

## Zinc-enriched (ZEN) Terminals in Onuf's Nucleus Innervating External Urethral Sphincter: HRP Tracing Method and Zinc Selenium Autometallography

Boye Lee, Yi Suk Kim, Boeby Lee<sup>1</sup>, Hyun Sook Lee<sup>1</sup>, Gye-Rae Tak<sup>2</sup>,  
Young Il Lee<sup>3</sup>, Jeoug Yeol Lee<sup>4</sup> and Seung Mook Jo\*

Department of Anatomy, Gachon Medical School

<sup>1</sup>Department of Anatomy, College of Medicine, Konkuk University

<sup>2</sup>Division of Biomedical Engineering, College of Medicine, Konkuk University

<sup>3</sup>Department of Anatomy, College of Medicine, Dankook University

<sup>4</sup>Graduate School, College of Medicine, Hallym University

(Received November 27, 2006; Accepted December 22, 2006)

### 바깥요도조임근을 지배하는 Onuf 핵에서 관찰된 Zinc 함유 신경종말: HRP 추적법 및 zinc selenium 조직화학법

이보예, 김이석, 이법이<sup>1</sup>, 이현숙<sup>1</sup>

탁계래<sup>2</sup>, 이영일<sup>3</sup>, 이정열<sup>4</sup>, 조승묵\*

가천의과대학교 해부학과, <sup>1</sup>건국대학교 의과대학 해부학교실,

<sup>2</sup>건국대학교 의료생명대학 의학공학부, <sup>3</sup>단국대학교 의과대학 해부학교실,

<sup>4</sup>한림대학교 의과대학 대학원

#### ABSTRACT

Onuf's nucleus, which is located in the ventral horn, has been known to innervate the striated muscles of the urethral and anal sphincter muscles via the pudendal nerve. Onuf's nuclei are resistant to pathologic condition such as poliovirus. The reason why the motor neurons in Onuf's nucleus are less degenerated is not certain until now.

The present study aims at updating the microscopical characteristics including its location the Onuf's nucleus innervating the external urethral sphincter, and ultrastructures of the zinc-enriched (ZEN) terminals synaptically-contacting with Onuf's motor neurons in the rat spinal gray matter by using HRP tracing method and zinc selenium autometallography, respectively.

Based on HRP tracing method, Onuf's nuclei were located adjacent lateral dendritic projections of the ventral horn. Their shape was almost round at lumbar level, but oval at sacral segment of spinal cord. In size, their somata were smaller than that of other motor nuclei.

In AMG stained sections, Onuf's nuclei were innervated by highly concentrated ZEN terminals, and con-

This work was supported by Korea Research Foundation Grant (R01-2006-000-11131-0).

\* Correspondence should be addressed to Dr. Seung Mook Jo, Department of Anatomy, Gachon Medicine and Science University, 1198, Kuwol-Dong Namdong-Gu, Incheon 405-760, Korea. Ph.: (032) 460-2159, FAX: (032) 460-3824, E-mail: josm@gachon.ac.kr

tained small and middle-sized ZEN, but totally void of large ZEN terminals. AMG silver grains were confined to presynaptic ZEN terminals against dendritic elements and somata of the Onuf's motor neurons. A majority of the ZEN terminals contained flattened synaptic vesicles and made symmetrical synaptic specializations.

**Keywords :** HRP, AMG, ZEN, Onuf, Spinal cord, Rat

## INTRODUCTION

Zinc-enriched (ZEN) terminals in the spinal cord are dispersed throughout the gray matter. The superficial dorsal horn (laminae I, III, IV) and lamina X, involved in sensory transmission, contain relatively high concentrations of ZEN terminals (Jo et al., 2000; Schroder et al., 2000). These results have led to a functional consideration of zinc ions being a possible neurotransmitter or neuromodulator of sensory transmission and motor control.

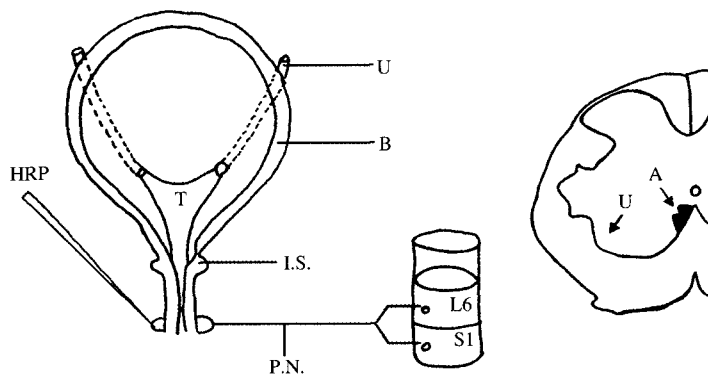
Onuf's nucleus, which is located in the ventral horn, has been known to innervate the striated muscles of the urethral and anal sphincter muscles via the pudendal nerve (Sato et al., 1978; Nagashima et al., 1979; Kuzuhara et al., 1980; Roppolo et al., 1985). According to light microscopic findings, the concentration of the longitudinally running dendrites into bundles is prominent in the motoneuron of Onuf's nucleus (Dekker et al., 1973).

The present study demonstrated that neurons in Onuf's nucleus were retrogradely labeled from the vesical striated sphincter and that the motor neurons were preferentially located ventrolaterally rather than dorsomedially in the nucleus.

## MATERIALS AND METHODS

### 1. HRP tracing method

Adult Sprague-Dawley rats were used in present study. We examined the topographic distribution of neurons in Onuf's nucleus innervating the vesical striated sphincter muscles using HRP tracing method (Fig. 1). Each rat was anesthetized with an i.p. injection of sodium pentobarbital (50 mg/kg). After exposing the vesical striated sphincter muscle in retropubic space, 1  $\mu$ L of 30% solution of HRP dissolved in sterile 0.9% saline was stereotaxically injected into the middle portion of external sphincter muscle by pressure through a glass micropipette coupled to a 5  $\mu$ L Hamilton microsyringe. After a



**Fig. 1.** Schematic drawing demonstrating HRP injection into external urethral sphincter muscle of the rat urinary bladder (B). IS, internal sphincter; PN, pudendal nerve; T, trigone; U, ureter.

survival period by 24~48 h, the rats were deeply re-anesthetized with an i.p. injection of sodium pentobarbital (100 mg/kg) and then perfused through the ascending aorta with 0.5 liters of a solution composed of 6% formaldehyde and 1% picric acid in 0.1 M phosphate buffered saline (pH 7.4). After the perfusion, the spinal cord was immediately removed and placed in the same buffer containing 30% sucrose at 4°C for several days. Then, the spinal cord segments containing Onuf's nucleus (L6-S1) were then cut into transverse sections of 30  $\mu$ m thickness on a freezing microtome. The sections were collected in the same buffer containing 0.9% sodium chloride (pH 7.4), and were immediately processed according to the cobalt chloride-intensified DAB (diaminobenzidine) method of Adams to mark HRP-labelled neurons with black reaction products. Immunolabeled peroxidase was visualized by placing the sections in an incubation medium that was composed of 0.05% diaminobenzidine (DAB) and 0.01% hydrogen peroxidase in 0.1 M phosphate buffer (pH 7.4) for 5~10 min at room temperature.

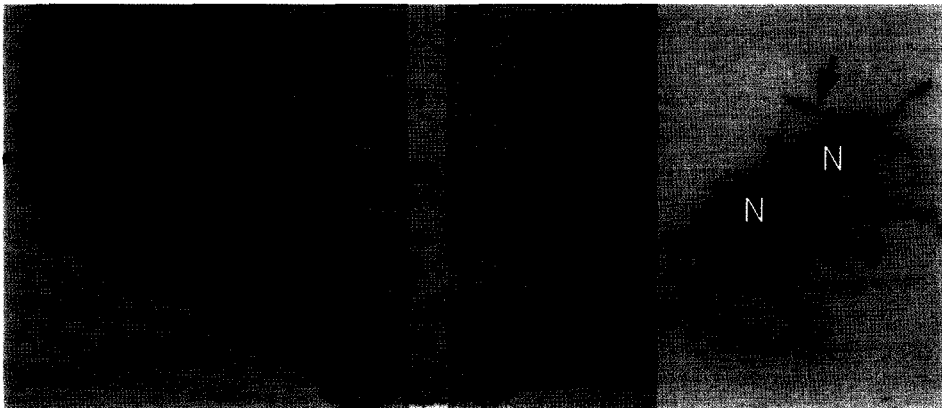
## 2. ZnSe autometallography (ZnSe<sup>AMG</sup>) procedure

As a separate study, rats were anesthetized and i.p. injected with sodium selenite (10 mg/kg). After 1 h, they

were re-anesthetized and transcardially perfused with saline followed by 3% glutaraldehyde in 0.1 M PB (pH 7.4).

For light microscopy (LM), the samples were rinsed in PB, placed in 30% sucrose until they sank to the bottom of the container, and frozen with CO<sub>2</sub> gas. Cryostat sections, 10  $\mu$ m thick, were cut and placed on Farmer (9 parts 10% sodium thiosulphate, 1 part 10% potassium ferrocyanide) rinsed glass slides, dipped in a 0.5% gelatine solution and placed in vials. The AMG developer was poured into the developing vials and placed in a 26°C water bath, and finally the whole set-up was covered by a light-tight hood as described by Danscher et al. (1982).

For electron microscopy (EM), 100  $\mu$ m sections of the samples were cut on a vibratome and stained floating in the AMG developer. Areas to be ultrastructurally analyzed were selected and fixed with 1% osmium tetroxide (OsO<sub>4</sub>) in PB for 30 min, dehydrated in a series of alcohols and embedded in Epon. Sections, 3  $\mu$ m thick, were cut and counterstained with toluidin blue and analyzed in a light microscope. Some of these sections were selected for ultrastructural studies. They were re-embedded on top of blank Epon blocks and cut into 80~100 nm ultrathin sections that were stained with uranyl ace-



**Fig. 2.** Light micrographs showing HRP-labelled cell bodies in the onuf's nucleus (N of onuf) innervating the external urethral sphincter. Arrowhead indicate anterior median fissure in transverse section of the rat spinal cord. HRP-labelled cell bodies is enlarged in the right panel. Note cell processes from the cell bodies.

tate (20 min) and lead citrate (5 min). Finally they were examined with a Philips 208 transmission electron microscope (Eindhoven, The Netherlands).

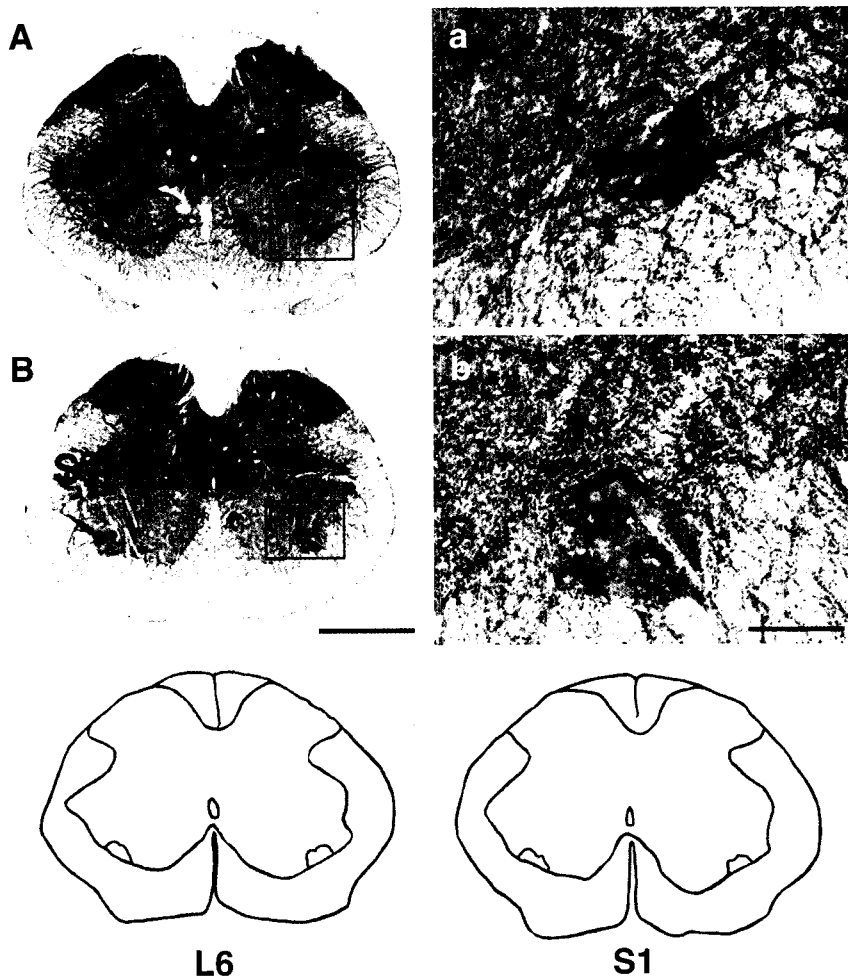
## RESULTS

### 1. Topographical localization of the Onuf's nucleus

Neurons labeled with HRP were observed exclusively

in Onuf's nucleus in the L6 and S1 ventral horn (Fig. 2). Most HRP labeled neurons were located in its ventrolateral part of Onuf's nucleus. Individual sections generally showed Onuf's to be round to oval in cross section. These findings are shown schematically in Fig. 3.

Three to five labelled neurons were observed in a cross sectional area of Onuf's nucleus. Their neuronal shape ranged between approximately circular through ellipsoid to elongated spindle shaped. The diameter of neuronal somata was measured 15~20  $\mu\text{m}$ .



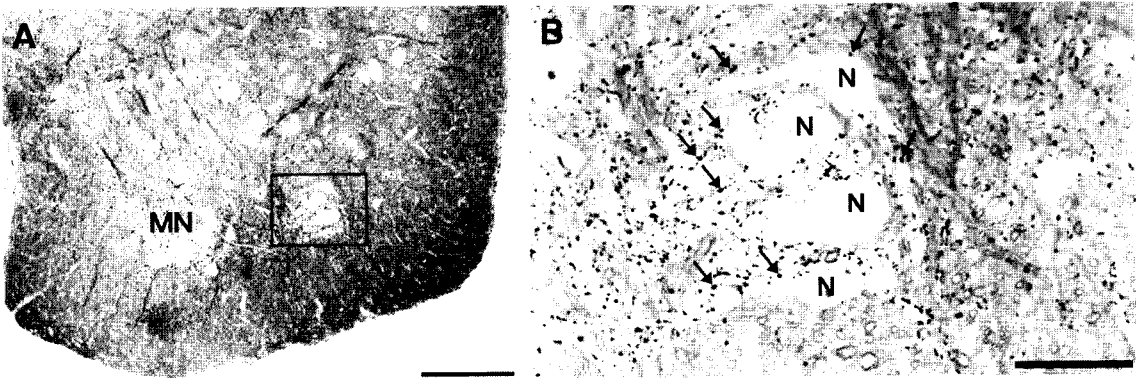
**Fig. 3.** Light photomicrographs of transverse 30- $\mu\text{m}$  cryosections stained with  $\text{ZnSe}^{\text{AMG}}$  of the rat spinal cord at lower lumbar and upper sacral segments. Intense staininess pronounced in the Onuf's nuclei containing motor neurons in the ventral horn. Note that lower panel is schematic drawing indicating the corresponding sites of Onuf's nucleus in upper panel. Magnification is the same in A-b. Scale bar=200  $\mu\text{m}$ .

## 2. Distribution of ZEN terminals in Onuf's nucleus

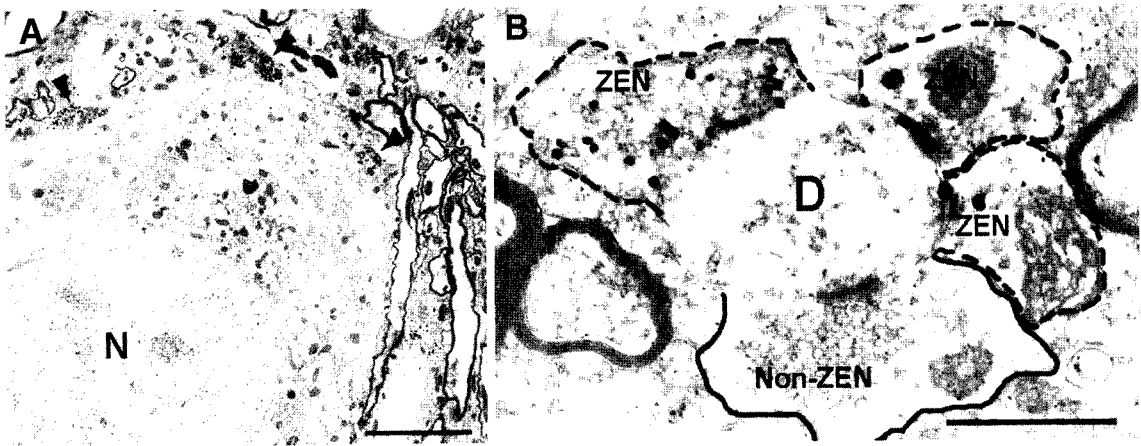
The rat Onuf's nucleus was characterized by closely grouped neurons surrounded by a pale stained neuropils of axially oriented dendrites. The neuropils throughout the spinal cord contained AMG silver grains demonstrat-

ing zinc selenide clusters. The white matter was unstained apart from rows of ZnSe<sup>AMG</sup> puncta along dendritic projections radiating from the gray matter (Fig. 4a).

Onuf's nuclei contained small and middle-sized ZEN terminals and was totally void of large ZEN terminals. motor neurons in the Onuf complex presented a unique pattern being devoid of the very large grains (Fig. 4b).



**Fig. 4.** Light micrographs from a 3  $\mu\text{m}$  thick Epon section of the ventral horn of the sacral segment in the rat spinal cord. Fig. 4A depict motor neuropil (MN) including Onuf's nucleus (rectangle), which is enlarged in B. Note numerous AMG grains (arrows) closely related to somata of Onuf's nuclear neurons (N). All sections are counterstained ding with 0.1% toluidine blue. Scale bars in A & B indicate 200 & 50  $\mu\text{m}$ , respectively.



**Fig. 5.** Electron micrographs showing ZEN terminals found in the Onuf nuclear neuron. ZEN terminals make axo-somatic (arrowheads) and axo-dendritic symmetrical synaptic contacts to neuronal somata (N) and dendritic elements (D). Most ZEN terminals contains heterogenous synaptic vesicles, and typically make symmetrical synaptic contact, while non-ZEN terminal (Non-ZEN) are absolutely void of AMG grains in clear round synaptic vesicles making unsymmetrical synaptic contacts with a dendritic element (D). Note AMG grains in the synaptic cleft. Scale bars: A=5  $\mu\text{m}$ , B=1  $\mu\text{m}$ .

### 3. Ultrastructural localization of ZnSe<sup>AMG</sup>

The dotted distribution of ZnSe<sup>AMG</sup> puncta in both ventral and dorsal horns corresponded ultrastructurally to ZEN terminals. AMG silver grains were located in presynaptic terminals. A majority of the ZEN terminals in the rat spinal cord contained flattened synaptic vesicles and made symmetrical (Fig. 5a).

ZEN terminals making symmetric synapses contained ZnSe<sup>AMG</sup> silver grains in flattened synaptic vesicles. The less abundant ZEN terminals making asymmetric contacts contained homogeneous round synaptic vesicles. Both kinds of terminals had AMG grains in only a fraction of the synaptic vesicles, and AMG grains were found in synaptic clefts. AMG silver grains were often concentrated close to the synaptic specializations (Fig. 5b).

## DISCUSSION

The ZnSe<sup>AMG</sup> staining of the Onuf's nucleus appeared relatively dense at the light microscopic level as compared with that in other motor nuclei. This is due to a generally higher concentration of ZEN terminals in this area horn and to the fact that many of these are medium-sized ZEN terminals.

Onuf's nucleus, the motor neuron nucleus in L6 and S1 segment innervating vesical striated muscle, diverges from other motor neuron nuclei with respect to afferent connections. In the present study, this is confirmed as its ZEN terminals belong to a smaller type. These findings are consistent with our previous results (Schroder et al., 2000). In agreement with previous HRP studies in cat (Tashiro et al., 1989), Onuf's nucleus can be anatomically divided into dorsomedial and ventrolateral groups, which innervate the rectal striated sphincter and vesical striated sphincter, respectively.

Most of the ZEN terminals observed in the rat Onuf nucleus were located presynaptic to dendrites and neuronal somata. Our findings, therefore, support the notion

that at least two populations of ZEN terminals are present in the rat spinal cord, a subset of glutamatergic boutons as found in the brain (Frederickson and Danscher, 1990) and a subset of GABAergic terminals (Danscher et al., 2001).

EM studies have suggested that these terminals are inhibitory, and we hypothesize that the ventral horn ZEN neurons giving rise to big axo-somatic and axo-dendritic ZEN boutons constitute a local inhibitory system which is a component in the antagonist-inhibitor circuit. These finding might be a clue to explain the reason that the motor neurons in Onuf's nucleus are less degenerated and are resistant to poliovirus (Hideaki et al., 1989).

The lumbosacral transition discloses a unique pattern of ZEN terminals (Jo et al., 2000) and includes a dense terminal input to the Onuf complex known to innervate the pelvic sphincters. The same segment also shows a high ZEN neuron density suggesting that at this level the ZEN systems are involved in the complex regulation of the integrated visceral and somatic control of bladder.

The present findings suggest that ZEN terminals could be involved in the regulation of motor functions at the spinal cord level for micturition control.

## REFERENCES

- 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, Jo SM, Varea E, Wang Z, Cole TB, Schroder HD: Inhibitory zinc-enriched terminals in the mouse spinal cord. *Neuroscience* 105 : 941-947, 2001.
- Dekker J, Lawrence D, Kuypers H: The location of longitudinally running dendrites in the ventral horn of the cat spinal cord. *Brain Res* 51 : 319-324, 1973.
- Frederickson CJ, Danscher G: Zinc-containing neurons in hippocampus and related CNS structures. *Prog Brain Res* 49 : 157-197, 1990.
- Hideaki K, Yasushi F, Miri F, Yasunori F, Kazuo N: Onuf's motoneuron is resistant to poliovirus. *J Neurol Sci* 93 : 85-

- 92, 1989.
- Jo SM, Danscher G, Schroder HD, Won MH, Cole TB: Zinc-enriched (ZEN) terminals in mouse spinal cord : immunohistochemistry and autometallography. *Brain Res* 870 : 163-169, 2000.
- Kuzuhara S, Kanayawa I, Nakanishi T: Topographical localization of the Onuf's nuclear neurons innervating the rectal and vesical striated sphincter muscles: a retrograde fluorescent double labeling in cat and dog. *Neurosci Lett* 16 : 125-130, 1980.
- Nagashima T, Beppu M, Uono M, Yamada H: Demonstration of neuronal localization of Onufrowicz's cell group X in rabbit by double labeling method. *Acta Histochemica et Cytochemica* 12 : 369-391, 1979.
- Roppolo JR, Nadelhaft I, DeGroat WC: The organization of pudendal motoneurons and primary afferent projections in the spinal cord of the Rhesus monkey revealed by horseradish peroxidase. *J Comp Neurol* 243 : 475-488, 1985.
- Sato M, Muzuno M, Konishi A: A localization of motoneurons innervating perineal muscles: a HRP study in cat. *Brain Res* 140 : 149-154, 1978.
- Schroder HD, Danscher G, Jo SM, Su H: Zinc-enriched boutons in rat spinal cord. *Brain Res* 868 : 119-222, 2000.
- Tashiro T, Satoda T, Matsushima R, Mizuno N: Convergence of serotonin-, enkephalin- and substance P-like immunoreactive afferent fibers on single pudendal motoneurons in Onuf's nucleus of the cat: a light microscopical study combining the triple immunocytochemical staining technique with the retrograde HRP-tracing method. *Brain Res* 481 :

392-398, 1989.

### < 국문 초록 >

Onuf 핵이란 척수 앞회색질뿔에 위치하는 운동핵으로 음부신경을 통해 방광과 항문괄약근을 조절하는 운동핵의 하나이다. Onuf 핵은 앞회색질뿔내 다른 운동신경핵과는 달리 회색질척수염과 같은 병적인 상황에서도 상당기간 손상되지 않고 기능을 유지하며, 퇴행성변화의 정도가 미약한데 정확한 원인에 관해서는 논란의 여지가 많다.

본 연구는 흰쥐 척수회색질내 바깥요도조임근을 신경 지배하는 Onuf 핵의 위치를 HRP 추적법으로 확인하였으며, 이들 신경핵내 운동신경세포와 연결해 있는 zinc 함유 (ZEN) 신경종말의 미세구조를 zinc selenium 조직화학법 (AMG)으로 염색하여 관찰하였다.

HRP 추적법의 결과로는, Onuf 핵은 랫드 척수회색질앞뿔의 내측에서 가지돌기의 무리와 거의 맞닿고 있었으며, 모양은 대개 구형 또는 난원형을 띠었다. 이들 신경핵내 운동신경세포의 세포체의 크기는 다른 운동핵의 신경세포보다 다소 작았다. 한편 AMG로 염색한 표본에서는 Onuf 핵에 분포하는 ZEN 신경종말은 다른 운동핵의 ZEN 신경종말과 비교하여 매우 높은 밀집도를 보였으나, 크기면에서도 상대적으로 작았다. 미세구조 관찰로는 Onuf 핵내 ZEN 신경종말은 운동핵의 세포체 및 가지돌기와 신경연접은 이루고 있었다. 이들 ZEN 신경종말은 주로 납작한 연접소포를 함유하였으며, 대칭적인 신경연접구조를 이루고 있었다.