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This article was originally published as: Barron, A. , Cake, M., Verdile, G. , & Martins, R. N. (2009). Ovariectomy and 17 beta-Estradiol Replacement Do Not Alter beta-Amyloid Levels in Sheep Brain. Endocrinology, 150(7), 3228-3236. Original article available here
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Ovariectomy and 17β-Estradiol Replacement Do Not Alter β-Amyloid Levels in Sheep Brain

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The benefits of estrogen replacement as a preventative treatment for Alzheimer's disease (AD) are subject to debate. Because the effects of estrogen depletion and replacement on accumulation of the neurotoxic β-amyloid (Aβ) peptide in transgenic animal models of AD have been variable, we examined Aβ levels and oxidative stress in a nontransgenic animal model. Sheep have traditionally been used as a model for human reproduction; however because they share 100% sequence homology with the human form of Aβ, they may also have potential as a nontransgenic model for Aβ biology. The effect of ovariectomy and estrogen replacement administered for 6 months via slow-release implant was examined in the brain of 4.5-yr-old sheep. Aβ levels were measured by ELISA, and protein levels of the amyloid precursor protein (APP), APP C-terminal fragments (C100), and presenilin-1 were examined semiquantitatively by Western blot as markers of APP processing. Markers of oxidative stress were examined semiquantitatively by Western blot [4-hydroxy-2(E)-nonenal] and oxyblot (protein carbonyls). We found no effects of estrogen depletion and supplementation in terms of AD-related biochemical markers, including Aβ levels, APP processing, and oxidative stress levels. Evidence of a trend toward increased P450 side-chain cleavage enzyme levels in the hippocampus of ovariectomized and estrogen supplemented sheep suggests that neurosteroidogenesis may compensate for gonadal estrogen depletion; however, these findings cannot explain the lack of effect of estrogen supplementation on APP processing. It is possible that supraphysiological doses of estrogen are necessary to yield antiamyloidogenic and antioxidative benefits in ovariectomized sheep. **(*Endocrinology* 150: 3228–3236, 2009)**

The rapid hormonal changes occurring at menopause, including estrogen depletion and elevated LH levels, are thought to play a role in the increased susceptibility to Alzheimer's disease (AD) in women (1, 2). These hormonal changes are believed to promote the accumulation of the neurotoxic β-amyloid (Aβ) peptide and increase susceptibility to Aβ-induced neurotoxicity (3–6 and as reviewed in Ref. 7). Aβ is a proteolytic product of the amyloid precursor protein (APP) and is thought to have a critical role in the pathogenic events that lead to neuronal loss and dysfunction in AD (reviewed in Ref. 8). A small percentage of all AD is termed early onset and is linked with the autosomal dominant inheritance of mutations in three genes, APP, PS-1, and PS-2. The vast majority of all AD cases are late onset and idiopathic, although a number of risk factors have been identified.

Because rodent Aβ differs from the human form, many animal models for AD involve transgenic animals that express one or more mutations in genes associated with AD. However, the effect of these mutations may mask the potential subtle effects of other factors, such as hormonal changes, which may play a role in AD pathogenesis. Investigation of the effect of estrogen depletion and subsequent estrogen supplementation in animals that share Aβ-sequence homology with humans may provide insight into the role of estrogen in the regulation of Aβ accumulation in sporadic AD cases.

The benefits of hormone therapy (HT) as a potential preventative strategy for AD remain unclear due to the variable outcomes of human clinical studies (reviewed in Ref. 9). Whereas many small studies have reported improved cognition and re-

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.
Copyright © 2009 by The Endocrine Society
doi: 10.1210/en.2008-1252 Received August 27, 2008. Accepted March 3, 2009.
First Published Online March 12, 2009

Abbreviations: Aβ, β-Amyloid; AD, Alzheimer's disease; APOE, apolipoprotein; APP, amyloid precursor protein; C100, APP C-terminal fragment; 2-DE, two-dimensional electrophoresis; ERT, estrogen replacement therapy; FL, full-length; HNE, 4-hydroxy-2(E)-nonenal; HT, hormone therapy; NTF, N-terminal fragment; OVX, ovariectomized; PS1, presenilin 1; P450scc, P450 side-chain cleavage enzyme.

duced prevalence of AD among HT users (10–13), the large Women's Health Initiative Memory Study found detrimental effects of HT on both cognition and risk of AD (14, 15). There are a number of factors that need to be considered in the latter studies that may help explain these discrepancies. These include the age at initiation of hormone replacement therapy, source of hormone (equine estrogens and progesterone compared with synthetic human forms of these hormones), and mode of delivery (cyclic, continuous). Animal models in which potentially confounding factors including type, dose, and mode of estrogen delivery can be rigorously controlled and tested are invaluable in determining the potential preventative benefits of HT for AD. However, even in transgenic animal models for AD, the effects of estrogen depletion and supplementation on brain Aβ accumulation is unclear due to discrepancies between studies. Whereas some studies have reported no effect of ovariectomy or estrogen supplementation on Aβ accumulation (3, 16–18), others have found ovariectomy to significantly elevate Aβ levels, which can be attenuated with estrogen supplementation (5, 19, 20).

To date, the effect of ovariectomy and subsequent estrogen replacement on Aβ has been examined in only one animal model that shares Aβ sequence homology with human Aβ. Petanceska *et al.* (21) examined the effect of ovariectomy in young guinea pigs, finding significantly elevated Aβ levels in ovariectomized guinea pigs, which could be partially attenuated by short-term treatment with 17β-estradiol (8 d). In the current study, we aimed to build on these findings, examining the effect of ovariectomy and estrogen replacement therapy (ERT) in sheep, another animal that shares Aβ homology.

Sheep have proven very useful as a model for human reproduction and metabolism due to similarities in estrus periodicity, breeding, and metabolic rate. The sheep estrus cycle lasts approximately 17 d, more closely approximating the length of the human menstrual cycle than rodents, which have an estrus cycle lasting approximately 4 d. Furthermore, sheep, like humans, are generally limited to one or two offspring, unlike other mammals such as rodents or dogs that are prolific breeders. As such, sheep are thought to better reflect hormonal regulatory mechanisms of ovulation than prolific breeders. Ovariectomy of sheep has been a very successful model for the study of menopausal symptoms and conditions including hot flushes (22), heart disease (23), bone density loss (24), and arthritic changes (25).

Sheep have also been extensively used in the field of neuropathology including the neurodegenerative prion disease scrapie (reviewed in Ref. 26) and as a model for the effect of traumatic brain injury on APP expression (27). Sheep are one of the few animals that develop neurofibrillary tangles during normal aging closely resembling that seen in the AD brain (28–31), thus making them useful for the study of tau neuropathology. Additionally, there is a high homology between sheep and human forms of apolipoprotein (APOE) (32), unlike rodent APOE. In humans, polymorphisms of APOE are recognized as an important risk factor for the development of AD (33). Whereas no similar polymorphisms have been found in sheep APOE (32), the high homology between sheep and human APOE makes research easier to translate between the species.

Here sheep have been used as a nontransgenic, human-like model of AD to investigate the effects of ovariectomy and subsequent estrogen replacement on APP processing, Aβ accumulation, and Aβ-related neurotoxicity (oxidative stress).

Materials and Methods

Animals and surgical procedures

Merino ewes were obtained from a single source and selected for uniformity of size, conformation, body condition, and absence of lameness. At 4 yr of age, 30 sheep were divided among three groups: nonoperated controls (control; n = 12), ovariectomized (OVX; n = 12), and ERT (n = 12).

Sheep were ovariectomized via a small midline laparotomy incision under general anesthesia, induced by iv diazepam/ketamine and maintained by inhaled halothane (2%). ERT was administered via 3- × 1-cm SILASTIC brand tubing (Dow Corning Corp., Midland, MI) 17ß-estradiol (Sigma, St. Louis, MO) implants placed sc on the lateral thorax at time of ovariectomy according to previously described methods (34). Venous blood was collected while animals were under anesthesia.

After a brief recovery period, sheep were maintained on irrigated pasture without supplementary feeding. Blood was collected again 1 and 3 months after surgery, with final blood collection occurring at the time the animals were killed.

Animals were killed by intracardiac infusion of saturated KCl while under ketamine/diazepam general anesthesia at 4.5 yr of age after a 6-month treatment period. All animal procedures were approved by the Murdoch University Animal Ethics Committee.

Tissue collection and preparation

Venous blood samples were collected while under general anesthesia before the animals were killed, and plasma and serum were collected and stored at −80 C for later analysis. After the animals were killed, animals were decapitated and the brain was removed and bisected. The hippocampus and frontal cortex from the left hemisphere were snap frozen by submersion in liquid nitrogen and stored at −80 C. Tissue was homogenized in 3 ml/g 20 mM Tris buffer (pH 7.4) containing protease inhibitor cocktail (Roche, Basel, Switzerland). Aliquots of total homogenate were stored at −80 C for Western blot analysis. For two-dimensional oxyblot analysis, total homogenate was centrifuged at 14,000 rpm for 20 min at 4 C, and the supernatant was aliquoted and stored at −80 C for further processing. Protein concentration of total homogenate and supernatant for oxyblot analysis were determined by micro-BCA protein assay kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

Estrogen, progesterone, and LH assays

Plasma estradiol levels were assessed in a solvent extraction RIA. Samples were assayed blind, with two replicates of each sample extracted by adding ethyl acetate-hexane (3:2) and then drying the solvent layer under nitrogen. Estradiol (Sigma) was dissolved in ethanol and used to prepare standards ranging from 100 pg/ml to 0.1 pg/ml, which were dried under N2. Samples and standards were reconstituted in 0.1 M PBS containing 0.1% gelatin. Samples were incubated with the tracer (10,000 dpm of 1,2,6,7-[^{3}H] estradiol-17β; Amersham Biosciences, Buckinghamshire, UK) and the antiestradiol antibody (ICN Pharmaceuticals Inc., Aurora, OH) at 4 C for 24 h. After incubation, cold 0.05% dextran T70-coated charcoal (0.5%) was added to all tubes except those used to determine total counts. The tubes were incubated for 20 min and then centrifuged at 4 C at 2000 × *g* for 20 min. The supernatant was aspirated into counting vials with scintillation fluid (Starscint; Packard, Groningen, Netherlands) and counted in a liquid scintillation counter. The cross-reactions of the antibody were estradiol-17β (100%), estradiol (2.46%), estradiol-17α (1.32%), estrone (1.32%), estrone-sulfate

(0.21%), and ethinyl estradiol (0.11%). Mean percentage recovery for extraction for the assay was 91% and the sensitivity of the assay was 0.1 pg/tube. All samples were processed in a single assay with an intraassay coefficient of variation of 4.8%.

Plasma progesterone was measured using a double-antibody RIA as previously described by Gales *et al.* (35). Each assay contained six replicates each of three quality control samples containing 3.4, 1.6, and 0.8 ng/ml, which were used to estimate the intraassay coefficients of variation (5.1, 4.6, and 6%). The limit of detection was 0.1 ng/ml. Samples that had progesterone values below the limit of detection were assigned a value of zero. The averages of groups were calculated including samples assigned nil progesterone value.

Plasma LH was measured by a double-antibody RIA, which is described and validated by Martin *et al.* (36) with further modifications described by Martin *et al.* (37). Samples were assayed blind in duplicate and the limit of detection was 0.06 ng/ml. Each assay had a standard curve that included four total counts, four tubes for nonspecific binding, 11 replicates of B0, three replicates of each standard, and six replicates each of three quality control samples containing 2.2, 1.1, and 0.51 ng/ml, which were used to estimate the intraassay coefficients of variation (4.9, 6, and 6%).

Western immunoblotting

Full-length (FL)-APP, APP C-terminal fragment (C100), and presenilin 1 (PS1) N-terminal fragment (NTF) were examined as markers of APP processing. Human specific APP monoclonal antibody C1/1.6 directed against the last 20 residues of APP was used to probe for both FL-APP and C100 (donated by Professor Paul Matthews, Nathan Kline Institute, Orangeburg, NY). FL-APP and C100 blots were also probed with mouse monoclonal antibody WO2 directed against residues 5–8 of the human Aβ domain for comparison of immunoreactive bands detected by the two different antibodies.

Rabbit polyclonal PS1 antibody 14 was used to probe for PS1-NTF (donated by Professor Sam Gandy, Mt. Sinai School of Medicine, New York, NY), which is directed against a synthetic peptide akin to the first 25 amino acids of human PS1.

Hydroxy-2(E)-nonenal (HNE) is one of the most reliable markers of lipid peroxidation (38) and therefore was used as an indicator of oxidative stress; blots were probed with a commercially available goat antihuman polyclonal 4-HNE antibody (Chemicon, CA).

P450 side-chain cleavage enzyme (P450scc) is a steroidogenic enzyme catalyzing the conversion of cholesterol to pregnenolone, which is the precursor for all neurosteroids. The levels of this enzyme were semiquantitatively determined using Western immunoblotting with monoclonal P450scc antibody (Millipore, Temecula, CA).

Western immunoblotting was performed as described previously (39). Gels included a human familial AD sample for direct comparison with sheep proteins. A rodent testes and adrenal sample was included used as a positive control for P450scc. All blots were performed in duplicate. Films were scanned using Bio-Rad GS800 densitometer and quantified with Quantity One (Bio-Rad, Hercules, CA) software. All data are normalized to the control group.

Aβ sandwich ELISA

For Aβ ELISA, 300 μl of total homogenate were further processed according to the protocol of Schmidt *et al.* (40) and aliquots were stored at −80 C until ready for Aβ ELISA.

Aβ40 and Aβ42 levels were quantified by double-sandwich ELISA as previously described (41, 42) in plasma and brain homogenate from the hippocampus and frontal cortex. ELISA plates were coated with the capture antibody 6E10, and rabbit antiserum R208 (specific for Aβ40) or R226 (specific for Aβ42) was used as detection antibodies (provided by Professor Punkaj Mehta, New York Institute for Basic Research in Developmental Disabilities, New York, NY). The OD was measured at 450 nm using a FluoSTAR Optima microplate reader (BMG Labtech, Offenburg, Germany). Samples were run in duplicate and assays were carried out at least twice.

Two-dimensional electrophoresis oxyblot

Two-dimensional electrophoresis (2-DE) was combined with oxyblot protein oxidation detection kit (Chemicon Australia Pty. Ltd., Boronia, Victoria, Australia) to quantitatively and qualitatively compare protein oxidation in the hippocampus between treatment groups according to methods described previously (43). Two-dimensional blots were scanned using Bio-Rad GS800 densitometer and spot intensity and numbers were quantified using PDQuest (version 7.3; Bio-Rad) and Quantity One (Bio-Rad) software.

Statistical analysis

Parametric data were analyzed by one-way ANOVA in conjunction with least significant differences *post hoc* tests. The Mann-Whitney nonparametric test was used for data that did not fulfill ANOVA assumptions, as in the case of LH and progesterone levels. Correlations in nonparametric data were assessed with Spearman's Rho correlation, whereas correlations in parametric data were assessed using Pearson's correlation. Due to the repeated sampling of estrogen and progesterone, these data were examined using a linear mixed model with least significant differences *post hoc* tests.

Data were analyzed using the Statistical Package for Social Sciences (SPSS; version 11.5; SPSS Inc., Chicago, IL). All data are presented as mean ± SEM.

Results

Validating the experimental groups

Estrogen and progesterone levels were measured at time of surgery, 1 month, 3 months, and 6 months after surgery (Fig. 1). Significant differences were found in both estrogen [$F(3, 22) = 14.676$, $P < 0.001$] and progesterone [$F(3, 22) = 17.437$, $P < 0.001$] levels between treatment groups. The OVX group had significantly lower estrogen levels compared with both the control and ERT groups ($P < 0.001$). Estrogen levels were not significantly different between the control and ERT groups. Progesterone levels were significantly elevated in the control group compared with both the OVX and ERT groups ($P < 0.001$). There was no significant difference between progesterone levels in the OVX and ERT groups.

At the time the animals were killed, LH levels were significantly elevated in the OVX group (2.52 ± 0.25 ng/ml) compared with both the control group (0.45 ± 0.10 ng/ml; $P < 0.001$) and ERT group (0.69 ± 0.25 ng/ml; $P < 0.001$). As expected, a strong

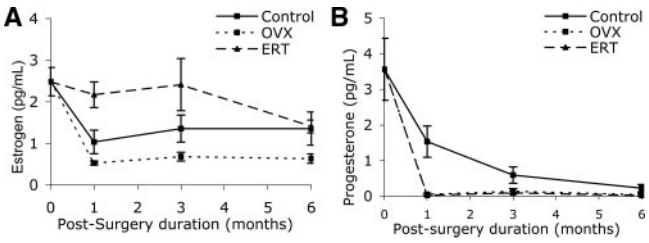

FIG. 1. Estrogen (A) and progesterone (B) hormone profiles across treatment duration in control (n = 6), OVX (n = 6), and ERT (n = 6) sheep. A gradual, nonsignificant decline in estrogen levels was observed in control animals in the last 3 months of treatment after surgery. An immediate and sustained depletion of estrogen was observed in OVX animals. Estrogen levels were significantly higher in ERT compared with OVX animals throughout treatment, although the levels were initially nonsignificantly lower than control animals (1–3 months after surgery). Progesterone levels were depleted to negligible levels in both OVX and ERT groups. Progesterone levels declined to negligible levels in the control group over the course of the 6-month study.

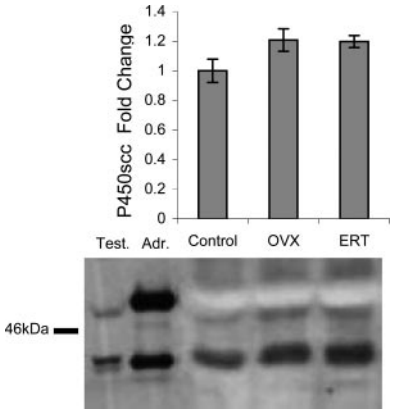

FIG. 2. P450scc expression in the hippocampus as assessed by Western blot in control (n = 12), OVX (n = 12), and ERT (n = 6) sheep. A trend toward elevated levels of the steroidogenic enzyme, p450scc, approached significance in the hippocampus of OVX and ERT sheep ($P = 0.080$). Representative blot shows rodent testes (Test.) and rodent adrenal (Adr.) positive controls for direct comparison (control: n = 12; OVX: n = 12; ERT: n = 6).

negative correlation was found between LH and estrogen levels at the time the animals were killed (Spearman's Rho = −0.735, $P = 0.001$). There was no significant correlation between progesterone and either estrogen or LH.

Effect of ovariectomy and estrogen supplementation on hippocampal levels of the steroidogenic enzyme P450scc

P450scc was semiquantitatively assessed by Western blot as a marker of neurosteroidogenic capability. In the sheep hippocampus, one main band was detected that corresponded in size to P450scc species observed in the control rodent testes and adrenal tissue (~42 kDa). A larger band at about 49 kDa was also observed in the rodent tissue but was not present in the sheep tissue. The 49-kDa species is detected by antibodies that recognize the carboxy-domain of P450scc, and this region is not thought to be conserved between the species (44). The 42-kDa species was quantified and a trend toward increase levels of P450scc in the hippocampus of OVX and ERT groups approached significance ($F = 3.007$, $P = 0.080$; Fig. 2).

Estrogen depletion or supplementation did not alter APP expression or metabolism in sheep hippocampus or frontal cortex

To determine whether estrogen status had any effect on APP expression or processing; FL-APP, APP-C100, and Aβ40 and Aβ42 were assessed in plasma, hippocampus, and frontal cortex. The levels of the NTF-PS1, thought to be a catalytic component of the γ-secretase enzyme that generates Aβ, were also measured.

After immunoblotting for APP, two immunoreactive bands were detected that directly corresponded in size to mature (~110 kDa) and immature (~100 kDa) FL-APP observed in the human AD control tissue (Fig. 3A) (45). The levels of FL-APP were unchanged between the treatment groups in both the hippocampus and frontal cortex, suggesting equal quantities of substrate for Aβ formation among the groups. Two C100 immunoreactive bands were also detected in sheep samples corresponding in size to the β-APP cleaving enzyme cut C-99 fragment and the α-secretase cut C-83 fragment observed in the human control tissue (Fig. 3B); these were quantified together as the C100 fragments (46). Again no significant differences in levels of C100 were observed between the groups in either the hippocampus or frontal cortex, indicating that BACE/α-secretase processing of APP processing was not altered.

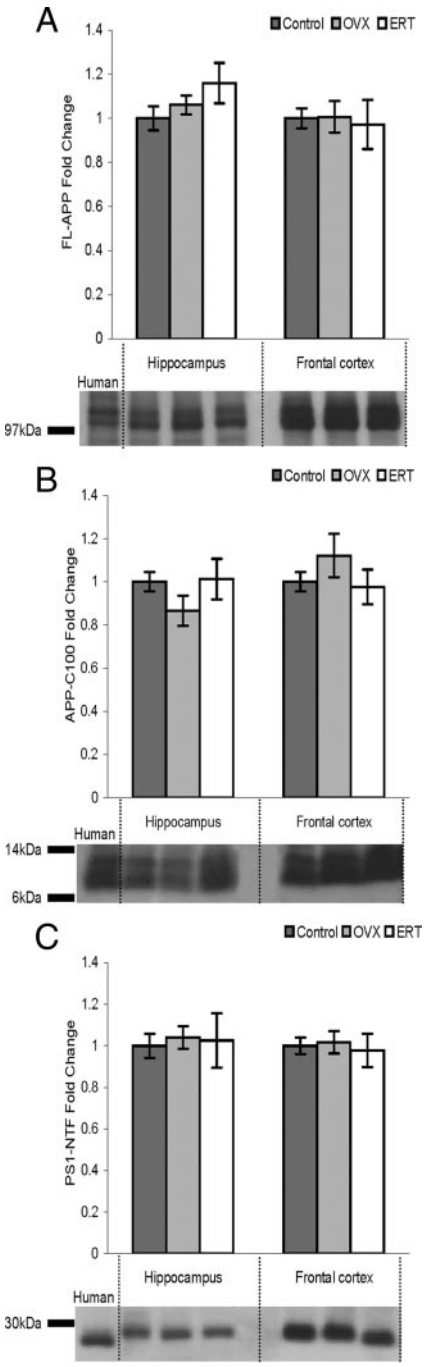

FIG. 3. No effect of OVX or subsequent ERT on FL-APP (A), C100 (B), or PS1-NTF (C) protein expression in the hippocampus and frontal cortex. FL-APP, C100, and PS1-NTF levels were measured by Western blot. Data presented as mean-fold change ± SEM. Fold change is normalized relative to the control group for each brain region. Representative blots are shown below for respective treatment groups (two way ANOVA; control: n = 12; OVX: n = 12; ERT: n = 6).

Although the levels of the PS1 N-terminal fragment were not altered between the treatment groups, it was interesting to note that compared with human PS1, sheep PS1 migrated to

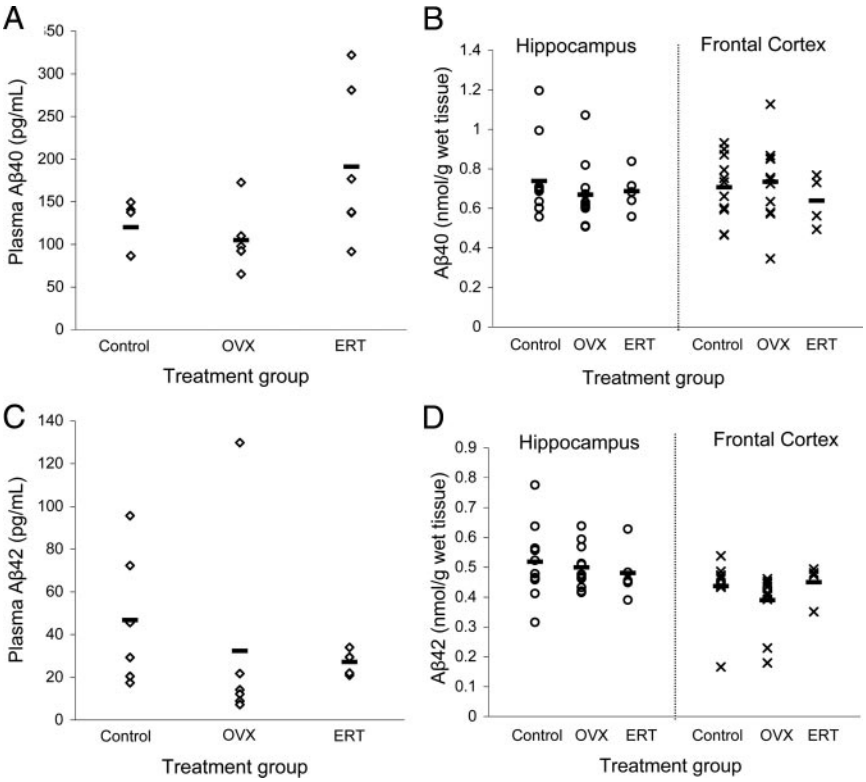

FIG. 4. No effect of ovariectomy or subsequent estrogen replacement on Aβ levels in the sheep plasma, hippocampus, or frontal cortex. Plasma levels of Aβ40 (A) and Aβ42 (C) were compared between control (n = 6), OVX (n = 6), and ERT (n = 6) groups. Hippocampal and frontal cortex levels of Aβ40 (B) and Aβ42 (D) were also compared between control (n = 12), OVX (n = 12), and ERT (n = 6) groups. No effect of treatment was observed on Aβ levels in the plasma, hippocampus, or frontal cortex.

a slightly larger size (Fig. 3C). Using two antibodies against different epitopes on PS1, a band corresponding to roughly 27 kDa in size was observed in the human tissue, whereas a NTF-PS1-like band (estimate of ~29 kDa) was observed in the sheep brain tissue.

Aβ40 and Aβ42 levels were examined in plasma, hippocampus, and frontal cortex. No significant differences in Aβ levels (Fig. 4) or the Aβ40:42 ratio (data not shown) were found among treatment groups in the plasma or either brain region. Furthermore, no significant correlations were found between Aβ40 or Aβ42 and estrogen, progesterone, or LH levels in the plasma or either of the brain regions.

No effect of estrogen status on levels of oxidative stress markers in sheep hippocampus

Hippocampal levels of oxidative stress were assessed 2-DE oxyblot and 4-HNE levels. The oxyblot kit was used in conjunction with 2-DE analysis to generate an oxidative proteome (Fig. 5, A–C). To determine whether there was up- or down-regulation of oxidative modification of specific proteins, spot numbers were compared between the treatment groups, with no significant effect of treatment found (Fig. 5D). 4-HNE adducts were measured by Western blot as a marker of lipid peroxidation; the adducts were observed as a smear on SDS-PAGE, and again no significant differences were detected between the treatment groups (Fig. 5E).

Discussion

This is the first study to examine APP processing and Aβ levels in a sheep model of menopause. No significant effect of ovariectomy or subsequent estrogen replacement on APP processing, Aβ levels, or oxidative stress was observed in the sheep model of menopause. Nor was any association between estrogen, progesterone, or LH levels and Aβ levels observed. These findings were surprising, given ovariectomy has been demonstrated to increase Aβ levels in guinea pigs (4) and transgenic mouse models of AD (5, 18, 19). However, there are many other studies in transgenic mouse models of AD that have also reported no effect of ovariectomy on Aβ levels (3, 16, 17). The reason for these discrepancies is unclear, and evidence of a trend toward increased hippocampal levels of the steroidogenic enzyme, P450scc, in the ovariectomized sheep leads to speculation that ovariectomy in otherwise healthy animals may be a poor model of brain estrogen deficiency due to local estrogen production via neurosteroidogenesis. Yet this would still not explain the lack of effect of estrogen supplementation observed on APP processing.

Validation of the sheep model of menopause (*i.e.* ovariectomy) for the study of AD

The effects of ovariectomy were studied between April and October, partly spanning the period of transition to seasonal anestrus characterized by lower ovarian activity. The gradual decline in estrogen levels observed in the control sheep over the last 3 months of the study coupled with a marked decline in progesterone levels over the course of the study most likely reflects this transition into anestrus.

Despite the decline in gonadal hormone levels in the control sheep, it is not believed that seasonality can account for the lack of effect of hormone manipulations in this study because estrogen levels remained significantly elevated in the control compared with ovariectomized sheep. These mild seasonal changes are typical of the Merino breed of sheep, which tend to gradually transition into a short period of anestrus in October to December lasting through to February. Furthermore, anestrus is typified by increased brain responsiveness to estrogen (reviewed in Ref. 47), with enhanced sensitivity to estrogen on negative feedback mechanisms in the hypothalamus (47). These changes in brain sensitivity to estrogen are specific to hypothalamic-pituitary-gonadal feedback systems because other feedback systems have been demonstrated to be unaffected by seasonality (47). The possibility that season could modulate the effects of ovariectomy on Aβ levels could be systematically addressed in future studies us-

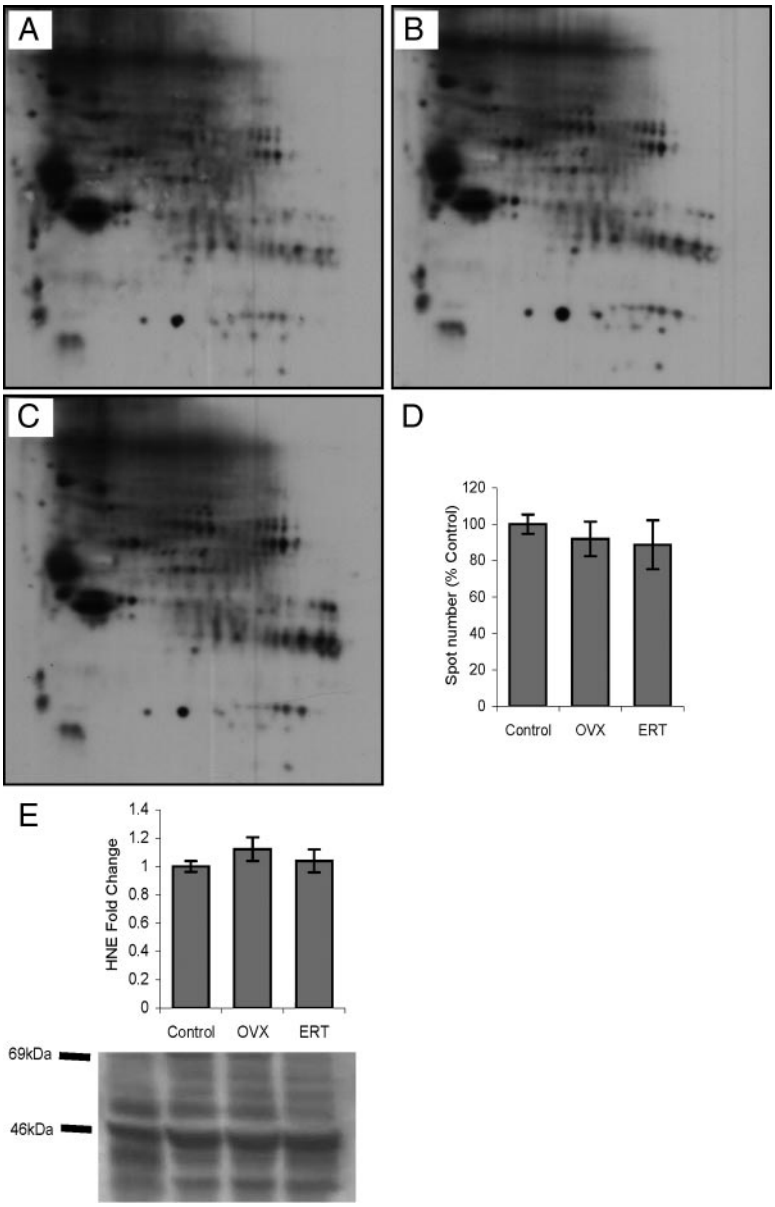

FIG. 5. Estrogen depletion and supplementation did not alter expression of markers of oxidative stress in sheep hippocampus. Representative oxyblots from the control (A), ovariectomized (B), and estrogen replacement (C) groups are shown. Spot numbers were quantified to compare the number of oxidatively modified proteins in each treatment group (D); no significant differences were found between the treatment groups. 4-HNE levels were also found to be unaltered by either OVX or ERT (E).

ing a breed of sheep that exhibits the traditional marked seasonal hormone variation.

The success of ovariectomy was confirmed, with depleted serum estrogen coupled with elevated LH levels, thus mimicking hormonal changes observed at menopause. The ERT dosage was sufficient to restore physiological serum concentrations of estrogen and LH, although, as expected, progesterone levels remained significantly depleted in the estrogen supplemented group.

It should also be noted that physiological levels of estrogen and progesterone are much lower (almost 10-fold) in sheep compared with humans; however, this is tempered by the absence of steroid binding globulin and transcortin, resulting in equivalent bioavailable gonadal hormone levels. Therefore, total hormone levels assessed in the current study are an excellent indicator of bioavailable hormone concentrations, as opposed to rodent models in which measurement of SHBG is necessary to accurately assess bioavailable hormone levels. In fact, it is suggested that free estrogen levels may be a better indicator of AD risk than total hormone levels (48–50) Nevertheless, although total levels of the gonadal hormones are severalfold lower in sheep, ovariectomized sheep have been successfully used as a model for the investigation of multiple menopausal conditions including hot flashes (22), heart disease (23), bone density loss (24), and arthritic changes (25).

Ovariectomy does not necessarily result in central nervous system estrogen depletion

Another factor to be considered when examining the effects of ovariectomy on Aβ levels is whether the hormone profile we observed in the serum reflects hormone status in the central nervous system. Evidence suggests that in some experimental models, ovariectomy is an inadequate model of brain estrogen deficiency, with *de novo* estrogen synthesis compensating for depletion of gonadal estrogen ablation (51, 52). High estrogen activity has been demonstrated in the brain of ovariectomized mice relative to other body regions using the estrogen-responsive element-luciferase mouse model, which has been engineered to express the nonmammalian luciferase protein in response to classical estrogen receptor activation (53).

The importance of brain estrogen levels has been highlighted by Yue *et al.* (54), who found decreased estrogen and aromatase levels in AD compared with control brains, whereas no differences were found in serum estrogen levels (54). Yue *et al.* hypothesized this estrogen deficiency reflected impaired estrogen production from precursor androgens by the enzyme aromatase in the brain, finding that ovariectomy alone did not alter plaque formation and was not sufficient to deplete brain estrogen levels in APP mice. In contrast, ovariectomy in aromatase knockout mice cross-bred with APP mice exhibited accelerated plaque formation.

Whereas brain estrogen levels were not directly determined in this study, levels of the steroidogenic enzyme, P450scc, were assessed in the hippocampus. P450scc catalyzes the conversion of cholesterol to pregnenolone, which is the rate-limiting step in neurosteroid production (reviewed 55). A trend toward increased levels of P450scc approached significance in the hippocampus of ovariectomized and estrogen supplemented groups compared with control sheep. This may reflect some degree of compensatory increases in neurosteroidogenesis in gonadal estrogen-depleted sheep. Further studies should comprehensively address the relationship between gonadal and brain estrogen levels.

Even though it is possible that the compensatory effects of neurosteroidogenesis may account for the lack of effect of ovariectomy on Aβ accumulation observed, several lines of evidence argue against a role for estrogen in the modulation of APP processing and Aβ accumulation in this study. First, plasma Aβ levels were also unaffected by ovariectomy or estrogen supplementation, and no relationship was observed between plasma Aβ and hormone levels, thereby confirming the observations in the brain (although it should be considered that the relationship between peripheral and central Aβ levels is not completely understood). Second, whereas the status of brain estrogen levels remains contentious in the ovariectomized sheep, exogenous administration of estrogen circumvents the issues of the role of neurosteroidogenesis, with no effect of estrogen supplementation observed on APP processing, Aβ accumulation, or oxidative stress.

Estrogen replacement therapy and Aβ metabolism

We did not observe any change in Aβ or oxidative stress levels after 6 months of 17β-estradiol treatment in OVX sheep. Other studies similarly reported no effect of estrogen supplementation on Aβ levels in OVX APP mice (17, 18) and APP/PS1 mice (16). In contrast, there are many studies that have reported significantly decreased Aβ levels after 17β-estradiol treatment in animals including guinea pigs (4), APP/PS1/Tau triple transgenic mice (20), APP/PS1 (5), and APP mice (3, 5). Methodological differences in the animal model, age of animals, duration of treatment, mode of estrogen delivery, type of estrogen, and estrogen dosage may help explain inconsistencies in the reported effect of estrogen replacement on Aβ levels.

The majority of *in vivo* studies reporting positive effects of exogenous estrogen on Aβ levels were administered high doses (3–5), whereas many of the studies administering lower estrogen doses reported no effect on Aβ (16–18). It is therefore possible that we may have observed an effect if estrogen had been administered at supraphysiological doses to the sheep [although a recent study administering physiological doses of estrogen to APP/PS1/Tau triple transgenic mice reported significant reductions in Aβ in this model (20)].

Future studies should address the effect of estrogen dosage on Aβ and oxidative stress in the sheep model of menopause. If the benefits of estrogen cannot be yielded at physiological concentrations, and then the costs of estrogen treatment may outweigh the benefits due to increased risk of breast cancer, pulmonary embolism, and stroke (56). Neuroactive selective estrogen receptor modulators that do not activate peripheral receptors and therefore can be safely used at high doses may be a potential alternative to estrogen for therapeutic intervention in AD.

APP processing in sheep

We used well-characterized APP and Aβ antibodies to demonstrate the presence of the main components of the APP processing pathways in sheep tissue.

It is interesting to note that unlike human and nonhuman primates, sheep (31) do not form Aβ plaques during normal aging, despite sharing a homologous form of Aβ. It has been hypothesized that sheep are efficient at clearing Aβ, supported by the observation that cerebral injections of Aβ were rapidly cleared from injection sites in the cerebral cortex of sheep (57). Comparative biological studies are necessary to verify this hypothesis; however, it could be speculated that the combination of ovariectomy with other epigenetic factors such as chronic stress or diet manipulations may be necessary to elicit effects on Aβ accumulation in sheep (58).

The current study has focused on the effect of estrogen depletion and supplementation on APP metabolism and oxidative stress. Whereas the AD research literature to date has focused primarily on the role of estrogen in the modulation of APP processing; estrogen may play a role in other important aspects of AD pathology including tau neuropathology, neuronal loss, neuroinflammation, and cognitive decline (reviewed in Ref. 59). Sheep may prove to be a powerful nontransgenic model for the study of AD because in addition to expressing a homologous form of Aβ, they form neurofibrillary tangles in normal aging. Furthermore, the recent advancement of a hippocampal-dependent memory test adds a novel dimension to this animal model for the study of AD (60).

One of the benefits offered by a large animal model such as sheep is that treatment delivery implantation devices designed for human use can be effectively tested in these animals. Here we administered 17β-estradiol via a commonly used slow release delivery device designed for use in sheep. In the clinical setting, slow-release implants avoid issues of compliance, which is particularly pertinent for dementia subjects. However, more complications including urinary retention, hydronephrosis, and increased mortality have been reported in animal models when estrogen is administered via slow release pellets as opposed to oral administration (61). Sheep may provide a good animal model for assessing the pharmacokinetics of estrogen replacement therapy and other potential AD treatments due to their large size and similar metabolic rate to humans, easing the translation of laboratory work to the clinical setting.

Conclusions

Sheep provide a useful model for the study of APP biology because they share a homologous form of Aβ with humans. Whereas transgenic mouse models have provided important advancements in our understanding of AD, sheep may help fill an important niche in AD research that helps to bridge the gap in understanding of the more common sporadic form of AD which is regulated by age-related factors and lifestyle. Here OVX sheep were used as a model of menopause to investigate the effects of estrogen depletion and supplementation on APP metabolism and oxidative stress. From the literature it is clear that under certain conditions estrogen can modulate Aβ levels; however, the circumstances necessary to elicit these effects and their relevance to the clinical setting remain unclear. We found no effects of ovariectomy and subsequent estrogen supplementation on APP metabolism or oxidative stress in sheep. Whereas neurosteroidogenesis may offer a potential explanation as to why no effect of ovariectomy was observed on APP processing or oxidative stress, the question remains as to why exogenous administration did not affect these parameters. It is possible that supraphysiological doses of estrogen are necessary to yield antiamyloidogenic and

antioxidative benefits; however, if this is the case, then any potential benefits will be overshadowed by serious, life-threatening side effects associated with these higher doses.

Acknowledgments

The authors thank Kevin Taddei and Georgia Martins (Edith Cowan University, Joondalup, Western Australia, Australia) for their assistance with the tissue collection; Karl DeRyuck (Edith Cowan University) for his assistance with the Aβ ELISAs; Linda Wijaya (Edith Cowan University) for her help with the assessment of P450scc; and Graeme Martin and Margaret Blackberry (University of Western Australia, Nedlands, Western Australia, Australia) for advice, supply of the hormone implants, and carrying out the LH and estradiol assays.

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This work was supported by the Sir James McCusker Foundation for Alzheimer's Disease Research. A.M.B. is funded through University of Western Australia Postgraduate Scholarship, GlaxoSmithKline Postgraduate Support Grant. R.N.M. is supported by grants from the Department of Veterans Affairs, National Health and Medical Research Council, and Hollywood Private Hospital. G.V. is generously supported by grants from Mr. Warren Milner (Milner English College, Perth, Western Australia, Australia) and Ms. Helen Sewell.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Zandi PP, Carlson MC, Plassman BL, Welsh-Bohmer KA, Mayer LS, Steffens DC, Breitner JCS** 2002 Hormone replacement therapy and incidence of Alzheimer disease in older women: the Cache County Study. JAMA 288:2123–2129
2. **Gao S, Hendrie HC, Hall KS, Hui S** 1998 The relationships between age, sex, and the incidence of dementia and Alzheimer disease: a meta-analysis. Arch Gen Psychiatry 55:809–815
3. **Levin-Allerhand JA, Lominska CE, Wang J, Smith JD** 2002 17α-Estradiol and 17β-estradiol treatments are effective in lowering cerebral amyloid-β levels in AβPPSWE transgenic mice. J Alzheimers Dis 4:449–457
4. **Petanceska SS, Nagy V, Frail D, Gandy S** 2000 Ovariectomy and 17β-estradiol modulate the levels of Alzheimer's amyloid β peptides in brain. Exp Gerontol 35:1317–1325
5. **Zheng H, Xu H, Uljon SN, Gross R, Hardy K, Gaynor J, Lafrancois J, Simpkins J, Refolo LM, Petanceska S, Wang R, Duff K** 2002 Modulation of Aβ peptides by estrogen in mouse models. J Neurochem 80:191–196
6. **Bowen RL, Verdile G, Liu T, Parlow AF, Perry G, Smith MA, Martins RN, Atwood CS** 2004 Luteinizing hormone, a reproductive regulator that modulates the processing of amyloid-β precursor protein and amyloid-β deposition. J Biol Chem 279:20539–20545
7. **Barron AM, Fuller SJ, Verdile G, Martins RN** 2006 Reproductive hormones modulate oxidative stress in Alzheimer's disease. Antioxid Redox Signal 8:2047–2059
8. **Verdile G, Fuller S, Atwood CS, Laws SM, Gandy SE, Martins RN** 2004 The role of β amyloid in Alzheimer's disease: still a cause of everything or the only one who got caught? Pharmacol Res 50:397–409
9. **Henderson VW** 2006 Estrogen-containing hormone therapy and Alzheimer's disease risk: understanding discrepant inferences from observational and experimental research. Neuroscience 138:1031–1039
10. **Henderson VW, Paganini-Hill A, Emanuel CK, Dunn ME, Buckwalter JG** 1994 Estrogen replacement therapy in older women. Comparisons between Alzheimer's disease cases and nondemented control subjects. Arch Neurol 51:896–900
11. **Kawas C, Resnick S, Morrison A, Brookmeyer R, Corrada M, Zonderman A, Bacal C, Lingle DD, Metter E** 1997 A prospective study of estrogen replacement therapy and the risk of developing Alzheimer's disease: the Baltimore Longitudinal Study of Aging. Neurology 48:1517–1521
12. **Tang MX, Jacobs D, Stern Y, Marder K, Schofield P, Gurland B, Andrews H, Mayeux R** 1996 Effect of oestrogen during menopause on risk and age at onset of Alzheimer's disease. Lancet 348:429–432
13. **Baldereschi M, Di Carlo A, Lepore V, Bracco L, Maggi S, Grigoletto F, Scarlato G, Amaducci L** 1998 Estrogen-replacement therapy and Alzheimer's disease in the Italian Longitudinal Study on Aging. Neurology 50:996–1002
14. **Shumaker SA, Legault C, Rapp SR, Thal L, Wallace RB, Ockene JK, Hendrix SL, Jones 3rd BN, Assaf AR, Jackson RD, Kotchen JM, Wassertheil-Smoller S, Wactawski-Wende J** 2003 Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women: the Women's Health Initiative Memory Study: a randomized controlled trial. JAMA 289:2651–2662
15. **Espeland MA, Rapp SR, Shumaker SA, Brunner R, Manson JE, Sherwin BB, Hsia J, Margolis KL, Hogan PE, Wallace R, Dailey M, Freeman R, Hays J** 2004 Conjugated equine estrogens and global cognitive function in postmenopausal women: Women's Health Initiative Memory Study. JAMA 291:2959–2968
16. **Heikkinen T, Kalesnykas G, Rissanen A, Tapiola T, Iivonen S, Wang J, Chaudhuri J, Tanila H, Miettinen R, Puoliväli J** 2004 Estrogen treatment improves spatial learning in APP+PS1 mice but does not affect beta amyloid accumulation and plaque formation. Exp Neurol 187:105–117
17. **Green PS, Bales K, Paul S, Bu G** 2005 Estrogen therapy fails to alter amyloid deposition in the PDAPP model of Alzheimer's disease. Endocrinology 146:2774–2781
18. **Golub MS, Germann SL, Mercer M, Gordon MN, Morgan DG, Mayer LP, Hoyer PB** 2008 Behavioral consequences of ovarian atrophy and estrogen replacement in the APPswe mouse. Neurobiol Aging 29:1512–1523
19. **Levin-Allerhand JA, Smith JD** 2002 Ovariectomy of young mutant amyloid precursor protein transgenic mice leads to increased mortality. J Mol Neurosci 19:163–166
20. **Carroll JC, Rosario ER, Chang L, Stanczyk FZ, Oddo S, LaFerla FM, Pike CJ** 2007 Progesterone and estrogen regulate Alzheimer-like neuropathology in female 3xTg-AD mice. J Neurosci 27:13357–13365
21. **Petanceska SS, Nagy V, Frail D, Gandy S** 2000 Ovariectomy and 17β-estradiol modulate the levels of Alzheimer's amyloid-β peptides in brain. Neurology 54:2212–2217
22. **MacLeay JM, Lehmer E, Enns RM, Mallinckrodt C, Bryant HU, Turner AS** 2003 Central and peripheral temperature changes in sheep following ovariectomy. Maturitas 46:231–238
23. **Gaynor JS, Monnet E, Selzman C, Parker D, Kaufman L, Bryant HU, Mallinckrodt C, Wrigley R, Whitehill T, Turner AS** 2000 The effect of raloxifene on coronary arteries in aged ovariectomized ewes. J Vet Pharmacol Ther 23:175–179
24. **Newton BI, Cooper RC, Gilbert JA, Johnson RB, Zardiackas LD** 2004 The ovariectomized sheep as a model for human bone loss. J Comp Pathol 130:323–326
25. **Cake MA, Appleyard RC, Read RA, Smith MM, Murrell GA, Ghosh P** 2005 Ovariectomy alters the structural and biomechanical properties of ovine femoro-tibial articular cartilage and increases cartilage iNOS. Osteoarthritis Cartilage 13:1066–1075
26. **Hunter N** 2007 Scrapie-Uncertainties, biology and molecular approaches. Biochim Biophys Acta 1772:619–628
27. **Anderson RW, Brown CJ, Blumbergs PC, McLean AJ, Jones NR** 2003 Impact mechanics and axonal injury in a sheep model. J Neurotrauma 20:961–974
28. **Nelson PT, Marton L, Saper CB** 1993 Alz-50 immunohistochemistry in the normal sheep striatum—a light and electron-microscope study. Brain Res 600:285–297
29. **Nelson PT, Saper CB** 1995 Ultrastructure of neurofibrillary tangles in the cerebral-cortex of sheep. Neurobiol Aging 16:315–323
30. **Braak H, Braak E, Strothjohann M** 1994 Abnormally phosphorylated tau-protein related to the formation of neurofibrillary tangles and neuropil threads in the cerebral-cortex of sheep and goat. Neurosci Lett 171:1–4
31. **Nelson PT, Greenberg SG, Saper CB** 1994 Neurofibrillary tangles in the cerebral-cortex of sheep. Neurosci Lett 170:187–190
32. **Komatsu Y, Horiuchi M, Ishiguro N, Matsui T, Shinagawa M** 1998 Characterization of the sheep apolipoprotein E (ApoE) gene and allelic variations of the ApoE gene in scrapie Suffolk sheep. Gene 208:131–138
33. **Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, Roses AD, Haines JL, Pericak-Vance MA** 1993 Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. Science 261:921–923
34. **Adams NR, Briegel JR, Sanders MR, Blackberry MA, Martin GB** 1997 Level of nutrition modulates the dynamics of oestradiol feedback on plasma FSH in ovariectomized ewes. Anim Reprod Sci 47:59–70

35. **Gales NJ, Williamson P, Higgins LV, Blackberry MA, James I** 1997 Evidence for a prolonged postimplantation period in the Australian sea lion (*Neophoca cinerea*). J Reprod Fertil 111:159–163
36. **Martin GB, Oldham CM, Lindsay DR** 1980 Increased plasma LH levels in seasonally anovular merino ewes following the introduction of rams. Anim Reprod Sci 3:125–132
37. **Martin GB, Tjondronegoro S, Blackberry MA** 1994 Effects of nutrition on testicular size and the concentrations of gonadotrophins, testosterone and inhibin in plasma of mature male sheep. J Reprod Fertil 101:121–128
38. **Esterbauer H, Schaur RJ, Zollner H** 1991 Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med 11:81–128
39. **Martins RN, Taddei K, Kendall C, Evin G, Bates KA, Harvey AR** 2001 Altered expression of apolipoprotein E, amyloid precursor protein and presenilin-1 is associated with chronic reactive gliosis in rat cortical tissue. Neuroscience 106:557–569
40. **Schmidt SD, Nixon RA, Mathews PM** 2005 ELISA method for measurement of amyloid-β levels. In: Sigurdsson EM, ed. Amyloid proteins: methods and protocols. Totowa, NJ: Humana Press Inc.; 279–297
41. **Mehta PD, Dalton AJ, Mehta SP, Kim KS, Sersen EA, Wisniewski HM** 1998 Increased plasma amyloid-β protein 1–42 levels in Down syndrome. Neurosci Lett 241:13–16
42. **Mehta PD, Pirttilä T, Mehta SP, Sersen EA, Aisen PS, Wisniewski HM** 2000 Plasma and cerebrospinal fluid levels of amyloid beta proteins1–40 and 1–42 in Alzheimer disease. Arch Neurol 57:100–105
43. **Bates KA, Martins RN, Harvey AR** 2007 Oxidative stress in a rat model of chronic gliosis. Neurobiol Aging 28:995–1008
44. **Roby KF, Larsen D, Deb S, Soares MJ** 1991 Generation and characterization of antipeptide antibodies to rat cytochrome P-450 side-chain cleavage enzyme. Mol Cell Endocrinol 79:13–20
45. **Verdile G, Fraser PE, St. George Hyslop P, Kwok JBJ, Schofield PR, Fisher C, Helmerhorst E, Martins RN** 1999 Decreased secretion of amyloid precursor protein in Chinese hamster ovary cells overexpressing presenilin1. Alzheimer Rep 2:231–239
46. **Verdile G, Groth D, Mathews PM, St George-Hyslop P, Fraser PE, Ramabhadran TV, Kwok JB, Schofield PR, Carter T, Gandy S, Martins RN** 2004 Baculoviruses expressing the human familial Alzheimer's disease presenilin 1 mutation lacking exon 9 increase levels of an amyloid beta-like protein in Sf9 cells. Mol Psychiatry 9:594–602
47. **Lehman MN, Goodman RL, Karsch FJ, Jackson GL, Berriman SJ, Jansen HT** 1997 The GnRH system of seasonal breeders: anatomy and plasticity. Brain Res Bull 44:445–457
48. **Muller M, Schupf N, Manly JJ, Mayeux R, Luchsinger JA,** Sex hormone binding globulin and incident Alzheimer's disease in elderly men and women. Neurobiol Aging, in press
49. **Schupf N, Winsten S, Patel B, Pang D, Ferin M, Zigman WB, Silverman W, Mayeux R** 2006 Bioavailable estradiol and age at onset of Alzheimer's disease in postmenopausal women with Down syndrome. Neurosci Lett 406:298–302
50. **Hoskin EK, Tang MX, Manly JJ, Mayeux R** 2004 Elevated sex-hormone binding globulin in elderly women with Alzheimer's disease. Neurobiol Aging 25:141–147
51. **Corpéchot C, Robel P, Axelson M, Sjövall J, Baulieu EE** 1981 Characterization and measurement of dehydroepiandrosterone sulfate in rat brain. Proc Natl Acad Sci USA 78:4704–4707
52. **Corpéchot C, Synguelakis M, Talha S, Axelson M, Sjövall J, Vihko R, Baulieu EE, Robel P** 1983 Pregnenolone and its sulfate ester in the rat brain. Brain Res 270:119–125
53. **Ciana P, Di Luccio G, Belcredito S, Pollio G, Vegeto E, Tatangelo L, Tiveron C, Maggi A** 2001 Engineering of a mouse for the *in vivo* profiling of estrogen receptor activity. Mol Endocrinol 15:1104–1113
54. **Yue X, Lu M, Lancaster T, Cao P, Honda S, Staufenbiel M, Harada N, Zhong Z, Shen Y, Li R** 2005 Brain estrogen deficiency accelerates Aβ plaque formation in an Alzheimer's disease animal model. Proc Natl Acad Sci USA 102: 19198–19203
55. **Mellon SH, Griffin LD, Compagnone NA** 2001 Biosynthesis and action of neurosteroids. Brain Res Rev 37:3–12
56. **Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson RD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockene J, Investigators WGftWsHI** 2002 Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. JAMA 288:321–333
57. **Nelson PT, Saper CB** 1996 Injections of okadaic acid, but not β-amyloid peptide, induce Alz-50 immunoreactive dystrophic neurites in the cerebral cortex of sheep. Neurosci Lett 208:77–80
58. **Fukuzaki E, Takuma K, Himeno Y, Yoshida S, Funatsu Y, Kitahara Y, Mizoguchi H, Ibi D, Koike K, Inoue M, Yamada K** 2008 Enhanced activity of hippocampal BACE1 in a mouse model of postmenopausal memory deficits. Neurosci Lett 433:141–145
59. **Maggi A, Ciana P, Belcredito S, Vegeto E** 2004 Estrogens in the nervous system: mechanisms and nonreproductive functions. Annu Rev Physiol 66: 291–313
60. **Lee C, Colegate S, Fisher AD** 2006 Development of a maze test and its application to assess spatial learning and memory in Merino sheep. Appl Anim Behav Sci 96:43–51
61. **Levin-Allerhand JA, Sokol K, Smith JD** 2003 Safe and effective method for chronic 17β-estradiol administration to mice. Contemp Top Lab Anim Sci 42:33–35