Reproductive Biology and Endocrinology

Research

Multiple luteinizing hormone receptor (LHR) protein variants, interspecies reactivity of anti-LHR mAb clone 3B5, subcellular localization of LHR in human placenta, pelvic floor and brain, and possible role for LHR in the development of abnormal pregnancy, pelvic floor disorders and Alzheimer's disease

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Abstract

Distinct luteinizing hormone receptor (LHR) protein variants exist due to the posttranslational modifications. Besides ovaries, LHR immunoreactivity (LHRI) was also found in other tissues, such as the brain, fallopian tube, endometrium, trophoblast and resident tissue macrophages. The 3B5 mouse monoclonal antibody was raised against purified rat LHR. In rat, porcine and human ovaries, the 3B5 identified six distinct LHR bands migrating at ~92, 80, 68, 59, 52 and 48 kDa. Characteristic LHRI was detected in rat, human and porcine corpora lutea. During cellular differentiation, subcellular LHR distribution changed from none to granular cytoplasmic, perinuclear, surface, nuclear and no staining. There were also differences in vascular LHR expression – lack of LHRI in ovarian vessels and strong staining of vessels in other tissues investigated. In normal human term placentae, villous LHRI was associated with blood sinusoids and cytotrophoblast cells, and rarely detected in trophoblastic syncytium. In all abnormal placentae, the LHRI of sinusoids was absent, and syncytium showed either enhanced (immature placental phenotypes) or no LHRI (aged placental phenotype). LHRI in human brain was identified in microglial cells (CD68+ resident macrophages). Protein extracts from human vaginal wall and levator ani muscle and fascia showed strong ~92 and 68 kDa species, and LHRI was detected in smooth muscle cells, fibroblasts, resident macrophages and nuclei of skeletal muscle fibers. Our observations indicate that, in contrast to the theory on the role of vascular hormone receptors in preferential pick up of circulating hormones, there is no need to enhance selective pick up rather only prevent LH/CG transport to inappropriate sites. Abnormal placental LHR expression may play a role in the development of abnormal pregnancy. Expression of LHR in the pelvic floor compartments suggests that high LH levels in postmenopausal women may contribute to the pelvic floor relaxation and increased incidence of pelvic floor disorders. Since chorionic gonadotropin increases secretion of a variety of cytokines by monocytes, and induces their inflammatory reaction and phagocytic activity, high LH levels in aging individuals may also activate microglia (mononuclear phagocyte system in the central nervous system) and contribute to the development of Alzheimer's disease and other inflammation-mediated neurodegenerative diseases.

Background

The luteinizing hormone receptor (LHR) plays a fundamental role in ovarian responsiveness to pituitary LH. The LHR consists of a 335 residue extracellular domain which contains six N-linked glycosylation sites [1]. Posttranslational changes in glycosylation and phosphorylation result in several LHR variants migrating between ~93 and 44 kDa [2–17]. Lower molecular weight forms (48 and 44 kDa species) appear to represent a glycosylated extracellular domain expressed in mammalian cells (truncated receptor) and retain hormone binding specificity. They are not secreted from cells, but remain trapped intracellularly [18]. In addition to various glycosylated LHR variants, western blotting also yielded a 170 kDa band representing an LHR dimer [19]. LH binds to LHR variants with different affinities, and highest affinity appears to be associated with the fully glycosylated receptor (~90 kDa) [19]. Chorionic gonadotropin (CG), which is important for corpus luteum (CL) rescue and maintenance of pregnancy, also binds to LHR, although with a 10-fold lower binding affinity compared with that of LH [20].

The mouse anti-rat LHR monoclonal antibody (mAb), clone 3B5, was developed against purified rat LHR [21]. The antibody showed immunoreactivity with rat granulosa cells of mature (preovulatory) follicles, ovarian thecal and interstitial cells, granulosa-lutein cells of developing, mature and regressing CL, and with testicular Leydig cells, and no reactivity with rat kidneys [22]. During the last ten years, affinity purified 3B5 antibody has been used in several immunohistochemical studies [23–26]. To our knowledge, however, no analysis of the 3B5 antibody by western blot has been reported.

In porcine ovaries, LHR expression was detected in granulosa and theca cells of preovulatory follicles, but not in granulosa lutein cells of the mature CL [27]. In human ovaries, LHR expression was also detected in granulosa and theca cells of preovulatory follicles, but mature CL showed strong expression in luteal cells, which disappeared during luteal regression. CL from early human pregnancies showed various intensities of LHR expression (from weak to intense) on the surface and in the cytoplasmic regions of luteal cells [24].

LHR expression was also detected in nonpregnant human uterus (glandular and luminal epithelial cells, stromal cells, myometrial cells and vascular smooth muscle cells), syncytiotrophoblast of midterm but not term human placenta, fetal membranes and decidua [28], human and rat brain [29,30], rat prostate [14,15], human trophoblast and syncytiotrophoblast from early pregnancies [31], porcine fallopian tubes and umbilical cord [16,32], ovarian, decidual, endometrial and luteal macrophages [22,33], and ovarian cancer cell cultures and tumor tissues [34].

Beside its involvement in the regulation of ovarian function, the LHR appears to be involved in some additional effects. LHR immunoreactivity in porcine fallopian tubes was confined to the epithelium and smooth muscle cells, and in vitro LH treatment caused relaxation of the oviduct [35]. Also, LHR stimulation plays a role in the induction and maintenance of myometrial quiescence [36–38]. Since smooth muscles belong among important compartments of the pelvic floor, high LH levels after menopause might be involved in the increased incidence of pelvic floor disorders, a common condition in postmenopausal women [39]. Since the brain was reported to express functional LHR [29,30], there is also a possibility that high LH levels in aging individuals may in some way participate in the development and progression of neurodegenerative process of several degenerative neurological diseases, including Alzheimer's disease.

The aim of the present study was to investigate subcellular LHR distribution and determine LHR protein variants identified by 3B5 mAb in various rat, human and porcine tissues. During cellular differentiation, a transition from cytoplasmic to perinuclear, surface, nuclear and no LHR immunoreactivity was detected. In protein extracts from rat, porcine and human ovaries six distinct LHR bands were observed, migrating between ~92 and 48 kDa.

Materials and Methods

Rats

Rats of the CII-ZV strain, born and maintained in the Laboratory of Biology of Reproduction, Zaragoza, Mexico, were maintained on a 14L-10D cycle (lights on 0500–1900 h). The postnatal rats were weaned at 21 days of age, and they attained sexual maturity during the 6th week of life. Ovaries were studied in proestrous rats, at the age of two months. Daily vaginal smears were collected for about ten days prior to sacrifice.

Porcine tissues

Twenty porcine ovaries with fallopian tubes and some additional porcine tissues were obtained from a local abattoir and tissue samples were frozen within one hour. With respect to the follicular and luteal development, porcine ovaries were macroscopically classified as follows: Early/mid follicular – medium sized antral follicles (3–5 mm) accompanied by shrinking fibrous corpora lutea of similar size; late follicular – large antral follicles (>5 mm) not accompanied by any luteal tissue; early/ mid luteal – large corpora lutea (~10 mm) characterized by yellowish color; and late luteal – CL of diminishing size (~7 mm) with massive bleeding into the central cavity (bluish color) and no accompanying follicles.

Human tissues

Frozen blocks of human tissues from previous studies were obtained as described previously [40–44]. Some tissues also originated from the Cooperative Human Tissue Network, Columbus, Ohio. Functional stage of ovaries was classified according to the endometrial morphology [45]. Ovaries and endometrial samples were obtained from hysterectomy specimens. We also utilized frozen blocks and protein extracts from human placental chorionic villi, and protein extracts from trophoblast cultures and cultured amniotic fibroblasts and placental mononuclear cells [46]. Placental mononuclear cells were collected from the first digestion, which contains no trophoblast cells [46]. The study was approved by the Institutional Animal Care and Use Committee and Institutional Review Board.

Tissue processing and peroxidase immunohistochemistry

All chemicals, except where specified otherwise, were purchased from Sigma Chemical Co., St. Louis, MO. Tissue samples were collected into cryomold biopsy vinyl specimen molds (Tissue-Tek Cryomold Biopsy, Miles Inc. Diagnostic Division, Elkhart, IN) and embedded in an optimum cutting temperature formulation of water-soluble glycols and resins (O.C.T. compound; Miles). The molds with specimens were frozen by floating on liquid nitrogen and stored at -80°C until use. Frozen tissues were sliced into 7 μm serial sections using a cryostat microtome with specimen retraction during return travel (Carl Zeiss Microm HM 505 E; MICROM Laborgeräte GmbH, Waldorf, Germany) and ten to twelve distinct tissue sections were placed on each slide. The slides were dried ~2 h at room temperature, fixed 5 min in acetone, dried 30 min, and stored at -80°C until immunoperoxidase staining was performed. Prior to staining, to prevent water condensation, the slides were transferred at -80°C into pre-chilled air protected boxes containing Drierite granules (W.A. Hammond Drierite Co, Ltd, Xenia, OH), and the boxes with slides were equilibrated to -20°C, +4 (cold room) and room temperature (20 min each step) before opening.

Slides were incubated overnight (cold room) with primary antibodies (see below). Specimens were then transferred to room temperature, extensively washed in freshly prepared PBS, pH 7.22, and incubated 20 minutes with swine anti-mouse IgG peroxidase conjugate (SwAM; SEVAC Praha, Prague, Czech Republic) diluted 1:20, and preabsorbed with rat kidney homogenate to remove nonspecific background [46]. Control slides were similarly processed, but primary antibody was replaced with PBS. Antigen-antibody complexes were detected by standard diaminobenzidine technique (brown color). Some slides were counterstained with hematoxylin, and all slides were dehydrated and mounted. Dual color immunohistochemistry experiments were performed as described previously [47].

Evaluation was performed on a Leitz DM RB (Leica Inc., Wetzlar, Germany) microscope equipped with differential interference contrast and a DEI-470 CCD Video Camera System (Optronics Engineering, Goleta, CA) with detail enhancement. The video images were captured by CG-7 color frame grabber (Scion Corporation, Frederick, MD) supported by Scion Image public software developed at the National Institutes of Health (Wayne Rasband, NIH, Bethesda, MD), and ported into Microsoft® Power-Point® 97 SR-2 (Microsoft Corporation, Redmont, WA).

Primary antibodies

For LHR immunohistochemistry, the 3B5 affinity purified antibody was used at 50 μg/ml. The antibody is IgG1 class, developed and characterized previously [21,22]. The antibody was stored at -80°C, either as a lyophylizate, or in diluted aliquots. For immunoblots, the antibody concentration was 10 μg/ml. We also used mouse anti-human Thy-1 mAb, clone F15-42-01 [48] (Dr. Rosemarie Dalchau, Institute of Child Health, London, UK), a marker of placental decidual cells (and fibroblasts and brain tissue) [46], mouse anti-rat Thy-1, clone OX-7 [49] (Dr Alan F. Williams, University of Oxford, Oxford, UK), mAbs against CD68, a marker of tissue macrophages, and CD14, a marker of monocytes (Dako Corporation, Carpinteria, CA), and leukocyte-common antigen, clone F10-89-4 [50] (Dr. John F. Fabre, Institute of Child Health, London, UK).

Tissue extracts and western blot analysis

For preparation of protein extracts from rat, human and porcine tissues for western blot analysis, 150 cryostat tissue sections (7 μm) were collected into microcentrifuge tubes and lysed by adding ice-cold lysis buffer (20 mM Tris pH 7.5, 200 mM NaCl, 0.25% Nonidet P-40; 400 μl/ 100 mg of tissue sections) containing 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride. Tissue culture cells were collected and processed as described previously [46].

After 15 min on ice, the tissue and cell lysates were sonicated using a SonicatorTM Cell Disruptor (Heat Systems-Ultrasonic, Inc., Plainview, NY) for 5 seconds, and centrifuged at 11000 × g for 20 min at 4°C. Supernatants were stored at -80°C. For western blot analysis, protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA). Equal amounts of unboiled protein (60 μg) were loaded onto reducing 10% SDS-Tris-glycine polyacrylamide gels, transferred to nitrocellulose (Bio-Rad), and processed as described previously [51], with some modifications.

Briefly, membranes were washed in Tris-buffered saline containing 0.05% Tween 20 (TBST) and non-specific binding sites were blocked by immersing the membrane in blocking reagent (0.5% casein in TBST) for 1 hour at room temperature on an orbital shaker. After blocking, the membranes were briefly rinsed in two changes of TBST, and washed once for 15 minutes and twice for 5 minutes in fresh changes of TBST. The membranes were subjected to overnight incubation (4°C) with mouse anti-rat LHR monoclonal antibody in blocking reagent.

The membranes were washed and incubated with peroxidase labeled secondary antibody – goat anti-mouse IgG and IgM (Jackson Immunoresearch, West Grove, PA) diluted 1:2000 – for 1 hour at room temperature. Before staining, the secondary antibody was diluted 1:60 (5 μl/300 μl), absorbed with rat kidney homogenate (150 mg/300 μl) for 20 min, centrifuged and diluted 1:2000 to the final concentration. This absorption eliminated any non-specific background. After incubation, the membranes were washed 1 × 15 min, 2 × 10 min and 4 × 5 min in fresh changes of TBST, and incubated for 1 hour in blocking buffer at room temperature. Bound antibodies were detected by a chemiluminescent detection system (ECL; Amersham Pharmacia Biotech, Piscataway, NJ) as recommended by the manufacturer's protocol. For exposure (1 min, 5 min, and up to 1 h) we used Kodak XAR film (Eastman Kodak, Rochester, NY).

For protein load control, the membranes were reprobed with actin mouse mAb, clone C4 (Boehringer Mannheim Corp., Indianapolis, IN) at 0.25 μg/ml, and developed as above.

Absorption studies
We also included absorption studies with porcine ovarian homogenates. Primary antibody (100 μg) in 2 ml PBS was incubated 20 min with 500 mg of porcine ovarian homogenate (late follicular phase) at room temperature. After centrifugation (11000 × g for 10 min at 4°C), the supernatant was diluted to 10 ml with blocking reagent and used for immunostaining of nitrocellulose membranes.

Results
Rat tissues (Figure 1)
In rat ovaries, the LHR immunoreactivity was detected in the mature and regressing CL (MCL and RCL, Fig. 1A) and ovarian interstitial cells (i), follicular theca interna (t, Fig. 1B), and mature granulosa cells (mg, Fig. 1B vs. control, 1C). Note a lack of immunoreactivity in ovarian vessels (v, Fig. 1A). The antibody also identified granular cytoplasmic LHR immunoreactivity in immature granulosa cells (ig, Fig. 1B) of preantral and early antral follicles, suggestive of early LHR synthesis. This may represent syn-

thesis of a receptor extracellular domain not yet associated with the cell surface, but remaining trapped intracellularly [18].

Maturation of preovulatory follicles was associated with a transition of scarce granular cytoplasmic LHR immunoreactivity (ig, Fig. 1D) to LHR accumulation in more differentiated granulosa cells (dg). LHR immunoreactivity was also found in surface epithelium of the rat uterine cavity, epithelium of uterine glands (se and ug, Fig. 1E), nuclei of stromal cells (arrowhead), and in uterine vessels (v). The latter contrasted with a lack of staining of ovarian vessels (see above).

Human tissues (Figure 2)
During follicular selection (mid follicular phase, as determined by endometrium morphology) dominant follicles in human ovaries showed granular cytoplasmic LHR expression in granulosa cells (g, Fig. 2A), similar to those in immature rat follicles (see above). Theca interna (ti) showed more distinct LHR immunoreactivity, characterized by accumulation of LHR cytoplasmic granules. Follicular vessels (vascular layer, vl, and thecal vessels, tv) were virtually unstained. Some LHR immunoreactivity was also detected in theca externa cells (te).

Early luteal development (fresh CL) was associated with very strong cytoplasmic LHR immunostaining of granulosa lutein cells migrating into the follicular cavity (gl, Fig. 2B). Adjacent theca-lutein cells (tl) also exhibited prominent staining, but showed distinct vacuolization. No LHR expression accompanied developing luteal vessels (v). In the mature CL, granulosa lutein cells showed diminution of cytoplasmic LHR immunoreactivity and strong surface staining (Fig. 2C). Luteal cells in the old CL (late luteal period) showed marked diminution of LHR expression (Fig. 2D). Regression of luteal tissue (CL investigated during the follicular phase) showed residual staining of theca-lutein cells and no staining of regressing granulosa lutein cells (Fig. 2E vs. ctr).

In CL of pregnancy (CLP; 3 months), less or more distinct granular cytoplasmic LHR immunoreactivity (arrowhead, Fig. 2F vs. ctr) and no surface staining was detected. This suggests that high CG levels accompanying early pregnancy may preserve CLP through the cytoplasmic receptor. In other words, selective pick up of CG through the surface LHR expression in granulosa lutein cells during the luteal phase after conception appears not to be required if the circulating CG is very high.

In fallopian tubes, distinct cytoplasmic LHR expression was associated with the luminal aspect of epithelial cells (arrowhead, Fig. 2G). In endometrium (late follicular phase – note stromal edema), LHR immunoreactivity was
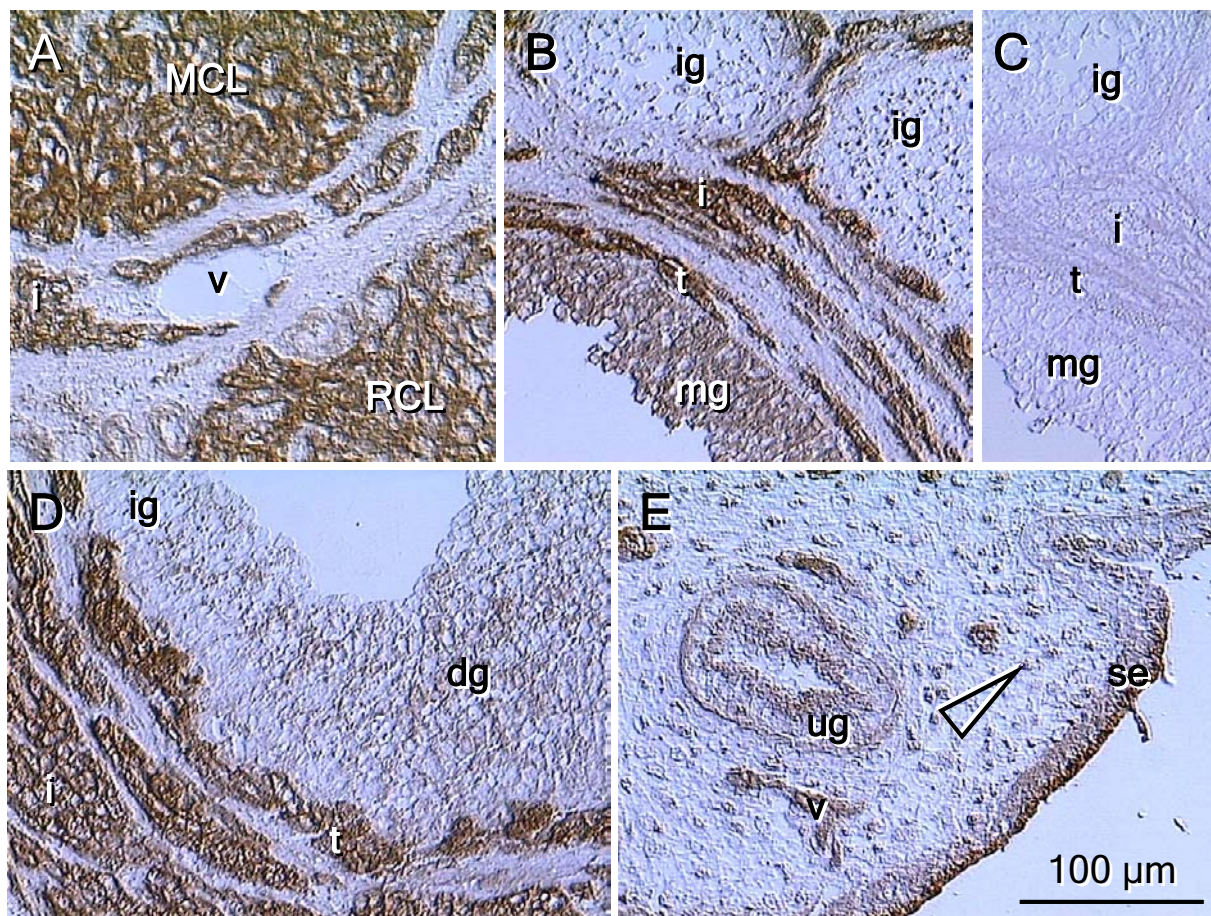
Figure 1
LHR expression in the rat ovaries and uterus. **A**) Strong LHR immunoreactivity is apparent in mature (MCL) and regressing CL (RCL) and interstitial cells (i). Note unstained vessels (v). **B**) Strong staining of theca interna (t) and weaker staining of mature granulosa cells (mg) in preovulatory follicle. Note scarce cytoplasmic granules with LHR immunoreactivity in immature granulosa cells (ig). **C**) Control. **D**) Increased staining during granulosa cell differentiation (dg) in large antral follicle. **E**) In the rat uterus, the LHR immunostaining is apparent in the surface epithelium (se), uterine glands (ug) and vasculature (v). Uterine stromal cells show nuclear staining (arrowhead). No hematoxylin counterstain.

associated with endometrial glands (g, Fig. 2H) and with the surface epithelium of the uterine cavity (se). There was also granular cytoplasmic expression in endometrial stromal cells (s), precursors of decidual cells, and similar granular staining was observed in smooth muscle cells of late follicular phase myometrium (data not shown). 3B5 antibody also stained human brain vessels (v, Fig. 2I) and showed strong LHR immunoreactivity with microglial cells (arrowhead; see Fig. 8).

Porcine tissues (Figure 3)
Porcine preovulatory follicles showed distinct LHR staining of theca interna, lack of LHR expression in vascular layer under the follicular membrane, and transition of granular cytoplasmic to surface LHR immunoreactivity in granulosa cells (Fig. 3A,3B,3C, vs. control D). Mature CL showed strong surface LHR immunoreactivity of luteal cells (Fig. 7E), and old corpora lutea showed diminution of LHR immunoexpression (Fig. 3F). Ovarian surface epithelium, the source of most ovarian tumors, was also
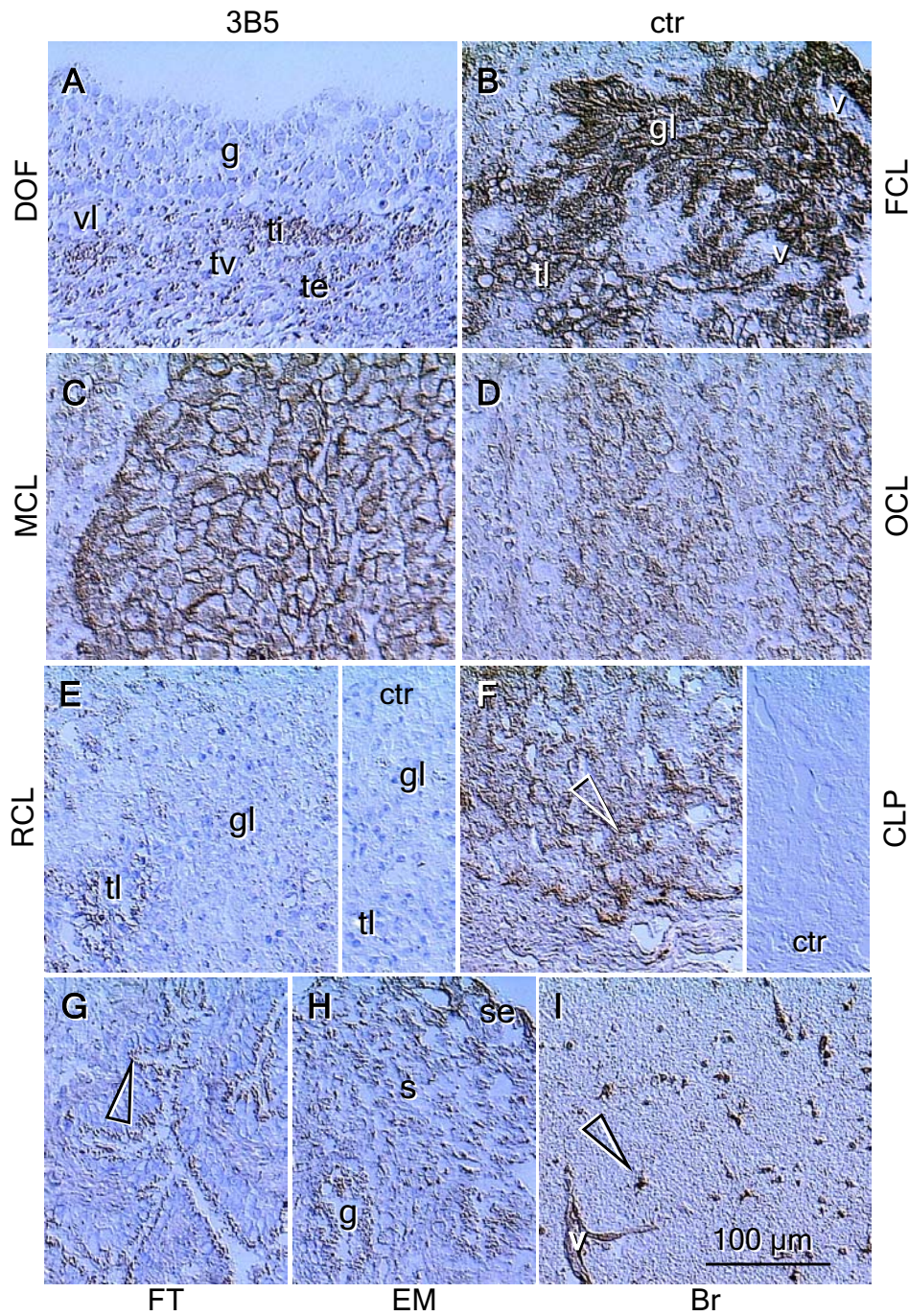
Figure 2
Reactivity of 3B5 with human tissues. **A**) Dominant follicle (DOF, midfollicular phase) – g, granulosa; vl, vascular layer; ti, theca interna; tv, thecal vessels; te, theca externa. **B**) Fresh CL – gl, granulosa lutein cells; tl, theca lutein cells; v, proliferating luteal vessels. **C**) Mature CL – midluteal phase. **D**) Old CL (OCL, late luteal phase). **E**) Regressing CL (RCL, mid-follicular phase of the subsequent cycle). **F**) Corpus luteum of pregnancy (CLP, 3rd month of pregnancy) Arrowhead indicates cytoplasmic LHR immunoreactivity. **G**) Fallopian tube. Arrowhead indicates LHR immunoreactivity at the luminal aspects of epithelial cells. **H**) Endometrium (EM, late follicular phase – note stromal edema) – se, surface epithelium; g, glands; s, endometrial stromal cells. **I**) Staining of brain microvasculature (v) and microglial cells (arrowhead – see Fig. 8). Insets show control staining (ctr). Hematoxylin counterstain except panel **F**.
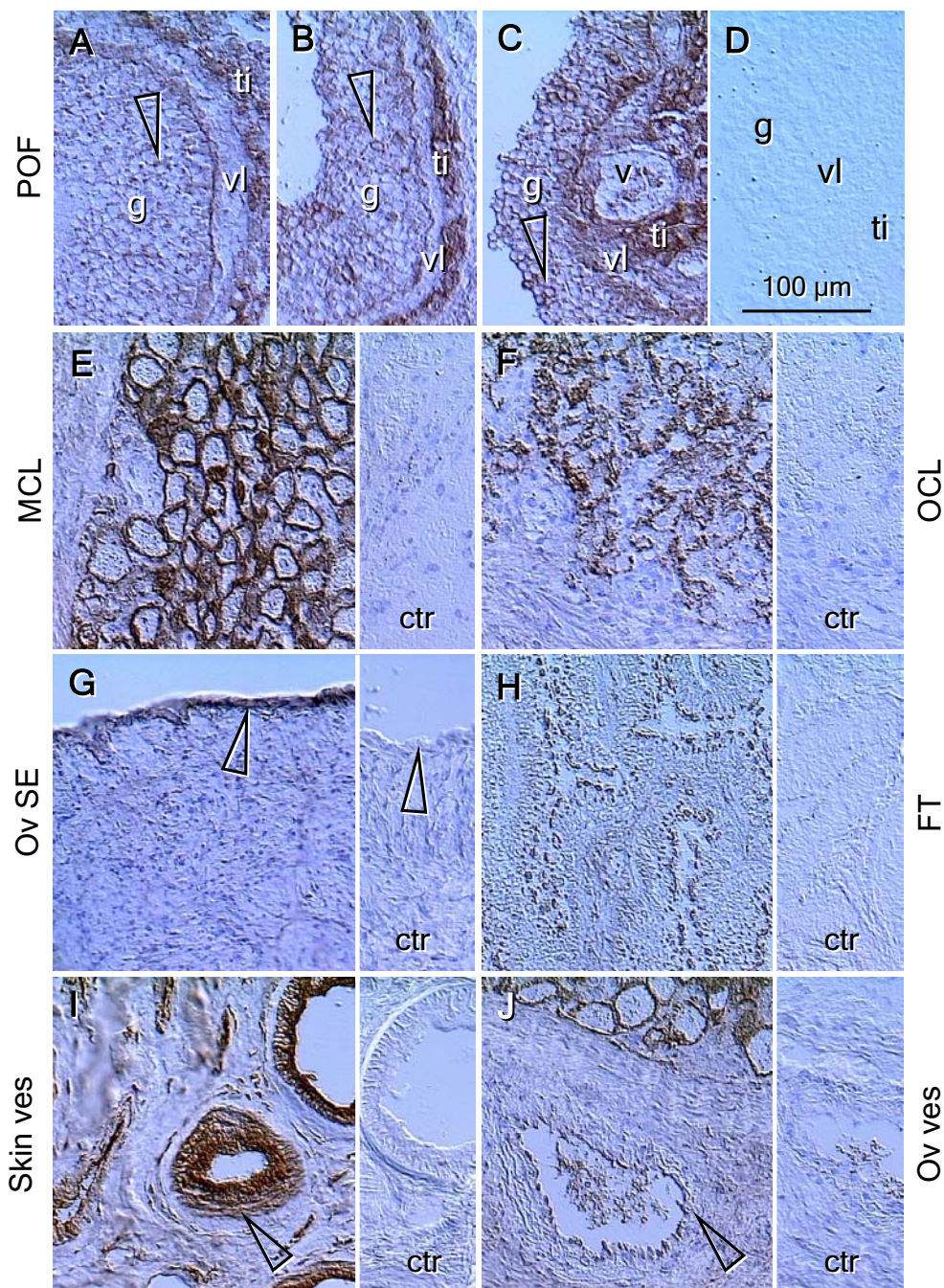
Figure 3
LHR immunostaining in porcine tissues. **A–C**) Preovulatory follicles (late follicular phase) show a transition from granular cytoplasmic to surface LHR immunoreactivity in granulosa cells (arrowheads), lack of staining of vascular layer, increase of LHR staining in theca interna and extreme dilatation of follicular microvasculature (v). **D**) Control for **C**. **E**) Mature CL. **F**) Old CL. **G**) Ovarian surface epithelium (arrowhead). **H**) Fallopian tube (FT). **I**) Skin vessels show strong LHR immunoreactivity of the entire vascular wall (arrowhead). **J**) In ovarian vasculature, the LHR immunoreactivity is limited to the nuclei of some endothelial cells (arrowhead) but other components of vascular wall are unstained. Insets in **E–J** show control staining. Details in text. Hematoxylin counterstain except panels **A–D**, **H** and **I**.
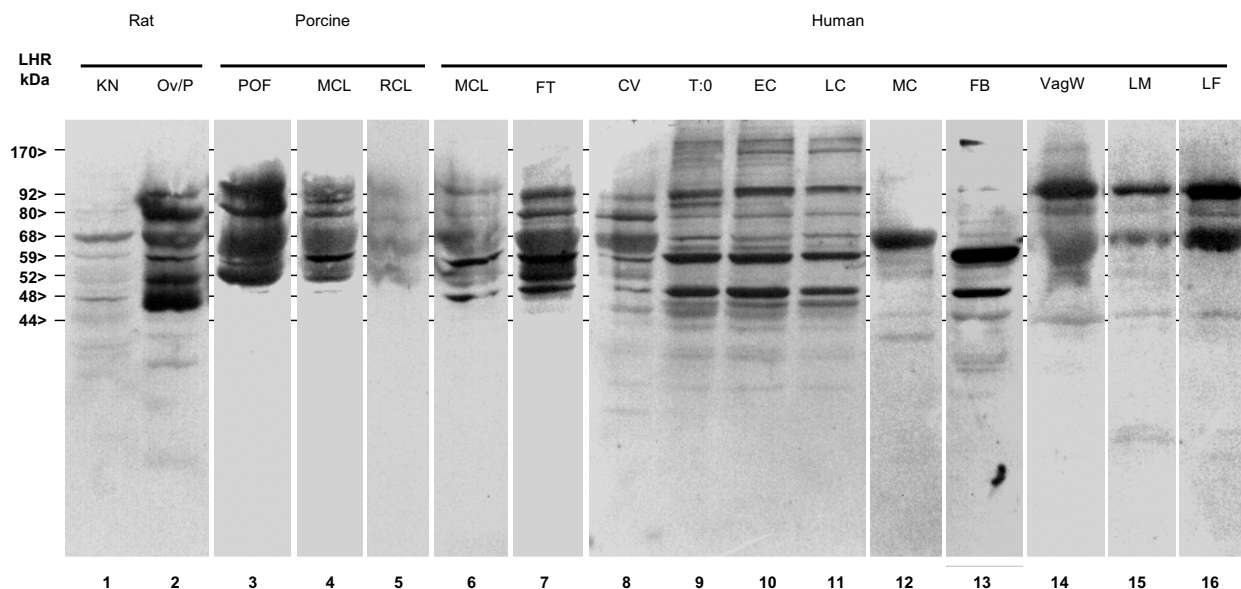
Figure 4
Western blot analysis of rat, porcine and human tissues with 3B5. KN, kidney; Ov/P, ovary in proestrus; POF, preovulatory follicle; MCL, mature CL; RCL, regressing CL (follicular phase); FT, fallopian tube; CV, chorionic villi (term placenta); T:0, time 0 trophoblast culture; EC, early culture (24 h); LC, late culture (72 h); MC, cultured placental mesenchymal cells; FB, cultured amniotic fibroblasts; VagW, vaginal wall (epithelium, connective tissue and smooth muscle bundles); LM, levator ani muscle; LF, levator ani fascia. Details in text.

stained (arrowhead, Fig. 3G). In porcine fallopian tube (Fig. 3H), the LHR immunostaining was similar to that in human oviduct. Strong LHR immunoreactivity in vascular walls was detected in the skin (Fig. 3I), which does not represent a classic LH/CG target. This contrasted with a lack of staining in ovarian vessels, except weak nuclear staining of endothelial cells (arrowhead, Fig. 3J). Insets in Fig. 7E,7F,7G,7H,7I,7J show the control procedure.

Observations of LHR immunoreactivity in porcine tissues indicate that LHR distribution is similar to that in human tissues. They also show LHR expression in the ovarian surface epithelium (not investigated in human ovaries) and astonishing differences in vascular walls of nonclassic (strong LHR in skin vessels) and classic LHR targets (week expression in ovarian vessels) – see Ref. [40] and Discussion beneath for a role of vascular LHR expression in the LH/CG delivery.

Multiple rat, human and porcine LHR protein variants (Figures 4 and 5)
Western blot analysis of protein extracts from normal rat ovaries and human and porcine mature CL with 3B5 antibody showed six distinct bands migrating between ~92

and ~48 kDa (lanes 2, 4 and 6, Fig. 4; see also Fig. 5A). Although weak, some LHR immunoreactivity was also detected in the rat kidney protein extract (lane 1). This observation is consistent with a lack of LHR immunoreactivity in rat kidney cryostat sections reported previously [22].

Distinct LHR expression was detected in rat proestrous ovaries (lane 2, Fig. 4), which contain LHR+ CL at various stages of development and regression, and developing and mature preovulatory follicles. However, in porcine preovulatory ovaries (late follicular phase), which contain numerous preovulatory follicles but no luteal tissue, very strong LHR immunoreactivity was also detected (lane 3, Fig. 4). Regression of porcine luteal tissue (RCL; lane 5) was associated with a marked decline of LHR expression. This confirmed immunohistochemical observations.

In human tissues, mature CL (MCL; lane 6) showed LHR bands similar to that in mature porcine CL. Very strong expression of all six LHR variants was detected in the fallopian tube (FT; lane 7), which is the site of first contact with the developing embryo secreting CG, a signal required for CL preservation.
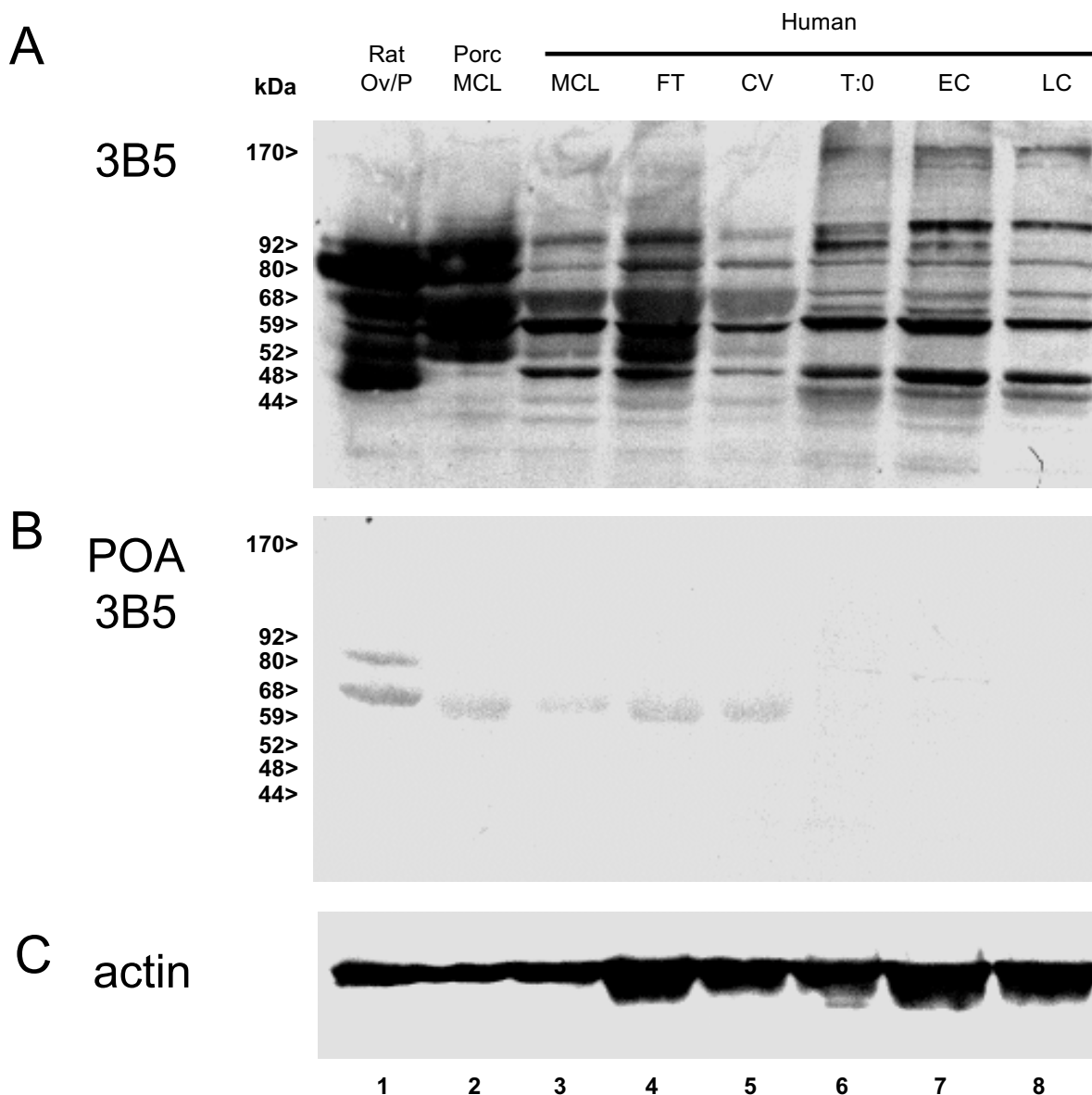
Figure 5
3B5 absorbed with porcine ovaries (western blot control). Reactivity of 3B5 in western blots (**A**) diminishes after preabsorption with porcine ovaries [porcine ovary absorbed (POA) 3B5] (**B**). C) Actin control for protein loading. For abbreviations see Fig. 4.

Placental chorionic villi (CV; lane 8) also showed six distinct bands similar to those detected in the rat, human and porcine ovaries. However, time 0 and early and late trophoblast cultures derived from the same placenta (lanes 9–11) showed additional high molecular weight LHR variants (~170 kDa), possibly LHR homodimers [19], which were barely detectable or undetectable in other tissues, including placental CV. Moreover, there

were additional bands detected in trophoblast cultures (~85, 60, and 46 kDa species) and a lack of a ~52 kDa variant as compared to the luteal, FT and CV protein extracts. The 44 kDa species, probably a partially degraded form from LHR turnover [18], was observed in placental cultures (lanes 9–13) and pelvic floor compartments (lanes 14–16).

We also investigated cultures of placental mesenchymal cells (MC), which are always associated with trophoblast cultures [40,41], and amniotic fibroblasts (FB). Placental mesenchymal cells showed a prominent LHR band at ~68 kDa (lane 12). Note the virtual lack of other bands characteristic of trophoblast cultures. Placental amniotic fibroblasts showed two distinct LHR bands migrating at ~59 and 48 kDa (lane 13). Similar ~59 and 48 kDa species were distinctly expressed in trophoblast cultures.

Western blot analysis of pelvic floor compartments showed strong expression of ~92 and 68 kDa species in protein extracts from the vaginal wall (VagW; lane 14), which consists of vaginal epithelium, longitudinal and circular smooth muscle cell bundles, and connective tissue. A less prominent band was detected at ~80 kDa. Three similar bands were also found in human levator ani muscle and fascia (lanes 15 and 16). These observations indicate that pelvic floor compartments represent potential targets for the LH/CG effect. Absorption with porcine ovaries resulted in severe diminution of 3B5 reactivity with rat, porcine and human tissues in western blots (Fig. 5B vs. 5A).

Altogether, these data indicate that the 3B5 antibody identifies six LHR protein variants showing distinct expression in rat, porcine and human tissues, including human placenta, with some variants also detected in human female pelvic floor compartments.

Human chorionic villi and placental membranes (Figure 6)
In normal placentae at term, the LHR immunoreactivity in mature chorionic villi was found in mononucleated cytotrophoblast cells under the syncytial layer (arrowheads, Fig 6A) and villous vascular sinusoids (v), but most of the syncytiotrophoblast was virtually unstained (white asterisks vs. black). Mononucleated trophoblast cells showed variable nuclear LHR immunoreactivity (Fig. 6B, detail from A). The most prominent nuclear staining was found in trophoblast cells merging with the syncytium (full arrowheads vs. white). The red arrowhead indicates diminution of LHR expression in the cell which joined the syncytial layer. Staining of villous sinusoids was associated with basal (abluminal) aspects of endothelial cells (arrowheads, Fig. 6C). Developing (immature) chorionic villi were occasionally observed and showed more pronounced granular cytoplasmic LHR immunoreactivity in

the syncytial layer (asterisk, Fig. 6D), a feature also detected in immature granulosa cells (see above).

In contrast to normal placentae, granular cytoplasmic staining of the syncytial layer dominated in some moderately abnormal placentae (asterisks, Fig. 6E), which showed mild compensatory dilatation of villous sinusoids (v) – see Ref. [52] for definition of placental abnormality. In such placentae, the LHR+ mononucleated trophoblast cells were barely detectable, as well as LHR expression in villous sinusoids (white arrowheads).

Differences between placental types were more obvious at lower magnification. Figure 6F (vs. control 6I), shows normal placenta with prominent LHR staining of villous sinusoids (full arrowhead) and no staining of syncytium (white asterisk). Figure 6G shows LHR immunostaining of syncytium (black asterisk) and lack of vascular LHR (white arrowhead), resembling immature chorionic villi (immature placental phenotypes, characteristic for intrauterine growth retardation and maternal diabetes [52] – note lack of dilatation of villous sinusoids). Figure 6H shows that neither surface (white asterisk) nor significant vascular LHR immunoreactivity (white arrowhead) was detected in placentae with extreme dilatation of villous sinusoids (aged placental phenotype [52]).

In placental membranes, occasionally found multilayered amniotic epithelium showed no or fine granular cytoplasmic staining of epithelial cells adjacent to the basement membrane (full arrowhead, Fig. 6J), followed by perinuclear (yellow arrowhead) and nuclear LHR immunoexpression (red arrowhead). Cells most distant from the basement membrane showed no staining (white arrowhead).

Extravillous trophoblast cells in placental membranes and placental basal plate (Fig. 6K) exhibited usually perinuclear (full arrowheads) and occasionally nuclear LHR expression (data not shown). In contrast, however, nuclear or no staining was prevalent in adjacent decidual cells (white arrowheads). Fig. 6L shows a semi-parallel section stained for Thy-1 glycoprotein, a characteristic marker of human decidual cells [46]. Note Thy-1+ decidual cells (white arrowhead) and unstained extravillous trophoblast cells (full arrowhead). Dual color immunohistochemistry for LHR (brown) and CD68 (blue; Fig. 6M) indicates that LHR+ decidual cells also show expression of CD68 (white arrowhead – note brown and blue color) of decidual macrophages (red arrowhead), i.e., marker also characteristic for regressing luteal cells [40]. Note a lack of macrophages among trophoblast cells.
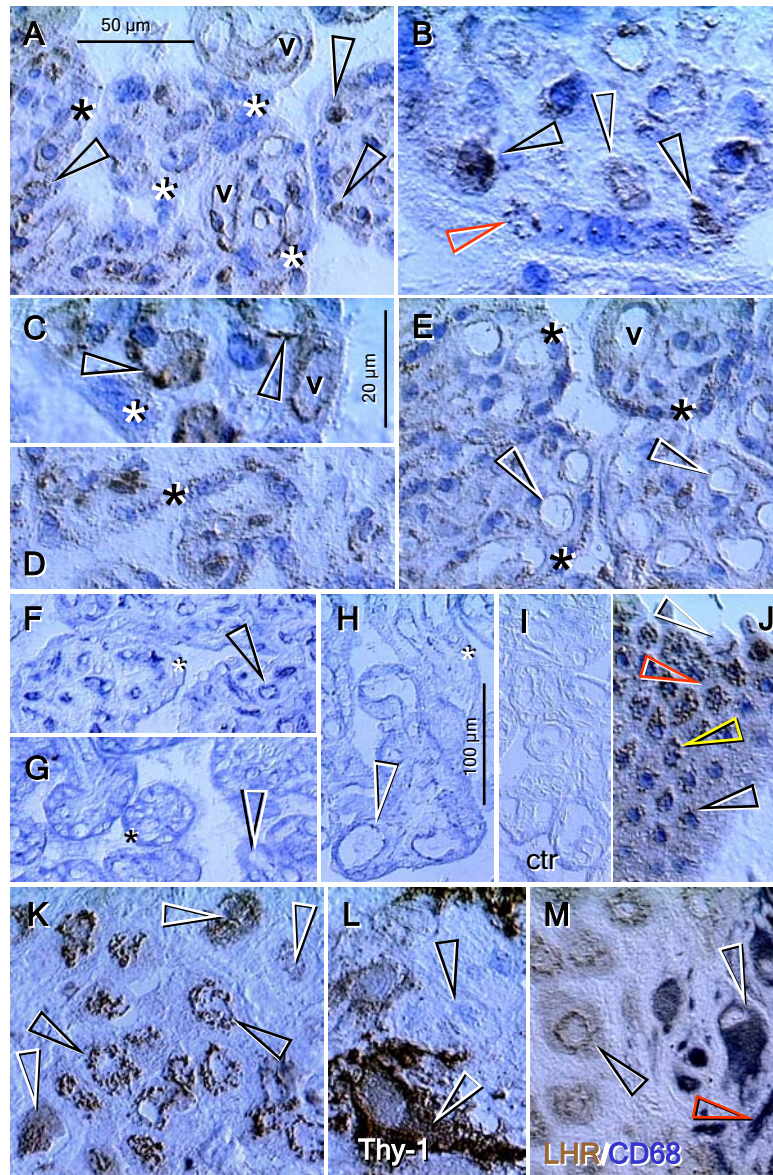
Figure 6
LHR immunoreactivity in chorionic villi and placental membranes. **A**) LHR+ mononucleated trophoblast (arrowheads) and villous vascular sinusoids (v) in normal term placentae. Note unstained syncytium (white asterisks) or sporadic LHR+ granules (black asterisk). **B**) Detail from (**A**) (turned 90°) shows cytotrophoblast cells merging with syncytium (full arrowheads vs. white arrowhead), followed by diminution of LHR immunoreactivity (red arrowhead). **C**) Villous sinusoids. **D**) Immature (developing) villus in normal placenta. **E**) Abnormal placenta (note enhanced dilatation of all sinusoids – compare with **A**). Panels (**F–H**) show staining for LHR (DAB-Ni substrate) in various placental types (see text) vs. control (**I**). **J**) Occasionally detected multi-layered amniotic epithelium shows a transition from granular cytoplasmic (full arrowhead) to perinuclear (yellow arrowhead) and nuclear LHR immunoreactivity (red arrowhead). Note unstained surface cells (white arrowhead). **K**) Extravillous trophoblast cells (basal plate) with strong perinuclear staining (full arrowheads) variable LHR immunoreactivity in decidual cells (white arrowheads). **L**) Parallel section shows Thy-1+ decidual cells (white arrowhead) and unstained trophoblast cells (full arrowhead). **M**) Dual color immunohistochemistry: extravillous trophoblast expressing LHR (full arrowhead), decidual cells expressing both markers (white arrowhead) and decidual macrophages expressing CD68 only (red arrowhead). Hematoxylin counterstain except panels **F–I** and panel **M** (dual color immunohistochemistry). Bar in **C**, for **B** and **C**; bar in **H**, for **F–I**. For other panels see bar in **A**.
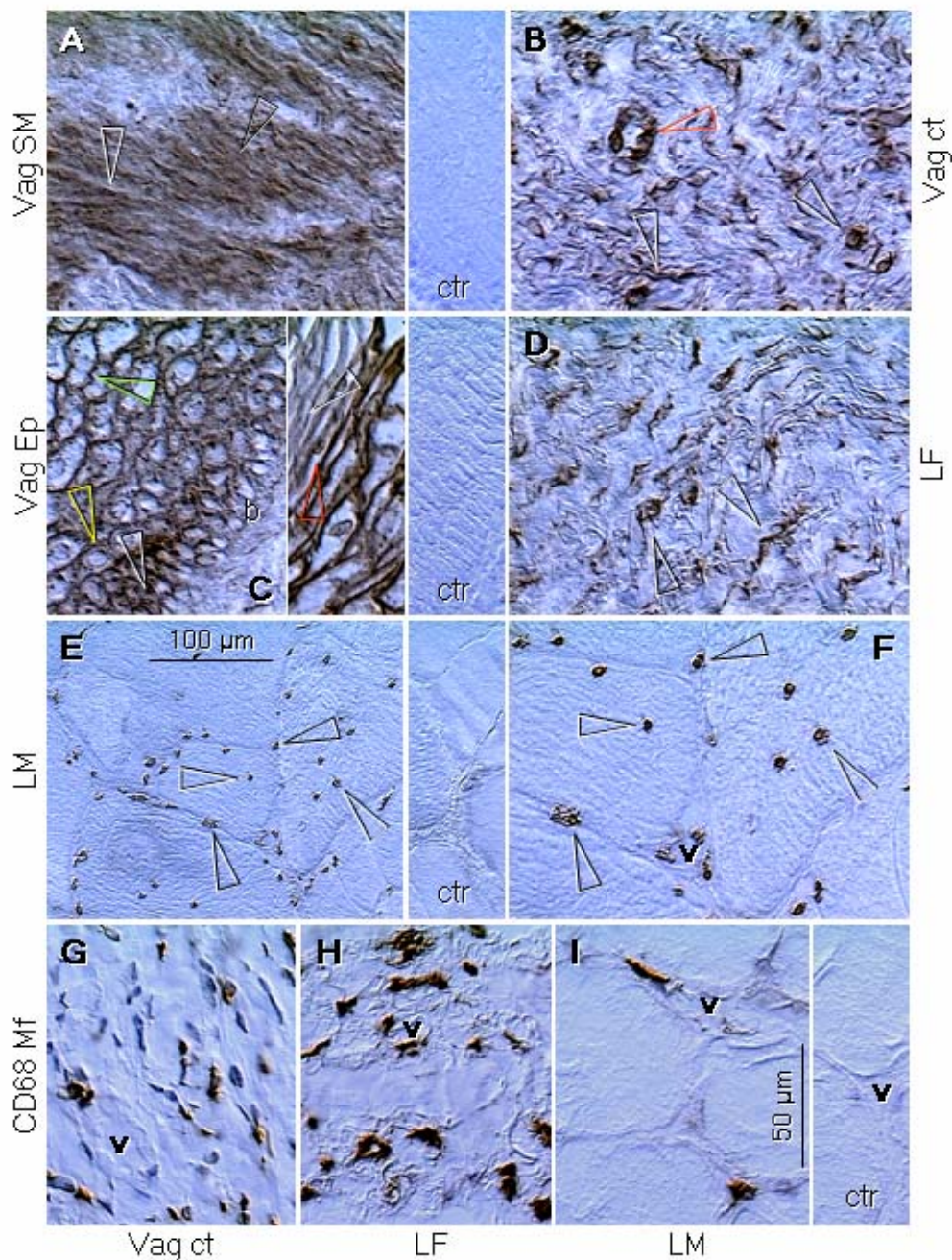
Figure 7
LHR immunoreactivity and resident macrophages in the female pelvic floor. **A**) Vaginal smooth muscle (Vag SM): cytoplasmic (full arrowhead) and nuclear LHR immunoreactivity (white arrowhead). **B**) Vaginal connective tissue (Vag ct): fibroblast type cells (white arrowhead), rounded mesenchymal cells (full arrowhead) and vessels (red arrowhead). **C**) Vaginal epithelium (Vag Ep): basal (stem) cells (b), parabasal cells (full arrowhead) and intermediate cells (yellow and green arrowheads). Superficial layer (middle inset): nuclear staining (red arrowhead) and diminution of staining in the surface cells (white arrowhead). **D**) Levator ani fascia (LF): principal cells (fibroblasts; white arrowhead) and rounded mesenchymal cells (full arrowhead). **E**) Levator ani muscle (see also **F** for detail): mesenchymal cells among muscle fibers (full arrowhead) and muscle cell nuclei (white arrowheads). CD68 staining in vaginal connective tissue (**G**), levator fascia (**H**) and levator muscle (**I**). Insets indicated as ctr show control staining. No hematoxylin counterstain except panels **G–I**. Bar in **I** for all panels except **E**.
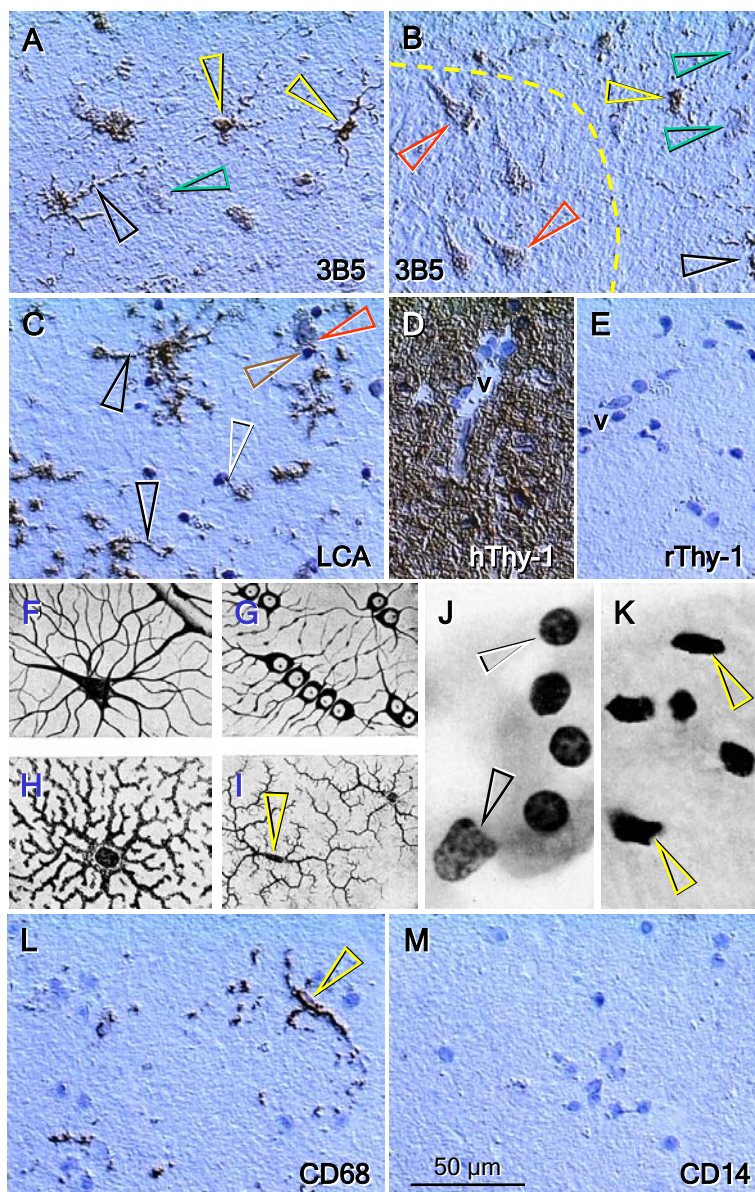
Figure 8
LHR immunostaining in the gray matter of the human brain parietal lobe. **A**) LHR+ microglial cells with characteristic dendritic processes (full arrowhead) and flattened nuclei (yellow arrowheads). Green arrowhead indicates unstained nerve cell. **B**) Area occupied by microglia (full and yellow arrowheads) contains unstained nerve cells (green arrowheads). However, area lacking microglia contains LHR+ nerve cells (red arrowheads). **C**) Staining for leukocyte common antigen (LCA) reveals picture similar to that in (**A**). Note dendritic processes of microglial cells (full arrowheads), and interaction with neuronal (red arrowhead) and other glial elements (white arrowhead). **D**) Anti human Thy-1 (hThy-1) shows diffuse staining of brain tissue except for the vascular endothelium (v). **E**) Control staining with anti-rat Thy-1 (rThy-1). **F–K**) Schematic drawings of interstitial (glial) cells in the human brain and differences in their nuclei (from Ref. [54]). **F**) Fibrous astrocytes – white matter. **G**) Oligodendroglial cells – satellites to nerve cells. **H**) Protoplasmic astrocytes – grey matter. **I**) Microglial cells – mesodermal origin, gray and white matter. Yellow arrowhead indicates flattened nucleus of microglial cell. **J**) Irregularly oval nuclei of astrocytes (full arrowhead) and oval nuclei of oligodendroglia (white arrowhead). **K**) Small, often flattened nuclei of microglial cells. **L**) CD68 staining shows flattened nucleus of microglial cell (yellow arrowhead). M) No staining was observed with CD14, a marker of monocytes and immature tissue macrophages. Hematoxylin counterstain: weak in panels **A** and **B**, moderate in **C-E, L** and **M**. Bar in **M**, for **A-E, L** and **M**.

Pelvic floor (Figure 7)

Previously documented LH-dependent relaxation of the porcine oviduct [53] raises a question if high LH levels in postmenopausal women do not contribute to the relaxation of the pelvic floor, and hence to the increased incidence of pelvic floor disorders in these females. We investigated connective tissues and muscular compartments of the pelvic floor, in order to determine cellular equivalents to LHR bands detected in western blots and possible cellular targets for the LH effect.

Strong cytoplasmic and nuclear LHR immunoreactivity was found in smooth muscle cells in the vaginal wall (Fig. 7A vs. ctr). Vaginal connective tissue fibroblasts (white arrowhead, Fig. 7B) and other stromal elements (full arrowhead), as well as vaginal vessels (red arrowhead), also showed distinct LHR immunoreactivity.

In vaginal epithelial layers, a model for differentiation of epithelial cells [42], basal (stem) cells were unstained (b, Fig. 7C), dense cytoplasmic staining was found in parabasal (immature) cells (full arrowhead), followed by perinuclear (yellow arrowhead) and cell surface LHR immunoreactivity (green arrowhead). Nuclear staining was found in lower superficial cells (red arrowhead in adjacent panel), and cornified superficial cells showed overall diminution of LHR immunoreactivity (white arrowhead).

Additional pelvic floor structure, the levator ani fascia, was studied and exhibited nuclear LHR immunoreactivity in principal cells (fibroblasts; white arrowhead, Fig. 7D) and other mesenchymal cells with rounded nuclei (full arrowhead), most probably resident macrophages (see below). Mesenchymal cells in levator ani muscle also showed LHR immunoreactivity (full arrowheads, Fig. 7E and 7F). In addition, nuclear LHR staining was also detected in levator ani striated muscle fibers, some of which were centrally (abnormally) located in this sample (white arrowheads, Fig. 7E and 7F). Note unstained muscle vessels (v) accompanied by LHR+ stromal cells.

Distribution of resident macrophages, as shown in Fig. 7G,7H,7I, suggests that rounded mesenchymal cells showing LHR immunoreactivity in pelvic floor connective tissues might be represented by this type of cells. Note association of some resident macrophages with vasculature (v).

These observations indicate that principal components of the pelvic floor (smooth muscle cells, fibroblasts and striated muscle fibers), which are responsible for pelvic floor integrity, are potential targets for LH/CG hormonal effects. Additional cells exhibiting LHR immunoreactivity in pelvic floor connective tissues appear to be resident macrophages.

Human brain (Figure 8)

Neurodegenerative diseases, including Alzheimer's disease, also show increased incidence in aging individuals. Therefore, a question arises if the rise in LH, reflecting diminution of sex steroids, can also contribute to the initiation and progression of neurodegeneration. We studied LHR expression in the gray matter of the human parietal lobe cortex. Strong LHR immunoreactivity was associated with small cells with flattened nuclei (yellow arrowheads, Fig. 8A) and numerous spiny processes (full arrowhead). Adjacent nerve cells were unstained (green arrowheads, Fig. 8B), but distant nerve cells showed moderate staining (red arrowheads).

Staining for the leukocyte common antigen (Fig. 8C) and observation for characteristic morphology and nuclear shape (Fig. 8I vs. 8F,8G,8H; Fig. 8K vs. 8J – adapted from Ref. [54]) as well as expression of CD68 of resident macrophages (Fig. 8L) indicate that LHR immunoreactivity in Fig. 8A identifies microglial cells of mesenchymal (mesodermal) origin. Yet, Fig. 8M indicates that these cells do not express CD14 of monocytes (and primitive tissue macrophages [42]). This suggests that they are highly differentiated dendritic type cells. Figure 8D shows diffuse expression of Thy-1 in the cortex, identified with anti-human Thy-1 antibody. Figure 8E is a control using the anti-rat Thy-1 antibody (not reacting with human tissues).

These data indicate that microglial cells and some nerve cells in the gray matter of the human brain cortex may be influenced by LH/CG proteohormones. Yet, loss of gray matter from areas of the parietal and temporal lobes is the most obvious consequence of Alzheimer's disease [55,56].

Discussion

Our observations indicate that the 3B5 anti-LHR antibody reacts with rat, porcine and human tissues. In western blots, the 3B5 antibody identifies six distinct bands migrating between ∼92 and 48 kDa, and this reactivity with protein extracts from all three species diminishes when the antibody is preabsorbed with porcine ovaries. We also show changes in subcellular LHR distribution during differentiation of various cell types. To our knowledge, LHR expression in fibroblasts, striated muscle cells, and microglial cells (CD68+ resident macrophages) in the central nervous system is presented for the first time. We also report tissue differences in vascular LHR expression.

During cellular differentiation, subcellular LHR distribution changed from granular cytoplasmic to perinuclear, surface and nuclear staining; virtually no staining was

detected in stem cells and most differentiated and aged cells. Yet, cell surface staining was observed only on classic LH/CG targets, granulosa and theca cells of preovulatory follicles and mature luteal cells, but also in the vaginal epithelium. This indicates that these cells are prepared for the selective accumulation of LH/CG signals from the circulation.

There was also a striking difference in vascular LHR expression between ovaries and other tissues. In ovarian (and also striated muscle) vessels the LHR expression was virtually absent (Fig. 1A, 2A, 3J), while other tissues investigated, including uterus, brain, skin and vagina, showed distinct vascular LHR immunoreactivity. We also reported previously that in the rat the endothelial LHR expression is absent in testicular vessels, and vessels in other tissues involved in LH/CG transport into (pituitary) and from the blood (kidney) [22]. Saturation of LHR in ovarian targets may be dependent on the precise delivery of small amounts of circulating LH/CG from the blood into the extravascular space. A receptor mediated endothelial transport of hCG has been suggested to represent a model for general involvement of specific receptors in transport of other plasma proteins [57,58]. Yet, application of this theory on our data will indicate that the LH/CG will be delivered to the uterus, brain, skin and vagina rather than to the ovary. Hence, the opposite mechanism should be considered – endothelial LHR expression may suppress the transport of LH/CG from vessels to the extravascular space. The LH/CG molecule is smaller than that of albumin or IgG, i.e., proteins exhibiting ubiquitous distribution, so there is no need to enhance rather only prevent LH/CG transport to inappropriate sites. Binding of LH/CG to endothelial cells expressing LHR may prevent LH/CG transport from circulation by electrostatic forces, and the lack of endothelial LHR expression may be associated with LH/CG transport mediated by a general mechanism involved in protein exchange [40].

We report that normal term placentae show virtually no LHR expression in trophoblastic syncytium while syncytium of some abnormal placentae (immature phenotypes) exhibits LHR immunoreactivity. This resembles high LHR expression in first trimester placenta [28], which is a source of high hCG levels [59]. Yet, hCG levels fall during the second trimester, and elevated maternal midtrimester hCG is associated with higher rates of maternal and neonatal complications (pregnancy-induced hypertension, preeclampsia, gestational diabetes, and perinatal death) [60–63].

Hence, some abnormal term placentae appear to preserve syncytium in a younger state, accompanied by high secretion of hCG. In contrast, vascular LHR expression was detected in sinusoids of chorionic villi from normal term placentae, but was virtually absent in all abnormal placentae. If the vascular LHR expression represents a barrier for hCG transport from chorionic villi to the fetal blood (see above), lack of this barrier may cause high levels of fetal hCG resulting in perinatal morbidity and mortality [63], regardless of normal or abnormal hCG production.

Western blot analysis of placental villi and trophoblast cultures indicates that cultured trophoblast cells show additional bands, including ~170 kDa species, possibly an LHR homodimer [19]. Although the ~170 kDa species persisted in late cultures, other (additional) LHR variants diminished. This suggests an activation of isolated trophoblast cells and enhanced LHR synthesis with formation of additional glycosylated LHR variants, particularly in time 0 and early cultures. When compared to chorionic villi, trophoblast cultures showed stronger ~59 and 48 kDa species, characteristic for cultured fibroblasts. Indeed, it has been reported, that differentiation of cultured trophoblast is associated with activation of accompanying fibroblasts [64].

Early differentiation of granulosa, luteal, and other cell types expressing LHR was associated with cytoplasmic LHR immunoreactivity, reflecting receptor synthesis prior its surface expression. In addition, the CL of pregnancy, the function of which is highly dependent on CG, also showed cytoplasmic expression. Hence, it is possible that high levels of circulating LH/CG, e.g., during pregnancy and after menopause, may influence not only cells with surface LH expression, but also cells with cytoplasmic, and perhaps exclusively nuclear LHR expression (pelvic floor compartments, including striated muscle). In other words, cells lacking surface LHR may not be influenced by temporary increase of LH production during the preovulatory period of the ovarian cycle due to the "cell membrane LHR barrier," and the LH effect is probably also prevented by the "vascular LHR barrier." Yet, high CG levels during pregnancy may pass the "cell membrane barrier" and act through the cytoplasmic LHR in the CL of pregnancy and other cells with cytoplasmic expression, and such effect could be enhanced if the "vascular LHR barrier" is absent – abnormal placentae of aged phenotype and similar putative age-related vascular changes in other tissues.

But the LH/CG action through the cytoplasmic/perinuclear and nuclear LHRs opens a possibility of more direct LH/CG action within the cells, which may not require a classic second messenger pathway (cyclic AMP dependent signaling mechanism) involvement, or this system can act through the intracellular LHRs. Evidence is accumulating that a number of other factors modulate the actions of gonadotropins in the ovary and testis via activation of alternative signaling pathways and via LHR protein vari-

ants, and alternative second messenger pathways for the transmission of the LHR activation effect exist, which may not include stimulation of cyclic AMP levels [65–69]. A question also arises if the nuclear LHR expression in terminally differentiated cells (e.g., striated muscle fibers) may not indicate a possibility of the direct effect of LH/CG on modulation of protein synthesis, reflecting certain effects of sex steroids on their nuclear receptors.

LH/CG causes a relaxation of smooth muscle cells, which express LHR [36–38] (Fig. 7A). The data presented also indicate that additional principal cell types in the pelvic floor show LHR expression, including stromal and fascial fibroblasts and striated muscle cells. This is associated with relatively high expression of LHR protein in western blots, and a fully glycosylated ~92 kDa species in particular. Interestingly, strong ~59 and 48 kDa proteins expressed in cultured fibroblasts were not detected in pelvic floor lysates, but a distinct ~68 kDa band from mesenchymal cells was apparent. We speculate that a ~92 kDa species in the pelvic floor is a result of interaction of resident macrophages with pelvic floor fibroblasts.

The hCG secreted by trophoblastic syncytium during pregnancy may play an important role in the physiologic adaptation of the body, and preparation of the pelvic floor for labor in particular. However, when compared to hCG, LH has a 10-fold higher LHR binding affinity [20]. Consequently, high LH levels after menopause may cause pathologic relaxation of the pelvic floor resulting in pelvic floor disorders.

Strong LHR expression in microglial cells in the brain cortex is of particular interest. Microglial cells are resident macrophages in the central nervous system, and LHR expression has been described in other types of human resident macrophages (ovary, decidua, endometrium and corpora lutea) [22,33]. Pathological activation of microglia has been reported in a wide range of conditions such as Alzheimer's disease, cerebral ischemia, prion diseases, multiple sclerosis, AIDS dementia, and other degenerative neurological diseases [70,71]. Some of these degenerative diseases are associated with advanced age and high levels of circulating LH. High LH levels might pass the presumptive LH barrier of brain vessels expressing LHR, or vascular LHR expression might diminish with age, as in abnormal term placentae. Yet, elevated maternal mid-trimester chorionic gonadotropin is associated with fetal cerebral blood flow redistribution and adverse perinatal outcome [63]. In addition, estrogens, which are known to cause a diminution of high LH levels in postmenopausal women, have been reported to be effective in the prevention and treatment of Alzheimer's disease [72–74]. Microglia belong among cells of the mononuclear phagocyte system [75]. Since CG increases secretion of a variety of cytokines

by monocytes, and induces their inflammatory reaction and phagocytic activity [76–79], high LH levels in aging individuals may also activate resident macrophages in the central nervous system and contribute to the development of Alzheimer's disease and other inflammation-mediated neurodegenerative diseases.

Outside of the area occupied by microglial cells in the grey matter of the cerebral cortex, weaker LHR expression was also detected in some nerve cells. This suggests that such nerve cells could be directly influenced by LH/CG proteohormones (with unknown consequences), while the nerve cells among microglial branching processes are protected from such an effect.

Conclusion
In conclusion, our observations concur with and expand current knowledge on LHR expression in gonadal and nongonadal tissues reported by other investigators. We show that the 3B5 antibody identifies six distinct LHR protein variants in three different mammalian species. Subcellular LHR localization varies during cellular differentiation. In contrast to the theory on the role of vascular hormone receptors in preferential pick up of circulating hormones, there may be no need to enhance selective pick up rather only prevent LH/CG transport to inappropriate sites. Abnormal placental LHR expression may play a role in the development of abnormal pregnancy and fetal outcome, and expression of LHR in the pelvic floor compartments suggests that high LH levels in postmenopausal women may contribute to pelvic floor relaxation and increased incidence of pelvic floor disorders. High LH levels in aging individuals may also participate in the development of inflammation-mediated neurodegenerative diseases, including Alzheimer's disease.

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