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Histochemical and Fluorescent Labelling of Neurons Projecting to Nucleus Accumbens: the Relation to Pain Processing

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Abstract: This study was undertaken to examine the laminar distribution of spinotelencephalic tract neurons in the rat, by means of retrograde fluorescent labelling and to estimate the role of NADPH-diaphorase-reactive spinal cells as sources of spinosupraspinal pathways in the rat. The localization, size and shape of some neurons were similar in both labelling methods. Cells were mostly multipolar in the medial part of nucl. proprius, and fusiform and spindle-shaped in lamina I and X. It is suggested that some of the NADPH-d (+) neurons in the deep dorsal horn and lamina X are sources of a spinosupraspinal pathway to nucleus accumbens and the excitability and spontaneous activity of these dynamic neurons may be higher when spinally mediated hyperalgesia is developed.

Key Words: Nucleus accumbens, Nociception, Hyperalgesia, NADPH-diaphorase, Fluoro-Gold

Introduction

The basal ganglia are associated with the motor functions of the brain, although it is becoming clear that they may subserve many non-motor functions as well. Recent neurophysiological, clinical and behavioral experiments have indicated that nucleus accumbens (Acb) also processes non-noxious somatosensory information. Basal ganglia are rich in many different neuroactive chemicals that may be involved in the modulation of pain processing also (1). Stein et al. (2) and Ma et al. (3) have found that Acb contains opioid receptors. Jin et al. (4) have shown that the microinjection of morphine into Acb produces antinociception. These data emphasize that Acb plays an important role in pain processing.

It has been acccepted that nitric oxide (NO) plays a role in synaptic transmission in both the central and periferic nervous systems (5-9). NO is a small gaseous molecule easily able to pass through neuronal membranes with a very short half-life. It has been suggested that it acts as a retrograde transmitter and also as a classical neurotransmitter. It can also act in the neuron where it is produced. NO has been found to have a role in nociceptive transmission (8). It has later been shown that NO plays an important role in the mediation of nociceptive processes such as thermal hyperalgesia (10). Aanosen (11, 12), Raigordsky and Urca (13), Dickenson and Aydar (14), Woolf and Thompson (15), Kitto et al.(16) and Meller et al.(6, 17) have reported that the NMDA receptor is responsible in nociceptive transmission.

It is known that NOS requires NADPH as a cofactor, so it has been suggested that NADPH-diaphorase is a form of NOS. NADPH-diaphorase activity has been determined in different regions of the central nervous system (8, 9) and it has been suggested that this activity reflects the activity of NOS. Both NADPH-d and NOS have been found in similar regions of the spinal cord (8, 9, 10, 17, 18).

As shown in previous studies (19, 20), there are direct projections from the spinal cord to telencephalon, including Acb and septal nuclei. As a working hypothesis, it has been proposed that these projections might transmit information to the areas involved in the control of motivational-affective responses to noxious stimuli. It was also found that NO-synthase involved in nociceptive processing in the spinal cord and in spinally mediated hyperalgesia (16, 17).

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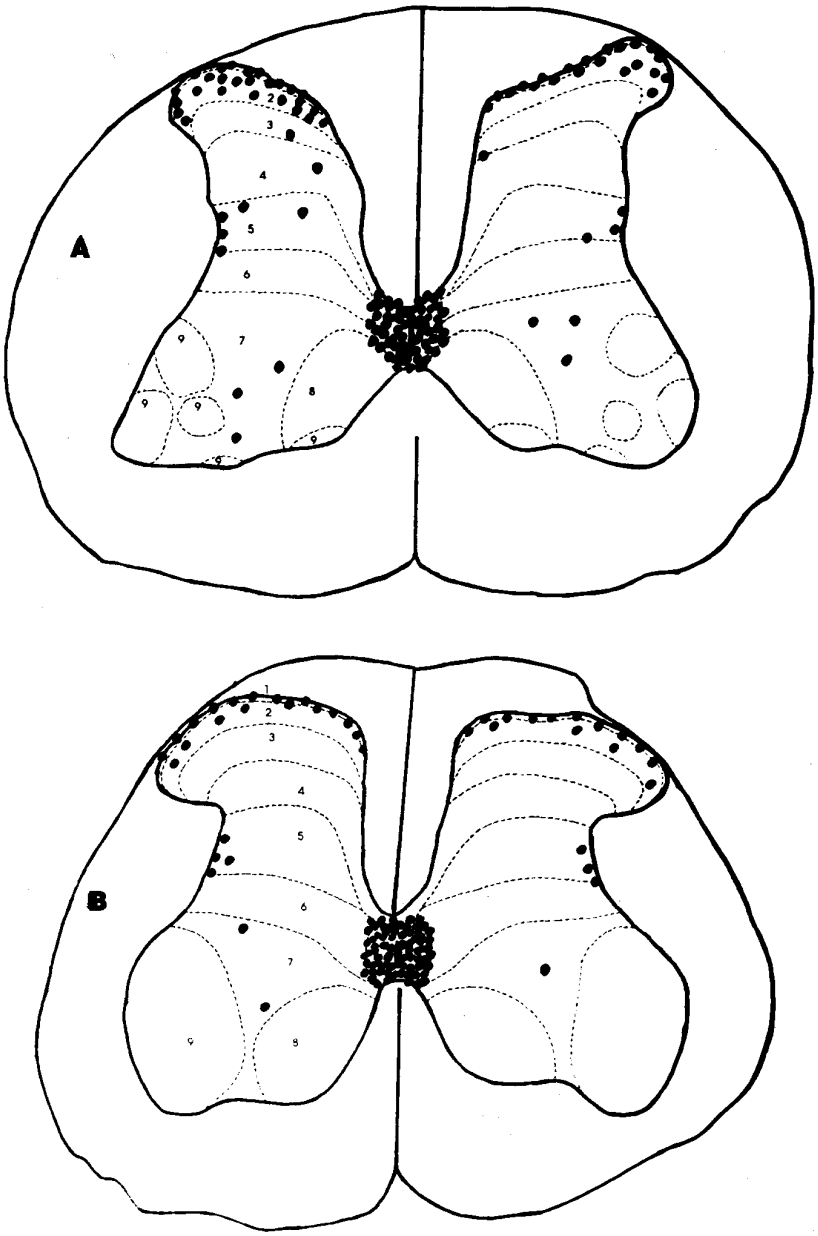

Figure 1. Schematic representation of the distribution of NADPH-d (+) cells in the cervical (a) and the lumbar (b) enlargements.

This study was undertaken to estimate the role of NOS-containing neurons, as sources of spinocerebral pathways, which may be involved in nociceptive transmission, spinally mediated hyperalgesia and pain modulation.

Material and Methods

The experiments were performed on nine male Sprague-Dawley rats weighing about 250-300 g. The NADPH-d histochemical reaction was assayed on the forebrain, and spinal cord sections taken from six experimental cases. The staining procedure was modified from the method of Vincent and Kimura (9). Rats were deeply anaesthetized with Nembutal (60 mg/kg, i.p.), intracardially perfused with 100 ml of heparinized saline (25 000 U/l) followed with 500 ml fixative (4-5 % paraformaldehyde in 0.1 M phosphate buffer, pH 7.3). After perfusion, the brains were transferred to PB and one day later were blocked and sectioned with vibratome at 50 µm. Coronal sections were collected in PB, rinsed 1-2 hs, and incubated in 0.1 M PB (pH 7.3) containing 0.3 % Triton X-100,

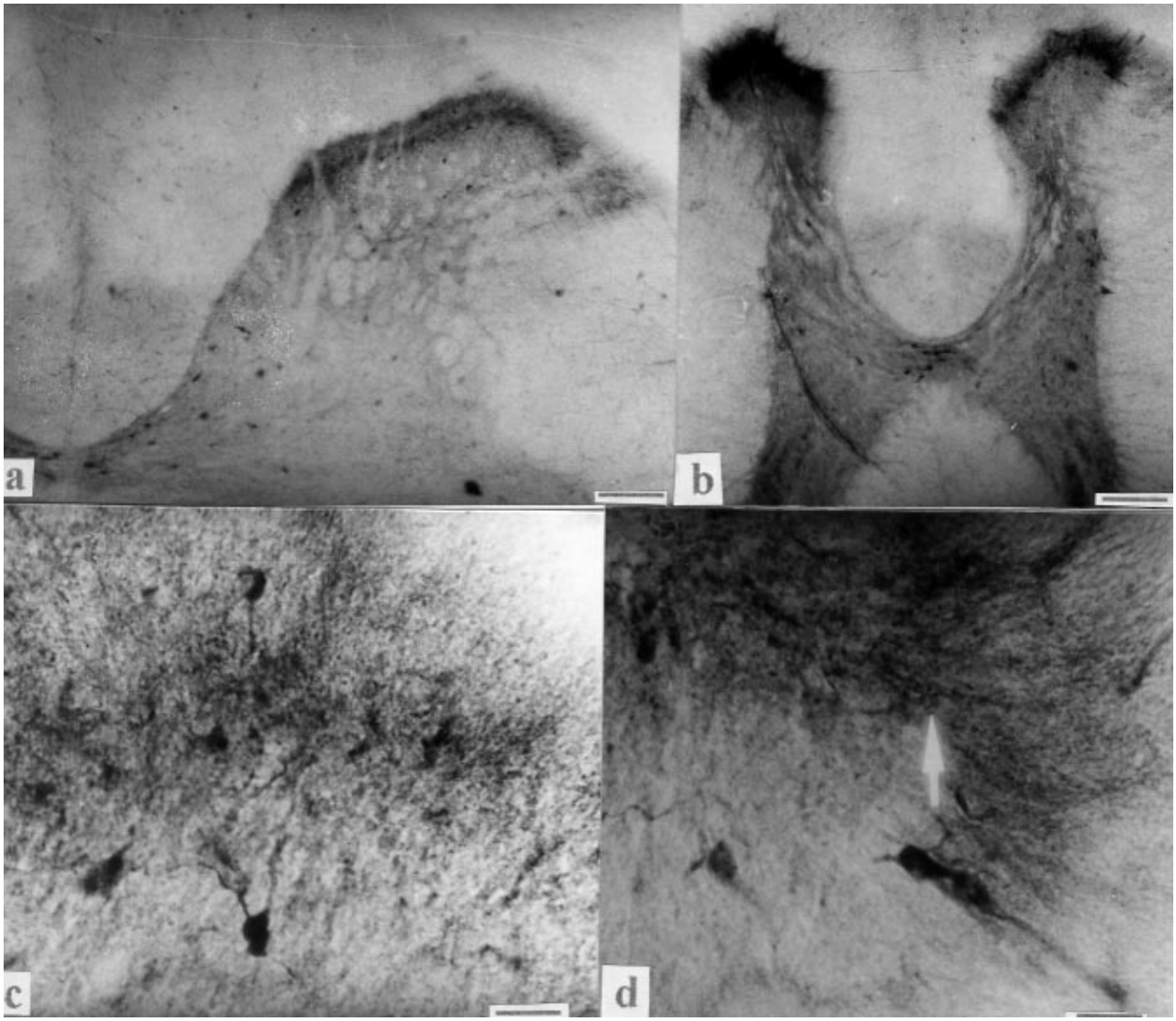

Figure 2. Photomicrographs of NADPH-d (+) cells a. NADPH-d (+) cells in the cervical enlargement. b. NADPH-d cells in the thoracic segment. c. NADPH-d (+) cells in the cervical segment, lamina IIo. d. NADPH-d (+) cells in the thoracic segment, lamina I. (Scale bar=200 µm, 100 µm, 10 µm, 10 µm, a-d, respectively)

0.5 mg/ml nitroblue tetrazolium (Sigma, USA) and 1.0 mg/ml NADPH tetrasodium salt (Sigma, USA) at 37°C for 30-60 min. Following the reaction, sections were rinsed in PB, mounted on gelatin coated slides and left to dry at room temperature. Mounted sections were then immersed in 100 % alcohol cleared in xylene and cover-slipped directly with Entellan.

The fluorescent labelling study was performed on seven male rats. The animals were anaesthetized with nembutal (45 mg/kg, i.p.) and placed in a stereotaxic frame. Three injections (200 nl each) of Fluoro-Gold (FG, fluorochrome INC, USA, prepared 2% solution) were administered to Acb (Fr +1, 2 according to the stereotaxic atlas [25]). Injections were made manually through a glass micropipette (tip diameter = 50-80 (µm) attached to the needle of a 10 µl Hamilton syringe. After a survival time of 7 days, the animals were intracardially perfused with 4% paraformaldehyde solution. The brain and spinal cord segments were removed and embedded in paraffin. The mean intensity of compression of the tissue during embedding in paraffin was calculated as 12.8 %. 20-µm-thick transverse and longitudinal sections were taken

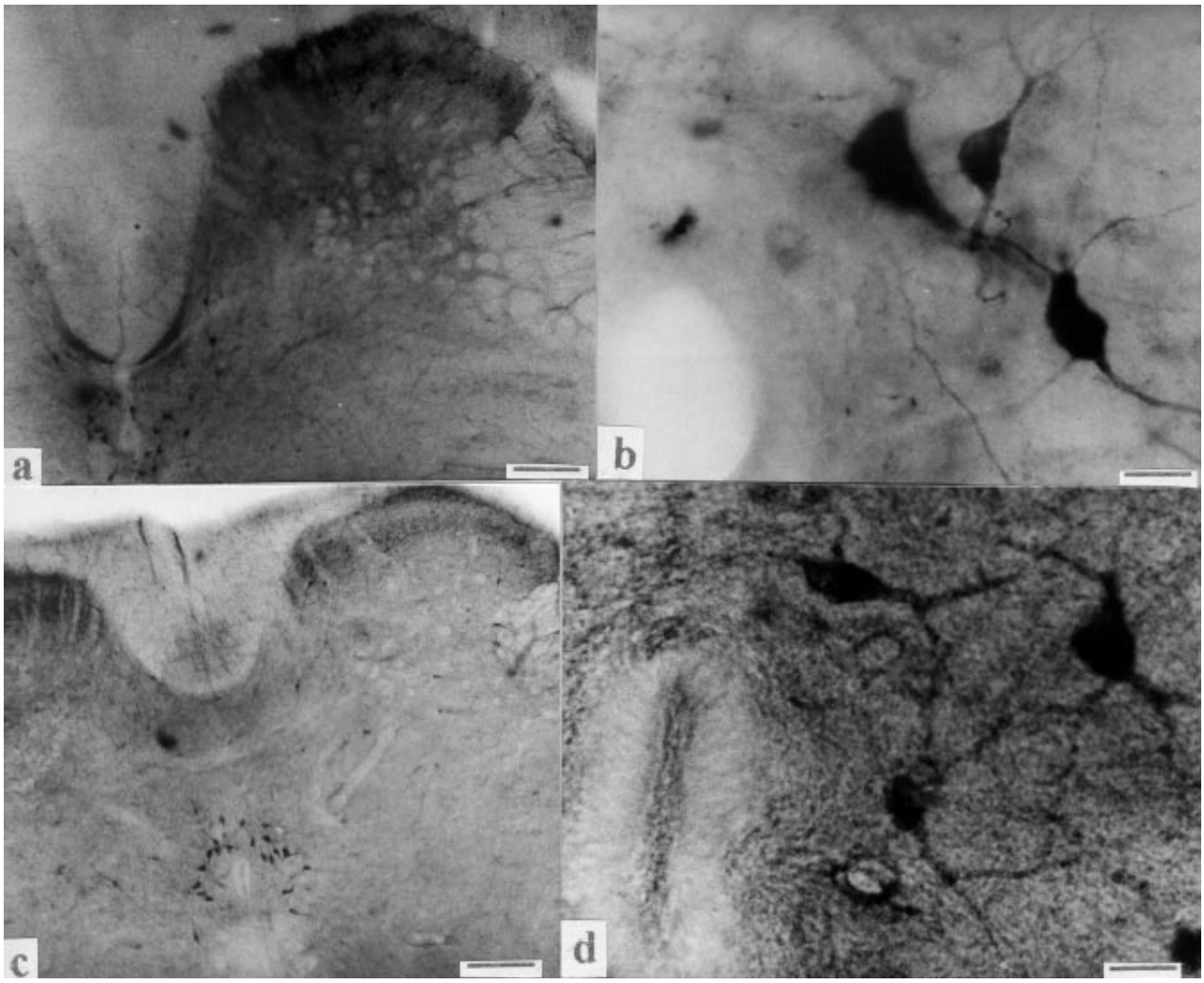

Figure 3. NADPH-d (+) cells a. in the lumbar segment b. lumbar segment Lamina X c. Sacral segment d. neuron within lamina X, sacral segment. (Scale bar =200μm, 10μm, 200μm, 10μm a-d, respectively).

with a microtome from the cervical (C6-C8), thoracic (Th5-Th7), lumbar (L3-L5) and sacral (S1-S3) segments. Sections were mounted on glass slides, dried overnight, dehydrated in ethanol, cleared in toluene and coverslipped with Entellan. In the other cases (n=5), the brains and spinal cord segments were sectioned with vibratome (50-(μm-thick) and also dried, dehydrated, cleared and coverslipped with Entellan. Sections were examined under a fluorescence microscope. Fluorescence was excited by UV light (wavelength 360-420 nm) and filtered through BG 12 + BG 3 filters. FG was noted as intense yellow fluorescence in the cytoplasm and proximal dendrites of the some spinal neurons. These FG-labelled neurons were plotted on diagrams according to the stereotaxic atlas (19), combining many sections. Measurements of the parameters of identified NADPH-d (+) and FG (+) spinal neurons in different laminae and LSN were made on microscopic images under an X90 objective. All measured and calculated values of the parameters were expressed as mean ±SEM (standard error of mean). Parameters of the cells were compared using correlation fields and differences between populations of neurons at the four levels of the spinal cord were characterized using ANOVA.

The localization of NADPH-d (+) and FG (+) cells on coronal sections were charted with the aid of an x-y plotter. Nuclear borders and fiber pathways in the diagrams were checked and denoted also according to the stereotaxic atlas (21).

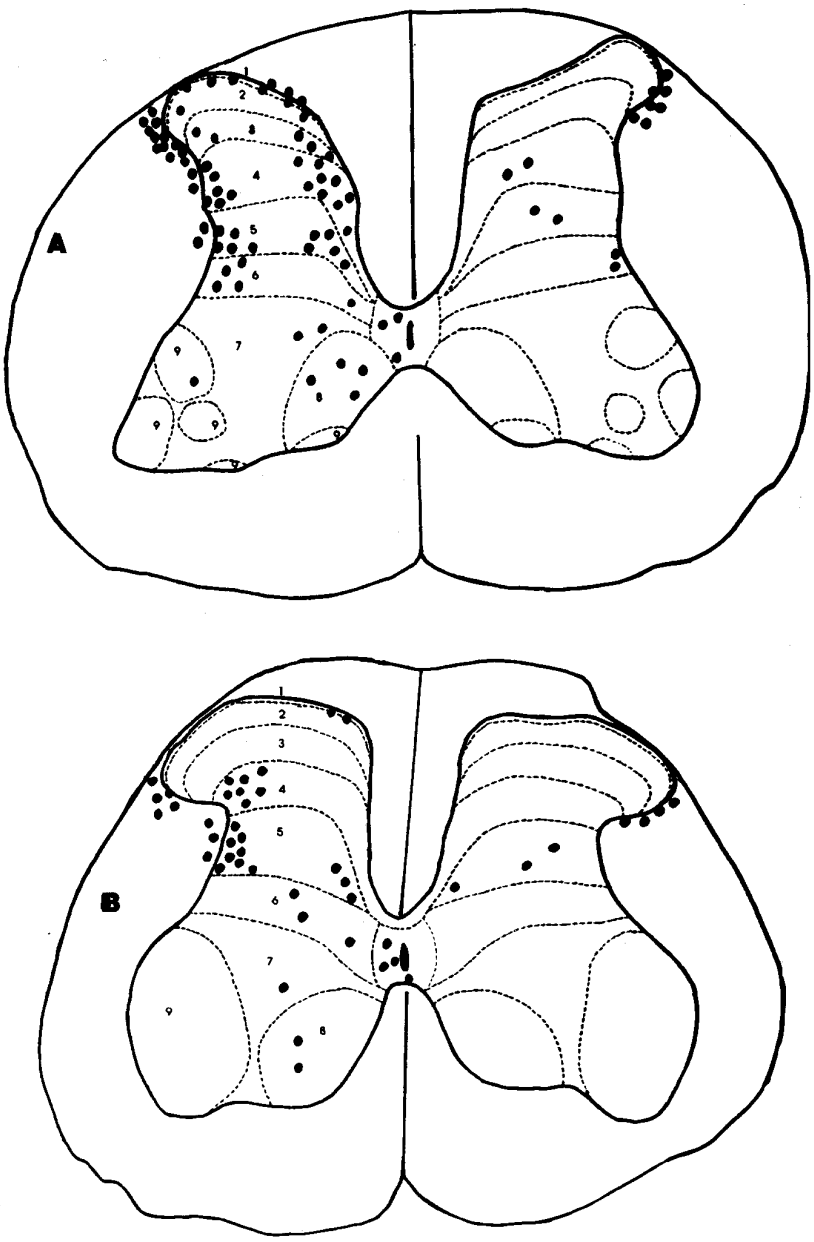

Figure 4. Schematic representation of the distribution of FG (+) cells in the cervical (a) and the lumbar (b) enlargements.

Results

In the histochemical studies, NADPH-d (+) neurons were mostly observed in lamina IIo, deep dorsal horn, lateral spinal nucleus (LSN) and especially within lamina X. In the thoracic cord a lot of these cells were also found in the intermediolateral nucleus. Schematic representation of the distribution of NADPH-d (+) cells in the cervical and lumbar enlargements is shown in Figures 1 a, b.

Positively stained neurons in lamina IIo were mostly fusiform in shape. The long axes of these cells measured 11.80 ± 0.03 µm and the short axes 6.76 ± 0.02 µm. NADPH-d (+) cells with long axes measuring 11.6 ±0.25 µm and short axes 6.51 ± 0.11 were noted in the deep dorsal horn and also the lateral reticulated area. The detected NADPH-d (+) neurons in lamina X had spindle-shaped profiles with long axes measuring 21 ± 0.05 µm and the short axes 11.38 ± 0.03 µm.

The average numbers of NADPH-d (+) cells per 50 µm-thick section were 78 and 79 for the cervical and lumbars enlargements, respectively. The average number of NADPH-d (+) cells in the thoracal and sacral segments

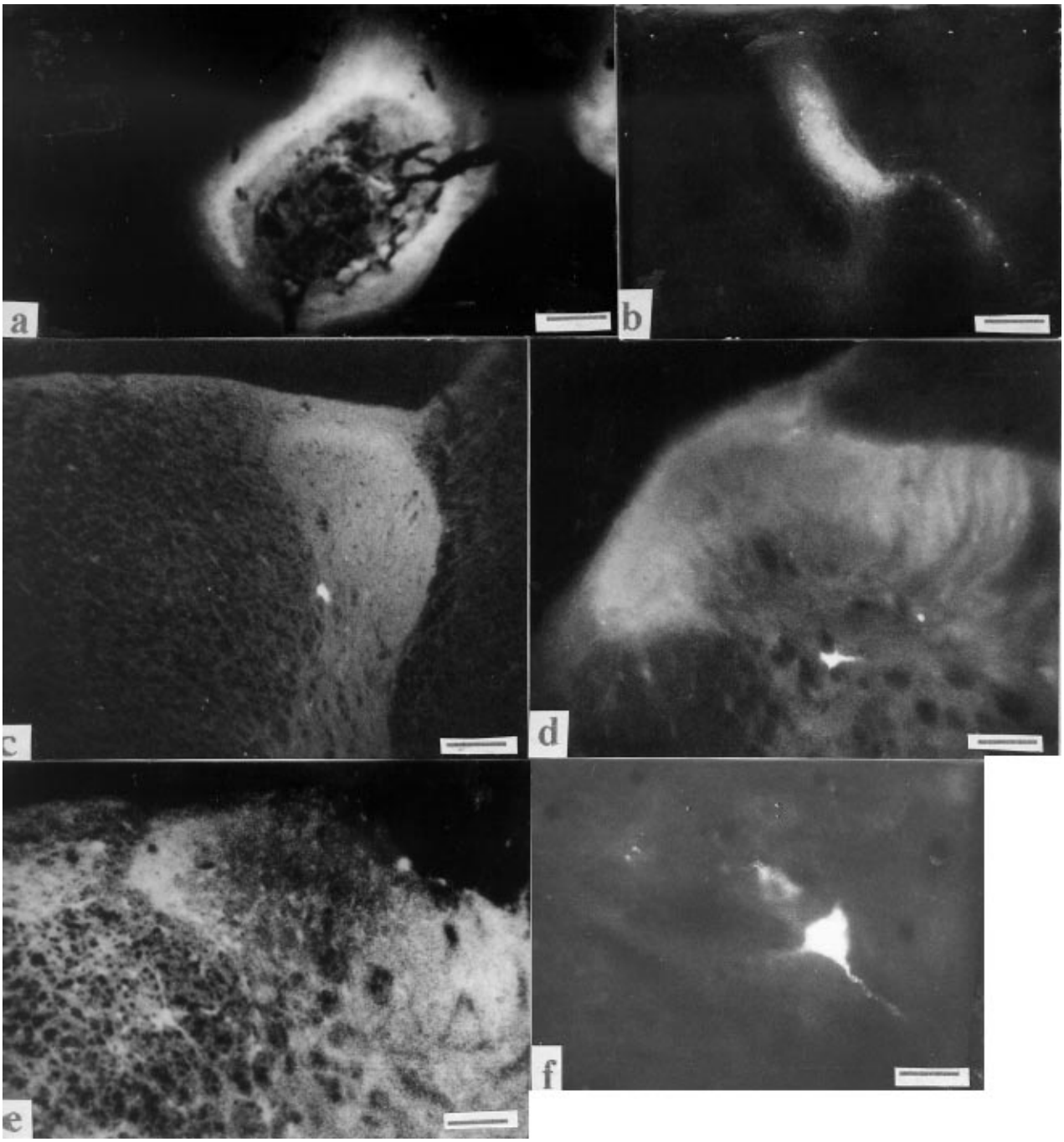

Figure 5. Photomicrographs of FG-labelled neurons in spinal cord. a. Site of FG injection (nucl. accumbens) b. FG (+) cell (lamina IV) in the cervical enlargement c. Lamina V, thoracic segment d. lumbar segment, lamina IV external zone, e. lumbar segment f. sacral segment, lamina X. Scale bar = 1250, 10, 200 and 10 (m, respectively.

were 43 and 56, respectively. Thus, NADPH-d (+) cells in all segments were mostly fusiform and spindle-shaped in lamina IIo, multipolar in the medial parts of nucl. proprius and spindle-shaped in lamina X (Fig. 2 a-d, Fig. 3 a-d).

In the fluorescent labelling studies, the necrotic core of the FG injection was centered in Acb, and an extracellular halo of dye (diffusion zone) covered a small region within the septal nuclei (Fig. 5a). Acb-projecting neurons

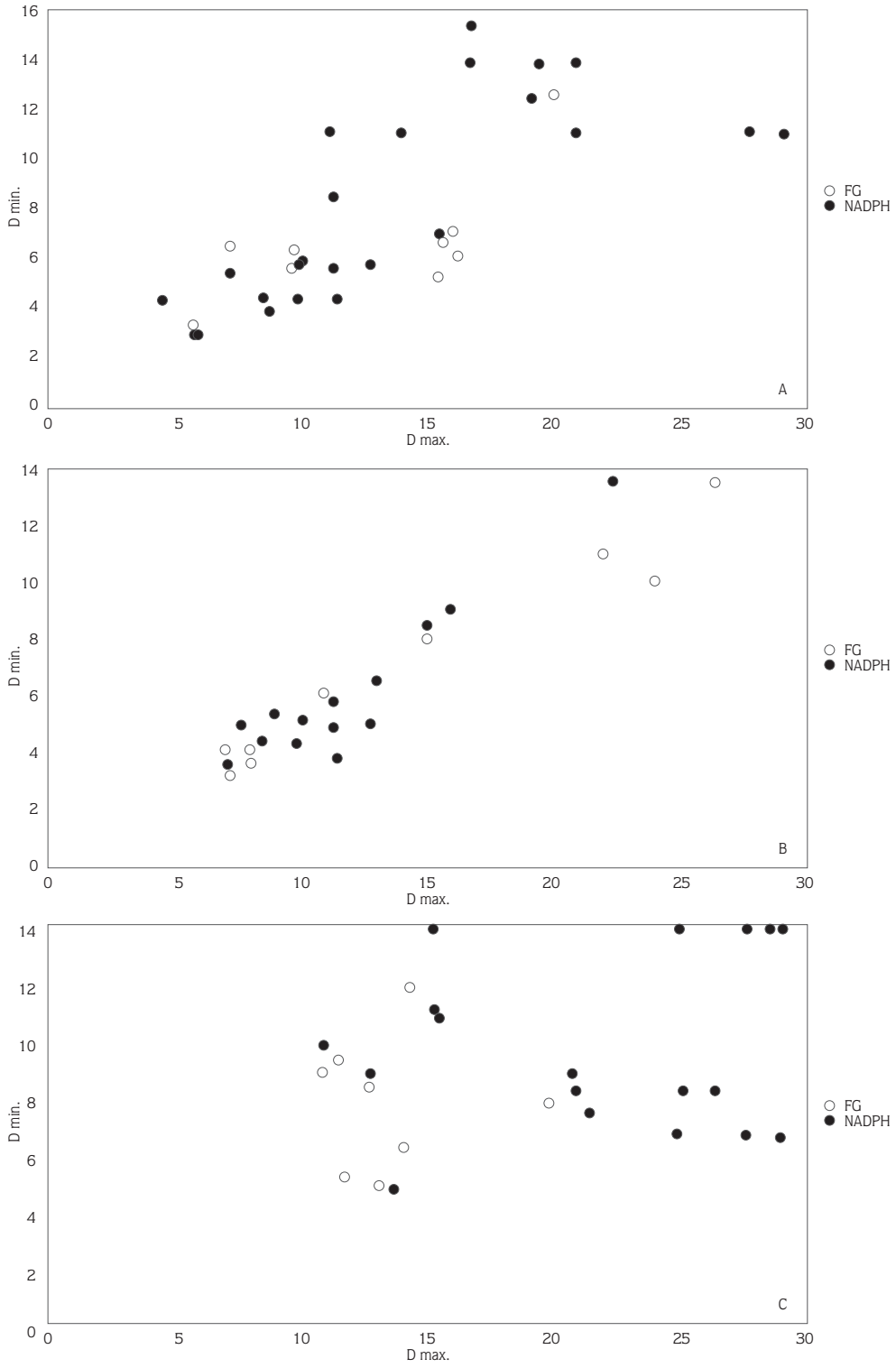

Figure 6. Correlation fields of the values of large and small diameters and forms of NADPH-d (+) spinal neurons in the cervical enlargement a. laminae I-II b. laminae IV-VI c. lamina X. Dots and circles represent parameters of NADPH-d (+) and FG (+) cells, respectively for the cervical enlargement of the spinal cord. D max.: large diameter of cells (abscissa) D min.: small diameter of cells (ordinate) in µm.

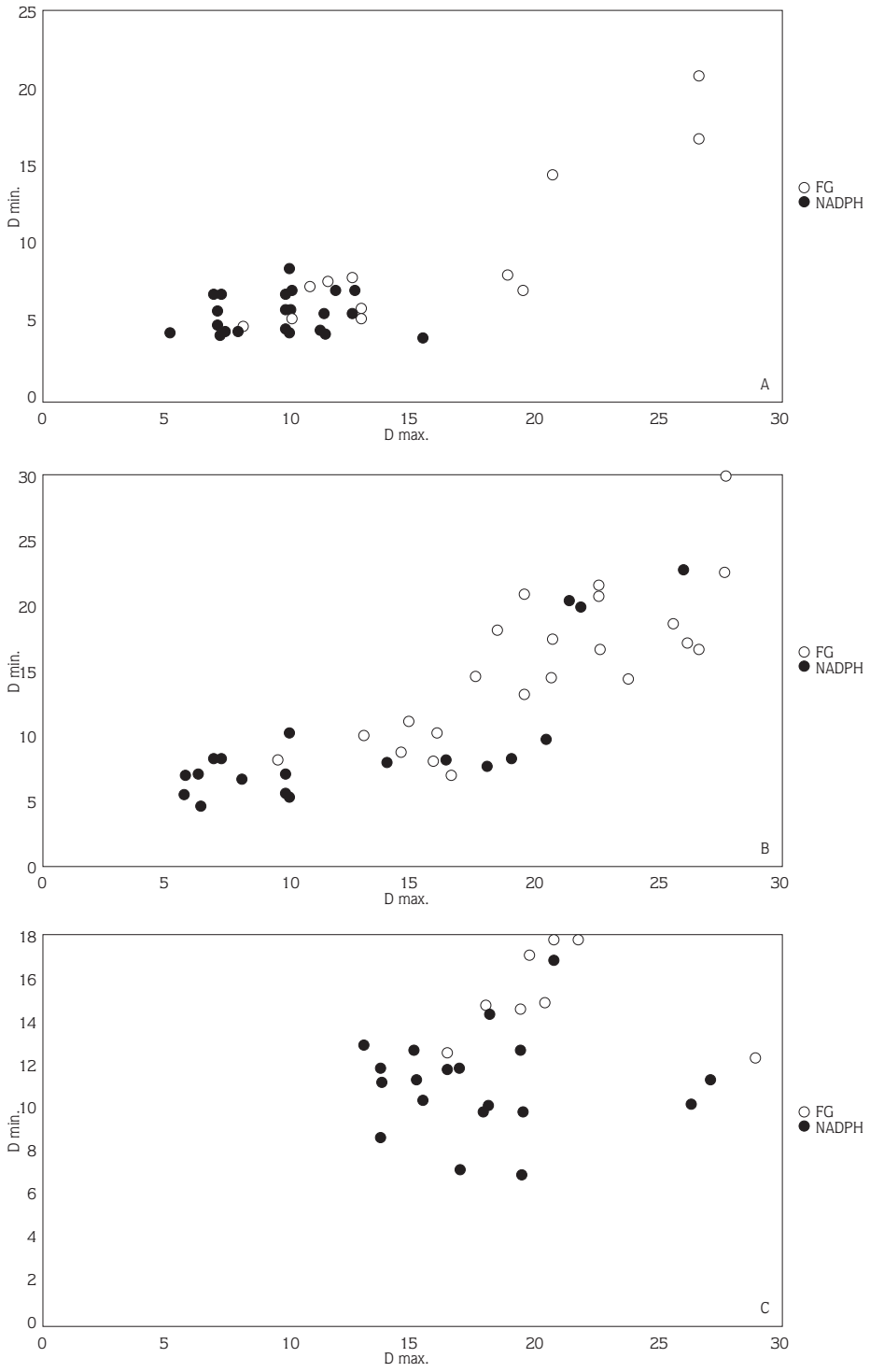

Figure 7. Correlation fields of the values of large and small diameters and forms of NADPH-d (+) spinal neurons in the lumbar enlargement a. lamina I-II b. laminae IV-VI c. lamina X.

were observed mostly in lamina I (the marginal zone) and the deep dorsal horn. The area around the central canal (lamina X) and in the dorsolateral funiculus at both sides also contained approximately 4.5 % and 27.9 % of labelled neurons respectively (Fig. 4 a-b). FG (+) neurons in lamina IIo were small spindle-shaped cells measuring 13 ± 0.58 µm in the long axis and 6.16 ± 0.22 µm in the short axis. FG (+) neurons within the deep dorsal horn (laminae IV-VI) were mostly multipolar, but also elliptic or fusiform in shape. In the cervical enlargement, these cells measured 22.04 ± 0.22 µm in the long axis and 12.4 ± 0.07 µm in the short axis. In the lumbar enlargement, Acb-projecting cells were slightly larger in size ($p< 0.5$) compared with the cervical enlargement, measuring 22.48 ± 0.22 µm in the long axis and 15 ± 0.20 µm in the short axis. FG (+) neurons in Lamina X measured 12.08 ± 0.21 µm in the long axis and 6.44 ± 0.32 µm in the short axis (Fig. 5 a-f).

The average number of Acb-projecting cells per 50-µm-thick section in the cervical, thoracal, lumbar and sacral segments were 0.46, 0.30, 0.42 and 0.08, respectively. In LSN and lamina X FG-labelled neurons were distributed bilaterally, and in lamina I were concentrated exclusively contralaterally. Figure 6 and figure 7 represent correlation fields of the values of diameters of NADPH (+) neurons and FG (+) cells in the different laminae of the spinal cord. Some NADPH-d (+) cells and Acb-projecting neurons overlap in size within LSN, laminae I-II, IV-VI and X.

Discussion

Acb is thought to play a significant role in the initiation of several types of motivational-affective behavioral responses to pain (22, 23, 24). Burstein et al. were the first to inject FG into Acb and found that many spinal neurons were labelled with this fluorochrome (19). Thus, these cells are the elements of an ascending spinocerebral pathway that has been suggested to be involved in the transmission of nociceptive information and this pathway is an ascending part of a complex loop which plays a role in the modulation of nociception.

Our recent study of retrograde double-labelling of cells with FG and Primuline O showed that sources of spino-supraspinal pathways to the upper centers of the brain stem, Acb and septal nuclei were mixed together in the marginal zone, deep dorsal horn (laminae IV-VIII), lamina X and the LSN, but had their own discrete projection fields (20).

In the present study, it was found that the pattern of distribution of FG (+) and NADPH-d (+) neurons was the same. They were observed to be mixed together in the gray matter throughout the spinal cord mostly within the dorsal horn, LSN and especially in lamina X. The cells were mostly fusiform and spindle - shaped in laminae I-II and X but multipolar within nucl. proprius (laminae IV-VI).

Quantitative analysis of histochemically revealed neurons showed that there were 78 and 79 NADPH-d (+) cells in the cervical and lumbar enlargements respectively, while this number was 0.46 and 0.42 for FG (+) cells in 50 µm-thick sections. Teherefore, the number of NADPH-d (+) cells were found to be much higher than the FG (+) cells.

Herdegen et al. (25) investigated NOS activity in neurons of the lumbar spinal cord of adult rats following s.c. injection of formalin in one hindpaw. In the untreated rat, NOS immunoreactivity and NADPH-d activity was present predominantly in laminae II-III and in the deep dorsal horn, and also predominantly in lamina X. After formalin injection, the number of neurons labelled with NOS and NADPH-d (+) cells increased in the dorsal horn. Double-labelling demonstrated that in the superficial dorsal horn most of the NADPH-d (+) neurons show a close spatial relationship to fibers and varicosities immunoreactive for substance P and c GRP. It is interesting to note that NOS-immunoreactive neurons were found within Acb in the monkey (26). Vincent and Kimura (9) found many scattered medium-sized neurons throughout Acb in their study on rats.

Recently Gear et al. have shown that suppression of tonic activity in an ascending supraspinal pathway disinhibits or activates a descending supraspinal antinociceptive circuit with an opioid link in Acb. Thus, permanent inhibition of supraspinal opioid-dependent antinociceptive mechanism by ascending tonic activity in a spino-supraspinal pathway implies that the main effect of the spinal input into the limbic system is to facilitate nociceptive sensitivity but not to suppress nociceptive transmission at the level of the spinal cord (27).

Another interesting phenomenon in the spinal cord is named 'diffuse noxious inhibitory control' (DNIC). Continued noxious input from the first stimulus is expected to decrease the efficacy of the second noxious input in exciting spinal neurons because of its activation of DNIC (28, 29). DNIC was observed as a great inhibitory effect produced by continuous repetitive high-intensity stimulation in the cat (30).

However, there is hyperalgesia at the level of the spinal cord also. When there is a low frequency input to

the spinal cord, synaptic transmission is mediated by non-NMDA receptors. However in tonic and chronic pain, where high frequency or sustained afferent input produces a prolonged depolarization, the Mg^{+2} block on spinal NMDA receptors is removed, allowing for NMDA receptor activation, an influx of Ca^{+2} and production of NO in NOS containing neurons. This results in potentiation of synaptic transmission that would manifest hyperalgesia, facilitation, an expression of receptive fields, central sensitization and/or wind-up (5, 6, 10, 15).

During the manifestation of hyperalgesia on the level of the spinal cord, the result is a higher level of neuronal activity within nucl. proprius, LSN and lamina X, where many Acb projecting neurons and NADPH-d (+) cells are distributed.

It can be suggested that Acb-projecting cells exist among NOS-containing neurons in the LSN, the deep dorsal horn and the area around the central canal. It has been shown that NO, when released, rapidly diffuses to the nearby neurons at a distance up to 500 μm away and modulate the excitability of neurons mixed together with NOS-containing cells (31). Analyzing our data, it is possible to say that some of the FG (+) cells also contain NOS, because the size, shape and localization of these cells overlap (see correlation fields, Fig. 6 a-b and Fig. 7 a-b).

Some neurons in LSN, the deep dorsal horn and lamina X are sources of spino-supraspinal pathways to Acb and septal nuclei (cells of origin of spinotelencephalic tract). Therefore, excitability and spontaneous activity of these dynamic neurons would be higher when spinally mediated hyperalgesia is developed (16). Thus, a higher level of tonic activity in the ascending spinotelencephalic pathways to the Acb will inhibit the opioidergic output of the Acb neurons and will suppress the activity of a descending supraspinal antinociceptive circuit (27). It will imply the facilitation of nociceptive sensitivity or will lift the level of spinally mediated hyperalgesia. Thus, the mechanism of hyperalgesia is more complicated than proposed previously (6, 10, 16).

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