BOR Papers in Press. Published on July 8, 2015 as DOI:10.1095/biolreprod.115.130617

Analysis of the expression of tachykinins and tachykinin receptors in the rat uterus during early pregnancy[1]

Francisco M. Pinto,[3] Aixa R. Bello,[4] Manuel Gallardo-Castro,[3] Francisco Valladares,[5] Teresa A. Almeida,[4] Manuel Tena-Sempere,[6] and Luz Candenas[2,3]
[3]Instituto de Investigaciones Químicas, CSIC, Sevilla, Spain
[4]Instituto de Enfermedades Tropicales y Salud Pública y Departamento de Biología Celular, Universidad de La Laguna, La Laguna, Tenerife, Spain
[5]Departamento de Anatomía, Anatomía Patológica e Histología, Universidad de La Laguna, Campus de Ofra s/n, La Laguna, Tenerife, Spain
[6]Departamento de Biología Celular, Fisiología e Inmunología, Universidad de Cordoba; CIBER Fisiopatología de la Obesidad y la Nutrición, ISCiii and Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC)/Hospital Universitario Reina Sofía, Cordoba, Spain

Runing Title: Tachykinins in the uterus of early pregnant rats

Summary Sentence: The tachykinin family participates in the uterine events that occur around implantation in the early pregnant rat.

Keywords: Tachykinins; tachykinin receptors; early pregnancy; decidua; rat uterus.

[1] Supported by grants from Ministerio de Economía y Competitividad (CTQ2011-25564 and BFU2005-04495-C02-01), Spain, with joint financing by FEDER funds from the European Union.
[2]Correspondence: Luz Candenas. Instituto de Investigaciones Químicas, Avda. Americo Vespucio 49, 41092 Sevilla, Spain. E-mail**:** luzcandenas@iiq.csic.es

ABSTRACT
The peptides of the tachykinin family participate in the regulation of reproductive function acting at both central and peripheral levels. Our previous data showed that treatment of rats with a tachykinin NK3R antagonist caused a reduction of litter size. In the present study, we analyzed the expression of tachykinins and tachykinin receptors in the rat uterus during early pregnancy. Uterine samples were obtained from early pregnant rats (days 1-9 of pregnancy) and from non-pregnant rats during the proestrus stage of the ovarian cycle, and real-time quantitative RT-PCR, immunohistochemistry and Western blot studies were used to investigate the pattern of expression of tachykinins and tachykinin receptors. We found that all tachykinins and tachykinin receptors were locally synthesized in the uterus of early pregnant rats. The expression of substance P, neurokinin B, and the tachykinin receptors NK1R and NK3R mRNAs and proteins underwent major changes during the days around implantation and they were widely distributed in implantation sites, being particularly abundant in decidual cells. These findings support the involvement of the tachykinin system in the series of uterine events that occur around embryo implantation in the rat.

INTRODUCTION
Differentiation of the uterus to the receptive stage and cross-talk between the blastocyst and uterine luminal epithelium are essential for embryo implantation [1-3]. In the rat, the formation

of blastocysts occurs on pregnancy day 4 and their attachment is produced between days 4 and 5 [2]. This is followed by the differentiation of endometrial stromal cells into decidual cells that proliferate surrounding the implanting conceptus, a process that is essential to support subsequent development of the embryo and placenta [1,2]. However, the molecular mechanisms involved in these events remain poorly understood [1-3].

Increasing evidences suggest that tachykinins (TKs) participate in the regulation of reproduction, a role that has been conserved throughout evolution [4-12]. TKs are a family of peptides including substance P (SP), neurokinin A (NKA), neurokinin B (NKB) and hemokinin-1 (HK-1) [5-12]. In the rat, SP and NKA are encoded by the *Tac1* gene, while NKB and HK-1 are encoded by the genes *Tac3* and *Tac4*, respectively [6,9-13]. Tachykinin effects are mediated by three tachykinin receptors (TKRs), named NK1 (NK1R), NK2 (NK2R) and NK3 (NK3R), which are encoded by the genes *Tacr1*, *Tacr2* and *Tacr3* [4-13]. NK1R is the preferred receptor for SP and HK-1; NK2R for NKA and NK3R for NKB. However, endogenous tachykinins are not highly selective and are able to activate all TKRs [6,9-12].

TKs and TKRs are widely distributed in different types of male and female reproductive cells at both central and peripheral levels [4-26]. The essential role of one of the tachykinins, NKB, and its cognate receptor, NK3R, in reproduction was confirmed by the finding that mutations in *TAC3* and *TACR3* were associated with human normosmic hypogonadotrophic hypogonadism (nHH), a disease characterized by the failure of sexual maturation and infertility [8,19,23,24]. Besides its role in nHH, the expression of NB and/or NK3R was found to be dysregulared in other reproductive disorders such as preemclampsia [13] and uterine leiomyomata [26].

TKs appear to act as important intercellular signaling molecules within the mammalian uterus. All known TKs and TKRs are abundantly expressed in the uterus of different mammalian species, including humans, rats and mice, where their expression vary with age, during the ovarian cycle and during pregnancy and is tightly controlled by ovarian steroids [11,16,17,26-35]. In the rat uterus, *Tacr3* mRNA levels were higher during early pregnancy, in comparison with late pregnant animals, while *Tacr1* increased in late pregnancy [34]. However, the expression of TKs and TKRs during the days around implantation has not been investigated. In this study, we analyzed the presence of TKs and TKRs in the rat uterus during the 9 initial days of pregnancy and showed, to our knowledge for the first time, that the TK family is widely distributed in implantation sites (ISs), being particularly abundant in decidual cells.

MATERIALS AND METHODS

Animals

Ethical approval for these studies was obtained from the Ethics Committee of CSIC (Spain). Three month-old virgin female Wistar rats were purchased from Charles River Laboratories (Barcelona, Spain). Animals were maintained in an air-conditioned room at 22ºC under controlled lighting (12 h light/12 h dark), with free access to food and water. Pregnancy was induced by mating proestrus rats with male rats overnight. Vaginal smears were checked daily to determine the stage of the estrous cycle in non-pregnant animals and to confirm pregnancy in mated females. The day on which sperm was observed in the vaginal lavage was defined as day 0 (D0) of gestation. Uteri were obtained from rats on days 1, 3, 4, 5, 6, 7, 8 and 9 (D1-D9) of pregnancy and from virgin rats in the proestrus stage of the ovarian cycle. Rats were killed at 10:00 h. by decapitation following exposure to CO_2 and the uterine horns were rapidly removed, trimmed of surrounding connective tissue and opened longitudinally.

For the initial studies, samples were randomly chosen from different uterine regions in order to analyze the expression of all target genes in samples representative of the entire uterus. In a second set of experiments, samples from non-pregnant rats and from D1, D3, D4 and D5 pregnant animals were specifically chosen from the cervical, the middle or the ovarian section of both uterine horns, to investigate the expression of TKs and TKRs in different uterine regions. In a third series of experiments, uterine samples from D6, D7, D8 and D9 pregnant rats were obtained from inter-implantation and implantation sites. In the later, the conceptus was untouched (for histochemical analyses) or carefully removed (for RT-PCR and Western blot studies). Uterine samples were immediately immersed in RNAlater (Ambion) and stored at -80ºC until use.

RNA extraction and real-time quantitative polymerase chain reaction (qPCR)

Total RNA was isolated using the RNA/Protein purification kit (Norgen Biotek Corp., Ontario, Canada) and residual genomic DNA was removed by incubating the RNA samples with RNase-free DNase I and RNasin (Promega, Madison, WI). Complementary DNAs (cDNAs) were synthesized using the Quantitect Reverse Transcription kit (Qiagen, Venlo, The Netherlands).

Real-time qPCR was used to quantify the expression of the test genes in the different uterine samples, which was carried out by using the $2^{-\Delta\Delta C}{}_T$ method [26,29,33]. qPCR was performed on a Bio-Rad iCycler iQ real-time detection apparatus (Hercules, CA) using FastStart SYBR Green Master (Roche Diagnostics GmbH, Manheim, Germany). Cycling conditions were 5 sec at 95ºC, 10 sec at 60ºC and 10 seconds at 72ºC, for 50 cycles. The sequences of the specific primer pairs designed to amplify each target gene are shown in Supplemental Table S1 (Supplemental Data are available online at www.biolreprod.org). Supplemental Table S1 also shows the primers used to amplify β-actin (*Actb*); glyceraldehyde-3-phosphate-dehydrogenase (*Gapdh*); protein phosphatase-1 catalytic subunit beta-isoform (*Pp1cb*), and polymerase (RNA) II (DNA directed) polypeptide A (*Polr2a*), which were chosen as reference genes for normalizing the PCR data on the basis of previous studies in the human, mouse and rat uterus [26,29,30,33].

The fold change of the target gene expression was calculated relative to the geometric mean mRNA expression of the four reference genes in each sample [26,33]. A pool of uterine cDNAs from virgin proestrus rats was used as a control sample throughout the study and the ratio of each target gene mRNA/geometric mean of the four reference genes mRNA in this control sample was designated as 1.

Immunohistochemistry

Uterine samples from four D4 (pre-implantation) and four D7 (post-implantation) pregnant rats were fixed in 10% buffered formalin, embedded in paraffin and cut in 3-µm thick sections. The sections were deparaffined, rehydrated, hematoxylin–eosin stained and evaluated for histology.

For indirect immynohystochemistry, deparaffined sections were rehydrated in 0.05 M Tris-buffered saline (TBS; Trizma base 0.05 M, NaCl 0.9%, pH 7.4) and incubated overnight at room temperature with one of the following primary antibodies: a) goat anti-NKB (sc-14109; Santa Cruz Biotechnology, Santa Cruz, CA, USA); b) rabbit anti-NK3R (sc-28952, Santa Cruz); c) rabbit anti-SP (8450-0004, Bio-Rad) or d) goat anti-NK1R (sc-5218; Santa Cruz). All primary antibodies were diluted 1:50 in TBS containing 0.05% Triton X-100. After rinsing, the sections were incubated with a biotinylated goat-anti-rabbit or rabbit anti-goat (1:1000) followed by a streptavidin-peroxidase conjugate (1:1000; Jackson ImmunoResearch, West Grove, PA, USA), both for 60 min at room temperature. Peroxidase activity was detected using 0.005% 3,3'-

diaminobenzidine tetrahydrochloride (DAB) (Sigma) and 0.001% hydrogen peroxide followed by hematoxylin staining contrast. Immunostaining specificity was assessed by replacing the specific antisera by normal serum, omitting one step of the reaction or following preabsorption of the antisera with the corresponding antigens.

Western Blot experiments

Total proteins were extracted from uterine tissue by using the RNA/Protein purification kit (Norgen Biotek Corp., Ontario, Canada) and approximately 20 μg of protein were loaded on precast 4-20% gradient stain-free gels (Bio-Rad). Proteins were separated by electrophoresis, transferred to polyvinyldifluoride (PVDF) membranes and non-specific staining blocked by incubation with 1% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TTBS). The membranes were incubated overnight with goat anti-NK1R (sc-5218), rabbit anti-NK2R (sc-28951, Santa Cruz) or rabbit anti-NK3R antibody (sc-28952). Immunoreactivity was detected by treatment with appropriate HRP-conjugated secondary antibody and developed with the Amersham advance enhanced chemiluminescence (ECL) kit (Buckinghamshire, UK). Primary antibody dilution was 1:2500 and for the secondary antibody it was 1:50000 (goat anti-rabbit IgG) or 1:20.000 (rabbit anti-goat IgG). Stain-free total protein measurements were used to normalize NK1R, NK2R and NK3R protein expression using the total protein loading control method and ImageLab Software (Bio-Rad).

Statistical analysis

All values are expressed as means ± SEM and were obtained by pooling individual data. *n* represents the number of experiments in uterine samples from *n* different rats. Statistical analyses were performed using Mann–Whitney's U (for comparison of mean ranks between two groups) or Kruskal–Wallis followed by Dunn´s (to compare more than two groups) non-parametric tests. These procedures were undertaken using Graphpad PRISM (version 5.0) program. $P<0.05$ values were considered significant.

RESULTS

mRNA expression of tachykinins and tachykinin receptors during early pregnancy in the rat uterus

The genes encoding tachykinins (*Tac1*, *Tac3* and *Tac4*) and tachykinin receptors (*Tacr1*, *Tacr2* and *Tacr3*) were all expressed in the uterus of early pregnant rats (D1-D9).

During pregnancy days 1-3, *Tac1* mRNA levels were similar to those observed in the uterus of non-pregnant proestrous rats (Figs. 1A and 2A). *Tac1* increased by 6-fold in D4 and by 16-fold in D5 and then returned to levels similar to those in D1 and D3, which were however moderately higher than those in the proestrous rat (Figs. 1A and 2A). While *Tac1* expression was increased in all D5 samples analyzed, it showed a great variability in pregnancy D4, with 5 samples showing low levels, similar to those in D1 and D3, and 5 samples showing high levels, similar to those in pregnancy D5. *Tac3* increased by 2.5-fold in D4 and D5, by 9-fold in pregnancy D6, showed a sharp increase, by 40-fold, in D7 and remained highly elevated in D8 and D9 (Figs. 1B and 2B). *Tac4* uterine expression did not change during the initial 9 days of pregnancy (Fig. 1C).

The expression of *Tacr1* and *Tacr3* also changed during early pregnancy (Fig. 1D,E and 2C,D). In parallel with *Tac1*, *Tacr1* increased by 5-fold in D4 and by 2-fold in D5, in comparison with mRNA levels in the uterus of proestrus rats (Fig. 1D). *Tacr3* was already elevated in D1, with levels about 10-fold higher than those found in proestrous rats. A decrease in *Tacr3* was

observed in D5, although its expression remained elevated, by 4-9 fold, in days 5-9, relative to mRNA levels in proestrus rats (Figs. 1E, 2D). Uterine *Tacr2* expression did not change during the initial 9 days of pregnancy (Fig. 1F).

We next analyzed whether the changes in mRNA were restricted to a particular uterine section or took place along the whole uterine horn. During the initial 5 days of pregnancy each uterine horn was subdivided in three different segments: ovarian, middle and cervical. We found that the mRNA levels of each TK and TKR in D1, D3, D4 and D5 were virtually identical in the distinct parts of the uterus and expression changes were homogeneously produced along the uterine horn (Fig. 2, not shown for *Tac4* and *Tacr2,* only results in uterine cervical and middle sections are presented).

In days 6-9, implantation sites were clearly visualized and we analyzed the expression of TKs and TKRs in ISs and in the adjacent inter-implantation sites, randomly chosen from any part of the uterine horn. The expression of *Tac1* was similar in implantation and inter-implantation sites (Fig. 2A). In contrast, expression of *Tac3* was mainly located in implantation sites, with mRNA levels being between 5- and 14-fold higher than in inter-implantation sections (Fig. 2B). For *Tacr1*, there was a tendency towards a higher expression in inter-implantation sites which, however, did not reach statistical significance (Fig. 2C). *Tacr3* expression was between 5- and 20-fold higher in inter-implantation sites, compared with mRNA levels in the adjacent ISs (Fig. 4D).

Protein expression of tachykinins and tachykinin receptors during early pregnancy in the rat uterus

Immunohistochemistry was used to determine the tissue and cell distribution of SP, NKB, NK1R and NK3R proteins in the uterus of D4 and D7 pregnant rats.

In D4, i.e. before implantation, SP immunoreactivity (ir) was observed in luminal epithelial cells lining the uterus as well as in glandular epithelial cells. Strong immunoreactivity was also visible in the stromal compartment, particularly in the superficial endometrial stroma, and in myometrial smooth muscle cells (Fig. 3a). NKB, NK1R and NK3R immunostaining was found in similar localizations and was weaker than that for SP (Fig. 3e,i,m). In uterine inter-implantation sites from D7 rats, SP-, NKB-, NK1R- and NK3R-ir was also found in luminal and glandular epithelial cells, stromal cells and myometrium (Fig. 3b,f,j,n). Intense immunoreactivity for both TKs and TKRs was detected in implantation sites in the uterus from D7 pregnant rats, where positive immunostaining was mainly located in decidual cells. It is remarkable the strong immunoreactivity observed for NKB, NK1R and NK3R in implantation sites (Fig. 3g,h,k,l,o,p). SP, NKB, NK1R and NK3R were also expressed abundantly in the conceptus (data not shown).

Tachykinin receptor protein levels during the initial 8 days of pregnancy were further analyzed by Western blot. Immunoreactive bands of the size expected for NK1R, NK2R and NK3R were detected in all uterine samples assayed. NK1R levels began to increase in D6 and were significantly higher in ISs, compared with the adjacent inter-implantation sites, in D7 and D8 (Fig. 4A and Supplemental Fig. S1). The levels of NK2R protein remained stable during the initial days of pregnancy in the rat (Fig. 4B and Supplemental Fig. S1). NK3R protein increased in D4, remained elevated during D5, D6 and D7 and began to decrease in D8 (Fig. 4C and Supplemental Fig. S1). In D7, NK3R protein levels were higher in ISs, in comparison with adjacent inter-implantation sites (Fig. 4C and Supplemental Fig. S1).

DISCUSSION

Despite its essential role in pregnancy initiation, the molecular mechanisms underlying endometrial receptivity are not fully elucidated jet [1-3]. In the present study, we demonstrate that all TKs and TKRs are expressed-in the uterus of early pregnant rats, with strikingly different profiles of expression of SP, NKB, NK1R and NK3R around the implantation window, and an abundant presence in implantation sites, particularly in decidual cells. These findings provide the molecular basis for a putative role of the TK system in the regulation of uterine functions in the early pregnant rat.

NKB and its cognate receptor, NK3R, have recently emerged as essential regulators of reproduction acting at both central and peripheral levels [13-36]. Our current data show that NKB and NK3R are expressed, at both the mRNA and protein levels, in the uterus of early pregnant rats. Notably, *Tac3* expression and NKB content were markedly increased from D6 onwards, showing a preferential localization in implantation sites. NKB-ir was particularly abundant in implantation sites in D7 rats, being mainly located in the decidual layer, the differentiating endometrial stromal cells that surround the implanting blastocyst (see Fig. 3). The observation that NKB is also abundant in the conceptus (present study) and placental trophoblasts [13] strongly argues for a role of NKB in the regulation of implantation and placentation, acting at both the maternal and embryo sides.

A role for NK3R in early pregnancy has been recently suggested by our previous findings, showing that treatment of rats with a selective NK3R antagonist caused a reduction in litter size [17], a fact that was subsequently confirmed in *Tacr3* null mice [37]. In the rat uterus, NK3R mRNA expression and function increase during early pregnancy and are reduced in late pregnancy [34]. The present data show that *Tacr3* levels are persistently elevated during early pregnancy (D1 to D9), a phenomenon that might be linked to relatively low E_2 levels at the initiation of pregnancy, as estradiol strongly down-regulates *Tacr3* expression in the rat uterus [16,27,35]. As observed for NKB, NK3R protein was mainly expressed in implantation sites and was particularly abundant in the decidua. In contrast, *Tacr3* levels were higher in inter-implantation sites than in adjacent ISs at D6-D9 (see Fig. 2). This observation discloses a complex dynamics between mRNA and protein expression for NK3R, which might suggest accumulation of NK3R protein at implantation sites, even against low mRNA expression levels. Alternatively, high expression of *Tacr3* at inter-implantation sites might not drive NK3R synthesis in the absence of appropriate signal(s), of as yet unknown nature, that could trigger protein translation. Further studies will help to clarify this issue.

Compelling evidence suggests that not only NKB/NK3R, but the whole TK family participates in the regulation of uterine function. *Tac4* and *Tacr2* are widely distributed in the mammalian uterus, although their expression is not apparently regulated by ovarian steroids [11,27,33,34]. In contrast, E_2 up-regulates the expression of *Tacr1* in the rat and mouse uterus and exerts a time-dependent dual (stimulatory/inhibitory) effect on *Tac1* expression in the mouse [27,33]. Our present data show that SP and NK1R undergo important expression changes, at the mRNA and protein level, during the initial days of pregnancy. Moreover, SP and NK1R proteins were co-expressed with NKB and NK3R and were highly abundant in the decidua and the conceptus at implantation sites. These data strongly suggest that SP, NK1R, NKB and NK3R may act in a coordinated manner to modulate embryo implantation and placentation in the rat.

In the human uterus, NKB and NK3R are present in luminal and glandular epithelia, endometrial stromal cells and myometrial smooth muscle cells [31]. SP, NKB, NK1R and NK3R showed a similar localization in the rat uterus (Fig. 3). NK2R is the major TK receptor mediating uterine contractions in humans and rats while NK1R seems to be the predominant receptor in the mouse

uterus [16,29,30,34]. Uterine expression of NK3R increased during early pregnancy in the rat but it was undetectable in early pregnant mice [30]. Moreover, treatment with a selective NK3R antagonist decreased fertility in rats but had no effect in mice [17]. Besides these species differences, we have noticed that blockade of NK3R causes an increase in the expression and function of NK1R in the rat uterus (Pinto and Candenas, unpublished data). Altogether, these data argue for the existence of a redundancy within the TK system, with different TKs and TKRs being able to fulfil a similar function, in order to preserve fertility function [7,37]. This is also suggested by the observation that the loss of function of the NKB/NK3R system in mammals evokes a pubertal delay, but not complete infertility [10,17,19,21,23,36-38] and may explain recent findings in mice bearing mutations in *Tac2* or *Tacr3*, showing that NKB/NK3R signaling can be dispensable for adult reproductive function [37,38].

In conclusion, this study shows that all TKS and TKRs are widely distributed in the uterus of early pregnant rats, with variable profiles of SP, NK1R, NKB and NK3R during the initial days of pregnancy and abundant expression in the decidual layer at the implantation sites. A summary of these findings is shown in Fig. 5. The present data support and provides the molecular basis for the involvement of the TK system in the series of uterine events that regulate embryo implantation in the rat.

REFERENCES

1. Zhang P, Tang M, Zhong T, Lin Y, Zong T, Zhong C, Zhang B, Ren M, Kuang H. Expression and function of kisspeptin during mouse decidualization. PLoS One 2014; 9:e97647.

2. Johnson MH. Initiating Pregnancy. In: Johnson MH (ed.), Essential Reproduction, 7th ed. Masachusset, USA: Blackwell Publishing, 2013:207-224.

3. Dominguez F, Yáñez-Mó M, Sanchez-Madrid F, Simón C. Embryonic implantation and leukocyte transendothelial migration: different processes with similar players? FASEB J 2005; 19:1056-1060.

4. Navarro VM, Bosch MA, León S, Simavli S, True C, Pinilla L, Carroll RS, Seminara SB, Tena-Sempere M, Rønnekleiv OK, Kaiser UB. The integrated hypothalamic tachykinin-kisspeptin system as a central coordinator for reproduction. Endocrinology 2015; **156:**627-637.

5. Debeljuk L, Lasaga M. Modulation of the hypothalamo-pituitary-gonadal axis and the pineal gland by neurokinin A, neuropeptide K and neuropeptide gamma. Peptides 1999; 20:285-299.

6. Almeida TA, Rojo J, Nieto PM, Pinto FM, Hernandez M, Martin JD, Candenas ML. Tachykinins and tachykinin receptors: structure and activity relationships. Curr Med Chem 2004; 11:2045-2081.

7. Pennefather JN, Lecci A, Candenas ML, Patak E, Pinto FM, Maggi CA. Tachykinins and tachykinin receptors: a growing family. Life Sci 2004a; 74:1445-1463.

8. Topaloglu AK, Reimann F, Guclu M, Yalin AS, Kotan LD, Porter KM, Serin A, Mungan NO, Cook JR, Ozbek MN, Imamoglu S, Akalin NS, et al. TAC3 and TACR3 mutations in familial hypogonadotropic hypogonadism reveal a key role for Neurokinin B in the central control of reproduction. Nature Genet 2009; 41:354-358.

9. Satake H, Aoyama M, Sekiguchi T, Kawada T. Insight into molecular and functional diversity of tachykinins and their receptors. Protein Pept Lett 2013; 20:615-627.

10. Hu G, Lin C, He M, Wong AO. Neurokinin B and reproductive functions: "KNDy neuron" model in mammals and the emerging story in fish. Gen Comp Endocrinol 2014; 208:94-108.

11. Page NM. Hemokinins and endokinins. Cell Mol Life Sci 2004; 61:1652-1663.

12. Steinhoff MS, von Mentzer B, Geppetti P, Pothoulakis C, Bunnett NW. Tachykinins and their receptors: contributions to physiological control and the mechanisms of disease. Physiol Rev 2014; 94:265-301.
13. Page NM, Woods RJ, Gardiner SM, Lomthaisong K, Gladwell RT, Butlin DJ, Manyonda IT, Lowry PJ. Excessive placental secretion of neurokinin B during the third trimester causes pre-eclampsia. Nature 2000; 405:797-800.
14. Jayasena CN, Comninos AN, Stefanopoulou E, Buckley A, Narayanaswamy S,Izzi-Engbeaya C, Abbara A, Ratnasabapathy R, Mogford J, Ng N, Sarang Z, Ghatei MA, Bloom SR, Hunter MS, Dhillo WS. Neurokinin B Administration Induces Hot Flushes in Women. Sci Rep 2015; 5:8466.
15. Brown ER, Harlan RE, Krause JE. Gonadal steroid regulation of substance P (SP) and SP-encoding messenger ribonucleic acids in the rat anterior pituitary and hypothalamus. Endocrinology 1990; 126:330-340.
16. Pennefather JN, Patak E, Pinto FM, Candenas ML. Mammalian tachykinins and uterine smooth muscle: the challenge escalates. Eur J Pharmacol 2004b; 500:15-26.
17. Pintado CO, Pinto FM, Pennefather, JN, Hidalgo A, Baamonde A, Sanchez T, Candenas ML. A role for tachykinins in female mouse and rat reproductive function. Biol Reprod 2003; 69:940-946.
18. Rance NE. Menopause and the human hypothalamus: Evidence for the role of kisspeptin/neurokinin B neurons in the regulation of estrogen negative feedback. Peptides 2009; 30:111-122.
19. Topaloglu AK, Semple RK. Neurokinin B signalling in the human reproductive axis. Mol Cell Endocrinol 2011; 346:57-64.
20. Lehman MN, Coolen LM, Goodman RL. Minireview: kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: a central node in the control of gonadotropin-releasing hormone secretion. Endocrinology 2010; 151:3479-3489.
21. Navarro VM, Ruiz-Pino F, Sánchez-Garrido MA, García-Galiano D, Hobbs SJ, Manfredi-Lozano M, León S, Sangiao-Alvarellos S, Castellano JM, Clifton DK, Pinilla L, Steiner RA, Tena-Sempere M. Role of neurokinin B in the control of female puberty and its modulation by metabolic status. J Neurosci 2012; 32:2388-2397.
22. Aoyama M, Kawada T, Fujie M, Hotta K, Sakai T, Sekiguchi T, Oka K, Satoh N, Satake H. A novel biological role of tachykinins as an up-regulator of oocyte growth: identification of an evolutionary origin of tachykininergic functions in the ovary of the ascidian, Ciona intestinalis. Endocrinology 2008; 149:4346-4356.
23. Gianetti E, Tusset C, Noel SD, Au MG, Dwyer AA, Hughes VA, Abreu AP, Carroll J, Trarbach E, Silveira LF, Costa EM, de Mendonça BB, et al. TAC3/TACR3 mutations reveal preferential activation of gonadotropin-releasing hormone release by neurokinin B in neonatal life followed by reversal in adulthood. J Clin Endocrinol Metab 2010; 95:2857-2867.
24. Young J, Bouligand J, Francou B, Raffin-Sanson ML, Gaillez S, Jeanpierre M Grynberg M, Kamenicky P, Chanson P, Brailly-Tabard S, Guiochon-Mantel A. TAC3 and TACR3 defects cause hypothalamic congenital hypogonadotropic hypogonadism in humans. J Clin Endocrinol Metab 2010; 95:2287-2295.
25. de Croft S, Boehm U, Herbison AE. Neurokinin B activates arcuate kisspeptin neurons through multiple tachykinin receptors in the male mouse. Endocrinology 2013; 154:2750-2760.
26. Cañete H, Dorta I, Hernández M, Cejudo Roman A, Candenas L, Pinto FM, Valladares F, Báez D, Montes de Oca F, Bello AR, Almeida TA. Differentially regulated expression of

neurokinin B (NKB)/NK3 receptor system in uterine leiomyomata. Hum Reprod 2013; 28:1799-1808.
27. Pinto FM, Armesto CP, Magraner J, Trujillo, M, Martín JD, Candenas ML Tachykinin receptor and neutral endopeptidase gene expression in the rat uterus: characterization and regulation in response to ovarian steroid treatment. Endocrinology 1999; 140:2526-2532.
28. Crane LH, Williams MJ, Nimmo AJ, Hamlin GP. Estrogen-dependent regulation of neurokinin 3 receptor-mediated uterine contractility in the rat. Biol Reprod 2002; 67:1480-1487.
29. Patak E, Candenas ML, Pennefather JN, Ziccone S, Lilley A, Martin JD, Flores C, Mantecón AG, Story ME, Pinto FM. Tachykinins and tachykinin receptors in human uterus. Br J Pharmacol 2003; 139:523-532.
30. Patak E, Pinto FM, Story ME, Pintado CO, Fleming A, Page NM, Pennefather JN, Candenas ML. Functional and molecular characterization of tachykinins and tachykinin receptors in the mouse uterus. Biol Reprod 2005; 72:1125-1133.
31. Cejudo Roman A, Pinto FM, Dorta I, Almeida TA, Hernández M, Illanes M, Tena-Sempere M, Candenas L. Analysis of the expression of neurokinin B, kisspeptin and their cognate receptors, NK_3R and KISS1R in the human female genital tract. Fertil Steril 2012; 97:1213-1219.
32. Pinto FM, Cintado CG, Devillier P, Candenas ML. Expression of preprotachykinin-B, the gene that encodes neurokinin B, in the rat uterus. Eur J Pharmacol 2001; 425:R1-R2.
33. Pinto FM, Pintado CO, Pennefather JN, Patak E, Candenas L. Ovarian steroids regulate tachykinin and tachykinin receptor gene expression in the mouse uterus. Reprod Biol Endocrinol 2009; 7:77.
34. Candenas ML, Magraner J, Armesto CP, Anselmi E, Nieto PM, Martín JD, Advenier C, Pinto FM. Changes in the expression of tachykinin receptors in the rat uterus during the course of pregnancy. Biol Reprod 2001; 65:538-543.
35. Cintado CG, Pinto FM, Devillier P, Merida A, Candenas ML. Increase in neurokinin B expression and in tachykinin NK3 receptor-mediated response and expression in the rat uterus with age. J Pharmacol Exp Ther 2001; 299:934-938.
36. Pinilla L, Aguilar E, Dieguez C, Millar RP, Tena-Sempere M. Kisspeptins and reproduction: physiological roles and regulatory mechanisms. Physiol Rev 2012; 92:1235-1316.
37. Yang JJ, Caligioni CS, Chan YM, Seminara SB. Uncovering novel reproductive defects in neurokinin B receptor null mice: closing the gap between mice and men. Endocrinology 2012; 153:1498-1508.
38. True C, Nasrin Alam S, Cox K, Chan YM, Seminara S. Neurokinin B is critical for normal timing of sexual maturation but dispensable for adult reproductive function in female mice. Endocrinology 2015; 156:1386-1397.

FIGURE LEGENDS

Fig. 1. A-F) Real-time quantitative RT-PCR analysis of expression of the genes encoding tachykinins (*Tac1*, *Tac3* and *Tac4*) and tachykinin receptors (*Tacr1*, *Tacr2* and *Tacr3*) in the rat uterus during the initial 9 days of pregnancy. Values are expressed relative to mRNA levels in non-pregnant proestrus rats. Each point represents the mean ± SEM in 10 different animals. *$P<0.05$, significant difference vs. mRNA levels in uteri from proestrus rats, Mann-Whitney test. $^{a,b,c,d,e,f,g,h}P<0.05$ significant difference vs. mRNA levels in uteri from day 1,3,4,5,6,7,8 and 9 pregnant rats, respectively, Kruskal-Wallis followed by Dunn's test.

Fig. 2. A-D) Real-time quantitative RT-PCR analysis of expression of *Tac1*, *Tac3*, *Tacr1* and *Tacr3* in the rat uterus during the initial 9 days of pregnancy. mRNA levels in middle (M) and cervical (C) uterine sections from pregnancy days 1, 3, 4 and 5 and in implantation sites (IS) and adjacent inter-implantation sites (N) from day 6, 7, 8 and 9 pregnant rats. Values are expressed relative to mRNA levels in non-pregnant proestrus rats. Each bar represents the mean ± SEM in 5 different animals. **P*<0.05, significant difference vs. mRNA levels in uteri from adjacent segment (pregnancy days 1, 3, 4 and 5) or implantation site (pregnancy days 6, 7, 8 and 9), Mann-Whitney test.

Fig. 3. Immunolocalization of SP, NKB, NK1R and NK3R in the rat uterus during early pregnancy. In pregnancy day 4, brown immunostaining was located in endometrial luminal and glandular epithelium (arrows) stromal (stars) and myometrial (asterisks) cells (**a**,**e**,**i**,**m**). In pregnancy day 7, SP, NKB, NK1R and NK3R signals had a similar localization in inter-implantation sites (Int. IS, **b**,**f**,**j**,**n**). Intense immunoreactivity for both TKs and TKRs was detected in implantation sites (IS, **c**,**g**,**k**,**o**) with positive immunostaining being very strong in decidual cells (**d**,**h**,**l**,**p**). Bar = 100μm.

Fig. 4. A-C) Western blot analysis of the expression of tachykinin receptors in the rat uterus during the initial 8 days of pregnancy. NK1R, NK2R and NK3R protein levels in middle (M) and cervical (C) uterine sections from pregnancy days 1, 3, 4 and 5 and in implantation sites (IS) and adjacent inter-implantation sites (N) from day 6, 7 and 8 pregnant rats. For each tachykinin receptor, protein expression was normalized against the stain-free total protein measurement from the blot and expressed relative to protein levels in non-pregnant proestrus rats. Each bar represents the mean ± SEM in 4 different animals. **P*<0.05, significant difference vs. protein levels in uteri from adjacent segment (pregnancy days 1, 3, 4 and 5) or inter-implantation site (pregnancy days 6, 7 and 8), Mann-Whitney test. ^{a,b,c,d}*P*<0.05 significant difference vs. protein levels in uteri from day 1, 3, 4 and 5 pregnant rats, respectively, Kruskal-Wallis followed by Dunn's test.

Fig. 5. Schematic representation showing the time-course changes in expression of tachykinins and tachykinin receptors in the rat uterus during early pregnancy. All members of the tachykinin family are expressed in the uterus of day 1 (D1) to day 8 (D8) pregnant rats, but only those who demonstrate a differential expression pattern are included in the figure. IS, implantation site; N, inter-implantation site; ICM, inner cell mass; D, decidua.

Figure 1

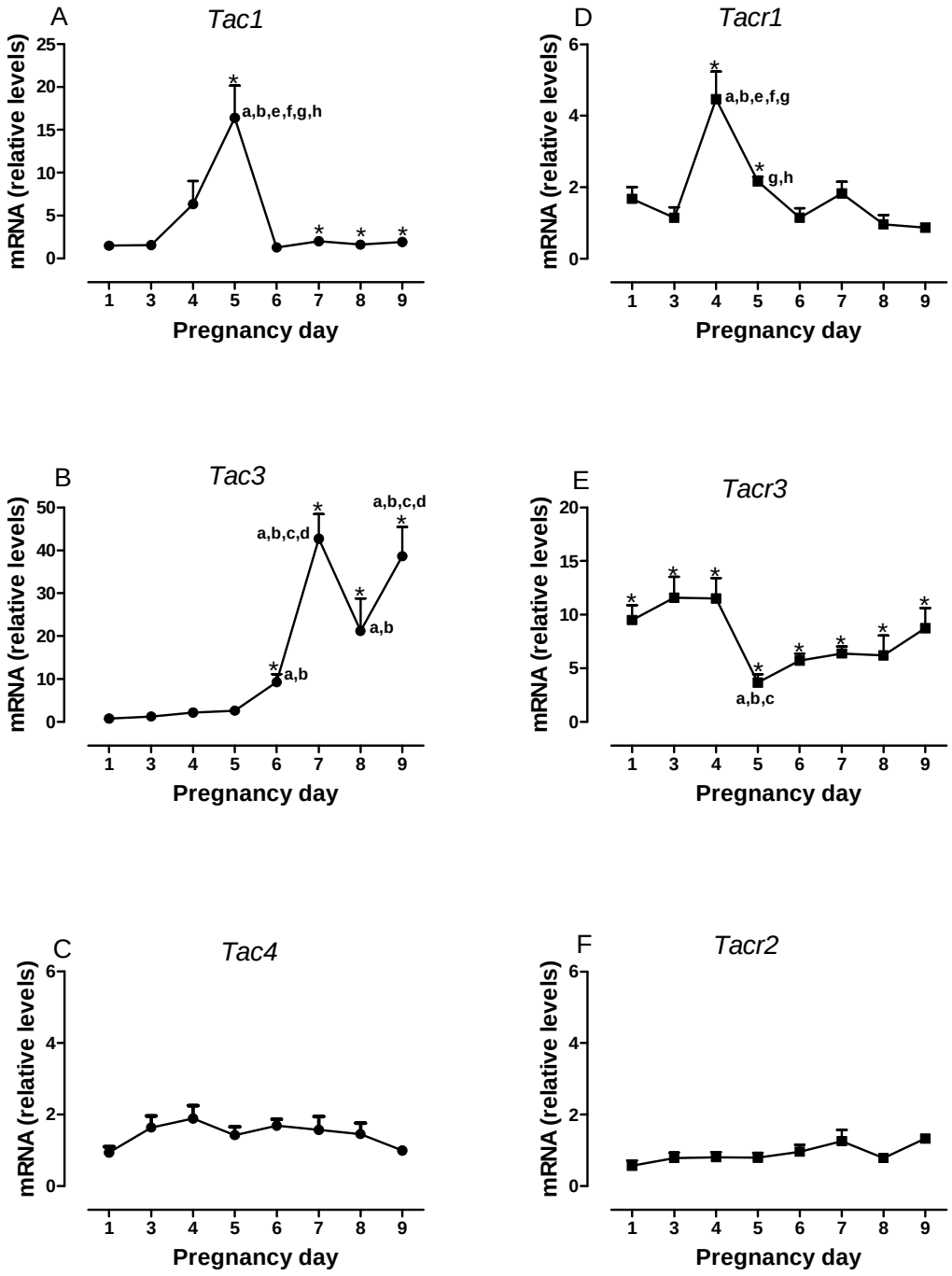
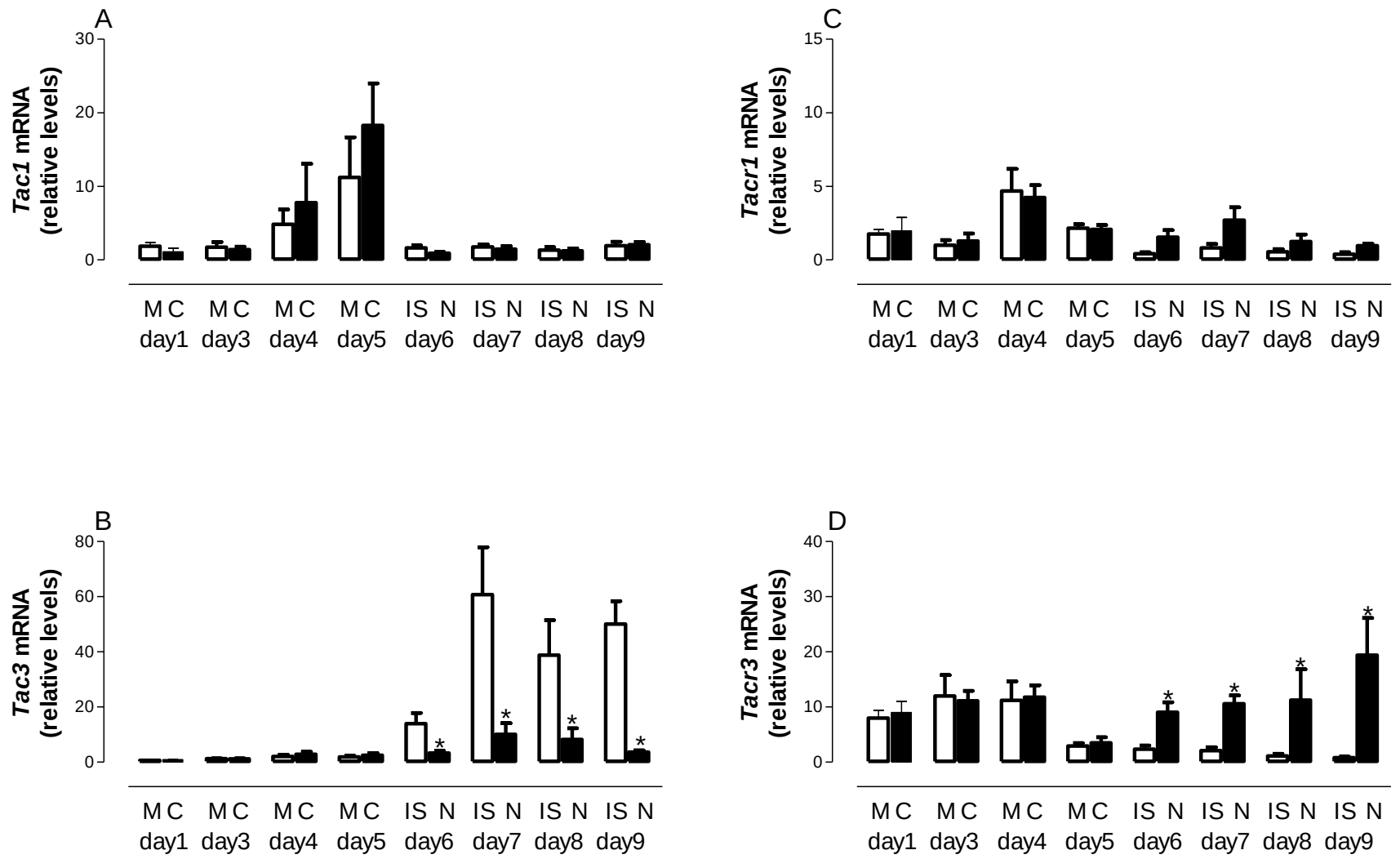

A
Tac1
mRNA (relative levels)
Pregnancy day
a,b,e,f,g,h
D
Tacr1
a,b,e,f,g
g,h
B
Tac3
a,b,c,d
a,b
E
Tacr3
a,b,c
C
Tac4
F
Tacr2

Figure 2


A
Tac1 mRNA (relative levels)
30
20
10
0
M C day1
M C day3
M C day4
M C day5
IS N day6
IS N day7
IS N day8
IS N day9
C
Tacr1 mRNA (relative levels)
15
10
5
0
B
Tac3 mRNA (relative levels)
80
60
40
20
0
D
Tacr3 mRNA (relative levels)
40
30
20
10
0
*

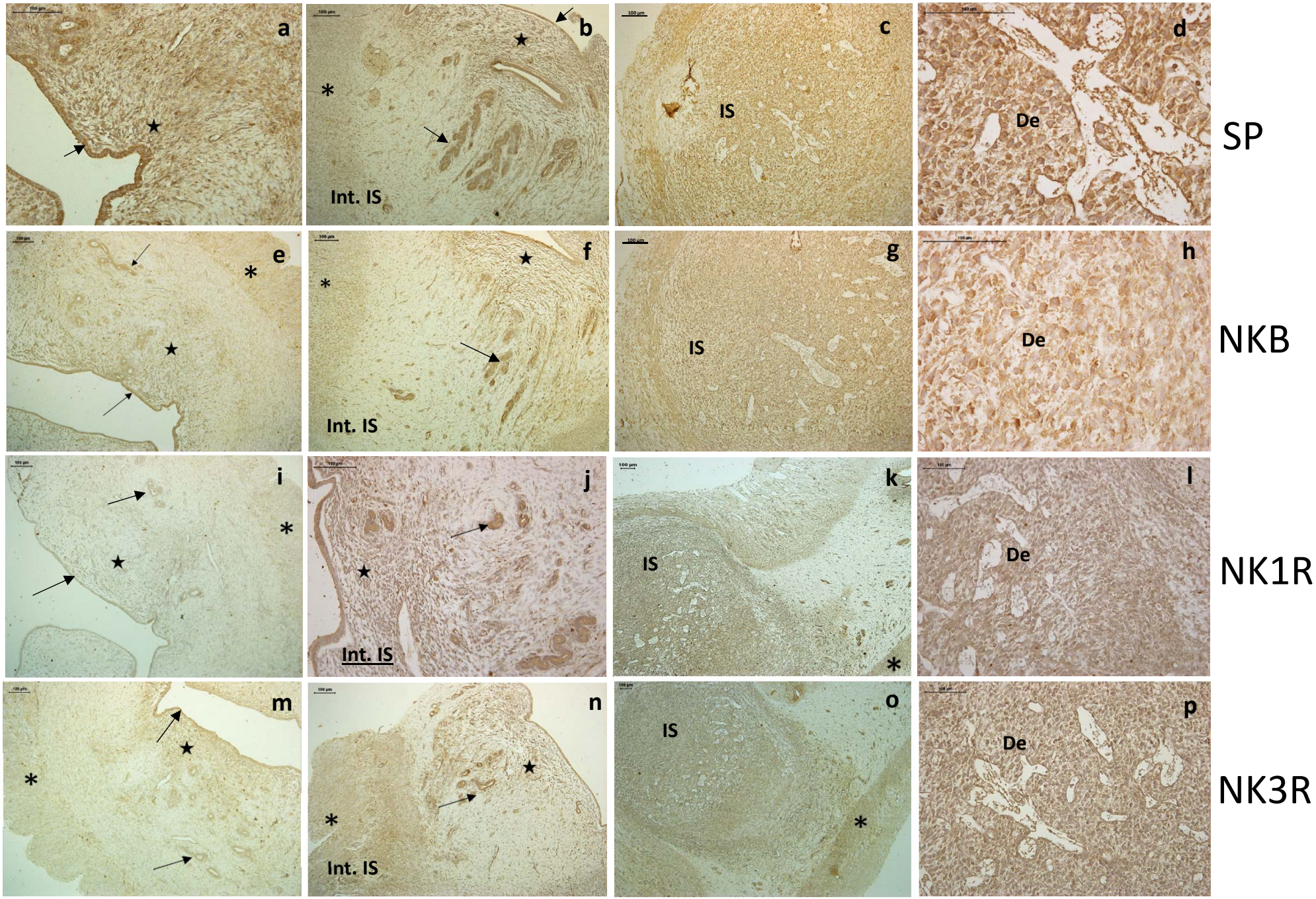
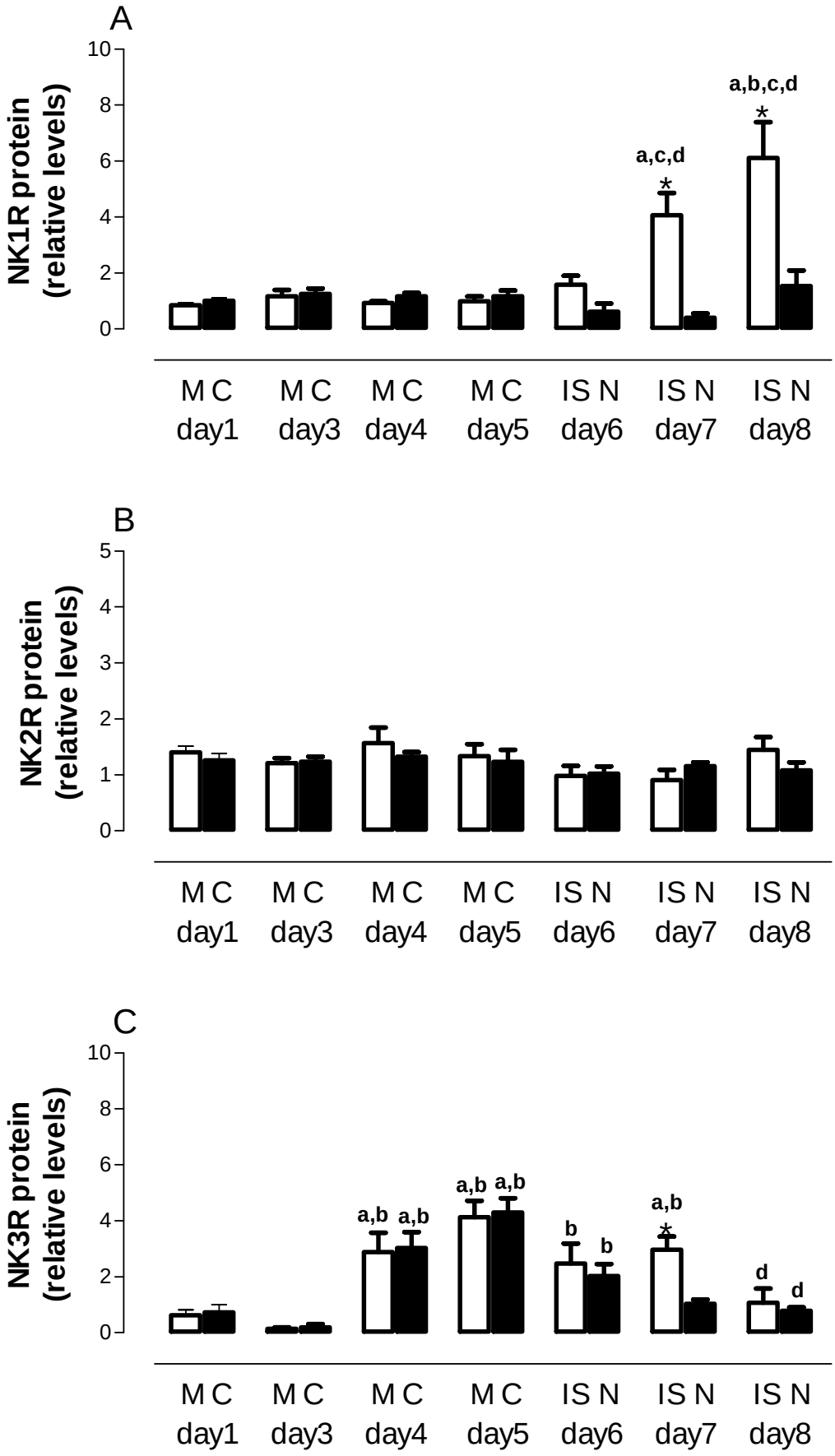

a
b
Int. IS
c
IS
d
De
SP
e
f
Int. IS
g
IS
h
De
NKB
i
j
Int. IS
k
IS
l
De
NK1R
m
n
Int. IS
o
IS
p
De
NK3R

Figure 3

Figure 4


A
NK1R protein (relative levels)
10
8
6
4
2
0
a,c,d
*
a,b,c,d
*
M C day1
M C day3
M C day4
M C day5
IS N day6
IS N day7
IS N day8
B
NK2R protein (relative levels)
5
4
3
2
1
0
M C day1
M C day3
M C day4
M C day5
IS N day6
IS N day7
IS N day8
C
NK3R protein (relative levels)
10
8
6
4
2
0
a,b
a,b
a,b
a,b
b
b
a,b
*
d
d
M C day1
M C day3
M C day4
M C day5
IS N day6
IS N day7
IS N day8

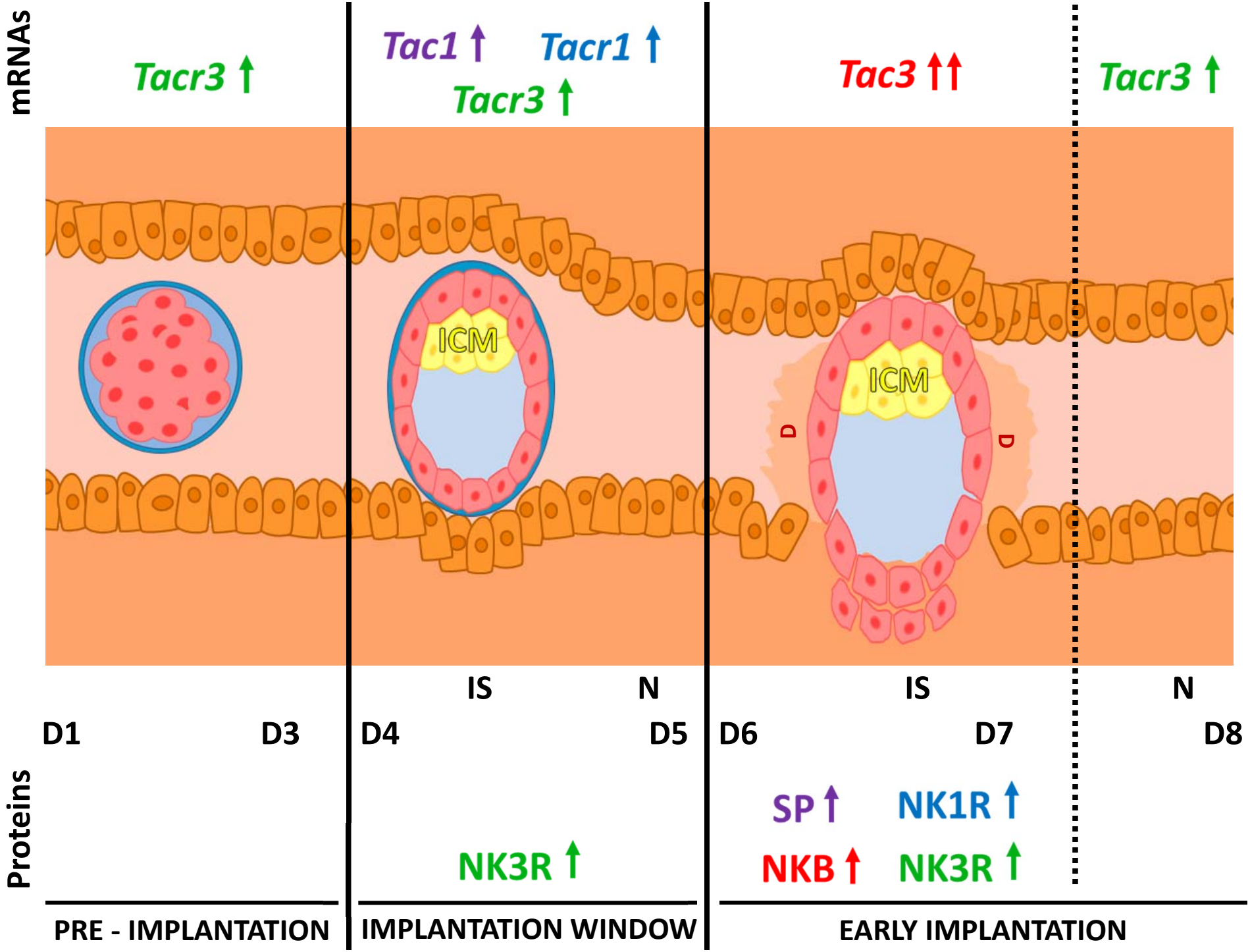

mRNAs
Tacr3 ↑
Tac1 ↑
Tacr1 ↑
Tacr3 ↑
Tac3 ↑↑
Tacr3 ↑
ICM
ICM
D
D
IS
N
IS
N
D1
D3
D4
D5
D6
D7
D8
Proteins
NK3R ↑
SP ↑
NK1R ↑
NKB ↑
NK3R ↑
PRE - IMPLANTATION
IMPLANTATION WINDOW
EARLY IMPLANTATION

Figure 5