr1+/−, Cnr1+/−, and Cnr1−/− sperm. A, Through aniline blue staining, three classes of stained sperm head were observed and reported as percentage of white and light and dark blue. B, Flow cytometry analysis of sperm stained with OA. Representative histograms of AO-stained sperm in M1 gate. Intensely green (FL1-H > 10^3) fluorescing DNA and total (green + red) fluorescing DNA were used to analyze. C, HDS (used as index of uncondensed chromatin) values. D, DD (used as index of DNA susceptibility to acid denaturation) values. Graphs were representative of three sperm samples relative to separate animals (three animals for genotype). Data were expressed as the mean ± SEM (different letters are statistically significant at P < 0.01). SPZ, Spermatozoa.
tor to sustain Tnp2 levels. Accordingly, we found that both WT and Cnr1+/+ animals injected with AEA alone showed increased Tnp2 mRNA levels, whereas the selective CNR1 antagonist, AM281, counteracted AEA effect. The selective CNR2 antagonist, AM630, was ineffective, excluding CNR2 involvement. In vitro experiments clearly demonstrated PKA and PKC involvement in AEA/CNR1 signaling. Such findings demonstrated CNR1-dependent Tnp2 turnover and strongly suggested endocannabinoid activity on spermatids because CNR1 protein (9) and Tnp2 mRNA (29) both make their debut in these cells at round stage. Interestingly, Tnp2 mRNA storage occurs for some days in spermatids during elongation (29). In particular, the mRNA is preserved in translationally inert ribonucleoprotein particles; afterward, it is translated in elongating-condensing spermatids (stages 10–15 of spermiogenesis) and then degraded, after translation (45, 50). Because our results indicated that AEA increased Tnp2 mRNA, the endocannabinoid could have affected mRNA levels by regulating either transcription or stability.

Interestingly, spermatids produce, degrade, and bind AEA (15). Furthermore, AEA regulates transcriptional activity inducing DNA methylation during keratinocyte differentiation (51). Therefore, chronic exposure to high AEA levels or exocannabinoids may be detrimental for male fertility because temporal and stage-specific appearance of TNP2 is regulated by Tnp2 mRNA (45) and it is prerequisite for the correct differentiation of round spermatids into mature and motile sperm with fertilizing capability (52). In agreement, Sun et al. (53) have reported that high AEA levels in Faah−/− mice reduced motility and the fertilizing capability of sperm.

Previous reports on Tnp2−/− mice indicated that TNP1/2 partially complement each other. Therefore, TNP2 absence will not necessarily affect the levels of Tnp1 or Prm1/2 transcripts (28). Our results confirmed that Tnp1 and Prm1/2 mRNA levels did not change, although Tnp2 levels, either mRNA or protein, were decreased in Cnr1−/− animals. Moreover, similarly to WT, Cnr1−/− mice showed heterogeneous sperm population because of variable histone content. Indeed, we identified white and light and dark blue cells. Although the percentage of white sperm was lower as compared with those of WT, their detection indicated that in the presence of low Tnp2 levels, histone displacement still occurred in some sperm. Our data are in agreement with Zhao’s results showing that Tnp2 mutation, either Tnp2++/− or Tnp2−/−, did not affect histone displacement (28). Collectively, these results may suggest that histone displacement was independent of Tnp2 expression. Accordingly, it has also been proposed that the displacement starts before initiation of TNP accumulation in the nucleus (27). However, TNP2 absence in the majority of Cnr1+/− and Cnr1−/− tubules at stages 14 and 15 may alternatively suggest that blue sperm are generated in the absence of TNP2.

Cnr1−/− germ cells exhibited greater histone retention than the WT group. Indeed, the percentage of light and dark blue sperm (cells with low and high histone retention, respectively) was higher in Cnr1−/− than in WT mice. Therefore, CNR1 loss caused a double alteration on spermiogenesis: it decreased Tnp2 levels and increased the percentage of sperm retaining histones. These alterations confirmed again that CNR1 activity regulated Tnp2 expression and histone displacement/retention. Furthermore, in Cnr1+/− animals, data on Tnp2 levels did not match histone retention (number of aniline blue-stained sperm). In fact, Tnp2 mRNA levels were 50% lower in either Cnr1+/− or Cnr1−/− compared with WT testes, whereas the percentage of dark blue sperm (cells with high histone retention) was lower in Cnr11+/− than in Cnr1−/−, and the former

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% of DNA damage (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caput</td>
<td></td>
</tr>
<tr>
<td>Cnr1+/+</td>
<td>4.61 ± 0.84a</td>
</tr>
<tr>
<td>Cnr1−/−</td>
<td>4.76 ± 1.12a</td>
</tr>
<tr>
<td>Cnr1+/−</td>
<td>5.56 ± 0.95b</td>
</tr>
<tr>
<td>Cauda</td>
<td></td>
</tr>
<tr>
<td>Cnr1+/+</td>
<td>15.16 ± 4.01a²</td>
</tr>
<tr>
<td>Cnr1−/−</td>
<td>17.12 ± 4.02a²</td>
</tr>
<tr>
<td>Cnr1+/−</td>
<td>27.80 ± 4.01a²</td>
</tr>
</tbody>
</table>

Data are representative of three separate experiments and are expressed as mean values of percentage of DNA damage ± SEM (a vs. a′, P < 0.01; a vs. b, P < 0.01; b vs. b′, P < 0.01; a′ vs. b′, P < 0.001).

### Table 4. Reproductive performance of Cnr1−/− male mice

<table>
<thead>
<tr>
<th></th>
<th>WT females (n)</th>
<th>Mating events (n)</th>
<th>Gestating females (n)</th>
<th>Litter size (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cnr1+/+</td>
<td>11</td>
<td>14</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>Cnr1−/−</td>
<td>12</td>
<td>13</td>
<td>14</td>
<td>10</td>
</tr>
</tbody>
</table>

Total number of animals (male and female), mating events, and gestating females are reported. Litter size values are reported as number of pups per gestating female.
was closer to WT values. However, no difference between Cnr1+/− and Cnr1−/− was found when the light blue sperm were considered. These results demonstrated that, in Cnr1−/− mice, Tnp2 levels were identical to those of Cnr1+/−, whereas histone displacement/retention was close to normal. Haploinsufficiency (54) in spermatids might explain the Tnp2 picture in Cnr1+/− mice: spermatids are genetically haploid (50% WT and 50% knockout) and phenotypically diploid; they develop in syncytial clones, share intracellular signals, mRNA, and small proteins through cytoplasmic bridges (55). Therefore, in Cnr1+/− animals, CNR1 signal transduction could spread to either normal or null spermatid and be inefficient to sustain Tnp2 transcripts. In contrast, histone displacement/retention was affected to a lesser extent.

During spermiogenesis, histone displacement is followed by replacement of proteins whose role is to bind and condense DNA to protect its integrity (56). Consequently, disrupted histone displacement would impair chromatin packaging and therefore afford less defense against DNA assault. Our results, obtained using AO staining (revealing HDS and DD values, indicative of uncondensed chromatin and high DNA susceptibility to acid denaturation, respectively) and the Comet assay (revealing DNA fragmentation), supported our hypothesis. In fact, all values were significantly higher in Cnr1−/− than in WT sperm. Unexpectedly, chromatin quality and DNA integrity of Cnr1+/− and WT sperm were similar. Perhaps histone displacement in Cnr1+/− mice, although partially disrupted as compared with WT animals, was still sufficient to promote condensation to protect DNA in white and light blue sperm. Therefore, it was conceivable that DNA quality data (HDS and DD) of the whole sperm sample masked the slight contribution of blue cell population. According to a previous report (57), we concluded that DNA damage was primarily linked to disrupted histone displacement rather than 50% lower Tnp2 levels. Indeed, chromatin remodeling appeared disrupted to a greater extent in Cnr1−/− compared with Tnp2+/− mice. An additional effect of CNR1 loss may be envisaged in germ cells. Anyway, a direct role of TNP2 in DNA quality preservation cannot be excluded. Indeed, TNP2 controls DNA integrity by affecting 1) DNA nick repairing activity (58); and 2) ultimate degree of chromatin condensation (59), with the former event accomplished during spermiogenesis, whereas the latter occurs in the epididymis. Here, a tightly compacted protamine structure is required to stabilize and protect DNA (21) by nucleases (60) or reactive oxygen species (49). In all examined groups collected from the epididymis, sperm DNA was damaged strikingly between caput and cauda suggesting inadequate protaminization to protect DNA fully even in the WT mice. The vulnerability of epididymal sperm was supported by studies showing that DNA damage was lowest in testicular sperm, increasing in caudal epididymal and ejaculated sperm (30, 61–64). Defective sperm showed an increased susceptibility to DNA damage during epididymal passage (65). Interestingly, we found that Cnr1−/− sperm, carrying an abnormal chromatin, showed increased DNA damage in caput compared with cauda.

To determine the effects of such damage on male fertility, we examined the litter size produced by Cnr1−/− males. Surprisingly, no substantial difference was found in the ability of WT and Cnr1−/− males to produce offspring. This suggested that 27% (mean value) of sperm DNA damage did not compromise male fertility. Of course, because Cnr1−/− mice produced sperm with heterogeneous histone content (white and light and dark blue sperm), we cannot comment on which sperm reached and fertilized the oocytes. However, the loss of CNR1 did not impair fertility. This conclusion was supported by results (53) showing that the compromised fertility of Faah−/− sperm (probably due to high AEA level exposure) was rescued in double null mutation Faah−/− / Cnr1−/− animals. Therefore, sperm populations of Cnr1−/− animals, on the whole, retained their fertilizing capability.

Endocannabinoids act on Leydig cells regulating testosterone production via CNR1; Cnr1−/− mice have low testosterone levels (9, 13). We show that CNR1 activity influenced spermiogenesis. It might occur by direct action of endocannabinoids on spermatids or, indirectly, via Sertoli or Leydig cells. Interestingly, CNR1 first appears in testicular tubules during spermiogenesis in both spermatids and Sertoli cells (9). Although we cannot exclude Sertoli and/or Leydig cell influence, we suggest that testosterone withdrawal cannot be responsible for these defects (66). Indeed, the number of spermatozoa and epididymal epithelium morphology, although they are both dependent on testosterone (67), are not affected by loss of CNR1 (39).

It is important to note that, although the Cnr1−/− mouse is a powerful model to investigate the relationship between endocannabinoids and sperm maturation, mouse model does not reflect the much poorer quality and greater vulnerability of human sperm. Even the “fertile” human male has higher basal levels of sperm DNA damage and lower conception, implantation, and fecundity rates (30). Thus, additional alterations in chromatin remodeling and further sperm DNA damage caused by endocannabinoid dysfunction or recreational exocannabinoid use have much greater potential to affect human male reproductive capacity adversely.

In conclusion, our results indicated that CNR1 signaling may be required for full Tnp2 expression, ensuring
proper spermatid chromatin maturation. In particular, we have shown for the first time that CNR1 plays a dual role in spermatid maturation through the regulation of both Tnp2 levels and histone displacement that in turn impacts on sperm DNA quality. New perspectives about endocannabinoid involvement in chromatin remodeling may be envisaged.

Acknowledgments

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