Estrogen Decreases Zinc Transporter 3 Expression and Synaptic Vesicle Zinc Levels in Mouse Brain*

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Previous studies suggest that female sex hormones modulate synaptic zinc levels, which may influence amyloid plaque formation and Alzheimer’s disease progression. We examined the effects of ovariectomy and estrogen supplement on the levels of synaptic zinc and zinc transporter protein Znt3 in the brain. Ovariectomy was performed on 5-month-old mice, and 2 weeks later, pellets containing vehicle, low (0.18 mg/pellet), or high dose (0.72 mg) 17β-estradiol were implanted. After 4 weeks, animals were decapitated, and blood and brain were collected for analysis. Blood analysis indicated that estrogen implants altered plasma estrogen levels in a dose-dependent manner. Analysis of brain tissue showed that ovariectomy raised hippocampal synaptic vesicle zinc levels, whereas estrogen replacement lowered these zinc levels. Western blots revealed that Znt3 levels in the brain were modulated in parallel with synaptic zinc levels, whereas no change was detected in the levels of Znt3 mRNA, as determined by Northern blot and reverse transcriptase-PCR analysis. However, mRNA levels of the α subunit of adaptor protein complex (AP)-3, which modulates the level of Znt3 levels, were altered by estrogen depletion or replacement. These data demonstrate that estrogen alters the levels of Znt3 and synaptic vesicle zinc in female mice, probably through changing AP-3 α expression. Since synaptic zinc may play a key role in neuronal death in acute brain injury as well as in plaque formation in Alzheimer’s disease, and since estrogen may be beneficial in both conditions, our results may provide new insights into the effects of estrogen on the brain.

The prevalence of Alzheimer’s disease (AD)1 is substantially higher in postmenopausal females than in males of the same age (1–3). Consistent with the idea that changes in sex hormone levels underlie this phenomenon, there are evidences that estrogen replacement therapy reduces the incidence and severity of AD in postmenopausal women (4–6). Although the sex dependence of AD pathogenesis is intriguing, how it arises and how estrogen might protect against AD is poorly understood.

Animal models such as the human Swedish mutant amyloid precursor protein transgenic (Tg2576) mouse (7) may be useful in investigations of AD gender difference since the plaque load in Tg2576 mice shows the same intersex disparity as in humans (8, 9). These findings suggest that AD gender correlations may derive from differences in the rate of amyloid plaque formation. Consistent with these observations, it appears estrogen may affect various steps in β-amyloid (Aβ) metabolism, such as synthesis (10, 11) and degradation (12, 13).

Recent evidence indicates that endogenous metal ions such as zinc, copper, and iron contribute to Aβ aggregation and plaque accumulation. Zinc and copper were shown to induce rapid aggregation of synthetic Aβ in an aqueous environment (14, 15), probably by binding to Aβ histidine residues (16). In addition, concentrations of transition metals including zinc and copper are elevated in brains of AD patients (17, 18), more so within plaques (19–21). Furthermore, treatment with metal chelators resulted in the dissolution of aggregated Aβ from AD brain extracts (22) and inhibited accumulation of amyloid plaques in Tg2576 mice (23).

Endogenous zinc in glutamatergic synaptic vesicles constitutes 10–30% of total brain zinc (24). This zinc appears to play a disproportionately large role in Aβ aggregation and plaque formation since removal of synaptic vesicle zinc by deleting the Znt3 gene (24) resulted in a 70–80% decrease in the levels of insoluble Aβ and congophilic plaques in Tg2576 mice (9). Furthermore, the level of synaptic zinc is greater in aged female than male Tg2576 mice (9). Since the gender difference in plaque burden in Tg2576 mice disappeared when synaptic vesicle zinc was removed, it appears that different synaptic zinc levels may explain differences between plaque formation and AD occurrence in males and females (9).

In the present study, we investigated whether manipulation of estrogen levels by ovariectomy, or ovariectomy plus estrogen replacement, would alter synaptic zinc levels in the mouse brain. In addition, we examined whether changes in estrogen levels altered Znt3 expression since Znt3 is the protein solely responsible for zinc transport into synaptic vesicles.

**EXPERIMENTAL PROCEDURES**

*Animals—Production and genotyping of Znt3-null mice was performed as described previously (9, 24). Wild-type (Znt3+/−) and null-mutant (Znt3−/−) mice were bred and maintained using a C57Bl6/129sv
Ovariectomy and Estrogen Replacement—Both ovaries of female mice at 5 months of age were surgically removed under ketamine-xylazine anesthesia. After 2 weeks, a pellet containing either 17β-estradiol (E2; 0.18 or 0.72 mg/pellet) or placebo vehicles was implanted subcutaneously at the scruff of the neck. The pellets were designed to release their contents continuously for 60 days (Innovative Research of America, Sarasota, FL). The pellet-implanted animals were housed in a controlled animal facility for 4 weeks.

Quantitative Determination of Plasma 17β-Estradiol—On the 29th day after pellet implantation, blood was withdrawn from the inferior vena cava into a heparinized syringe under anesthesia. Blood plasma was collected following centrifugation, and plasma 17β-estradiol concentrations were determined using an E2 enzyme-linked immunosorbent assay kit (BioSorce, Camarillo, CA).

Tissue Preparation—Following blood collection, the brain was rapidly removed, dissected into two hemispheres, and frozen in liquid nitrogen. After dissecting out the cerebellum, the left hemisphere of the cerebrum was weighed and stored at −80 °C for metal assay. For histological evaluation, coronal sections (12-μm thickness) of the right hemisphere were obtained using a cryostat (Leica, Nussloch, Germany) and mounted on poly-t-lysine-coated glass slides.

Metal Analysis—The left hemisphere was lyophilized, digested in ultrapure nitric acid, and then dried by evaporation. The appropriate dilution of 1% HNO3 was added to brain samples prior to triplicated metal analysis by inductively coupled plasma mass spectrometry (ICP-MS) using an Ultramass 700 spectrophotometer (Varian, Victoria, Australia).

Histofluorescent Zinc-Specific Staining and Measurement of Vesicular Zinc—Unfixed brain sections were stained with a zinc-specific fluorescence probe, Zn-1,6,7,8-tetraethyl-5(6)-oxo-1,5,5a,6,7,8-hexahydro-1,6,8-triazino[4,5-c]quinoline-6-sulfonylamine (TQS, 4.5 μM; Molecular Probes, Eugene, OR) in 140 mM sodium barbitol and 140 mM sodium acetate buffer (pH 10.0) for 90 s (25). After washing with saline, TQS-stained sections were examined under a fluorescence microscope (dichroic mirror, 400 nm; excitation filter, 330–385 nm; barrier filter, 420 nm) (BX60, Olympus, Tokyo, Japan) and photographed with a digital camera (Camedia C2900-Z; Olympus).

The quantification of vesicular zinc was conducted as described previously (9). Fluorescence intensity in the mossy fiber region of the dentate gyrus was assessed using a 20× objective. The 20× objective was used to image TQS-stained sections and then photographed using a bright-field microscope (Olympus).

Protein Analysis by Western Blots—The cerebellum was removed from the brain, and the cerebrum was dissected into two hemispheres. The right hemisphere was homogenized in radioimmune precipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% Triton X-100, 25 mM NaF, 1 mM dithiothreitol, and 1 mM Na2VO4; pH 7.4) supplemented with a protease inhibitor mixture (Roche Applied Science), centrifuged at 14,800 × g, and the supernatant protein concentration was determined using the BCA method (Pierce). Proteins (120 μg) were separated on 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Amersham Biosciences) in Tris-glycine buffer (25 mM Tris, 192 mM glycine, containing 20% methanol). The membranes were then blocked in 5% fat-free dry milk in phosphate-buffered saline, 0.1% Tween 20. After overnight incubation with antibodies specific for Znt3 (26), synaptophysin (Santa Cruz Biotechnology, Santa Cruz, CA) or β-actin (Santa Cruz Biotechnology) (1:1000 dilution in 3% fat-free milk-

| Group                      | 17β-Estradiol, mean ± S.E. | pg/ml |
|----------------------------|----------------------------|-------|
| Control (6 months)         | 13.9 ± 0.5                 |       |
| Control (15 months)        | 10.9 ± 0.5                 |       |
| OVX + placebo              | 5.6 ± 0.2                  |       |
| OVX + E2 (0.18 mg)         | 127.4 ± 3.6                |       |
| OVX + E2 (0.72 mg)         | 810.8 ± 18.6               |       |

*OVX, ovariectomy.*

Analysis of Znt3 mRNA—Total RNA was isolated from the left hemisphere of the cerebrum using TRIzol (Invitrogen), and RNA (1 μg) was reverse-transcribed into cDNA using a Superscript™ One-Step RT-PCR kit (Invitrogen). The following oligonucleotides were synthesized as primers for use specific mouse Znt3 (GenBank™ accession number NM-011773) as primers specific for mouse Znt3; 5′-ACT CTC CTC TAC CTC GCC TT-3′ (Znt3 sense); 5′-GATT GGA GAT CAT GAT TGT CT-3′ (Znt3 antisense); 5′-CCG ATC TTC TCC CTC CCT GGA-3′ (β-actin sense) and 5′-CTT ACG ATG TCT GGT GCA-3′ (β-actin antisense); 5′-GGA GTT CCT CAA GCT AAT GG-3′ (Ap1g sense) and 5′-GTA CCT GAA AAA AGG GTT CA-3′ (Ap1g antisense); 5′-CCA ACG CAA TGG TAT CTG TG-3′ (Ap3d sense) and 5′-CCT TGG GAT TCT CAT CCT CA-3′ (Ap3d antisense); and 5′-AGC CCA CAG CAA ATC AG-3′ (Ap3s sense) and 5′-TAT AAA GAG GGC AGG TTG GT-3′ (Ap3s antisense). After PCR amplification, agarose gel electrophoresis was used to identify Znt3 (493 bp), β-actin (440 bp), Ap1g (848 bp), Ap3d (509 bp), and Ap3s (500 bp) fragments.

In addition, we evaluated mRNA expression by Northern blot analysis. Total RNA (10 μg) was resolved on 1.2% agarose/formaldehyde gels and transferred to nylon membranes (Hybond™-N+; Amersham Biosciences). After immobilizing RNA by UV cross-linking, membranes were prehybridized in a hybridization solution (ExpressHyb; Clontech) and then hybridized with radio labeled cDNA probes specific for Znt3, β-actin, Ap1g, Ap3d, or Ap3s in the same solution. cDNA probes corresponding to the above RT-PCR products were labeled with [α-32P]dCTP using a random priming kit (Amersham Biosciences). Membranes were washed at 65 °C in 2× SSC, 0.1% SDS solution and subjected to autoradiography.

Statistical Analysis—All evaluations were performed in an evaluator-blinded manner. All values represent mean ± S.E. Differences between groups were examined using one-way analysis of variance followed by Student-Newman-Keuls test. A p value of less than 0.05 was considered significant.

RESULTS

Ovariectomized female Znt3+/− mice were surgically removed at 5 months of age, and mice were then divided into three groups of 10. Two weeks after ovariectomy, mice were subcutaneously implanted with sustained release (60 day) pellets containing vehicle only or low dose 17β-estradiol (0.18 mg) or high dose 17β-estradiol (0.72 mg). Four weeks after pellet implantation, blood samples and brains were harvested for analysis.

Plasma levels of 17β-estradiol were determined (Table I). In non-operated controls, the levels of 17β-estradiol were about 13–14 pg/ml in 6-month-old female mice and 10–11 pg/ml in 15-month-old female mice, indicating that estrogen levels decrease with age (p < 0.05 by analysis of variance). When compared with non-operated controls, 6-month-old ovariectomized mice had substantially lower 17β-estradiol plasma levels. In contrast, 6-month-old ovariectomized mice that received low or high dose 17β-estradiol had dose-dependently higher 17β-estradiol plasma levels when compared with both control and ovariectomized mice.

Mice brains were assayed for synaptic vesicle zinc levels. In
non-operated mice, we found that 15-month-old female mice had slightly higher synaptic vesicle zinc levels when compared with 6-month-old female mice ($p < 0.05$) (Fig. 1A), consistent with our previous data demonstrating an age-dependent increase in the levels of synaptic zinc in female mice (9). Although the levels of total brain zinc in ovariectomized mice were only about 10% higher than those in age-matched control females ($p < 0.05$) (Fig. 1B), the levels of synaptic vesicle zinc were about 50% higher, as determined by TSQ fluorescence, an indicator of synaptic vesicle zinc ($p < 0.05$) (Figs. 1B and 2). Since synaptic vesicle zinc comprises 10–30% of total brain zinc (24), most changes in total zinc could be attributed to the
increase in synaptic vesicle zinc. In support of this concept, in Znt3−/− mice that completely lack synaptic vesicle zinc, neither ovariectomy nor estrogen replacement altered total zinc levels (Fig. 1C). In contrast, in ovariectomized Znt3+/− mice, estrogen replacements substantially reduced the levels of total and synaptic zinc in an estrogen dose-dependent manner (Figs. 1B and 2). High dose estrogen treatment caused synaptic vesicle zinc levels to fall below those in control non-operated mice.

Since Znt3 protein is essential for sequestration of synaptic vesicle zinc (24, 26), we examined whether ovariectomy or estrogen replacements altered Znt3 expression. Western blots revealed that ovariectomy slightly increased the levels of Znt3 protein (Fig. 3). In contrast, Znt3 levels were markedly reduced by estrogen replacement in ovariectomized mice. Indeed, Znt3 levels in ovariectomy plus estrogen-supplemented mice were lower than those in control mice (Fig. 3), consistent with the higher levels of estrogen in 17β-estradiol-supplemented mice than in control mice (Table I). Similar changes in Znt3 expression were noted following immunohistochemical staining. Anti-Znt3 antibody immunoreactivity in the hippocampus, particularly in mossy fibers, was increased in ovariectomized mice. Estrogen replacements down-regulated Znt3 levels to below controls (Fig. 3).

Other reports have shown that estrogen replacement affects synapse number (27, 28), which may contribute to changes in overall Znt3 levels. Using Western blotting, we examined the level of synaptophysin, a synapse-specific protein. The level of synaptophysin in the brain was unaffected by ovariectomy or estrogen replacements (Fig. 3), indicating that changes in Znt3 levels following ovariectomy or estrogen supplement were not caused by changes in synapse numbers.

To understand more about the changes in Znt3 protein levels, we examined Znt3 mRNA levels using both RT-PCR and Northern blot assays. We found that neither ovariectomy nor ovariectomized plus estrogen treatment altered the levels of Znt3 transcripts (Fig. 4). Brain tissues from Znt3−/− and Znt3+/− (heterozygotes) mice showed no and intermediate levels of Znt3 transcripts, respectively. These data suggest that estrogen affects Znt3 levels through post-transcriptional events.

Previous studies (29, 30) have demonstrated that both Znt3 and synaptic vesicle zinc were remarkably reduced in the brain of mocha mice, in which the entire AP-3 complex was completely depleted by the genetic ablation of δ subunit. Thus, we examined whether estrogen depletion or replacement modulate expression of adaptor protein complexes in transcriptional level. Indeed, Ap3d transcript level in the brain of ovariectomized Znt3+/− mice was higher than that in controls. Conversely, estrogen replacement lowered the level of Ap3d mRNA in ovariectomized rat brains (Fig. 5). On the other hand, transcript levels of the other AP subunits (Ap3α and Ap1g) were not altered by ovariectomy or estrogen replacement.

**FIG. 2.** Histofluorescent synaptic zinc in the hippocampus. Fluorescence photomicrographs of TSQ-stained hippocampi sections from Znt3−/− mice (A), or from non-operated/untreated (B), ovariectomized/placebo-treated (C), and ovariectomized/17β-estradiol (0.72 mg/pellet)-treated (D) Znt3+/− mice are shown. Scale bar, 50 μm. mf, mossy fiber terminals.

**FIG. 3.** Effects of ovariectomy (OVX) and ovariectomy plus estrogen supplement on levels of Znt3 in the cerebrum. A–D, bright field photomicrographs of anti-Znt3 antibody-stained hippocampi sections from Znt3−/− mice (A) or from non-operated/untreated (B), ovariectomized/placebo-treated (C), and ovariectomized/estrogen-treated (D) Znt3+/− mice. Scale bar, 50 μm. E, representative Western blots showing Znt3, β-actin, or synaptophysin expression in samples obtained from cerebrum of Znt3−/− and Znt3+/− mice that were untreated, ovariectomized, or ovariectomized and received low or high dose estrogen supplement. F, densitometric measurements of the above Western blots. The optical density of Znt3 in each group was normalized to that of β-actin as an internal control, and then values relative to untreated Znt3+/− mice were calculated (mean ± S.E., n = 3). Asterisks represent a significant difference between the corresponding comparisons (p < 0.05).

**FIG. 1.** Western blots showing Znt3 levels in cerebrum of ovariectomized/placebo-treated (A), ovariectomized/17β-estradiol (0.72 mg/pellet)-treated (B) and control (C) mice. (D) Densitometric measurements of the above Western blots. The optical density of Znt3 in each group was normalized to that of β-actin. Scale bar, 50 μm.

**DISCUSSION**

Whether estrogen should be used for AD treatment appears unclear since some studies have concluded that estrogen replacement may reduce the incidence or severity of AD (4–6), whereas others suggested it may have negative or even harmful effects (31, 32). In animal AD models, estrogen clearly reduces the burden of Aβ and amyloid plaques (10–13, 33). Although the precise mechanisms underlying these effects are unknown, estrogen may reduce Aβ production by inhibiting generation (10, 11) or facilitating clearance (12, 13) of the protein. The present results suggest an additional mechanism of action of estrogen. Our data indicate that estrogens may reduce the formation of mature plaques by down-regulating the levels of synaptic zinc, which contributes to the transformation...
of soluble Aβ into insoluble forms (9). This effect appeared to be due to a reduction in the level of Znt3, the protein required for accumulation of synaptic vesicle zinc.

Endogenous metals such as zinc and copper have been implicated as promoters of Aβ aggregation in AD (14, 15). The levels of zinc and copper are higher in brains of AD patients (17, 18), especially in and around amyloid plaques (19, 21). Moreover, histochemical staining with zinc-specific fluorescent dyes revealed high levels of labile zinc in Aβ plaques of Tg2576 mice (20) and AD patients (34). Of various pools of brain zinc, synaptic vesicle zinc, which constitutes 10–30% of total brain zinc, may play a disproportionately critical role since removal of synaptic vesicle zinc results in a 70–80% reduction in amyloid plaques (9).

Synaptic vesicle zinc is a special pool of zinc in the brain. It is localized mainly inside glutamatergic synaptic vesicles of the forebrain (35), and probably because of its liability, can be identified using simple histochemical techniques such as Timm’s staining or dye fluorescence (25, 36). Like other neurotransmitters, zinc in synaptic vesicles is released with neuronal activity (37, 38) and may play diverse physiological roles in synaptic transmission upon release (39, 40). Furthermore, zinc may gain access into neuron cytoplasm and serves signaling functions (41).

Synaptic vesicle zinc may also have pathological roles. In addition to the above-mentioned role in Aβ aggregation, excessive release of synaptic zinc may contribute to neuron death in various models of acute brain injury (42–44). Hence, understanding the mechanisms regulating synaptic vesicle zinc levels appears important. Znt3 appears to be the membrane protein necessary for synaptic vesicle zinc transport since genetic deletion of Znt3 results in complete disappearance of synaptic vesicle zinc without altering the levels of the major protein-bound zinc pool (24). In the brain, expression of Znt3 is developmentally regulated (45). Other than this developmental modulation in expression, little information is available as to the mechanisms regulating Znt3 levels in the brain.

In a previous study, we noted increased synaptic vesicle zinc levels in female Tg2576 mice, which have higher plaque burdens than male mice (9). Since the sex-dependent difference in plaque burden disappeared in Znt3-null mice, we hypothesized that sex-dependent factors such as estrogen may modulate the levels of synaptic vesicle zinc by regulating Znt3 expression. The present results indicate that brain levels of synaptic vesicle zinc are affected by changes in the level of estrogen. Whereas ovariectomy increased the levels of synaptic vesicle zinc in the brain, estrogen replacement reduced levels. Since total zinc levels in the brain did not alter greatly, estrogen may specifically modulate the levels of synaptic zinc.

Our data indicate that the effect of estrogen on synaptic zinc levels was mediated by changes in Znt3 levels. Ovariectomy increased the levels of Znt3, whereas estrogen replacement reduced Znt3 levels, indicating that estrogen regulates Znt3 levels in the brain. Increases in synaptic vesicle zinc in 12-month-old female mice (9) might be the result of a rapid decline in estrogen levels and the consequent increase in Znt3 levels around that age. In the present study, estrogen regulation of Znt3 did not appear to be the result of changes in the synaptic integrity since synaptophysin levels in the cerebrum were not altered by ovariectomy or estrogen replacement. We did not detect any change in Znt3 mRNA levels by RT-PCR or Northern blot analysis, suggesting that modulation of Znt3 by estrogen occurs at the level of protein metabolism.

Recent studies have demonstrated that the genetic ablation of the AP-3 subunit in mocha mice caused the lack of Znt3 with less alteration of synaptic integrity in the brain (29, 30).

In the mocha mice, whereas the transcript level of another AP-3 subunit α (Ap3α) (and presumably also the other subunits) was normal, the AP-3 subunit peptides (β, μ, and α) disappeared, perhaps due to the instability (29). In our study, changes in the level of estrogen are inversely correlated with the levels of both Znt3 protein and Ap3α transcripts but not with those of Znt3 or Ap3α transcripts. These results suggest that estrogen modulates the levels of AP-3 complex in the brain via altering the expression of Ap3α transcripts, which in turn results in changes in the levels of Znt3 and synaptic vesicle zinc. It would be interesting to see whether other proteins that are under the control of the AP-3 complex (29, 30) are also modulated by estrogen.

Despite increasing interest in the neurobiology of synaptic vesicle zinc (46, 47), little information is available as to its
regulation. The present results show for the first time that estrogen modulates Znt3 levels, the key protein for synaptic vesicle zinc transport. Since synaptic vesicle zinc contributes to neuron death in certain models of acute brain injury (46), the protective effects of estrogens in those models (48, 49) may result in part from the reduction of vesicle zinc. In addition, estrogen-dependent reduction of amyloid plaques in Tg2576 mice may also result partly from reduced levels of Znt3 and synaptic vesicle zinc. Further studies appear warranted to understand the mechanisms regulating Znt3 function and the pathophysiological significance of such regulation.

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