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

Yi Suk Kim, Sang Hyun Kim, Beob Yi Lee[1], Hyun Sook Lee[1],
Sung Joo Kim[2] and Seung Mook Jo*

Department of Anatomy, Gachon Medical Graduate School
[1]Department of Anatomy, Konkuk Medical Graduate School
[2]Department of Obstetrics and Gynecology, Hallym Sacred Heart Hospital
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중추신경계통 내 분포하는 zinc 이온의 조직화학적 동정법 비교: I. 광학현미경수준에서

김이석, 김상현, 이법이[1], 이현숙[1], 김성주[2], 조승묵*

가천의과학대학교 의학전문대학원 해부학과,
[1]건국대학교 의학전문대학원 해부학교실,
[2]한림대학교 한림대성심병원 산부인과

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-

gering/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; Palmiter 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; Gordey 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.

Palmiter 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 $ZnSe^{AMG}$ 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 to 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 ($ZnSe^{AMG}$)

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 & Moller-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 CO_2 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 26°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 ($ZnT3^{IHC}$)

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 immuno-labelling 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 (H_2O_2) 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% H_2O_2 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 CO_2 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. ZnSe^AMG

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 ZnSe^AMG staining for zinc ions revealed a clear demarcation between concentric layers of cells and neuropil in the olfactory bulb. Strongly intense ZnSe^AMG staining was found in the granular cell and olfactory glomerular layers. On the other hand, moderate ZnSe^AMG 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).
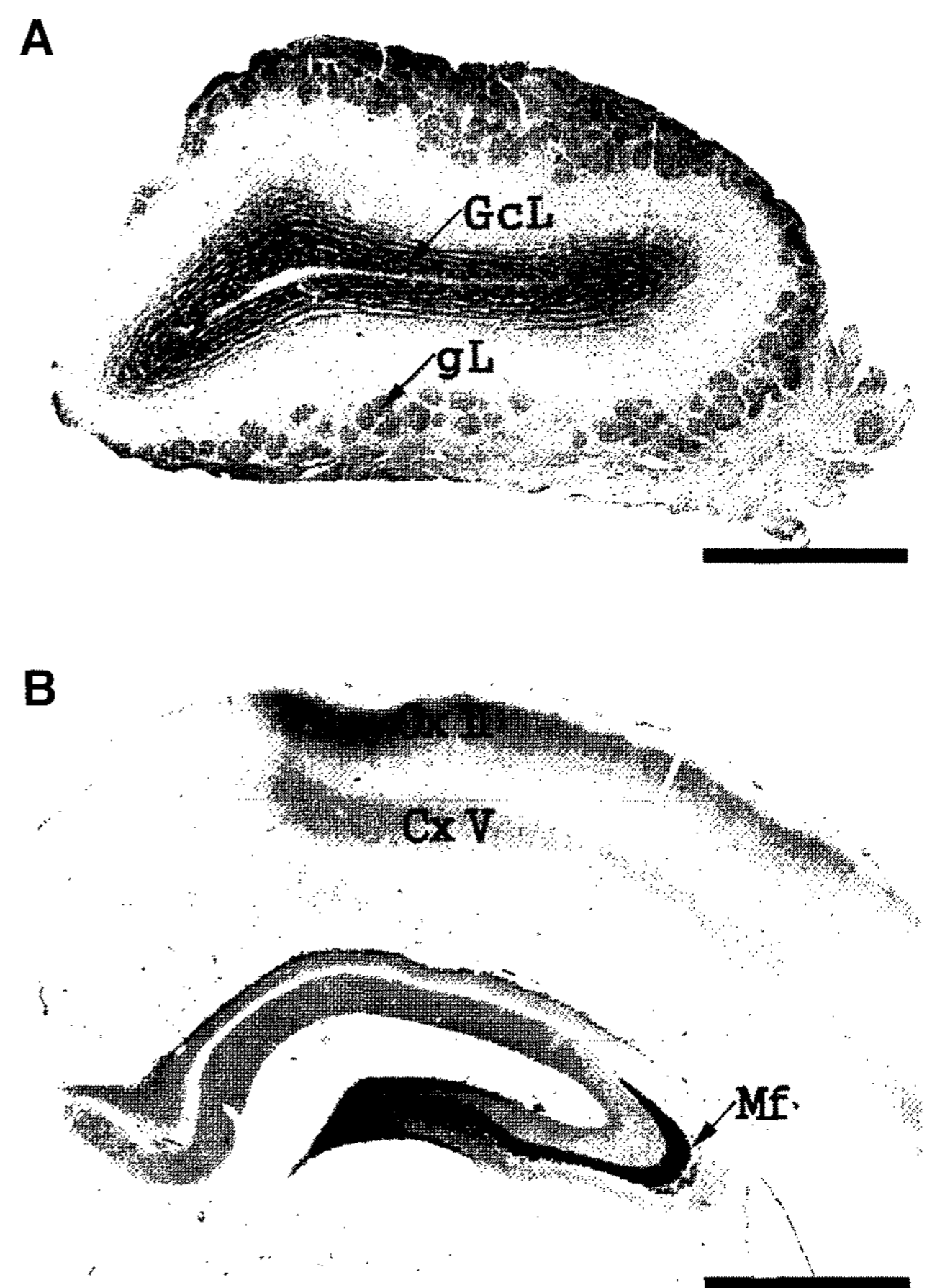

Fig. 1. Light micrographs showing autometallographic (AMG) staining for zinc in the olfactory bulb (A) and cerebral cortex including hippocampus (B). Fig. 1A & B are taken from 30 μm-thick cryo-section. Note dense staining in the granular cell layer (GcL) and glomerular layer (gL) of the olfactory bulb, and cortical layers Cx II and V of the cerebral cortex, and Mossy fiber (Mf) of the hippocampal complex. Scale bars: (A) 500 μm; (B) 1 mm.

AMG for Zinc in the CNS

A

B
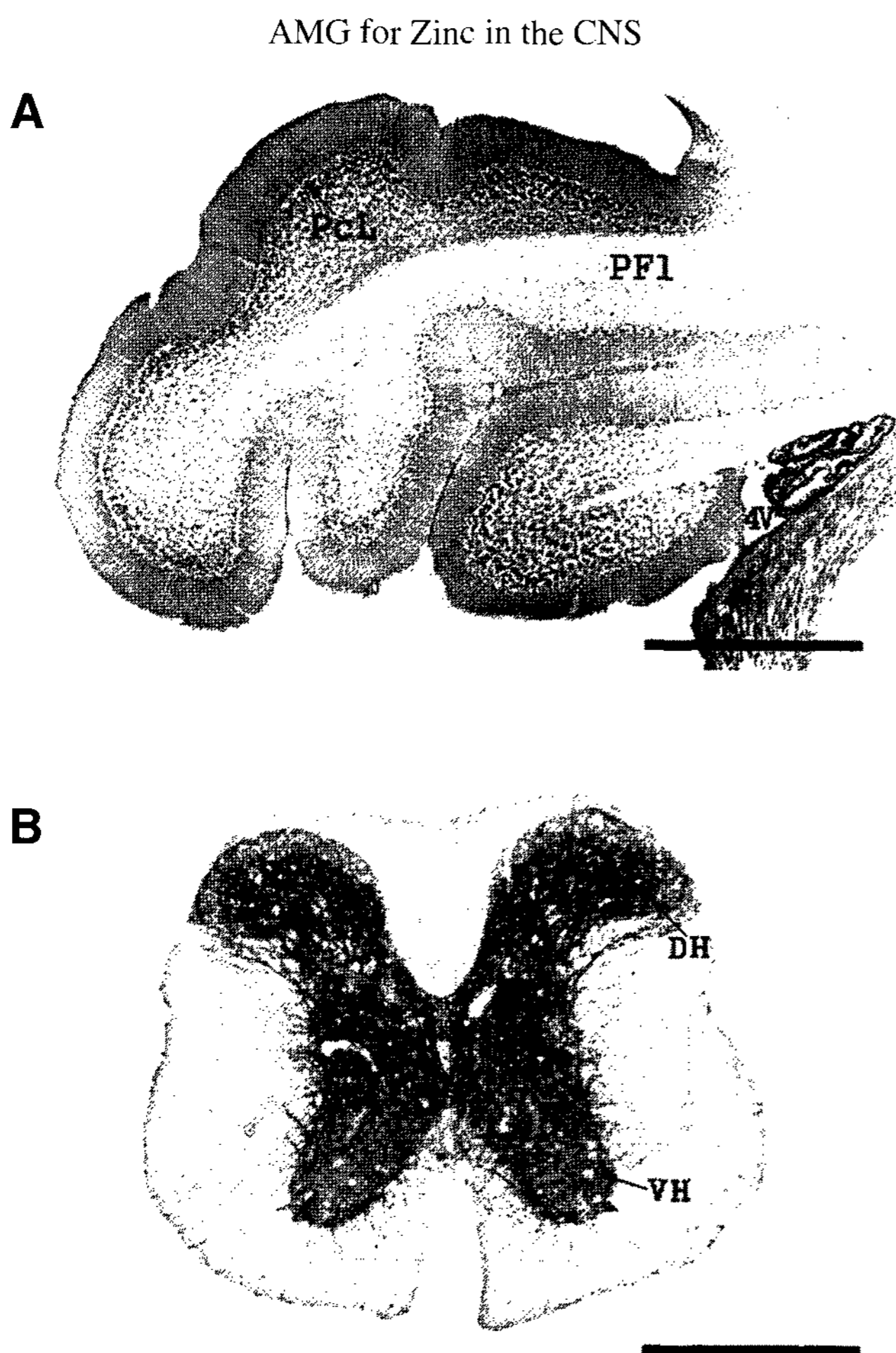
Fig. 2. Light micrographs showing autometallographic (AMG) staining for zinc in the cerebellum (A) and spinal cord (B). Fig. 2A is taken from 30 μm-thick cryosection of parafloccular lobe (PFl). Note that the cortical layer including Purkinje cell layer (PcL, arrow) is the most stained, while medulla is almost unstained. Fig. 2B shows laminar pattern of the AMG staining in the grey matter. Note intense staining in the superficial dorsal horn and regions around the central canal, and relatively weak staining is seen in ventral horn. Asterisk indicates part of 4th ventricle with darkly stained choroid plexus. DH, dorsal horn; VH, ventral horn. Scale bars: (A) 400 μm; (B) 100 μm.

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).

Different staining patterns
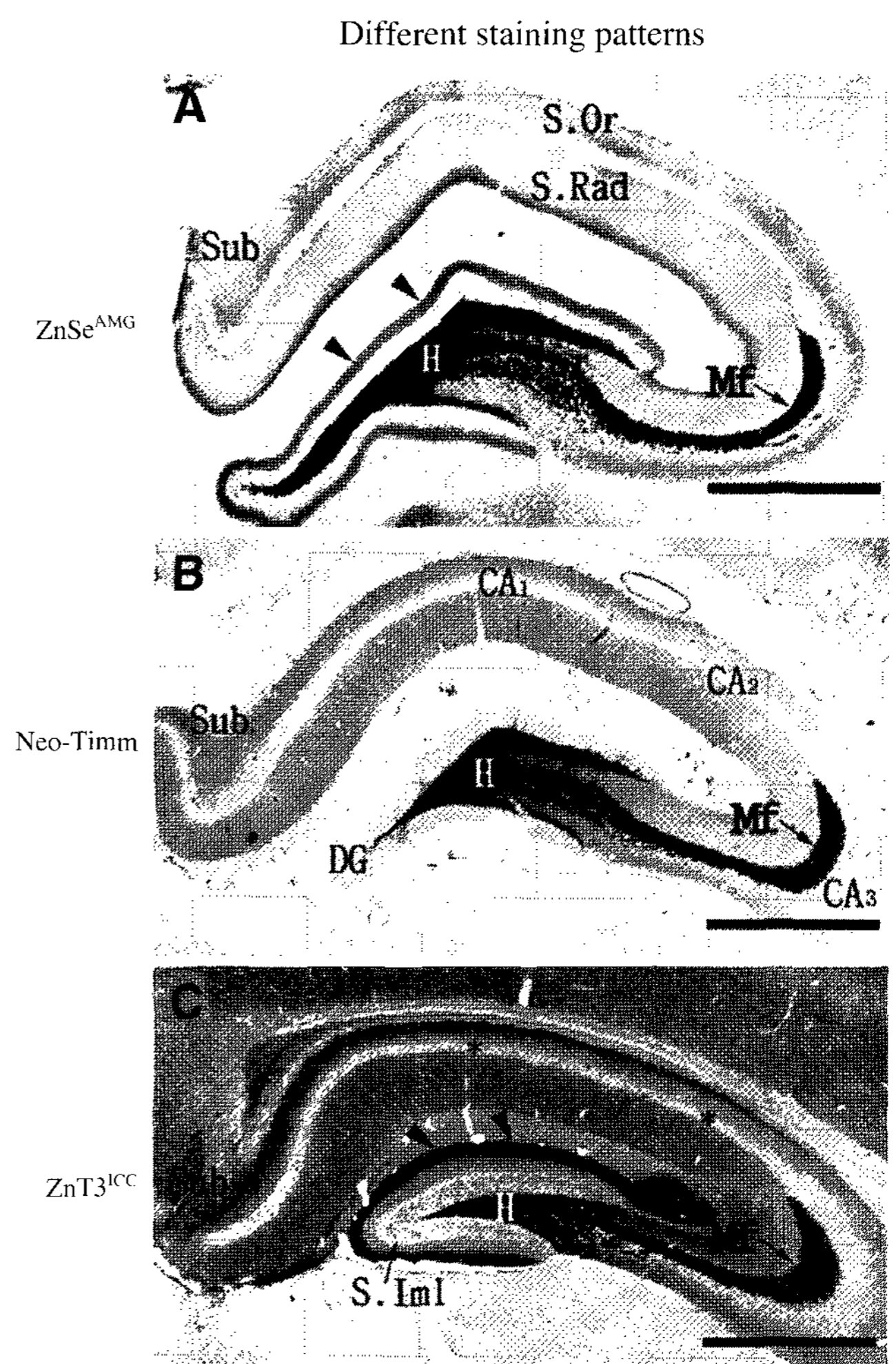
Fig. 3. Light micrographs showing different staining patterns according to different staining methods for zinc detection in the rat hippocampus. Fig. 3A, B, and C correspond to zinc staining with ZnSe^{AMG}, Neo-Timm, and zinc ZnT3^{IHC}. The general staining pattern is almost identical, that is, most intense staining is found in hilus (H) and Mossy fiber (Mf) in CA$_3$ whereas moderate staining is observed in the stratum oriens (S. Or) and radiatum (S. Rad) through CA$_1$ and CA$_2$ of the hippocampus. However, darkly stained area (arrowheads), dentate inner molecular layer (iml), in Fig. 3A is unstained in the sections stained by Neo-Timm and ZnT3^{ICC}, and darkly stained area (arrowheads), dentate outer molecular layer (oml), in Fig. 3C don't presence in the sections stained by ZnSe^{AMG} and Neo-Timm. DG, dentate gyrus; S. Iml, dentate inner molecular layer; Sub, subiculum. Scale bars: (A ~ C) 400 μm.

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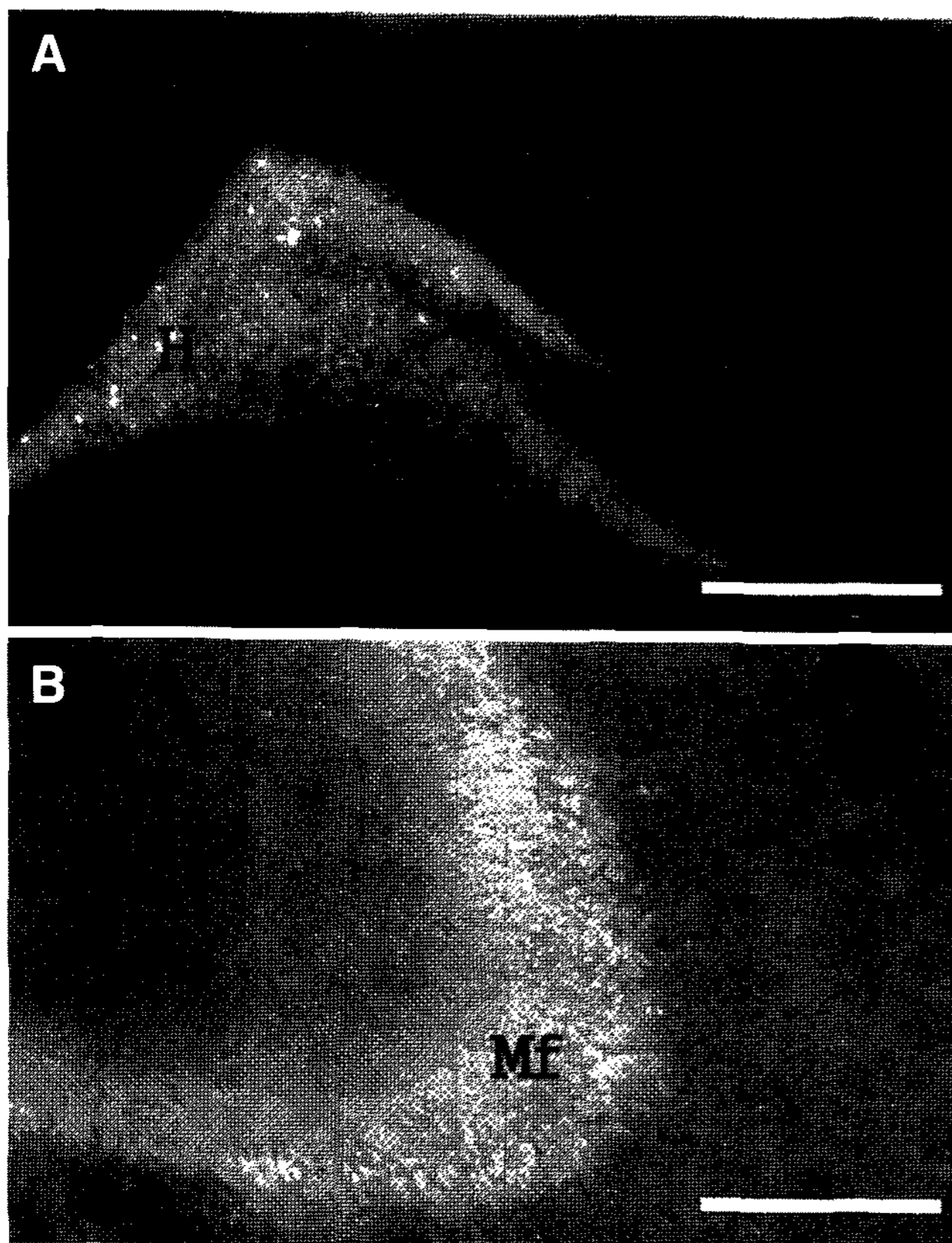
Fig. 4. Fluorescent micrographs taken from 30 μm thick-cryosection of the hippocampus, showing dense fluorescence of TSQ, a specific marker for zinc concentrated in the hilus (H) and Mossy fiber (Mf). Note vivid fluorescent staining of the mossy fiber plexus and hilus. Scale bars: (A) 40 μm; (B) 40 μm.

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 ZnSe^AMG 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, ZnSe^AMG 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 been 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 ZnT3^IHC, and darkly stained area, dentate outer *molecular layer was not present in the sections stained by* ZnSe^AMG 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 ZnSe^AMG 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

Assaf SY, Chung SH: Release of endogenous Zn^{2+} from brain tissue during activity. Nature 308 : 734-736, 1984.

Coleman JE: Zinc proteins: Enzymes, storage proteins, transcription factors, and replication proteins. Annu Rev Biochem 61 : 897-946, 1992.

Danscher G: Exogenous selenium in the brain. A histochemical technique for light and electron microscopical localization of catalytic selenium bonds. Histochemistry 76 : 281-293, 1982.

Danscher G, Juhl S, Stoltenberg M, Krunderup B, Schrøder HD, Andreasen A: Autometallographic silver enhancement of zinc sulfide crystals created in cryostat sections from human

brain biopsies. A new technique that makes it feasible to demonstrate zinc ions in tissue sections from biopsies and early autopsy material. J Histochem Cytochem 45 : 1503-1510, 1997.

Danscher G, Moller-Madsen B: Silver amplification of mercury sulfide and selenide: a histochemical method for light and electron microscopic localization of mercury in tissue. J Histochem Cytochem 33 : 219-228, 1985.

Danscher G, Stoltenberg M, Juhl S: How to detect gold, silver and mercury in human brain and other tissues by autometallographic silver amplification. Neuropathol Appl Neurobiol 20 : 454-467, 1994.

Ebadi M, Elsayed MA , Aly MHM: The importance of zinc and metallothionein in brain. Boil Signals 3 : 123-126, 1994.

Fraker PJ, Telford WG: A reappraisal of the role of zinc in life and death decisions of cells. Proc Soc Exp Biol Med 215 : 229-236, 1997.

Frederickson CJ: Neurobiology of zinc and zinc containing neurons. Int Rev Neurobiol 31 : 145-238, 1989.

Frederickson CJ, Hernandez MD, McGinty JF: Translocation of zinc may contribute to seizure-induced death of neurons. Brain Res 480 : 317-321, 1989.

Frederickson CJ, Kasarskis EJ, Ringo D, Frederickson RE: A quinoline fluorescence method for visualizing and assaying the histochemically reactive zinc (bouton zinc) in the brain. J Neurosci Meth 20 : 91-103, 1987.

Frederickson CJ, Moncrieff DW: Zinc-containing neurons. Biol Signals 3 : 127-139, 1994.

Geneser FA, Holm IE, Slomianka L: Application of the Timm and selenium methods to the central nervous system. Neurosci Prot 93-050-15 : 1-14, 1993.

Gordey M, Kang MH, Olsen RW, Spigelman I: Zinc modulation of GABAA receptor-mediated chloride flux in rat hippocampal slices. Brain Res 691 : 125-132, 1995.

Howell GA, Welch MG, Frederickson CJ: Stimulation-induced uptake and release of zinc in hippocampal slices. Nature 308 : 736-738, 1984.

Jo SM, Danscher G, Schrøder HD, Won MH, Cole TB: Zinc-enriched (ZEN) terminals in mouse spinal cord: immunohistochemistry and autometallography. Brain Res 870 : 163-169, 2000.

Martinez-Guijarro FJ, Soriano E, Del Rio JA, Lopez-Garcia C:

Zinc-positive boutons in the cerebral cortex of lizards show glutamate immunoreactivity. J Neurocytol 20 : 834-843, 1991.

Masters BA, Quaife CJ, Erickson JC, Kelly EJ, Froelick GJ, Zambrowicz BP, Brinster RL, Palmiter RD: Metallothionein III is expressed in neurons that sequester zinc in synaptic vesicles. J Neurosci 14 : 5844-5857, 1994.

Palmiter RD, Cole TB, Quaife CJ, Findley SD: ZnT-3, a putative transporter of zinc into synaptic vesicles. Proc Natl Acad Sci USA 93 : 14934-14939, 1997.

Schionning JD, Danscher G, Christensen MM, Ernst E, Moller-Madsen B: Differentiation of silver-enhanced mercury and gold in tissue sections of rat dorsal root ganglia. J Histochem 25 : 107-111, 1993.

Vallee BL, Coleman JE, Auld DS: Zinc fingers, zinc clusters, and zinc twists in DNA-binding protein domains. Proc Natl Acad Sci, USA 88 : 999-1003, 1991.

Westbrook GL, Mayer MLn: Micromolar concentrations of Zn^{2+} antagonize NMDA and GABA responses of hippocampal neurons. Nature 328 : 640-643, 1987.

< 국문초록 >

본 연구자는 Zinc Selenium autometallography ($ZnSe^{AMG}$) (Danscher et al., 1997) 염색법을 중심으로 중추신경계통 내 zinc (Zn^{2+})의 분포와 이들을 함유하고 있는 신경종말, 소위 ZEN (zinc-enriched) terminals의 미세구조에 관하여 보고한 바 있다.

이번 연구에서는 다른 몇 가지, 즉 Neo-Timm staining (Danscher, 1982), TSQ fluorescence staining (Frederickson et al., 1987), Zinc transporter-3 Immunohistochemistry ($ZnT3^{IHC}$) (Palmiter et al., 1997) 염색법으로 흰쥐 해마복합체에 분포하는 Zn^{2+}를 염색한 후 이들의 염색패턴에서 차이점을 밝히고자 하였다.

$ZnSe^{AMG}$염색법은 Zn^{2+}에 대한 특이성은 다소 떨어지나 광학 및 전자현미경하에서 관찰이 가능하며, 반영구적인 표본으로 보관이 가능하다는 장점이 있었고, TSQ는 Zn^{2+}에 대한 특이성이 매우 높을 뿐 아니라 그 염색법이 매우 간단하다는 장점이 있는 반면 형광물질의 안정성과 표본보관이 용이하지 않다는 단점이 있다. 그 외 Neo-Timm 염색법은 TSQ형광염색법과 유사한 염색 패턴을 보였으며, $ZnT3^{IHC}$ 염색법은 오히려 $ZnSe^{AMG}$에 가까운 염색의 결과를 보였다.

본 연구의 결과는 중추신경계통 내 Zn^{2+}에 관한 형태학적 연구에서 기초자료로 활용될 수 있을 것이다.