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Autometallography for Zinc Detection in the Central Nervous System

Seung Mook Jo, Danscher Gorm[1], Sung Jun Kim, Seung-Kook Park, Tae-Cheon Kang and Moo Ho Won*
Dept. Anat., Coll. Med., Hallym Univ., Chunchon 200-702, Korea
[1]Dept Neurobiol., Inst Anat., Aarhus Univ., DK 8000, Aarhus C, Denmark
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중추신경계통내 분포하는 Zinc의 조직화학적 동정

조승묵, Danscher Gorm[1], 김성준, 박승국, 강태천, 원무호*
한림대학교 의과대학 해부학교실
[1]Dept Neurobiol., Inst Anat., Aarhus Univ., DK 8000, Aarhus C, Denmark

ABSTRACT

Zinc is one of the most abundant oligoelements in the living cell. It appears tightly bound to some metalloproteins and nucleic acids, loosely bound to some metallothioneins or even as free ion. Small amounts of zinc ions (in the nanomolar range) regulate a plentitude of enzymatic proteins, receptors and transcription factors, thus cells need accurate homeostasis of zinc ions. Zinc is an essential catalytic or structural element of many proteins, and a signaling messenger that is released by neural activity at many central excitatory synapses. Growing evidences suggest that zinc may also be a key mediator and modulator of the neuronal death associated with transient global ischemia and sustained seizures, as well as perhaps other neurological disease states. Some neurons have developed mechanisms to accumulate zinc in specific membrane compartment ("vesicular zinc") which can be evidenced using histochemical techniques.

Substances giving a bright colour or emitting fluorescence when in contact with divalent metal ions are currently used to detect them inside cells; their use 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". This study is a short report on the autometallograhical approaches for zinc detection in the central nervous system (CNS) by means of a modified selenium method.

Key words : AMG, CNS, Mouse, Rat, Selenium, ZEN, Zinc

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* Correspondence should be addressed to Dr. Moo Ho Won, Department of Anatomy, College of Medicine, Hallym University, Okchon-1-Dong Chunchon 200-702, Korea. Ph.: 033-240-1614, FAX: 033-256-1614, E-mail: mhwon@sun.hallym.ac.kr

INTRODUCTION

Zinc is one of the most abundant cations in the nervous system (0.15~0.12 mM in the mammalian brain) (Ebadi et al., 1994), where it is found associated to nucleic acid and a large variety of proteins, either bound to then by strong or labile bonds, or forming part of the molecule. Among the zinc-associated proteins, some of them are mainly extracellular (metalloproteins, growth factors), where others are intracellular (a great variety of cytoplasmic enzymes) and even intranuclear (nuclear replication, transcriptional enzymes and transcriptional regulators) (Vallee et al., 1991; Coleman, 1992). In the intracellular environment, free ionic zinc interacts with many regulatory enzymes and similarly to calcium, may act as a secondary messenger triggering/inhibiting important cellular processes, e.g. cell death and apoptosis (Fraker & Telford, 1997). Nerve cells may accumulate some divalent cations in normal physiological conditions (e.g. ionic zinc). In addition, acute or chronic experimental exposures (or environmental contamination) to some heavy metals (mercury, lead, gold, etc.) result in a dramatic acculmulation of these substances in nerve cells.

Therefore, it is acceptable that intracellular zinc homeostasis occurs. Perhaps this is the reason why a diversity of neurons have developed vesicular components with specific sequestering and storage mechanisms (Masters et al., 1994; Palmiter et al., 1997). These are the so-called zinc enriched (ZEN) neurons (Frederickson, 1989; Frederickson & Moncrieff, 1994), which accumulate glutamate and zinc inside their synaptic vesicles (Martinez-Guijarro et al., 1991) and release it 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 as well as excitatory synaptic effects (Westbrook & Mayer, 1987; Gordey et al., 1995). In spite of the high mobility of these ions and their strong interaction with an additional variety of proteins, it is possible to detect them by means of histo-cyto-chemical methods.

Direct methods may be used "in vivo" and for quantitative-microanalytical purposes although they do not allow usually good histological preservation. Indirect methods, although not very-suitable for quantitation, are much more sensitive and compatible with light and electron microscopy. Two widely used methodologies for the zinc autometallography (AMG) have been the addition of sulphide ions in the fixative (Timm and Neo-Timm methods) or the injection of selenium ions (Danscher selenium method) to the animal immediately before tissue fixation. It is very important that both sulphide and selenium salt precipitates remain insoluble along the histological process. Then, metal salt particles in the tissue sections are evidenced by AMG.

This study is a short report on the autometallograhical approaches for zinc detection in the central nervous system by means of a modified selenium method ($ZnSe^{AMG}$).

MATERIALS AND METHODS

Male Wistar rats (10 wks) and BALB/c mice (10 wks) were used for zinc autometallography in the pre-sent study. Animals were injected i.p. under Halothane anaesthesia with sodium selenide (10 mg/kg) dissolved in phosphate buffer (PB). One and a half hours later the animals were sacrificed by transcardial perfusion with 3% glutaraldehyde (GA) solution in PB. The brain and spinal cord segments were removed and placed in the same fixatives for 3 hrs in the refrigerator (4°C). The specimens were cryoprotected in 30% sucrose, followed by freezing with CO_2 gas, cutting at 20 μm thick on a Cryostat, and placement on rinsed slides with Farmer's solution (Danscher et al., 1985). The AMG silver enhancement was performed with the original silver lactate developer (Danscher 1982). After air drying the slides were dipped in 0.5% gelatine solution and allowed to dry for 10 min, then placed in jars with the AMG devel-

oper for 60 min. The AMG development was stopped by replacing the developer with 5% sodium thiosulphate solution. 10 min later the jars were placed under running warm tap water (40°C) for 10 min in order to remove the gelatine film, dipped twice in distilled water. The stained sections were dehydrated in alcohol, cleared in xylene, coverslipped with mounting media, then examined under light microscope.

Sections prepared for EM were cut 100 μm in thickness on a Vibratome. The AMG development were processed using the same methods for LM described above. Areas for EM analysis were isolated, placed in small jars and fixed in 1% OsO_4 in PB for 30 min. The small tissue blocks were then embedded in Epon 812, and survey sections were cut at 3 μm thick and counterstained with 0.1% toluidine blue. Sections selected for EM were re-embedded on top of a blank Epon block, from which ultrathin sections (90 nm) were cut. The ultrathin sections were double stained with uranyl acetate (30 min) and lead citrate (5 min), then examined on a Philips

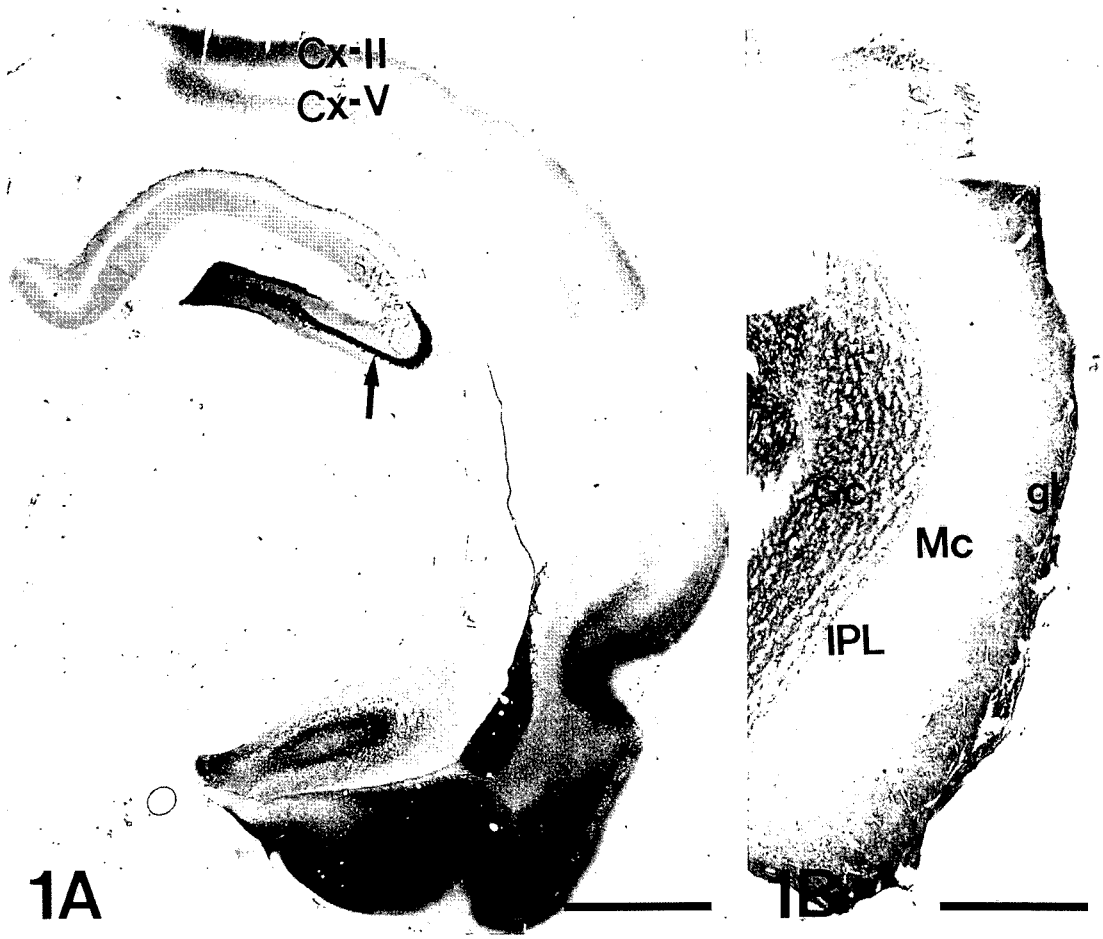

Fig. 1. Photographs showing the distribution of punctate AMG staining ($ZnSe^{AMG}$) on 20 μm-thick coronal cryosection of the rat brain (1A), and mouse olfactory bulb (MOB, 1B). Intense staining is present in neocortical layers I-III and V, hippocampus. Note that the laminar differences in AMG staining are clearly demonstrated. Granule cell layer (Gc) and olfactory glomerular layer (gl) show strong staining with less intense staining in the internal plexiform layers (IPL) and mitral cell layer (Mc). Scale bars: 2 mm in 1A, 500 mm in 1B.

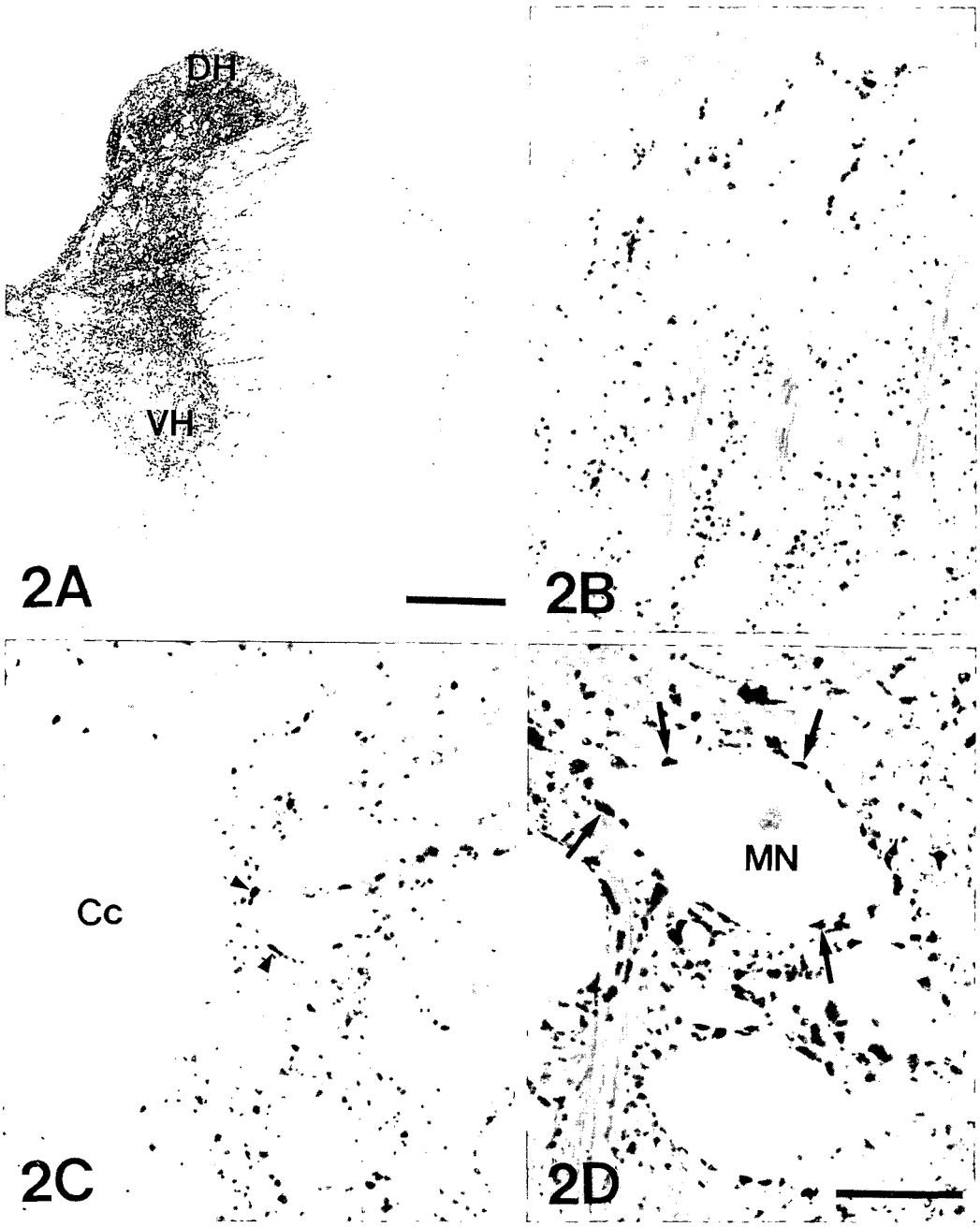

Fig. 2. Photographs taken from 20 μm-thick cryosection (2A) and 2 mm-thick epon section (2B-D) of thoracolumbar segment in the mouse spinal cord. Note that the different sizes of $ZnSe^{AMG}$ in neuropil of the gray matter (2B-D). 2B: dorsal horn (DH) laminae contain a lot of fine staining elements with a few coarse granules. 2C: many big-sized granules (arrowheads) are distributed around the central canal (Cc). 2D: a number of biggest grains (arrows) are distributed in the motor nucleus in the ventral horn (VH), especially around motor neurons (MN). Arrows indicate punctate $ZnSe^{AMG}$ closely related to somata and dendrites. All epon sections are counterstained with 0.1% toluidine blue. 2B-D are the same magnification. Scale bars: 200 μm in 2A, 50 μm in 2D.

(Eindhoven, The Netherlands) 208 electron microscope.

RESULTS

"Vivid" zinc staining is present in neocortical layers I-III and V, hippocampus (especially mossy fibers, CA 1-4, stratum radiatum and oriens, and dentate gyrus) (Fig. 1A), subiculum, amygdala, thalamus, striatum, and olfactory bulb; much less staining is found in cerebellum, brain stem; moderate staining in the spinal cord.

In the mouse olfactory bulb, staining for zinc ions with a selenium method revealed a clear demarcation between concentric layers of cells and neuropil in the mouse olfactory bulb (Fig. 1B). Light microscopy disclosed a dense staining in the gray matter with a characteristic segmental laminar pattern, while the white matter was unstained apart from rows of stained dots-like structures along what looks like dendritic projection radiating from the gray matter (Fig. 2). Reaction products of the $ZnSe^{AMG}$ appeared black dots with variable sizes and densities in a given lamina or area. The neuropil throughout the spinal cord exhibited AMG staining in different concentrations, showing a typical lamination in the gray matter.

Utrastructural studies have indicated that chelatable neuropil zinc highly localized to synaptic boutons, indeed possibly to a minority subpopulation of clear round vesicles with excitatory synaptic boutons. The ZEN terminals contained a large number of homogeneous round synaptic vesicles, and typically made asymmetrical synapses. $ZnSe^{AMG}$ grains could occasionally be found in the synaptic cleft (Figs. 3A-B). These terminals exhibited morphological characteristics such as electron dense axoplasm and densely packed spherical vesicles, and established synaptic contacts with thick postsynaptic thickening (Fig. 3B).

The dotted distribution of $ZnSe^{AMG}$ staining in both ventral and dorsal horns of the mouse spinal cord was corresponded ultrastucturally to ZEN terminals, where synaptic vesicles containing AMG silver was localized

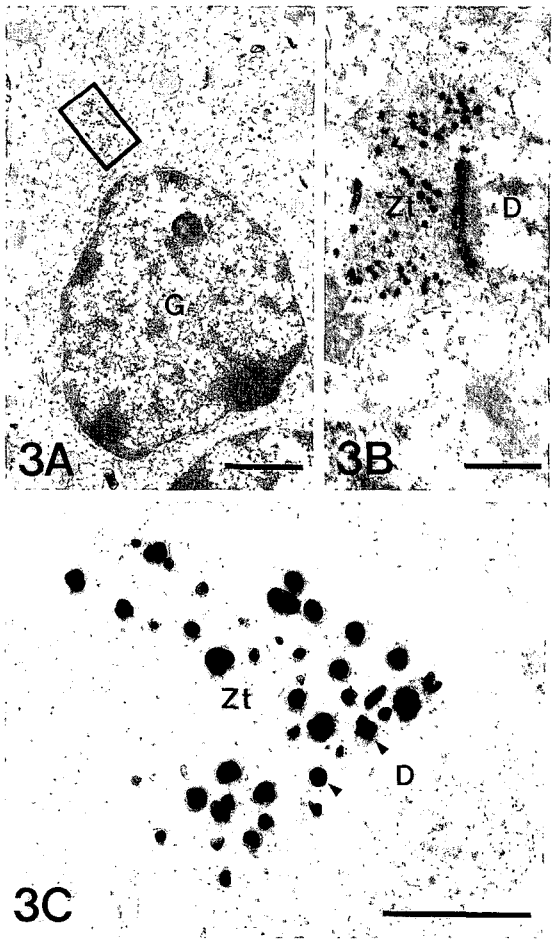

Fig. 3. Electron micrographs showing synaptic localization of $ZnSe^{AMG}$ in the granule cell (G) layer of the MOB (3A-B) and showing axodendritic synapses found in dorsal horn laminae II-IV of the mouse spinal cord (3C). Note that ZEN terminals (rectangle) found in the MOB are shown to make asymmetric synapses with dendrites in the granule cell layer, while a majority of the ZEN terminals (Zt) observed in the mouse spinal cord contain flattened synaptic vesicles and made symmetrical contacts to dendritic (D) elements. Arrowheads indicate the $ZnSe^{AMG}$ in the synaptic cleft (3C). Scale bars: (3A) 2 μm; (3B) 5 μm; (3C) 10 μm.

in presynaptic terminals. Most of ZEN terminals found in dorsal horn contained flattened synaptic vesicles and made symmetrical contacts, but a minority of ZEN terminals contained clear round (Fig. 3C), and made asymmetrical synaptic contacts. In general, big ZEN termi-

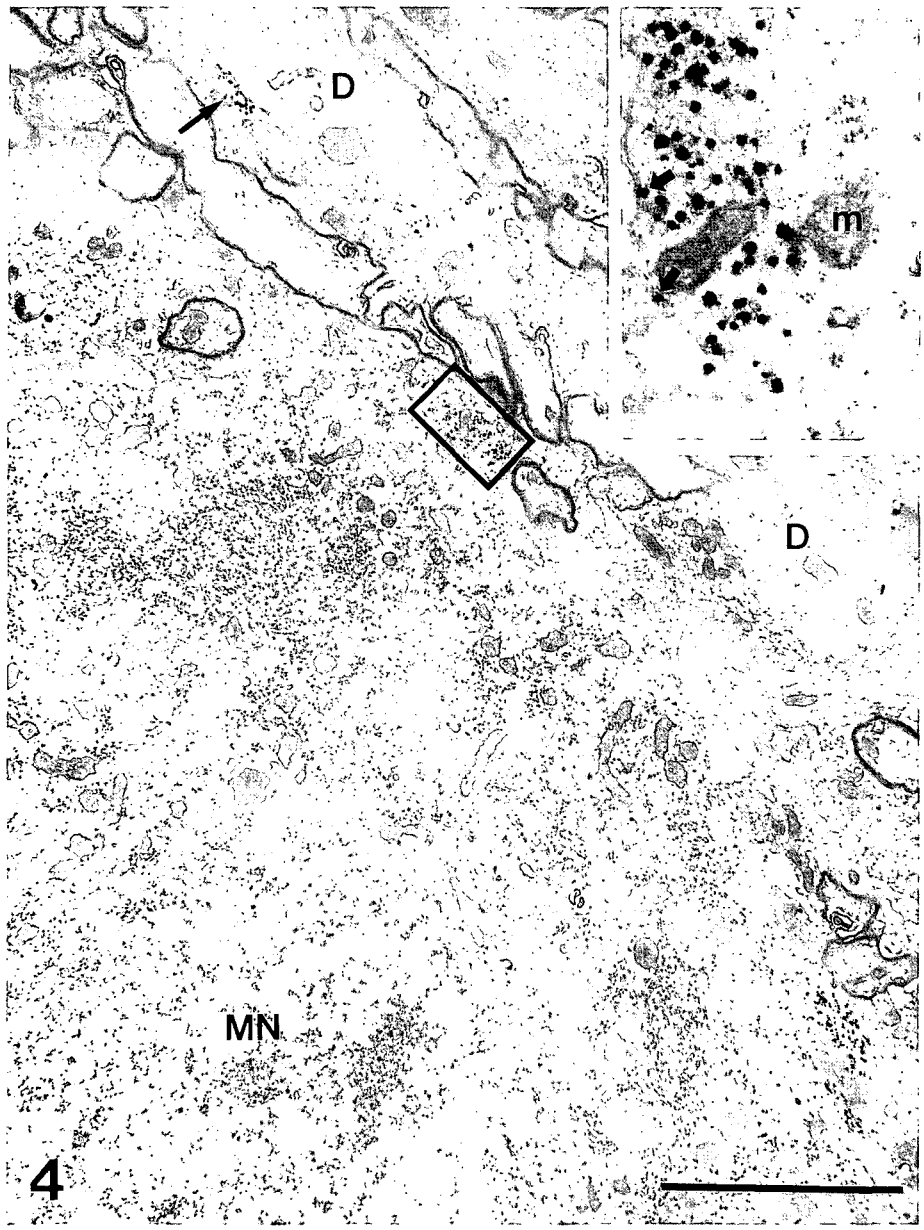

Fig. 4. Electron micrograph showing biggest-sized ZEN terminals (rectangle) contacting with motor neuron (MN) in upper thoracic spinal segment, and containing a number of the $ZnSe^{AMG}$ with mitochondria (m). Most of them make axo-dendritic (arrow) and axo-somatic symmetrical synaptic contacts to motor neurons. Note that a few $ZnSe^{AMG}$ are close to synaptic specializations (arrows, Inset). Inset is magnified five times of the rectangle. Scale bars indicate 5 μm.

nals were presynaptic to dendritic elements or cell bodies, while small ones to small calibered dendrites. A few axo-axonal synaptic complex were observed in the superficial dorsal horn. ZEN terminals found in ventral neuropil were generally bigger than those in dorsal horn. Most of ZEN terminals were presynaptic to both

large and small dendritic profiles as well as motoneuronal somata (Fig. 4).

DISCUSSION

The present study demonstrated the presence of ZEN terminals in the central nervous system of the rat and mouse, through the use of a specific staining method for ionic zinc ($ZnSe^{AMG}$).

Silver autometallography (Liesegang, 1911) offers the opportunity of revealing very small metal salt particles which catalyze the reduction of silver ions to metallic silver by hydroquinone. In this way, a shell of metallic silver is formed around the catalytic center; this silver shell may grow continuously until it is detected by light and electron microscopy. In short, intraperitoneal injection of a variety of selenium compounds (usually sodium selenite) in live animals, followed by a short survival time prior to aldehyde perfusion results in precipitation of metallic cations. Selenium metal salts precipitates may be evidenced by autometallographic development of tissue sections, using the developing solution of the Neo-Timm method (Danscher, 1982). It is generally accepted that zinc evidenced by the selenium method is the same that is labeled by dithizone.

The staining pattern was consistent with the idea that histochemically-reactive zinc is present on synaptic vesicles. In the nervous system, $ZnSe^{AMG}$ has been used in the study of zinc enriched 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 demonstrated mercury, gold and cadmium in section (Danscher & Moller-Madsen, 1985) using elegant strategies to differentiate between silver, gold or mercury deposits (Danscher et al., 1994). Similarly to the Neo-Timm method, the selenium method may also been used in combination with other histochemical and immunocytochemical techniques (Geneser et al., 1993).

The differences in size and density of ZEN terminals along the brain regions are intriguing. These different size of the ZEN terminals may emanate from distinct subpopulations of ZEN neurons, or perhaps from the same ZEN neuron, but along a different arborization. Selenium metal salt precipitates are far more stable than those of sulphur; this facilitates histological processing of samples. However, since selenite compounds show differential toxicity among experimental animals, it is necessary to find the correct intraperitoneal doses and the optimum survival time before sacrifice for every animal species, and even for a defined ages. Sometimes, inappropriate selenite doses may give artifactual results, e.g., differential precipitation of metal ions around blood vessels (Howell et al., 1989) or even incomplete precipitation of metal ions around fields populated by structures bearing large amounts of metal ions. In the present study, most of ZEN terminals were localized on soma and proximal dendrites. These findings support the idea that ZEN terminals are likely to have a profound influence on their counterparts in the CNS. In addition, $ZnSe^{AMG}$ provided very good ultrastructural preservation of the tissue. In the case of synaptic vesicles, the electron dense precipitates appear adhered to inner-side of the membrane. These precipitates may even be seen in the synaptic cleft after vesicle exocytosis.

In conclusion, the ZEN terminals are clearly visualized by $ZnSe^{AMG}$. Functionally the present data suggest that ZEN terminals may be involved in a variety of neurobiological roles throughout various regions of the CNS.

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<국문초록>

Zinc는 인체 내에 철(Fe^{2+}) 다음으로 많은 trace element로서 200여개 효소의 기능에 필수적일 뿐만 아니라 신경계통내에서는 신경조절물질로 작용한다. 뿐만 아니라 허혈, 간질 및 퇴행성 뇌질환의 주요 병리기전에도 관여되어 있다. 그러나 대부분의 Zinc는 단백질에 결합되어(bound form) 신경세포의 세포질 및 핵질내에 존재하고, 10% 이하의 Zinc는 이온상태(free form, Zn^{2+})로 신경종말(Zinc enriched terminal)에 있는데, 후자만이 조직화학법으로 가시화된다. 최근까지 새로 개발된 조직화학법으로 Zinc enriched (ZEN) neurons의 분포에 관한 연구가 각광받고 있으나, 국내에서는 이에 대한 연구가 전무한 실정이다. 이에 본 연구자는 고전적인 조직화학법의 기본 원리를 소개하고, 랫드 중추신경계통내 Zinc의 분포를 광학 및 전자현미경으로 관찰하고자 하였다.

본 연구에서 사용된 실험동물은 Wistar 계통의 랫드(10주령)와 BALB/c 마우스이며, 마취제로는 Pentobarbital (50 mg/kg)을 이용하였다. 생체 뇌조직내 이온상태의 Zinc (Zn^{2+})를 침전시키기 위하여 selenium (10 mg/kg, i.p.)을 처리하였고, 1시간 후 3% Glutaraldehyde 액으로 관류고정하여 동물을 희생시켰다. 뇌와 척수를 꺼내어 sucrose에 가라앉을때 까지 담가두었다가 Dry Ice를 이용하여 얼리고, Freezing microtome 위에서 30 μm 두께의 절편을 작성하였다. 조직절편내 Zn^{2+}을 동정하기 위한 조직화학법으로는 autometallography (AMG) (Danscher, 1985)를 이용하였다.

광학현미경하에서 밝혀진 Zinc의 분포는 해마복합체

를 비롯한 종뇌의 여러부위에 고농도로 분포하였고, 척수에는 중간정도, 그리고 소뇌 및 뇌간에는 매우 낮은 농도로 분포하였다. 전자현미경에서 관찰된 AMG 염색과립(silver grains)은 신경종말에 있는 연접소포에 국한되었으며, 이러한 ZEN terminals은 주위 여러 신경세포의 돌기(dendrites) 및 세포체(soma)에 특이한 연접을 이루고 있었다. 즉 후각망울을 포함한 종뇌에서는 주로 비대칭연접 (asymmetrical synapses)이 관찰되었던 반면에, 척수에서는 대칭연접(symmetrical synapses)을 이루고 있었다.

이상의 결과를 종합하면, 신경종말내 연접소포에 Zinc를 함유하고 있는 소위 ZEN terminals은 중추신경계통에 광범위하게 분포하고 있으며, 또한 신경부위에 따라 다양한 분포와 미세구조의 차이를 보였다. 이러한 사실은 중추신경계통내에서 Zinc가 영위하는 신경생물학적 기능이 신경부위에 따라 다양할 것임을 시사한다.