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Original Article

AVPV *Kiss1* neuron-specific knockdown of purinergic P2X2 receptor suppresses LH surge and ovulation in *Kiss1-Cre* rats

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Abstract. Ovulation disorders are a major cause of low pregnancy rates and infertility in humans and livestock. Kisspeptin neurons located in the anteroventral periventricular nucleus (AVPV) are responsible for the generation of the gonadotropin-releasing hormone (GnRH)/luteinizing hormone (LH) surge and the consequent ovulation in female rodents. The present study aimed to examine whether purinergic neurons are direct upstream stimulators of AVPV kisspeptin neurons that trigger the GnRH/LH surge and consequent ovulation in *Kiss1-Cre* rats. We specifically knocked down the mRNA expression of the *P2rx2* purinergic receptor in AVPV kisspeptin neurons by administering an adeno-associated virus (AAV) vector containing Cre-dependent *P2rx2* short hairpin RNA (shRNA) into the AVPV region of ovariectomized (OVX) *Kiss1-Cre* rats treated with a proestrus level of estradiol-17β (OVX + high E2) or ovary-intact *Kiss1-Cre* rats. The E2-induced afternoon LH surge was significantly suppressed by AVPV kisspeptin neuron-specific knockdown of *P2rx2* in OVX + high E2 *Kiss1-Cre* rats compared with scrambled shRNA-treated control OVX + high E2 *Kiss1-Cre* rats. Furthermore, the specific knockdown of *P2rx2* in AVPV kisspeptin neurons largely disrupted the estrous cycle, spontaneous LH surge, and ovulation in ovary-intact *Kiss1-Cre* rats. These findings suggest that purinergic neurons directly stimulate AVPV kisspeptin neurons via P2X2 receptors (P2RX2) to induce the GnRH/LH surge and consequent ovulation in female rats.
Key words: ATP, GnRH, Kisspeptin, Purinergic neuron

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Ovulation disorders are a major cause of infertility and low pregnancy rates in humans and livestock. Reproduction in mammals is controlled by a complex network comprising the hypothalamus–pituitary–gonadal (HPG) axis, including the neuroendocrine system and its central controllers. Hypothalamic kisspeptin neurons regulate the HPG axis by directly stimulating the gonadotropin-releasing hormone (GnRH) neurons in mammals, including rodents, ruminants, and primates [1–4]. Mutations or deletions in *GPR54*/*Gpr54* (the kisspeptin receptor gene) or *KISS1*/*Kiss1* (the kisspeptin gene) result in infertility and hypogonadotropic hypogonadism in humans and rodents [5–9]. Kisspeptin neurons located in the anteroventral periventricular nucleus (AVPV) in rodents and the preoptic area (POA) in other species, such as primates, ruminants, pigs, and musk shrews, are responsible for the generation of the GnRH/luteinizing hormone (LH) surge and consequent ovulation [10–16]. Another major population of kisspeptin neurons located in the arcuate nucleus (ARC) is considered to regulate tonic (pulsatile) GnRH/LH release and folliculogenesis in female mammals, as reviewed elsewhere [2, 17]. In female rodents, an increase in the levels of circulating estrogen secreted from mature follicles at the proestrus stage exerts a positive feedback effect on AVPV kisspeptin neurons, consequently inducing a GnRH/LH surge. Preovulatory levels of estradiol-17β (E2) upregulate c-Fos (a neuronal activation marker) levels and *Kiss1* gene expression in AVPV kisspeptin neurons of ovariectomized (OVX) rats and mice [10, 18, 19]. Notably, diestrous levels of E2, which cannot induce an LH surge, upregulated AVPV *Kiss1* expression in OVX female rats [10], suggesting that upstream stimulatory neuronal activation by preovulatory estrogen levels are required to trigger AVPV kisspeptin neuronal activation.

Our recent study demonstrated that signaling by the adenosine 5′-triphosphate (ATP)-P2X2 receptor (P2RX2, encoded by the *P2rx2* gene), an ionotropic purinergic receptor, could be an upstream stimulatory regulator of AVPV kisspeptin neurons to induce the GnRH/LH surge and consequent ovulation in female rats [20]. This is because the estrogen-induced LH surge was blocked in wild-type OVX rats upon administering a purinergic receptor antagonist, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), into the AVPV region, wherein P2RX2-expressing kisspeptin neurons are located. Furthermore, the administration of ATP into the AVPV region immediately caused a surge-like increase in plasma LH levels in the morning in OVX wild-type rats, but not in OVX *Kiss1* knockout (KO) rats treated with preovulatory levels of E2. Moreover, proestrous levels of E2, but not diestrous E2 levels, activated purinergic neurons in the hindbrain. These findings suggest that AVPV kisspeptin neurons, activated by ATP derived from hindbrain purinergic neurons, induce the GnRH/LH surge and consequent ovulation in the presence of preovulatory levels of estrogen [20]. However, whether ATP directly stimulates AVPV kisspeptin neurons via purinergic receptor activity remains unclear.

Therefore, the present study aimed to examine whether purinergic neurons "directly" activate AVPV kisspeptin neurons to trigger

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the GnRH/LH surge and consequent ovulation in the presence of preovulatory levels of E2. For this, we specifically knocked down *P2rx2* mRNA expression in AVPV kisspeptin neurons by administering an adeno-associated virus (AAV) vector containing *P2rx2* short hairpin RNA (shRNA) into the AVPV region in genetically modified *Kiss1-Cre* female rats [21]. We then determined whether AVPV *Kiss1*-specific *P2rx2* knockdown attenuated the E2-induced LH surge in OVX *Kiss1-Cre* rats as well as the spontaneous LH surge and ovulation in ovary-intact *Kiss1-Cre* rats.

Materials and Methods

Animals

Kiss1-Cre male and female rats were generated in our laboratory, and the *Kiss1*-specific Cre recombinase activity was validated in our previous studies [21, 22]. Briefly, *Kiss1-Cre*-activated tdTomato rats, obtained by crossing *Kiss1-Cre* heterozygous and *Cre*-dependent td-Tomato reporter rats [LE-Tg(Gt(ROSA)26Sor-CAG-tdTomato)24Jfhy] [23], showed co-expression of *Kiss1* and tdTomato in the AVPV and ARC regions [21]. Furthermore, *Kiss1*-specific *Kiss1* KO male and female rats, obtained by crossing *Kiss1-Cre* heterozygous and *Kiss1*-floxed rats, lacked *Kiss1* expression in the brain and exhibited hypogonadotropic hypogonadism [21, 22]. The female *Kiss1-Cre* heterozygous rats (aged 8–16 weeks; 200–320 g body weight) used herein were obtained by crossing *Kiss1-Cre* male and wild-type Iar:Wistar-Imamichi female rats (Institute for Animal Reproduction, Kasumigaura, Japan), and were kept under controlled conditions of temperature (22 ± 3°C) and lighting (14 h light and 10 h darkness; lights on at 0500 h and off at 1900 h) with unrestricted access to standard laboratory chow (CE-2; Clea, Tokyo, Japan) and water. Female *Kiss1-Cre* rats with at least two consecutive regular 4-day estrous cycles were used in this experiment. Brain surgery was performed under anesthesia using an intraperitoneal injection of a mixture of 0.375 mg/kg medetomidine (Domitol; Nippon Zenyaku Kogyo Co., Ltd. Fukushima, Japan), 2 mg/kg midazolam (Midazolam; Sandoz, Tokyo, Japan), and 2.5 mg/kg butorphanol (Vetorphale; Meiji Animal Health Co., Ltd., Tokyo, Japan). Other surgical procedures were performed under anesthesia using an intraperitoneal injection of 26.7 mg/kg ketamine (Ketamin Inj. 5%; Fujita Pharmaceutical Co., Ltd., Tokyo, Japan) and 5.3 mg/kg xylazine (Seractal 2%; Elanco Japan Inc., Tokyo, Japan) mixture followed by inhalation of 1–3% isoflurane (Isoflurane Inhalation Solution; Vitaris Pharmaceuticals Inc., Tokyo, Japan) or isoflurane alone. All animal experiments were approved by the Committee on Animal Experiments of the Graduate School of Bioagricultural Sciences, Nagoya University.

Experimental design to examine the effects of AVPV kisspeptin neuron-specific knockdown of P2rx2 mRNA expression on estrogen-induced LH surge, estrous cyclicity, spontaneous LH surge, and ovulation

To examine the effects of AVPV kisspeptin neuron-specific knockdown of *P2rx2* mRNA expression on E2-induced LH surge, *Kiss1-Cre* female rats were bilaterally injected with AAV-U6-*P2rx2*-shRNA-flox-CMV-EGFP (AAV-*P2rx2*-shRNA-EGFP, 3.1×10^{13} viral genome/ml; n = 8) or AAV-U6-scrambled-shRNA-flox-CMV-EGFP (AAV-scrambled-shRNA-EGFP, 2.5×10^{13} viral genome/ml; n = 4) into the AVPV region through a cannula (C315I, Plastics One, Roanoke, VA, USA) with its tip at 0.12 mm posterior to the bregma, ± 0.5 mm from the midline, and 8.2 mm below the surface of the skull according to the coordinates of a rat brain atlas [24]. A total volume of 1 µl AAV per injection site was injected at a rate of 0.25 µl/min to each side using a microinfusion pump (EP-60, EICOM, Kyoto, Japan) and syringe (Hamilton Company, Reno, NV, USA). Fourteen days after the AAV injection, the *Kiss1-Cre* rats were OVX and then immediately received a subcutaneous implant of Silastic tubing (inner diameter 1.57 mm, outer diameter 3.18 mm, and length 25 mm; Dow Corning, Midland, MI, USA) filled with E2 (Sigma-Aldrich, St. Louis, MO, USA) dissolved in peanut oil (Sigma-Aldrich) at 20 µg/ml to produce a negative-feedback level of plasma E2 (low E2) [25]. Five days after the low E2 implantation, the tubing was replaced with another Silastic tubing (28 mm in length) filled with E2 dissolved in peanut oil at 1000 µg/ml to produce a positive-feedback level of plasma E2 (high E2) to induce the LH surge [20, 26]. Two days after the high E2 implantation, the OVX + high E2 *Kiss1-Cre* rats were subjected to blood sampling, and blood samples (100 µl) were collected every 1 h from 1000 h to 2100 h through a silicon cannula (inner and outer diameters being 0.5 mm and 1.0 mm, respectively; Shin-Etsu Polymer, Tokyo, Japan) inserted into the right atrium via the jugular vein on the day before the blood sampling. Plasma samples (25 µl) were obtained by immediate centrifugation and stored at –20°C until assaying for LH. On the day after blood sampling, the animals were deeply anesthetized with sodium pentobarbital (40 mg/kg; Tokyo Chemical Industry, Tokyo, Japan) and perfused with 4% paraformaldehyde (Sigma-Aldrich). The brains were immediately removed and post-fixed in the same fixative overnight at 4°C, followed by immersion in 30% sucrose in 0.05 M phosphate buffer at 4°C until the brains sank. According to the rat brain atlas [24], serial 50-µm coronal sections of the hypothalamus, including the AVPV (from 0.12 mm anterior to 0.84 mm posterior to the bregma) or ARC (1.72–4.36 mm posterior to the bregma) regions, were made using a cryostat (CM1800, Leica Biosystems, Wetzlar, Germany). The injection sites of the AAV vectors were evaluated by direct observation of enhanced green fluorescent protein (EGFP) under a fluorescence microscope (ApoTome.2, Carl Zeiss, Oberkochen, Germany).

To determine the effects of *P2rx2* knockdown on the estrous cycle, spontaneous LH surge, and ovulation, ovary-intact *Kiss1-Cre* rats were injected with AAV-*P2rx2*-shRNA-EGFP (n = 4) or AAV-scrambled-shRNA-EGFP (n = 5) into the AVPV region. Vaginal smears were examined daily for at least three weeks after AAV injection to identify the estrous stage of the animals. Blood samples (100 µl) were then collected every 1 h between 1000 h and 2100 h on the day of proestrus or the corresponding day in AVPV scrambled shRNA-treated control *Kiss1-Cre* and AVPV *P2rx2* shRNA-treated *Kiss1-Cre* rats. Plasma samples (25 µl) were collected and stored as described above. Ovaries were retrieved and weighed on the next day of blood sampling. The oocyte–cumulus complexes were collected from the oviduct ampullae and counted under a stereomicroscope (SZ40; Olympus, Tokyo, Japan). The ovaries were examined under a stereomicroscope to evaluate the presence of the corpora lutea (CLs). The brains were collected to inspect the AAV vector-injection sites, as described above.

Double staining for Kiss1 and EGFP using in situ hybridization for Kiss1 and immunohistochemistry for EGFP

To evaluate the transfection efficacy of the AAV vectors in the AVPV kisspeptin neurons, we performed *in situ* hybridization for *Kiss1* mRNA and immunohistochemistry for EGFP in the AVPV of OVX + high E2 *Kiss1-Cre* rats injected with either AAV-*P2rx2*-shRNA-EGFP or AAV-scrambled-shRNA-EGFP. Free-floating brain sections that included the AVPV region (every second section, 10 sections in total) were incubated with anti-EGFP antibody (1:1000; Abcam, Cambridge,

UK, Cat# 13970, RRID: AB_300798) for 2 nights at 4°C. The brain sections were then fixed again with 10% neutral buffer formalin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for 10 min and rinsed with 0.05 M PBS containing 0.2% glycine. The sections were incubated with 1 µg/ml digoxigenin (DIG)-labeled *Kiss1* complementary RNA (cRNA) probe (position 33–349, AY196983) overnight at 60°C, as previously described [26]. The hybridized DIG-labeled probe was detected using a peroxidase (POD)-conjugated anti-DIG antibody (Roche Diagnostics, Mannheim, Germany) and the TSA Plus Biotin System (1:100; Akoya Bioscience, Marlborough, MA, USA). Sections were then incubated with DyLight 594-conjugated streptavidin (1:500; Thermo Fisher Scientific, Waltham, MA, USA, Cat# 21842) for 20 min at room temperature (RT) to detect *Kiss1* mRNA, followed by Alexa Fluor 488 goat anti-chicken IgY secondary antibody (1:800; Thermo Fisher Scientific, Cat# A11039, RRID: AB_2534096) incubation for 2 h at RT to detect the EGFP protein. Images of cells with *Kiss1*-positive and EGFP-immunopositive signals were captured using a fluorescence microscope (ApoTome.2, Carl Zeiss). The numbers of *Kiss1*-expressing and *Kiss1*-expressing EGFP-immunopositive cells were counted at least twice bilaterally, and the average was calculated.

Radioimmunoassay for LH

Plasma LH concentrations were measured by a double-antibody radioimmunoassay, as previously described [27], using a rat LH radioimmunoassay kit including rabbit anti-rat LH antiserum (RRID: AB_2665533) provided by the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA, USA) and were expressed in terms of the National Institute of Diabetes and Digestive and Kidney Diseases rat LH reference preparation-3. The lowest detectable LH concentration was 3.9 pg/tube for 25-µl plasma samples, and the intra- and inter-assay coefficients of variation were 5.6% and 6.4% at 2.3 ng/ml and 1.4 ng/ml, respectively.

Evaluation of P2rx2-shRNA candidates using in vitro analysis for P2rx2 knockdown by P2rx2 siRNAs

Three small interfering RNA (siRNA) constructs targeting mouse *P2rx2* (Table 1) were designed using the siDirect software (http://sidirect2.rnai.jp) [28]. The MISSION siRNA Universal Negative Control (siRNA-NC; Sigma-Aldrich) was used as the negative control for transfection [29]. The mHypoA-51 immortalized mouse neuronal cells isolated from the hypothalamus of adult female C57BL/6 mice [30, 31] were used to evaluate the *P2rx2* siRNAs. The cells were cultured in phenol red-free DMEM (Sigma-Aldrich) supplemented with 4 mM L-glutamine (Sigma-Aldrich), 1% penicillin/streptomycin (Gibco, Thermo Fisher Scientific), and 5% charcoal-stripped fetal bovine serum (Biowest, Kansas City, MO, USA). One of the three *P2rx2*-siRNAs (1 nM) or siRNAs-NC (1 nM) was transfected into the cells using the Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific). Twenty-four or 48 h after transfection, the cells were treated with E2 (1 nM) for 4 h according to previous studies [20, 31], and the cells were collected for analysis of *P2rx2* mRNA expression.

Real-time RT-PCR analysis for P2rx2 mRNA expression

P2rx2 expression in mHypoA-51 cells was measured using real-time RT-PCR as previously described [20]. Total RNA was purified from mHypoA-51 cells using ISOGEN (Nippon Gene, Tokyo, Japan), and ReverTra Ace (TOYOBO, Osaka, Japan) was used to synthesize full-length cDNA. The obtained cDNA was used as a template in a PCR with primers for *P2rx2* and *Actb* with the following sequences: forward and reverse primers for mouse *P2rx2*, 5′-GCGTTCTGGGACTACGAGAC-3′ and 5′-GATCCCCTTGACTTTGGTGA-3′ (GenBank accession no. AY044240.2) and for mouse *Actb*, 5′-GGTGGGAATGGGTCAGAAGG-3′ and 5′-GTACATGGCTGGGGTGTTGA-3′ (GenBank accession no. NM_007393.5), respectively. Real-time RT-PCR analysis was performed using an ABI 7500 real-time system (Thermo Fisher Scientific) with the THUNDERBIRD qPCR Mix (TOYOBO). The cycling protocol was as follows: predenaturation for 1 min at 95°C, and 40 amplification cycles of 15 sec at 95°C and 1 min at 60°C. The specificity of the amplification products was confirmed by dissociation curve analysis (60–95°C) after the 40-cycle amplification. The relative expression levels of the *P2rx2* mRNA were normalized to those of *Actb* and the fold changes were calculated using the $2^{-\Delta\Delta CT}$ method.

Plasmid construction and AAV vector production for Cre-dependent knockdown of P2rx2 expression

DNA constructs encoding Cre-activated *P2rx2*-targeted shRNA were designed using the target sequences of siRNA No. #2, based on the results of the *in vitro* siRNA experiments described above

Table 1. DNA and RNA sequences of siRNAs against mouse *P2rx2*

siRNA No.	Target sequences (21 nt) +2 nt overhang	Antisense (guide sequence) +2 nt overhang	Sense (passenger sequence) +2 nt overhang	GeneBank Accession No.
#1	cagggaaattcagtctcattccc	gaaugagacugaauuucccug	gggaaauucagucucauuccc	AY044240.2
#2	gggattcgaattgacgttattgt	aauaacgucaauucgaauccc	gauucgaauugacguuauugu	AY044240.2
#3	ttgccaaatattacaagataaac	uuaucuuguaauauuuggcaa	gccaaauauuacaagauaaac	AY044240.2

The sequences are shown from 5' to 3', left to right.

Table 2. DNA sequences of shRNAs against rat *P2rx2*

shRNA type	Target sequence	Antisense (guide sequence)	Sense (passenger sequence)	GeneBank Accession No.
P2rx2 shRNA	ggattcgaatcgatgttat	ataacatcgattcgaatcc	ggattcgaatcgatgttat	NM_053656.3
Scrambled shRNA		attaatcggagttatcgtg	cacgataactccgattaat	

The sequences are shown from 5' to 3', left to right.

(Table 2 and Fig. 1). The scrambled shRNA for negative control was constructed using the GeneScript scrambled shRNA design system (https://www.genscript.com) and selected using GGGenome (gggenome.dbcls.jp) based on the number of mismatches in the rat genome. DNA constructs were synthesized from the pMK plasmid using the GeneArt Strings DNA fragment service (Thermo Fisher Scientific). The pMK plasmid backbone was replaced with pMA-AAV-U6-CMV-EGFP (Vector Biolabs, Malvern, PA, USA). The loop sequence, including the floxed stop cassette between the antisense and sense sequences for *P2rx2*-targeted and scrambled shRNAs, was obtained from a previous study [32]. The sequence of loop, loxP, and stop cassette fragment was (loop-sites in bold, loxP sites underlined) 5′-**gaagct**ataacttcgtatagcatacattatacgaagttatgggaagctataacttcgtatag catacattatacgaagttatagcttggtagcgcggtgtattatacttttggaaagaattcactg gccgtcgttttacaacgtcgtgactgggaaaaccctggcgttacccaacttaatcgccttgcag cacatccccctttcgccagctggcgtaatagcgaagaggcccgcaccgatcgcccttcccaa cagttgcgcagcctgaatggcgaatggcgcctgatgcggtatttttctccttacgcatctgtgcg gtatttcacaccgcatatttttttataacttcgtatagcatacattatacgaagttatagcttg**agcttg**-3′.

AAV was prepared as described previously [33]. Briefly, human embryonic kidney (HEK) 293T cells (American Type Culture Collection, Manassas, VA, USA) were maintained in DMEM (Thermo Fisher Scientific) containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Plasmids for AAV-DJ, pHelper (Cell Biolabs Inc., San Diego, CA, USA), and AAV-U6- shRNA-flox-CMV-EGFP were cotransfected into HEK293T cells using a polyethyleneimine (Sigma-Aldrich) transfection reagent. Three days after transfection and culture at 37°C in a humidified atmosphere containing 3% CO_2, the viral vectors were purified following a gradient purification method using iodixanol OptiPrep (Alere Technologies AS, Oslo, Norway), and the titer was quantified using a QuantStudio 3 real-time PCR system (Thermo Fisher Scientific), as described above. Forward and reverse primers for *P2rx2* and scrambled shRNA vectors were 5′-AACGTCGTGACTGGGAAAAC-3′ and 5′-GGTGTGAAATACCGCACAGA-3′, for *EGFP* 5′-GACGTAAACGGCCACAAGTT-3′ and 5′-AAGTCGTGCTGCTTCATGTG-3′ (GenBank accession no. NC_025025.1), and for *AmpR* 5′-TTCATTCAGCTCCGGTTCCC-3′ and 5′-CTGACAACGATCGGAGGACC-3′. AAV vectors carrying *P2rx2* and scrambled shRNA were stored at –80 °C for future use.

Statistical analysis

Statistical differences in plasma LH levels at each time point (1-h intervals) between *P2rx2* shRNA- and scrambled shRNA-treated *Kiss1-Cre* rats were determined by two-way repeated measures analysis of variance (ANOVA) followed by simple main effects. Differences in the area under the curve (AUC) of plasma LH concentrations (1000–2100 h) and the number of *Kiss1*-expressing and *Kiss1*-expressing EGFP-immunopositive cells were compared between *P2rx2* shRNA- and scrambled shRNA-treated control OVX + high

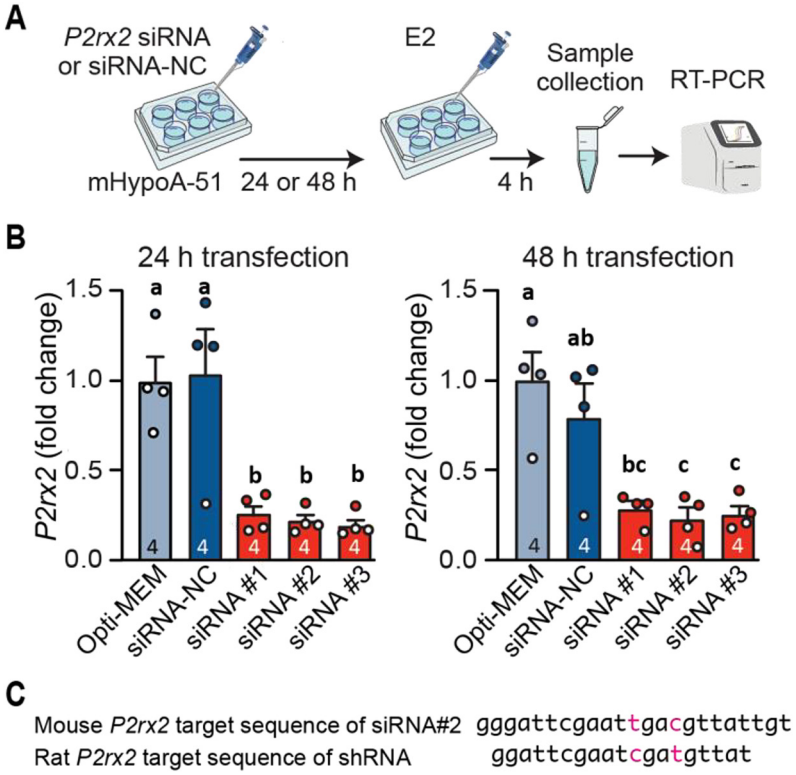

Fig. 1. Evaluation of the efficacy of siRNA in suppressing *P2rx2* mRNA expression in the mHypo-A51 cell line. **A**: mHypoA-51 cells were transfected with one of three *P2rx2* siRNA constructs (siRNA#1, #2, or #3) or negative-control siRNA (siRNA-NC). The cells were incubated for 24 or 48 h and then treated with E2 for 4 h prior to collection of the cells to analyze *P2rx2* mRNA levels. *P2rx2* mRNA expression levels were measured by real-time RT-PCR. **B**: *P2rx2* mRNA expression levels in mHypo-A51 cells 24 or 48 h after the transfection of *P2rx2* siRNA or siRNA-NC. Opti-MEM, non-transfected control. Values are the mean ± SEM. Numbers in each column indicate the number of samples used. Circles on the bar charts indicate the individual data. Different letters indicate statistically significant differences ($p < 0.05$; one-way ANOVA followed by the Bonferroni test). **C**: The target sequence of the rat *P2rx2* shRNA corresponding to the mouse *P2rx2* target sequence of siRNA#2. The base-mismatches are highlighted in magenta. The sequences are shown from 5' to 3', left to right. siRNA, small interfering RNA; SEM, standard error of the mean; ANOVA, analysis of variance.

E2 *Kiss1-Cre* rats using Student's *t*-test. Statistical differences in *P2rx2* mRNA expression levels in mHypoA-51 cells were determined by one-way ANOVA, followed by the Bonferroni post-hoc test for multiple comparisons. All analyses were performed using the SAS OnDemand for Academics software (https://welcome.oda.sas.com).

Results

Evaluation of the siRNA efficacy to suppress P2rx2 mRNA expression using mHypo-A51 cell line

To evaluate the efficacy of the siRNA sequence, three *P2rx2* siRNA constructs were each transfected into a mouse hypothalamus-derived model of AVPV kisspeptin neurons, the mHypoA-51 cell line (Fig. 1A). Real-time RT-PCR analysis revealed that all three *P2rx2* siRNAs (siRNA#1, #2, and #3) significantly reduced *P2rx2* mRNA levels ($P < 0.05$, Fig. 1B) in the mHypoA-51 cell line compared with that observed in the control groups (cells cultured only in medium (Opti-MEM), or with non-specific siRNA as a negative control (siRNA-NC)) 24 h after transfection. In addition, *P2rx2* siRNA#2 and #3 significantly reduced *P2rx2* mRNA levels ($P < 0.05$, Fig. 1B) compared with that observed in the control groups cultured with Opti-MEM or siRNA-NC 48 h after transfection. The *P2rx2* siRNA#2 sequence was selected for shRNA preparation for *in vivo Kiss1*-specific knockdown of *P2rx2* mRNA expression using *Kiss1-Cre* rats. The correspondence between the mouse *P2rx2* target sequence of siRNA#2 and rat *P2rx2* target sequence of shRNA is shown in Fig. 1C. A schematic sequence diagram of AAV and recombination using the Cre/LoxP system is illustrated in Fig. 2A.

AVPV kisspeptin neuron-specific knockdown of P2rx2 mRNA expression attenuated E2-induced LH surge in OVX Kiss1-Cre rats

We investigated the effect of AVPV kisspeptin neuron-specific *P2rx2* mRNA knockdown on the E2-induced LH surge in OVX *Kiss1-Cre* rats (Fig. 2A). EGFP expression derived from AAV transfection was found in the AVPV-POA region, but not in the ARC, where another major population of kisspeptin neurons is located, in all *Kiss1-Cre* rats, as observed in brain sections from representative *Kiss1-Cre* rats treated with AAV-scrambled-shRNA-EGFP or AAV-*P2rx2*-shRNA-EGFP (Fig. 2B), indicating that AAV was precisely transfected into the AVPV-POA region but not in the ARC of the animals. Figure 2C shows *Kiss1* mRNA expression and EGFP immunoreactivity in the AVPV of representative OVX + high E2 *Kiss1-Cre* rats treated with scrambled shRNA or *P2rx2* shRNA. Quantitative analysis revealed that approximately two-fifth (40.8 ± 6.6%) of *Kiss1*-expressing cells in *Kiss1-Cre* rats with the AVPV injection of scrambled shRNA and approximately one-fourth (22.3 ± 7.0%) of *Kiss1*-expressing cells in *Kiss1-Cre* rats with the injection of AVPV *P2rx2* shRNA showed EGFP immunoreactivity in the AVPV. No significant difference was observed in the number of *Kiss1*-expressing and *Kiss1*-expressing EGFP-immunopositive cells between the scrambled shRNA and *P2rx2* shRNA groups (Fig. 2D, $P = 0.116$ and $P = 0.054$, respectively). *Kiss1*-specific knockdown of *P2rx2* mRNA in AVPV kisspeptin neurons attenuated the E2-induced LH surge in OVX *Kiss1-Cre* rats, whereas an E2-induced LH surge was observed in control OVX *Kiss1-Cre* rats treated with scrambled shRNA (Fig. 2E). Plasma LH levels were significantly lower in *Kiss1-Cre* rats injected with AVPV *P2rx2* shRNA than in AVPV-scrambled shRNA-injected control rats between 1700 h–2000 h ($P < 0.05$, Fig. 2E), and the AUC of plasma LH levels was significantly lower in the AVPV *P2rx2* knockdown group than in the scrambled shRNA-treated controls ($P = 0.0129$; Fig. 2F).

AVPV kisspeptin neuron-specific knockdown of P2rx2 mRNA expression disrupted estrous cyclicity, spontaneous LH surge, and ovulation in Kiss1-Cre rats

Next, we determined the effects of AVPV kisspeptin neuron-specific knockdown of *P2rx2* on the estrous cycle, spontaneous LH surge, and ovulation in ovary-intact *Kiss1-Cre* rats (Fig. 3A). Four of the five ovary-intact control *Kiss1-Cre* rats (animal no. #1–#4) showed normal 4-day estrous cycles throughout the experimental period, whereas the remaining individual (#5) showed a diestrus phase during the last 4 days of the experiment. In contrast, three of four ovary-intact AVPV kisspeptin neuron-specific *P2rx2* knockdown *Kiss1-Cre* rats showed persistent diestrus, which started approximately 2 weeks after AVPV *P2rx2* shRNA injection (Fig. 3B), whereas the remaining one (#7) showed a normal estrous cycle. *Kiss1-Cre* control rats treated with AAV-scrambled-shRNA-EGFP in the AVPV (#1–#4) showed a spontaneous LH surge in the afternoon during the proestrus stage (Fig. 3C), whereas *Kiss1-Cre* rat treated with AAV-*P2rx2*-shRNA-EGFP (#7) showed a disruption of the spontaneous LH surge despite being in the proestrus stage (Figs. 3B and D). Plasma LH levels were almost undetectable during the sampling period in AVPV *P2rx2* shRNA-treated *Kiss1-Cre* rat (#7), resulting in a relatively low AUC of plasma LH levels in *P2rx2* shRNA-treated ovary-intact *Kiss1-Cre* rat ($n = 1$), compared with scrambled shRNA-treated *Kiss1-Cre* control rats ($n = 4$, Fig. 3E). Furthermore, *P2rx2* shRNA injection into the AVPV blocked spontaneous ovulation in ovary-intact *Kiss1-Cre* rat (#7), whereas scrambled shRNA-treated control rats (#1–#4) showed ovulation, resulting in ovulated oocytes in the control group but not in the *P2rx2* shRNA-treated rat (#7) (Fig. 3F). Notably, many CLs were observed in the ovaries of control *Kiss1-Cre* rats injected with scrambled shRNA, whereas only a few were observed in the ovary of *Kiss1-Cre* rat injected with *P2rx2* shRNA (#7) (Fig. 3G). Ovarian weights were similar between the groups (Fig. 3H).

Discussion

The present study demonstrates that purinergic neurons may directly stimulate AVPV kisspeptin neurons via P2RX2 to play a critical role in the generation of the GnRH/LH surge and consequent ovulation in female rats. This is because AVPV kisspeptin neuron-specific knockdown of *P2rx2* mRNA expression significantly suppressed the E2-induced afternoon LH surge in OVX *Kiss1-Cre* female rats, and *P2rx2* knockdown disrupted the estrous cycle in ovary-intact *Kiss1-Cre* rats. The direct stimulation of AVPV kisspeptin neurons by purinergic neurons is largely consistent with our previous study showing that the administration of PPADS, a non-selective antagonist for P2X receptors, into the AVPV region attenuated the E2-induced afternoon LH surge in wild-type OVX female rats, and that the administration of PPADS into the AVPV at the proestrus stage significantly decreased the number of ovulated oocytes in ovary-intact wild-type rats [20]. The current results further demonstrated that P2RX2 is specifically involved in the E2-induced GnRH/LH surge, as *Kiss1*-specific *P2rx2* knockdown attenuated the E2-induced LH surge. Furthermore, in our previous study, the administration of ATP, an endogenous ligand of P2RX2, into the AVPV region immediately caused a surge-like increase in plasma LH levels in the morning in OVX + high E2 wild-type rats but not in *Kiss1* KO rats [20]. Taken together, the current findings reveal that purinergic neurons projecting to the AVPV region "directly" stimulate kisspeptin neurons via P2RX2 to induce GnRH/LH surges and consequent ovulation in female rats.

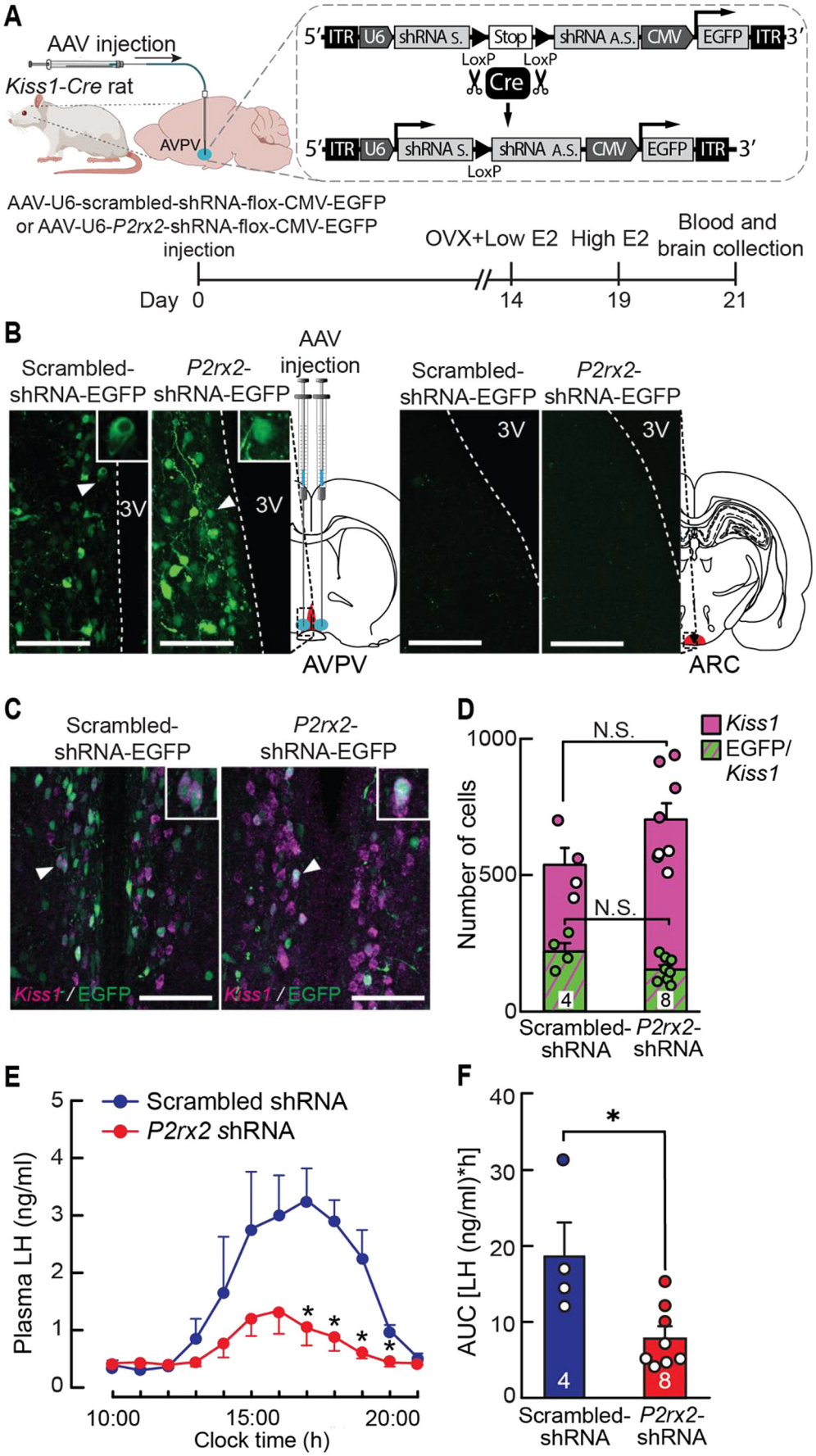
A
AAV injection
Kiss1-Cre rat
AVPV
5′ ITR U6 shRNA S. Stop shRNA A.S. CMV EGFP ITR 3′
LoxP LoxP
Cre
5′ ITR U6 shRNA S. shRNA A.S. CMV EGFP ITR 3′
LoxP
AAV-U6-scrambled-shRNA-flox-CMV-EGFP
or AAV-U6-P2rx2-shRNA-flox-CMV-EGFP
injection
OVX+Low E2
High E2
Blood and brain collection
Day 0
14
19
21
B
Scrambled-shRNA-EGFP
P2rx2-shRNA-EGFP
AAV injection
3V
AVPV
ARC
C
Kiss1 / EGFP
D
Kiss1
EGFP/ Kiss1
N.S.
Number of cells
Scrambled-shRNA
P2rx2-shRNA
E
Scrambled shRNA
P2rx2 shRNA
Plasma LH (ng/ml)
Clock time (h)
F
AUC [LH (ng/ml)*h]

Fig. 2.

Fig. 2. AVPV kisspeptin neuron-specific knockdown of *P2rx2* suppressed E2-induced LH surge in OVX *Kiss1-Cre* female rats. **A**: AAV vectors carrying *P2rx2*-specific or scrambled shRNA were injected into the bilateral AVPV regions of *Kiss1-Cre* female rats. The area outlined by the dotted line indicates the Cre-dependent expression of shRNAs in AVPV kisspeptin neurons of *Kiss1-Cre* rats. The AAV vectors were designed to express either *P2rx2*-specific shRNA or non-specific scrambled shRNA under the human U6 promoter in a Cre-dependent manner, along with an EGFP reporter under a separate CMV promoter. The shRNA is conditionally expressed in AVPV kisspeptin neurons through Cre-mediated deletion, which excises the stop cassette positioned between the sense (shRNA S.) and antisense (shRNA A.S.) sequences. Fourteen days after the injection of AAV-U6-*P2rx2*-shRNA-flox-CMV-EGFP (AAV-*P2rx2*-shRNA-EGFP, n = 8) or AAV-U6-scrambled-shRNA-flox-CMV-EGFP (AAV-scrambled-shRNA-EGFP, n = 4), the *Kiss1-Cre* rats were ovariectomized (OVX) and implanted with diestrus levels of estradiol-17β (low E2) for 5 days and proestrus levels of E2 (high E2) for 2 days. Blood samples were collected from freely moving rats every 1 h from 1000 h to 2100 h. **B**: Images of brain sections of representative *Kiss1-Cre* rats transfected with AAV-scrambled-shRNA-EGFP or AAV-*P2rx2*-shRNA-EGFP showing EGFP expression in the AVPV-preoptic region but not in the ARC. Arrowheads indicate magnified EGFP-expressing cells in the insets. 3V, third ventricle; scale bars, 100 μm. **C**: Representative images of *Kiss1*-expressing (magenta) and EGFP-immmunopositive (green) cells in the AVPV of *Kiss1-Cre* rats injected with scrambled shRNA (left) or *P2rx2* shRNA (right). Insets show magnified images of *Kiss1*-expressing EGFP-immunopositive cells indicated by arrowheads. **D:** The number of *Kiss1*-expressing (magenta) and *Kiss1*-expressing EGFP-immunopositive (striped) cells in the AVPV of *Kiss1-Cre* rats injected with scrambled shRNA or *P2rx2* shRNA. The number of *Kiss1*-expressing cells, as well as the number of *Kiss1*-expressing EGFP-immunopositive cells in the AVPV of OVX + high E2 *Kiss1-Cre* rats in the *P2rx2* shRNA group (n = 8) and scrambled shRNA group (n = 4), were not significantly different (P = 0.116 and P = 0.054, respectively; Student's *t*-test). **E:** Plasma LH profiles in OVX + high E2 *Kiss1-Cre* rats treated with AVPV administration of *P2rx2* shRNA (n = 8, indicated by red circles) or scrambled shRNA (n = 4, indicated by blue circles). Values with * in the *P2rx2*-shRNA-treated group were significantly lower than scrambled-shRNA-treated controls at each time point (P < 0.05, Two-way repeated measures ANOVA followed by simple main effects). **F**: The AUC of plasma LH levels was significantly lower (* P = 0.0129, Student's *t*-test) in *Kiss1-Cre* rats injected with *P2rx2* shRNA compared to the corresponding value of the control *Kiss1-Cre* rats injected with scrambled shRNA. Values represent the mean ± SEM. Numbers in each column indicate the number of animals used. Circles on the bar chart indicate the individual data. AVPV, anteroventral periventricular nucleus; LH, luteinizing hormone; AAV, adeno-associated virus; shRNA, short hairpin RNA; EGFP, enhanced green fluorescent protein; ARC, arcuate nucleus; AUC, area under the curve; ANOVA, analysis of variance; SEM, standard error of the mean.

To the best of our knowledge, the present study is the first to show that purinergic neurons are direct upstream stimulators of AVPV kisspeptin neurons that trigger estrogen-induced and spontaneous GnRH/LH surges, and consequent ovulation.

It is likely that hindbrain A1 and A2 purinergic neurons are the source of ATP and are responsible for the estrogen-induced LH surge, because our previous study showed that c-Fos (a marker of activated neurons) expression in purinergic neurons in the A1 and A2 regions, but not in other brain regions, was induced by preovulatory levels of E2 in wild-type OVX rats just before the occurrence of the LH surge [20]. Furthermore, in the previous study, retrograde tracing using FluoroGold suggested the direct projection of purinergic neurons from the A1 and A2 regions of the hindbrain to the AVPV region of female rats. Moreover, immunohistochemistry revealed that estrogen receptor α (ERα) is expressed in the majority of A1 and A2 purinergic neurons in OVX rats [20]. These findings suggest that circulating proestrus level of estrogen directly activates ERα-expressing purinergic neurons in the hindbrain A1 and A2 purinergic neurons that project to the AVPV region, consequently triggering the GnRH/LH surge via direct activation of AVPV kisspeptin neurons through P2RX2. Circadian signals from the suprachiasmatic nucleus (SCN), which projects to AVPV kisspeptin neurons, also play a role in the timing of the LH surge [34, 35]. Importantly, it is unlikely that the SCN is the source of purinergic neurons that stimulate AVPV kisspeptin neurons to induce a GnRH/LH surge, because our previous study found no vesicular nucleotide transporter (a marker of purinergic neurons)-immunopositive cells in the SCN of female rats [20]. Further studies are required to clarify how the hindbrain purinergic input and SCN circadian signals collectively cooperate to trigger the GnRH/LH surge by regulating the AVPV kisspeptin neurons in female rodents.

It is speculated that ATP-P2RX2 signaling may activate AVPV kisspeptin neurons and promote kisspeptin release through calcium ion influx in female rats. Our previous study demonstrated that ATP administration elevates intracellular Ca^{2+} levels in mHypoA-51 cells, a model of rodent AVPV kisspeptin neurons, and that ATP injection into the AVPV region immediately increase plasma LH levels in female rats [20]. Indeed, P2RX2 is known as a ligand-gated, cation-selective ion channel [36], and calcium influx through the P2RX2 activates mitogen-activated protein (MAP) kinases, specifically extracellular signal-regulated kinase (ERK) 1 and ERK2, in rat pheochromocytoma-derived PC12 cells [37]. Furthermore, ATP-P2RX2 signaling stimulates arginine vasopressin (AVP) release by increasing intracellular Ca^{2+} levels in isolated posterior pituitary nerve terminals in rat and mice [38, 39]. Therefore, in the current study, the extracellular ATP-activated Ca^{2+} influx through P2RX2 may have resulted in AVPV kisspeptin neuronal activation and subsequent kisspeptin release, resulting in the induction of a GnRH/LH surge and consequently, ovulation. Notably, ATP-P2RX2 signaling may not be involved in the regulation of AVPV *Kiss1* mRNA expression in female rats because no significant difference was found in the number of AVPV *Kiss1*-expressing cells between *P2rx2* knockdown and control groups using OVX + high E2 *Kiss1-Cre* rats.

The present study showed that AVPV kisspeptin neuron-specific knockdown of *P2rx2* largely disrupted estrous cyclicity in ovary-intact *Kiss1-Cre* rats. This disruption was observed approximately two weeks after AAV-*P2rx2*-shRNA treatment and thereafter. Notably, one individual (#7) in the AAV-*P2rx2*-shRNA-injected ovary-intact group, which showed a normal estrous cycle, failed to exhibit an endogenous LH surge or ovulation, even though the animal was in the proestrus phase. In contrast, all four individuals (#1–#4) in the AAV-scrambled shRNA-injected group, which showed normal estrous cycles, exhibited endogenous LH surges and ovulation in the proestrus phase. These findings suggest that P2RX2 signaling is involved in the induction of spontaneous LH surges and ovulation in female rats. Moreover, a number of CLs were observed in the ovaries of scrambled shRNA-treated control *Kiss1-Cre* rats, whereas relatively few CLs were found in the ovary of AVPV *P2rx2*-knockdown *Kiss1-Cre* rat (#7) in the estrus phase, providing further evidence that AVPV kisspeptin neuron-specific knockdown of *P2rx2* disrupts the GnRH/LH surge and ovulation. Unexpectedly, the majority (three of four) of *Kiss1-Cre* rats treated with AVPV *P2rx2* shRNA showed persistent diestrus but not persistent estrus, implying that both ovulation and folliculogenesis/steroidogenesis were disrupted by the AVPV kisspeptin neuron-specific knockdown of *P2rx2*. Theoretically, the blockade of the GnRH/LH surge may cause persistent estrus. Future studies reporting that the current AVPV *Kiss1*-specific *P2rx2* knockdown causes a decrease in P2RX2 protein levels only in AVPV kisspeptin

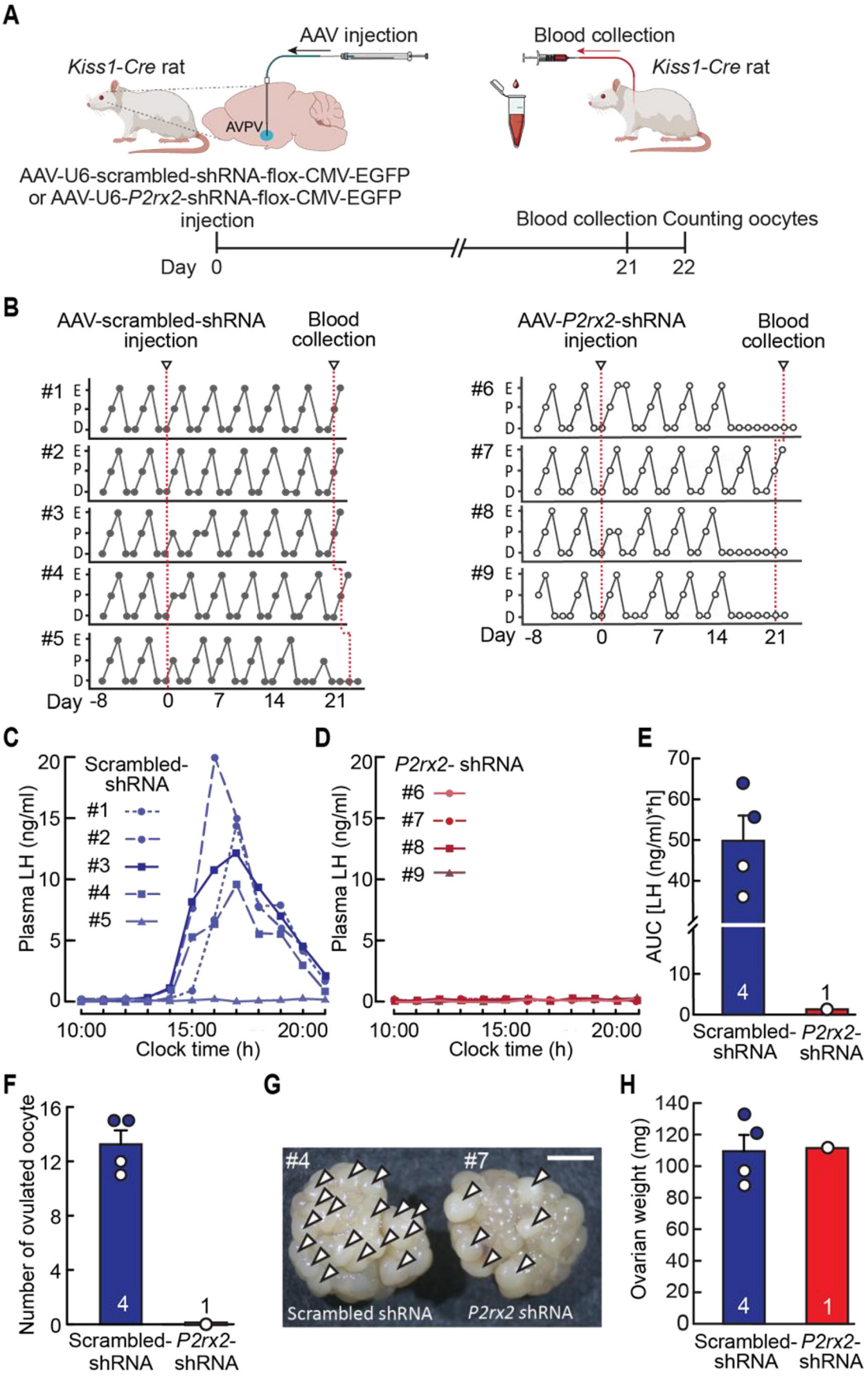
A
AAV injection
Kiss1-Cre rat
AVPV
Blood collection
Kiss1-Cre rat
AAV-U6-scrambled-shRNA-flox-CMV-EGFP
or AAV-U6-P2rx2-shRNA-flox-CMV-EGFP
injection
Blood collection Counting oocytes
Day 0
21
22
B
AAV-scrambled-shRNA injection
Blood collection
AAV-P2rx2-shRNA injection
Blood collection
#1
#2
#3
#4
#5
#6
#7
#8
#9
E
P
D
Day -8 0 7 14 21
C
Scrambled-shRNA
#1
#2
#3
#4
#5
Plasma LH (ng/ml)
Clock time (h)
D
P2rx2- shRNA
#6
#7
#8
#9
E
AUC [LH (ng/ml)*h]
Scrambled-shRNA
P2rx2-shRNA
F
Number of ovulated oocyte
G
#4
#7
Scrambled shRNA
P2rx2 shRNA
H
Ovarian weight (mg)

Fig. 3.

Fig. 3. AVPV kisspeptin neuron-specific knockdown of *P2rx2* disrupted spontaneous LH surge, ovulation, and estrous cyclicity in ovary-intact *Kiss1-Cre* female rats. **A**: After the AVPV injection of AAV-*P2rx2*-shRNA-EGFP or AAV-scrambled-shRNA-EGFP, vaginal smears were monitored daily for at least 21 days, and the blood samples were collected to examine plasma LH levels on the day of proestrus or corresponding day and ovulated oocytes were collected on the day after the blood sampling. **B**: Estrous cyclicity from 8 days before AAV injection until at least 3 weeks after AAV scrambled shRNA or *P2rx2* shRNA injection in *Kiss1-Cre* rats. Four out of five ovary-intact control *Kiss1-Cre* rats showed normal 4-day estrous cycles throughout the experimental period, while three of four ovary-intact AVPV kisspeptin neuron-specific *P2rx2* knocked-down *Kiss1-Cre* rats showed persistent diestrus. E, estrus; P, proestrus; D, diestrus. Individual plasma LH profiles measured in ovary-intact rats treated with either AVPV scrambled shRNA (C) or *P2rx2* shRNA (D). **E**: The AUC of plasma LH levels in *Kiss1-Cre* rats that were in proestrus on the day of blood sampling in scrambled shRNA- (animal #1–#4, n = 4) or *P2rx2* shRNA-treated *Kiss1-Cre* rat (#7, n = 1). **F**: AVPV administration of *P2rx2* shRNA blocked ovulation in the ovary-intact *Kiss1-Cre* rat (#7, n = 1), whereas control rats (#1–#4, n = 4) showed ovulation at estrus. **G**: Images of ovaries in representative *Kiss1-Cre* rats with AVPV injection of scrambled shRNA (#4, left) or *P2rx2* shRNA (#7, right). Scale bar, 2 mm. Arrowheads, corpora lutea. **H**: Ovarian weights (total of left and right) of the *Kiss1-Cre* rat injected with *P2rx2* shRNA (#4, n = 1) and the control rats (#1–#4, n = 4) injected with scrambled shRNA. Values are represented as mean ± SEM. Circles on the bar charts indicate the individual data. AVPV, anteroventral periventricular nucleus; LH, luteinizing hormone; AAV, adeno-associated virus; shRNA, short hairpin RNA; EGFP, enhanced green fluorescent protein; SEM, standard error of the mean.

neurons would strengthen our interpretation. Moreover, further studies are required to clarify whether the disruption of AVPV kisspeptin neuronal function by AVPV *Kiss1*-specific knockdown of *P2rx2* affects tonic GnRH/gonadotropin release to regulate folliculogenesis/steroidogenesis in the ovaries.

In conclusion, findings of the present study suggest that purinergic neurons directly activate AVPV kisspeptin neurons through P2RX2 to trigger estrogen-induced spontaneous GnRH/LH surges and consequent ovulation in female rats.

Conflict of Interest: The authors declare that they have no competing interests.

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