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Vasopressin regulates hypothalamic GnRH synthesis: Histomorphological evidence in hypothalamus and biological effects in GT1-7 cells

Zhu Zhu[a,b], Xiaozhen Zhao[a,c], Feng Huang[a], Feng Wang[a], Wei Wang[a,d,*]

[a] Department of Human Anatomy and Histoembryology, School of Basic Medical Sciences, Fujian Medical University, Fuzhou 350122, China
[b] Department of Pathology, Mengchao Hepatobiliary Hospital of Fujian Medical University, Fuzhou, Fujian, China
[c] Key Laboratory of Brain Aging and Neurodegenerative Diseases of Fujian Provincial Universities and Colleges, Fuzhou 350122, China
[d] Research Center for Neurobiology, School of Basic Medical Sciences, Fujian Medical University, Fuzhou 350122, China

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ABSTRACT

Aims: To investigate the direct histomorphological clues and observe the biological effects of VP acting on gonadotropin-releasing hormone (GnRH) secretion.

Main methods: Immunofluorescence was conducted to investigate the expressions of GnRH and VP in experimental left varicocele (ELV) rats and ELV repair rats. The colocalization of GnRH and VP was observed by electron microscopy immunohistochemistry. The protein-protein interaction between GnRH and VP was tested by co-immunoprecipitation (co-IP) and the proximity ligation assay (PLA). The effects of intracellular and extracellular VP on GnRH and relative transcription factors (Oct-1, Otx2, Pbx1b and DREAM) were respectively evaluated in VP overexpressed and VP treated GT1-7 cells.

Key findings: Both hypothalamic GnRH and VP decreased in ELV rats and recovered by ELV repair. The overlapped immunolocalizations of GnRH and VP mainly distributed in the lateral part of the arcuate nucleus (ArcL) and median eminence (ME) with a Manders' overlap coefficient of 0.743 ± 0.117. Immunoreactive substances of GnRH and VP existed in the same and adjacent terminals. VP overexpression did not cause any significant effects on the expressions of GnRH and Oct-1, as well as GnRH promoter activity. While 50–200 pg/ml VP treatments increased GnRH mRNA levels in a dose- and time-dependent manner in GT1-7 cells. Additionally, 200 pg/ml VP triggered a marked promotion of expressions of GnRH, Oct-1, Oxt2 Pbx1b and DREAM, as well as GnRH promoter activity ($P < 0.05$).

Significance: The results reveal the colocalization and interaction of VP and GnRH, which will be conducive to explain the effects and mechanisms of VP acting on reproduction.

1. Introduction

Currently, 10–15% of reproductive-age couples worldwide suffer from infertility. For over 50% of infertile couples, infertility is caused by the male partner. As a reproductive centre, the hypothalamus collects and integrates a large amount of reproductive and metabolic information *in vivo*, and the synthesis and release of gonadotropin-releasing hormone (GnRH) represent the final signal in hypothalamus-mediated reproductive regulation.

GnRH is a major neurohormone that is secreted in a periodic pulsatile manner by GnRH neurons mainly located in the preoptic-hypothalamic region and projecting fibres to the median eminence (ME) [1,2]. GnRH is responsible for the secretion of gonadotropins by the anterior pituitary, along with spermatogenesis and steroidogenesis [3]. The genesis of GnRH pulse generation is a complex biological process regulated by many factors. Octamer-binding transcription factor-1 (Oct-1) and orthodenticle homeobox2 (Otx2) are conserved across several vertebrate species and have been early identified up-regulating the transcription of the *GnRH-I* gene *in vivo* and *in vitro* [4]. And cofactors, such as Pre-B-cell leukemia homeobox 1b (Pbx1b), are crucial for the interactions between Oct-1 and the regulatory regions of the GnRH promoter [5]. Additionally, GnRH neurons shows a distinctive feature of generating activity-dependent, long-duration intracellular calcium ion concentration ($[Ca^{2+}]_i$) transients. Calcium influx and a novel Ca^{2+}-binding protein, downstream regulatory element antagonist modulator (DREAM), have been reported to act as facilitators of GnRH secretion in several ways, such as upregulating calcium-dependent transcription factors and controlling burst firing dynamics [6]. In

* Corresponding author at: Department of Human Anatomy and Histoembryology, Fujian Medical University, No 1, North Xuefu Road, University New District, Fuzhou 350122, China.
E-mail address: wwfjmu@163.com (W. Wang).

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addition to intracellular factors, the synthesis and release of GnRH are regulated by many neuropeptides, steroid hormones, and neurotransmitters.

Arginine vasopressin (AVP, also referred to as vasopressin, VP) is synthesized in the parvocellular cells of the supraoptic nuclei (SON) and paraventricular nuclei (PVN), which send projections to impact feeding, blood glucose regulation, maternal care, aggression, and locomotor activity [7–9]. In addition to its principal roles, VP has been reported to be linked to male-typical behaviours of rodents and fish and stimulate mating-related neuronal activity and reproductive behaviour in leeches [10,11]. Additionally, VP has been indicated in stimulating seminiferous tubule contraction and decreasing sperm count and motility [12–14]. VP also has been reported to increase the concentration of spermatozoa and the accumulation of cytosolic cAMP in vas deferens epithelial cells and to modulate ion transport [15,16]. Parvocellular VP has been suggested to project to kisspeptin (Kp) neurons located in the anteroventral periventricular nuclei, which induce GnRH1 gene transcription [17,18]. VP V1a2 receptors were reported co-localize with GnRHI on neurons in the preoptic anterior hypothalamus, which may identify a structural linkage between the VP and GnRHI [19], but the evidence of direct effects of VP on GnRH secretion and the mechanisms is still unknown. Thus, the definite association between VP and reproduction is still a great source of concern.

Recently, higher-grade varicoceles have been reported to be associated with higher nocturia levels [20], and we observed an interesting phenomenon in which the urine volume/water intake ratio dramatically increased in the rat model of experimental left varicocele (ELV); the decreases in hypothalamic VP levels occurred along with decreases in the GnRH level in this classic animal model of reproductive dysfunction. To confirm whether VP directly affects hypothalamic GnRH neurons, the distributions of GnRH and VP in the hypothalamus were examined, and the possible interaction between GnRH and VP was explored by electron microscopic immunohistochemistry and an *in situ* proximity ligation assay (PLA). Furthermore, serial cytological experiments were conducted with an immortalized GnRH-expressing cell line, GT1-7, to verify the effects of VP on GnRH neurons. The results of the present study provide a preliminary reference for the exploration of the direct effect of VP on GnRH neurons, which may be benefit studies on reproductive regulation.

2. Experimental procedure

2.1. Animals and cells

Sixty mature male Sprague-Dawley (SD) rats weighing 200 ± 20 g were obtained from the Laboratory Animal Centre of Fujian Medical University, China (No. SCXK(Min)2012-0001) and were maintained at one per cage at a constant temperature (22 ± 2 °C) and humidity (50 ± 10%) under a 12-h light/12-h dark cycle during the experiment. Animal experimentation was performed in compliance with the local ethics committee (No. 2015-29), and the animals were treated in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines.

GT1-7 cells (immortalized hypothalamic neurons [21]) were kindly provided by Dr Chen Xiaochun (Union Hospital, Fujian). GT1-7 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (11965-092, Gibco) with 10% foetal bovine serum (FBS) (16000-044, Gibco) at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Observations VP and GnRH expression in a rat model of reproductive injury

Sixty rats were randomly divided into four groups (n = 15): a sham-operated group (SO group), an experimental left varicocele group (ELV group), an ELV repair group, ELV sham repair group. An experimental left varicocele rat model was created by partially ligating the left renal vein, as described by Saypol [22]. Rats in the SO group underwent a similar operation without any ligation. Two weeks after ligation, rats in ELV repaired group received ELV repair, during which the spermatic veins were ligated and cut [23]. Rats in the ELV sham repair group underwent a similar blunt separation. The severity of varicocele and changes in the kidney were examined prior to sampling. Rats were excluded if renal atrophy occurred, and these selection criteria were implemented to eliminate possible interference caused by renal atrophy.

Eight weeks after the first surgery, 24-hour urine samples from each group were collected using metabolic cages (3M12B440, Tecniplast, Italy). The rats were warmed in a hot box at 38 °C for 5 min, and then placed in a restraining apparatus which was also kept at 38 °C to adapt to the environment for 5 min. Animals' blood pressures, including the systolic blood pressure (SBP), mean blood pressure (MBP), and diastolic blood pressure (DBP), were examined by the tail-cuff method (BP-98A, Softron Biotechnology, Japan). Brains were harvested, fixed, dehydrated, and cut into frozen sections (15 μm) using a cryostat (CM 1950, Leica, Germany). The sections of hypothalamus were incubated with antibodies for VP (dilution 1:5000, AB1565, Merck Millipore, USA) and GnRH (dilution 1:100, sc-32292, Santa Cruz, USA). The controls were incubated with 10% donkey serum but not primary antibodies. After incubating with the fluorescein-conjugated secondary antibodies and staining with DAPI, the sections were imaged and analysed with a laser scanning confocal microscope (LSM800, Zeiss, USA). The mean Manders' overlap coefficient was analysed to evaluate the overlapping immunolocalizations of GnRH and VP respectively in lateral part of the arcuate nucleus (ArcL), medial part of the arcuate nucleus (ArcM) and ME. Total number of the overlapped immunolocalizations of GnRH and VP (the colocalization sites) were counted and recorded by two individuals.

2.3. Investigation of histomorphological correlation between VP and GnRH

Localization of GnRH and VP in the ArcL at the ultra-structural level was observed by electron microscopy immunohistochemistry. Oscillated sections were incubated with primary antibodies targeting GnRH (1:50 dilution) and VP (1:5000 dilution) diluted with 10% donkey serum for 48 h at 4 °C. The control slides were incubated with one of the two primary antibodies individually. Then, the sections were incubated with the following antibodies and reagents, in turn: nanogold anti-rabbit (1:100 dilution, #2003, Nanoprobes, USA), biotin anti-mouse (1:200 dilution, AP192B, Merck Millipore, USA), a rabbit ABC kit (Vector Laboratories, USA), and the HQ Sliver Enhancement Kit (25F065, Nanoprobes, USA). Thin sections stained with silver were collected on formvar-coated slot grids, stained with lead citrate, and examined with a JEM-1400 electron microscope with a digital camera system (Veleta, Olympus, Japan).

In situ PLA was performed to detect the potential direct correlation between VP and GnRH. Frozen brain sections of normal SD rat hypothalamus were permeabilized with 0.3% Triton-X 100, blocked using a blocking solution (DUO92002, Sigma-Aldrich, USA), and incubated with primary antibodies for GnRH and VP diluted with Antibody Diluent (DUO92002, Sigma-Aldrich, USA). The controls were incubated with blocking solution but not primary antibodies. The diluted PLA probe solution was mixed with anti-rabbit PLUS (DUO92002, Sigma-Aldrich, USA) and anti-mouse MINUS (DUO92004, Sigma-Aldrich, USA). The signals were detected using the Duolink *In Situ* Detection Reagent (DUO92014, Sigma-Aldrich, USA).

2.4. Protein-protein interaction between VP and GnRH

The GT1-7 cells were transiently transfected with the VP and GnRH expression plasmids (pCMV-HA-VP and pCMV-Myc-GnRH, respectively). The cells were lysed 48 h after transfection. Whole cell extracts and complexes were immunoprecipitated using an anti-HA monoclonal

antibody (Mab) (ab18181, Abcam, MA, USA) and subjected to immunoblotting with an anti-Myc MAb (ab9132, Abcam, MA, USA); then, the complexes immunoprecipitated with the anti-Myc MAb were immunoblotted with an anti-hemagglutinin (HA) MAb. The antibody used in the control reaction was anti-mouse IgG (ab190475, Abcam, MA, USA). The complex-bound resin (20422, Thermo Fisher, MA, USA) was added to Electrophoresis Loading Buffer (39000, Thermo Fisher, MA, USA). The samples were evaluated by Tricine-SDS-PAGE.

2.5. Examining the effects of intracellular and extracellular VP on GnRH secretion

GT1-7 cells (cultured in 10-cm culture dish, 1×10^6 per dish) were either transfected with pCMV-HA-GFP-VP (20 μg per dish) (pCMV-HA-GFP-VP group) or administered 200 pg/ml (a concentration close to serum VP levels in male rats) VP in the culture supernatant (VP200 group). GT1-7 cells transfected with pCMV-HA-GFP (pCMV-HA-GFP group) and cultured in complete medium (control group) served as controls. A double dose (40 μl per dish) of Lipofectamine 2000 was used to transfect GT1-7 cells at 80% confluency. Half of the medium was replaced after 12 h, and all the medium was replaced 24 h after transfection. The infection efficiency was determined by fluorescent microscopy 48 h after transfection. The cells were stained with anti-VP (AB1565, Merck Millipore, Darmstadt, Germany) followed by FITC conjugated goat anti-rabbit IgG antibody (AP187F, Merck Millipore) and examined by FACS analysis. Cells stained with FITC conjugated goat anti-rabbit IgG antibody served as control. Additionally, the effects of different dosages of VP ranging from 25 pg/ml to 8000 pg/ml (25, 50, 100, 200, 400, 800, 1000, 2000, 4000, 6000, 8000 pg/ml) on GT1-7 cells were also evaluated in the following experiments. GT1-7 cells treated with high-glucose DMEM with 10% FBS were used as a control.

The cells and supernatant were collected and evaluated 48 h post-transfection or 6 h post-treatment with (Arg^8)-AVP (ab120175, Abcam, USA). The expression levels of GnRH in the supernatant were examined using EIA kits (S-1217, LHRH-EIA Kit, Peninsula Laboratories International, USA) following the manufacturer's protocol. GT 1-7 cells (1×10^6 per dish) cultured in 10 cm dishes were lysed by RIPA (1 ml), and 20 μg of total protein was loaded onto Tricine-SDS-PAGE gels to examine the expression of intracellular GnRH, GnRH relative transcription factors and proteins. The primary antibodies targeted GnRH (1:100 dilution), Oct-1 (1:1000 dilution, ab178869, Abcam, USA), Otx2 (1:1000 dilution, ab114138, Abcam, USA), Pbx1b (1: 500 dilution, 4342, CST, USA) and DREAM (1:500 dilution, ab61770, Abcam, USA).

Total RNA from GT1-7 cells was extracted with Trizol and converted to cDNA using the PrimeScript RT reagent kit (RR047A, Takara, Japan). Real-time PCR was conducted with primers and cDNA templates mixed with the SYBR Premix Ex Taq II kit (DRR081, Takara, Japan). Gene-specific primers for GNRH, OCT1, Otx2, and GAPDH were designed (Table 1) and synthesized by Sangon Biotech (China). The gene transcript levels were normalized to that of GAPDH in the same sample. The data were analysed and quantified using the $2^{-\Delta\Delta Ct}$ method.

A total of 100 ng of pGL3-Basic plasmid (#E1751, Promega, USA) with inserts of the GNRH1 promoter sequence (−1800–+50) were transfected into GT1-7 cells (administered and blank control groups) using Lipofectamine 2000 along with 10 ng of the Renilla luciferase pRL-TK plasmid (#E2241, Promega, USA). GT1-7 cells in the pCMV-HA-VP group were simultaneously transfected with pCMV-HA-VP, and GT1-7 cells in the pCMV-HA group were simultaneously transfected by pCMV-HA. Forty-eight hours after the transfections, GT1-7 cells in the administered group and cells administered different dosages of VP were administered VP for 6 h, while cells in the blank group were treated with the same volume of culture medium. A dual luciferase assay was performed using the Dual-Luciferase® Reporter Assay System (#E1910, Promega, USA). Luciferase activity was measured as the ratio of firefly luciferase signal to Renilla luciferase signal. Each experiment was performed in triplicate.

To investigate the dynamic effects of extracellular VP on GT1-7 cells, the proliferation of GT1-7 cells administrated by dosages of VP ranging from 25 pg/ml to 8000 pg/ml was evaluated with a Cell Counting Kit-8 (96992, Sigma, USA). The cell viability was evaluated by the examination of intracellular ATP using a luminescent cell viability assay kit (G7570, Promega, USA). The intracellular Ca^{2+} levels were detected using a calcium assay kit (640176, BD, USA) with a FlexStation 3 (Molecular Devices, Sunnyvale, America).

Table 1
Primer sequences for qRT-PCR.

Gene	Primer sequence
GnRH	F 5′-AACAATGCGTCTCTTGAGCA-3′ R 5′-TACCCATATATAAGTGGGTCG-3′
Oct-1	F 5′-AACATTAAGTGAAGGCGCCGA-3′ R 5′-AAATAATGGCCTCGATTAAGC-3′
Otx2	F 5′-CCGGTACCCAGACATCTTCA-3′ R 5′-TTGGCCACTTGTTCCACTCT-3′

Note: F, forward; R, reverse.

2.6. Statistical analysis

Immunofluorescence intensities and the overlap coefficients were measured using Image-Pro Plus version 6.0 (Media Cybernetics, Rockville, USA). The data were analysed with SPSS version 20.0 (IBM, USA). All data were normally distributed, as assessed by the One Sample Kolmogorov-Smirnov test, and are presented as the means ± standard deviations (SDs). Differences between groups were evaluated by analysis of variance (ANOVA), and comparisons between groups were made using the Bonferroni test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. GnRH and VP expression levels in the hypothalamus in the presence of reproductive impairment

Eight weeks after the first ELV surgery, 12 rats in ELV group and 13 rats in ELV sham repair group developed typical experimental left varicocele as shown in Table 2. At the end of experiments, the urine volume/water intake ratio of ELV rats dramatically increased ($P < 0.05$), while the blood pressure levels, including SBP, MBP, and DBP, decreased compared with the SO group and the ELV repair group ($P < 0.05$). No significant differences were detected between ELV group and ELV sham repair group. There were no significant differences between the SO group and the ELV repair group, as shown in Table 2.

As shown in Fig. 1A, the fluorescence intensities of immunoreactive substances of both GnRH and VP in the ME plane of the hypothalamus obviously decreased in the ELV group compared with the SO and ELV repair groups ($P < 0.05$), and the fluorescence intensities of VP obviously decreased in the ELV repair group compared with the SO group ($P < 0.05$. Fluorescence intensities of GnRH: SO group 85.23 ± 18.41, ELV group 36.61 ± 12.73, ELV sham repair group 34.44 ± 9.21, ELV repair group 72.31 ± 14.38; fluorescence intensities of VP: SO group 43.36 ± 8.38, ELV group 17.98 ± 4.71, ELV sham repair group 15.28 ± 5.02, ELV repair group 31.28 ± 5.79).

3.2. Histomorphological clues of a direct correlation between VP and GnRH in the hypothalamus

Double-immunofluorescence labelling of GnRH and VP in the ME plane of the hypothalamus of SO group revealed that the immunolocalization of GnRH was in ArcL, ArcM and ME of SO rats (Fig. 1A). The overlapped immunolocalizations of GnRH and VP were distributed throughout ArcL, ArcM and ME, and > 80% overlapped

Table 2
Urine/water ratio, blood pressures of experimental groups.

	SO group (n = 15)	ELV group (n = 12)	ELV sham repair group (n = 13)	ELV repair group (n = 14)
Water intake (ml)	26.38 ± 1.81	36.41 ± 1.65[a]	35.28 ± 2.13[a]	29.41 ± 3.65[b]
Urine volume (ml)	9.20 ± 1.07	16.91 ± 1.46[a]	17.41 ± 1.72[a]	10.32 ± 1.46[b]
urine volume/water intake	0.35 ± 0.02	0.48 ± 0.04[a]	0.49 ± 0.05[a]	0.37 ± 0.06[b]
SBP (mm Hg)	112.45 ± 8.21	92.62 ± 7.17[a]	90.87 ± 6.32[a]	109.55 ± 7.01[b]
MBP (mm Hg)	106.83 ± 9.45	83.21 ± 7.31[a]	82.25 ± 5.31[a]	105.33 ± 8.23[b]
DBP (mm Hg)	79.53 ± 8.28	67.73 ± 6.67[a]	65.91 ± 6.08[a]	81.81 ± 6.32[b]
HR (bpm)	365.22 ± 26.53	371.37 ± 25.56	370.83 ± 23.42	368.82 ± 24.38

[a] $P < 0.05$, compared to SO group.
[b] $P < 0.05$, compared to ELV group.

immunolocalizations were in ME (Fig. 1B). The mean Manders' overlap coefficient of GnRH and VP in Arc and ME was 0.743 ± 0.117. The Manders' overlap coefficient in ArcL, ArcM and ME were 0.782 ± 0.132, 0.631 ± 0.108 and 0.843 ± 0.141 respectively. The mean Manders' overlap coefficient in ELV group and ELV repair group showed no significant changes compared with SO group.

The colocalization sites of GnRH and VP were presented as white points in images in 4th column of Fig. 1A. Compared to SO group, the number of colocalization sites in both Arc and ME significantly decreased in ELV group ($P < 0.05$). Compared to ELV group, the number of colocalization sites in both ArcL and ME of ELV repair group significantly increased (Fig. 1C, $P < 0.05$). While the colocalization sites in ArcM showed no significant changes after ELV repair.

An ultrastructural electron microscopy survey of GnRH and VP immunoreactivity in the ArcL revealed many compact deposits (labelled GnRH, Fig. 2A-a) and silver-enhanced gold particles (labelled VP, Fig. 2A-b). Silver-enhanced gold particles were found in the axon terminals stained by compact deposits and tanycyte processes close to the terminals stained by compact deposits (Fig. 2A-c & d).

In situ PLA was performed to detect direct VP-GnRH interactions in brain sections, and PLA-positive signals (green fluorescence) mainly located in ArcL and ME as shown in Fig. 2B. Additionally, PLA-positive signals appeared in the cytoplasms and dendrites of neurons in ArcL (Fig. 2B).

3.3. Protein-protein interaction between VP and GnRH

After transfection and immunoprecipitation, Western blotting with anti-HA revealed that HA-tagged VP was precipitated by the anti-Myc antibody but not the control without Myc-GnRH. Similarly, the reciprocal experiment with the anti-Myc antibody indicated that Myc-GnRH was specifically coprecipitated with HA-VP (Fig. 2C).

Forty-eight hours after transfection, 93.77 ± 4.21% of GT1-7 cells in pCMV-HA-GFP group and 92.53 ± 5.10% of GT1-7 cells in pCMV-HA-GFP-VP group were GFP positive as shown in Fig. 3A. Flow cytometry analysis showed high GFP expressions in pCMV-HA-GFP and pCMV-HA-GFP-VP transfected cells. 90.10 ± 3.83% cells transfected by pCMV-HA-GFP-VP expressed VP, while no VP expressed in pCMV-HA-GFP transfected cells (Fig. 3A). Based on the Western blotting analysis, the expression level of Oct-1 in the GT1-7 cells transfected with pCMV-HA-GFP-VP increased by approximately 25% ($P < 0.05$), and the expressions of Otx2 increased by 15% (Fig. 3B, $P < 0.05$). After treatment with 200 pg/ml of VP for 4 h, Pbx1b and DREAM significantly increased in the GT1-7 cells by approximately 100% ($P < 0.05$), and the expressions of Oct-1 and Otx2 respectively increased by 80% and 150% ($P < 0.05$). The GnRH expression in the GT1-7 cells treated by 200 pg/ml of VP detected by Western blotting increased by up to 150% after treatment (Fig. 3B, $P < 0.05$).

Significant differences in Oct-1, Otx2, Pbx1b, DREAM and GnRH expressions were detected between the pCMV-HA-GFP-VP transfected cells and the 200 pg/ml VP-treated cells (Fig. 3B, $P < 0.05$).

3.4. The effects of intracellular and extracellular VP on GnRH synthesis and secretion in GT1-7 cells

The expression levels of GnRH in the supernatants of the VP200 group significantly increased compared with the control group (Fig. 3C, $P < 0.05$). The supernatant GnRH levels of the pCMV-HA-GFP-VP group were higher than those of the pCMV-HA-GFP group ($P = 0.071$) but lower than those of the VP200 group (Fig. 3C, $P < 0.05$). As detected by real-time PCR, there were significant differences between the VP200 group and the pCMV-HA-GFP-VP group with respect to the transcription levels of GnRH, Oct-1, and Otx2 (Fig. 3D, $P < 0.05$). The Otx2 mRNA levels in the pCMV-HA-GFP-VP group were higher than those in the pCMV-HA group ($P < 0.05$) but lower than those in the VP200 group (Fig. 3D, $P < 0.05$). The luciferase activity of the reporter with the GNRHI promoter sequence was highly promoted in the VP200 group ($P < 0.05$), while no obvious change in the luciferase activity was observed in the pCMV-HA-GFP-VP group (Fig. 3E).

3.5. The optimum concentration of extracellular VP promoting GnRH synthesis

After VP treatment in the supernatant (concentrations ranged from 0 to 8000 pg/ml), intracellular Ca^{2+} in GT1-7 cells gradually increased in the first 6 h and reached apex levels at 6 h post-treatment (Fig. 4A & B). Among different dosages ranging from 0 to 6000 pg/ml, 200 pg/ml was most effective in increasing intracellular Ca^{2+} (Fig. 4C, $P < 0.05$). Intracellular Ca^{2+} levels in cells treated with 7000 and 8000 pg/ml VP significantly increased compared to 200 pg/ml VP treated group (Fig. 4C, $P < 0.05$). A CCK-8 assay also detected supernatant VP (25 pg/ml–200 pg/ml), which significantly promoted GT1-7 cells proliferation compared with control cells (Fig. 4D, $P < 0.05$). A cell viability assay showed that 200 pg/ml VP significantly increased GT1-7 intracellular ATP levels 6 h after exposure compared with other dosages (Fig. 4E, $P < 0.05$). While 2000–8000 pg/ml VP significantly inhibited GT1-7 cells proliferation and decreased intracellular ATP levels compared with control cells ($P < 0.05$).

EIAs showed that 200 pg/ml VP resulted in the most significant increase of GnRH in the supernatant, and supernatant GnRH levels were positively correlated with VP exposure concentrations ranging from 0 to 200 pg/ml (Fig. 4F, $R^2 = 0.836$, $P < 0.05$). Dual-luciferase assay detected the activity of the reporter with the GNRHI promoter sequence were significantly upregulated after 200 pg/ml VP exposure (Fig. 4G, $P < 0.05$). QPCR found the transcription levels of GnRH mRNA, OCT-1 mRNA and Otx2 mRNA significantly upregulated after 200 pg/ml VP exposure (Fig. 4H, $P < 0.05$). VP at 200 pg/ml was most effective in boosting GnRH transcription, and the GnRH transcription levels were positively correlated with the exposure time at first 6 h (Fig. 4I, $R^2 = 0.909$, $P < 0.05$).

4. Discussion

Due to the vital role played by GnRH neurons in the hypothalamic-

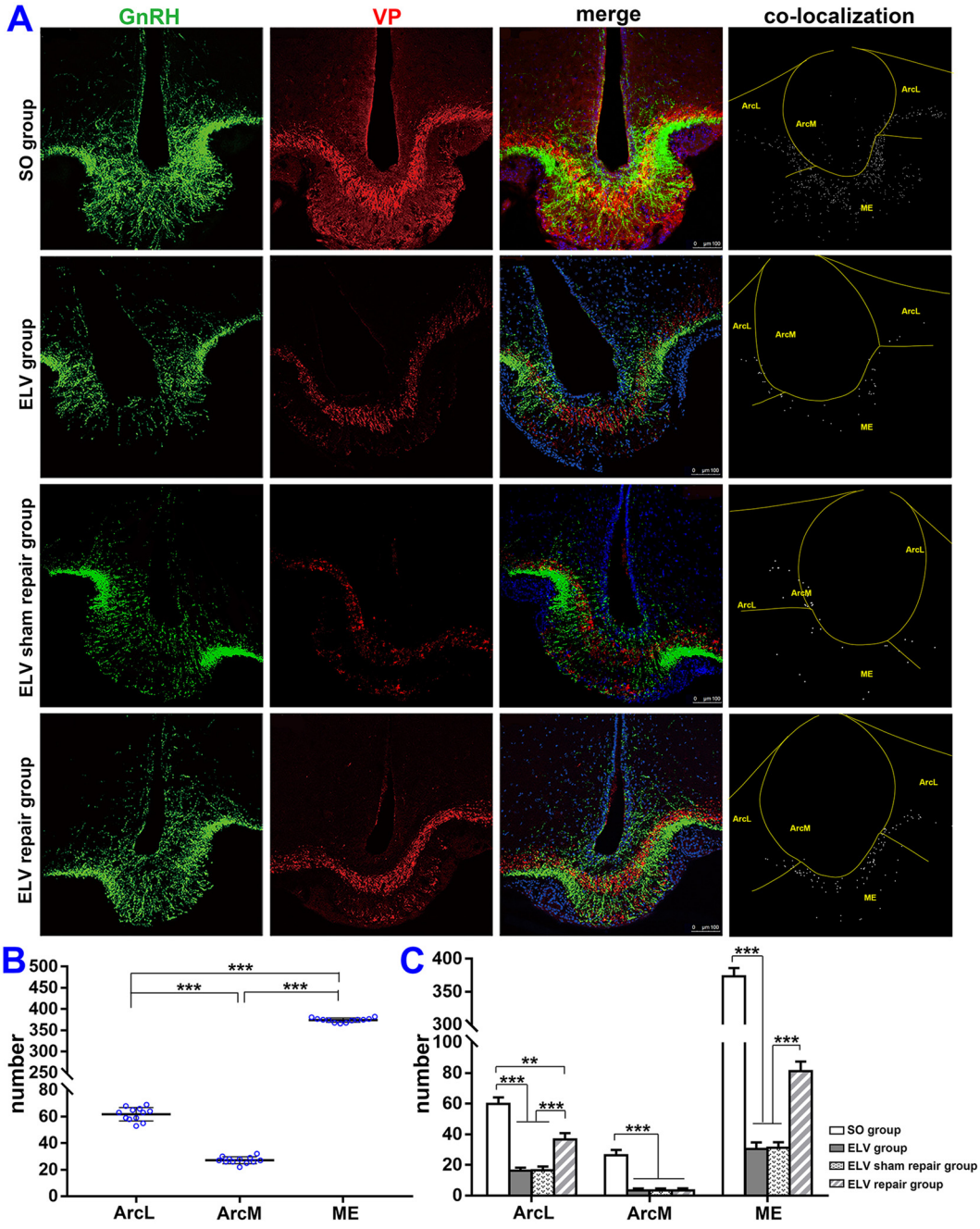

Fig. 1. The distributions and expressions of GnRH and VP in the hypothalamus of ELV rats. A) Double-immunofluorescence labelling of GnRH (green), VP (red), and nuclei (blue) in the hypothalamus of the SO group, ELV group, ELV sham repair group and ELV repair group. The 4th column showed the colocalization sites of GnRH and VP (white points) based on photos in 3rd column. B) The distribution of colocalization sites of GnRH and VP in ArcL, ArcM and ME of SO rats. C) The changes of total number of colocalization sites of GnRH and VP in ArcL, ArcM and ME in experimental groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pituitary-gonadal axis, the definite effects of VP on GnRH secretion have gained widespread attention. Based on the interesting phenomenon that the urine volume/water intake ratio dramatically increased but the blood pressure levels decreased in experimental varicocele rats, this study reported the characteristics of hypothalamic GnRH and VP expression, found histomorphological clues of a direct correlation between VP and GnRH neurons, and detected the effects of intracellular and extracellular VP on GnRH synthesis and secretion *in vitro*. The results of the present study provide a preliminary reference for the direct effect of VP on GnRH neurons and suggest that extracellular VP can effectively promote GnRH synthesis.

The ELV rat model is a classic reproductive injury model mimicking the pathology of male varicocele. Our previous study indicated that ELV establishment significantly attenuated the hypothalamic GnRH expression and spine density in the Arc, implying that the changes in GnRH neurons participate in ELV progression [24]. Additionally, the ratio of urine output to water intake was calculated in the present study to equilibrate the effects of various levels of water intake on urine output, although increased water intake directly leads to the dilution of urine and an increase in urine output. VP, also known as antidiuretic hormone (ADH), is essential for water regulation and has negative effects on blood pressure [25]. The higher urine volume/water intake ratio and the lower blood pressure levels in ELV rats made VP become a focus of research. The decreases in the fluorescence intensities of

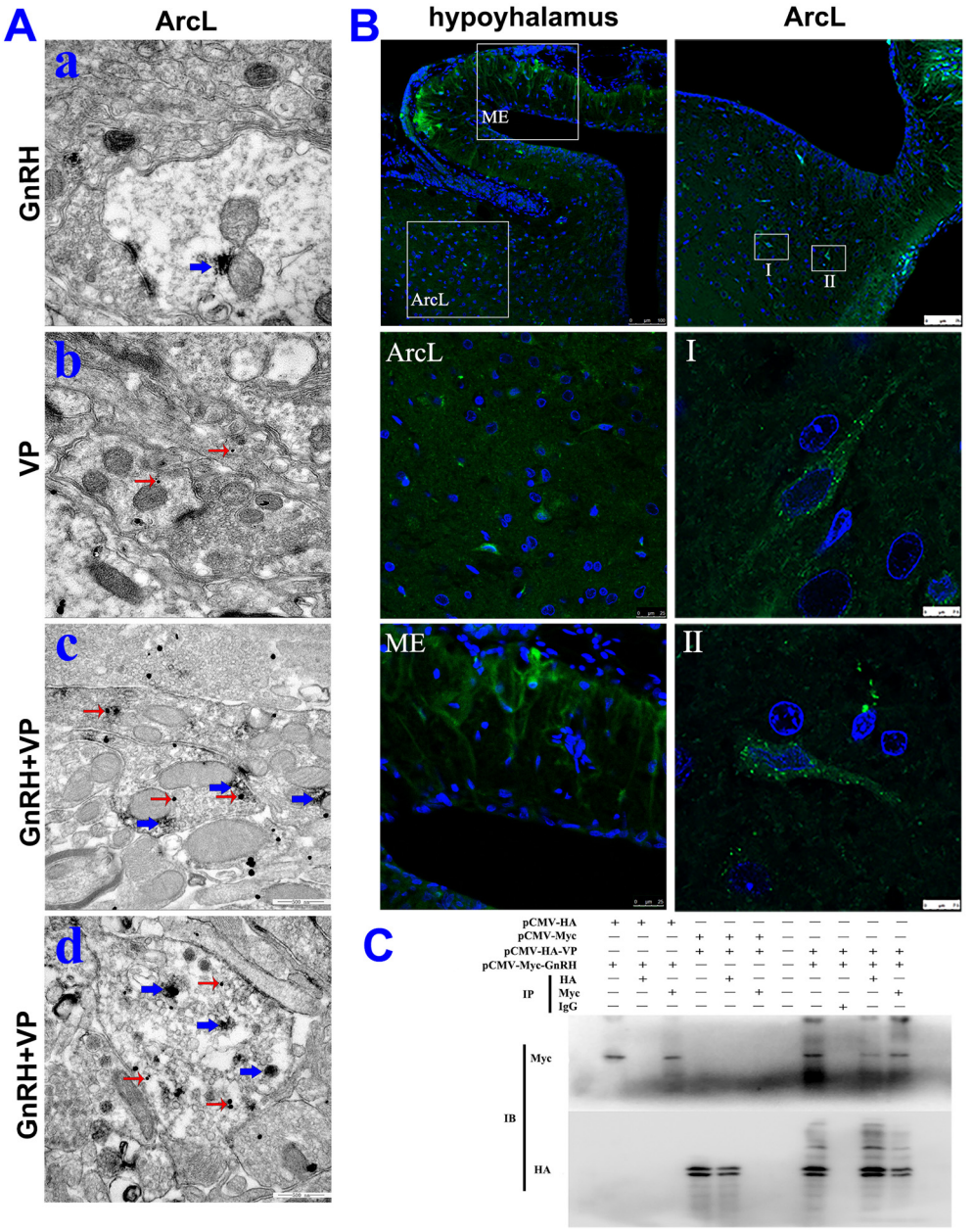

Fig. 2. The histomorphological clues of direct correlation between VP and GnRH in hypothalamus. A) Electron microscopy of GnRH and VP immunoreactivity in the ArcL. Red arrowheads show blue arrowheads show compact deposits (labelled GnRH, a), and silver-enhanced gold particles (labelled VP, b), which are found in the same and adjacent neuronal terminals (c). VP also exists in the tanycyte processes close to the terminals expressing compact deposits (d). B) Detection of GnRH-VP complexes by *in situ* PLA. The images in 1st column show the GnRH-AVP complex with DAPI-counterstained nuclei in the rat ArcL. PLA-positive signals (green fluorescence) appeared in ArcL and ME under 400× magnification. PLA-positive signals mainly located in the cytoplasm and dendrites of neurons in ArcL (I, II) under 1000× magnification as shown in images in 2nd column. C) Detected by immunoprecipitation, the indicated bands correspond to the GnRH-Myc and VP-HA proteins showed the interaction between GnRH and VP. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

immunoreactive substances of both GnRH and VP in the Arc and the ME plane of the hypothalamus in ELV rats were restored to normal levels by ELV repair, indicating that the relationship between hypothalamic GnRH and VP is one of co-variation. For ArcL adjacent to ME is the key nucleus where GnRH neurons scatters, we supposed VP projected to ME have some effects on GnRH synthesis and secretion through extracellular or intracellular pathway.

It has been reported in the literature that VP may promote the secretion of GnRH by activating the Kiss1-GPR54 system [26,27]. However, it is not yet known whether VP is directly related to GnRH neurons and GnRH secretion. Immunofluorescence histochemical staining of hypothalamic sections revealed that GnRH and VP were distributed throughout the ArcL and ME and were localized in a somewhat overlapping fashion. The results showed the expressions of GnRH and VP, as well as the colocalization sites, decreased after ELV and restored by ELV repair. The covariation and colocalization of GnRH and VP spark speculation about the interactions between them two. To further verify the existence of interactions between VP and GnRH, *in situ* PLA, and electron microscopic immunohistochemistry were employed to examine whether there is a closely adjacent relationship or a coexistent relationship that has been deemed as the basis of interactions between hypothalamic VP and GnRH. *In situ* PLA, a straightforward process for identifying protein interactions in their natural context that has rarely been used in hypothalamic sections [28], was used to observe the co-localization region, ArcL, of GnRH and VP. PLA-positive signals were found in the cytoplasm and neurites of neurons in the ArcL, consistent with the electron microscopic immunohistochemistry results revealing immunoreactive substances of GnRH and VP in the same and adjacent axon terminals. The proximity of protein locations is a prerequisite for protein-protein interactions; therefore, experiments were conducted in GT1-7 cells to investigate whether AVP influences the synthesis and release of GnRH.

GT1-7 cells were treated in two ways; specifically, they were either dosed or transfected with VP, reflecting the extracellular and intracellular actions of VP, respectively. A dose of 200 pg/ml VP in culture supernatants was effective in stimulating GnRH in the supernatant and cell extracts; this concentration was close to the concentration of VP in the rat serum of the SO group. After preliminarily investigating

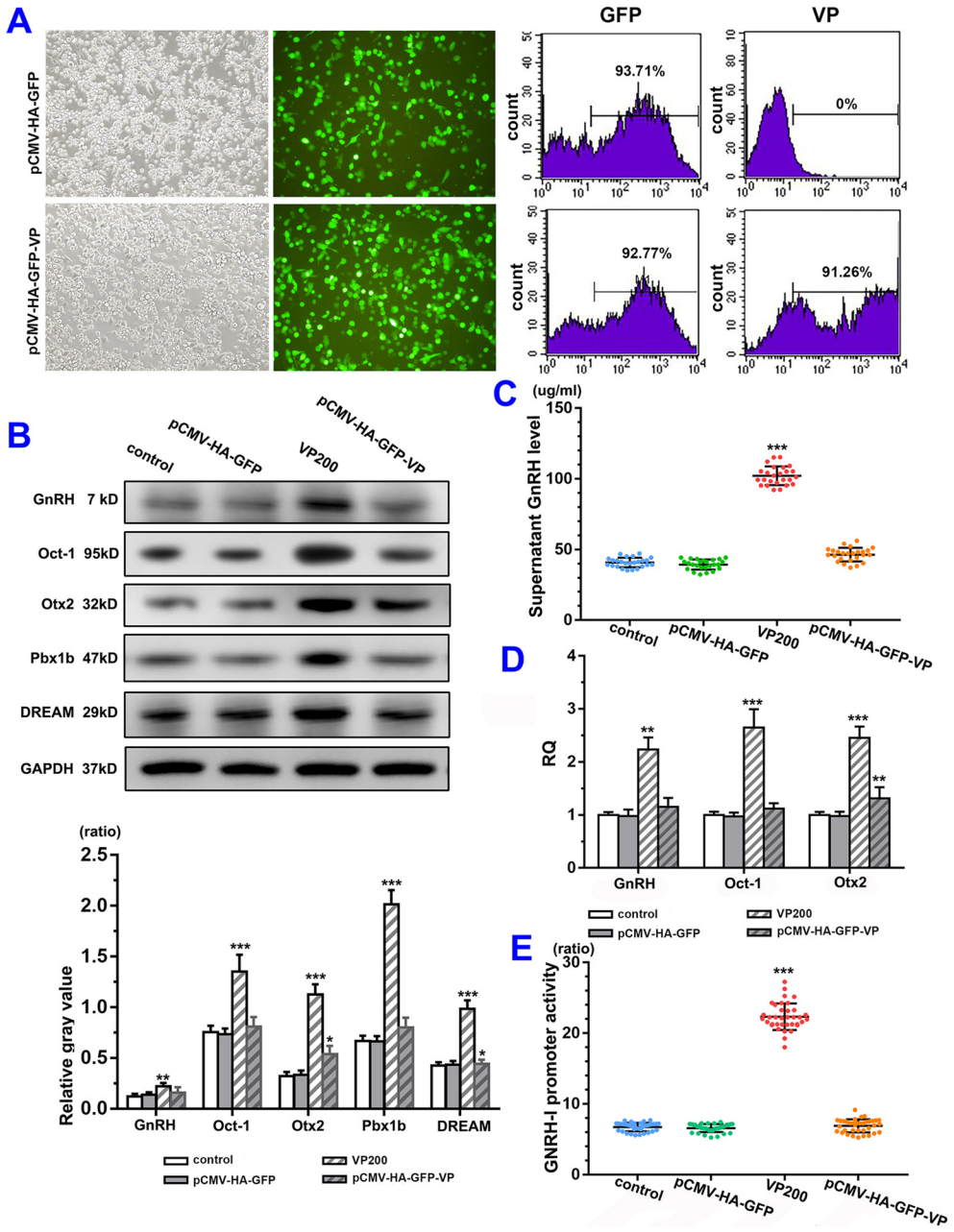

Fig. 3. The effects of intracellular and extracellular VP on GnRH synthesis and secretion in GT1-7 cells. A) The expressions of GFP and VP in pCMV-HA-GFP and pCMV-HA-GFP-VP transfected GT1-7 cells detected by flow cytometry assay. B) Immunoblots of GnRH and relative transcription factors in GT1-7 cells with different treatments. Compared with cells transfected with the empty plasmid (lane 2), the GnRH, Otx2 expression levels were increased in the cells transfected with a plasmid carrying VP (lane 4) and were lower than the levels in the cells treated with 200 pg/ml of VP (lane 3). C) Supernatant VP at 200 pg/ml significantly increased the supernatant GnRH level. D) Supernatant VP at 200 pg/ml significantly increased the transcription levels of GnRH, Oct-1 and Otx2 in GT1-7 cells. E) Supernatant VP at 200 pg/ml significantly promoted the luciferase activity of GnRH promote. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

the effects of VP on the transcription of GnRH and some relevant transcription factors, including Oct-1 and Otx2, which have been shown to bind to the regulatory regions of the GnRH gene and boost GnRH neuron pulse generation [29], the experiments demonstrate that extracellular administration of VP is more effective in stimulating GnRH and relevant transcription factors than transfecting the cells with VP. We surmise that the presence of paracrine-generated or projected VP in close proximity to GnRH neurons may promote the synthesis and secretion of GnRH. The evidence also suggests that the stimulation effects of VP mostly occur through extracellular stimulation, but it is not clear whether VP can act through presynaptic release, although there is a possibility that VP could enter GnRH neurons, considering the intracytoplasmic-positive PLA signal in neurons of the Arc.

A series of cell experiments were conducted to study the optimum concentration of extracellular VP promoting GnRH synthesis. Significant dose-effect correlations were detected between supernatant GnRH and VP administration and between VP and intracellular Ca^{2+}, indicating that the effective concentration range of extracellular VP is 25–200 pg/ml. The time-effect correlation indicates the promoting effects of VP on GnRH neurons achieving their apex levels at 6 h post-treatment, lasting approximately 3 h. The phenomenon indicates that the stimulation of VP on GnRH secretion is not immediate; rather, it is durative. Most GnRH neurons generate $[Ca^{2+}]_i$ transients throughout development, and calcium influx has been indicated to act as vital facilitators of GnRH secretion. As for the results showing the effects of 200 pg/ml extracellular VP on promoting GT1-7 proliferation and viability, as well as intracellular Ca^{2+}, stimulation on intracellular Ca^{2+} may be one of the key mechanisms of extracellular VP on GnRH secretion. VP in concentrations as low as 25 pg/ml could increase cell viability, and VP in concentrations as low as 50 pg/ml could increase cell proliferation. While only VP concentrations higher than 50 pg/ml promoted GnRH transcription and promoter activity, and higher than 200 pg/ml VP showed effective on stimulating intracellular Ca^{2+}. These results indicate the promotion on cell viability which is one index of energy mechanisms may be one of the early processes of the effects of VP on GT1-7 cells, and the promotion on intracellular Ca^{2+} may be

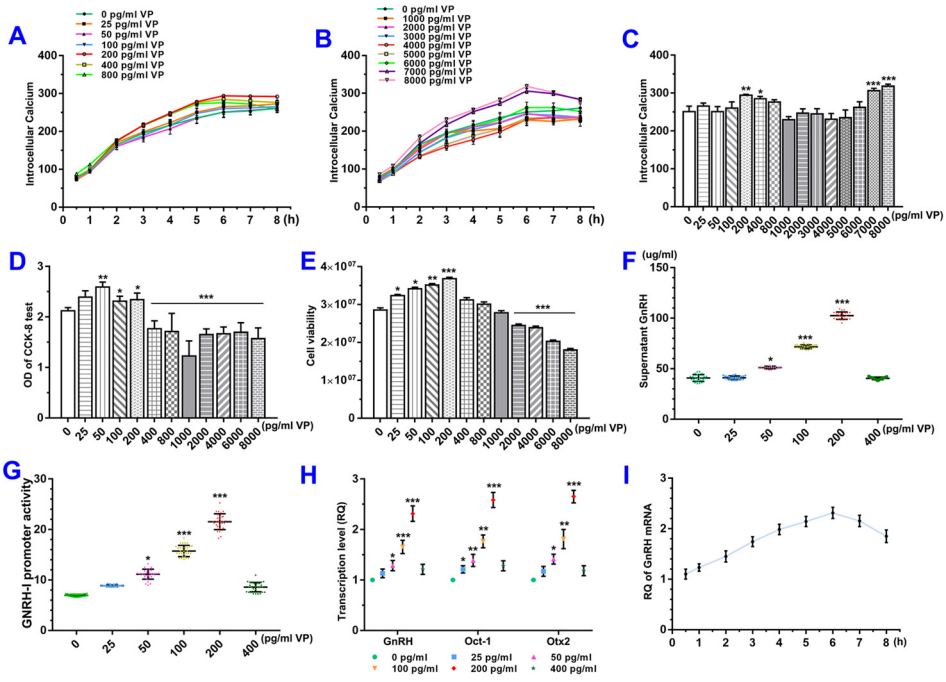

Fig. 4. VP at 200 pg/ml was most effective in boosting GnRH synthesis. After VP exposure, intracellular Ca^{2+} gradually increased in the first 6 h and reached apex levels at 6 h post-treatment (A, B). Among different dosages ranging from 0 to 6000 pg/ml, 200 pg/ml was most effective in increasing intracellular $Ca2^{+}$ (C), promoting cell proliferation (D) and viability (E), increasing GnRH level in supernatant (F), promoting the luciferase activity of GnRH reporter (G) and upregulating the transcription levels of GnRH mRNA, OCT-1 mRNA and Otx2 mRNA (H). The GnRH transcription levels were positively correlated with the exposure time at first 6 h in 200 pg/ml VP treated cells (I). *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

partially responsible for the stimulatory effects of 200 pg/ml VP. In addition, the *in vitro* experiments showed VP in concentrations higher than 400 pg/ml decreased the cell proliferation, and VP in concentrations higher than 2000 pg/ml decreased the intracellular ATP. While VP in concentrations higher than 7000 pg/ml increased Ca^{2+}, which may indicate some cytotoxicity of high concentration of extracellular VP on GT1-7 cells. As for the sensitive changes of intracellular ATP and Ca^{2+} in accordance with concentration of VP, we speculate that extracellular VP upregulates the synthesis of GnRH through activating energy metabolism and increasing intracellular Ca^{2+}, and further experiments will be conducted to investigate the definite effects and mechanisms of extracellular VP on $[Ca^{2+}]_i$ transients and energy metabolism. It is worth noting that VP concentrations > 1000 pg/ml decreased GnRH transcription and secretion without decreasing the expression levels of relevant transcription factors, suggesting that the upregulation of Oct-1 and Otx2 may facilitate the activation of GT1-7 cells but is not crucial for the GnRH stimulatory effects of extracellular VP. In summary, the effects of VP on the synthesis activity of GT1-7 cells based on histomorphological clues of direct correlations were reported for the first time in this study, but the concrete underlying mechanisms have not yet been revealed. These mechanisms will be the research focus of future studies.

5. Conclusion

Starting from the increased urine volume/water intake ratio and the decreased blood pressure levels following experimental varicocele, this study reported the coexistence and co-variation of VP and GnRH in ArcL, discovered histomorphological evidence of direct correlations between VP and GnRH neurons, and detected dose- and time-effect correlations between extracellular VP and GnRH synthesis in GT1-7 cells. The results of the present study provide a preliminary reference for the direct effect of VP on GnRH neurons and suggest that extracellular VP promotes GnRH synthesis. The results supply new opinions for basic and clinical studies on the regulation of GnRH secretion and will be conducive to explain the effects and mechanisms of VP acting on reproduction.

Author contributions

Zhu Zhu performed the genetic studies and drafted the manuscript. Xiaozhen Zhao cultured the cells and conducted the transduction. Feng Huang took the immunofluorescence and PLA assays. Feng Wang established the experimental left varicocele rat model and sampled. Wei Wang conceived of the study, participated in its design, and helped to revised draft the manuscript. All authors read and approved the final manuscript.

Competing interests

All authors declare no competing interests.

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References

[1] R. Burgus, et al., Primary structure of the ovine hypothalamic luteinizing hormone-releasing factor (LRF) (LH-hypothalamus-LRF-gas chromatography-mass spectrometry-decapeptide-Edman degradation), Proc. Natl. Acad. Sci. U. S. A. 69 (1) (1972) 278–282.
[2] P.H. Seeburg, et al., The mammalian GnRH gene and its pivotal role in reproduction, Recent Prog. Horm. Res. 43 (1987) 69–98.
[3] K. Maeda, et al., Neurobiological mechanisms underlying GnRH pulse generation by the hypothalamus, Brain Res. 1364 (2010) 103–115.
[4] V.H. Lee, et al., Gonadotropin-releasing hormone: regulation of the GnRH gene, FEBS J. 275 (22) (2008) 5458–5478.
[5] N. Rave-Harel, et al., TALE homeodomain proteins regulate gonadotropin-releasing hormone gene expression independently and via interactions with Oct-1, J. Biol. Chem. 279 (29) (2004) 30287–30297.
[6] C.L. Jasoni, et al., Calcium dynamics in gonadotropin-releasing hormone neurons, Front. Neuroendocrinol. 31 (3) (2010) 259–269.
[7] C.H. Wideman, H.M. Murphy, Modulatory effects of vasopressin on glucose and

protein metabolism during food-restriction stress, Peptides 14 (2) (1993) 259–261.
[8] T. Tsunematsu, et al., Vasopressin increases locomotion through a V1a receptor in orexin/hypocretin neurons: implications for water homeostasis, J. Neurosci. 28 (1) (2008) 228–238.
[9] M.M. Lim, L.J. Young, Neuropeptidergic regulation of affiliative behavior and social bonding in animals, Horm. Behav. 50 (4) (2006) 506–517.
[10] Z.R. Donaldson, L.J. Young, Oxytocin, vasopressin, and the neurogenetics of sociality, Science 322 (5903) (2008) 900–904.
[11] D.A. Wagenaar, et al., A hormone-activated central pattern generator for courtship, Curr. Biol. 20 (6) (2010) 487–495.
[12] G.C. Harris, H.D. Nicholson, Characterisation of the biological effects of neurohypophysial peptides on seminiferous tubules, J. Endocrinol. 156 (1) (1998) 35–42.
[13] S.J. Assinder, et al., Oxytocin and vasopressin expression in the ovine testis and epididymis: changes with the onset of spermatogenesis, Biol. Reprod. 63 (2) (2000) 448–456.
[14] B.G. Kasson, et al., Identification and characterization of arginine vasopressin-like substances in the rat testis, J. Biol. Chem. 260 (9) (1985) 5302–5307.
[15] T.M. Hagedorn, et al., Oxytocin and vasopressin stimulate anion secretion by human and porcine vas deferens epithelia, Biol. Reprod. 77 (3) (2007) 416–424.
[16] B.L. Smarr, et al., Oestrogen-independent circadian clock gene expression in the anteroventral periventricular nucleus in female rats: possible role as an integrator for circadian and ovarian signals timing the luteinising hormone surge, J. Neuroendocrinol. 25 (12) (2013) 1273–1279.
[17] B. Vida, et al., Evidence for suprachiasmatic vasopressin neurones innervating kisspeptin neurones in the rostral periventricular area of the mouse brain: regulation by oestrogen, J. Neuroendocrinol. 22 (9) (2010) 1032–1039.
[18] R. Piet, et al., Estrogen permits vasopressin signaling in preoptic kisspeptin neurons in the female mouse, J. Neurosci. 35 (17) (2015) 6881–6892.
[19] R.J. Kline, et al., Arginine vasotocin V1a2 receptor and GnRH-I co-localize in preoptic neurons of the sex changing grouper, Epinephelus adscensionis, Gen. Comp. Endocrinol. 225 (2016) 33–44.
[20] H. Han, et al., The prevalence and association of varicoceles on male patients with benign prostatic hyperplasia/lower urinary tract symptoms, Urology 90 (2016) 97–100.
[21] P.L. Mellon, et al., Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis, Neuron 5 (1) (1990) 1–10.
[22] D.C. Saypol, et al., Influence of surgically induced varicocele on testicular blood flow, temperature, and histology in adult rats and dogs, J. Clin. Invest. 68 (1) (1981) 39–45.
[23] M.I. Ozturk, et al., Increased sperm DNA damage in experimental rat varicocele model and the beneficial effect of varicocelectomy, Int. J. Fertil. Steril. 6 (2) (2012) 95–100.
[24] Z. Zhu, et al., Morinda officinalis polysaccharides stimulate hypothalamic gnrh secretion in varicocele progression, Evid. Based Complement. Alternat. Med. 2017 (2017) 9057959.
[25] V.E. Torres, Vasopressin in chronic kidney disease: an elephant in the room? Kidney Int. 76 (9) (2009) 925–928.
[26] S. Higo, et al., Mapping of Kisspeptin Receptor mRNA in the whole rat brain and its co-localisation with oxytocin in the paraventricular nucleus, J. Neuroendocrinol. 28 (4) (2016).
[27] X. Liu, A.E. Herbison, Kisspeptin regulation of neuronal activity throughout the central nervous system, Endocrinol. Metab. (Seoul) 31 (2) (2016) 193–205.
[28] S. Pierre, K. Scholich, Toponomics: studying protein-protein interactions and protein networks in intact tissue, Mol. BioSyst. 6 (4) (2010) 641–647.
[29] M.L. Givens, et al., Phylogenetic footprinting reveals evolutionarily conserved regions of the gonadotropin-releasing hormone gene that enhance cell-specific expression, Mol. Endocrinol. 18 (12) (2004) 2950–2966.