Expression and distribution of GPR55 and GPR119 during the development of rat testis

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ABSTRACT

G Protein-Coupled Receptors, GPR55 and GPR119 are widely distributed throughout the body and exert important biological functions. However, little is known about their roles in testis. This study aimed to examine the expression and distribution of GPR55 and GPR119 during the development of the rat testis. Sixty male Sprague Dawley rats (180–240 g) were divided into 10 groups as 7, 14, 21, 28, 35, 42, 49, 56, 63, and 70 postnatal days of age (PND) (six animals per group). The testicular expression of GPR55 and GPR119 has been investigated by immunohistochemistry, Western blot, and quantitative RT-PCR methods. We observed that GPR55 and GPR119 are expressed throughout the rat testis development from PND 7 to 70. However, no difference was observed between the groups in terms of expression levels, except for GPR55 mRNA expression in the group of PND 7. Immunohistochemistry analysis showed that GPR55 is expressed in spermatids and spermatocytes in the mid-term tubules and spermatocytes in the late-stage tubules in groups of PND 56, 63, and 70. For GPR119, very intense positivity was observed only in spermatids in the mid-term (stage VII-VIII) tubules in the groups of PND 56, 63, and 70. No significant difference was observed in the number of GPR55 and GPR119 positive cells in testes from PND 56 through PND 70. Taken together, both GPR55 and GPR119 receptors are expressed throughout the rat testis development (PND 7 to 70). These results suggest that GPR55 and GPR119 are involved in the modulation of male reproductive function.

Introduction

Endocannabinoid system (ECS) consists of two established cannabinoid receptors, cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2) and some putative cannabinoid receptors including GPR55 and GPR119. Despite the low structural similarity to CB1 and CB2 receptors, the pharmacology of GPR55 and GPR119 displays an overlap with the established cannabinoid receptors (24). A wide variety of ligands, including phytocannabinoids and endocannabinoids, activate these established cannabinoid receptors, several of which activate putative cannabinoid receptors. GPR55 can be activated by cannabinoids such as Δ^9-Tetrahydrocannabinol (THC) and cannabidiol, endocannabinoids including N-Arachidonoylethanolamine (anandamide, AEA) and 2-Arachidonoylglycerol (2-AG), and endocannabinoid-like compounds including N-Palmitoylethanolamine (PEA) and N-Oleoylethanolamine (OEA) (31). GPR119 can be activated by several compounds including AEA, OEA and N-oleoyldopamine (13, 21). Numerous selective agonists and antagonists for GPR55 and GPR119 have also been synthesized and characterized for the targeted induction or inhibition of these receptors for research and therapy purposes (39). GPR55 is coupled to $G\alpha_{12/13}$ and $G\alpha_q$ proteins and mediates the activation of ras homolog gene family member A (RhoA) and Rho-associated protein kinase (ROCK). Then, ROCK can either stimulate phospholipase C (PLC) activity which leads to increased

intracellular Ca^{2+}, or activates mitogen-activated protein kinases (MAPKs) such as extracellular signal- regulated kinase (ERK) which regulates different transcription factors (18, 28, 40, 48). GPR55 expression has been observed in numerous tissues/organ systems, including the brain, lung, spleen, placenta, white adipose tissue, adrenal glands, gastrointestinal tract, liver, uterus, bladder, kidney, prostate, bones, testes and others (23, 27, 35, 37, 42, 47). GPR55 has been implicated in a wide range of pathophysiological conditions, including motor coordination, neural development (6), nervous system disorders (32), vascular function (19), platelet and endothelial cell function (25), bone metabolism (47), angiogenesis (50), immunity (7), cancer development (4), inflammatory and neuropathic pain (44).

GPR119 is coupled to the signal transducer Gαs whose activation is followed by adenylate cyclase action and results in a rise in intracellular cyclic adenosine monophosphate (cAMP) levels (20). GPR119 is primarily expressed in gastrointestinal tract (duodenum, stomach, jejunum, ileum and colon) (8, 34), and pancreatic β-cells (41). GPR119 expression has also been reported in testes, brain, skeletal muscles, cardiac muscles and liver (43). GPR119 has been associated with pathophysiological conditions including obesity (12), diabetes mellitus (36), cancer (22) and metabolic dysfunction associated with fatty liver disease (51). The wide distribution of GPR55 and GPR119 throughout the body suggests that they might be involved in diverse biological functions. Although GPR55 and GP119 expressions have been observed in normal rat testes (49), very little is known about their physiological roles in testes. THC and AEA, ligands for cannabinoid receptors, have previously been reported to reduce the plasma luteinizing hormone (LH) levels in male rats and mice, respectively (33, 46), which was accompanied by a fall in serum levels of testosterone produced by Leydig cells in the presence of LH in testes. Moreover, chronic exposure to THC caused a weight reduction in prostate, seminal vesicles and epididymis and the impairment of spermatogenesis in rats (11, 29). These findings suggest that induction of GPR55 and GPR119-mediated signaling may have a role in testis development. However, post-natal development of GPR55 and GPR119 expression in testis is unknown. This study aimed to examine the expression and distribution of GPR55 and GPR119 at the transcript (mRNA) and protein levels during the development of rat testes.

Materials and Methods

Animals: Male Sprague Dawley rats (180–240 g) ranging from postnatal day (PND) 7 to 70 were used in this study (Experimental Animals Production and Experimental Research Center). All animals were kept under a 12 h light/dark cycle at a constant temperature and humidity, with free access to food and water. All animal procedures were performed according to a protocol approved by Animal Experiments Local Ethics Board in Burdur Mehmet Akif Ersoy University (13.03.2019/501). Sixty male Wistar rats were divided into 10 groups of 7, 14, 21, 28, 35, 42, 49, 56, 63 and 70 days of age (six animals per group). The rats were randomly assigned to each group and did not receive any treatment regimen. Prior to necropsy, clinical observations were recorded for each animal. Animals were euthanized by decapitation without anesthesia at PND 7 through PND 28 and by ketamine/xylazine injection at PND 35 and above. Testis tissues were removed and either fixed in buffered-formalin for histopathology and immunohistochemistry (right testis), or rapidly frozen in liquid nitrogen for Western blot and qRT-PCR analysis (left testis).

***Histopathology and Immunohistochemistry*:** For the histopathological examination, testicular tissues taken from each animal were fixed in Modified Davidson's solution for 24 h, then passed through graded alcohol series and embedded in paraffin after routine histological procedures. Then, 4-5 µm thick sections were taken from paraffin blocks with microtome (Leica RM 2125). The sections were deparaffinized in xylol (Xylene - Merck Millipore:108661), then dehydrated in alcohol series, and stained with Hematoxylin & Eosin. Finally, the tissue sections were examined under a light microscope for the presence of any lesions.

For immunohistochemical staining, 4-5 µm-thick sections were mounted on poly-L-lysine-coated slides (Menzel Polysine Slides: J2800AMNZ) and the sections were deparaffinized in xylol and dehydrated in alcohol series, then antigen retrieval was performed by autoclaving (15 min, 120 °C) in citrate buffer (pH 6). After a 20-30 min cooling step, slides were rinsed in phosphate-buffered saline (PBS) solution for 5 min. After antigen retrieval, staining was continued with ImmPRESS Excel Amplified HRP Polymer Staining Kit (Anti-Rabbit IgG) according to the manufacturer's instructions. Briefly, the tissue sections were incubated in BLOXALL block solution for 10 min to abolish endogenous peroxidase activity, followed by a 5 min wash with PBS. The sections were then incubated with normal horse serum (2.5%) for 20 min and excess serum was removed. Next, the slides were incubated overnight with either GPR55 (1:500 dilution, ab203663, Abcam) or GPR119 (1:250 dilution, NBP2-47661, Novus) antibodies at 4 °C. After incubation with primary antibodies, the slides were washed with PBS for 5 min and incubated with amplifier antibody solution. Following a 5-min-PBS wash, ImmPRESS Polymer Reagent was applied to the slides. After two PBS washes,

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slides were incubated with ImmPACT DAB EqV working solution and the staining status was observed under a light microscope. After staining was observed in the positive control, the slides were washed first with PBS, then with tap water. For counterstaining, the slides were kept in Mayer's hematoxylin stain (Merck) for 25 sec and then submerged in water. After air drying, the slides were covered using entellan, examined under a light microscope and images were captured during the evaluations.

Western Blot Analysis: Freshly collected tissue samples were immediately frozen in liquid nitrogen and stored at -80 °C until western blot was performed. The testicular tissue samples for each group were weighed and homogenized (TissueLyser LT, Qiagen) in radioimmunoprecipitation assay (RIPA) buffer (sc-24948, Santa Cruz Biotechnology). Tissue homogenates were centrifuged at 13,000 x g for 15 min at 4 °C and the resulting supernatants were transferred to tubes. Total protein concentration was measured by BCA assay (9300A, Takara). For each sample, 25 µg total protein extract was resolved using SDS-PAGE with 10% polyacrylamide gel and then protein bands were transferred to a polyvinylidene fluoride (PVDF) membrane (Immun-Blot PVDF, IPVH00010, Merck Millipore). After blocking the membrane with 5% skimmed milk for 2 h at room temperature (RT), the blots were incubated with primary antibodies for GPR55 (1:200, ab203663, Abcam), GPR119 (1:500, NBP2-47661, Novusbio), and β-actin monoclonal antibody (1:5000, 66009-1-Ig, Proteintech) overnight at 4 °C. The next day, the blots were washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit IgG: ab205718 and goat anti-mouse IgG: ab205719, 1:10,000 dilution, Abcam) for 1 h at RT, and then washed with Tris-buffered saline with Tween solution (TBST:50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween-20). Signals were detected by chemiluminescence substrate using Clarity Western ECL Substrate (#1705061, Bio-Rad) were quantified by the density of protein bands using Image J analysis program (Image J Version 1.52).

qRT-PCR Analysis: Freshly frozen rat testis tissues were weighed and maximum 100 mg tissue samples were excised. After finely mincing, tissues were homogenized in 1 ml Tri-reagent (Sigma, T9424) to obtain a single cell suspension. Then, samples were further processed according to the manufacturer's instructions to obtain total cell RNA. For each sample, 1 µg Dnase I (Thermo)-treated total RNA was converted to cDNA in a 20 µl reaction volume using iScript Reverse Transcription Supermix (Biorad). For qRT-PCR, following primer and probes were used to amplify target gene transcripts in a dublex PCR settings: rGPR55 forward primer TGTCTTCAC CATCTGCTTCATC, rGPR55 reverse primer TTCCAACCCATACCAGCATC, rGPR55 prob (5'-HEX, 3'-BHQ1) TCCTCGCCATCCAGTACCCTCTT; rGPR119 forward primer TGATACCTTGATTGGCG TGG, rGPR119 reverse primer CATGATCTGGAAGTAACGG AGG, rGPR119 prob (5'-HEX, 3'-BHQ1) AAGACCTT GTGTAGCCTTCGGATGG; rGAPDH forward primer AACCCATCACCATCTTCCAG, rGAPDH reverse primer CACGACATACTCAGCAC CAG, rGAPDH Prob (5'-FAM, 3'-BHQ1) ACCCCATTTGATGTTAGC GGGATCTC. PCR mix was prepared in a 10 µl volume containing iQ Multiplex Powermix (1X), 0.5 µl cDNA, 300 nM of each primer and 200 nM of each prob. Reactions were performed using CFX96 Touch (Biorad) thermal cycler with the following settings: 95°C for 2 min, then 40 cycles of 95°C for 10 sec and 60°C for 45 sec. During primer optimization process and each PCR run, cDNAs prepared from adult rat brain tissues were used as a positive control along with a no template control. Target and reference genes were run as single and duplex reactions and confirmed that their threshold cycles (Ct) were similar in both conditions.

For each sample, difference in the threshold cycles (dCt) was calculated by subtracting the Ct value of GAPDH from the Ct of GPR55 or GPR119. The calculated mean dCts from groups were first analyzed for normal distribution. Since the mean GPR55 dCt of each group displayed a normal distribution (Kolmogorov-Smirnov test, $P=0.258$) with unequal variances ($P<0.05$), the data were evaluated by Welch's ANOVA test. Mean dCts for GP119 followed a normal distribution (Kolmogorov-Smirnov test, $P=0.540$) with homogeneous group variance and were analyzed by Fischer's test. Using group 1 (PND 7) as calibrator, 2^ddCT indicating fold difference in gene expression was calculated according to Livak's method.

***Statistical Analysis*:** One-way ANOVA was used to compare the means of 10 independent groups. Tukey or Games-Howel (in cases of heterogeneous variance) tests were used as post-hoc tests. First, the normality of the data collected by RT-PCR and western blot methods were examined with tests (Anderson-Darling, Kolmogorov-Smirnov and Shapiro Wilk), scatter charts and quantile-quantile (Q-Q) plots. The homogeneity of variance, which is another important assumption of ANOVA, was also checked with Levene and Bartlett tests, and in cases of unequal variances, Welch test was used instead of Fischer's test. P values smaller than 0.05 were considered statistically significant. All statistical analyses were

performed using Jamovi program. Graphs were prepared in Graphpad Prism 9.

Results

Histopathology and Immunohistochemistry: Histopathologically, micrographs of H&E-stained testis sections were evaluated for the presence of germ cell loss, degeneration/apoptosis, germ cell exfoliation, macro/microtubular vacuolation and necrosis, and no pathological changes were observed. Strong positive staining for GPR55 was observed in spermatids (residual bodies of spermatids and round spermatids) and spermatocytes in mid-term tubules and spermatocytes (zygotene and pachytene) in late-stage tubules at the 8th, 9th, and 10th weeks. However, no GPR55-positive cells were found at the 7th week and before (Figure 1 and Figure 3). For GPR119, very intense positivity was observed only in spermatids (residual bodies of spermatids and round spermatids) in mid-term (stage VII-VIII) tubules at the 8th, 9th and 10th weeks. No positive signal was observed in the other weeks (Figure 2 and Figure 3). In addition, there was no significant difference in the number of GPR55 and GPR119 positive cells between weeks 8, 9 and 10.

Western Blot Analysis Findings: The protein levels of both GPR55 and GPR119 were determined in the rat testis using Western blot analysis. Both GPR proteins were detected in all testes samples and the blotting results confirmed the immunohistochemistry data. The relative expression levels of these GPRs in the developing testis are shown in Figure 4. For the statistical analysis of Western blot measurements, normality assumption was checked for both proteins; normality was achieved in both (K-S P=0.258 and P=0.110, respectively, QQ plots are normal); homogeneity of variance was provided for GPR55 (both tests P>0.05); not available for GPR119 (both tests P<0.01). As a result of the Fischer's test for GPR55, there was no difference between the groups (P=0.464). Although there was a difference between the groups (P=0.031) as a result of the Welch test for GPR119, no differing groups were found in the Post-hoc test.

RT-PCR Analysis: Both GPR55 and GPR119 mRNA transcripts were detected in testis tissues of all groups. However, according to their Ct values (Figure 5A and 5D), which is inversely correlated with the starting transcript levels, overall GPR119 expression (Ct>30) in each group was lower than GPR55 expression (Ct<30). Group 1 (PND 7) had the least GPR55 mRNA level (P<0.0001, compared to other groups except group 2, PND 14) (Figure 5B and 5C), then the transcript levels exhibited a gradual increase in the second and third weeks by 7-fold and 14-fold, respectively (Figure 5C). Despite minimal differences, GPR55 mRNA levels showed no significant change between weeks 3 and 10.

GPR119 was expressed in all developmental stages even though at a lower level than GPR55. The GPR119 expression level varied within and between groups (Figure 5E and 5F). Nonetheless, the Fischer's test analysis of the mean dCts for the groups showed that there was no significant difference between the groups (P=0.181).

Discussion and Conclusion

Spermatogenesis, the development of sperms from male germ cells, is a complex biological process including mitotic and meiotic divisions followed by differentiation stages. These events are modulated by endocrine hormones produced in the hypothalamus and pituitary and by a complex network of locally produced factors within the testis (17, 26). Endocannabinoid system is one of these factors and is known to regulate sperm development (16). AEA, an endocannabinoid, is synthesized by germ cells, Sertoli cells and spermatozoa in the testis (9, 45). Both GPR55 and GPR119 receptors can be activated by endocannabinoids including AEA and endocannabinoid-like compounds such as OEA (13, 21, 31). Cannabinoids and their endogenous counterparts bind to cannabinoid receptors, CB1 and CB2, and non-cannabinoid receptors, GPR55 and GPR119, of the ECS; thus regulate several aspects of male reproduction (31, 38). The functional importance of ECS in spermatogenesis has been shown by the ECS knockout mouse models. Detection of a higher number of motile sperms in the epididymis of CB1-deficient mice and impaired fertilizing ability of sperms in fatty acid amide hydrolase (FAAH)-knockout mice which have a high amount of AEA, indicated that depending on their expression level, ECS might have a negative regulatory role in sperm development. Another ECS receptor, vanilloid receptor TRPV1, can also react to AEA and inhibits acrosome reaction (30). Conversely, CB2 receptor signaling triggered by 2-AG promotes spermatogenic differentiation from spermatogonia (15). A previous study showed that endocannabinoid receptors and ligands, AEA and 2-AG, exist in both mRNA and protein levels in resting and capacitated mouse sperms. Besides, their concentrations further increased in capacitated sperms, suggesting an active role for endocannabinoids in the regulation of spermatogenesis and sperm functions (5). Altogether, ECS influences different stages of sperm development and function. Despite a growing number of studies on CB1, CB2 and TRPV1-mediated regulation of sperm development, the role of GPR55 and GPR119-mediated signaling in spermatogenesis has not been defined well.

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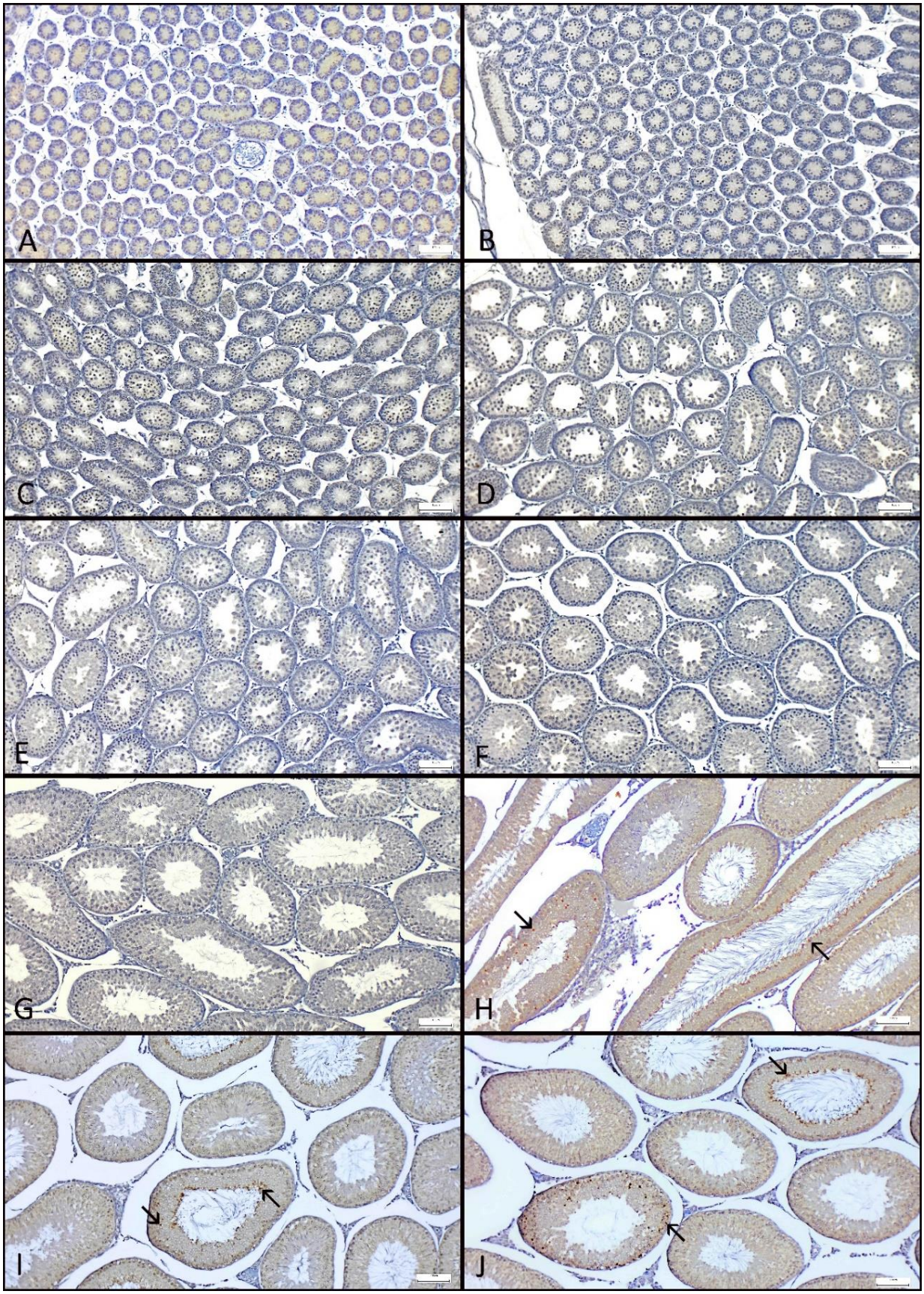

Figure 1. GPR55 immunohistochemistry. GPR55 negativity at weeks 1-7 (A-G). At week 8 (H), positivity of spermatids in the mid-stage (stage VII-VIII) tubule (right), and spermatocytes in the late-stage tubule (left) (arrows). At 9th week (I), positivity of spermatocytes and spermatids in mid-term (stage VII-VIII) tubule (arrows). At week 10 (J), positivity of spermatids in mid-stage (stage VII-VIII) tubule (right) and spermatocytes in late-stage tubules (left) (arrows). GPR55, DAB, Bar: 100 µm.

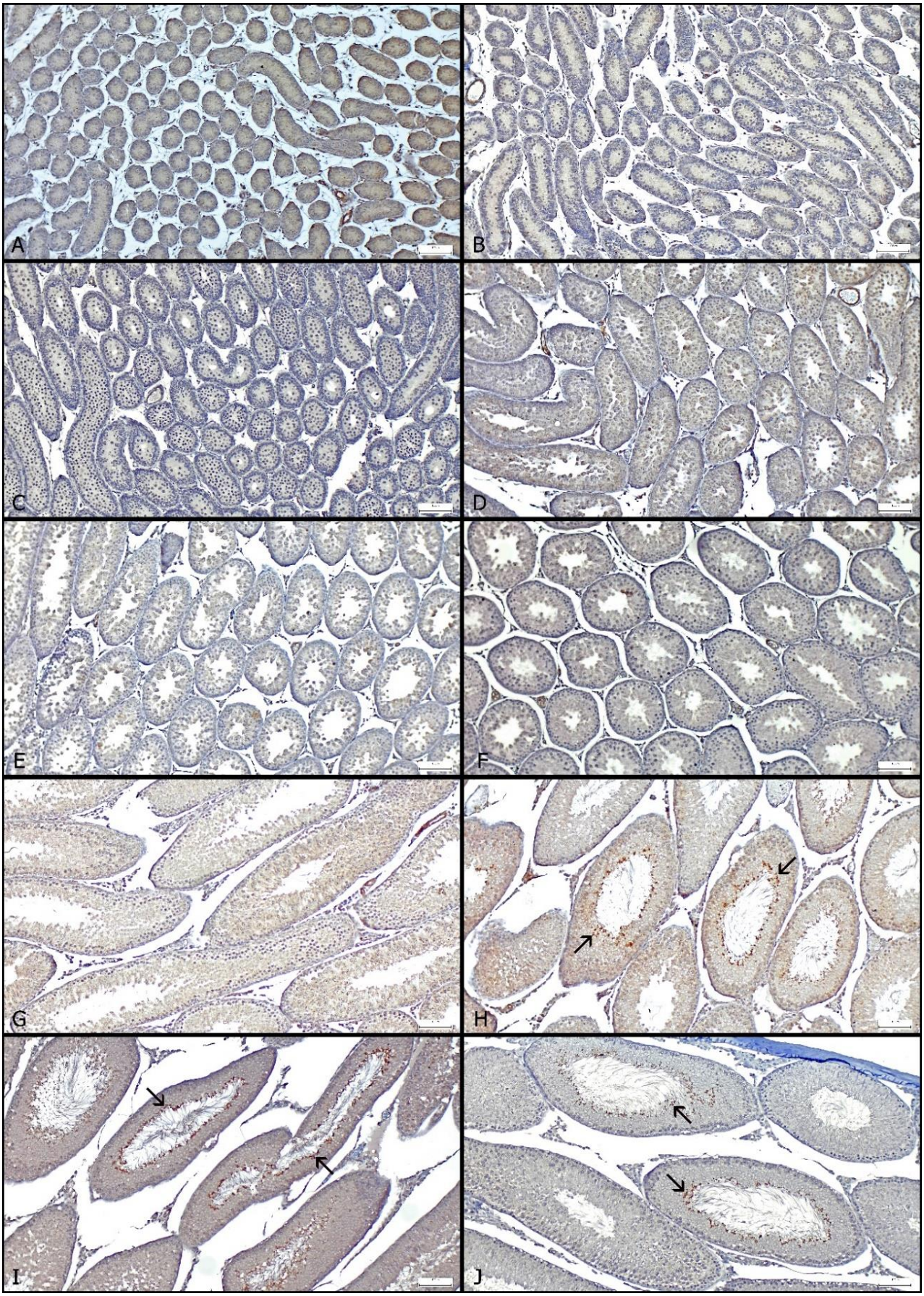

Figure 2. GPR119 immunohistochemistry. GPR119 negativity at weeks 1-7 (A-G). Intense positivity of spermatids in mid-term (stages VII-VIII) tubules at 8th, 9th and 10th weeks (H-J) (arrows). GPR119, DAB, Bar: 100 µm.

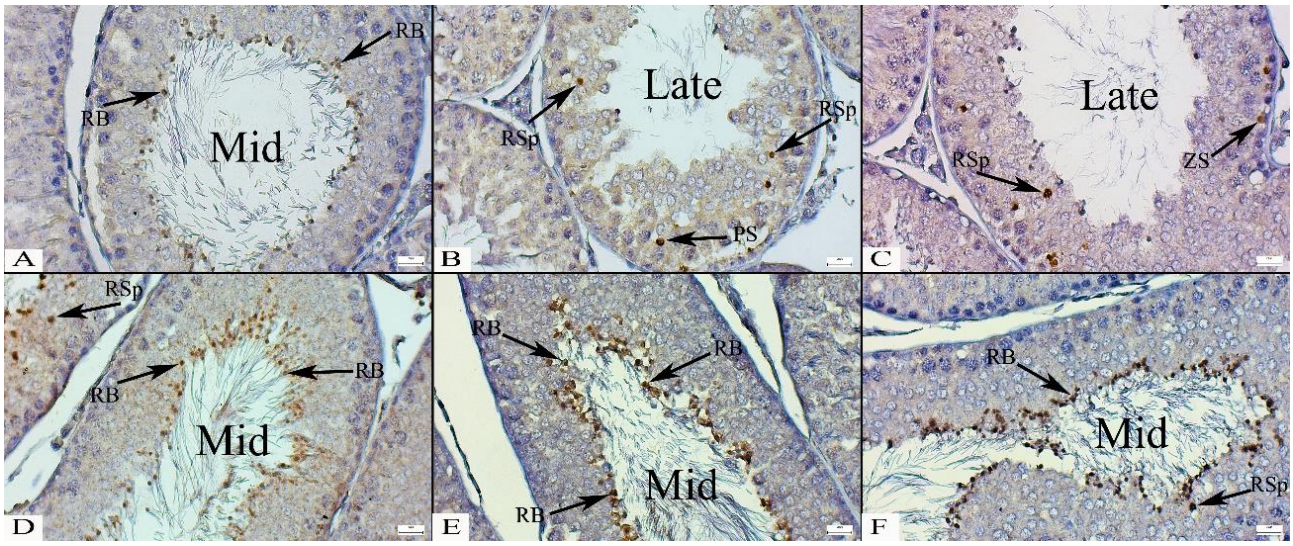

Figure 3. GPR55 and GPR119 immunohistochemistry. GPR55 positivity in residual bodies (RB) of spermatids in the mid-stage (stage VII-VIII) tubule (A) at 8th week, and pachytene spermatocytes (PS), round spermatids (RSp) and zygotene spermatocytes (ZS) in the late-stage tubules in 9th (B) and 10th (C) weeks. GPR55, DAB, Bar: 20 µm. GPR119 positivity of residual bodies of spermatids and round spermatids in mid-term (stages VII-VIII) tubules at 8th (D), 9th (E) and 10th (F) weeks. GPR119, DAB, Bar: 20 µm.

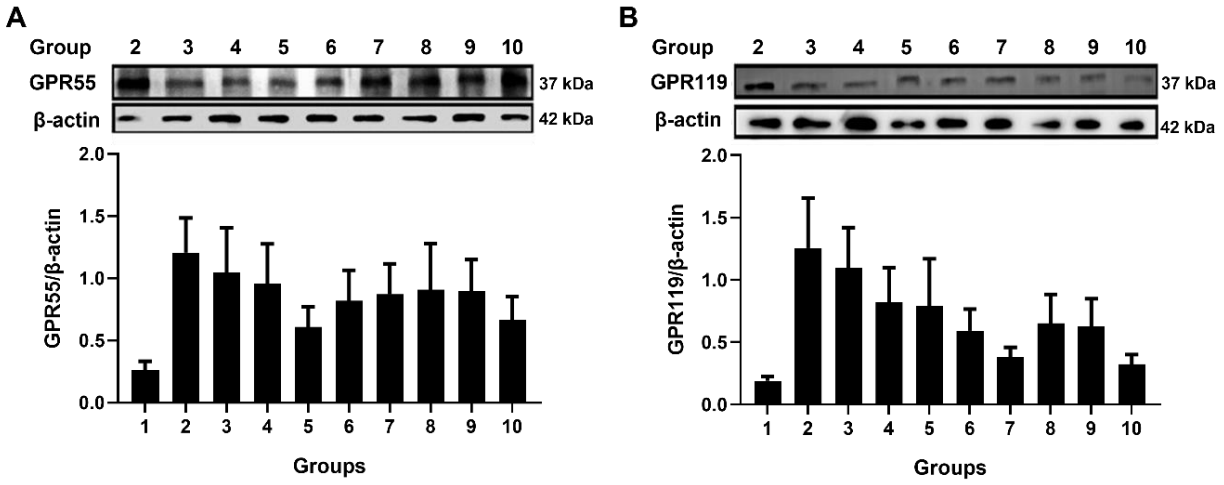

Figure 4. Expression levels of GPR55 and GPR119 proteins in rat testis tissues. Representative western blot images display A) GPR55 and B) GPR119 protein expression in one sample per group. For the quantification of protein abundance, blot images from separate experiments were analyzed by densitometry and the data was plotted as mean GPR55: β actin ratios ± SEM for each group (n = 6, 3 replicates/group).

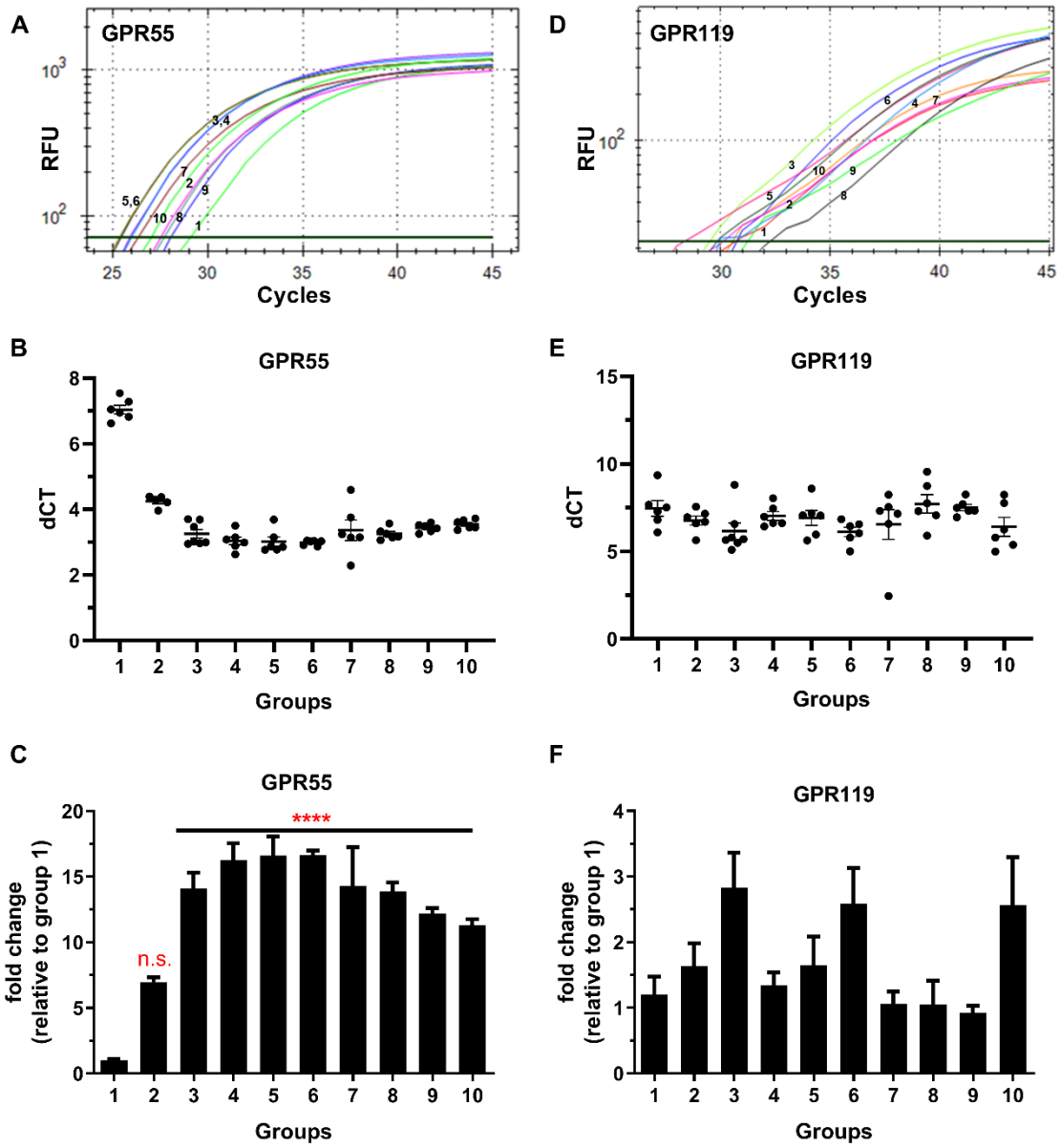

Figure 5. GPR55 and GPR119 mRNA levels at different developmental stages of rat testis. Representative amplification curves of A) GPR55 and D) GPR119 transcripts, labelled with their pertaining group numbers, show the detected florescence level in each reaction cycle. RFU means relative fluorescence unit. dCt (GPR55 Ct or GPR119 Ct- GAPDH Ct) values of each transcript plotted in B) and E) as the mean dCt of each group with SEM. n=6 for all, except n=7 for group 3 and n=5 for group 2 due to exclusion of an outlier in GPR55 data. Reactions were run as two technical replicates for GPR55 and three for GPR119. Welch's ANOVA test was applied. **** denotes P<0.0001, n.s. means not significant. The data were also shown as fold change (2^-ddCt) in gene expression C) and F) calculated by Livak method compared to group 1.

In the current study, we detected the expression of both GPR55 and GPR119 throughout the development of rat testis from PND 7 to 70 by two different methods, qRT-PCR and western blotting. The abundance of GPR55 transcript and protein was at the lowest levels at PND 7, then increases at PND 14 (not significant). From PND 14 and thereafter, GPR55 expression was easily detectable, but exist at variable amounts. Interestingly, immunohistochemical staining shows that GPR55 protein signal was detectable only in 56, 63 and 70 post-natal day rat tissues but undetectable in others. Similarly, GPR119 was expressed at the lowest amount at PND7 compared to other time points, then shows a shifting protein and mRNA abundance in the later weeks. In line with GPR55, positive signal for GPR119 protein was detectable only at PND 56, 63 and 70 but not before that. Even though both IHC and western blotting are used to detect protein targets, their detection sensitivity might differ by the abundance of target protein and steps of sample processing, which might the reason of negative IHC staining for GPR55 and GPR119 at earlier than PND56.

In rats, one spermatogenesis cycle takes about 8 weeks during which the sperm begins to develop and completes 8 different developmental stages until to become ready to be released into the tubular lumen (10). GPR119 immunoreactivity was only seen in spermatids (residual bodies of spermatids and round spermatids) in stage VII and VIII tubules which have mature spermatozoa ready to be released. However, GPR55 signal was observed both in spermatids as well as in spermatocytes at late stage tubules. Immunohistochemically, the presence of a strong positivity for both GPR55 and GPR199 at the 8th week suggests that both receptor signaling might be modulating the completion of spermatogenesis process. In line with our findings, a previous study from Amoako et al. indicated that GPR55 mRNA is present in human sperms and related with the normal sperm count, motility and morphology (1). Sperms with decreased motility have significantly lower GPR55 transcript levels than normal sperm samples (1). Furthermore, the engagement of GPR55 with a lipid ligand, palmitoylethanolamide (PEA), lead to increased mobility in human sperms (2). On the other hand, GPR119 transcripts were previously detected in normal human testis, but the role of GPR119 in testis and spermatogenesis still remains to be investigated (43). However, lower levels of OEA, a GPR119 receptor ligand, in human seminal plasma has been associated with abnormal sperm count, morphology and motility (3). Conversely, *in vitro* OEA-treatment of normal human sperms led to increased survival and motility, suggesting that these effects might be through GPR119 receptor (3). With findings from our study displaying an active GPR119 expression throughout the rat testis development, GPR119-mediated signaling might influence multiple stages of male gametogenesis.

Overall, CB1 and GPR55 receptors are extensively expressed in the central nervous system and the ECS is known to regulate gonadal function via the hypothalamic-pituitary-gonadal (HPG) axis (14). In addition to their endocrine regulatory effects, ECS components are expressed in the testis and might directly contribute to the sperm development and function. Unraveling the developmental regulation of the receptors and ligands of the ECS will enlighten both the molecular mechanisms of normal testis physiology and their involvement in male reproductive system pathologies such as infertility and cancer. Testicular expression of GPR55 and GPR119 receptors from early to late developmental stages shown by our study corroborates the possible role of these receptors in sperm development. Further investigation of GPR55 and GPR119 signaling through functional studies are necessary to clarify their role in key stages of spermatogenesis.

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Conflict of interest

The authors declare that there is no conflict of interest.

Author Contributions

HT and VI conceived and planned the experiments. VI performed histopathology and immunohistochemistry experiments. MT carried out western blot analysis. HT, AK, and MMK performed RT-PCR experiment. HT, VI, and MMK contributed to preparation of testicular samples. HT, VI, MT, AK, and MMK contributed to the interpretation of the results. HT took the lead in writing the manuscript. All authors read and approved the final version of the manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

All animal procedures were performed in accordance with the Burdur Mehmet Akif Ersoy University Animal Experiments Local Ethics Board (13.03.2019/501)'s regulations and approval.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

References

1. **Amoako AA, Marczylo EL, Willets JM, et al** (2011): Differential expression of the orphan G-protein coupled receptor Gpr55 in human spermatozoa. 67th Annual Meeting of the American Society for Reproductive Medicine, Orlando, FL, United States, 15-19 October 2011. Philadelphia, PA, United States: Elsevier Inc.
2. **Amoako AA, Willets JM, Marczylo TH, et al** (2011): Activation of GPR55 by palmitoylethanolamide increases human sperm motility. 58th Annual Scientific Meeting of the Society for Gynecologic Investigation (SGI), Miami Beach, FL, United States, 16-19 March 2011. Thousand Oaks, CA, United States: Sage Publications.
3. **Amoako AA, Marczylo TH, Elson J, et al** (2014): *Relationship between seminal plasma levels of anandamide congeners palmitoylethanolamide and oleoylethanolamide and semen quality*. Fertil Steril, **102**, 1260-1267.
4. **Andradas C, Caffarel MM, Perez-Gomez E, et al** (2011): *The orphan G protein-coupled receptor GPR55 promotes cancer cell proliferation via ERK.* Oncogene, **30**, 245-252.

5. **Catanzaro G, Battista N, Rossi G, et al** (2011): *Effect of capacitation on the endocannabinoid system of mouse sperm*. Mol Cell Endocrinol, **343**, 88-92.
6. **Cherif H, Argaw A, Cécyre B, et al** (2015): *Role of GPR55 during axon growth and target innervation*. Eneuro, **2**, 1-20.
7. **Chiurchiù V, Lanuti M, De Bardi M, et al** (2015): *The differential characterization of GPR55 receptor in human peripheral blood reveals a distinctive expression in monocytes and NK cells and a proinflammatory role in these innate cells.* Int Immunol, **27**, 153-160.
8. **Chu ZL, Carroll C, Alfonso J, et al** (2008): *A role for intestinal endocrine cell-expressed g protein-coupled receptor 119 in glycemic control by enhancing glucagon-like Peptide-1 and glucose-dependent insulinotropic Peptide release.* Endocrinology, **149**, 2038-2047.
9. **Cobellis G, Cacciola G, Scarpa D, et al** (2006): *Endocannabinoid system in frog and rodent testis: type-1 cannabinoid receptor and fatty acid amide hydrolase activity in male germ cells.* Biol Reprod, **75**, 82-89.
10. **Creasy DM** (1997): *Evaluation of testicular toxicity in safety evaluation studies: the appropriate use of spermatogenic staging*. Toxicol Pathol, **25**, 119–131.
11. **Fujimoto GI, Morrill GA, O'Connell ME, et al** (1982): *Effects of cannabinoids given orally and reduced appetite on the male rat reproductive system.* Pharmacology, **24**, 303–313.
12. **Fyfe MC, McCormac JG, Overton HA, et al** (2008): *GPR119 agonists as potential new oral agents for the treatment of type 2 diabetes and obesity*. Expert Opin Drug Discov, **3**, 403-413.
13. **Godlewski G, Offertáler L, Wagner JA, et al** (2009): *Receptors for acylethanolamides—GPR55 and GPR119*. Prostaglandins Other Lipid Mediat, **89**, 105-111.
14. **Gorzalka BB, Dang SS** (2012): *Minireview: Endocannabinoids and Gonadal Hormones: Bidirectional Interactions in Physiology and Behavior*. Endocrinology, **153**, 1016-1024.
15. **Grimaldi P, Orlando P, Di Siena S, et al** (2009): *The endocannabinoid system and pivotal role of the CB2 receptor in mouse spermatogenesis*. Proc Natl Acad Sci USA, **106**, 11131–11136.
16. **Grimaldi P, Di Giacomo D, Geremia R** (2013) *The endocannabinoid system and spermatogenesis*. Front Endocrinol, **4**, 192.
17. **Acharyya S** (2021). *Immune and endocrine aspects of the testis and its relation to male infertility.* Chem Biol Lett, **8**, 144-161.
18. **Henstidge CM, Balenga NA, Ford LA, et al** (2009): *The GPR55 ligand L-α-lysophosphatidylinositol promotes RhoA-dependent Ca2+ signaling and NFAT activation.* The FASEB Journal, **23**, 183-193.
19. **Hiley CR, Kaup SS** (2007): *GPR55 and the vascular receptors for cannabinoids.* Br J Pharmacol, **152**, 559-561.
20. **Huan Y, Jiang Q, Li G, et al** (2017): *The dual DPP4 inhibitor and GPR119 agonist HBK001 regulates glycemic control and beta cell function ex and in vivo.* Sci Rep, **7**, 1-11.
21. **Im DS** (2021): *GPR119 and GPR55 as Receptors for Fatty Acid Ethanolamides, Oleoylethanolamide and Palmitoylethanolamide*. Int J Mol Sci, **22**, 1034.
22. **Im JH, Kang KW, Kim SY, et al** (2018): *GPR119 agonist enhances gefitinib responsiveness through lactate-mediated inhibition of autophagy*. J Exp Clin Cancer Res, **37**, 1-15.
23. **Imbernon M, Whyte L, Diaz-Arteaga A, et al** (2014): *Regulation of GPR55 in rat white adipose tissue and serum LPI by nutritional status, gestation, gender and pituitary factors.* Mol Cell Endocrinol, **383**, 159-169.
24. **Irving A, Abdulrazzaq G, Chan SL, et al** (2017): *Cannabinoid receptor-related orphan G protein-coupled receptors.* Adv Pharmacol, **80**, 223-247.
25. **Kargl J, Brown AJ, Andersen L, et al** (2013): *A selective antagonist reveals a potential role of G protein–coupled receptor 55 in platelet and endothelial cell function*. J Pharmacol Exp Ther, **346**, 54-66.
26. **Khanehzad M, Abbaszadeh R, Holakuyee M, et al** (2021): *FSH regulates RA signaling to commit spermatogonia into differentiation pathway and meiosis.* Reprod Biol Endocrinol, **19**, 1-19.
27. **Kremshofer J, Siwetz M, Berghold VM, et al** (2015): *A role for GPR55 in human placental venous endothelial cells.* Histochem Cell Biol, **144**, 49-58.
28. **Lauckner JE, Jensen JB, Chen HY, et al** (2008): *GPR55 is a cannabinoid receptor that increases intracellular calcium and inhibits M current*. Proc Natl Acad Sci USA, **105**, 2699-2704.
29. **Lewis SEM, Paro R, Borriello L, et al** (2012): *Long-term use of HU210 adversely affects spermatogenesis in rats by modulating the endocannabinoid system.* Int J Androl, **35**, 731–740.
30. **Maccarrone M, Barboni B, Paradisi A, et al** (2005): *Characterization of the endocannabinoid system in boar spermatozoa and implications for sperm capacitation and acrosome reaction.* J Cell Sci, **118**, 4393–4404.
31. **Maccarrone M, Rapino C, Francavilla F, et al** (2021): *Cannabinoid signalling and effects of cannabis on the male reproductive system.* Nat Rev Urol, **18**, 19-32.
32. **Marichal-Cancino BA, Fajardo-Valdez A, Ruiz-Contreras AE, et al** (2017): *Advances in the Physiology of GPR55 in the Central Nervous System.* Curr Neuropharmacol, **15**, 771–778.
33. **Murphy LL, Steger RW, Smith S, et al** (1990): *Effects of delta-9-tetrahydrocannabinol, cannabinol and cannabidiol, alone and in combinations, on luteinizing hormone and prolactin release and on hypothalamic neurotransmitters in the male rat.* Neuroendocrinology, **52**, 316-321.
34. **Odori S, Hosoda K, Tomita T, et al** (2013): *GPR119 expression in normal human tissues and islet cell tumors: evidence for its islet-gastrointestinal distribution, expression in pancreatic beta and alpha cells, and involvement in islet function.* Metabolism, **62**, 70-78.
35. **Oka S, Kimura S, Toshida T, et al** (2010): *Lysophosphatidylinositol induces rapid phosphorylation of p38 mitogen-activated protein kinase and activating transcription factor 2 in HEK293 cells expressing GPR55 and IM-9 lymphoblastoid cells.* J Biochem, **147**, 671-678.
36. **Overton HA, Fyfe MCT, Reynet C** (2008): *GPR119, a novel G protein-coupled receptor target for the treatment of type 2 diabetes and obesity*. Br J Pharmacol, **153**, 76-81.
37. **Patil KC, McPherson L, Daly CJ** (2015): *Co-localization of alpha1-adrenoceptors and GPR55: a novel prostate cancer paradigm?* J Pharm Pharmacol, **6**, 212-220.

38. **Payne KS, Mazur DJ, Hotaling JM, et al** (2019): *Cannabis and male fertility: a systematic review*. J Urol, **202**, 674–681.
39. **Pertwee RG, Howlett AC, Abood ME, et al** (2010): *International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB1 and CB2.* Pharmacol Rev, **62**, 588-631.
40. **Ryberg E, Larsson N, Sjögren S, et al** (2007): *The orphan receptor GPR55 is a novel cannabinoid receptor.* Br J Pharmacol, **152**, 1092-1101.
41. **Sakamoto Y, Inoue H, Kawakami S, et al** (2006): *Expression and distribution of Gpr119 in the pancreatic islets of mice and rats: predominant localization in pancreatic polypeptide-secreting PP-cells.* Biochem Biophys Res Commun, **351**, 474-480.
42. **Shi QX, Yang LK, Shi WL, et al** (2017): *The novel cannabinoid receptor GPR55 mediates anxiolytic-like effects in the medial orbital cortex of mice with acute stress.* Mol Brain, **10**, 1-11.
43. **Soga T, Ohishi T, Matsui T, et al** (2005): *Lysophosphatidylcholine enhances glucose-dependent insulin secretion via an orphan G-protein-coupled receptor.* Biochem Biophys Res Commun, **326**, 744-751.
44. **Staton PC, Hatcher JP, Walker DJ, et al** (2008): *The putative cannabinoid receptor GPR55 plays a role in mechanical hyperalgesia associated with inflammatory and neuropathic pain.* Pain, **139**, 225-236.
45. **Sugiura T, Kondo S, Sukagawa A, et al** (1996): *Enzymatic synthesis of anandamide, an endogenous cannabinoid receptor ligand, through N-acylphosphatidylethanolamine pathway in testis: involvement of Ca2+-dependent transacylase and phosphodiesterase activities.* Biochem Biophys Res Commun, **218**, 113-117.
46. **Wenger T, Ledent C, Csernus V, et al** (2001): *The central cannabinoid receptor inactivation suppresses endocrine reproductive functions.* Biochem Biophys Res Commun, **284**, 363–368.
47. **Whyte LS, Ryberg E, Sims NA, et al** (2009): *The putative cannabinoid receptor GPR55 affects osteoclast function in vitro and bone mass in vivo.* Proc Natl Acad Sci USA, **106**, 16511-16516.
48. **Yang H, Zhou J, Lehmann C** (2016): *GPR55–a putative "type 3" cannabinoid receptor in inflammation.* J Basic Clin Physiol Pharmacol, **27**, 297-302.
49. **Yu Y, Fuscoe JC, Zhao C, el al** (2014): *A rat RNA-Seq transcriptomic BodyMap across 11 organs and 4 developmental stages.* Nat Commun, **5**, 1-11.
50. **Zhang X, Maor Y, Wang JF, et al** (2010): *Endocannabinoid-like N-arachidonoyl serine is a novel pro-angiogenic mediator.* Br J Pharmacol, **160**, 1583-1594.
51. **Zhao J, Zhao Y, Hu Y, et al** (2021): *Targeting the GPR119/incretin axis: a promising new therapy for metabolic-associated fatty liver disease.* Cell Mol Biol Lett, **26**, 1-28.