**Distribution of Muscarinic Cholinergic (mACh) Receptors
in the Rat Spinal Cord:
In vitro Quantitative Autoradiographic Investigation**

By

**Kiminao MIZUKAWA, Nagayasu OTSUKA, Kumiko HABA*
and Norio OGAWA***

Department of Anatomy and *Institute for Neurobiology,
Okayama University Medical School,
Okayama 700, Japan

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Summary: The binding of ^3H -labeled L-quinuclidinylbenzilate (QNB) sites was studied in cryosections of the rat spinal cord (cervical, thoracic and lumbar cords) by *in vitro* macro-autoradiography in an attempt to elucidate the distribution of muscarinic cholinergic (mACh) receptors. In this study, transverse sections as well as longitudinal sections of the thoracic cord were employed. A moderately high density of QNB binding sites could be seen in the gray matter of the spinal cord, especially in the marginal regions of the dorsal horn and around motor neurons in the ventral horn. In the lateral horn of the thoracic cord, a moderately high density of QNB binding sites was segmentally observed. By image analysis with a RAS 1000 (Amersham), color coded autoradiographic images which enhanced the small differences in optical density could be easily obtained, and quantitative analysis of the QNB binding sites in small-sized regions of the spinal cord was also performed. The highest densities of mACh receptors in the spinal cord were localized in the substantia gelatinosa of the dorsal horn and the region of motor neurons of the ventral horn. These findings suggest that while mACh receptors are related with motor functions, they are deeply related with sensory and autonomic functions.

Several neuroanatomical and neuropharmacological studies have demonstrated that acetylcholine functions as a neurotransmitter, and the acetylcholine system has been clarified more in the mammalian spinal cord (Aquilonius *et al.*, 1981; Kimura *et al.*, 1981; Nagata *et al.*, 1982; Ryall, 1983; Silver and Wolstencroft, 1971). During the last few years, progress in radioligand and receptor

autoradiographic analysis systems for cholinergic receptors has facilitated studies on the precise distribution of several cholinergic receptors in the mammalian spinal cord and under several experimental and pathological conditions (Charlton *et al.*, 1981; Geary *et al.*, 1985; Gillberg and Aquilonius, 1985; Gillberg *et al.*, 1984; Gillberg and Wiksten, 1986; Kayaalp and Neff, 1980; Palacios *et al.*, 1981;

Send correspondence to: Kiminao Mizukawa, M.D. Department of Anatomy, Okayama University Medical School, 2-5-1 Shikata-cho, Okayama City, Okayama 700, Japan

Scatton *et al.*, 1984; Seybold, 1985; Seybold and Elde, 1984; Unnerstall *et al.*, 1982; Villiger and Faull, 1985; Wamsley *et al.*, 1984; Whitehouse *et al.*, 1983).

The aim of the present study was to elucidate the distribution of muscarinic cholinergic (mACh) receptors in transverse as well as longitudinal sections of the rat spinal cord by the *in vitro* macro-autoradiography method employing ^3H -labeled L-quinuclidinylbenzilate (QNB) as the ligand for the mACh receptors. Quantitative evaluation of the regional distribution of mACh receptors was also performed using an image analysis system.

Materials and Methods

Four adult male albino rats (Wistar strain; body weight, 200-300 g) were used in this study. They were anesthetized by intraperitoneal injection of Nembutal (0.1 mg/100 g body weight) and killed by decapitation. The spinal cord was removed immediately, dissected into the cervical, thoracic and lumbar cords, rapidly frozen on crushed dry-ice and cut into 12 μm thick sections on a cryostat in transverse and longitudinal directions. These sections were thaw-mounted on chrome alum gelatin coated slides and dried completely. The sections on the slide glasses were preincubated twice for 2-4 min in ice-cold 50 mM Tris/HCl buffer (pH 7.6). After the preincubation, they were incubated in 50 mM Tris/HCl buffer containing 1 nM ^3H -QNB (specific activity, 46 Curies/mmol; Amersham, Japan), a potent muscarinic cholinergic antagonist, at 20°C for 2 h with or without 1 μM atropine. Non-specific binding was determined by incubating the sections and the ^3H -QNB in the presence of excess (1 μM) atropine. After this incubation, the slide glasses were rinsed twice with the same Tris/HCl buffer, and the slides were dried in a stream of cold air. They were

secured with strips of double-sided adhesive tape so that the backs of the slides adhered to a hard board in a cassette. A sheet of Hyperfilm- ^3H (Amersham, CEA, AB, Sweden) was pressed so as to contact the tissue sections and autoradiographic micro-scales (Amersham, Japan) on the slides, and the cassette was closed in a dark room. After exposure for 3 weeks at 4°C in the dark room, the film was developed for 7 min at 20°C in Microdol X (Kodak), fixed for 10 min, washed in running water and dried in air. The distribution of mACh receptors was revealed on the films. The films were placed on a photographic illumination apparatus, the autoradiograms were processed with a receptor autoradiography image analysis system (Amersham, RAS 1000) and several color coded autoradiographic images were obtained. Furthermore, quantitative analysis of the concentration of QNB binding sites in the dorsal, lateral and ventral horns of each of the segmental spinal cords was performed respectively.

Results

We observed a moderately high density of QNB binding sites in the gray matter of the spinal cord, but could not obtain positive autoradiograms for QNB binding in the white matter. Fig. 1 shows that the QNB binding sites for mACh receptor were most densely distributed in the dorsal horn, especially in the marginal regions (i.e. substantia gelatinosa), and ventral horn in transverse sections of the cervical, thoracic and lumbar spinal cord, respectively. The patterns of distribution of QNB binding sites were similar in the cervical, thoracic and lumbar spinal cords. Fig. 2 shows autoradiograms of transverse (A) and longitudinal sections (B) of the thoracic cord. In longitudinal sections of the thoracic cord, it was apparent that the densities of QNB binding sites occurred segmentally in

Table 1. Concentration of QNB binding sites for mACh receptors in the rat spinal cord

	(n)	Specific binding sites of each region (DPMS/mg)		
		Dorsal horn	Lateral horn	Ventral horn
Cervical cord	(16)	41,747 ± 4,699	—	40,438 ± 4,017
Thoracic cord	(16)	41,980 ± 7,335	39,321 ± 9,488	36,928 ± 7,173
Lumbar cord	(16)	48,403 ± 8,563	—	43,513 ± 5,400

n is the number of sections used for quantitative analysis from 4 experimental animals. Each value is expressed as the mean ± S.E.M.

patches along the lateral aspect of the lateral horn (Fig. 2B). Fig. 3 shows color coded autoradiographic images of thoracic and lumbar spinal cords processed with the image analysis system. In this way, small differences in the optical densities could be enhanced, so facilitating easy recognition of the differences in optical densities. Quantitative analysis of the concentration of QNB binding sites in the dorsal horn, ventral horn and lateral horn of the cervical, thoracic and lumbar spinal cords was undertaken respectively (Table 1). The highest concentration was observed in the dorsal horn, followed by the ventral horn. The lateral horn of the thoracic cord exhibited the widest range of variance which reflected a segmental distribution of QNB binding sites.

Discussion

Previous studies (Gillberg *et al.*, 1984; Scatton *et al.*, 1984; Seybold, 1985; Seybold and Elde, 1984; Villiger and Faull, 1985; Wamsley *et al.*, 1984) have demonstrated that QNB binding sites are distributed in the dorsal horn and ventral horn of the spinal cord of several mammalian species including human

beings. Most of our findings agreed well with the results of these previous investigations. In the present study, furthermore, we clearly revealed a segmental distribution of QNB binding sites in the lateral horn of the rat thoracic cord. Such segmental high densities of QNB binding sites were very similar to the beaded arrangement of acetylcholinesterase staining observed in the intermedio-medial nucleus (Silver and Wolstencroft, 1971). The densities of QNB binding sites in the gray matter of the spinal cord are not so high in comparison with those of the cerebral cortex and striatum areas (Mizukawa *et al.*, 1987). The quantitative analysis undertaken in this study showed that the densest distribution of QNB binding sites for mACh receptors was the dorsal horn, especially in the substantia gelatinosa area, followed by the lateral horn of the thoracic cord and the ventral horn. By employing *in vitro* macro-autoradiography, resolution for two dimensions becomes higher than that in conventional radiolabeled assay. As a result of image analysis with the RAS 1000, color coded autoradiographic images, which enhanced small differences in optical density, could be easily obtained and showed

the differences in an attractive manner. Moreover, with the progress of computer image analysis and the use of standard micro-scales for autoradiography (Geary *et al.*, 1985; Palacios *et al.*, 1981; Unnerstall *et al.*, 1982), it has become easy to perform quantitative analysis for very small regions which are difficult to punch out, such as the substantia gelatinosa or lateral horn of the thoracic cord, for biochemical assay and/or radiolabeled assay in receptor binding studies.

It has been demonstrated that acetylcholine exists in the motor neurons of the ventral horn by both enzyme histochemistry and immunohistochemistry. Acetylcholine has been considered to act mainly for motor function in the motor neurons of the ventral horn, so that motor function is deeply related with the mACh receptor. According to our results, the mACh receptor is more deeply related with sensory function in the rat spinal cord, simultaneously.

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Explanation of Figures

Plate I

Fig. 1. Autoradiograms of QNB binding sites in transverse sections of the rat spinal cord. Bar = 0.5 mm. C: cervical cord, T: thoracic cord, L: lumbar cord, DH: dorsal horn; LH: lateral horn; VH: ventral horn. A high concentration of QNB binding sites for mACh receptors is observed in the dorsal horn, especially in the substantia gelatinosa, and in the ventral horn.

Plate II

- Fig. 2. Autoradiograms of QNB binding sites for mACh receptors in the thoracic cord. Bars = 0.5 mm.
DH: dorsal horn; VH: ventral horn; LH: lateral horn; *: central canal.
A: Transverse section.
B: Longitudinal sections of the lateral horn of the thoracic cord.
Note the high concentration of QNB binding sites found segmentally in the lateral horn, as indicated by the arrowheads.

Plate III

Fig. 3, Color coded autoradiographic images of the thoracic and lumbar cords.

A: Transverse sections of the thoracic cord.

B: Longitudinal section of the thoracic cord.

C: Transverse section of the lumbar cord.

The concentration of QNB binding sites increases with the color sequence shown on the right-hand scale:
white- purple- blue- green- yellow- orange- red- black.