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Nociceptin is upregulated by FSH signaling in Sertoli cells in murine testes

Ko Eto *, Masahiro Shiotsuki, Tomomi Sakai, Shin-ichi Abe

Department of Biological Sciences, Graduate School of Science and Technology, Kumamoto University, 2-39-1 Kurokami, Kumamoto 860-8555, Japan

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ABSTRACT

In postnatal testes, follicle-stimulating hormone (FSH) acts on somatic Sertoli cells to activate gene expression directly via an intracellular signaling pathway composed of cAMP, cAMP-dependent protein kinase (PKA), and cAMP-response element-binding protein (CREB), and promotes germ cell development indirectly. Yet, the paracrine factors mediating the FSH effects to germ cells remained elusive. Here we show that nociceptin, known as a neuropeptide, is upregulated by FSH through cAMP/PKA/CREB pathway in Sertoli cells in murine testes. Chromatin immunoprecipitation from Sertoli cells shows that CREB phosphorylated at Ser133 associates with prepronociceptin gene encoding nociceptin. Analyses with Sertoli cells and testes demonstrates that both prepronociceptin mRNA and the nociceptin peptide are induced after FSH signaling is activated. In addition, the nociceptin peptide is induced in testes after 9 days post partum following FSH surge. Thus, our findings may identify nociceptin as a novel paracrine mediator of the FSH effects in the regulation of spermatogenesis.

1. Introduction

Spermatogenesis is a highly organized process dependent on the local microenvironment within testes. A self-renewing population of diploid spermatogonia undergoes mitotic divisions and differentiation into tetraploid spermatocytes, which then undergo two successive rounds of meiosis, a specialized cell division program, and become haploid spermatids; the spermatids undergo spermiogenesis, a specialized cell differentiation program, and become spermatozoa.

Follicle-stimulating hormone (FSH), a glycoprotein produced in the pituitary, plays an essential role in the initiation and maintenance of pubertal spermatogenesis that results in the production of normal sperm [1]. The total levels of FSH in serum increase during the first spermatogenic wave in mice from 8 dpp onward [2]. When FSH binds to a Gs protein-coupled seven-transmembrane receptor, adenylyl cyclase (AC) is activated, resulting in an increased concentration of intracellular cAMP, a second messenger; subsequently cAMP-dependent protein kinase (PKA) is activated and phosphorylates multiple substrates such as cAMP-response element-binding protein (CREB), a transcription factor, at Ser133, leading to the activation of gene expression [1]. As FSH receptor is exclusively expressed in Sertoli cells [3,4], it is considered that the ultimate effects of FSH on germ cells are mediated through not only intimate interactions of Sertoli cells with germ cells but also paracrine interactions within seminiferous tubules at multiple steps of germ cell differentiation [5]. Thus, FSH regulates Sertoli cell functions directly and germ cell development indirectly [6,7]; however, few of the critical players secreted from Sertoli cells to germ cells have been identified to mediate the FSH effects.

In the current study, we demonstrated that nociceptin, known traditionally as a neuropeptide, is upregulated by FSH signaling in Sertoli cells within seminiferous tubules of postnatal murine testes, suggesting the existence of a novel paracrine mechanism mediating the FSH effects on spermatogenesis.

2. Materials and methods

2.1. Cell culture, treatments, and transfection

A Sertoli cell line (Sertoli B) established from 7-dpp murine testes [8] was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C under an atmosphere of 5% CO_2. Cells were seeded in 24-well plates at 6×10^4 cells/well for Western blotting or 100-mm dishes at 3×10^6 cells/dish for RT-PCR, and 1 day later deprived of serum for 16 h and treated for the last 2 h with the vehicle (water) or an inhibitor for PKA (H-89, 200 nM, Sigma–Aldrich) prior to treatment with the vehicle (DMSO) or dibutyryl cAMP (db-cAMP, 200 μM, Sigma–Aldrich). For forced expression of FSH receptor, cells were seeded in 24-well plates at 3×10^4 cells/well for Western blotting or 30-mm dishes at 1.5×10^5 cells/plate for RT-PCR. The next day the cells were transfected with 0.4 or 2 μg of the empty vector or the expression plasmid for murine FSH receptor (kindly provided by Dr. Hämäläinen) using 1.25 or 5 μl of FuGENE HD (Roche) in 30 or 100 μl of OPTI-MEM (Invitrogen), respectively [9]. The cells were cultured for 30 h after transfection,

* Corresponding author.
E-mail address: etoko@gpo.kumamoto-u.ac.jp (K. Eto).

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serum-starved for 16 h, and treated for the last 2 h with the vehicle or H-89 prior to treatment with the vehicle (water) or FSH (200 ng/ml, National Hormone & Peptide Program, Dr. Albert F. Parlow).

2.2. Animals, organ culture, and treatments

Seminiferous tubules derived from testes of male mice (C57B/6) at 7 dpp were prepared in DMEM and treated at 32 °C for 2 h with the vehicle or H-89 prior to culture with the vehicle or FSH. The Institutional Animal Care and Use committee of Kumamoto University approved protocols for the animal experiments.

2.3. Chromatin immunoprecipitation (ChIP)

The experiments were performed with a kit (Imgenex) according to the manufacturer's protocol. Briefly, Sertoli B cells, grown in 100-mm dishes at 5×10^6 cells/dish, were treated for 10 min at 37 °C with the vehicle or db-cAMP and processed for ChIP with a rabbit antibody to CREB phosphorylated at Ser133 (2 μg; Cell Signaling Technology) or an unrelated rabbit antibody (2 μg). The precipitated DNA was subjected to PCR as the template for murine prepronociceptin proximal promoter containing a consensus CRE site (282 bp) using the forward primer 5′-TTG GGC TGT TTG TTT GAT GA-3′ and the reverse primer 5′-CAG CCA CAG TGG ACA AAA GA-3′ [10] for 50 cycles at 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 45 s. The PCR products were applied to agarose gel electrophoresis and visualized by ethidium bromide staining, and verified by nucleotide sequencing using an Applied Biosystems model 310 automated DNA sequencer (Life Technologies).

2.4. Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA (5 μg, each) was extracted from Sertoli B cells and testes with ISOGEN (Nippon Gene), treated with RNase-free DNase I (Takara), and reverse-transcribed with Superscript III (Invitrogen) using random hexamers as described previously [9,11]. The reaction mixture was diluted at 10-fold with RNase-free water and subjected to PCR as follows: for 35 or 45 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s using primers 5′-ATG AAA ATC CTC TTT TGT GA-3′ and 5′-CTA CAC ATT ACC ATT CTG GT-3′ for prepronociceptin (564 bp); for 25 cycles at 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 45 s using primers 5′-TGG GCC GCG TCT CCT TCG AG-3′ and 5′-GGG GAA TGA GGA AAA TAT GG-3′ for cyclophilin (584 bp). The PCR products were applied to agarose gel electrophoresis and visualized by ethidium bromide staining, and verified by nucleotide sequencing.

2.5. Western blotting

Sertoli B cells, cultured in 24-well plates, were washed in phosphate-buffered saline and lysed in 50 μl/well of 2× SDS–PAGE sample buffer (100 mM Tris/HCl, pH 6.8, 4% SDS, 12% β-mercaptoethanol, 0.08% bromophenol blue). After the extracts (10 μl, each) were subjected to SDS–10%PAGE, proteins were transferred to a polyvinylidene difluoride membrane (Immobilon, Millipore) and blocked with 5% non-fat milk in Tris-buffered saline (pH 7.4) containing 0.1% Tween20 (TBS–T). The membrane was probed with a primary antibody specific for CREB phosphorylated at Ser133 (43 kDa; 1:1000, diluted with TBS–T containing 5% bovine serum albumin (BSA), Cell Signaling Technology), CREB (43 kDa; 1:1000, TBS–T containing 5% BSA, Cell Signaling Technology), FSH receptor (75 kDa; 1:3000, TBS–T containing 5% non-fat milk, kindly provided by Dr. Walker), or β-actin (45 kDa; 1:2000, TBS–T containing 5% non-fat milk, Millipore), detected with the appropriate secondary antibodies conjugated to horseradish peroxidase, and developed with an enhanced chemiluminescence system (Immobilon Western Chemiluminescent HRP substrate, Millipore).

2.6. Peptide extraction and detection

Extraction of endogenous peptides from tissues was performed as reported [12]. Testes derived from 7- to 20-dpp mice or the 7-dpp testes cultured were washed twice in 0.9% (w/v) NaCl, boiled for 3 min, and homogenized thoroughly in 5 volumes/weight of freshly prepared 0.1 M acetic acid. The homogenates were incubated for 2 h at room-temperature with occasional mixing and centrifuged twice at 2200*g* for 20 min at room-temperature to remove higher molecular weight of proteins. The supernatants were spotted at 2 μg/2 μl onto a nitrocellulose membrane (GE Healthcare), followed by Western blotting with an antibody to the nociceptin peptide (17 amino acid residues; 1:500, diluted with TBS–T containing 5% non-fat milk, Yanaihara Institute, Inc.). The synthetic nociceptin peptide (2 μl, each) was also spotted as the standard to final doses of 1 (2 pmol), 3 (6 pmol), 10 (20 pmol), or 30 μM (60 pmol). For immunodepletion, the antibody (1 μg/0.5 μl) was incubated for 2 h at room-temperature with 0.9 μg of the synthetic nociceptin peptide in 50 μl of TBS–T containing 5% non-fat milk, and processed for Western blotting of peptide extracts as described above.

2.7. Statistics

Data were obtained as the mean ± SE. For statistical comparison, Student's *t* test was used. *P* values less than 0.05 were considered to be statistically significant.

2.8. Others

Protein concentrations were estimated by Coomassie dye binding (Bio-Rad) using BSA as a standard. For some experiments, signals detected in the RT-PCR and Western blot analyses were quantified by densitometry using Image J software (NIH).

3. Results

3.1. Phosphorylation of CREB following cAMP stimulation in a Sertoli cell line

CREB is expressed and phosphorylated in germ and Sertoli cells during the early phase of murine spermatogenesis [13]. To verify Sertoli cell-specific phosphorylation of CREB by cAMP/PKA pathway, we used Sertoli B cells, a Sertoli cell line. The time course of CREB phosphorylation was examined in cells after dibutyryl cAMP (db-cAMP), a membrane-permeable analog of cAMP, was added to the cultures by Western blotting for CREB phosphorylated on Ser133. The phosphorylation reached the maximum level at 10 min after the addition of db-cAMP, and then returned to the basal level by 60 min (Fig. 1A and C); however, this was suppressed to the basal level by an inhibitor for PKA (H-89) (Fig. 1B and C).

We validated the presence of the FSH-operated intracellular signaling pathway mediating CREB phosphorylation in Sertoli B cells. Because endogenous FSH receptor was undetectable in the cells, we transfected them with an expression plasmid for murine FSH receptor and detected its exogenous expression by Western blotting (Fig. 1D). In cells overexpressing FSH receptor, CREB phosphorylation was enhanced after FSH was added to the cultures; however, this was suppressed to the basal level by H-89 (Fig. 1E and F). These results indicated that CREB is a target for FSH signaling comprising cAMP/PKA pathway in Sertoli B cells.

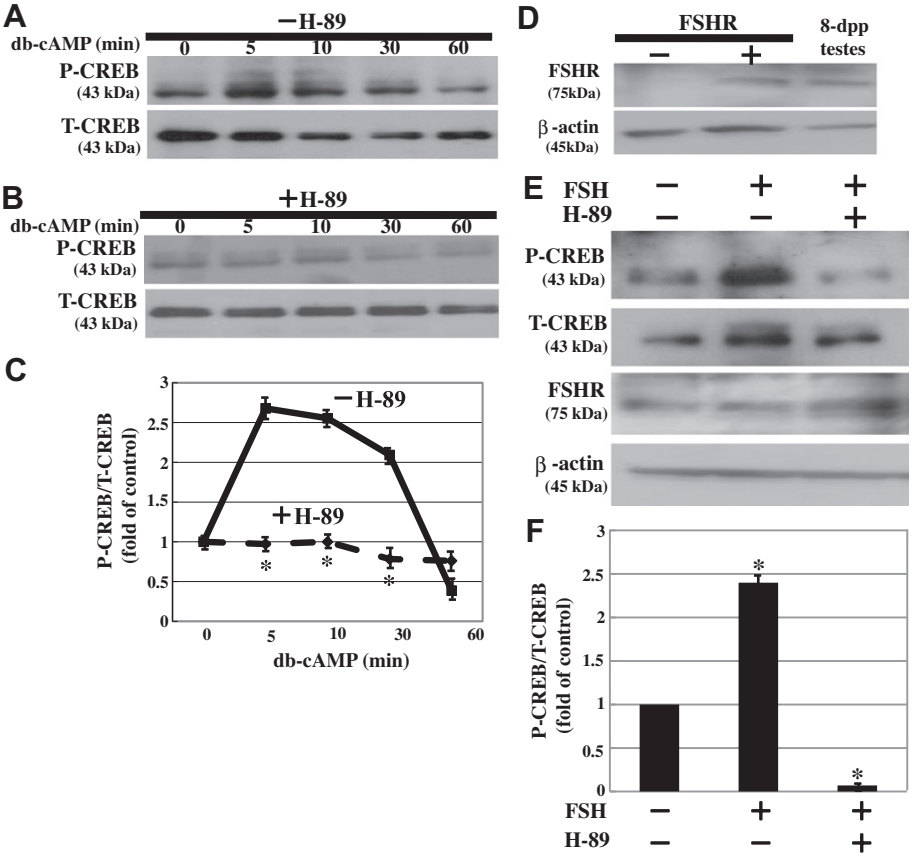

Fig. 1. Phosphorylation of CREB following the activation of FSH signaling in Sertoli cells. (A–C) Sertoli B cells were pretreated for 2 h with water (−) (A) or H-89 (+) (B), followed by treatment with db-cAMP for the indicated periods, and analyzed by Western blotting for phosphorylated CREB (P-CREB) and total CREB (T-CREB) as the loading control. Results are representative of three independent experiments (A,B) and quantified by densitometry as a fold of P-CREB normalized to T-CREB in cells treated for each period with db-cAMP, relative to that in those without db-cAMP, in the absence (solid line) or presence (dashes) of H-89 (C). $^{*}p < 0.01$ vs cells treated for the respective period with db-cAMP in the absence of H-89. (D) Sertoli B cells were transfected with the empty vector (−) or an expression plasmid for murine FSH receptor (FSHR) (+) and analyzed by Western blotting for FSHR and β-actin as the loading control. The 8-dpp testes are a positive control for FSHR expression. (E,F) Sertoli B cells transfected with the FSHR expression plasmid were pretreated for 2 h with water (−) or H-89 (+), followed by treatment for 10 min with water (−) or FSH (+), and analyzed by Western blotting for the indicated proteins. Results are representative of three independent experiments (E) and quantified by densitometry as a fold of P-CREB normalized to T-CREB in cells treated with FSH alone or FSH in the presence of H-89, relative to that in those without FSH in its absence (F). $^{*}p < 0.01$.

3.2. Identification of prepronociceptin gene associating cAMP-dependently with phosphorylated CREB

To identify novel genes that are regulated by CREB phosphorylated at Ser133 in Sertoli cells, we performed chromatin immunoprecipitation (ChIP) from Sertoli B cells. After cells were stimulated for 10 min with db-cAMP, extracts were prepared and processed for ChIP with the same antibody to phosphorylated CREB. We screened by PCR several genes, whose proximal promoter regions associate with phosphorylated CREB, and investigated murine prepronociceptin [10]. The proximal promoter of murine prepronociceptin gene has one functional CRE site in a different location from the human promoter [10,14]. The DNA fragment from the putative transcription start site to the ATG translation start codon (252 bp) was detected only in cells treated with db-cAMP but not in untreated cells (Fig. 2A, upper panel). None could be detected from immunoprecipitates with an unrelated antibody (Fig. 2A, middle panel). Nucleotide sequencing of the detected DNA fragment confirmed the presence of a consensus CRE sequence (CGTCA) at 30 bp upstream of the ATG translation start codon in the proximal promoter of murine prepronociceptin gene as reported [10] (Fig. 2B). These results indicated that phosphorylated CREB associates with the proximal promoter region of prepronociceptin gene in Sertoli B cells. This gene encodes a precursor protein of prepronociceptin, from which a mature nociceptin peptide consisting of 17 amino acid residues is produced. Nociceptin, also known as orphanin FQ, is a neuropeptide belonging to the opioid peptide family and shares the identical amino acid sequence between mice and other species.

3.3. Effects of cAMP and FSH on the expressions of prepronociceptin mRNA and the nociceptin peptide in Sertoli cells and testes

To determine whether the association of phosphorylated CREB with the proximal promoter region of prepronociceptin gene activates the transcription in Sertoli cells, we cultured Sertoli B cells with db-cAMP for 12, 24, or 48 h and analyzed by RT-PCR (Fig. 3A). The prepronociceptin mRNA was transiently induced 24 h after db-cAMP addition to the cultures; however, this was completely suppressed by H-89. Interestingly, none was seen in cells exposed for 12 or 48 h to db-cAMP.

We determined by RT-PCR whether FSH activates the expression of prepronociceptin mRNA in Sertoli cells. Here we used for organ cultures the 7-dpp testes just before total levels of serum FSH rise in mice [2] because we wanted to ignore the effect of endogenous FSH on the mRNA expression. The mRNA was clearly induced when testes were cultured for 24 h following FSH addition; however, this was suppressed by H-89 (Fig. 3B and C).

We then examined the effect of FSH on the expression of the nociceptin peptide in organ cultures of testes. After the 7-dpp tes-

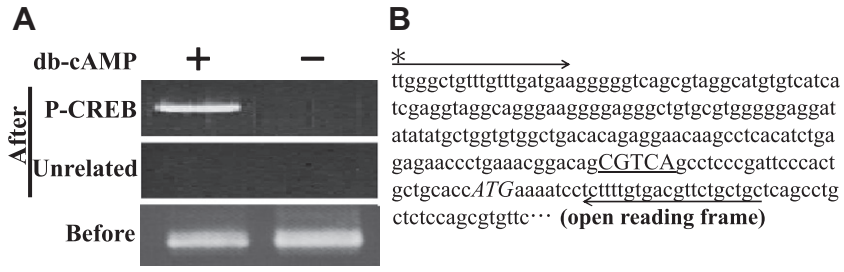

Fig. 2. Identification of prepronociceptin gene, whose proximal promoter associates with phosphorylated CREB, from Sertoli cells. (A) Sertoli B cells were either left untreated (−) or treated for 10 min with db-cAMP (+) and subjected to chromatin immunoprecipitation (ChIP) (After) with an unrelated antibody (middle panel) or an antibody to P-CREB (upper panel). The precipitates were amplified by PCR using primers flanking the prepronociceptin proximal promoter. Before (lower panel) represents the PCR analyses of the cell extracts used for each ChIP. (B) The nucleotide sequence of the DNA fragment detected by PCR is shown. A consensus CRE sequence is indicated in capital letters underlined; primer sequences used for PCR, arrows; a putative transcription start site, an asterisk; and the ATG translation start codon, italic capital letters. Results are representative of three separate experiments.

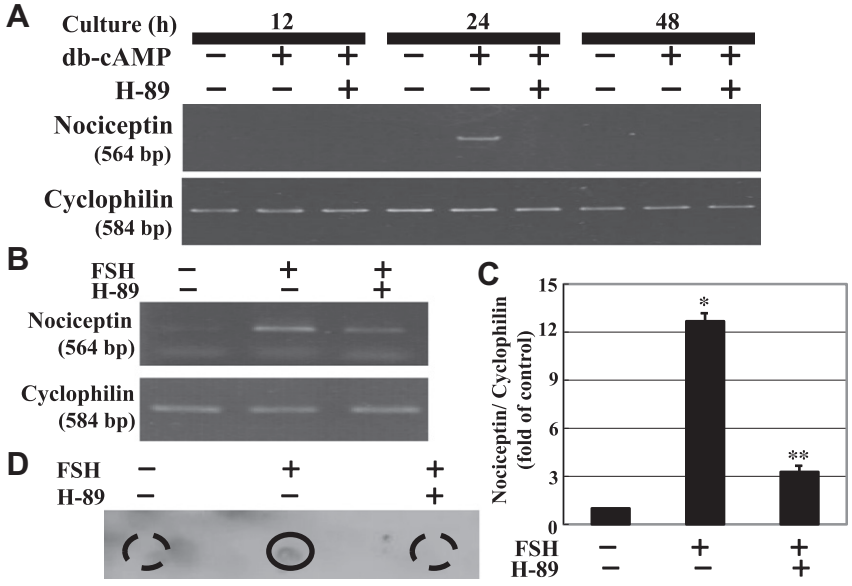

Fig. 3. Induction of nociceptin following the activation of FSH signaling in Sertoli cells. (A) Sertoli B cells were pretreated for 2 h with water (−) or H-89 (+), followed by treatment with DMSO (−) or db-cAMP (+) for the indicated periods, and analyzed by PCR for nociceptin and cyclophilin as the internal control. (B,C) The 7-dpp testes were pretreated for 2 h with water (−) or H-89 (+), followed by treatment for 24 h with water (−) or FSH (+), and analyzed by RT-PCR for nociceptin and cyclophilin. Results are representative of three independent experiments (B) and quantified by densitometry as a fold of nociceptin expression normalized to cyclophilin expression in cells treated with FSH alone or FSH in the presence of H-89, relative to that in those without FSH in the absence of H-89 (C). * $p < 0.01$; ** $p < 0.05$. (D) The experiment was performed as in (B), except that the extracts, prepared by acetic acid from each of the testes cultured, were spotted at 2 μg/2 μl onto a nitrocellulose membrane and analyzed by Western blotting for the nociceptin peptide.

tes were exposed for 24 h to FSH, their extracts containing endogenous peptides were prepared and analyzed by Western blotting. The peptide was induced by FSH stimulation; however, this was completely suppressed by H-89 (Fig. 3D). To evaluate the specificity of the nociceptin antibody reactivity, the antibody was immunodepleted by incubating with the synthetic nociceptin peptide, followed by Western blotting of the same samples as above. No immunopositive signal was detected, revealing that the antibody recognized the nociceptin peptide specifically (data not shown). These results indicated that FSH activates the transcription of prepronociceptin gene through cAMP/PKA/CREB pathway, resulting in an increased production of the nociceptin peptide, in Sertoli cells.

3.4. Expression of the endogenous nociceptin peptide in testes

We finally examined the expression of the endogenous nociceptin peptide in testes. The 7- to 20-dpp testes were used because a drastic FSH surge, differentiation of premeiotic spermatogonia into meiotic spermatocytes, and their differentiation into postmeiotic spermatids occur during this age range. Endogenous peptides were prepared from testes at each age and analyzed by Western blotting. Strikingly, the nociceptin peptide started to increase drastically

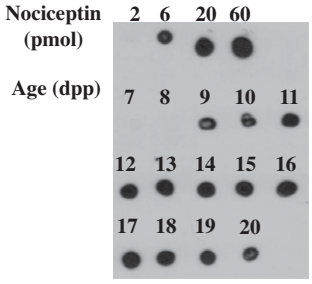

Fig. 4. Expression of the endogenous nociceptin peptide in testes. The experiment was performed as in Fig. 3D, except for the use of testes at various ages indicated. Each of the extracts at 2 μg/2 μl and 2, 6, 20, or 60 pmol/2 μl of the synthetic nociceptin peptide were spotted.

after 9 dpp and the amounts in the extracts were estimated as less than 20 pmol (Fig. 4).

4. Discussion

4.1. Prepronociceptin gene is transcriptionally regulated by FSH signaling in Sertoli cells

In response to FSH, its receptor may trigger multiple signaling pathways that regulate Sertoli cell functions including gene expression directly and lead to germ cell development indirectly [1,6,7,15]. However, which signaling pathways are activated in Sertoli cells and how the signals affect germ cells are poorly understood. CREB is highly expressed and phosphorylated in Sertoli cells during the early phase of spermatogenesis [13]. In addition, cAMP/PKA system phosphorylating CREB exists in mammalian Sertoli cells [1,15]. That is why we used Sertoli B cells [8] to gain insights into Sertoli cell-specific signal transduction and gene expression. We revealed that Sertoli B cells exhibit the same physiological responses to FSH via cAMP in at least PKA activation and CREB phosphorylation/activation as testicular Sertoli cells excluding the undetectable level of FSH receptor expression. Our ChIP assay demonstrated that cAMP/PKA pathway may regulate the association of phosphorylated CREB with a putative consensus CRE sequence in the proximal promoter of murine prepronociceptin gene, which seems to be highly conserved between Sertoli cells in testes and neurons in brains [10,14]. Moreover, we showed for the first time by RT-PCR analyses of Sertoli B cells and testes and Western blot analyses of peptide extracts from testes that the expressions of prepronociceptin mRNA and the nociceptin peptide may be upregulated by FSH signaling via cAMP/PKA/CREB pathway in Sertoli cells. We found that it takes 24 h for FSH to induce nociceptin, consistent with previous reports showing that gene and/or protein induction for cellular responses to FSH is generally delayed by at least 24 h [16,17], although the reason remains unknown.

Interestingly, this is the first report on the expression of nociceptin in testes. The opioid peptide family members such as proopiomelanocortin, preprodynorphin, and preproenkephalin are transcriptionally regulated in a similar fashion by raising the level of intracellular cAMP in brains [18,19], consistent with our result for the FSH-activated transcription of prepronociceptin gene in Sertoli cells. Other peptides than nociceptin that might be expressed and function in testes are the focus of our future investigations. Overall, these data suggested that the FSH-operated cAMP/PKA/CREB pathway transcriptionally upregulates prepronociceptin expression, leading to the production of the nociceptin peptide, in Sertoli cells.

4.2. Nociceptin is a paracrine factor mediating the FSH-regulated germ cell development

The functional roles of FSH in spermatogenesis have been addressed in mammals [1], but more information is required. The FSH effects on germ cells are mediated through the receptor expressed restrictedly in Sertoli cells [1,3,4], whereby paracrine factors are produced and secreted locally to act on germ cells. In mammalian testes, stem cell factor (SCF) is expressed and upregulated by FSH stimulation in Sertoli cells and is critical for proliferation of differentiating type A spermatogonia [20–22]. However, few paracrine factors are known beyond SCF, which are essential for spermatogenesis. There is a dramatic surge of FSH in serum after 8 dpp in mice [2] and we found that the nociceptin peptide is hardly detected in the 7- and 8-dpp testes; therefore we considered that these are correlated with a constant induction of nociceptin in testes after 9 dpp following FSH surge, consistent with our *in vitro* results showing that FSH signaling is capable of inducing nociceptin in testes.

Northern blot analyses for the distribution of prepronociceptin mRNA in rat tissues demonstrated that it is abundantly present in spinal cords and brains but barely in testes [23]. However, our analyses with Sertoli B cells and testes demonstrated a transient and local increase in the expression of nociceptin in Sertoli cells following the activation of FSH signaling. This may be necessary for determining the temporal and spatial specificity for nociceptin-mediated paracrine functions during spermatogenesis within seminiferous tubules in the postnatal testes. Overall, nociceptin has a potential role in the FSH-regulated spermatogenesis. Identification of nociceptin described here provides new cue for understanding the molecular mechanisms underlying spermatogenesis. The question concerning the physiological significance and functional roles of nociceptin still remains unanswered and is now under investigation with *in vitro* and *in vivo* analyses.

In conclusion, the present study reports that nociceptin is induced in Sertoli cells through the FSH-activated intracellular signaling pathway and provides insights into a novel paracrine regulation of spermatogenesis in postnatal murine testes.

Acknowledgments

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