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Authors: Aizawa, Tohru, Ikata, Takaaki, and Katoh, Shinsuke

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Double-Immunolabeling Studies of Glucocorticoid Receptors in Enkephalinergic Neurons of the Rat Spinal Cord

Tohru Aizawa*, Takaaki Ikata and Shinsuke Katoh

Department of Orthopedic Surgery, School of Medicine, The University of Tokushima, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan

ABSTRACT—Using double-immunolabeling, we attempted to localize the glucocorticoid receptor (GR) and methionine-enkephalin-octapeptide (ENK-8) in the rat spinal cord. The 2nd to 8th cervical segments were analyzed in intact and in colchicine-injected animals. GR immunoreactivity was detected in the nuclei of neurons in all segments, and was most intense in small neurons in the superficial layers (laminae 1–3) of the dorsal horn. Medium-sized neurons in laminae 4–6 and the reticular formation, as well as the large ventral horn neurons, all showed moderate immunoreactivity. Double-immunolabeling revealed that, in all regions examined except the ventral horn, some of the GR-positive neurons were also ENK-8-positive. These findings suggest that enkephalinergic neurons in the spinal cord may be regulated by glucocorticoids, each of which is presumably related to the neuronal antinociceptive mechanism in the dorsal horn.

INTRODUCTION

The receptors of adrenal steroids are heterogeneous and show a region-specific localization in the central nervous system (CNS) (Fuxe *et al.*, 1985a; McEwen *et al.*, 1986; Ahima and Harlan, 1990). Two receptor systems for glucocorticoids have been verified through the ligand binding property. Type I receptors have a high affinity to corticosterone; in contrast, type II receptors (the classical glucocorticoid receptors, GR) are characterized by a high affinity to dexamethasone. It has been demonstrated by immunohistochemistry that type I receptor immunoreactivity (IR) is localized in hippocampal pyramidal neurons and septal neurons (Ahima *et al.*, 1991). GR-IR is distributed more widely in neurons of the hippocampus, hypothalamus, brainstem, and spinal cord (Fuxe *et al.*, 1985a, b; Hisano *et al.*, 1988). Immunohistochemical analysis of the spinal cord has shown that GR-IR is localized in motor and dorsal horn neurons (Ahima and Harlan, 1990). These findings suggest that adrenal steroids influence both the sensory and motor functions of the spinal cord (Hall, 1982). Physiological studies have also demonstrated steroidal action on spinal neurons (De Nicola *et al.*, 1989).

Neuronal peptides in the spinal cord have been documented as intrinsic mediators of primary sensation and its modulation. Chao and McEwen (1990) revealed that preproenkephalin mRNA synthesis was regulated by glucocorticoid in rat strial neurons. Furthermore, Yin and Howells (1992) showed that dexamethasone has stimulatory effects on proenkephalin gene expression in cultured rat glioma cells. Of the peptides, opioids which include enkephalin are known to participate in antinociception. Immunohistochemical analysis of the opioid system has demonstrated that enkephalin-IR exists in dorsal horn neurons (Finley *et al.*, 1981; Katoh *et al.*, 1988). Therefore, the dorsal horn may be one of the sites for steroid-opioid interaction in the spinal cord. In the present study, we used double-immunolabeling to examine the neuroanatomical relationship between GR, which is described as having a high affinity to dexamethasone-positive neurons, and enkephalinergic neurons in the rat spinal cord.

* Corresponding author: Tel. +81-88-633-7240; FAX. +81-88-633-0178.

MATERIALS AND METHODS

Animals and fixation

Twenty adult male Wistar rats (250–350 g body weight) were used. Under chloral hydrate anesthesia (45 mg/100 g body weight), 10 animals received colchicine (100 μg/10 μl/rat) (Nakarai, Kyoto, Japan) by injection into the cisterna cerebellomedullaris 24 hr before sacrifice to facilitate the detection of enkephalinergic neurons. The intact and the colchicine-injected animals were fixed by transaortic perfusion with 0.1 M sodium cacodylate buffer (pH 7.4) followed by a fixative (4% paraformaldehyde, 0.15 % glutaraldehyde, and 0.2% picric acid in the same buffer). The 2nd to 8th cervical segments of the spinal cord were dissected out and immersed in fresh fixative overnight at 4°C. The tissues were cut into 30 μm-thick vibratome sections and stored in cold fixative (4% paraformaldehyde, and 0.2% picric acid in the same buffer) until immunostaining.

Antisera

To immunostain GR, two anti-GR antibodies were used. The first was a mouse monoclonal antibody (BuGR-2) that recognizes the 17 amino acid sequence (407–423) near the DNA-binding portion of the rat liver GR (Gametchu and Harrison, 1984). The second was a polyclonal antibody (PolyGR) purchased from Affinity BioReagents (Golden, CO, USA). This antibody, raised in rabbits against a 22 amino acid synthetic peptide (amino acids 346–367 of the human GR), is

reported to recognize, by Western Blot, a single 97 kDa band corresponding to the rat GR (Cidlowski *et al.*, 1990). The immunohistochemical specificity of both antibodies has also been evaluated by other studies (Hisano *et al.*, 1988; Ahima and Harlan, 1990; Cidlowski *et al.*, 1990). For the present immunolabeling, the antibodies were diluted (1:500 for BuGR-2; 1:1,000 for PolyGR) with 0.02 M phosphate buffered saline (PBS, pH 7.4) containing 10% normal horse or goat serum (Cappel, West Chester, PA, USA) and 0.1% sodium azide. To immunostain enkephalinergic neurons, anti-methionine-enkephalin-octapeptide (ENK-8) antiserum (Peninsula Laboratories, Inc. San Carlos, CA, USA) was used. The specificity of the antiserum was characterized according to the same absorption test as previously described (Katoh *et al.*, 1988). The antiserum was diluted (1:5,000) with PBS containing liver extract and a carrier protein for immunostaining.

Immunohistochemistry

Sections were first washed with PBS and immersed in 10% normal horse or goat serum in PBS for 60 min at 25°C to suppress background antibody binding onto the tissues. For single-immunolabeling of GR-IR, the sections were processed as follows: (*a*) BuGR-2 (60 hr, 4°C) or PolyGR (15 hr, 4°C); (*b*) biotinylated horse anti-mouse IgG (1:100, 2 hr, 32°C, Vectastain ABC Kit, Vector, Burlingame, CA, USA) for BuGR-2 or biotinylated goat anti-rabbit IgG (1:200, 2 hr, 32°C, Vector) for PolyGR; and (*c*) avidin-biotin-peroxidase complex (2 hr, 32°C, Vector). After each incubation with the immunoreagents, the sections were thoroughly washed with PBS. The immunoreaction was revealed with either diaminobenzidine tetrahydrochloride (DAB) (Hisano *et al.*, 1988) or DAB-nickel ion solution (Ni-DAB, Hancock, 1984). For double-immunolabeling, only the sections from colchicine-injected animals were used.

After revealing GR-IR with Ni-DAB, the sections were further processed for immunolabeling of ENK-8 as follows: (*a*) anti-ENK-8 serum (36 hr, 4°C); (*b*) biotinylated goat anti-rabbit IgG (1:200, 2 hr, 32°C, Vector); and (*c*) avidin-biotin-peroxidase complex (2 hr, 32°C, Vectastain ABC Elite Kit, Vector). The immunoreaction for ENK-8 was revealed with DAB, so that GR-IR was labeled with Ni-DAB (dark blue) and ENK-8-IR with DAB (brown). The vibratome sections were not treated with hydrogen peroxide (which suppresses the reaction of endogenous peroxidase), to prevent interference with the immunoreactivity of BuGR-2 and PolyGR. To serve as negative controls to assess the immunoreaction with BuGR-2 or PolyGR, sections were incubated with the diluents alone. These preparations showed no immunoreaction. In this study, the absorption control experiment was not performed because of the difficulty of obtaining the immunogens. The staining specificity was previously examined (Hisano *et al.*, 1988; Ahima and Harlan, 1990; Cidlowski *et al.*, 1990).

From each of 10 colchicine-injected rats, we sampled 10 sections of cervical spinal cord at random. The labeled neurons per section were counted. The mean and standard deviation (SD) were calculated for the number of GR-positive neurons and ENK-8-positive neurons for each section.

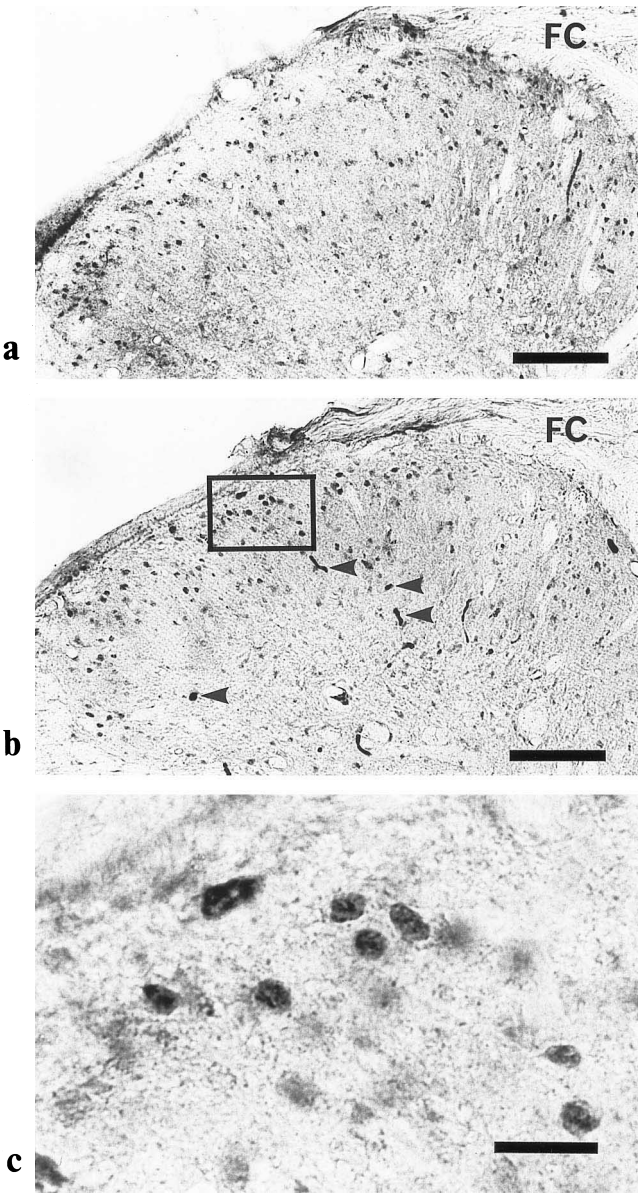

Fig. 1. GR-positive neurons in the dorsal horn of the cervical spinal cord (C5). a. PolyGR staining in an intact animal revealed with DAB chromogen. GR-positive neurons are localized predominantly in laminae 1–3. FC fasciculus cuneatus. Scale bar: 100 μm. b. BuGR-2 staining in a colchicine-injected animal revealed with DAB chromogen. The distribution of the GR-positive neurons is nearly identical to that in PolyGR staining (a). Arrowheads indicate erythrocytes. FC fasciculus cuneatus. Scale bar: 100 μm. c. Magnified view of the framed area in b. GR-IR is confined to the nuclei of GR-positive neurons. Scale bar: 20 μm.

RESULTS

Localization of GR-immunoreactivity

Both antibodies (BuGR-2 and PolyGR) brought about almost the same results in the demonstration of GR-IR (Fig. 1a, b). At any location within the spinal cord, GR-IR was confined to the nuclei of positive neurons and glia-like cells. The immunoreaction was consistently slightly more intense in sections stained with PolyGR than in those stained with BuGR-2. By the Ni-intensification stronger signals were obtained than those with DAB alone. Positive signals detected by Ni-DAB were found as small particles in the cell nuclei, while such particles were unclear by DAB alone (Figs. 1c, 2, 3, 4, and 5).

In both intact and colchicine-injected animals, many GR-positive neurons were revealed throughout the cervical spinal cord. The distribution and intensity of the immunoreaction did not differ between the two groups. In the dorsal horn, a large population of small GR-positive neurons was revealed in laminae 1–3, chiefly in lamina 2 (Fig. 1). In laminae 4–6 and the reticular formation, medium-sized neurons were moderately,

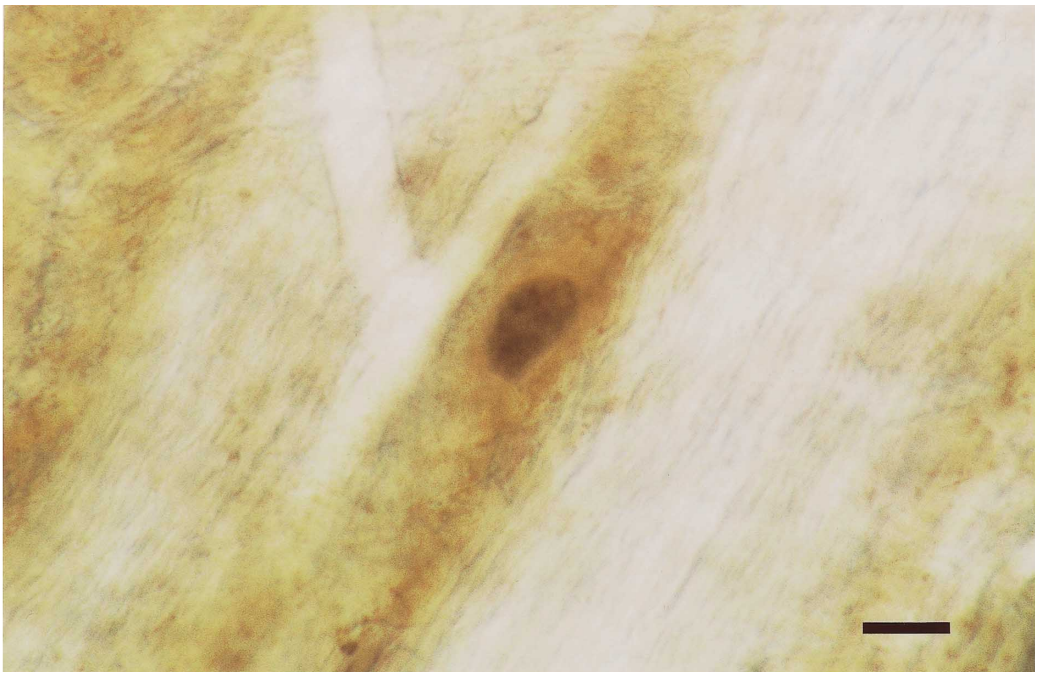

Fig. 2. Double-immunolabeling for GR (dark blue) and ENK-8 (brown) in the cervical spinal cord (C3) in lamina 2 of the dorsal horn. An example of small GR-positive neuron that contains dark blue signals as GR-IR within the nucleus Scale bar: 1 μm.

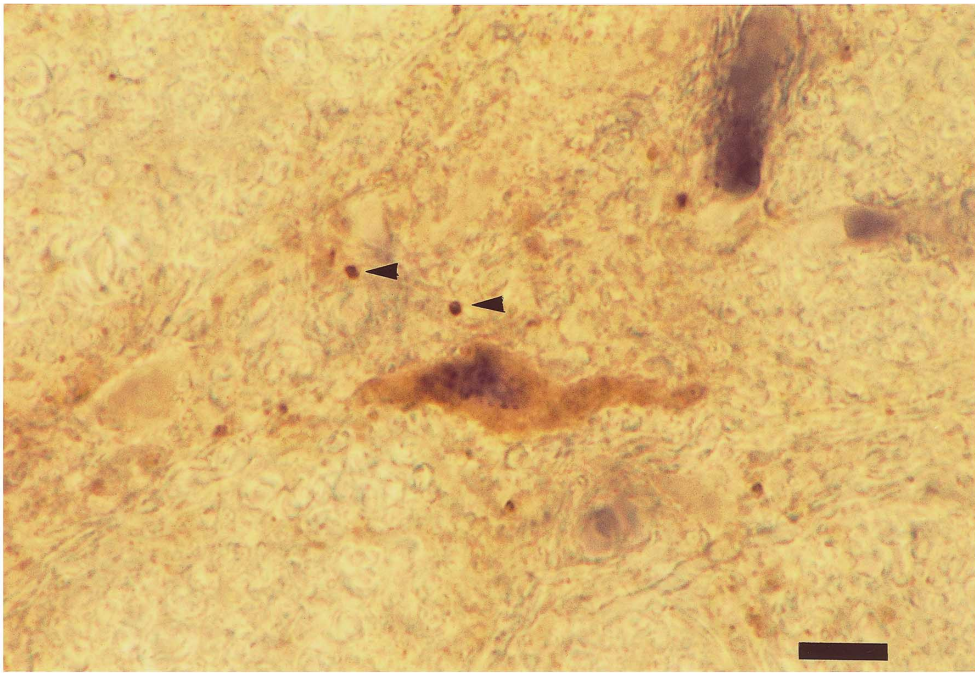

Fig. 3. Double-immunolabeling for GR (dark blue) and ENK-8 (brown) in the cervical spinal cord (C3) in lamina 4. A medium-sized, double-labeled neuron whose nucleus is heavily marked with dark blue particles. The brown dots (arrowheads) indicate ENK-8-positive fibers. Scale bar: 1 μm.

sometimes strongly, immunoreactive for GR. In lamina 9 of the ventral horn, nuclei of almost all large neurons (probably motor neurons) expressed moderate to strong GR-IR. In addition, the nuclei of glia-like cells were usually immunostained throughout the white matter and in the pia mater.

Double-immunolabeling for GR and ENK-8

We examined only colchicine-injected rats because it was easier to detect ENK-8-positive neurons in them. In the dorsal horn of all cervical segments, double-labeled neurons were frequently encountered in the dorsal horn (5.0±0.9 neurons, mean±SD per section), chiefly in lamina 2 (Fig. 2). ENK-8-positive fibers were also revealed. GR-positive neurons were

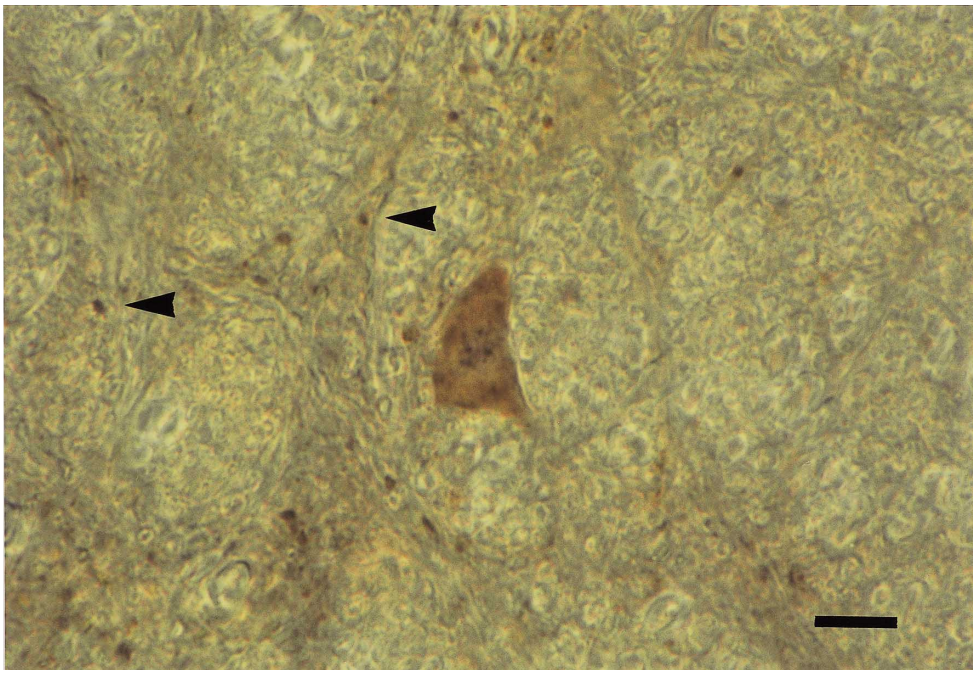

Fig. 4. Double-immunolabeling for GR (dark blue) and ENK-8 (brown) in the cervical spinal cord (C3) in the reticular formation. A medium-sized, double-labeled neuron is detected. Several dark blue particles can be seen in the nucleus. Arrowheads indicate ENK-8-positive fibers. Scale bar: 1 μm.

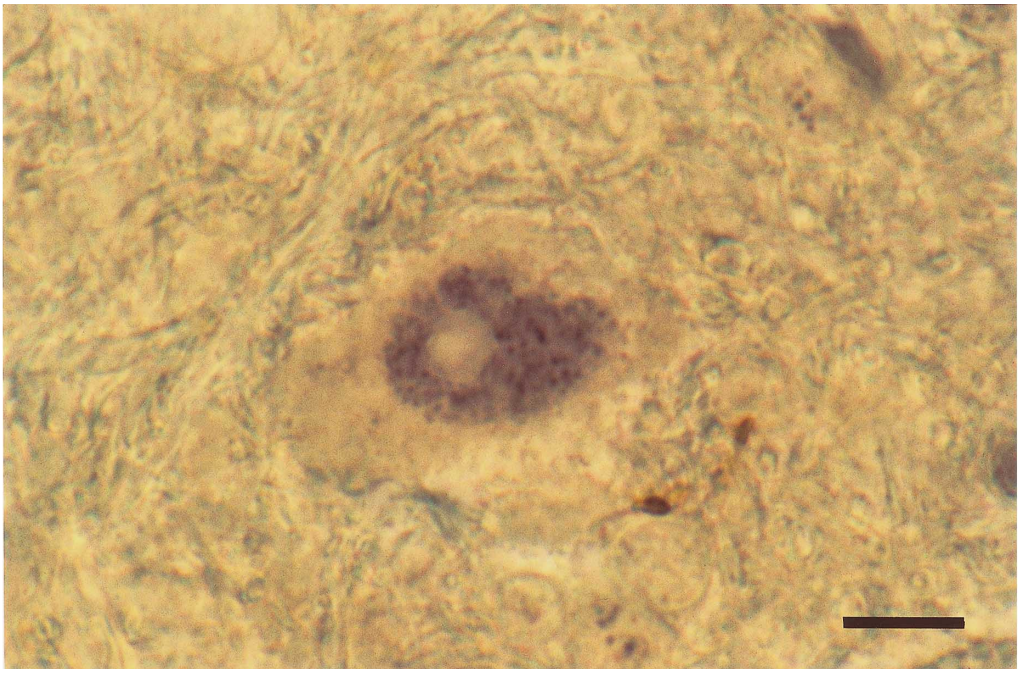

Fig. 5. A large GR-positive (dark blue) neuron in the ventral horn. GR-IR can be observed in the nucleus but in neither the nucleolus nor the cytoplasm. Scale bar: 1 μm.

25.0±2.7 neurons, and ENK-8-positive neurons were 5.5±1.1 neurons in the dorsal horn per section.

Double-labeled neurons accounted for 20. 0% of GR-positive neurons and 91.0% of ENK-8-positive neurons. In laminae 4–6 (Fig. 3) and in the reticular formation (Fig. 4), a few medium-sized GR-positive neurons were immunoreactive for ENK-8. About a half of ENK-8-positive neurons in these regions were also positive for GR. In the ventral horn, all the GR-positive neurons (2.6±0.8 neurons per section) were large-sized and did not contain ENK-8 (Fig. 5).

DISCUSSION

The present study shows the co-localization of GR-IR and

ENK-8-IR in neurons of the cervical spinal cord in the rat. Previous immunohistochemical studies have described GR-positive neurons in the superficial layers of the dorsal horn in the spinal cord (Fuxe *et al.*, 1985b; Ahima and Harlan, 1990). Using BuGR-2, Ahima and Harlan (1990) demonstrated that GR-positive neurons are located in lamina 2 of the dorsal horn and in laminae 8 and 9 of the ventral horn at cervical, thoracic, and lumbar levels. Anti-GR antibody (PolyGR) and BuGR-2, each of which recognizes a different domain of the receptor protein, were used in the present study. The distribution of GR-positive neurons was identical for the two antibodies and consistent with previously reported data (Fuxe *et al.*, 1985b; Ahima and Harlan, 1990). Therefore, the present study not only reconfirms the existence of glucocorticoid receptors in lamina 2, 8, and 9, but also documents their existence in lamina 1, 3, 4–8, the reticular formation, and the glia-like cells of the spinal cord.

Coexistence of GR and enkephalin has been shown in the basal ganglia and paraventricular nucleus of the hypothalamus (Cintra *et al.*, 1991; Ceccatelli *et al.*, 1989), and it was suggested that glucocorticoid may influence pain and the mood of the animals through the neurons in the hypothalamus. However, previous studies have not identified the neuroactive substances that GR-positive neurons produce in the spinal cord. The most salient result of the present study is that some of the GR-positive neurons were enkephalinergic at least in the cervical segments of the spinal cord.

The co-localization of GR-IR and ENK-8-IR was most frequently detected in neurons of the dorsal horn and sometimes in laminae 4–8 of the cervical spinal cord. This suggests that glucocorticoids regulate a certain function of the enkephalinergic neurons, probably at the genomic level. By a molecular biological approach in cultured rat glioma cells, Yin and Howells have recently reported that dexamethasone stimulates proenkephalin expression (1992). Furthermore, the rat proenkephalin gene has been reported to contain a nucleotide sequence highly homologous to a glucocorticoid response element (Beato, 1989; Muller and Renkawitz, 1991). Using the *in situ* hybridization technique, Chao and McEwen (1990) demonstrated that glucocorticoids regulate preproenkephalin mRNA synthesis in rat striatal neurons. In the sympathoadrenal system of the rat, dexamethasone produces effects at the level of enkephalin (Stachowiak *et al.*, 1988). Increased glucocorticoid levels in response to stress lead to increased sympathetic enkephalin levels (Henion *et al.*, 1993). Thus, glucocorticoids may also influence peptide production in enkephalinergic neurons of the spinal cord.

Enkephalinergic neurons in the dorsal horn are thought to mediate antinociception to noxious stimuli from peripheral regions (Hökfelt *et al.*, 1977; Glazer and Basbaum, 1983). Therefore, glucocorticoids may influence this function of enkephalinergic neurons. Analgesia is known to be evoked under some stressful conditions. In stress-induced analgesia, glucocorticoids are considered critical factors in a neuronal analgesic pathway within the brain following activation of the hypothalamic-pituitary-adrenal axis (MacLennan *et al.*, 1982; Ratka *et al.*, 1988). At present, there is no neurophysiological evidence for a positive regulation by glucocorticoids of the release of enkephalins from dorsal horn neurons under stressful conditions. However, by taking into consideration the molecular biological evidence obtained by Yin and Howells (1992), the present data may suggest that the opioid interacts with the steroid, and the interaction relates to stress-induced analgesia in the spinal cord.

Recent immunohistochemical studies have described the co-localization of ENK-8 and substance P (Katoh *et al.*, 1988) or gamma-aminobutyric acid (Todd *et al.*, 1992) in dorsal horn neurons of the rat. Therefore, glucocorticoids may be involved in the synthesis of such neuroactive substances in enkephalinergic neurons. The results of the present double-immunolabeling analysis suggest the presence of GR-positive "non-enkephalinergic" neurons in the dorsal horn, though the technical limitation of immunohistochemistry should be kept in mind. This may indicate that glucocorticoids regulate neurons other than the enkephalinergic kind. After peripheral nerve stimulation, c-Fos-related proteins are expressed in dorsal horn neurons (Hunt *et al.*, 1987), some of which are positive for dynorphin (Noguchi *et al.*, 1991). Molecular biological studies on cultured cells have suggested a glucocorticoid-mediated down-regulation of c-Fos mRNA (Yin and Howells, 1992) and the negative influence of glucocorticoids on the expression of collagenase through an interaction of GR and the AP-1 complex (c-Fos/c-Jun) (Jonat *et al.*, 1990). Thus, it may be possible that adrenal steroids affect the dorsal horn through various neuronal functions including those of ENK-8.

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REFERENCES

Ahima RS, Harlan RE (1990): Charting of Type II glucocorticoid receptor-like immunoreactivity in the rat central nervous system. Neuroscience 39: 579–604

Ahima R, Krozowski Z, Harlan R (1991): Type I corticosteroid receptor-like immunoreactivity in the rat CNS: distribution and regulation by corticosteroids. J Comp Neurol 313: 522–538

Beato M (1989): Gene regulation by steroid hormones. Cell 56: 335–344

Ceccatelli S, Cintra A, Hökfelt T, Fuxe K, Wikstrom A-C, Gustafsson JA (1989): Coexistence of glucocorticoid receptor-like immunoreactivity with neuropeptides in the hypothalamic paraventricular nucleus. Exp Brain Res 78: 33–42

Chao HM, McEwen BS (1990): Glucocorticoid regulation of preproenkephalin messenger ribonucleic acid in the rat striatum. Endocrinology 126: 3124–3130

Cidlowski JA, Bellingham DL, Powell-Oliver FE, Lubahn DB, Sar M (1990): Novel antipeptide antibodies to the human glucocorticoid receptor: recognition of multiple receptor forms in vitro and distinct localization of cytoplasmic and nuclear receptors. Mol

Endocrinol 4: 1427–1437
Cintra A, Fuxe K, Solfrini V, Agnati LF, Tinner B, Wikström A-C, Staines W, Okret S, Gustafsson J-A (1991): Central peptidergic neurons as targets for glucocorticoid action. Evidence for the presence of glucocorticoid receptor immunoreactivity in various types of classes of peptidergic neurons. J Steroid Biochem Molec Biol 40: 93–103
De Nicola AF, Moses DF, Gonzalez S, Orti E (1989): Adrenocorticoid action in the spinal cord: some unique molecular properties of glucocorticoid receptors. Cell Mol Neurobiol 9: 179–192
Finley JCW, Maderdrut JL, Petrusz P (1981): The immunocytochemical localization of enkephalin in the central nervous system of the rat. J Comp Neurol 198: 541–565
Fuxe K, Wikström A-C, Okret S, Agnati LF, Härfstrand A, Yu Z-Y, Granholm L, Zoli M, Vale W, Gustafsson JA (1985a): Mapping of glucocorticoid receptor immunoreactive neurons in the rat tel- and diencephalon using a monoclonal antibody against rat liver glucocorticoid receptor. Endocrinology 117: 1803–1812
Fuxe, K, Härfstrand A, Agnati LF, Yu Z-Y, Cintra A, Wikström A-C, Okret S, Cantoni E, Gustafsson J-A (1985b): Immunocytochemical studies on the localization of glucocorticoid receptor immunoreactive nerve cells in the lower brain stem and spinal cord of the male rat using a monoclonal antibody against rat liver glucocorticoid receptor. Neurosci Lett 60: 1–6
Gametchu B, Harrison RW (1984): Characterization of a monoclonal antibody to the rat liver glucocorticoid receptor. Endocrinology 114: 274–279
Glazer EJ, Basbaum AI (1983): Opioid neurons and pain modulation: an ultrastructural analysis of enkephalin in cat superficial dorsal horn. Neuroscience 10: 357–376
Hall ED (1982): Glucocorticoid effects on central nervous excitability and synaptic transmission. Int Rev Neurobiol 23: 165–195
Hancock MB (1984): Visualization of peptide-immunoreactive processes on serotonin-immunoreactive cells using two-color immunoperoxidase staining. J Histochem Cytochem 32: 311–314
Henion PD, Landis SC (1993): Modulation of the enkephalinergic phenotype of rat sympathetic neurons by hormonal and transynaptic mechanisms. J Neurobiol 24: 1243–1251
Hisano S, Kagotani Y, Tsuruo Y, Daikoku S, Chihara K, Whitnall MH (1988): Localization of glucocorticoid receptor in neuropeptide Y-containing neurons in the arcuate nucleus of the rat hypothalamus. Neurosci Lett 95: 13–18
Hökfelt T, Ljungdahl A, Terenius L, Elde R, Nilsson G (1977): Immunohistochemical analysis of peptide pathways possibly related to pain and analgesia: enkephalin and substance P. Proc Natl Acad Sci USA 74: 3081–3085
Hunt SP, Pini A, Evan G (1987): Induction of c-fos-like protein in spinal cord neurons following sensory stimulation. Nature 328: 632–634
Katoh S, Hisano S, Kawano H, Kagotani Y, Daikoku S (1988): Light- and electron-microscopic evidence of costoring of immunoreactive enkephalins and substance P in dorsal horn neurons of rat. Cell Tiss Res 253: 297–303
Khachaturian H, Watson SJ, Lewis ME, Coy D, Goldstein A, Akil H (1982): Dynorphin immunocytochemistry in the rat central nervous system. Peptides 3: 941–954
Jonat C, Rahmsdorf HJ, Park K-K, Cato ACB, Gebel S, Ponta H, Herrlich P (1990): Antitumor promotion and anti-inflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. Cell 62: 1189–1204
MacLennan AJ, Drugan RC, Hyson RL, Maier SF, Madden J IV, Barchas JD (1982): Corticosterone: a critical factor in an opioid form of stress-induced analgesia. Science 215: 1530–1532
Magoul R, Onteniente B, Geffard M, Calas A (1987): Anatomical distribution and ultrastructural organization of the GABAergic system in the rat spinal cord. An immunocytochemical study using anti-GABA antibodies. Neuroscience 20: 1001–1009
McEwen BS, De Kloet ER, Rostene W (1986): Adrenal steroid receptors and actions in the nervous system. Physiol Rev 66: 1121–1188
Muller M, Renkawitz R (1991): The glucocorticoid receptor. Biochim Biophys Acta 1088: 171–182
Noguchi K, Kowalski K, Traub R, Solodkin A, Iadarola MJ, Ruda MA (1991): Dynorphin expression and Fos-like immunoreactivity following inflammation induced hyperalgesia are colocalized in spinal cord neurons. Mol Brain Res 10: 227–233
Ratka A, Sutanto W, De Kloet ER (1988): Long-lasting glucocorticoid suppression of opioid-induced antinociception. Neuroendocrinology 48: 439–444
Stachowiak MK, Lee PHK, Rigual RJ,Viveros OH, Hong JS (1988): Roles of the pituitary-adrenocortical axis in control of the native and cryptic enkephalin levels and proenkephalin mRNA in the sympathoadrenal system of the rat. Mol Brain Res 3: 263–274
Todd AJ, Spike RC, Russell G, Johnston HM (1992): Immunohistochemical evidence that Met-enkephalin and GABA coexist in some neurons in the rat dorsal horn. Brain Res 584: 149–156
Yin J, Howells RD (1992): Glucocorticoid-mediated down regulation of c-fos mRNA in C6 glioma cells: lack of correlation with proenkephalin mRNA. Mol Brain Res 12: 187–194

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