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Adrenalectomy-induced ZnT3 downregulation in mouse hippocampus is followed by vesicular zinc depletion

Sang Won Suh[a,b,*], Seung Mook Jo[a,c], Zsolt Vajda[a], Gorm Danscher[a]

[a] *Department of Neurobiology, Institute of Anatomy, University of Aarhus, DK-8000 Aarhus C, Denmark*
[b] *Department of Neurology, University of California, San Francisco, CA 94121, USA*
[c] *Department of Anatomy, Gachon Medical School 1198, Kuwol-Dong, Namdong-Gu, Inchon 405-760, South Korea*

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Abstract

The effects of adrenalectomy (ADX) on the vesicular zinc content of zinc-enriched (ZEN) terminals in mouse hippocampus were investigated at light microscopic levels using zinc transporter-3 immunohistochemistry ($ZnT3^{IHC}$) and zinc selenium autometallography ($ZnSe^{AMG}$). ZnT3 resides in the synaptic vesicle membranes of ZEN neurons and is believed to move zinc ions into the vesicles. $ZnT3^{IHC}$ staining closely corresponds to the $ZnSe^{AMG}$ staining, but in the present study we present evidence of a delayed decrease of ZEN zinc, as compared to downregulation of the ZnT3 protein following ADX. Twenty-four hours after adrenalectomy the level of $ZnT3^{IHC}$ was visibly reduced while the $ZnSe^{AMG}$ staining intensity seemed unchanged. After 10 and 30 days, however, downregulation of ZnT3 was paralleled by a distinct reduction in $ZnSe^{AMG}$ staining. The total protein concentration of ZnT3 was reduced by about 53%, and the total zinc concentration in the hippocampus of the same mice was reduced by 43–64%, 30 days after the adrenalectomy. The present results support previous results suggesting that ZnT3 is responsible for transport of zinc ions into a pool of synaptic vesicles in ZEN terminals.

Keywords: Zinc; Zinc transporter; Adrenalectomy; Hippocampus; AMG (autometallography); Immunohistochemistry

Histochemically reactive zinc is present in many regions of the central nervous system [8,9,13,22] and is especially abundant in the olfactory bulb, amygdala and the hippocampal mossy fiber region [2,5,15]. Despite the abundance of free and loosely bound zinc ions in the brain and papers that focus on the toxic potentials of the substantial zinc pool in the terminals of a vast number of brain and spinal cord neurons [11,14,16,18,28–30], the mechanisms controlling vesicular zinc in the CNS is still not well described. An understanding of the mechanisms responsible for maintaining vesicular zinc homeostasis is a clinically important step towards developing therapeutic intervention techniques that could be valuable in preventive or curative measures of Alzheimer's disease, stroke, seizure, head trauma and hypoglycemia-induced neuronal death.

In a previous study, we showed that adrenalectomy (ADX) decreases the vesicular zinc concentration, opening the possibility that zinc transporters are influenced by the level of corticosteroids [31]. Recently, a ZnT3 antibody has been cloned and the ZnT3 protein found to be located in the vesicular membrane of ZEN terminals [21]. ZnT3 belongs to a family of mammalian zinc transporters (ZnT1–9) [3]. ZnT3 is localized in the terminal fields of ZEN pathways and the immunohistochemical staining pattern of ZnT3 is identical to the pattern of zinc–sulphur nanocrystals seen with the Neo-Timm [6] or immersion autometallographic [17] techniques, or the pattern of zinc–selenium nanocrystals seen with the in vivo selenium technique [7,27]. These facts, including the lack of AMG staining in ZnT3 knockout mice, strongly suggest that ZnT3 is responsible for transporting zinc into synaptic vesicles [21,33]. Recently, Lee et al. showed that exogenously administered estrogen has the ability to decrease synaptic zinc levels. The mechanism by which estrogen acts to decrease synaptic zinc levels is by decreasing the ZnT3

* Corresponding author. Tel.: +1 415 221 4810; fax: +1 415 750 2011.
E-mail address: swsuh@itsa.ucsf.edu (S.W. Suh).

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protein levels [19]. When an ovarectomy is performed, the opposite effect of estrogen administration occurs and the levels of ZnT3 protein increase. The increase is followed by an increased concentration of zinc ions in the mouse brains. The present study suggests that the surgical removal of an endocrine organ or the manipulation of hormone levels are able to modulate zinc transporter protein levels, especially ZnT3, and that this manipulation can cause changes in the levels of zinc ions in the synaptic vesicles of ZEN neurons in brain and spinal cord.

Stress hormone glucocorticoids are located in the adrenal glands, and during acute stress this adrenal steroid hormone is released in excessive amounts into the blood stream and transported into the brain. If the concentration of glucocorticoids in the brain is either too high or too prolonged, hippocampal neurons will experience deleterious effects [23,25]. The hippocampal neurons contain a high density of glucocorticoid receptors, and as a result the hippocampus is one of the most vulnerable areas of the brain during severe stress [20,23]. Higher blood glucocorticoid concentrations potentiate the vulnerability of hippocampal neurons after ischemia [25] and kainate-induced seizures [24]. Interestingly, vesicular zinc is highly localized to the presynaptic terminals of hippocampal mossy fibers and is excessively released after restraint-induced stress [12].

Vesicular zinc has also been suggested to be related to stress-induced neuronal injury in addition to the neuronal death that is seen following ischemia [16], seizure [11], head trauma [28] and hypoglycemia [29]. We suggested previously a collaborating role between vesicular zinc and glucocorticoids on stress-induced neuronal death. We found that a decreased concentration of vesicular zinc ions in the hippocampus following ADX could decrease kainate-induced neuronal death [31]. However, the study did not show how the vesicular zinc content could be decreased after ADX.

The present study was undertaken to test the hypothesis that vesicular zinc depletion after ADX is mediated by a decrease in the level of ZnT3 proteins in the ZEN terminals.

All animal surgeries were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. The male BALB/c mice (8–10 weeks old, 30–35 g) used in the experiments were purchased from Møllegaard Breeding Center, Denmark. Animals were housed two or three per cage in a temperature-controlled room (22–24 °C) with a light/dark cycle of 12 h/12 h (light on at 6.00 a.m.). Food and tap water were available ad libitum. Adrenalectomy (ADX, $N = 30$) and sham laparotomy (control, $N = 16$) were performed with the animal under halothane inhalation anesthesia (induction 3% and maintained 1.5%) using the dorsal approach [26]. Surgical operations were performed between 10.00 a.m. and 12.00 a.m. After adrenalectomy, the animals had access to 0.9% NaCl water ad libitum. Animals were sacrificed at 1 day (ADX, $N = 3$ and Cont, $N = 3$), 10 days (ADX, $N = 3$ and Cont, $N = 3$), 30 days (ADX, $N = 12$ and Cont, $N = 10$) or 3 months (ADX, $N = 2$) after sham or ADX. Only animals that failed to gain weight after the ADX were included in the actual samples.

To confirm the effectiveness of the ADX surgery, the plasma corticosterone level was measured from sham-operated or adrenalectomized mice at 24 h after surgery. Blood was collected in chilled tubes containing EDTA by heart puncture, and immediately centrifuged at $3000 \times g$ for 15 min at 4 °C. Plasma was collected and stored at −70 °C until the assay. The autoDELFIA cortisol assay was used, which is a solid phase time-resolved fluoroimmunoassay based on the competitive reaction between europium-labeled cortisol and sample cortisol for a limited amount of binding sites on cortisol-specific, biotinylated monoclonal antibodies (derived from mice). The method was published in details before [1,31].

The sham-operated and adrenalectomized mice were anesthetized with pentobarbital (50 mg/kg, i.p.) and perfused transcardially with 50 ml isotonic saline, followed by 200 ml fixative. The mice were perfused with 4% paraformaldehyde in 0.1 M Phosphate-Buffered Saline (PBS, pH 7.4). The brains were removed and postfixed in the same fixative (4 h, 4 °C). The mouse brains were cryoprotected with 30% sucrose in 0.1 M PBS (12 h, 4 °C) and frozen with CO_2 gas. Coronal serial sections (30 μm) were made on a cryostat. An affinity-purified rabbit antibody specific for ZnT3 (provided by Dr. R. Palmiter) was used for immunocytochemical localization. The immunolabeling procedures were performed in accordance with the routine ABC technique. Sections were rinsed in 0.1 M Tris-Buffered Saline (TBS) (0.05 M TB/0.15 M NaCl, pH 7.4), then endogenous peroxidases were inactivated by treatment with 1% H_2O_2 in methanol for 15 min. The sections were rinsed three times with TBS containing 1% Triton X-100 (Triton), and treated with 1% BSA, 3% goat serum and triton in TBS for 1 h to reduce unspecific staining. The sections were rinsed in TBS for 30 min and incubated for 2 days at 4 °C in ZnT3 antiserum, diluted 1:100 in TBS containing 3% goat serum, and 1% BSA and triton. After a rinse for 45 min in TBS containing triton, the sections were incubated in biotinylated goat anti-rabbit IgG (diluted 1:200) for 1 h at room temperature (RT, 22 °C), rinsed 30 min in TBS and then incubated in ABC (ABC Kit; DAKO), diluted 1:100 in 1% BSA and TBS for 1 h at RT. Sections were rinsed in TB (pH 7.6) and incubated for 15 min in 0.025% 3,3′-DAB (diaminobenzidine) with 0.0033% H_2O_2. Stained sections were rinsed in TBS followed by alcohol dehydration and xylene clearance. Finally they were mounted on 0.5% gelatin-coated slides, coverslipped, and observed and photographed with a Zeiss photomicroscope (Axiophot). To assess nonspecific effects a few sections in every experiment were incubated in a buffer without primary antibodies. This procedure always resulted in a complete lack of immunoreactivity.

Tissues were homogenized in an Ultra-Turrax T8 homogenizer (IKA Labortechnik) on ice in buffer containing 0.25 M sucrose, 1 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml diisopropyl fluorophosphate, and 4 μM/ml leupeptin. The homogenate was centrifuged in an Eppendorf 5403 centrifuge