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Reproductive hormones regulate the selective permeability of the blood-brain barrier ☆

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ABSTRACT

Reproductive hormones have been demonstrated to modulate both gap and tight junction protein expression in the ovary and other reproductive tissues, however the effects of changes in reproductive hormones on the selective permeability of the blood-brain barrier (BBB) remain unclear. Age-related declines in BBB integrity correlate with the loss of serum sex steroids and increase in gonadotropins with menopause/andropause. To examine the effect of reproductive senescence on BBB permeability and gap and tight junction protein expression/localization, female mice at 3 months of age were either sham operated (normal serum E_2 and gonadotropins), ovariectomized (low serum E_2 and high serum gonadotropins) or ovariectomized and treated with the GnRH agonist leuprolide acetate (low serum E_2 and gonadotropins). Ovariectomy induced a 2.2-fold increase in Evan's blue dye extravasation into the brain. The expression and localization of the cytoplasmic membrane-associated tight junction protein zona occludens 1 (ZO-1) in microvessels was not altered among groups indicating that the increased paracellular permeability was not due to changes in this tight junction protein. However, ovariectomy induced a redistribution of the gap junction protein connexin-43 (Cx43) such that immunoreactivity relocalized from along the extracellular microvascular endothelium to become associated with endothelial cells. An increase in Cx43 expression in the mouse brain following ovariectomy was suppressed in ovariectomized animals treated with leuprolide acetate, indicating that serum gonadotropins rather than sex steroids were modulating Cx43 expression. These results suggest that elevated serum gonadotropins following reproductive senescence may be one possible cause of the loss of selective permeability of the BBB at this time. Furthermore, these findings implicate Cx43 in mediating changes in BBB permeability, and serum gonadotropins in the cerebropathophysiology of age-related neurodegenerative diseases such as stroke and Alzheimer's disease.
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1. Introduction

The blood-brain barrier (BBB) maintains brain homeostasis by limiting entry of substances to the central nervous system [1] through interaction of transmembrane and intracellular proteins that make up endothelial cell tight junctions and gap junctions. Although complex, tight junction proteins such as zona occludens 1 (ZO-1) and ZO-2, occludin and claudin-5, and gap junction proteins such as connexin-43 (Cx43), Cx45, Cx32 and Cx26 between endothelial cells maintain the selective permeability of the BBB [2]. The importance of gap junction proteins is indicated by the neurodevelopmental and deficits observed in Cx knockout mice [3]. Therefore, the identification of factors regulating the expression and function of junctional proteins is crucial for understanding the maintenance of BBB function during health and alterations during disease.

Failure of the BBB leads to neuroinflammatory diseases such as encephalitis and meningitis [4], and is associated with neurological diseases such as stroke, cerebral amyloid angiopathy and Alzheimer's disease (AD; [5–9]). The selective permeability of the BBB appears to be compromised during the normal course of aging [10] and numerous associated changes have been reported in the aging cerebrovasculature including decreased microvascular density, loss of endothelium, increased tortuosity, twisted/string vessels, fragmentation of the microvasculature, loss of the fine perivascular neural plexus, and lumpy vessels. Such changes are far more pronounced in the neurodegenerative condition of

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AD (reviewed in [11]), where fibrinogen leakage from microvessels is significantly greater than in control brain [12].

The incidence of neuroinflammatory diseases is increased during development and senescence, and correlates with the marked hormonal changes that occur during these times. Interestingly, dysregulation of the expression of gap junction and tight junction proteins, and alterations in paracellular permeability and junctional communication, are mediated by reproductive hormones. In particular, it has been reported that sex steroids can modulate changes in BBB permeability of female rats [13]. In this context, luteinizing hormone (LH), which becomes markedly elevated with reproductive senescence, has been demonstrated to potently down-regulate, via PKA/MAPK signaling pathways, the expression of Cx43 in large preovulatory follicles, theca cells and follicles undergoing atresia [14–18]. The developmental hormone, human chorionic gonadotropin (hCG), which has 83% homology to LH and binds the same receptor (LH/hCG receptor), also potently decreases Cx43 expression and morphological gap junctions in human myometrial smooth muscle cells [19]. In addition, LH decreases Cx43 expression and endometrial thickness in the human endometrium [20], promotes germinal vesicle breakdown possibly through a reduction of Cx43 in cumulus cells [21] and induces a decrease in the integrity of gap junctions in apoptotic human granulosa cells [22]. Recently, LH was shown to induce oocyte maturation via the interruption of cell-to-cell communication within the ovarian follicle, a process possibly mediated by the phosphorylation of Cx43 [23].

The decline in serum sex steroids with reproductive senescence may also alter BBB permeability. Hypothalamic Cx43 expression has been demonstrated to be regulated by steroid hormones in a brain-region-specific and sexually dimorphic manner in rats [24]. 17β-estradiol has been shown to modulate the expression of occludin, transendothelial resistance and paracellular permeability in human vascular endothelial cells [25] and human cervical epithelial cells [26]. Strong expression of endometrial Cx43 and Cx26 has been correlated with high serum concentrations of 17β-estradiol and progesterone, respectively, while gonadotropin-releasing hormone (GnRH) agonist treatment suppresses the expression of both connexins [27]. There also is evidence that ZO-1 expression is regulated by reproductive hormones [28].

Surprisingly, the role of reproductive hormones in the expression of these proteins required for the maintenance of the BBB is largely unexplored. Due to the facts that 1) gonadotropins become markedly elevated with menopause [29,30]/andropause [31], and 2) receptors for LH/hCG have been localized to endothelial cells and smooth muscle cells of the vasculature [32,33] and to neuronal cells [34–38], we hypothesized that menopause-related changes in serum reproductive hormones may alter the expression and/or function of tight and gap junctional proteins leading to the deterioration of the BBB associated with aging and neurodegenerative diseases. We report that ovariectomy increases the permeability of the BBB in young mice, and that changes in the expression and distribution of the gap junction protein Cx43 are mediated by gonadotropins. Thus, elevated serum gonadotropins following reproductive senescence may be one explanation for the loss of the selective permeability of the BBB with aging.

2. Materials and methods

2.1. Animals

Female B6/SJL mice (~3 months; Jackson Laboratory, Bar Harbor, Maine) and Tg2576 mice (Taconic, Germantown, New York) housed in the Animal Resource Center at Case Western Reserve University, Cleveland, OH were provided *ad libitum* access to food and water and maintained on a 12 h light/dark cycle. All procedures performed on animals were reviewed and approved by the Animal Resource Center of Case Western Reserve University, Cleveland, OH.

2.2. Experiment 1

Young female B6/SJL mice were either ovariectomized (n=5) or left intact (n=5). Young mice were used to avoid possible complications that might arise from post-estrous animals where the BBB might already be compromised or where other compensatory mechanisms might complicate data interpretation. Vaginal smears were performed post-surgery to examine the histology of epithelial cells to confirm that ovariectomy was successful. Two weeks after surgery, prior to sacrifice, mice were injected in the tail vein with a 2% Evan's blue dye solution to examine BBB permeability.

2.2.1. Quantitation of BBB integrity

The integrity of the BBB was assessed in female B6/SJL mice by a quantitative assay of Evan's blue dye (Sigma, St. Louis, MO). A 2% Evan's blue dye in saline solution was injected via the tail vein of anesthetized mice (4 ml/kg/mouse) 1 h prior to sacrifice. Animals were then transcardially perfused with phosphate buffered saline (PBS; Gibco, pH 7.4), the animals decapitated, brains removed and the cerebral hemispheres separated. One hemisphere was frozen, while the other was dropped fixed in methacarn. The frozen hemisphere was later homogenized in 400 ml of dimethyl formamide (Sigma, St. Louis, MO) and incubated for 72 h in a 50 °C water bath. The samples were then centrifuged at 1500 *g* for 10 min and the supernatant was analyzed at 620 nm using a spectrophotometer. Results were calculated as μM of Evan's blue dye per hemisphere as compared to a standard Evans blue dye curve. The other hemisphere of tissue was processed for histological analysis (as described below).

2.3. Experiment 2

Tg2576 mice at ~7.5 months of age were either bilaterally sham ovariectomized, ovariectomized or ovariectomized and treated with leuprolide acetate for 9 months (until ~16.5 months of age). Ovariectomized mice were left untreated or injected with leuprolide acetate. Table 1 summarizes the experimental design and predicted effects of each treatment on blood hormone levels. In the ovariectomized vehicle group, blood LH and FSH levels become elevated while estrogen levels decline; in the ovariectomized plus leuprolide-treated group, blood LH, FSH and estrogen levels all decline. This experimental design differentiates between whether end-point changes are due to sex steroids or gonadotropins.

2.3.1. GnRH agonist treatment

Leuprolide acetate, a well-known GnRH agonist (Lupron Depot, TAP Pharmaceuticals Inc., Lake Forest) was used for the experiments. Leuprolide acetate is a synthetic GnRH agonist that lowers LH/FSH levels by desensitizing GnRH receptor signaling that subsequently leads to decreased LH/FSH secretion and a decrease in the serum concentration of sex steroids. Animals were injected intramuscularly with vehicle or leuprolide acetate (1.5 mg/kg) biweekly during the first month and monthly thereafter up to 9 months. Leuprolide acetate was mixed with a diluent in a pre-filled dual-chamber syringe containing sterile lyophilized microspheres to form a suspension. Leuprolide is gradually released from microspheres over a 4-week period. Continuous treatment produces initial stimulation of the pituitary and an increase in blood gonadotropins, followed by a suppression of blood gonadotropins and sex steroids to castrate/post-menopausal levels within ~1 week. In females, both ovarian estrogen and androgen syntheses are inhibited.

2.3.2. Tissue collection

Blood was collected from B6/SJL mice pre-treatment and at 14 days post-surgery from an orbital sinus after mice were anesthetized with an IP injection of mouse anesthesia cocktail (0.1 ml/25 g body weight, containing ketamine HCl (9 mg/ml); xylazine HCl (1.7 mg/ml); acepromazine (0.3 mg/ml) in sterile saline). Blood was collected from Tg2576 mice at ~7.5 months of age and then again at the end of the 1st and 3rd months from an orbital sinus after mice were anesthetized as above. The final blood at 9 months was collected by heart puncture. Blood was collected from animals prior to their next dose of leuprolide acetate. Blood (100–400 μl) was collected into EDTA-coated tubes, which were centrifuged at 1000 *g* for 3 min at 4 °C, followed by centrifugation at 5000 *g* for 10 min prior to the collection of plasma, which was stored at −80 °C. Mice were sacrificed at 16 months of age with an injection of concentrated sodium barbital (50 μl each, 392 mg/ml, IP). Blood was collected at this time by heart puncture, the animals were then perfused with PBS, the animals decapitated and the brain removed from the skull. One brain hemisphere was fixed in methacarn (6 parts ethanol, 3 parts chloroform, and one part glacial acetic acid) for 24 h and then paraffin embedded for immunocytochemical studies. The other brain hemisphere was frozen in liquid nitrogen and stored at −80 °C for biochemical studies.

2.3.3. Hormone assays

Plasma levels of LH and FSH (Tg2576 mice) were assayed by radioimmunoassay at the National Hormone and Peptide Program (Torrance, CA).

Table 1
Summary of animals for hormonal modulation experiments

Group number	Group name	Gonadotropin level c.f. control	Estrogen level c.f. control	Number of mice	Age at euthanasia (month)
1	Sham OVX+vehicle	Normal	Normal	9	16
2	OVX+vehicle	High	Low	6	16
3	OVX+leuprolide	Low	Low	6	16

2.3.4. Immunohistochemistry

Paraffin embedded and methacarn fixed brain hemispheres were serially sectioned (8 μm in thickness) in the sagittal plane with a Leica CA 1900 cryostat. The sections were deparaffinized with xylene and hydrated through 100%, 95%, 70%, and 50% ethanol for 5 min in each, and then incubated in Tris-buffered saline (TBS) for 10 min. Endogenous peroxidase activity was eliminated with a 30 min incubation in 3% H_2O_2 in methanol. Following placement of the sections in citrate buffer (1 mM, pH 6.0), slides were autoclaved at 121 °C for 30 min and then rinsed well. Non-specific binding sites were blocked with 10% normal goat serum (NGS) in TBS for 30 min before application of anti-connexin-43 rabbit polyclonal antibody (1:100 dilution, Cell Signaling Technologies, Danvers, MA) overnight at 4 °C. After a series of washes, tissue sections were incubated with both the goat affinity purified antibody to rabbit IgG (1:50 dilution, 30 min) and the rabbit PAP (1:250 dilution, 60 min). Immunostaining was then developed using diaminobenzidine (DAKO Corporation, Carpinterie, CA) for 10 min, slides mounted using Permount (Fisher Chemicals, Fair Lawn, NJ) and staining analyzed under an inverted microscope (Zeiss Axiophot, Thornwood, NY). For ZO-1, an anti-ZO-1 rabbit polyclonal antibody (1:50 dilution; Invitrogen, Carlsbad, CA) was applied overnight at 4 °C, followed by washes as described above and immunofluorescent detection using a goat-anti rabbit IgG antibody conjugated to fluorescein isothiocyanate (FITC; Santa Cruz Biotechnologies, Santa Cruz, CA; 1:100 dilution, 2 h). To control for non-specific secondary antibody binding, control tissue sections were treated with secondary antibody alone.

2.3.5. Immunoblot analyses

The cerebral part of the frozen hemisphere of the mouse brain was homogenized on ice in lysis buffer (20 mmol/l Tris–HCl, pH 7.6, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 1% sodium dodecyl sulfate (SDS), and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), and then centrifuged for 10 min at 10 000 *g* at 4 °C. Following protein assay (Bicinchoninic Acid Protein assay kit; Pierce, Rockford, IL, USA), equal amounts of protein were loaded onto 10–20% tricine gels (Invitrogen, Carlsbad, CA) for SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was fixed with glutaraldehyde (4%, v/v in TBST for 30 min), blocked with milk (10%, w/v in TBST for 2 h) and then probed with a rabbit polyclonal antibody against Cx43 (dilution 1:1000, Cell Signaling Technologies, Danvers, MA) or GAPDH (dilution 1:1000, Santa Cruz Biotechnologies, Santa Cruz, CA) overnight at 4 °C. The blot was then incubated with the corresponding horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at 22 °C, washed and developed with enhanced chemiluminescence reagent (Santa Cruz Biotechnology, Santa Cruz, CA) as per the manufacturer's instructions. The chemiluminescent signal was captured on autoradiographs (Eastman Kodak, Rochester, NY, USA), which were scanned and the intensity of the autoradiograph signals (including a blank region) was determined using the NIH Image J Software. Control and treatment values were corrected for blank values, normalized to their respective GAPDH band intensity and the results then expressed as a fold change over sham levels.

2.4. Statistical analyses

ANOVA analyses and Student's *t*-tests were used to assess statistically significant differences between treatment groups.

3. Results

To assess the role of reproductive hormones in the maintenance of the BBB, young mice were ovariectomized and 2 weeks later the animals were injected with Evan's blue dye and the brains were removed for analysis. A 2.2-fold increase in Evan's blue dye extravasation into the brains of ovariectomized female mice compared to controls was noted (Fig. 1).

The ovariectomy-induced changes in the selective permeability of the BBB may result from increases in paracellular permeability due to changes in tight or gap junction proteins of microvessels. To examine whether gap proteins involved in the maintenance of the BBB were altered following ovariectomy, we examined the microvasculature for alterations in the staining of Cx43. There was a clear redistribution of the Cx43 labeling of the microvascular endothelium in ovariectomized compared with control animals (Fig. 2). Staining was clearly evident along the extracellular endothelium of microvessels in the cortex of the brain. However, following ovariectomy, there was a redistribution of immunoreactivity from the endothelium to specific endothelial cells. This ovariectomy-induced partitioning of Cx43 may alter endothelial barrier function, and thus the permeability of the BBB.

Since ovariectomy decreases serum estradiol but markedly increases serum gonadotropins levels in mice [39,40], these results suggest that hormonal changes associated with ovariectomy alter the biophysical characteristics of the BBB. To distinguish between whether the decline in serum sex steroids or the increase in gonadotropins was responsible for alterations in gap junction proteins that maintain the integrity of the BBB, mice were sham operated, ovariectomized (low estradiol, high gonadotropins) or ovariectomized and treated with the GnRH agonist leuprolide acetate (low estradiol, low gonadotropins). To confirm that ovariectomy and leuprolide acetate treatments modulated blood sex hormone levels, plasma collected from mice at 0, 1, 3 and 9 months (and also 6 months for vehicle and leuprolide-treated groups) post-treatment were analyzed for LH and FSH concentrations. The concentration of plasma LH increased 2.2-fold between 7 and 16 months of age in sham ovariectomized animals (0.44±0.11 ng/ml vs 0.96±0.07 ng/ml, respectively; mean±SEM, $P<0.05$, $n=9$). Ovariectomy induced an 8.4-fold increase in the concentration of plasma LH by 9 months post-surgery (16 months of age) compared to time 0 (7 months of age; 0.59±0.08 ng/ml vs 4.92±0.65 ng/ml, respectively; $P<0.001$, $n=9$). Leuprolide acetate treatment of ovariectomized mice completely suppressed the increase in the concentration of plasma LH from 1 month post-treatment to 9 months post-treatment (0.54±0.09 ng/ml vs 0.70±0.05 ng/ml; ns, $n=9$). Likewise, the concentration of plasma FSH did not alter significantly between 0 and 9 months post-surgery in sham ovariectomized mice, (7.8±2.2 ng/ml vs 12.9±2.2 ng/ml; $n=6$). Ovariectomy induced a significant 10.9-fold increase in the concentration of plasma FSH from 4.8±1.0 ng/ml pre-surgery to 52.6±3.1 ng/ml ($P<0.05$, $n=6$) at 1 month post-surgery and the concentration of plasma FSH remained at this level to 9 months post-surgery. Leuprolide acetate treatment of mice that were ovariectomized suppressed the increase in plasma FSH at 1 month (16.2±1.9 ng/ml; $n=6$) and FSH remained around sham ovariectomy levels through to 9 months post-surgery (8.4±1.0 ng/ml; $n=6$). Overall, these results indicate that leuprolide acetate suppresses plasma LH and FSH, and that the ovariectomy induced increases in plasma LH and FSH is suppressed by leuprolide acetate.

To examine how differences in sex steroids and gonadotropins affect the integrity of the BBB, we next examined the expression of the gap junction protein Cx43. An increase in total Cx43 expression was observed in the mouse brain following ovariectomy (low serum sex steroids, high serum gonadotropins) compared to sham-operated controls (Fig. 3), while a significant decrease in Cx43 expression was observed in ovariectomized mice that were treated with leuprolide acetate (low serum sex steroids and gonadotropins). The decrease in Cx43 expression following leuprolide acetate treatment suggests that gonadotropins (Figs. 2 and 3), and not sex steroids regulate Cx43 expression.

To examine whether reproductive hormones also altered the distribution of tight junction proteins, we probed tissue sections with a polyclonal antibody against ZO-1, a cytoplasmic membrane-

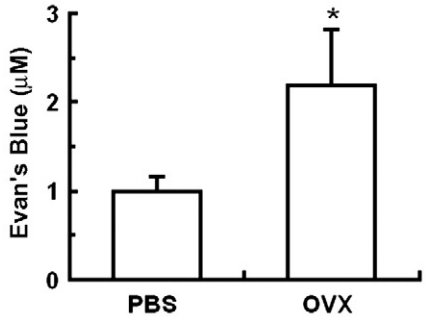

Fig. 1. Quantitation of Evan's blue extravasation. Female B6/SJL mice were either ovariectomized ($n=5$) or left intact ($n=5$). Two weeks after surgery mice were injected with a 2% Evan's blue dye solution to examine BBB permeability. There was a 2.2-fold increase in Evan's blue dye extravasation into the brains of ovariectomized female mice compared to control (mean±SEM, $P<0.05$).

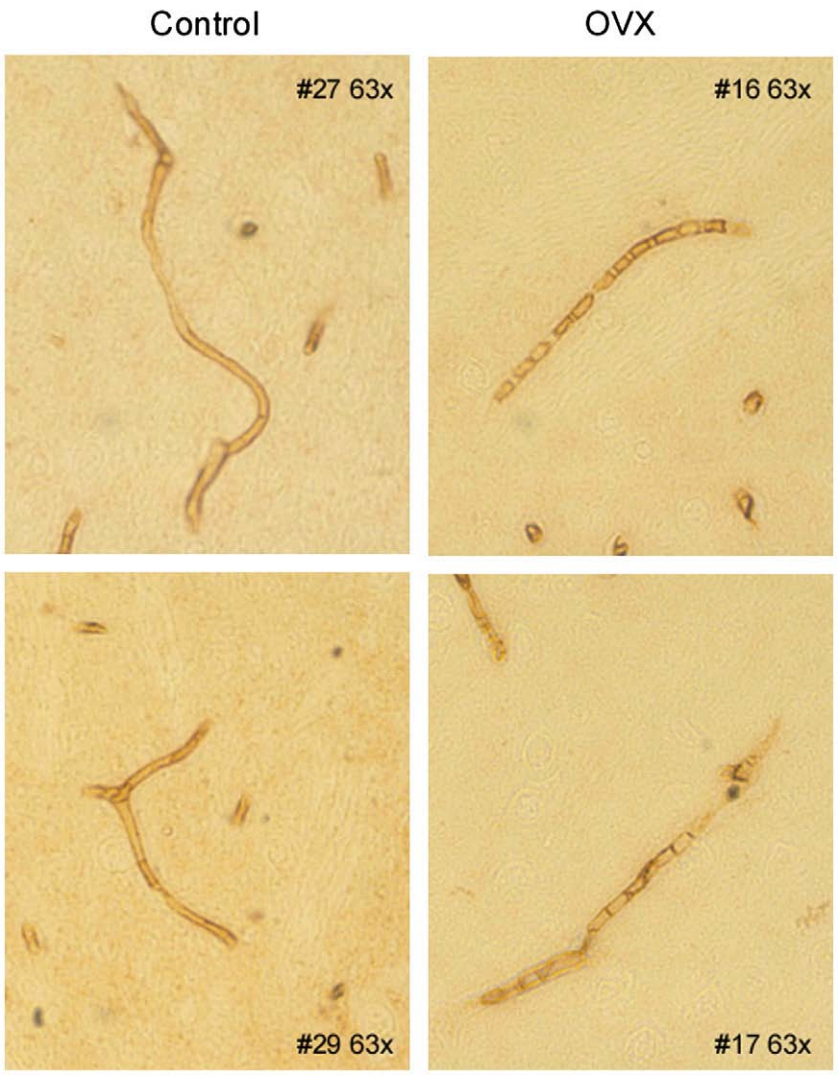

Fig. 2. Ovariectomy alters the immunolocalization of the gap junction protein connexin-43 in cortical microvessels. Ovariectomized and control brain sections were immunocytochemically labeled with a rabbit polyclonal antibody against connexin-43. Results indicated a clear redistribution of the Cx43 labeling of the microvascular endothelium in ovariectomized compared with control animals. Two representative control sections are shown on the left (#27 and #29) and 2 representative ovariectomized brain sections are shown on the right (#16 and #17). All pictures are shown at objective magnification 63×.

associated protein known to have a role in the development and stability of tight junctions. At objective magnifications of 40× and 63×, no differences in ZO-1 localization were observed between OVX mice and controls using immunofluorescence (Fig. 4) or diaminobenzidine staining (data not shown). To examine whether reproductive hormones also altered the expression of ZO-1, we immunoprobed the blot described in Fig. 3. Immunoblot analyses of the 225 kDa ZO-1 band indicated no change in expression between the sham, ovariectomized, and ovariectomized and leuprolide acetate-treated groups (Fig. 5) suggesting that reproductive hormones do not alter ZO-1 expression and distribution.

4. Discussion

The selective permeability of the BBB is maintained by complex junctional interactions between endothelial cells of brain capillaries [41–43]. While this selective permeability normally excludes the passage of molecules ≥100 Da, our results indicate that ovariectomy alters the selective permeability of the BBB, allowing molecules ≥961 Da, the molecular mass of Evan's blue dye, to cross the BBB (Fig. 1). At the molecular level, we observed no change in the expression of the tight junction protein ZO-1 (Figs. 4 and 5), suggesting that the abrupt

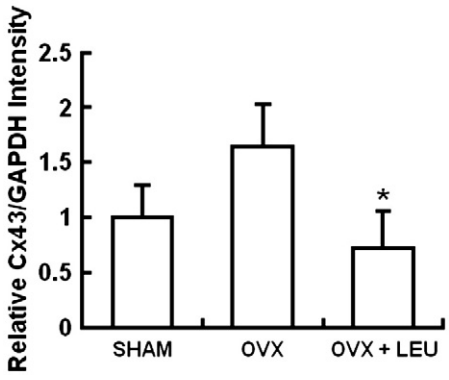

Fig. 3. Gonadotropins modulate the brain expression of connexin-43. Immunoblots of homogenized brain tissue samples from control (sham operated), ovariectomized, and ovariectomized and leuprolide acetate-treated Tg2576 mice (n=4 each) were probed with the polyclonal connexin-43 polyclonal antibody used in Fig. 2. The expression of connexin-43 increased following ovariectomy (low sex steroids, high gonadotropins) but decreased to sham levels when treated with leuprolide acetate (low sex steroids, low gonadotropins), indicating that changes in the serum gonadotropins, and not the sex steroids, regulate Cx43 expression (mean ± SEM, P<0.05).

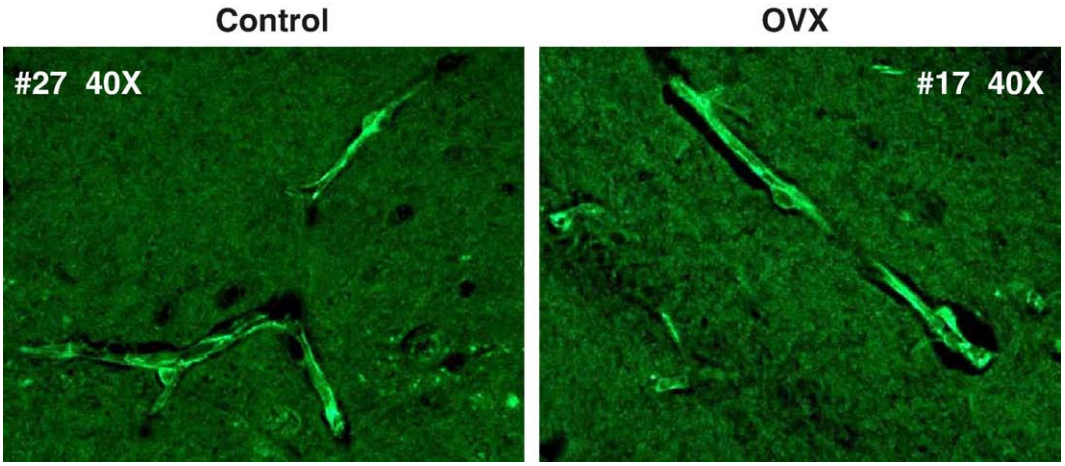

Fig. 4. Reproductive hormones do not alter cortical microvessel ZO-1 localization. Ovariectomized (#17) and control (#27) brain sections were immunofluorescently labeled with a rabbit polyclonal antibody against ZO-1. No differences in ZO-1 localization were observed between ovariectomized and control mice. All pictures are shown at objective magnification 40×. Non-specific staining of pyramidal neurons also was detected (not shown).

hormonal changes associated with ovariectomy were mediated via changes in the expression and/or distribution of other junctional proteins. Indeed, we observed both a redistribution and increase in the expression of the gap junction protein Cx43 following ovariectomy (Figs. 2 and 3), supporting previous research that Cx43 may influence endothelial barrier function [2]. Whether the increase in Cx43 expression is a protective response to the ovariectomy-induced decrease in selective permeability requires further research. Irrespective, the redistribution of Cx43 expression in ovariectomized animals potentially increases paracellular pathway diffusion, and also likely alters both the electrical communication and the free diffusion of molecules and ions, including small intracellular signaling molecules, between cells. Such changes may be the biochemical underpinning of the morphological changes observed in the cerebrovasculature with aging.

Our experimental paradigm manipulating reproductive hormones of the hypothalamic–pituitary–gonadal axis using ovariectomy and leuprolide acetate indicated that gonadotropins were more likely than sex steroids to modulate the expression of the Cx43 gap junction protein and its distribution (Figs. 2 and 3), consistent with previous studies in other systems demonstrating that reproductive hormones modulate Cx43 expression [14–23]. Alterations in vascular integrity appear to result from the LH-induced phosphorylation/dephosphorylation and down-regulation of Cx43 expression [14–18] in the endothelium, mediated via LH/hCG receptors during increases in gonadotropin concentration. The functionality of endothelial LH/hCG receptors in mediating barrier integrity has been demonstrated in the uterus where hCG administration decreases vascular resistance and plays a role in the peri-implantation period by increasing uterine blood flow through vasodilation [32]. Since LH can cross the BBB [44] and LH is expressed by [45], and accumulates intracellularly in the pyramidal neurons of AD compared with age-matched control brains [46], neural LH expression might influence Cx43 expression/distribution around astrocytes, microglia and neurons. FSH also has been shown to up-regulate Cx43 expression [18], and also could possibly induce vascular changes in the BBB.

While our studies suggest that gonadotropins are important for changes in Cx43, it is possible that changes to the BBB are mediated by the decline in sex steroids via other mechanisms. Support for this notion is indicated by the findings that progesterone promotes the reconstitution of the BBB after traumatic brain injury (reviewed in [47]). Likewise, 17β-estradiol has been shown to modulate the expression of occludin, transendothelial resistance and paracellular permeability in human vascular endothelial cells [25] and human cervical epithelial cells [26].

It is possible that GnRH also modulates Cx43 expression or localization [48] since ovariectomy greatly increases GnRH secretion [49,50]. In this connection, a rare complication from the administration of GnRH agonists to men for the treatment of prostate cancer is pituitary apoplexy [51,52], a hemorrhage of a subclinical pituitary adenoma resulting in acute symptoms of headache, vomiting, meningismus, visual impairments such as diplopia from opthalmoplegia, hormone dysfunction and alterations of consciousness. These symptoms appear within hours of GnRH agonist administration during the increase in serum gonadotropins [53], and suggest that either gonadotropins or GnRH signaling alters vascular integrity. Cx43 has been immunolocalized to gap junctions in the pituitary [54].

AD is characterized by a chronic inflammatory response that is suggestive of alterations in BBB integrity. While the etiology of AD neuroinflammation is unclear, it is known that experimental induction of brain inflammation by injection of complete Freund's adjuvant into the right plantar hindpaw in female Sprague–Dawley rats increases BBB permeability as determined by sucrose uptake and major changes in junctional protein expression (−60% occludin, 450% claudin-3, and 615% claudin-5; [55]). In AD, where serum LH/FSH levels are elevated compared with age-matched control individuals [56,57], Cx43 immunoreactivity is elevated in cortical areas containing Aβ plaques, and Cx43 is localized to astrocytic gap junctions in AD brain [58]. These results are consistent with the increases in Cx43 observed in our study following ovariectomy (Fig. 3). In this

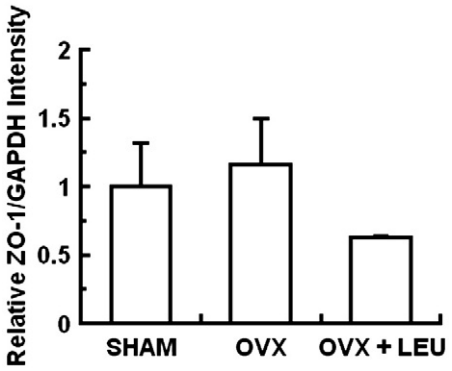

Fig. 5. Reproductive hormones do not alter brain ZO-1 expression. Immunoblots from Fig. 3 were probed with the ZO-1 polyclonal antibody used in Fig. 4. No difference in ZO-1 expression was detected between groups (mean ± SEM, $n = 4$, ns.

connection, LH induces processing of AβPP towards the amyloidogenic pathway [37] and its deposition in the vasculature has been postulated to be a vascular sealant [11,59,60]. Whether LH induces neuroinflammation via alteration of the BBB remains to be tested.

Tight junctions are well known to change in the mammary gland from 'leaky' during pregnancy to 'tight' at the commencement of lactation [61,62]. Physiological and ultrastructural evidence [61,63] supports the concept that the junctional complexes of the secretory epithelium change from 'leaky' to 'tight' at parturition. Birth is of course associated with a marked reduction in progesterone and hCG, the fetal equivalent of LH that binds the same receptor, providing further support for a role of gonadotropins and/or sex steroids in modulating the BBB. In this respect, maturation of tight junctions as indicated by the direct analysis of the cleft index of individual junctions has been reported during development [64,65].

In the ovary, the LH surge is thought to interrupt cell-to-cell communication within the ovarian follicle, leading to a decrease in intraoocyte concentrations of cAMP followed by resumption of meiosis [66]. The gonadotropin-induced redistribution of Cx43 (Fig. 2) is consistent with an interruption of cell-to-cell communication, and intriguingly, LH has been postulated to promote the aberrant reentry of neurons into the cell cycle in AD [67]. Thus, elevated LH levels with menopause/andropause may alter Cx43 expression and redistribution required to initiate cell division. In this respect, transfection expression of the Cx43 gene has been shown to induce E-cadherin overexpression and an inhibition of LH(7) cell proliferation indicating a significant role of Cx43 in the regulation of cell proliferation [68].

Thus, our results indicate that change in reproductive hormone signaling following ovariectomy induces an increase in the expression and relocalization of endothelial Cx43 that is accompanied by an increase in the permeability of the BBB. Given that ovariectomy induces changes in reproductive hormones similar to that of menopause/andropause, these data suggest that reproductive hormones regulate the selective permeability of the BBB via changes in junctional proteins. The exact compliment of junctional proteins altered by reproductive hormones awaits further study. These results have important implications for explaining changes in the BBB with aging [69–71], and in the development of the pathophysiology of age-related neurodegenerative/neuroinflammatory diseases such as stroke and AD [72,73].

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References

[1] P. Elrich, Das Sauerstoffbeduerfnis des organismus: Einus farbenanalytiche studie, 8, 1885, p. 167.
[2] K. Nagasawa, H. Chiba, H. Fujita, T. Kojima, T. Saito, T. Endo, N. Sawada, Possible involvement of gap junctions in the barrier function of tight junctions of brain and lung endothelial cells, J. Cell Physiol. 208 (2006) 123–132.
[3] T. Nakase, C.C. Naus, Gap junctions and neurological disorders of the central nervous system, Biochim. Biophys. Acta 1662 (2004) 149–158.
[4] R. Aspinall, Age-related changes in the function of T cells, Microsc. Res. Tech. 62 (2003) 508–513.
[5] W. Scholz, Studien zur Pathologic der Hirngef~13e IL Die drusige Entartung der Hirnarterien und -capillaren, Z. Ges. Neurol. Psychiatr. 162 (1938) 694–7159.
[6] R.N. Kalaria, Cerebral vessels in ageing and Alzheimer's disease, Pharmacol. Ther. 72 (1996) 193–214.
[7] R. Deane, B.V. Zlokovic, Role of the blood-brain barrier in the pathogenesis of Alzheimer's disease, Curr. Alzheimer. Res. 4 (2007) 191–197.
[8] Y. Matsumoto, D. Yanase, M. Noguchi-Shinohara, K. Ono, M. Yoshita, M. Yamada, Blood-brain barrier permeability correlates with medial temporal lobe atrophy but not with amyloid-beta protein transport across the blood-brain barrier in Alzheimer's disease, Dement. Geriatr. Cogn. Disord. 23 (2007) 241–245.
[9] B.D. Zipser, C.E. Johanson, L. Gonzalez, T.M. Berzin, R. Tavares, C.M. Hulette, M.P. Vitek, V. Hovanesian, E.G. Stopa, Microvascular injury and blood-brain barrier leakage in Alzheimer's disease, Neurobiol. Aging 28 (2007) 977–986.
[10] P.A. Stewart, K. Hayakawa, C.L. Farrell, R.F. Del Maestro, Quantitative study of microvessel ultrastructure in human peritumoral brain tissue. Evidence for a blood-brain barrier defect, J. Neurosurg. 67 (1987) 697–705.
[11] C.S. Atwood, R.L. Bowen, M.A. Smith, G. Perry, Cerebrovascular requirement for sealant, anti-coagulant and remodeling molecules that allow for the maintenance of vascular integrity and blood supply, Brain Res. Brain Res. Rev. 43 (2003) 164–178.
[12] M. Fiala, Q.N. Liu, J. Sayre, V. Pop, V. Brahmandam, M.C. Graves, H.V. Vinters, Cyclooxygenase-2-positive macrophages infiltrate the Alzheimer's disease brain and damage the blood-brain barrier, Eur. J. Clin. Invest. 32 (2002) 360–371.
[13] S. Bake, F. Sohrabji, 17beta-estradiol differentially regulates blood-brain barrier permeability in young and aging female rats, Endocrinology 145 (2004) 5471–5475.
[14] J.F. Wiesen, A.R. Midgley Jr., Changes in expression of connexin 43 gap junction messenger ribonucleic acid and protein during ovarian follicular growth, Endocrinology 133 (1993) 741–746.
[15] I. Granot, N. Dekel, Phosphorylation and expression of connexin-43 ovarian gap junction protein are regulated by luteinizing hormone, J. Biol. Chem. 269 (1994) 30502–30509.
[16] I. Granot, N. Dekel, Developmental expression and regulation of the gap junction protein and transcript in rat ovaries, Mol. Reprod. Dev. 47 (1997) 231–239.
[17] U. Wehrenberg, G.M. Rune, Spontaneous luteinization of antral marmoset follicles in vitro, Mol. Hum. Reprod. 6 (2000) 504–509.
[18] Y. Kalma, I. Granot, D. Galiani, A. Barash, N. Dekel, Luteinizing hormone-induced connexin 43 down-regulation: inhibition of translation, Endocrinology 145 (2004) 1617–1624.
[19] G. Ambrus, C.V. Rao, Novel regulation of pregnant human myometrial smooth muscle cell gap junctions by human chorionic gonadotropin, Endocrinology 135 (1994) 2772–2779.
[20] I. Granot, N. Dekel, E. Bechor, I. Segal, S. Fieldust, A. Barash, Temporal analysis of connexin43 protein and gene expression throughout the menstrual cycle in human endometrium, Fertil. Steril. 73 (2000) 381–386.
[21] M. Shimada, T. Terada, FSH and LH induce progesterone production and progesterone receptor synthesis in cumulus cells: a requirement for meiotic resumption in porcine oocytes, Mol. Hum. Reprod. 8 (2002) 612–618.
[22] R. Sasson, A. Amsterdam, Stimulation of apoptosis in human granulosa cells from in vitro fertilization patients and its prevention by dexamethasone: involvement of cell contact and bcl-2 expression, J. Clin. Endocrinol. Metab. 87 (2002) 3441–3451.
[23] S. Sela-Abramovich, E. Chorev, D. Galiani, N. Dekel, Mitogen-activated protein kinase mediates luteinizing hormone-induced breakdown of communication and oocyte maturation in rat ovarian follicles, Endocrinology 146 (2005) 1236–1244.
[24] M. Gulinello, A.M. Etgen, Sexually dimorphic hormonal regulation of the gap junction protein, CX43, in rats and altered female reproductive function in CX43+/− mice, Brain Res. 1045 (2005) 107–115.
[25] L. Ye, T.A. Martin, C. Parr, G.M. Harrison, R.E. Mansel, W.G. Jiang, Biphasic effects of 17-beta-estradiol on expression of occludin and transendothelial resistance and paracellular permeability in human vascular endothelial cells, J. Cell Physiol. 196 (2003) 362–369.
[26] R. Zeng, X. Li, G.I. Gorodeski, Estrogen abrogates transcervical tight junctional resistance by acceleration of occludin modulation, J. Clin. Endocrinol. Metab. 89 (2004) 5145–5155.
[27] P.A. Regidor, M. Regidor, A.E. Schindler, E. Winterhager, Aberrant expression pattern of gap junction connexins in endometriotic tissues, Mol. Hum. Reprod. 3 (1997) 375–381.
[28] F.S. Khan-Dawood, J. Yang, M.Y. Dawood, Localization and expression of zonula occludens-1 tight junction-associated protein in baboon (*Papio anubis*) corpora lutea, Hum. Reprod. 11 (1996) 1262–1267.
[29] B. Couzinet, G. Schaison, The control of gonadotrophin secretion by ovarian steroids, Hum. Reprod. Suppl. 2 (1993) 97–101.
[30] S. Chakravarti, W.P. Collins, J.D. Forecast, J.R. Newton, D.H. Oram, J.W. Studd, Hormonal profiles after the menopause, Br. Med. J. 2 (1976) 784–787.
[31] W.B. Neaves, L. Johnson, J.C. Porter, C.R.J. Parker, C.S. Petty, Leydig cell numbers, daily sperm production, and serum gonadotropin levels in aging men, J. Clin. Endocrinol. Metab. 59 (1984) 756–763.
[32] P. Toth, H. Lukacs, G. Gimes, A. Sebestyen, N. Pasztor, F. Paulin, C.V. Rao, Clinical importance of vascular LH/hCG receptors—a review, Reprod. Biol. 1 (2001) 5–11.
[33] S. Berndt, S. Perrier d'Hauterive, S. Blacher, C. Pequeux, S. Lorquet, C. Munaut, M. Applanat, M.A. Herve, N. Lamande, P. Corvol, F. van den Brule, F. Frankenne, M. Poutanen, I. Huhtaniemi, V. Geenen, A. Noel, J.M. Foidart, Angiogenic activity of human chorionic gonadotropin through LH receptor activation on endothelial and epithelial cells of the endometrium, Faseb. J. 20 (2006) 2630–2632.
[34] Z.M. Lei, C.V. Rao, J.L. Kornyei, P. Licht, E.S. Hiatt, Novel expression of human chorionic gonadotropin/luteinizing hormone receptor gene in brain, Endocrinology 132 (1993) 2262–2270.
[35] A.A. al-Hader, Z.M. Lei, C.V. Rao, Neurons from fetal rat brains contain functional luteinizing hormone/chorionic gonadotropin receptors, Biol. Reprod. 56 (1997) 1071–1076.
[36] A.A. al-Hader, Y.X. Tao, Z.M. Lei, C.V. Rao, Fetal rat brains contain luteinizing hormone/human chorionic gonadotropin receptors, Early Pregnancy 3 (1997) 323–329.
[37] R.L. Bowen, G. Verdile, T. Liu, A.F. Parlow, G. Perry, M.A. Smith, R.N. Martins, C.S. Atwood, Luteinizing hormone, a reproductive regulator that modulates the processing of amyloid-beta precursor protein and amyloid-beta deposition, J. Biol. Chem. 279 (2004) 20539–20545.
[38] T. Liu, J. Wimalasena, R.L. Bowen, C.S. Atwood, Luteinizing hormone receptor mediates neuronal pregnenolone production via up-regulation of steroidogenic acute regulatory protein expression, J. Neurochem. 100 (2007) 1329–1339.

[39] F.H. Bronson, The regulation of luteinizing hormone secretion by estrogen: relationships among negative feedback, surge potential, and male stimulation in juvenile, peripubertal, and adult female mice, Endocrinology 108 (1981) 506–516.
[40] S.I. Naik, L.S. Young, H.M. Charlton, R.N. Clayton, Pituitary gonadotropin-releasing hormone receptor regulation in mice. II: Females, Endocrinology 115 (1984) 114–120.
[41] T.S. Reese, M.J. Karnovsky, Fine structural localization of a blood-brain barrier to exogenous peroxidase, J. Cell Biol. 34 (1967) 207–217.
[42] M.W. Brightman, T.S. Reese, Junctions between intimately apposed cell membranes in the vertebrate brain, J. Cell Biol. 40 (1969) 648–677.
[43] Z. Nagy, H. Peters, I. Huttner, Fracture faces of cell junctions in cerebral endothelium during normal and hyperosmotic conditions, Lab. Invest. 50 (1984) 313–322.
[44] H. Lukacs, E.S. Hiatt, Z.M. Lei, C.V. Rao, Peripheral and intracerebroventricular administration of human chorionic gonadotropin alters several hippocampus-associated behaviors in cycling female rats, Horm. Behav. 29 (1995) 42–58.
[45] A.C. Wilson, M.S. Salamat, R.J. Haasl, K.M. Roche, A. Karande, S.V. Meethal, E. Terasawa, R.L. Bowen, C.S. Atwood, Human neurons express type I GnRH receptor and respond to GnRH I by increasing luteinizing hormone expression, J. Endocrinol. 191 (2006) 651–663.
[46] R.L. Bowen, M.A. Smith, P.L. Harris, Z. Kubat, R.N. Martins, R.J. Castellani, G. Perry, C.S. Atwood, Elevated luteinizing hormone expression colocalizes with neurons vulnerable to Alzheimer's disease pathology, J. Neurosci. Res. 70 (2002) 514–518.
[47] D.G. Stein, D.W. Wright, A.L. Kellermann, Does progesterone have neuroprotective properties? Ann. Emerg. Med. (2007).
[48] S. Tsukahara, F. Maekawa, H. Tsukamura, K. Hirunagi, K. Maeda, Morphological characterization of relationship between gap junctions and gonadotropin releasing hormone nerve terminals in the rat median eminence, Neurosci. Lett. 261 (1999) 105–108.
[49] A.C. Gore, E. Terasawa, A study of the hypothalamic pulse-generating mechanism responsible for LH release: electrical stimulation of the medial basal hypothalamus in the ovariectomized guinea pig, Brain Res. 560 (1991) 268–275.
[50] A.C. Gore, B.M. Windsor-Engnell, E. Terasawa, Menopausal increases in pulsatile gonadotropin-releasing hormone release in a nonhuman primate (*Macaca mulatta*), Endocrinology 145 (2004) 4653–4659.
[51] P. Chanson, G. Schaison, Pituitary apoplexy caused by GnRH-agonist treatment revealing gonadotroph adenoma, J. Clin. Endocrinol. Metab. 80 (1995) 2267–2268.
[52] N. Hernandez Morin, D. Huet, M. Hautecouverture, [Two cases of non-functional gonadotroph adenoma pituitary apoplexy following GnRH-agonist treatment revealing gonadotroph adenoma and pseudopituitary apoplexy after GnRH administration], Ann. Endocrinol. (Paris). 64 (2003) 227–231.
[53] Physicians' Desk Reference, 61st ed., Thomson PDR, Montvale 2007.
[54] N. Shirasawa, Y. Mabuchi, E. Sakuma, O. Horiuchi, T. Yashiro, M. Kikuchi, Y. Hashimoto, Y. Tsuruo, D.C. Herbert, T. Soji, Intercellular communication within the rat anterior pituitary gland: X. Immunohistocytochemistry of S-100 and connexin 43 of folliculo-stellate cells in the rat anterior pituitary gland, Anat. Rec. A. Discov. Mol. Cell Evol. Biol. 278 (2004) 462–473.
[55] T.A. Brooks, B.T. Hawkins, J.D. Huber, R.D. Egleton, T.P. Davis, Chronic inflammatory pain leads to increased blood-brain barrier permeability and tight junction protein alterations, Am. J. Physiol. Heart. Circ. Physiol. 289 (2005) H738–H743.
[56] R.L. Bowen, J.P. Isley, R.L. Atkinson, An association of elevated serum gonadotropin concentrations and Alzheimer disease? J. Neuroendocrinol. 12 (2000) 351–354.
[57] R.A. Short, R.L. Bowen, P.C. O'Brien, N.R. Graff-Radford, Elevated gonadotropin levels in patients with Alzheimer disease, Mayo Clin. Proc. 76 (2001) 906–909.
[58] J.I. Nagy, W. Li, E.L. Hertzberg, C.A. Marotta, Elevated connexin43 immunoreactivity at sites of amyloid plaques in Alzheimer's disease, Brain Res. 717 (1996) 173–178.
[59] C.S. Atwood, G.M. Bishop, G. Perry, M.A. Smith, Amyloid-beta: a vascular sealant that protects against hemorrhage? J. Neurosci. Res. 70 (2002) 356.
[60] C.S. Atwood, G. Perry, M.A. Smith, Cerebral hemorrhage and amyloid-beta, Science 299 (2003) 1014 author reply 1014.
[61] J.L. Linzell, M. Peaker, Changes in colostrum composition and in the permeability of the mammary epithelium at about the time of parturition in the goat, J. Physiol. 243 (1974) 129–151.
[62] M. Peaker, Recent advances in the study of monovalent ions movements across the mammary epithelium: relation to onset of lactation, J. Dairy Sci. 58 (1975) 1042–1047.
[63] D.R. Pitelka, S.T. Hamamoto, J.G. Duafala, M.K. Nemanic, Cell contacts in the mouse mammary gland. I. Normal gland in postnatal development and the secretory cycle, J. Cell Biol. 56 (1973) 797–818.
[64] P.A. Stewart, E.M. Hayakawa, Interendothelial junctional changes underlie the developmental 'tightening' of the blood-brain barrier, Brain Res. 429 (1987) 271–281.
[65] C. Schulze, J.A. Firth, Interendothelial junctions during blood-brain barrier development in the rat: morphological changes at the level of individual tight junctional contacts, Brain Res. Dev. Brain Res. 69 (1992) 85–95.
[66] K. Kawamura, J. Kumagai, S. Sudo, S.Y. Chun, M. Pisarska, H. Morita, J. Toppari, P. Fu, J.D. Wade, R.A. Bathgate, A.J. Hsueh, Paracrine regulation of mammalian oocyte maturation and male germ cell survival, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 7323–7328.
[67] C.S. Atwood, S.V. Meethal, T. Liu, A.C. Wilson, M. Gallego, M.A. Smith, R.L. Bowen, Dysregulation of the hypothalamic–pituitary–gonadal axis with menopause and andropause promotes neurodegenerative senescence. J. Neuropathol. Exp. Neurol. 64 (2005) 93–103.
[68] Y.X. Zhang, H.T. Xu, F.J. Qi, E.H. Wang, [Expression of connexin 43 in lung cancer and its correlation with E-cadherin], Zhonghua BingLiXue ZaZhi 35 (2006) 339–343.
[69] T. Morita, Y. Mizutani, M. Sawada, A. Shimada, Immunohistochemical and ultrastructural findings related to the blood-brain barrier in the blood vessels of the cerebral white matter in aged dogs, J. Comp. Pathol. 133 (2005) 14–22.
[70] M.Y. Su, E. Head, W.M. Brooks, Z. Wang, B.A. Muggenburg, G.E. Adam, R. Sutherland, C.W. Cotman, O. Nalcioglu, Magnetic resonance imaging of anatomic and vascular characteristics in a canine model of human aging, Neurobiol. Aging 19 (1998) 479–485.
[71] G.N. Shah, A.D. Mooradian, Age-related changes in the blood-brain barrier, Exp. Gerontol. 32 (1997) 501–519.
[72] M. Ujiie, D.L. Dickstein, D.A. Carlow, W.A. Jefferies, Blood-brain barrier permeability precedes senile plaque formation in an Alzheimer disease model, Microcirculation 10 (2003) 463–470.
[73] R.N. Kalaria, Cerebrovascular degeneration is related to amyloid-beta protein deposition in Alzheimer's disease, Ann. N. Y. Acad. Sci. 826 (1997) 263–271.