Detection Methods of Histochemically-reactive Zinc in the CNS at the Light Microscopical Level

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명추신경계동 내 분포하는 zinc 이온의 조직화학적 동정법 비교: Ⅰ. 광학현미경수준에서

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ABSTRACT

Small amounts of zinc ions regulate a plentitude of enzymatic proteins, receptors and transcription factors, thus cells need accurate homeostasis of zinc ions. Some neurons have developed mechanisms to accumulate zinc in specific membrane compartment ("vesicular zinc"), which can be evidenced using histochemical techniques. These neurons are the so-called zinc enriched (ZEN) neurons, which accumulate glutamate and zinc inside their synaptic vesicles and release it during synaptic transmission. In the present paper we have studied the distribution of the ZEN terminals in the rat hippocampus using ZnSe autometallography, Neo-Timm staining, ZnT3 immunohistochemistry and TSQ fluorescence staining.

Keywords : Ionic zinc, ZEN terminals, Autometallography, Rat, Hippocampus

INTRODUCTION

Zinc is one of the most abundant cations in the nervous system (0.12 ~ 0.15 mM in the mammalian brain) (Ebadi et al., 1994), where it is found associated to nucleic acids and a large variety of proteins, either bound to them by strong or labile bonds, or formed part of the molecule. Among the zinc-associated proteins, some of them are mainly extracellular (metalloproteins, growth factors), whereas others are intracellular (a great variety of cytoplasmic enzymes) and even intranuclear (nuclear replication enzymes, transcription enzymes, and transcriptional regulators) (Vallee et al., 1991; Coleman, 1992). In the intracellular environment, free ionic zinc interacts with many regulatory enzymes and, similar to calcium, may act as a secondary messenger trig-
ginger/inhibiting important cellular processes, e.g. cell death and the apoptosis (Fraker & Telford, 1997).

Therefore, it is conceivable that intracellular zinc homeostasis occurs. Perhaps this is the reason why a diversity of neurons has developed vesicular compartments with specific sequestering and storage mechanisms (Masters et al., 1994; Palmier et al., 1997). These are the so-called zinc enriched neurons (Frederickson, 1989; Frederickson & Moncrieff, 1994), which accumulate glutamate and zinc inside their synaptic vesicles (Martinez-Guijarro et al., 1991) and release them during synaptic transmission (Assaf & Chung, 1984; Howell et al., 1984). In the extracellular space, ionic zinc interacts with GABA and NMDA receptors, thus modulating the inhibitory synaptic effects as well as the excitatory synaptic effects (Westbrook & Mayer, 1987; Gordy et al., 1995). In spite of the high mobility of these ions and their strong interactions with an additional variety of proteins, it is possible to detect them by means of histo-cyto-chemical methods.

Palmier et al. (1997) identified a zinc transporter, ZnT3, which is essential for the transport of zinc into synaptic vesicles in ZEN terminals throughout the brain. The immunohistochemical staining pattern for ZnT3 is identical to that seen with the NeoTimm staining for vesicular zinc in mouse and monkey hippocampus. The ZnT3 has a distribution in the spinal cord almost identical to that seen by the zinc ion-specific ZnSeAMG technique, suggesting that ZEN terminals are involved in both sensory and motor activity at the spinal cord level (Jo et al., 2000).

Substances giving a bright color or emitting florescence when they are in contact with divalent metal ions are currently used to detect them inside the cells; the use of these methods leads to the so-called “direct” methods. The fixation and precipitation of metal ions as insoluble salt precipitates, their maintenance along the histological process and, finally, their demonstration after autometallographic development are essential steps for other methods, the so-called “indirect methods” (Neo-Timm method). Direct method may be used “in vivo” and for quantitative-micro analytical purposes although they do not allow usually good histological preservation. Although indirect methods are not very suitable for quantification, they are much more sensitive and compatible with light and electron microscopy.

This study is a short report on the different direct and indirect experimental approaches for zinc detection in the rat CNS.

**MATERIALS AND METHODS**

1. Zinc Selenium autometallography (ZnSeAMG)

Six rats were injected i.p. under halothane anesthesia with sodium selenide (10 mg/kg) dissolved in phosphate buffer (PB). After 1.5 h the animals were killed by transcardial perfusion with a 3% glutaraldehyde solution in PB. The olfactory bulb, cerebral cortex including hippocampus segments were removed and placed in the same fixatives for 3 h in the refrigerator (4°C). Sections, 100 μm thick, were cut on a vibratome and collected in the vial for AMG development. The AMG silver enhancement was performed with the original silver lactate developer (Danscher, 1982; Danscher & Møller-Madsen, 1985). In short, sections were placed in jars floating in the AMG developer for 60 min. AMG was stopped by replacing the developer with a 5% sodium thiosulfate solution. Ten min later the sections were rinsed with distilled water. The stained sections were dehydrated in alcohol, cleared in xylene, coverslipped with mounting media (DePeX), and examined under the light microscope.

2. Neo-Timm staining

Six rats were transcardially perfused with 0.1% NaS in 3% glutaraldehyde in 0.1 M phosphate buffer (PB) for 10 min. The brains including hippocampus were allowed to post-fix in 3% glutaraldehyde for 1 h, placed in a solution of 30% sucrose until they sank to the bottom of the vial, and frozen with CO2 gas. The frozen sections, 30-μm thick, were placed on glass slides cleaned in a 10% Farmer solution. The sections were dried for 15 min, fixed in 90% ethanol, re-hydrated and coated with 0.5% gelatin. In daylight, the newly prepared AMG developer was poured into Farmer-rinsed jars containing the slides, and the jars were placed in a 25°C water bath. At this temperature 30 μm cryostat sections were developed for 60 min. Following development, the slides were first rinsed in running tap water at 40°C for 20 min in order to remove the gelatin coat, and then dipped twice in distilled water. After rinsing and dehydration the sections were mounted with a DePex mounting medium (BDH Laboratory Supplies, Poole, UK) (Danscher et al., 1997).

3. Zinc transporter-3 Immunohistochemistry (ZnT335C)

Six rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and perfused transcardially with 50 mL isotonic saline,
followed by 200 mL 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Hippocampus were removed and postfixed using the same fixatives for 4 h (4°C).

Segmental pieces of the hippocampus were placed in PB and 10-μm thick paraffin sections were cut on a microtome, and followed by mounting on the gelatin-coating slides. An affinity-purified rabbit antibody specific for ZnT3 (provided by Dr. Richard Palmiter, Department of Biochemistry, University of Washington, USA) was used for the immunohistochemical localization. The immunolabelling procedures were performed in accordance with the routine avidin-biotin-peroxidase complex (ABC) method. Sections were rinsed in 0.1 M Tris-buffered saline (TBS: 0.05 M, Tris buffer (TB) in 0.15 M NaCl, pH 7.4), and endogenous peroxidases were then inactivated by treatment with 1% hydrogen peroxide (H2O2) in pure methanol for 15 min. The sections were rinsed three times in TBS and treated with 1% bovine serum albumin (BSA) and 3% goat serum in TBS containing 0.25% dimethyl sulfoxide (DMSO) for 2 h to reduce nonspecific 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, 1% BSA and 0.25% DMSO. Following rinses for 45 min in TBS, the sections were incubated in biotinylated goat anti-rabbit IgG (diluted 1:500) for 1 day at 4°C and rinsed for 30 min in TBS. The ABC kit (DAKO) was applied to visualize the reaction sites for 1 h at room temperature (RT). The ABC solution was diluted 1:100 in BSA 1% in TBS. After the sections had been rinsed in 0.1 M Tris buffer (TB, pH 7.6) and left in 0.025% 3,3’-diaminobenzidine (DAB) with 0.0033% H2O2 for 15 min at RT, the brown color appeared in the sections. Staining was stopped by rinsing with TB, followed by mounting and air-drying at RT.

4. TSQ fluorescence staining

For the TSQ (Molecular Probes, Eugene, OR, USA) fluorescence histochemical evaluations of zinc ions, six rats were euthanized with a lethal dose of halothane inhalation, and the brains were removed, frozen with CO2 gas, sectioned horizontally at 30 μm in a -14°C cryostat, thawed and dried onto gelatin-subbed slides. For TSQ fluorescence, the sections were stained with TSQ as described previously (Frederickson et al., 1987).

RESULTS

1. ZnSeAMG

The detailed pattern of ZEN terminals can be seen by an intraperitoneal injection of sodium selenide after 1.5 h. The densest staining patterns were found in hippocampus, moderate staining in cerebral cortex and olfactory bulb, and spinal cord and cerebellum showed relatively weak staining.

The ZnSeAMG staining for zinc ions revealed a clear demarcation between concentric layers of cells and neuropil in the olfactory bulb. Strongly intense ZnSeAMG staining was found in the granular cell and olfactory glomerular layers. On the other hand, moderate ZnSeAMG staining was seen in the internal plexiform layer and mitral cell layer (Fig. 1A).

The ZEN terminals was concentrated in telencephalic structures such as neocortical layers I-III and V (Fig. 1B). Strong staining was detected in the mossy fiber plexuses, in hilus, and in stratum lucidum of CA3 (Fig. 3A).
In the cerebellum, the molecular layer contained homogeneously distributed zinc precipitates. More intensely stained reaction products were located between molecular layer and Purkinje cell layer above the fourth ventricle (Fig. 2A).

Zinc-enriched (ZEN) terminals in the spinal cord were dispersed throughout the gray matter. The superficial dorsal horn (laminae I, III, IV) and lamina X, involved in sensory transmission, contained relatively high concentrations of ZEN terminals (Fig. 2B).

2. Neo-Timm staining

“Vivid” zinc staining was present in neocortical layers I-III and V. and hippocampus, especially mossy fibers, CA 1-4, stratum radiatum and oriens, and dentate gyrus (Fig. 3B).

Reaction products appeared black dots with variable sizes and densities in a given lamina or area. The neuropil
TSQ for Zinc in the Hippocampus

![Image of fluorescent micrographs](image)

**Fig. 4.** Fluorescent micrographs taken from 30 μm thick-cryosection of the hippocampus, showing dense fluorescence of TSQ, a specific marker for zinc concentrated in the hilus (H) and Mossy fiber (Mf). Note vivid fluorescent staining of the mossy fiber plexus and hilus. Scale bars: (A) 40 μm; (B) 40 μm.

throughout the spinal cord exhibited AMG staining in different concentrations, showing a typical lamination in the gray matter (data not shown).

3. ZnT3 IHC

The general staining pattern was almost identical, that is, most intense staining is found in hilus and Mossy fiber in CA3 whereas moderate staining was observed in the stratum oriens and radiatum through CA1 and CA2 of the hippocampus (Fig. 3C).

4. TSQ staining

The TSQ fluorescence pattern matched the ZnSeAMG staining both in the brain and spinal cord. TSQ-induced fluorescence persisted for hours and showed enhanced emission at a neutral pH compared to an acidic pH. The strongest fluorescence was found in the Mossy fiber and hilar area, but neuronal perikarya are unstained (Fig. 4).

**DISCUSSION**

In the nervous tissue, ZnSeAMG has been used in the study of ZEN synaptic fields, giving a pattern, which is similar to that of the Neo-Timm method. In addition, the selenium method has also been used to demonstrate mercury, gold and cadmium in sections (Danscher & Moller-Madsen, 1985; Schionning et al., 1993) using elegant strategies to differentiate between sliver, gold or mercury deposits (Danscher et al., 1994). Similarly to the Neo-Timm method, the selenium method may also be used in combination with other histochemical and immunocytochemical techniques (Geneser et al., 1993).

In the present study we have shown that there is a good correlation between immunohistochemical localization of ZnT3 and the distribution of zinc ions. Chemical distribution of ZnT3 and the AMG labeling indicates that both methods accurately label ZEN terminals in the rat hippocampus. However, darkly stained area, dentate inner molecular layer was unstained in the sections stained by Neo-Timm and ZnT3HC, and darkly stained area, dentate outer molecular layer was not present in the sections stained by ZnSeAMG and Neo-Timm.

The strongest fluorescence was found in the Mossy fiber and hilar area, but neuronal perikarya are unstained. It is noteworthy that, in the nervous tissue, the TSQ-detectable zinc pool is the same as that detected with the ZnSeAMG staining. The TSQ fluorescence pattern matched both in the brain and spinal cord of the mouse (Frederickson et al., 1987; Frederickson et al., 1989).

**REFERENCES**


<국문초록>

본 연구자는 Zinc Selenium autometallography (ZnSeAMG) (Danscher, et al. 1997) 영색법을 중심으로 증추신경계통 내 zinc (Zn2+)의 분포와 이들을 함유하고 있는 신경종합물소, 소위 ZEN (zinc-enriched) terminals의 미세구조에 관하여 보고한 바 있다.

이번 연구에서는 다음 몇 가지, 즉 Neo-Timm staining (Danscher, 1982), TSQ fluorescence staining (Frederickson et al., 1987), Zinc transporter-3 Immunohistochemistry (ZnT3Imc) (Palmiter et al., 1997) 영색법으로 편의 해마복합체에 분포하는 Zn2+를 영색한 후 이들 영색체단에서 차이점에 관해보고자 하였다.

ZnSeAMG영색법은 Zn2+에 대한 특이성은 다소 떨어지지만 광학 및 전자현미경에서 관찰이 가능하며, 반응구역 내 피복으로 보관이 가능하다는 장점이 있었고 TSQ는 Zn2+에 대한 특이성이 매우 높을 뿐 아니라 그 영색법이 매우 간단하다는 장점이 있는 반면 형광물질의 안정성과 표본보관이 용이하지 않다는 단점이 있다. 그 외 Neo-Timm 영색법은 TSQ영색법과 유사한 영색체단을 보였으며, ZnT3Imc 영색법은 오히려 ZnSeAMG에 가까운 영색의 결과를 보였다.

본 연구의 결과는 증추신경계통 내 Zn2+에 관한 형태학적 연구에서 기초자료로 활용될 수 있을 것이다.