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Modifications in the distribution of met-enkephalin in the cat spinal cord after administration of clonidine. An immunocytochemical study

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Summary. We have studied the modifications in the distribution of methionine-enkephalin in the cat spinal cord after intravenous or intrathecal administration of clonidine by using an immunocytochemical technique. In animals not treated with the substance, a very high density of immunoreactive fibers was found in layers I and II; a high density in the dorso-lateral funiculus and in the reticular formation; a moderate density in layers III, IV and V; and a low density in layer VI. However, after intravenous or intrathecal administration of clonidine a decrease in fibers containing met-enkephalin was observed in layers I and II (high or moderate density), the dorso-lateral funiculus, and the reticular formation (moderate or low density), and in layers IV and V (low or very low density). In all cases, the decrease in the immunoreactivity was more marked when clonidine was administered intrathecally. Our results suggest that clonidine induces the release of met-enkephalin in the spinal cord. They further suggest that the opioid peptide released could be involved in the control of nociceptive transmission by inhibiting the release of neurotransmitters (e.g., substance P). In summary, our study shows that clonidine could be involved in antinociceptive mechanisms in the cat spinal cord.

Key words: Met-enkephalin, Clonidine, Spinal cord, Immunocytochemistry, Cat

Introduction

Pain is the most important human clinical symptom and a very unpleasant experience whose solution remains to be solved. The discovery and localization of endogenous opioid peptides in the nervous system (Hughes et al., 1975; Guillemin et al., 1976; Conrath-Verrier et al., 1983; Conrath et al., 1986) has opened new therapeutic possibilities for reducing the sensation of pain, since if endogenous peptides with opioid activity could be released by means of psychic, physical or pharmacological stimuli, it seems that this release of peptides could produce analgesia without any secondary effects.

In this sense, after low-frecuency analgesic electro-acupuncture, several studies have shown alterations in the distribution of met-enkephalin in the cat central nervous system (Vázquez et al., 1995) especially at sites related to nociceptive pathways (Vacca-Galloway et al., 1985; Cobos, 1988; Vázquez et al., 1990). In the present study we searched for changes in the enkephalinergic system of the cat spinal cord using a chemical stimulus; namely, clonidine. We chose this substance because it has been demonstrated, a) to have a potent analgesic effect (Yaksh and Reddy, 1981; Nakamura and Lico, 1988; Mastrianni et al., 1989; Ossipov et al., 1989), and b) to be very efficient as treatment during the opioid withdrawal syndrome (Tseng et al., 1975; Gold and Pottasch, 1981).

Clonidine apparently produces analgesia by releasing methionine-enkephalin from the terminals, inhibiting this neuroactive substance the release of neurotransmitters (e.g., substance P) in the nociceptive pathways (Jessel and Iversen, 1977). Moreover, it seems that during the opioid withdrawal syndrome, clonidine administration restores the normal levels of opiate peptides that are decreased, because their synthesis would be blocked by exogenous opioid peptide administration.

The aims of this work were therefore: 1) to compare the density of fibers containing met-enkephalin in the cat spinal cord of control animals and animals treated with clonidine; and 2) to compare two routes of clonidine administration (intravenous or intrathecal) in order to determine which route elicits the most alterations in immunoreactive fiber density.

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Materials and methods

Experimental procedure

Ten adult male cats (2-3 kg body weight) obtained from commercial sources were used in this study. The animals were divided into three groups: a) control cats (not treated with clonidine) (2 animals); b) cats with intravenous administration of clonidine (4 animals); and c) cats with intrathecal administration of clonidine (4 animals). The animals were deeply anaesthetized with ketamine (40 mg/kg) and 5 min after anaesthesia clonidine was administered intravenously by catheter into the saphena magna vein (15 μg/kg diluted in 3 ml of saline solution) (Eisenach and Grice, 1988) or administered intrathecally between the fourth and the fifth lumbar vertebrae (20 μg/kg diluted in 3 ml of saline solution) (Eisenach et al., 1987; Drasner and Fields, 1988). Animals were perfused for 30 min and 80 min respectively after intravenous or intrathecal clonidine administration.

Tissue processing

All animals were perfused transcardially with 500 ml of 0.9% NaCl and 3 l of 4% paraformaldehyde diluted in Sörensen buffer. Spinal cords were dissected out, postfixed overnight in the latter solution, and placed in sucrose baths of increasing concentration (10-30%) until they sank. Using a cryostat, 80 μm serial transversal sections of the cervical spinal cord were cut and processed for immunocytochemistry.

Immunocytochemical procedure

An indirect immunoperoxidase technique was used for the detection of met-enkephalin, as described previously (Conrath et al., 1986). Free-floating sections were pre-incubated for 30 min in 1% normal horse serum in Sörensen buffer containing 0.3% Triton X-100 in order to enhance antibody penetration. Sections were then incubated overnight at 4 °C in the same buffer containing anti-met-enkephalin antiserum, at a dilution of 1/1600. After a 30-min wash with Sörensen buffer, sections were incubated for 1 h with sheep anti-rabbit immunoglobulin coupled to horseradish peroxidase as the second antiserum, diluted 1/250 in Sörensen buffer. After washing in this buffer (30 min) and Tris-HCl buffer (pH 7.4) (10 min), tissue-bound peroxidase was developed by the 3,3'-diaminobenzidine method.

Antiserum specificity

The antiserum used in this study was purchased from Cambridge Research Biochemical (Cambridge, U.K.). Immunostaining specificity was checked by: 1) omission of the met-enkephalin antiserum in the first incubation bath (no residual immunoreactivity was observed); and 2) the absence of possible interference by endogenous peroxidase on staining some sections, beginning with the diaminobenzidine step (again, no reaction was observed). The term met-enkephalin-like immunoreactive (ME-ir) is henceforth used to describe the staining results in our material. Mapping was carried out according to the atlas of Rexed (1954).

Results

The results obtained are summarized in Table 1 and partially illustrated in Figure 1. Immunoreactivity was always found in neuronal cells (in fibers, not in cell bodies), but never in non-neuronal cells. In the control group (not treated with clonidine), we found a very high density of ME-ir fibers in layers I and II of Rexed; a high density in the dorso-lateral funiculus and in the reticular formation; a moderate density in layers III, IV and V; and a low density in layer VI.

After intravenous administration of clonidine, the density of ME-ir fibers was high in layers I and II; moderate in layer III, the dorso-lateral funiculus, and reticular formation, and low in layers IV, V and VI. When clonidine was intrathecally administered, a moderate density of ME-ir fibers was observed in layers I, II and III; a low density in the dorso-lateral funiculus and reticular formation; and a very low density in layers IV, V and VI.

In summary, after intravenous or intrathecal clonidine administration, we observed a decrease in ME-ir fibers in layers I, II, IV, and V, the dorso-lateral funiculus, and the reticular formation this decrease being more pronounced in animals administered intratecally with clonidine.

Discussion

Overall, the results found in the control cats are consistent with previous findings by Conrath-Verrier et al. (1983) concerning the distribution of ME-ir fibers in the cat cervical spinal cord. However, those authors found ME-ir cell bodies in the cervical spinal cords of their animals. In our study, we did not observe any ME-ir cell bodies. This discrepancy is due to the fact that

Table 1. Summary of met-enkephalin immunoreactivity.

LAYER	CONTROL	INTRAVENOUS CLONIDINE	INTRATHECAL CLONIDINE
I	+++++	++++	+++
II	+++++	++++	+++
III	+++	+++	+++
IV	+++	++	+
V	+++	++	+
VI	++	++	+
FDL	++++	+++	++
FR	++++	+++	++

FDL: dorso-lateral funiculus; FR: reticular formation. Density of immunoreactivity: +++++, very high; ++++, high; +++, moderate; ++, low; +, very low.

Conrath-Verrier et al. (1983) administered colchicine to their animals while we did not. Currently, it is known that, in general, colchicine administration is necessary to observe peptide-containing perikarya.

In the control animals, we observed the highest density of ME-ir fibers in layers I and II of Rexed (substantia gelatinosa of Rolando), the dorso-lateral funiculus, and in the reticular formation, as has been previously reported (Conrath-Verrier et al., 1983). Such findings indicate that these regions of the spinal cord are involved in the transmission and control of nociceptive stimuli. Thus, for example, the dorso-lateral funiculus is formed by fibers that convey nociceptive stimuli from the periphery to the sensitive neurons located in the superficialmost layers of the dorsal horn. Additionally, this funiculus contains propiospinal fibers that arise from

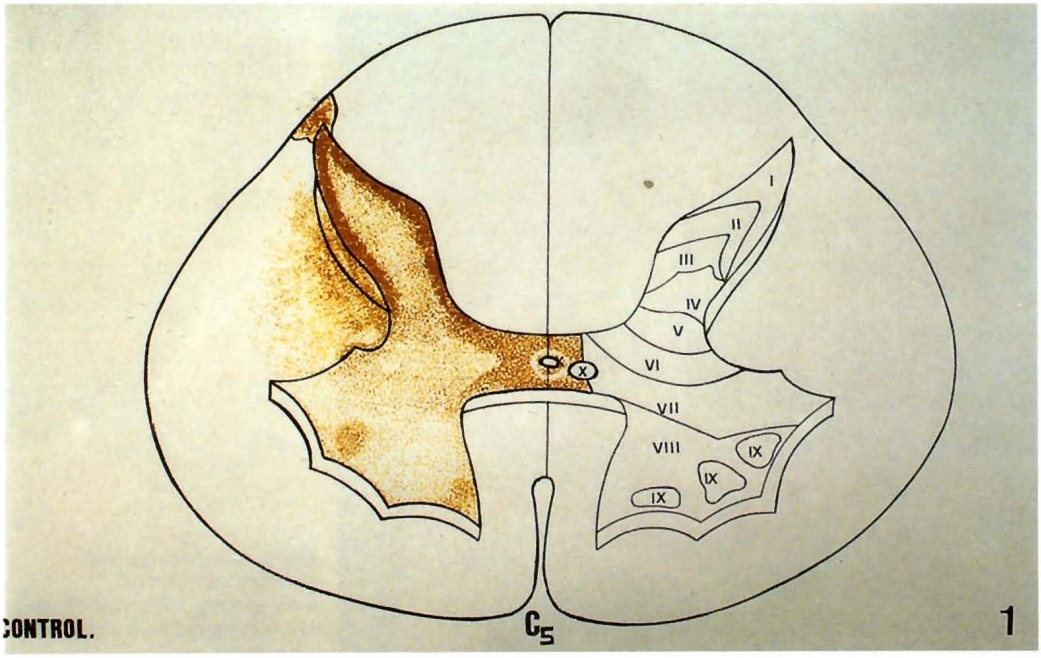

Fig. 1. Distribution of met-enkephalin immunoreactive fibers in the cat spinal cord corresponding to cervical level 5. These results were obtained from control animals (not treated with clonidine). The increasing intensity indicates the density of the immunoreactivity. Roman numbers indicate the layers of Rexed.

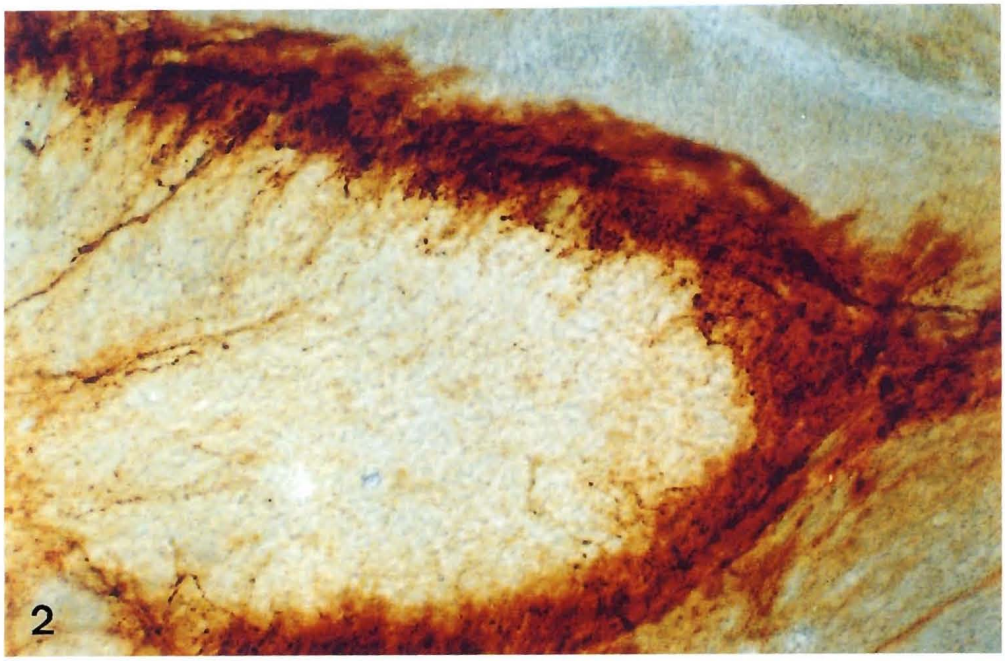

Fig. 2. Control animal. ME-ir fibers in layers I and II of Rexed in the dorsal horn. x 40

small neurons located in the substantia gelatinosa of Rolando (layers I and II of Rexed); these neurons are known to be intraspinal modulators of nociceptive afferences.

In comparison with control cats, the animals undergoing intravenous or intrathecal administration of clonidine showed a decrease in the density of ME-ir fibers, for example, in layers I, II and V of the cervical spinal cord; the decrease being more marked when clonidine was administered intrathecally. The decrease in the number of ME-ir fibers suggests that clonidine induces the release of ME from fibers and that the opioid peptides released could inhibit the release of neurotransmitters (e.g., substance P) from terminals rich in opiate receptors and involved in nociceptive transmission (Jessel and Iversen, 1977; Kuraishi et al., 1985).

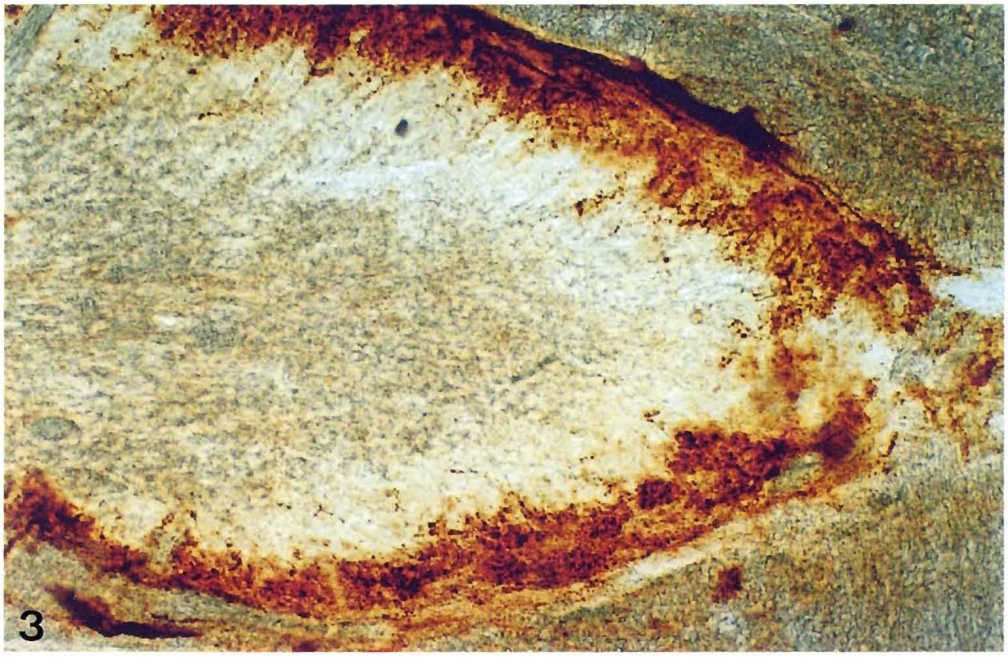

Fig. 3. Animal treated with clonidine administered intravenously. ME-ir fibers located in layers I and II of Rexed in the dorsal horn. x 40

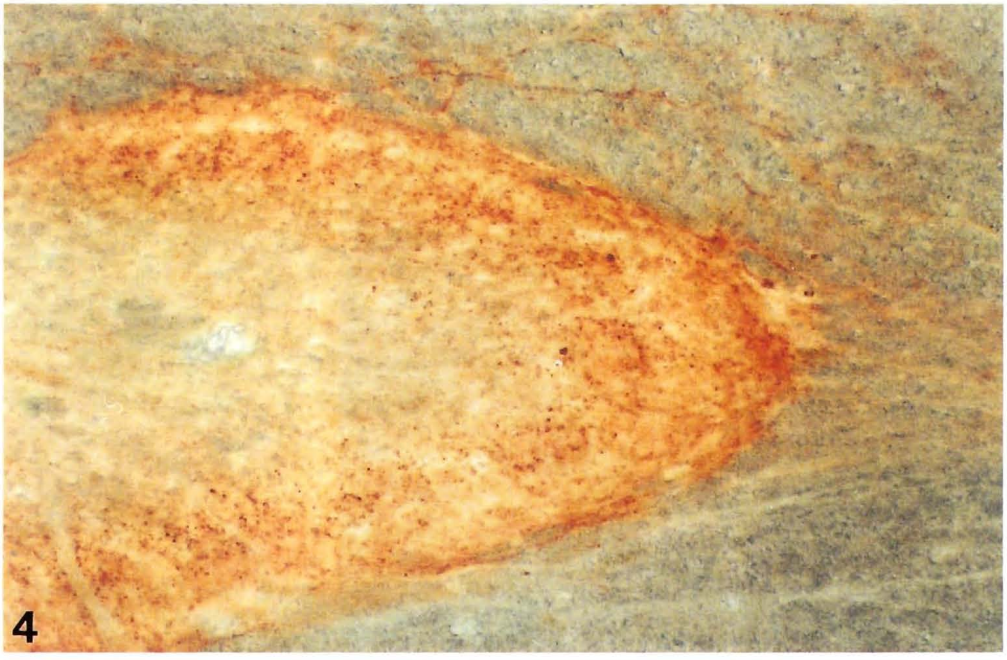

Fig. 4. Animal treated with clonidine administered intrathecally. Fibers containing met-enkephalin located in layers I and II of Rexed in the dorsal horn. x 40

In summary, the administration of clonidine could produce analgesia. The differences in the densities of ME-ir fibers found in animals intravenously or intrathecally administered with clonidine can be explained in terms of the idea that intrathecally administered clonidine has a local-regional effect on the spinal cord, reaching a higher concentration in the cerebrospinal fluid and nervous tissue than when it is administered intravenously (Castro and Eisenach, 1989).

The results found in animals treated with clonidine are very similar to those observed in the cat spinal cord after low-frequency analgesic electroacupuncture (Vacca-Galloway et al., 1985; Cobos, 1988). Taken together, these data suggest that both electroacupuncture and clonidine could produce analgesia by releasing met-enkephalin in the zones of the central nervous system involved in the transmission of nociceptive stimuli. The release of endogenous opiate peptides after electro-acupuncture or clonidine administration could explain the beneficial effects of therapies in subjects with the opiate withdrawal syndrome.

Finally, it has been indicated that clonidine exerts a partial agonist action on alpha-2 adrenergic presynaptic receptors (Haeusler, 1976; Raskind, 1988). Our data also suggest that the met-enkephalinergic system may be controlled by clonidine.

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