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Histochemically-reactive Zinc in the Rat Dorsal Root Ganglion (DRG) Neurons: Zinc Selenium Autometallography

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랫드 척수신경절내 zinc의 분포양상: Zinc Selenium Autometallography

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ABSTRACT

The present study was designed to demonstrate ionic zinc in the rat DRG by means of zinc selenium autometallography ($ZnSe^{AMG}$).

Ganglion cells varied in size from 15 to 100 μm. The smaller neurons were strongly stained with AMG, whereas the larger cells were weakly stained. Each large ganglion cell was surrounded by perineuronal satellite cells, showing apparent AMG staining. We demonstrated for the first time the existence of zinc-containing satellite cells in the rodent DRG.

Using electron microscopy, fine AMG grains were observed scattered in the somata of the DRG neurons, especially small cells. However, much lower concentrations of the AMG grains occupied in the large cells, and these were mostly localized in lysosome-like organelles.

These results indicate that zinc may be involved in sensory transmission in the DRG level.

Keywords : AMG, DRG, Lysosome, Rat, Satellite cell, Zinc

In the mammalian brain, less than 10% of the total zinc is loosely-bound zinc and these ions can be visualized either by autometallography (AMG) (Danscher, 1981) or the toluene sulfonamide quinoline (TSQ) fluorescence method (Frederickson et al., 1990). Terminals that contain zinc ions in a population of synaptic vesicles have been termed Zinc-ENriched (ZEN) terminals (Danscher, 1994).

The distribution of ZEN terminals is well-described in telencephalic structures such as neocortical layers I-III and V, hippocampus (Frederickson & Danscher, 1990), and amygdala (Pérez-Clausell & Danscher, 1985). Ultrastructurally, zinc ions are found by AMG to be localized in clear round vesicles in ZEN terminals making asymmetric synapses and being immunoreactive to glutamate (Martinez-Guijarro, 1991).

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Zinc is a small, hydrophilic, highly charged species, which cannot cross biological membranes by passive diffusion. Therefore, specialized mechanisms are required for both its uptake and its release. Recently, a murine zinc transporter called ZnT3 has been cloned by Palmiter et al. (1996). In the brain, ZnT3 appears to be limited to the ZEN terminals, suggesting that zinc plays an important role in those terminals.

We have recently demonstrated the existence of histochemically-reactive zinc in the DRG of the rat (Lee et al., 2005) under the light microscope. Ganglion cells vary in size from 15 to 100 μm. The smaller neurons are strongly stained with AMG, whereas the larger cells are not almostly stained. Each large ganglion cell is surrounded by perineuronal satellite cells, showing apparent AMG stainity. So far, however, peripheral nervous system (PNS) have no impressive ZEN systems. The present study was designed to further identify the existence of possible ZEN neurons in the DRG and fine structures of the DRG neurons containing zinc ions using zinc selenium autometallography ($ZnSe^{AMG}$).

Male Sprague-Dawley (SD) rats (200 ~ 225 g) were used. They were housed on a 12-h light/dark cycle with food and water *ad libitum*. Rats were allowed *ad libitum* access to food and water. They were housed in a room where temperature was controlled constantly at 20°C with a before tissue collection. All procedures for the animal treatment were carried out in accordance with the regulations of the Animal Ethical Committee at Gachon Medicine & Science University and the animal protection laws of Korea.

Rats were treated with 0.1% sodium selenite (10 mg/kg, i.p. dissolved in 0.1 M PB, pH 7.4) under ether anesthesia. The animals were allowed to survive for 1.5 h or 24 h, and then anesthetized with Pentobarbital and killed by a transcardial perfusion with 0.9% saline followed by 3% glutaraldehyde in 0.1 M PB. The DRGs (L5-S1) were dissected and postfixed with the same fixative for 3 h at 4°C. The samples were cryoprotected in 30% sucrose overnight, frozen with CO_2 gas, and cut into 30 μm thick sections. After air drying, sections were dipped in a 0.5% gelatin solution and allowed to dry for 10 min. Sections were placed in a vial and covered by the AMG developer (Danscher, 1981). The whole setup was covered by a dark hood through the 60 min development at 26°C. The AMG development was stopped by replacing the AMG developer by a 5% sodium thiosulfate solution. After 10 min, the sections were placed under 30°C running tap water for 10 min in order to remove the gelatin film. After several rinses in distilled water, the sections were dehydrated in alcohol, cleared in xylene, counterstained with 0.1%Toluidine Blue (TB), coverslipped with DEPEX and finally examined and photographed. Two rats without any treatment served as controls. No silver grains could be seen after AMG development. The DRGs were also stained by routine hematoxylin-eosin (H-E) preparation.

For electron microscopy, the samples were cut into 100 μm thick sections on a vibratome. These sections were incubated in the AMG developer for 60 min. The development was stopped by a thiosulfate solution for 10 min. After a careful rinse the sections were placed in 1% osmium acid in 0.1 M PB (pH 7.4) for 30 min. After embedding in Epon, semithin sections 2 μm thick were cut and also counterstained with TB for light microscopic analysis. Sections to be analyzed in the electron microscope were reembedded on top of a blank Epon block and ultrathin sections were cut and stained with uranyl acetate and lead citrate. The sections were analyzed and photographed in a Philips 208 electron microscope.

We used selenite stain to identify sensory neurons that are zinc-containing. The $ZnSe^{AMG}$ technique involves two steps: 1) the zinc ions are bound *in situ* in the live tissues as zinc selenide molecules that accumulate as zinc-selenium crystal lattices; 2) these nano-sized lattices are catalytic to AMG, i.e. if present in sections that are exposed to an AMG developer they will be silver-enhanced. The localization of zinc ions can then be observed both light and electron microscopically (Danscher and Montagnese, 1994).

One hour after an intraperitoneal injection of 20 mg/kg sodium selenite, rats were weak and had profound diarrhea similar to that previously described by Slomianka et al. (1990).

Ganglion cells varied in size from 15 to 100 μm. Within DRG, a single layer of neural crest-derived satellite cells usually surrounded, to form a continuous investment around, each neuron body. These cells were next to surface of ganglion somas, although an artifact in conventional paraffin sections often leaved an artificial space between the neuronal soma and satellite cell, and only their nuclei were typically visible in routine H-E preparations (Fig. 1).

The speckles of selenite stain in large cells were conspicuously absent, whereas there was more concentrated precipitate in small cells. That is, a population of small cell were AMG-positive. These ZEN neuronal somata were randomly spread throughout the ganglion. However, the perinuclear zones always stained most strongly (Fig. 2).

Each large cell is surrounded by a few satellite cells, showing apparent AMG stainity. We demonstrates, for the first time, the existence of zinc-containing satellite cells in the rodent

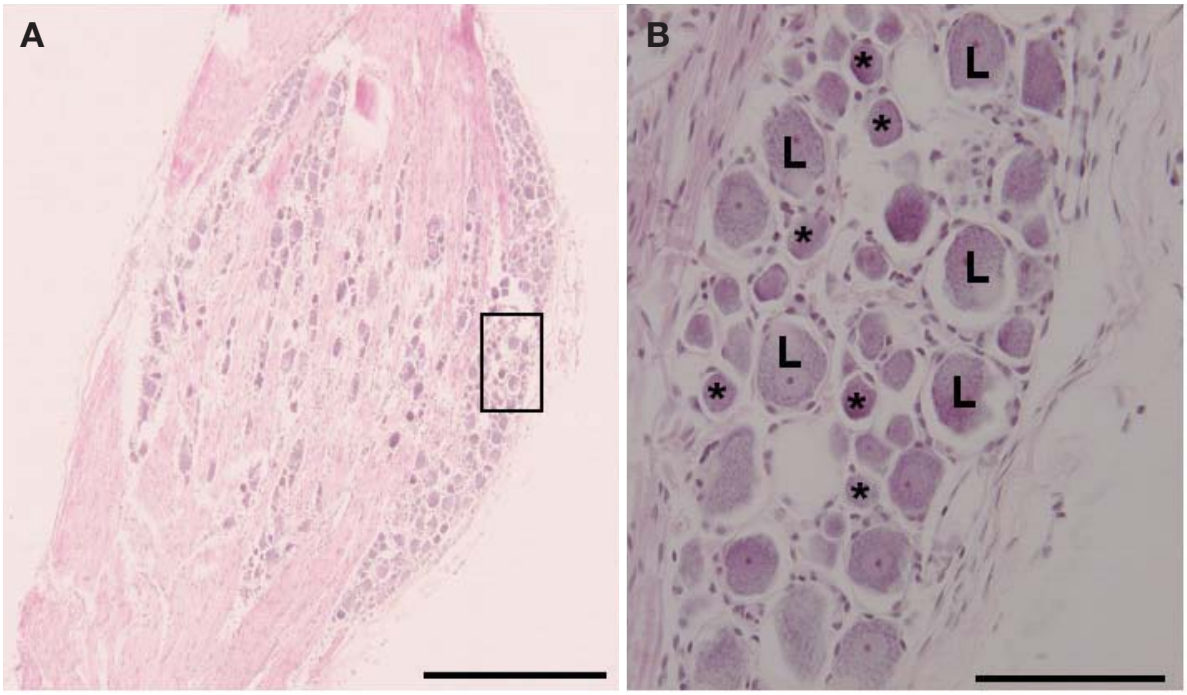

Fig. 1. Light micrographs taken from rat DRG stained by H-E. Sensory neurons within dorsal root ganglia are divided into two classes on the basis of perikaryal size. Asterisks show the darkly stained small ganglion cells, whereas the large cells (L) are faintly stained. The clear space around the neurons and the surrounding cells is an artifact caused by the tissue shrinkage during chemical preparation of the dorsal root ganglion. The rectangle on left panel is magnified on the right panel. Satellite cells are represented by the very small nuclei at the periphery of the neuronal cell bodies. Bars in Fig. 1A & 1B indicate 500 μm & 100 μm, respectively.

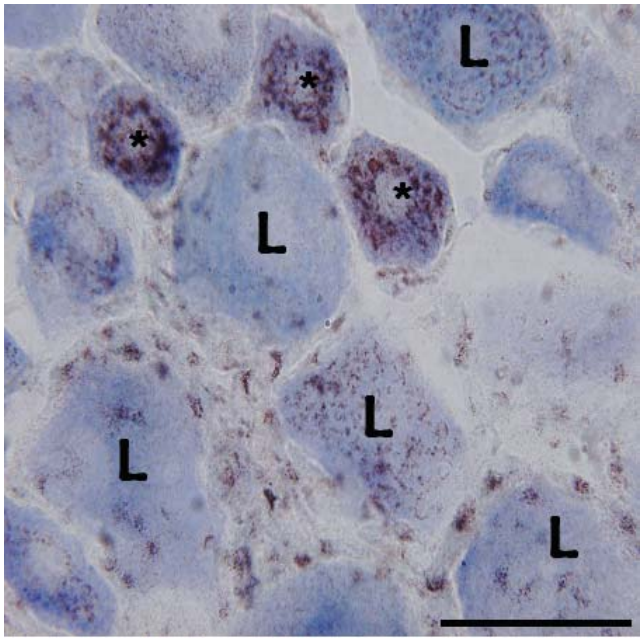

Fig. 2. Light micrograph taken from rat L5 DRG stained by $ZnSe^{AMG}$. Both small- (asterisks) and large (L)-diameter ganglion cells are stained in DRG tissue. A concentrated and uniform precipitate are more characteristic in small cells than those with large cells. The cellular stain observed in large-diameter neurons consisted of speckles of precipitate, often with a clear center. Scale bar: 100 μm.

DRG. At higher magnification, dot-like structures with strong AMG stainity could be seen in the perinuclear area of the perikarion (Fig. 3).

The satellite cells help to establish and maintain a controlled microenvironment around the neuronal body in the ganglion, providing electrical insulation as well as a pathway for metabolic exchanges. Thus in its functional role the satellite cell is analogous to the Schwann cell except that it does not make myelin. Satellite cells are specialized glial cells that surround the cell bodies found in DRG. (Ross & Pawlina, 2006; Ovalle et al., 2008; Kerr, 2010).

Zinc is transported into the brain via not only the blood-brain barrier but also the blood CSF barrier. Zinc is taken up by neurons, which may have two zinc uptake sites, i.e. the cell body and the neuron terminal, and also by glial cells, and it is then incorporated into zinc-binding proteins (Takeda, 2000).

One and one half hour after an i.p. injection of a sodium selenite solution, a subpopulation of DRG cells expressed varying degrees of AMG positive staining. The staining intensity of the ZEN neurons was related to the size or location of the ganglion cells. The AMG grains were located in the perinuclear zone confined to the Golgi complex and the vesicular

structure. At the electron microscopic level, the AMG grains were predominantly distributed in the perinuclear zone (Fig 4A). At higher magnifications AMG grains were found to be located primarily in membrane-enclosed structures in the Golgi region. Each large ganglion cell is surrounded by perineuronal satellite cells containing AMG grains (Fig. 4B). Some AMG grains were seen where an enclosing membrane could not be ensured. Additionally, AMG grains were located in unmyelinated axons. Few unspecific AMG grains were seen randomly located in the sections representing a sparse background staining (Data not shown).

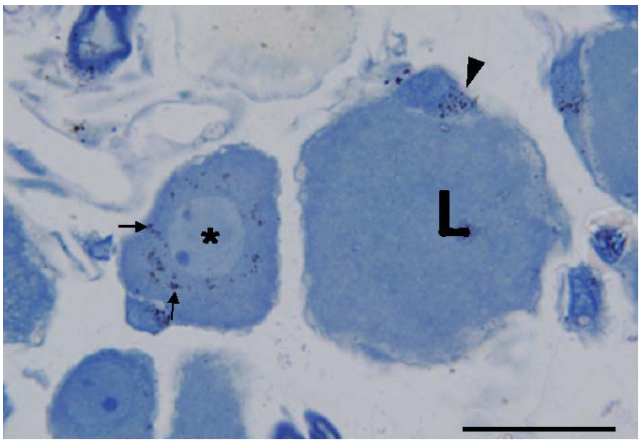

Fig. 3. Light micrograph taken from 2 μm-thick epon section stained by AMG, and counterstained with TB. Arrows indicate AMG grains (arrows) in the small ganglion cells (asterisk), Note each large ganglion cells are surrounded by perineuronal satellite cells, showing apparent AMG stainity (arrowheads). Scale bar: 50 μm.

Sensory neurons within dorsal root ganglia have been divided into two classes on the basis of perikaryal size. Both large and small cells were stained in DRG tissue. The cellular stain observed in large cells consisted of speckles of precipitate, often with a clear center. A concentrated and uniform precipitate was more characteristic in small cells than those with larger cells. This suggests that only small cells associated with processing of noxious thermal stimuli (Caterina et al., 1997) are different from larger cells. This intriguing possibility may be important in the modulation of pain transmission along large A-fibers by activity in C-fibers (Willer et al., 1983; Willer and Albe-Fessard, 1983).

Ultrastructural observations have shown that these AMG grains were predominantly distributed in the small cells. In comparison with the perinuclear distribution of strong AMG stainity in the small cells, pattern of the large cell was different from those of the small cells. That is, the AMG-positive staining zone was not restricted to the perinuclear regions and showed rather diffuse staining of the cytoplasm. In addition, general staining density were much weaker than those in the small cells.

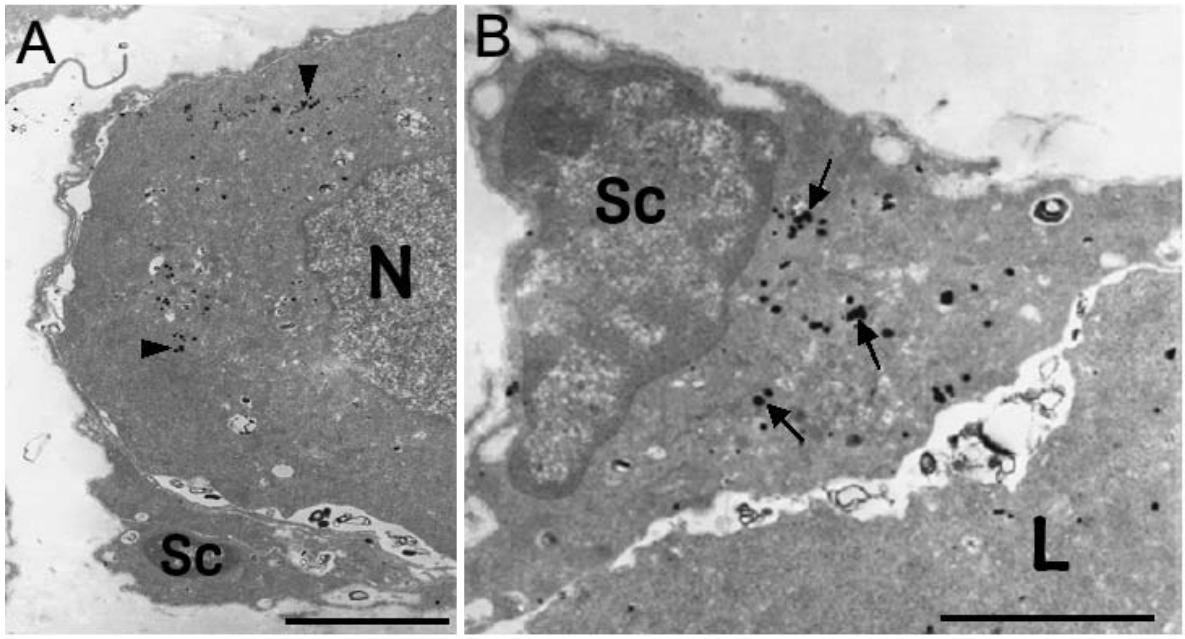

Fig. 4. Electron micrographs taken from the DRG stained by $ZnSe^{AMG}$. The AMG grains the AMG grains (arrowheads) are predominantly distributed in the perinuclear zone, and confined to the Golgi complex and the vesicular structure in the small ganglion cell (Fig. 4A). N indicate the nucleus of the small ganglion cell surrounded by the satellite cells (Sc). Note AMG grains in the satellite cells (Sc) surrounding the large ganglion cells (L). Scale bar: 5 μm.

Time dependent changes also take place in the PNS. $ZnSe^{AMG}$ grains are mainly located in small vesicles and in the Golgi complex 1～2 h after treatment with sodium selenide, but after 24 h also appear in lysosome-like organelles (Wang et al., 2003). This change in pattern might indicate that ZEN neurons in the PNS cannot accept the zinc-selenium clusters and try to remove/dissolve them. As mentioned above, the same process occurs in the CNS where zinc-selenium clusters are retrogradely transported through the axons and end up in lysosomes on the ZEN somata. The fact that $ZnSe^{AMG}$ grains are abundant in the Golgi complex areas might also indicate that the observed zinc ions are involved in the local packaging of proteins to be transported in the vesicles.

The presence of zinc-positive neurons in the DRG, an area known to be involved in sensation, indicates that zinc may plays a role in sensory perception.

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＜국문초록＞

이번 연구에서는 성체 랫드(Sprague-Dawley)의 척수신경절내 분포하는 zinc의 분포 관찰하기 위하여 zinc selenium autometallograpy (AMG)로 염색하였다. H-E염색표본에서 척수신경절 속에는 신경절세포들이 무리지어 분포하고 있었고, 세포체는 둥글거나 타원형이었으며, 작은 신경절세포, 큰 신경절세포로 대별되었다. 모든 신경절세포의 세포체는 한 층의 납작한 위성세포(satellite cell)에 의하여 둘러싸여 있었다. AMG 염색표본에서는 작은 신경절세포의 경우 강한 양성반응이 핵 주변부에서 관찰되었으나, 큰 신경절세포의 경우 전반적으로 미약한 AMG 양성반응을 보였고, 염색양상도 서로 달랐다. 그러나 큰 신경절세포를 둘러싸는 위성세포에서는 뚜렷한 AMG 양성반응이 관찰되었다. 이러한 조직화학적 본 연구의 결과는 zinc가 감각신경절인 랫드 척수신경절에서 통증을 포함한 감각의 전달과정에 의미있는 기능을 영위할 수 있음을 시사한다.