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ORIGINAL ARTICLE

Neurotensin induces sustainable activation of the ErbB–ERK1/2 pathway, which is required for developmental competence of oocytes in mice

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Abstract

Purpose: LH induces the expression of EGF-like factors and their shedding enzyme (ADAM17) in granulosa cells (GCs), which is essential for ovulation via activation of the ErbB–ERK1/2 pathway in cumulus cells (CCs). Neurotensin (NTS) is reported as a novel regulator of ovulation, whereas the NTS-induced maturation mechanism in oocytes remains unclear. In this study, we focused on the role of NTS in the expression of EGF-like factors and ErbBs, and ADAM17 activity, during oocyte maturation and ovulation in mice.

Methods: The expression and localization in GC and CC were examined. Next, hCG and NTS receptor 1 antagonist (SR) were injected into eCG-primed mice, and the effects of SR on ERK1/2 phosphorylation were investigated. Finally, we explored the effects of SR on the expression of EGF-like factors and ErbBs, and ADAM17 activity in GC and CC.

Results: NTS was significantly upregulated in GC and CC following hCG injection. SR injection suppressed oocyte maturation and ERK1/2 phosphorylation. SR also downregulated part of the expression of EGF-like factors and their receptors, and ADAM17 activity.

Conclusions: NTS induces oocyte maturation through the sustainable activation of the ERK1/2 signaling pathway by upregulating part of the EGF-like factor-induced pathway during oocyte maturation in mice.

KEYWORDS
neurotensin, ERK1/2, cumulus cells, granulosa cells, oocyte maturation

1 | INTRODUCTION

During ovulation in mammals, transient luteinizing hormone (LH surge) secreted from the pituitary gland stimulates granulosa cells of the preovulatory follicle, and a mature cumulus–oocyte complex (COC) is released into the oviduct. During this process, LH induces numerous physiological and morphological changes, such as granulosa cell luteinization, cumulus expansion, and meiotic resumption of the oocyte. LH stimulus induces cumulus expansion and oocyte meiotic resumption; however, LH receptor expression and localization

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are primarily restricted to granulosa cells.[1,2] Thus, a factor(s) that is expressed and released from LH-stimulated granulosa cells to cumulus cells and the oocyte is essential for the ovulation process.

The epidermal growth factor (EGF)-like factors, such as amphiregulin (AREG), epiregulin (EREG), and betacellulin (BTC), were first reported by Park et al.[3] as second messengers secreted from LH-stimulated granulosa cells. These EGF-like factors are expressed in granulosa cells following LH stimulation, which is secreted into the follicular fluid and serves as a transmitter of the LH stimulus from granulosa cells to cumulus cells to induce cumulus expansion, oocyte maturation, ovulation, and granulosa cell luteinization in mice.[3–6] Furthermore, our previous reports demonstrated the importance of other EGF-like factors, such as neuregulin 1 (NRG1), coordinately acting on AREG-induced oocyte maturation and ovulation.[7,8] Considering that all EGF-like factors are synthesized as type 1 transmembrane precursors, ectodomain shedding, which releases soluble ligands at the cell surface, is required for activating its own receptors (ErbBs).[9] This cleavage event is generally mediated by members of the disintegrin and metalloprotease (ADAM) family.[9] Our previous studies indicated that TACE/ADAM17 is a potent metalloprotease that releases the soluble form of EGF-like factors.[10,11] Specifically, AREG can bind to ErbB1; however, EREG, BTC, and NRG1 bind to other ErbBs. Furthermore, EREG and BTC can bind to and activate ErbB1, ErbB2, and ErbB4. NRG1 binds to ErbB2, ErbB3, and ErbB4.[12] ErbB2 has no ligand-binding site, whereas the signal is induced by heterodimerization with another ErbB receptor. ErbB3 has a ligand-binding site; however, it lacks receptor tyrosine kinase activity. Therefore, the ErbB3-induced signal is transduced in coordination with other ErbB family, primarily ErbB2.[13–15] During the ovulation process, we observed ErbB1, ErbB2, and ErbB3 expressions in granulosa cells, whereas ErbB4 expression had primarily increased in immature granulosa cells, 48 h following eCG injection and 4 h following hCG injection, but not 8 and 16 h following hCG injection.[7] The ErbB family induces the phosphorylation of ERK1/2 (also known as mitogen-activated protein kinase [MAPK]3/1) via Ras kinase, and phosphorylated ERK1/2 enhances the expression of COC expansion-related genes (*Has2*, *Tnfaip6*, and *Ptx3*) in cumulus cells and progesterone-related genes (*Star*, *Cyp11a*1, and *Hsd3b1*) in luteinizing granulosa cells.[16–18] Indeed, granulosa cells in specific knockout mice for *Erk1/2* failed to perform cumulus expansion, ovulation, and granulosa cell luteinization.[16] Thus, the EGF-like factor-induced activation of the ERK1/2 pathway via the ErbB family is crucial for the ovulation process.

Recently, it was reported that neurotensin (NTS) is expressed in the ovary following LH stimulation, and it plays a role in the regulation of ovulation. In response to hCG, NTS expression increased rapidly in granulosa cells of mice, rats, and cynomolgus macaques through the ErbB1, PKA, PI3K, and ERK1/2 signaling pathways.[19–21] NTS silencing experiments using an in vitro culture of granulosa cells revealed that NTS may affect the ovulatory process by regulating the expression of *Ell2*, *Rsad2*, *VPS37a*, and *Smtnl2*.[21] This finding is consistent with the result of transcriptome analysis of cumulus cells derived from hCG-injected mice conducted by our group, which showed a specific increase in NTS in cumulus cells in COCs following hCG injection.[4] In the present study, we examined the effects of NTS on the activation of the ERK1/2 pathway through the mRNA expression of EGF-like factors and the enzymatic activity of ADAM17 in granulosa cells, as well as the mRNA expression of ErbBs in cumulus cells, a pathway that is extremely important for oocyte maturation and ovulation.

2 | MATERIALS AND METHODS

2.1 | Materials and chemicals

Equine chorionic gonadotropin and human chorionic gonadotropins (eCG and hCG) were purchased from Asuka Animal Health (Tokyo, Japan). DMEM (low glucose, pyruvate) was purchased from Thermo Fisher Scientific (Waltham, MA, USA); penicillin–streptomycin was purchased from Nacalai Tesque (Kyoto, Japan); and fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific.

Amphiregulin (AREG; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMEM at 10 μg/mL and used at 100 ng/mL. Neurotensin (NTS; Phoenix Pharmaceuticals, Burlingame, CA, USA) was dissolved in DMEM at 100 μM and used at 1 μM. EGFR tyrosine kinase inhibitor AG1478 (Sigma-Aldrich), MEK inhibitor U0126 (Sigma-Aldrich), and NTSR1 inhibitor SR48692 (SR; Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO; FUJIFILM Wako Pure Chemical, Osaka, Japan) at 1 mM (AG1478 and U0126) or 7.5 mM (SR) and stored at −20°C until use. The final concentration of AG1478 (1 μM), U0126 (1 μM), or SR (7.5 μM) was obtained by dilution (1:1000) with DMEM. The final concentration of NTS and SR for in vitro experiment was referred in the previous paper.[22,23] The final concentration of DMSO was 0.1%, which did not affect the functioning of cumulus cells during meiosis.[24] Routine chemicals and reagents were obtained from Nacalai Tesque or Sigma-Aldrich.

For in vivo experiments, SR was suspended (1 mg/mL) with DMSO (10%) in saline and injected through intraperitoneal route at the dose of 0.1 mg/kg. The final concentration of SR was referred in the following paper.[25,26] Control was obtained by injection of only vehicle (10% DMSO in saline).

2.2 | Animals

Immature (3- to 4-week-old) female and adult (3- to 5-month-old) female C57BL/6 and adult male ICR mice were obtained from CLEA Japan (Tokyo, Japan). Immature female mice were injected intraperitoneally with 5 IU of eCG followed 48 h later and 6 IU hCG with or without SR. Animals were housed under a 12-h light/12-h dark cycle and provided food and water ad libitum. Mice were treated in accordance with the National Institutes of Health Guide for the Care, and the experiments were approved by the Committee for Ethics on Animal Experiments in the Prefectural University of Hiroshima (approval number 16SA007).

2.3 | Collection of ovaries, COCs, and granulosa cells for in vivo analysis

Immature female mice were primed with eCG for 48 h and then injected with hCG or hCG and SR (hCG + SR). The ovary contained multiple antral follicles or oviduct contained ovulated COCs was collected before or at 2, 4, 6, 8, 12, 16, or 48 h after hCG or hCG + SR injection. COCs were isolated from the antral follicles or oviducts by needle puncture and collected by pipette. After collecting COCs, granulosa cells were harvested from the residuum. Each sample was used for total RNA and protein isolation for in vivo examination.

2.4 | Measurement of the number of ovulated oocytes, litters, and corpus luteum

Immature female mice were primed with eCG for 48 h and then injected with hCG or hCG + SR; the number of ovulated COCs in the oviductal ampulla was measured at 16 h; and ovaries were collected at 48 h for corpora luteum count. Collected ovaries were fixed by 4% (w/v) paraformaldehyde (Tokyo Chemical Industry, Tokyo, Japan) for 4–5 h, dehydrated by staged ethanol series, cleaned in xylene, and embedded in paraffin. Fixed ovaries were cut to 5 μm thick in series on glass, deparaffinized in xylene, and then washed in PBS. To accurately measure the number of corpus luteum per ovary, ovary was sliced every 20 μm using a microtome Leica RM2125RTS (Leica Biosystems, Wetzlar, Germany), and all sliced sections were observed. The sections were treated with hematoxylin (Sakura Finetek Japan, Tokyo, Japan) for 1 min, and washed and stained with eosin (Sakura Finetek Japan) for 3 min. The samples were observed using a fluorescence microscope BZ-X700 (Keyence, Osaka, Japan).

Adult female mice were treated with SR (0.1 mg/kg) every 3 days for 10 days and mated with adult male mice, and the number of litters obtained was measured.

2.5 | Measurement of progesterone concentration

Ovaries 48 h after hCG or hCG + SR administration were collected and homogenized in 500 μL of whole-cell extract buffer (WCEB) [100 mM NaCl, 100 mM $Na_4P_2O_7$, 50 mM NaF, 0.1 mM $NaVO_4$, 1% (v/v) Triton X-100, 2.5 mM HEPES (pH 7.5), 10% (v/v) glycerol, 5 mM EDTA, and 5 mM EGTA], and protein concentration was measured according to the DC Protein Assay Regents Package (Bio-Rad Laboratories, Hercules, CA, USA) procedure. To saponify the sample, 600 μL of 0.3 N NaOH was added to extract steroids into a dichloromethane fraction. Extracted steroid hormones were placed in a vacuum, dried at 50°C to remove dichloromethane for 2 h, and then redissolved with 50% (v/v) methanol. Progesterone concentrations in the samples were detected using the Progesterone ELISA Kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manual. Progesterone measurement was performed using a microplate reader Varioskan Flash (Thermo Fisher Scientific).

2.6 | COC isolation and in vitro maturation of COCs

Ovaries of immature mice primed with eCG 48 h contain multiple preovulatory follicles. COCs were isolated from these follicles by needle puncture and selected nonexpanded COCs by pipette. A total of 50 or 100 COCs were cultured in separated wells of a 96-well plate in 100 μL of DMEM containing 1% FBS with AREG, NTS, AREG and NTS (AREG + NTS), or AREG, NTS, and SR (AREG + NTS + SR) at 2, 4, 6, 8, 12, or 16 h in 39°C humidified incubator (95% air and 5% CO_2). The cultured COCs were collected at selected intervals and used for total RNA isolation, protein isolation, or in vitro fertilization analysis. COC expansion was assessed by measuring the COC diameters with an eyepiece micrometer and phase-contrast microscopy (Nikon, Tokyo, Japan) with a 10× objective as previously described.[11,27–29] The diameter selected for measurement was defined as the greatest distance across the COC expanded matrix. Oocytes were separated from the cultured COCs, and the nuclear status was assessed as previously described.[11,27–29]

2.7 | In vitro fertilization

COCs, which were cultured for 16 h of in vitro maturation or collected from oviductal ampulla 16 h after injection of the hCG or hCG + SR, were placed into 100 μL of human tubal fluid (HTF) medium. Spermatozoa were collected from the cauda epididymis of ICR male mice in 400 μL of HTF medium. After 60 min of incubation, the spermatozoa were introduced into fertilization medium at a final concentration of 2×10^5 spermatozoa/mL and cultured for 6 h for insemination. After insemination, oocytes were washed thoroughly five times and then checked for the formation of pro-nuclei under a phase-contrast microscope. The gametes were further cultured in 50 μL of KSOM. The embryos with 2 pro-nuclei and 2 pore body at Day 1 were evaluated as fertilization, and the embryo with sufficient inner cell mass and trophoblast cells at Day 5 were evaluated as blastocyst.

2.8 | RNA extraction, reverse transcription, and quantitative PCR analysis

Granulosa cells and cumulus cells collected from eCG/hCG- or eCG/hCG + SR-injected mice or cumulus cells of COCs cultured with AREG, NTS, AREG + NTS, and AREG + NTS + SR in vitro condition were washed in PBS. Total RNA was extracted from the cells using a RNeasy Mini Kit (Qiagen, Venlo, Netherlands) according to the instruction manual and dissolved in nuclease-free water. Final RNA concentration of total RNA (10 ng/μL) was determined by microvolume spectrophotometer NanoDrop One (Thermo Fisher Scientific).

TABLE 1 Sequences of PCR primers used for RT-qPCR.

mRNA	Primer sequences	Product size	Annealing temperature	Accession no.
Rpl19	F: 5′-CTG AAG GTC AAA GGG AAT GTG-3′ R: 5′-GGA CAC AGT CTT GAT GAT CTC-3′	196	60	bc058135
Nts	F: 5′-GTG TGG ACC TGC TTG TCA GA-3′ R: 5′-TGC TTT GCT GAT CTT GGA TG-3′	177	60	NM_024435
Ntsr1	F: 5′-GCC CCT CCT CCT AAG AAA TG-3′ R: 5′-ACT CCA GGC TGA GTC CTT CA-3′	225	54	NM_018766
Ntsr2	F: 5′-GAA TGC ATC AGG GAA GGA AA-3′ R: 5′-GAG CTC ACT GGA AGG CAA AC-3′	165	64	AB056466
Ntsr3	F: 5′-GGG GAC CAA ACA ACA TCA TC-3′ R: 5′-CCT GGT CTG TTG ACA CAT GG-3′	219	62	AF175279
Star	F: 5′-GCA GCA GGC AAC CTG GTG-3′ R: 5′-TGA TTG TCT TCG GCA GCC-3′	249	60	NM_011485
Cyp11a1	F: 5′-GGG AGA CAT GGC CAA GAT GG-3′ R: 5′-CAG CCA AAG CCC AAG TAC CG-3′	279	60	BC068264
Hsd3b1	F: 5′-GGT GCA GGA GAA AGA ACT GC-3′ R: 5′-TGA CAT CAA TGA CAG CAG CA-3′	197	60	NM_001304800 XM_006501035
Has2	F: 5′-GAG CAC CAA GGT TCT GCT TC-3′ R: 5′-CTC TCC ATA CGG CGA GAG TC-3′	154	62	NM_008216
Tnfaip6	F: 5′-TTC CAT GTC TGT GCT GCT GGA TGG-3′ R: 5′-AGC CTG GAT CAT GTT CAA GGT CAA A-3′	330	64	NM_009398.2
Ptx3	F: 5′-GTG GGT GGA AAG GAG AAC AA-3′ R: 5′-GGC CAA TCT GTA GGA GTC CA-3′	190	64	NM_008987.3
Areg	F: 5′-CGG TGG AAC CAA TGA GAA CT-3′ R: 5′-TTT CGC TTA TGG TGG AAA CC-3′	198	62	NM_009704
Btc	F: 5′-GGA ACC TGA GGA CTC ATC CA-3′ R: 5′-TCT AGG GGT GGT ACC TGT GC-3′	227	60	NM_007568
Ereg	F: 5′-CCG TTT TCC TGG TAC ATG CT-3′ R: 5′-GCA TGT GTC CTT GTG TTT GC-3′	187	60	NM_007950
Nrg1	F: 5′-TGC ATT GCT GGC CTA AAG TG-3′ R: 5′-GTT CTT CCG GG TGG GTA CTG-3′	258	64	NM_001364422 XM_017312636
Erbb1	F: 5′-TGC CAA GGC ACA AGT AAC AG-3′ R: 5′-GTT GAG GGC AAT GAG GAC AT-3′	192	64	AF124513
Erbb2	F: 5′-CCC TAG GTA CTG GGA GCA CA-3′ R: 5′-GTC CTT TGG TTA CCC CCA CT-3′	186	60	L47239
Erbb3	F: 5′-AGT TCT CCC GTC CCA TCT CT-3′ R: 5′-GGG AGT AAG CAG GCT GTG TC-3′	197	60	L47240
Erbb4	F: 5′-CCC AGG CTT TCA ACA TAC CT-3′ R: 5′-CCT CTG GTA TGG TGC TGG TT-3′	150	60	L47241
Adam17	F: 5′-TTC AGG CAA TCA AAG CAG TG-3′ R: 5′-CAA ACG GTA AAC GCC TCA AT-3′	161	64	AH006905

Reverse transcription was performed as previously described.[27] Briefly, 10 ng/μL of total RNA was added to the 500 ng of Oligo(dT) primer (Promega, Madison, WI, USA) and 0.25 U of AMV reverse transcriptase (Promega) for 75 min at 42°C and for 5 min 95°C.

Quantitative PCR (qPCR) analysis was performed as previously described.[30] cDNA and specific primer pairs were added to the KAPA SYBR FAST Universal qPCR Kit (Kapa Biosystems, Wilmington, MA, USA) to give a total reaction volume of 15 μL. PCR was then performed using the Real-Time PCR Detection

System MiniOpticon (Bio-Rad Laboratories). The standard curve method was applied to determine the absolute quantity of *Ntsr1*, *Ntsr2*, and *Ntsr3*. A standard cDNA stock for absolute quantitation was prepared from the PCR products using a FavorPrep GEL/PCR Purification Mini Kit (Favorgen Biotech, Ping-Tung, Taiwan). qPCR condition was set to the following parameters: 30 s at 95°C, followed by 40 cycles each of 5 s at 95°C and 45 s at 60, 62, or 64°C. Specific primer pairs were selected and analyzed and indicated in Table 1. *Rpl19* was used as a control for reaction efficiency and variations in concentrations of mRNA in the original RT reaction. In this study, several housekeeping genes such as *Rpl19* and *Gapdh* were tested. *Rpl19* showed the least variation among treatment groups and was close to target gene; thus, *Rpl19* was used as the housekeeping gene in this study (data not shown). The results were first normalized to the expression levels of *Rpl19*. To avoid false-positive signals, dissociation curve analysis was performed at the end of amplification and the PCR products were applied to agarose gel electrophoresis to confirm the size. The data were calculated from relative values of at least 3 independent experiments.

2.9 | Immunofluorescence staining

Each ovarian section of eCG/hCG-treated mice was deparaffinized with xylene, quenched with 10% (v/v) hydrogen peroxide in methanol for 10 min, incubated in 0.3% (v/v) Triton X-100/PBS(−) for 30 min, microwaved in 10 mM citrate buffer, and then blocked with 5% (w/v) bovine serum albumin (BSA)/PBS(−) for 90 min. The samples were then incubated overnight at 4°C with primary antibodies against neurotensin or neurotensin receptor 1, followed by 2 h of incubation with Cy3-conjugated anti-rabbit IgG diluted 1:100. Samples were counterstained, mounted using VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Newark, CA, USA), and observed using a confocal microscope FLUOVIEW FV10i (Olympus, Tokyo, Japan). The Cy3 and DAPI signals were detected at wavelengths of 570 and 455 nm, respectively. Antibody information used in this study is shown in Table 2.

2.10 | Western blot analysis

Protein samples from cumulus cells were prepared by homogenization in WCEB, and protein extracts were stored at −80°C until use. Protein concentrations were determined by the DC Protein Assay Regents Package, according to the manufacturer's procedure, and then diluted by same volume of sample buffer solution with 2-ME (2×). After denaturing by boiling for 5 min, 10 μL of each protein samples (10 μg) was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 7.5% polyacrylamide gel and then transferred to PVDF membrane (GE Healthcare, Chicago, IL, USA). Membranes were blocked in Tris-buffered saline and Tween 20 [TBST; 10 mM Tris (pH 7.5), 150 mM NaCl, and 0.05% (v/v) Tween 20], containing 5% (w/v) nonfat dry milk (GE Healthcare). Blots were incubated overnight at 4°C with primary antibody. After washing in TBST for 1 h, the membranes were incubated for 1 h with a 1:2000 dilution of anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology, Danvers, MI, USA) or anti-mouse IgG HRP-linked antibody (Cell Signaling Technology) in 2.5% (w/v) nonfat dry milk in TBST at room temperature. After washing for 1 h with TBST, peroxidase activity was visualized using the ECL Plus Western Blotting Detection System (GE Healthcare), according to the manufacturer's instructions. The intensity of the objective bands was quantified by densitometric scanning using a Gel-Pro Analyzer (Media Cybernetics, Rockville, MD, USA). Antibody information used in this study is shown in Table 2.

2.11 | Measurement of the hyaluronic acid concentration

COCs, which were cultured for 16 h of in vitro maturation or collected from oviductal ampullae 16 h after injection of the hCG or hCG + SR, were homogenized in 30 μL of Cell Lysis Buffer 2 (R&D Systems, Minneapolis, MN, USA) and 30 μL of PBS, incubated for 30 min at room temperature, and centrifuged at 1000 *g* for 15 min, and the supernatant was collected and stored at −80°C until assayed. Hyaluronic acid concentration was measured by using Hyaluronan

TABLE 2 List of antibodies used for immunofluorescence staining and western blot analysis.

Antibody	Cat. No.	Company	Dilution used	
			WB	IF
NTS	AB5496	EMD Millipore		1:500
NTSR1	ab75555	Abcam		1:200
Cy3-conjugated anti-rabbit IgG	C2306	Sigma-Aldrich		1:100
Total-ERK1/2	4695	Cell Signaling	1:1000	
Phospho-ERK1/2	9106	Cell Signaling	1:1000	
ADAM17	3976	Cell Signaling	1:1000	
β-Actin	4967	Cell Signaling	1:10000	
Anti-rabbit IgG HRP-linked antibody	7074	Cell Signaling	1:2000	
Anti-mouse IgG HRP-linked antibody	7076	Cell Signaling	1:2000	

Quantikine ELISA Kit (R&D Systems) according to the manufacturer's instruction. Hyaluronan measurement was performed using Varioskan Flash.

2.12 | Measurement of ADAM17 activity

Granulosa cells were collected from eCG/hCG- or eCG/hCG + SR-injected mice. ADAM17 activity was measured by using SensoLyte 520 TACE (a-Secretase) Activity Assay Kit Fluorimetric (AnaSpec, Fremont, CA, USA) according to manufacturer's instruction. Briefly, collected granulosa cells were washed with PBS, and 60 μL of assay buffer of the kit with 0.1% (v/v) Triton X-100 was added. The cell suspension was incubated for 10 min at 4°C and centrifuged at 2500 *g* for 10 min at 4°C. The supernatant was collected and stored at −80°C until assayed. The protein concentration was measured according to the DC Protein Assay Regents Package, and 50 μL of each protein samples (10 μg) with assay buffer was used for the assay. ADAM17 activity measurement was performed using Varioskan Flash.

2.13 | Statical analysis

All data were obtained from at least 3 replications for comparison and were shown as the mean ± standard error of the mean (SEM). All percentage data were subjected to arcsine transformation before analysis to convert a binomial distribution to a normal distribution. All statistical analyses were performed using either the Student *t*-test or the one-way analysis of variance (ANOVA) followed by a Tukey multiple comparisons post-test for groups (StatView; Abacus Concepts, Berkley, CA, USA). $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Kinetic changes and localization of NTS and NTSR in the mouse ovary

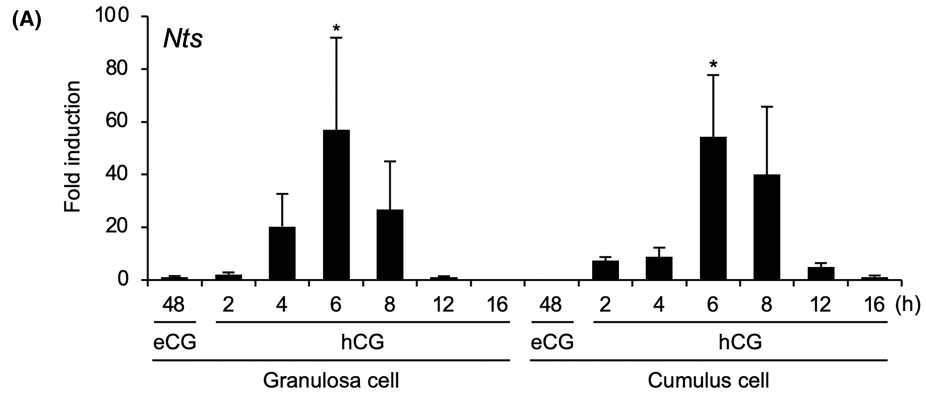
Nts mRNA was expressed at a low level 48 h following eCG injection; however, the expression level was significantly increased after hCG stimulation within 6 h in both granulosa and cumulus cells (Figure 1A). Because previous reports revealed that there are three types of receptors for NTS (NTSR1, NTSR2, and NTSR3),[31–33] we examined the kinetic changes in the expression of *Ntsr1*, *Ntsr2*, and *Ntsr3* in granulosa and cumulus cells following eCG and hCG injection in mice. Quantification by standard curve-based qPCR demonstrated that the expression of *Ntsr1* mRNA was significantly higher than that of *Ntsr2* and *Ntsr3* mRNA, especially in granulosa cells at 2, 6, and 12 h of hCG administration and in cumulus cells at 6–12 h of hCG administration (Figure 1B). Immunofluorescence analysis revealed that NTS protein was not detected before hCG stimulation; however, positive staining was observed in granulosa and cumulus cells 8 h following hCG administration (Figure 1C). Moreover, NTSR1 protein was also localized in granulosa and cumulus cells following hCG stimulation (Figure 1C).

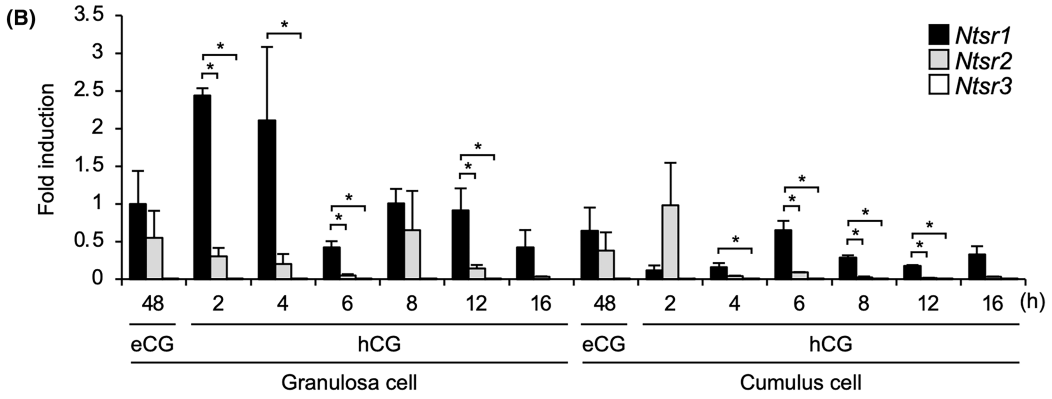
3.2 | Role of NTS in ovulation, fertility, and luteinization in vivo

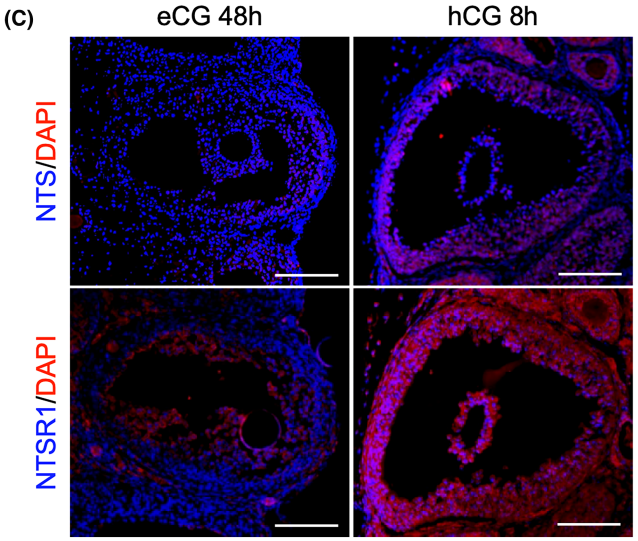
To determine the physiological role of NTS in COC during the ovulation process in vivo, we examined the pharmacologic effects of an NTSR1-selective antagonist (SR) on ovulation, oocyte maturation, and luteinization. We coinjected SR and hCG into mice 48 h following eCG injection, and COCs were collected from the oviduct 16 h following hCG or hCG and SR treatment of mice. In mice injected with hCG alone, the average number of ovulated COCs was 20.3 ± 2.1 and 57.7% of which developed into the blastocyst stage. In contrast, mice coinjected with hCG and SR exhibited a significantly reduced number of ovulated COCs (5.7 ± 4.7, Figure 2A) and percentage of embryos reaching the blastocyst stage (15.5%, Figure 2B). The average number of pups per litter was decreased in SR-primed mice (6.5 ± 0.5 pups number/litter) compared with that in vehicle-injected mice (9.0 ± 2.0 pups number/litter, Figure 2C).

As shown in Figure 2D–F, hCG-induced expression of *Star* and *Cyp11a1*, but not *Hsd3b1*, was significantly suppressed by the coinjection of hCG and SR. Similarly, production of hCG-induced progesterone in the ovary was attenuated by the coinjection of hCG and SR (Figure 2G). Furthermore, the number of corpus luteum was significantly decreased in hCG-stimulated ovaries compared with that in hCG- and SR-stimulated ovaries (12.2 ± 1.0 vs. 3.9 ± 0.3, Figure 2H,I).

FIGURE 1 Expression and localization of NTS and NTSRs in granulosa cells and cumulus cells of the ovary during the ovulation process. (A and B) Gene expression of *Nts* and *Ntsr1–3* in granulosa cells and cumulus cells collected at each time point after eCG/hCG was analyzed by qRT-PCR. The expression levels of genes were normalized according to that of *Rpl19*. Values are shown as the mean ± SEM of more than three replicates. (A) *Nts* expression of granulosa cells at eCG 48 h was set as 1, and values of *Nts* expression at each time point for both cells were presented as fold induction. *, significant differences were observed compared to eGG 48 h of each cell ($p < 0.05$). (B) *Ntsr1* expression of granulosa cells at eCG 48 h was set as 1, and values of all gene expression at each time point for both cells were presented as fold induction. *, significant differences were observed ($p < 0.05$). (C) Localization of NTS and NTSR1 was detected by immunofluorescence using anti-NTS and anti-NTSR1 antisera. Cross sections of mouse ovary recovered from eCG 48 h and hCG 8 h were stained with antiserum to visualize either NTS or NTSR1 (red) or nuclei (blue). Scale bars correspond to 100 μm. eCG, equine chorionic gonadotropin; hCG, human chorionic gonadotropin; NTS, neurotensin; NTSR, neurotensin receptor.







3.3 | Effect of NTS on AREG-induced cumulus expansion and oocyte maturation in in vitro cultured COCs

To determine the relationship between AREG and NTS in cumulus cells, eCG-stimulated COCs were cultured with AREG and/or NTS. The expression of cumulus expansion-related genes, the expansion status of cumulus cells, and the maturation and fertilization rate of the oocyte were measured. The expression levels of *Has2*, *Tnfaip6*, and *Ptx3* mRNA in cumulus cells of COCs cultured for 12 h in AREG- or NTS-containing medium were low; however, all gene expression levels were significantly upregulated following cotreatment with AREG and NTS. Furthermore, the high expression levels were significantly and completely suppressed by further addition

of SR to AREG- and NTS-containing medium (Figure 3A). NTS stimulus alone did not induce hyaluronan production in COC, whereas AREG induced hyaluronan accumulation in COC. Furthermore, cotreatment with AREG and NTS significantly increased the production compared with that in COCs that were cultured with NTS alone. The costimulation-induced increase of hyaluronan was significantly suppressed by further treatment with SR (Figure 3B). Moreover, the diameter of COC after cultivation with AREG and NTS was significantly increased compared with that of COC after cultivation with AREG or NTS alone. The COC diameter induced by cultivation with both AREG and NTS was significantly suppressed by further treatment of SR with AREG- and NTS-containing medium (Figure 3C,D). Furthermore, the MII rate, fertilization rate, and formation rate of the embryo reaching the blastocyst stage were low for oocytes in COCs cultured with NTS alone, whereas they were increased by AREG. Cultivation with AREG and NTS further increased these rates compared with AREG alone in the maturation medium. However, further supplementation of SR to AREG- and NTS-containing medium significantly decreased these rates (Figure 3E–G).

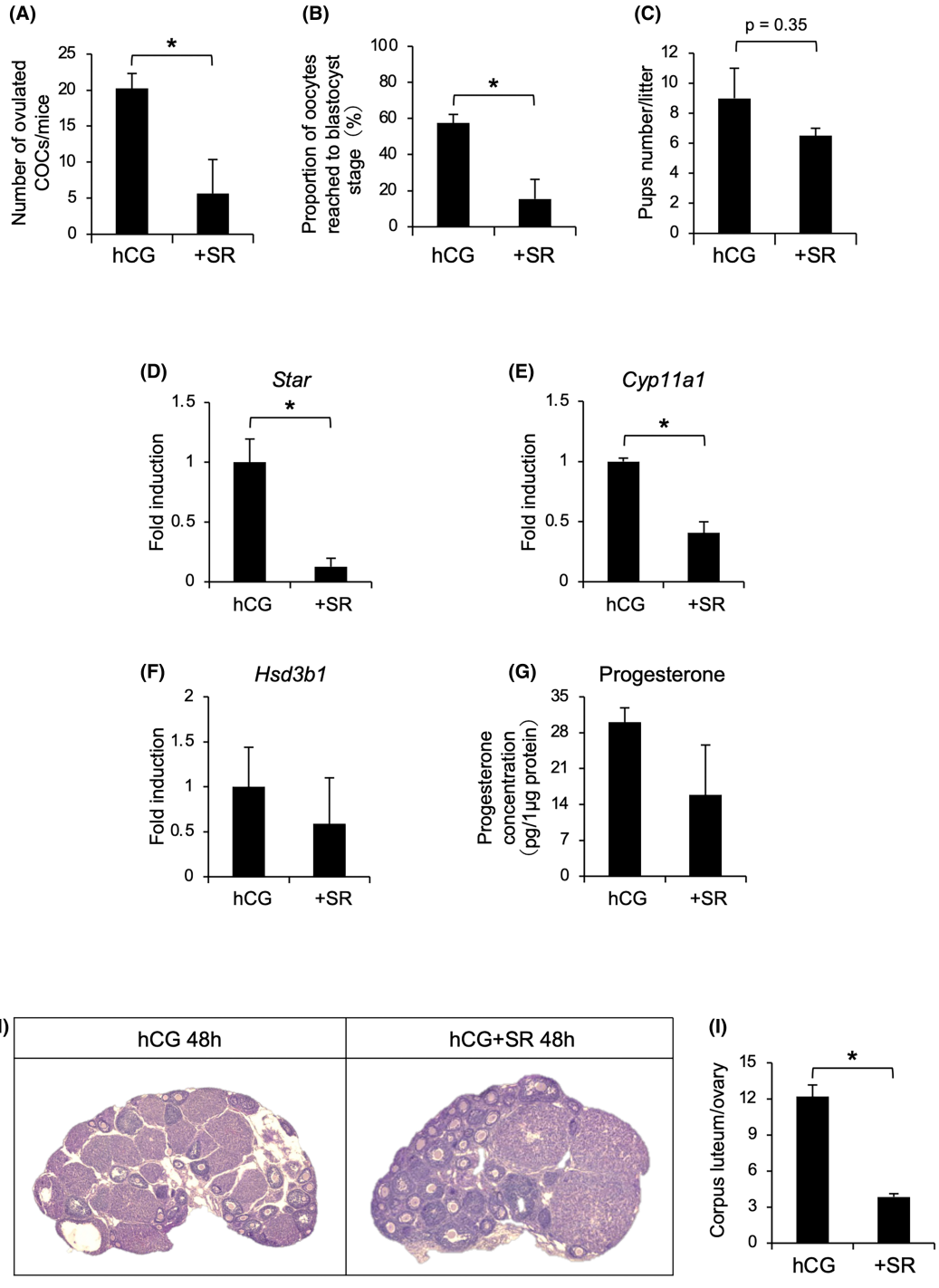


FIGURE 2 The role of NTS in ovulation, oocyte maturation, luteinization, and fertility. (A–C) The number of ovulated COCs (A), blastocyst rate (B), and pups born per litter (C) was impaired by inhibition of NTS. (A) The number of ovulated COCs in oviduct of immature mice at 16 h after primed with hCG or hCG and SR (NTSR1 inhibitor; 0.1 mg/kg) was counted. (B) Ovulated COCs were used for IVF test and analyzed the rate of oocytes reaching the blastocyst stage. (C) Numbers of pups born per litter in the adult female mice were administered vehicle or SR every 3 days for 10 days, and after mating with male mice, the number of offspring obtained was measured. The percentage data were subjected to arcsine transformation before analysis. *, significant differences were observed between the injection ($p < 0.05$). Values are shown as the mean ± SEM of more than three replicates. (D–F) The expression of luteal cell marker genes, *Star* (D), *Cyp11a1* (E), and *Hsd3b1* (F), in granulose cells was affected by NTS. Expression of each gene in granulosa cells prepared from mice at 16 h after primed with hCG or hCG and SR was analyzed by qRT-PCR. The expression levels of genes were normalized according to that of *Rpl19*. For reference, the hCG value was set as 1, and the data are presented as fold induction. *, significant differences were observed between the injection ($p < 0.05$). Values are shown as the mean ± SEM of more than three replicates. (G–I) The concentration of progesterone (G), luteinize formation (H), and the number of corpus luteum (I) in ovaries were inhibited by SR. (G) Progesterone concentration in ovaries collected from mice at 48 h after primed with hCG or hCG and SR was analyzed by ELISA. (H) Collected ovaries were used for histological analysis by staining of cross sections with hematoxylin and eosin. (I) Numbers of corpora lutea were counted in every 20 μm. *, significant differences were observed between the injection ($p < 0.05$). Values are shown as the mean ± SEM of more than three replicates. COC, cumulus–oocyte complex; hCG, human chorionic gonadotropin; IVF, in vitro fertilization; NTS, neurotensin.

3.4 | Role of NTS on the phosphorylation of ERK1/2 in cumulus cells in vivo and in vitro

We and others have shown that the ERK1/2 signaling pathway in cumulus and granulosa cells is essential for the success of ovulation, embryogenesis, and luteinization of granulosa cells by enhancing cumulus expansion and oocyte maturation.[16,34,35] To determine the effect of NTS on kinetic phosphorylation levels of ERK1/2, cumulus cells and granulosa cells were collected from the ovaries at 0, 2, 4, 6, 8, 12, or 16 h following hCG or hCG and SR injection. When cumulus cells were recovered from mice injected with hCG only, the phosphorylation of ERK1 and ERK2 was detected within 4 h and maintained up to 16 h. However, when cumulus cells were recovered from hCG and SR-primed mice, the phosphorylation of ERK1/2 was induced within 4 h, but ERK1 was significantly decreased at 12–16 h and ERK2 at 12 h (Figure 4A). On the contrary, the phosphorylation of ERK1 and ERK2 in granulosa cells was maintained throughout the ovulatory phase, and no significant changes were observed with SR administration (Figure S1).

Because NTS induced the sustainable activation of ERK1/2 in cumulus cells during the ovulation process in vivo, we examined the role of NTS more explicitly using an in vitro culture system. When COCs were cultured with AREG alone, ERK1/2 phosphorylation was induced within 2 h; however, this positive phospho-ERK1/2 was decreased following 12 h of cultivation. Cultivation with NTS alone also induced the phosphorylation of ERK1/2 within 6 h, but this phosphorylation was decreased after 12 h. By contrast, costimulation with AREG and NTS induced ERK1/2 phosphorylation within 2 h, and the induction was maintained up to 12 h (Figure 4B). This temporal phosphorylation pattern was similar to that in cumulus cells collected from the ovary following hCG injection (Figure 4A).

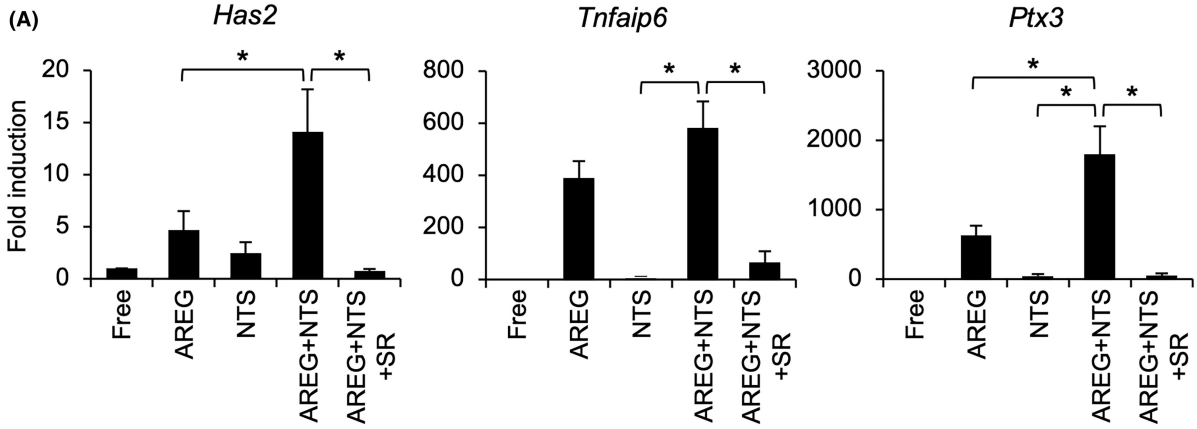
3.5 | NTS induces the expression of EGF-like factors and the activity of their shedding protease in granulosa cells and ErbBs in cumulus cells

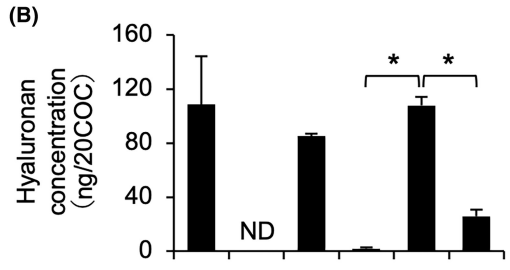
Because the sustained phosphorylation of ERK1/2 in cumulus cells was suppressed by the inhibition of NTS–NTSR in vivo and in vitro, we analyzed whether NTS was a stimulatory factor that induced the expression of EGF-like factors and/or ErbB family members. In validating this hypothesis, mice were injected with hCG or hCG + SR, and the expression of EGF-like factors, *Areg*, *Btc*, *Ereg*, and *Nrg1*, as well as the activity of their shedding protease, ADAM17, in granulosa cells and cumulus cells, was measured. In addition, we examined the receptor mRNA expression of EGF-like factors, *Erbb1*, *Erbb2*, *Erbb3*, and *Erbb4*, in granulosa cells and cumulus cells.

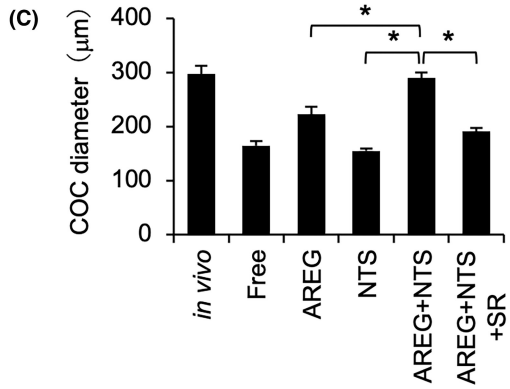
In granulosa cells, the expression level of *Areg* and *Btc* mRNA was increased following hCG stimulation within 2–4 h and decreased to less than or equal to basal levels at 6–16 h. The negative effect following coinjection of hCG and SR on the expression of these genes was not observed. Interestingly, the expression of *Ereg* and *Nrg1* was induced by hCG injection, whereas the high expression in granulosa cells was significantly suppressed by coinjection with hCG and SR (*Ereg*, 4 and 8 h; *Ngr1*, 4 h; Figure 5). In cumulus cells, as in granulosa cells, the expression level of *Areg* and *Btc* was not altered by SR injection, whereas the expression level of *Ereg* and *Nrg1* was significantly decreased by SR (*Ereg*, 4 and 6 h; *Nrg1*, 2 h; Figure S2A).

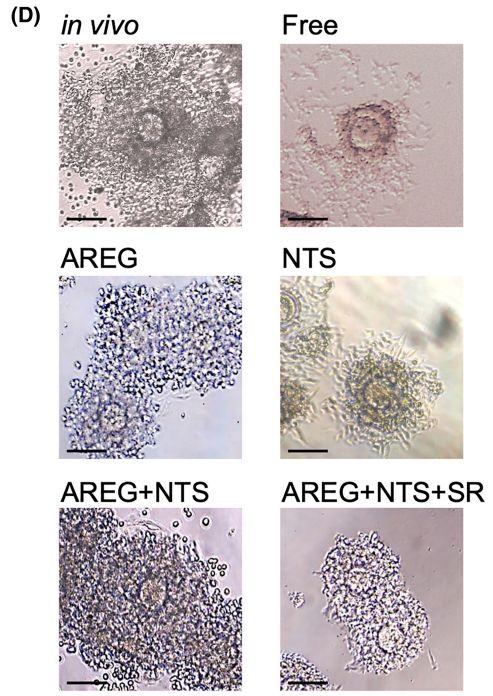
In granulosa cells, the expression level of *Adam17* mRNA was significantly increased 16 h following hCG administration compared with the coinjection of hCG + SR; however, the negative effect of the coinjection of hCG + SR within 12 h on *Adam17* mRNA expression was not observed (Figure 6A). The negative effect of the coinjection of hCG + SR on the protein expression of ADAM17 in granulosa cells was also not observed compared with that in granulosa cells stimulated by hCG alone (Figure 6B). On the contrary, ADAM17 activity in granulosa cells was significantly suppressed by coinjection with hCG + SR compared with that in granulosa cells stimulated by hCG alone at 8 h point (Figure 6C).

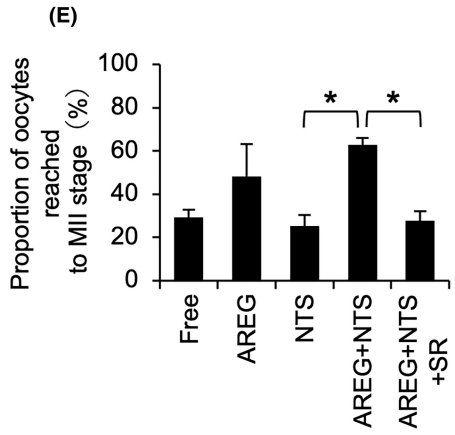
As a result of examining the effect of NTS on the expression of ErbB family members in cumulus cells, the expression level of *Erbb2* and *Erbb3* was markedly increased 8 h following hCG administration and was significantly suppressed to basal levels following coinjection with hCG and SR. On the other hand, the expression level of *Erbb1* and *Erbb4* was significantly increased by SR (*Erbb1*, 4 h; *Erbb4*, 12–16 h; Figure 7). In granulosa cells, the expression level of *Erbb1*, *Erbb2*, and *Erbb4* was not significantly altered by SR treatment. By contrast, the

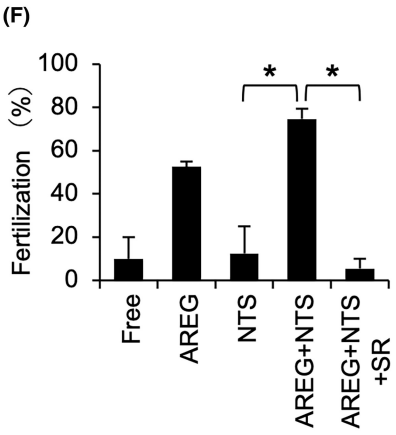

(A)
Has2
Tnfaip6
Ptx3
Fold induction
Free
AREG
NTS
AREG+NTS
AREG+NTS +SR


(B)
Hyaluronan concentration (ng/20COC)
ND


(C)
COC diameter (μm)
in vivo
Free
AREG
NTS
AREG+NTS
AREG+NTS +SR


(D)
in vivo
Free
AREG
NTS
AREG+NTS
AREG+NTS+SR


(E)
Proportion of oocytes reached to MII stage (%)
Free
AREG
NTS
AREG+NTS
AREG+NTS +SR


(F)
Fertilization (%)
Free
AREG
NTS
AREG+NTS
AREG+NTS +SR

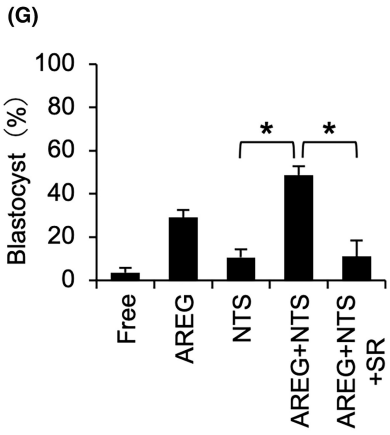

(G)
Blastocyst (%)
Free
AREG
NTS
AREG+NTS
AREG+NTS +SR

FIGURE 3 The role of NTS in cumulus cell functions and oocyte maturation in cultured COCs. (A) The expression of cumulus expansion marker genes, *Has2*, *Tnfaip6*, and *Ptx3* in cumulus cells, was affected by addition of NTS in the presence of AREG. The expression of each gene in cumulus cells collected from COCs, which is recovered from immature mice injected with eCG 48 h, cultured with Free (non-treated), AREG (100 ng/mL), NTS (1 μM), AREG + NTS, or AREG + NTS + SR (7.5 μM) for 12 h was analyzed by qRT-PCR. The expression levels of genes were normalized according to that of *Rpl19*. For reference, the Free value was set as 1, and the data are presented as fold induction. *, significant differences were observed compared to across treatment ($p < 0.05$). Values are shown as the mean ± SEM of more than three replicates. (B–D) Hyaluronan concentration (B), diameter (C), and morphology (D) of cultured COCs were promoted by addition of NTS in the presence of AREG compared to in vivo status. (B) Hyaluronan concentration in 20 COCs cultured with Free (non-treated), AREG, NTS, AREG + NTS, or AREG + NTS + SR for 16 h was analyzed by ELISA. (C and D) Diameters (C) and morphology (D) of COCs cultured Free (non-treated), AREG, NTS, AREG + NTS, or AREG + NTS + SR for 16 h were examined using an eyepiece micrometer and phase-contrast microscopy. *, significant differences were observed ($p < 0.05$). Values are shown as the mean ± SEM of more than three replicates. (E–G) Oocyte nuclear and cytoplasmic maturation of cultured COCs were promoted by NTS in the presence of AREG. MII (E), fertilization (F), and blastocyst (G) rates when COCs were cultured with Free (non-treated), AREG, NTS, AREG + NTS, or AREG + NTS + SR. MII rate was shown by oocyte reaching the metaphase II stage at 16-h culture (E). Fertilization rate (F) and blastocyst rate (G) were shown by oocyte reaching 2 pro-nuclei and 2 pore bodies or blastocysts, respectively, for 5 days of culture after IVF test. The percentage data were subjected to arcsine transformation before analysis. Values are shown as the mean ± SEM of more than three replicates. *, significant differences were observed compared to across treatment ($p < 0.05$). AREG, amphiregulin; COC, cumulus–oocyte complex; eCG, equine chorionic gonadotropin; IVF, in vitro fertilization; MII, metaphase II; NTS, neurotensin.

expression of *Erbb3* was significantly reduced to basal levels at 2 h of hCG administration by simultaneous SR administration (Figure S2B).

4 | DISCUSSION

NTS, which comprises 13 amino acids, is a hormone that was first isolated from the bovine hypothalamus, and the action is mediated by one of three receptors, two G-protein-coupled receptors, NTSR1 and NTSR2, or a single transmembrane domain sorting receptor, NTSR3.[31–33,36] The effects of NTS are primarily mediated by its high-affinity receptor, NTSR1.[37] NTS functions as a modulator of not only the dopaminergic system in the central nervous system[38,39] but also gastrointestinal motility and secretion in the small intestine and stomach.[40,41] Recently, it was reported that NTS and its receptor, NTSR3, were highly expressed in granulosa cells of macaques, humans, and rats after ovulation induction, suggesting their potential role in inducing ovulation.[19–21] However, the molecular mechanisms that induce ovulation phenomena, including cumulus expansion, oocyte maturation, and subsequent embryogenesis downstream of the NTS function, remain unclear.

In this study, we demonstrated for the first time that (1) NTS is selectively expressed in granulosa and cumulus cells of the ovary during ovulation, and (2) activation of the NTS–NTSR1 pathway promotes cumulus cell expansion, oocyte maturation, fertilization, and luteinization via sustained activation of the EGF-like factor–ErbB–ERK1/2 pathway. Furthermore, we found that as a mechanism for the sustained activation of this pathway during the ovulation period, NTS induces the expression of specific EGF-like factors, *Ereg* and *Nrg1*, and ADAM17 activity in granulosa cells, as well as *Erbb2* and *Erbb3*, in addition to *Ereg and Nrg1* in cumulus cells. This suggests that persistent ERK1/2 phosphorylation in cumulus cells following ovulation stimulation results from paracrine/autocrine stimulation by EGF-like factors released from granulosa cells and cumulus cells. These results indicate that NTS acts as a key regulator of the EGF-like factor–ErbB–ERK1/2 pathway during the ovulation process.

In the present study, we demonstrated that although *Nts* expression is initially induced by hCG/LH stimuli, it is also enhanced by LH-induced EGF-like factors. Indeed, when COCs collected from eCG-stimulated mice were cultured with AREG, *Nts* expression was markedly induced within 4 h, and it was completely suppressed by the addition of an ErbB1 (AG1478) or ERK1/2 (U0126) inhibitor (Figures S3 and S4). These results are consistent with previous reports that the inhibition of the EGF and ERK1/2 signaling pathways partially suppresses NTS expression using in vitro culture systems of granulosa cells in rats, mice, and humans.[20,21] Similarly, the inhibition of the PKA, p38MAPK, and PI3K pathways also resulted in a partial decrease in NTS expression, indicating that NTS is regulated via these pathways.[20,21] Following LH stimuli, AREG is rapidly induced in granulosa and cumulus cells through the p38MAPK signaling pathway within 2 h and induces phosphorylation of ERK1/2 by binding to ErbB1.[5] Furthermore, Fan et al.[16] reported that *Nts* expression was induced in wild-type granulosa cells after LH stimuli; however, its induction was decreased in granulosa cells of specific *Erk1/2* knockdown mice. Collectively, this suggests that the induction of NTS expression occurs through the EGF-like factor–ErbB1–ERK1/2 pathway during the ovulation process in cumulus cells and granulosa cells.

In this study, NTS primarily transmitted downstream signals via NTSR1. Interestingly, following ovulation stimulation, a notable increase in NTSR1 localization was observed in granulosa and cumulus cells, despite a consistent gene expression of *Ntsr1* (Figure 1B,C). It suggests that NTSR1 may enhance its presence on the plasma membrane via some post-translational modifications after ovulation stimuli. Palmitoylation, a post-translational modification, plays an important role in driving proteins to membrane rafts by adding palmitic acid to cysteine residues.[42–44] A previous study has reported that the addition of NTS increases the palmitoylation level of NTSR1.[45] In the present study, the NTSR1 localization signal increased following hCG administration without a significant change in *Ntsr1* mRNA expression, suggesting that the hCG-stimulated increase in NTS may have stabilized the membrane localization of NTSR1 via increased

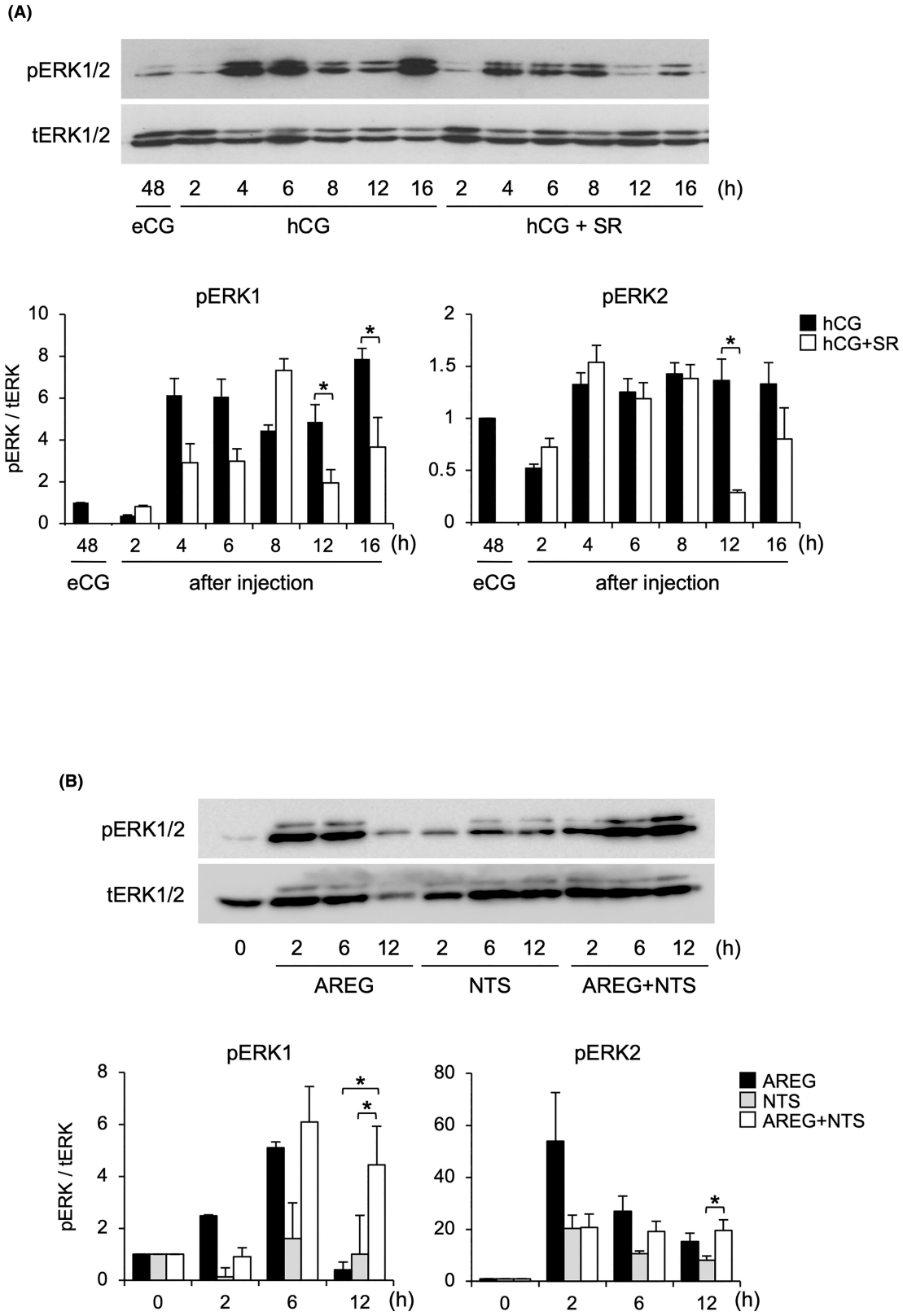


FIGURE 4 Effect of NTSR1 inhibitor on the phosphorylation of ERK1/2 in cumulus cells during ovulation process. (A) The activation of ERK1/2 in cumulus cells collected from ovaries of immature mice injected with eCG followed by 48 h later with hCG or hCG and SR was analyzed by western blotting. (B) The activation of ERK1/2 in cumulus cells of COCs, which were recovered from immature mice injected with eCG 48 h, cultured with AREG, NTS, and AREG + NTS up to 12 h was analyzed by western blotting. The intensity of the bands was analyzed using a Gel-Pro Analyzer. For reference, eCG 48 h value was set as 1, and the data were presented as fold induction. Values were shown as the mean ± SEM of more than three replicates. *, significant differences were observed compared to across treatment ($p < 0.05$). AREG, amphiregulin; COC, cumulus–oocyte complex; eCG, equine chorionic gonadotropin; hCG, human chorionic gonadotropin; NTS, neurotensin; NTSR, neurotensin receptor.

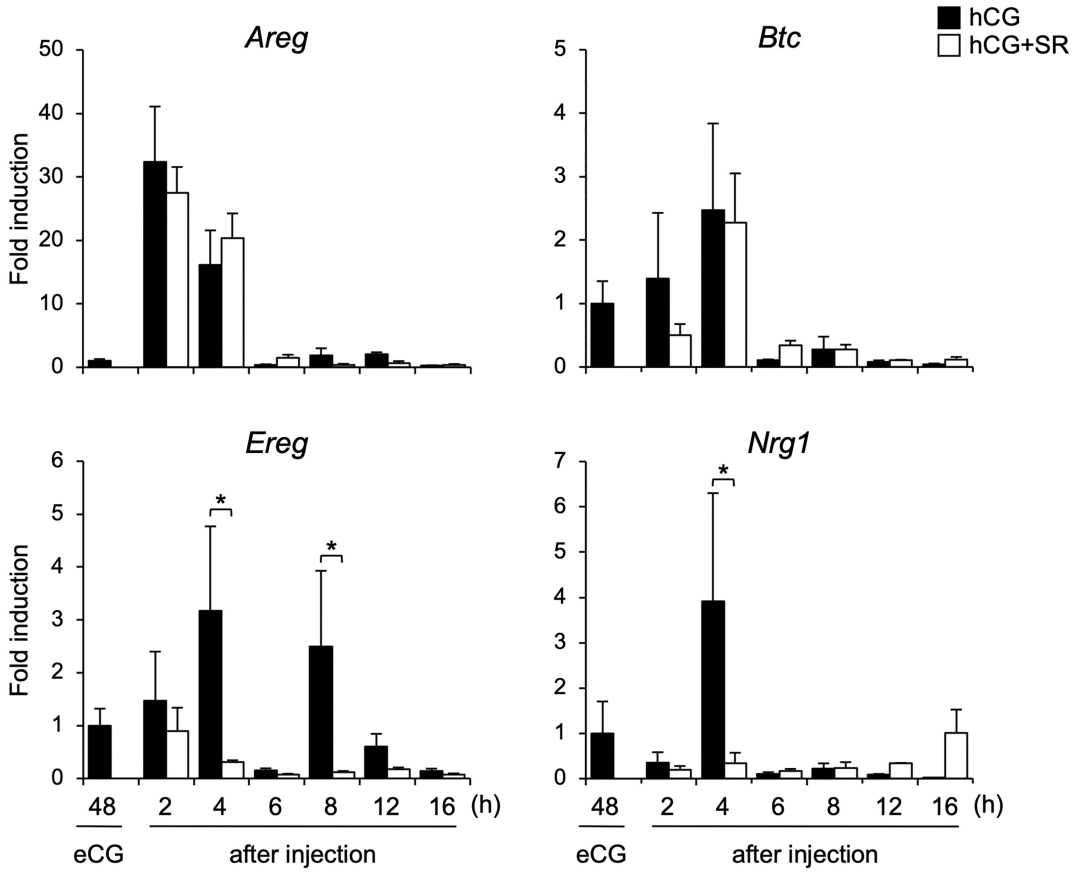


FIGURE 5 Effect of NTSR1 inhibitor on the mRNA expression of EGF-like factors in granulosa cells. The expression levels of genes were normalized according to that of *Rpl19*. For reference, the eCG 48 h value was set as 1, and the data are presented as fold induction. Values are shown as the mean ± SEM of more than three replicates. *, significant differences were observed compared to across treatment ($p < 0.05$). eCG, equine chorionic gonadotropin; EGF-like factor, epidermal growth factor-like factor; NTSR, neurotensin receptor.

palmitoylation levels. Further investigations into the specific post-translational modification and its dynamics will be considered to support and validate this hypothesis.

Furthermore, we revealed that the NTS–NTSR1 pathway plays an important role in promoting cumulus cell expansion, oocyte maturation, ovulation, and luteinization from the present in vivo and in vitro analysis. On the other hand, despite a significant decrease in the number of ovulations after SR administration, no significant difference in the number of pups was observed. This result may be attributed to the age difference among the female mice used in this study. Immature mice were used in experiments evaluating ovulation and blastocyst rate. However, during mating trials for the assessment of the number of pups, some female mice were lost because of the size disparity between immature females and mature males. Thus, we compared litter sizes by administering SR to mature females followed by mating with males. The use of mature females may have permitted endogenous LH to maintain NTS levels and thus offset the reduction in litter size following SR administration. In identifying the underlying mechanism of the effects of NTS, we focused on the phosphorylation of ERK1/2 in cumulus cells and granulosa cells, because ERK1/2 controls ovulation, COC expansion, oocyte maturation, and luteinization.[16] When COCs were cultured with AREG and NTS, ERK1/2 phosphorylation was enhanced and sustained during culture periods, whereas single treatment with NTS or AREG induced a transient, not consecutive, phosphorylation of ERK1/2 (Figure 4B). Furthermore, the continuous phosphorylation pattern of ERK1/2 was also observed in hCG-stimulated cumulus cells. Moreover, the inhibition of the NTS–NTSR1 pathway in vitro and in vivo abolished the sustainable phosphorylation of ERK1/2 (Figure 4A,B). Although the ERK1/2 activity in cumulus cells was inhibited following SR administration in the late ovulatory phase, no inhibitory effect was observed in granulosa cells following SR treatment (Figure S1). In granulosa cells, considering that SR administration reduced the expression level of specific EGF-like factors (*Ereg* and *Nrg1*; Figure 5), the receptor (*Erbb3*) mRNA (Figure S2B), progesterone production genes (Figure 2D,E), and ovulation process (Figure 2A,H,I), we estimated that NTS regulates ovulation process via progesterone-dependent manner in granulosa cells.

The present study showed that NTS increased the activity of ADAM17 without increasing the expression level of the ADAM17 gene and protein in granulosa cells. In fact, *Adam17* expression was significantly increased by SR treatment at 8 h, whereas ADAM17 activity was significantly decreased (Figure 6). This discrepancy between gene expression and protein activity may be due to the

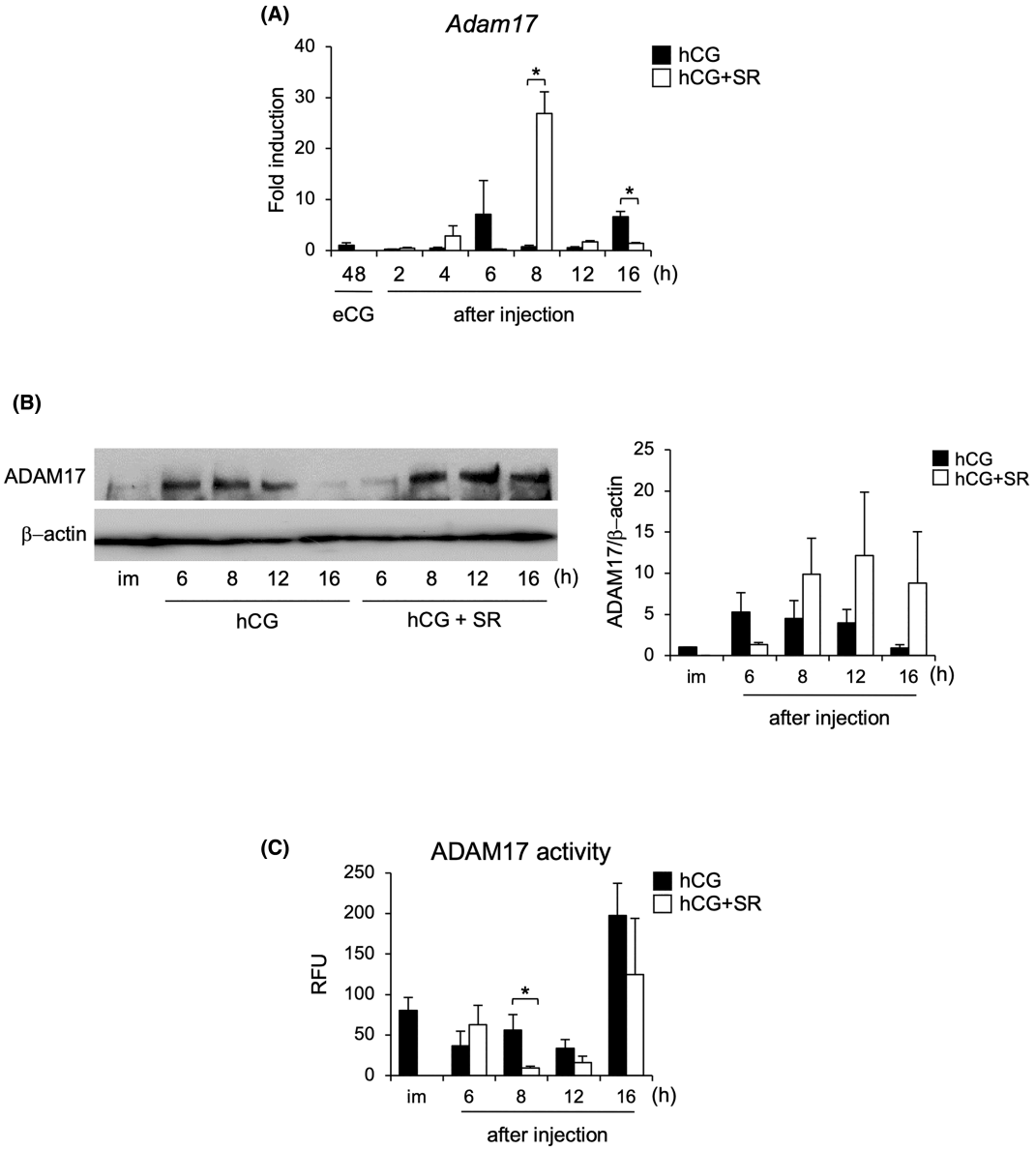


FIGURE 6 Effect of NTSR1 inhibitor on the expression of *Adam17* mRNA (A), ADAM17 protein (B), and enzyme activity of ADAM17 (C) in granulosa cells. (A) The expression levels of genes were normalized according to that of *Rpl19*. For reference, the eCG 48 h value was set as 1, and the data are presented as fold induction. (B) The expression of ADAM17 protein in granulosa cells collected from ovaries of immature (im) mice injected with eCG followed by 48 h later with hCG or hCG and SR was analyzed by western blotting. The intensity of the bands was analyzed using a Gel-Pro Analyzer. For reference, im value was set as 1, and the data were presented as fold induction. Values are shown as the mean ± SEM of more than three replicates. (C) The enzyme activity of ADAM17 in granulosa cells collected from ovaries of immature mice injected with eCG followed by 48 h later with hCG or hCG and SR was analyzed. Values are shown mean ± SEM of more than three replicates. *, significant differences were observed compared to across treatment ($p < 0.05$). ADAM17, a disintegrin and metalloprotease 17; eCG, equine chorionic gonadotropin; hCG, human chorionic gonadotropin; NTSR, neurotensin receptor.

differences in the mechanism of the induction of gene expression and activity. In our previous study, we reported that *Adam17* expression is induced via PKA and p38MAPK pathways, while its activity is strongly induced by PKC in addition to these pathways.[27] Furthermore, it is well reported that PKC is activated downstream of NTS.[22,46,47] Based on these results, we hypothesized that SR inhibits the NTS–PKC pathway after 6 h of hCG administration, when NTS is produced in the follicle, resulting in a significant decrease in ADAM17 activity at 8 h of hCG + SR coinjection.

NTSR1 is a type of GPCR and is known to induce the activation of ERK1/2 pathways in various types of cancer cells[48,49]; however, no detailed analyses have shown the mechanism of the NTS-induced phosphorylation of ERK1/2 in cumulus cells. EGF-like factors and EGF receptors are upstream activators of ERK1/2. Our results indicate that SR administration suppressed the expression of *Ereg* and *Nrg1*, but not *Areg* and *Btc*, in granulosa and cumulus cells. Furthermore, ADAM17 activity in granulosa cells and the expression of the EREG and NRG1 receptors, *Erbb2* and

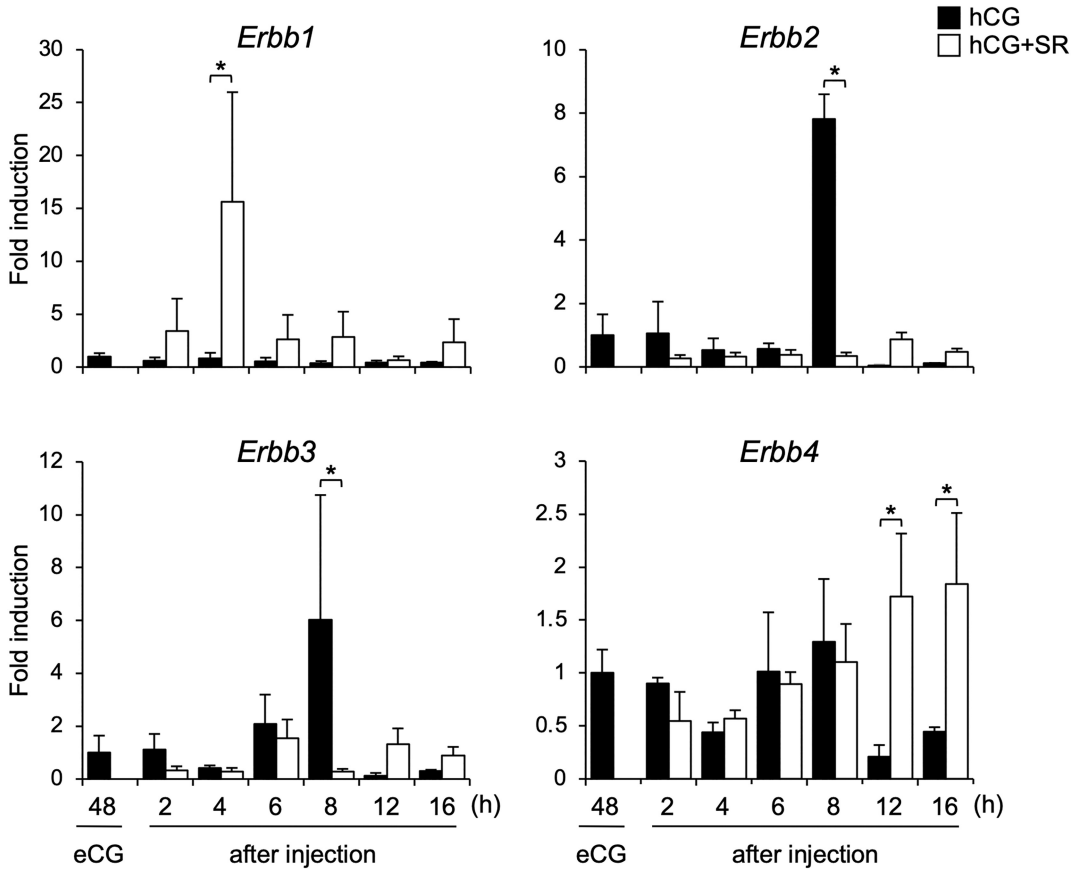


FIGURE 7 Effect of NTSR1 inhibitor on the expression of *Erbbs* in cumulus cells. The expression levels of genes were normalized according to that of *Rpl19*. For reference, the eCG 48 h value was set as 1, and the data are presented as fold induction. Values are shown as the mean ± SEM of more than three replicates. *, significant differences were observed compared to across treatment ($p < 0.05$). eCG, equine chorionic gonadotropin; NTSR, neurotensin receptor.

Erbb3, in cumulus cells were selectively suppressed by SR administration in mice. During the ovulation process, the main EGF-like factor changes sequentially. Previous reports showed that the expression of *Areg*, *Ereg*, and *Nrg1* mRNAs was initially upregulated followed by *Btc*. In addition, the expression level of *Areg*, *Nrg1*, and *Btc* in granulosa cells was transient, whereas *Ereg* mRNA was continuously maintained up to 12 h following hCG treatment.[3,7] EGF-like factors bind to their specific receptor, ErbB, in cumulus cells. Whereas AREG binds to ErbB1 alone, BTC and EREG bind to ErbB1, ErbB2, and ErbB4, and NRG1 binds to ErbB2, ErbB3, and ErbB4. The binding of an EGF family ligand to its cognate receptor results in the dimerization and activation of downstream targets.[12] It is likely that LH-dependent, but not NTS-dependent, AREG- and BTC-induced phosphorylation of ERK1/2 in cumulus cells occurs in the early phase of ovulation, and then, NTS enhances the continuous phosphorylation of ERK1/2 in cumulus cells up to the late phase of the ovulation process through the upregulation of EGF-like factors, EREG and NRG1, and their receptors, ErbB2 and ErbB3, in granulosa and cumulus cells.

In conclusion, we revealed for the first time that NTS is expressed in granulosa and cumulus cells and is upregulated by LH stimuli through the activation of the ERK1/2 signaling pathway. During the early phase of ovulation, LH-induced AREG and BTC promote the transient activation of ERK1/2 in granulosa and cumulus cells. Subsequently, NTS induced by ERK1/2 enhances the continuous phosphorylation of ERK1/2 up to the late phase of ovulation in cumulus cells through the upregulation of EGF-like factors, EREG and NRG1, and the activity of their shedding enzyme ADAM17, as well as their receptors ErbB2 and ErbB3. Based on these results, NTS induces the sustainable activation of the EGF-like factor–ErbB–ERK1/2 pathway, which ensures oocyte maturation and the developmental competence of oocytes during the ovulation process.

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CONFLICT OF INTEREST STATEMENT

Masayuki Shimada is an editorial board member of Reproductive Medicine and Biology and a co-author of this article. To minimize bias, they were excluded from all editorial decision-making related to the acceptance of this article for publication.

ANIMAL STUDIES

The study was conducted in accordance with the Guideline for the Care and Use of Laboratory Animals of Prefectural University of Hiroshima (approval number 16SA007).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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