

## **Modifications in the distribution of met-enkephalin in the pons of the cat, following the intravenous administration of clonidine: An immunocytochemical experimental study**

**J. Vázquez<sup>1</sup>, M. Muñoz<sup>2</sup> and J.C. García<sup>2</sup>**

<sup>1</sup>Department of Morphological Sciences, Faculty of Medicine, University of Sevilla and

<sup>2</sup>Department of Paediatrics, Children's Hospital, Sevilla, Spain

**Summary.** The distribution of met-enkephalin in the cat and its modifications following the stimulation by intravenous clonidine, was studied with indirect immunocytochemical techniques. We observed a decrease in the immunoreactivity of met-enkephalin following the administration of clonidine, relative to the controls, in the following structures: locus coeruleus, nucleus cuneiformis, formatio reticularis, nucleus gigantocellularis, nucleus reticularis lateralis, nucleus reticularis, nucleus reticularis parvocellularis, nucleus sensorius superior n. trigemini, nucleus raphes, substantia grisea periventricularis, nucleus eminentiae teretis, colliculus inferior, nucleus motorius n. trigemini, nucleus tracto spinalis n. trigemini, tractus spinalis n. trigemini and nucleus tracto mesencephalici n. trigemini. These experimental observations link the immunoreactivity changes to the structures that are associated with the direct action of clonidine and conclude that the pars metencephalinergeticus of the efferent pontine pathway of pain is in morpho-functional relationship with the analgesia produced by clonidine; an analgesic of an endogenous opiate character.

**Key words:** Met-enkephalin, Clonidine, Pons, Immunocytochemistry, Cat

### **Introduction**

Modern neurobiology, solidly based on the neuronal doctrine (Ramón y Cajal, 1972), in the doctrine of synapses (Sherrington, 1920), in the concept of neurosecretion (Scharrer, 1928), in the functional classification of nerve fibres (Erlanger et al., 1926) and, in the whole body, the constantly-advancing doctrine of

intercellular communication substances (Lembeck, 1953; de Robertis, 1964; Bargman, 1968; Simon et al., 1973; Terenius, 1973; Snyder and Simantov, 1977; Hökfett et al., 1980; Bloom, 1981) allows one to plan the therapeutic battle against pain on four fundamental fronts of the nervous system: 1st, the peripheral receptor; 2nd, the anatomical pathways of nociception; 3rd, the synaptic regions with the neurotransmitters and neuromodulators, and 4th, the cerebral cortex, the site of nociceptive integration. Convinced of the veracity of this quadruple plan-of-attack, the present experimental study was conducted to further the knowledge on the 2nd and 3rd of these fronts at the level of the pons of the cat.

Met-enkephalin (Hughes et al., 1975) is an endogenous pentapeptide opiate of the neurotransmitter-neuromodulator class localized in the telencephalon, mesencephalon, rhombencephalon (locus coeruleus, formatio reticularis, nucleus raphes, etc.) and the spinal medulla (Sar et al., 1978; Kuber and Wilbur, 1980; Pickel et al., 1980; Glazer and Basbaum, 1981; Haber and Else 1982; Somogy et al., 1982; Bouras et al., 1984). In many different ways and specifically on the efferent nociceptor pathway (Glazer et al., 1981; Jessell, 1983; García Robles et al., 1988) it has, among several other functions, that of acting on the presynaptic opiate receptors of the nociceptor pathway inhibiting the release of substance P (von Euler and Gaddum, 1931) in the spinal medulla (Vacca-Galloway et al., 1985) and at other sites of the central nervous system (Vázquez and Muñoz, 1989; Vázquez et al., 1990, 1993, 1995). The enkephalins control the transmission of the nociceptor information inhibiting the release of substance P (Jessell and Iversen, 1977; Luque, 1988; Vázquez et al., 1993). Clonidine, an imidazole derivative synthesised originally by Boehringer Ingelheim in 1962 (Hoefke and Kobinger, 1966), acts as a partial agonist of the  $\alpha$ -2-adrenergic receptors and possesses clear hypotensive and analgesic effects (Schmitt et al., 1974; Eisenbach et al., 1987; Max et al., 1988) as well as being efficacious in the treatment

of opiate withdrawal syndrome. To date, knowledge of the precise mechanism of the analgesic action is uncertain but it is thought that the endogenous opiates are implicated (Paalzoon and Paalzoon, 1976; Aghajanian, 1978; Fielding et al., 1978; Gold et al., 1978; Lavarly and Roth, 1980; Thoolen et al., 1981) by local stimulation of their release (Yaksh and Reddy, 1981; Goldstein, 1983; Yaksh, 1985; Nakamura and Ferreira, 1988) or by action on substance P (Kuraishi et al., 1985). We propose that clonidine produces analgesia by release of met-enkephalin in the sites of the efferent pathway of pain.

Indirect immunocytochemistry (Coons et al., 1942; Sternberger, 1979; Florez et al., 1987) is a suitable technique for evaluating the immunoreactive changes in met-enkephalin at the tissue level (Nakane and Pierce, 1966) since it facilitates direct comparison of cases and controls. The basis of our hypothesis is that, under the action of clonidine, a decrease in met-enkephalin would certainly be indicative of an increased transport and release of the pentapeptide into the synaptic spaces and, as such, that this present experimental study constitutes a further contribution to the knowledge of the anatomy of pain, particularly of its efferent discharge arm (Vázquez et al., 1995), and to the neurohistochemical basis of the analgesic action of intravenously-administered clonidine.

## Materials and methods

### *Experimental procedure*

We used 12 adult cats (2.5-4 Kg) divided into two groups: controls (4 cats) and experimental (8 cats). All the animals were anaesthetised with ketamine (40 mg/Kg body weight) (Conrath-Verrier et al., 1983, 1986; Conrath-Verrier, 1984). Clonidine (1 dose of 15 mg/Kg diluted in 5 ml of physiologic saline) was injected slowly via the saphena magna vein. Time lapses between ketamine and clonidine administrations and between clonidine and perfusion-sacrifice were 5 and 30 minutes, respectively.

### *Tissue processing*

Animals of both groups were perfused with buffer (500 ml) and then with 4% paraformaldehyde diluted in Sørensen buffer (Paese, 1962). The encephalon was obtained and postfixed in the same fixative for 12 h and then rinsed in several baths of saccharose in Sørensen buffer. After washing, the encephalon was frozen in liquid nitrogen and 80  $\mu$ m axial transverse sections were obtained in a cryostat.

### *Immunocytochemical staining*

For the immunocytochemical detection of met-enkephalin, indirect techniques were used (Nakane and Pierce, 1966; Falini and Taylor, 1983; Conrath-Verrier et

al., 1986). Tissue sections were immersed in 0.3% H<sub>2</sub>O<sub>2</sub> in methanol to eliminate endogenous peroxidase and, after hydration, incubated with 1% normal sheep serum in 0.3% triton X-100. Sections were then incubated overnight with rabbit anti-met-enkephalin antibody (1:1600, Cambridge Research Biochemicals, Cambridge, UK). Sheep anti-rabbit IgG horseradish peroxidase-conjugated antibodies at a dilution of 1:250 were used as secondary layer. Peroxidase was visualised with 3,3'-diaminobenzidine (DAB) and H<sub>2</sub>O<sub>2</sub>. The following controls were used: a) pre-absorption of the first antibody with met-enkephalin; b) omission of the different antibodies; and c) exclusive treatment with DAB. Mapping was carried out according to the stereotaxic atlas of Snider and Niemer (1961).

## Results

The results obtained are summarised in Table 1 and those corresponding to the P3.5 sectional plane are partially illustrated in Fig. 1. The most important findings were the following:

In the control group: intense immunoreactivity in colliculus inferior, nucleus cuneiformis, locus coeruleus and substantia grisea periventricularis; moderate immunoreactivity in formatio reticularis, nucleus motorius n. trigemini, nucleus tr. spinalis n. trigemini, nucleus reticularis gigantocellularis, nucleus reticularis lateralis, nucleus reticularis parvocellularis, nucleus sensorius superior n. trigemini and nucleus tr. mesencephalicis n. trigemini; very slight immunoreactivity in nucleus tr. spinalis n. trigemini; and negative immunoreactivity in tractus spinalis n. trigemini.

In the treated group: moderate immunoreactivity in colliculus inferior, locus coeruleus and substantia grisea periventricularis; slight immunoreactivity in nucleus cuneiformis, formatio reticularis, nucleus motorius n. trigemini, nucleus reticularis parvocellularis, nucleus sensorius superior n. trigemini and nucleus tr. mesencephalicis n. trigemini; very slight immunoreactivity in nucleus tr. spinalis n. trigemini; and negative immunoreactivity in tractus spinalis n. trigemini.

In summary, following the intravenous administration of clonidine, we observed a decrease in met-enkephalin immunoreactivity in the colliculus inferior, nucleus cuneiformis, formatio reticularis, locus coeruleus (Fig. 2), nucleus motorius n. trigemini, nucleus tr. spinalis n. trigemini, nucleus reticularis gigantocellularis, nucleus reticularis lateralis, nucleus reticularis parvocellularis, nucleus sensorius superior n. trigemini, substantia grisea periventricularis, nucleus tractus mesencephalicis n. trigemini and the absence or negativity of immunoreactivity in tractus spinalis n. trigemini.

## Discussion

In our study, the most important observations overall were of intense immunoreactivity in the mesencephalon

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in colliculus inferior, nucleus cuneiformis, locus coeruleus and substantia grisea periventricularis; moderate immunoreactivity in formatio reticularis, nucleus reticularis gigantocellularis, nucleus reticularis parvocellularis, nucleus reticularis lateralis, nucleus motorius n. trigemini, nucleus tr. spinalis n. trigemini,

nucleus sensorius superior n. trigemini and nucleus tr. mesencephalici n. trigemini; and slight immunoreactivity in tractus spinalis n. trigemini. Following the intravenous administration of clonidine, the intensity of immunoreactivity diminished in all of the pontine structures from intense to moderate, from moderate to

**Table 1.** Summary of the immunoreactivity of met-enkephalin

	P2		P2.5		P3		P3.5		P4		P5		P6		P7	
	Con	Clo	Con	Clo	Con	Clo	Con	Clo	Con	Clo	Con	Clo	Con	Clo	Con	Clo
Cin	++++	++	++++	++	++++	+++	0	0	0	0	0	0	0	0	0	0
Cu	++++	++	++++	++	0	0	0	0	0	0	0	0	0	0	0	0
DLI	++++	++	++	+	0	0	0	0	0	0	0	0	0	0	0	0
FR	+++	++	+++	++	++++	++	+++	++	++	++	+	+++	++	0	0	0
LDt	-	-	-	0	0	0	0	0	0	0	0	0	0	0	0	0
L1V	+	-	++	-	+++	++	0	0	0	0	0	0	0	0	0	0
PedM	-	-	-	-	-	-	-	-	-	-	-	-	0	0	0	0
Peds	+++	+	+++	+	+++	+++	+++	+++	+	-	-	-	-	-	-	-
PL	+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PM	-	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0
RA	++	++	++	++	++	0	0	++	++	++	++	++	++	+++	+++	++
SGPV	++++	++	++++	+++	++++	+++	++	+	-	0	0	0	0	0	0	0
TP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0
BcM	0	0	+++	++	+++	+++	++	+	-	0	0	0	0	0	0	0
CTR	0	0	-	-	-	-	-	-	-	0	0	0	0	0	0	0
CTRp	0	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FLM	0	0	-	0	0	0	0	0	0	0	0	0	0	0	0	0
TMtr	0	0	+++	++	+++	++	+++	++	0	0	0	0	0	0	0	0
LC	0	0	0	0	++++	+++	++++	+++	0	0	0	0	0	0	0	0
Rgc	0	0	0	0	-	-	-	-	-	-	-	-	-	-	+++	+++
TSr	0	0	0	0	++++	+++	+++	++	++	+	0	0	0	0	0	0
MT	0	0	0	0	0	0	+	+	+++	++	+++	++	0	0	0	0
OsM	0	0	0	0	0	0	+	+	+	-	+	-	+	-	0	0
RpV	0	0	0	0	0	0	-	-	-	-	0	0	0	0	+++	++
SST	0	0	0	0	0	0	+	+	++	+	+++	+++	0	0	0	0
TRI	0	0	0	0	0	0	-	-	0	0	++	++	0	0	0	0
CPT	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-
Vi	0	0	0	0	0	0	++	+	0	0	0	0	0	0	0	0
VM	0	0	0	0	0	0	0	0	+++	++	+++	++	-	-	-	-
VS	0	0	0	0	0	0	0	0	++	+	0	0	0	0	0	0
RL	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+++	++
Osl	0	0	0	0	0	0	0	0	0	0	+	-	+	-	0	0
TD	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F	0	0	0	0	0	0	0	0	0	0	++	-	-	-	0	0
VL	0	0	0	0	0	0	0	0	0	0	+++	+++	-	-	-	-
Abd	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Emt	0	0	0	0	0	0	0	0	0	0	0	0	+++	+++	0	0
NEt	0	0	0	0	0	0	0	0	0	0	0	0	+++	+++	+++	+
NNA	0	0	0	0	0	0	0	0	0	0	0	0	0	+++	++	+
TEt	0	0	0	0	0	0	0	0	0	0	0	0	++	-	+	-
GNF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+	+
NF	0	0	0	0	0	0	0	0	0	0	0	0	0	0	++++	+
Ph	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+++	++

Cin: colliculus inferior; Cu: nucleus cuneiformis; DLI: nucleus dorsalis lemnisci lateralis; FR: formatio reticularis; LDt: nucleus latero dorsalis tegmentii; LIV: nucleus ventralis lemnisci lateralis; PedM: pedunculus cerebellaris medius; PedS: pedunculus cerebellaris superior; PL: nucleus pontis lateralis; PM: nucleus pontis medialis; RA: nucleus raphes; SGPV: substantia grisea periventricularis; TP: tractus pyramidalis; BcM: marginal nucleus of the brachium conjunctivum; Ctr: nucleus corporis trapezoides; CTRp: corpus trapezoides; FLM: fasciculus longitudinalis medialis; TMtr: nucleus tr. mesencephalici n. trigemini; LC: locus coeruleus; Rgc: nucleus reticularis gigantocellularis; TSr: tractus spinocerebellaris ventralis; MT: nucleus motorius n. trigemini; OSM: nucleus olivaris superior medialis; RpV: nucleus reticularis parvocellularis; SST: nucleus sensorius superior n. trigemini; TRI: nervus trigeminus; CPT, corpus pontobulbare; Vi: nucleus vestibularis inferior; Vm: nucleus vestibularis medialis; Vs: nucleus vestibularis superior; RL: nucleus reticularis lateralis; Osl: nucleus olivaris superior lateralis; TD: nucleus tegmentalis dorsalis; F: nervus facialis; VL: nucleus vestibularis lateralis; Abd: nervus abducens; Emt: nucleus eminentiae teretis; NEt: nucleus tr. spinalis n. trigemini; NNA: nucleus n. abducentis; TEt: tractus spinalis n. trigemini; GNF: genu n. facialis; NF: nucleus n. facialis; Ph: nucleus praepositus hypogl. Intensity of staining: -, negative; +, very low; ++, low; +++, moderate; +++++, intense; ++++++, very intense; 0, this structure is not present at this site.

slight and from slight to negative, as shown in Table 1. This relates directly with the axonal transport and subsequent release of met-enkephalin into the synaptic space caused by the stimulus exercised by clonidine in the met-enkephalinergic system of the pons of the cat. We think that all of these structures are in morpho-functional relationship with the analgesia produced by clonidine as observed in clinical practice and in consequence, and that taking into account its place within the pain pathway (Oliveras et al., 1975; Proudipit and Anderson, 1975; Oleson et al., 1976; Pérez Casas and Bengoechea, 1977; Gonzalo, 1979; Florez and Martínez Lage, 1983), the efferent (analgesic) the arm of the pars met-enkephalinergica is linked into the overall arc of pain. Conversely, any met-enkephalinergic structure that, following the intravenous administration of clonidine, does not undergo modification in its grade of immunoreactivity, is not in functional relationship with the analgesia and, hence, must be associated with some other of the multiple functions of the pentapeptide (Fredrickson and Geary, 1982; Sitaram and Gillin, 1982; Akil et al., 1984; Arilla et al., 1986). We confirm, in view of the immunohistochemical data, the endo-genous opiate nature of the analgesic action of clonidine. Our previous studies using low-frequency electroacupuncture confirm the analgesic action of endogenous opiates at the level of the met-enkephalinergic structures; a decrease in the immunoreactivity of met-enkephalin (Valseca, 1987) and, at the level of peptidergic system (substance P), a clear increase in the immunoreactivity (Moreno, 1988). All these contribute to the linking of the structures studied to the anatomy of pain and, as such, confirm the role of pontine substance P following the administration of clonidine. This demonstrates that in the

asta posterior of the cervical medulla of the cat, following the intrarachidial administration of clonidine, substance P increases its immunoreactivity (Rubio, 1992) and is released in blocks (Kuraishi et al., 1985). Terminal and collateral route connections exist among the afferent spinoreticular and spino reticulo mesencephalergic nociceptors (Mehler et al., 1960; Levante and Abel-Fessard, 1972; Kerr, 1975; Pearl and Anderson, 1976; Glazer and Basbaum, 1981) and with the formatio reticular troncoencephalica. Based on the immunoreactivity changes that relate to the structural modifications in the different nuclei trigemini which are understood to be activated by pontine formatio reticularis and the substantia grisea periventricular, we propose the existence of met-enkephalinergic reticulo-trigeminal fibre modulators of cephalic pain in parallel with the known reticulospinal modulators of somato-visceral pain (Shetter and Atkinson, 1977; Nieuwenhuys et al., 1979). The locus coeruleus is a centre for the noradrenergic-met-enkephalinergic interaction that, following the co-transmission concept of Hökfelt (Hökfelt et al., 1980), now seems to be an important site of the efferent arm of the pain pathway at the pontine level and in which, possibly, met-enkephalin acts as a modulator of the release of noradrenalin in the synaptic space (Aghajanian, 1978), acting on the pre-synaptic opiate receptors, blocking the release of the neurotransmitter exciters of the noradrenalin type and of substance P. Clonidine inhibits the neuronal activity of the locus coeruleus (Svenson et al., 1975).

Finally, based on the immunohistochemical data of our present study and that of other studies, we hypothesise and propose, albeit with caution, the involvement of the following morphostructures in the

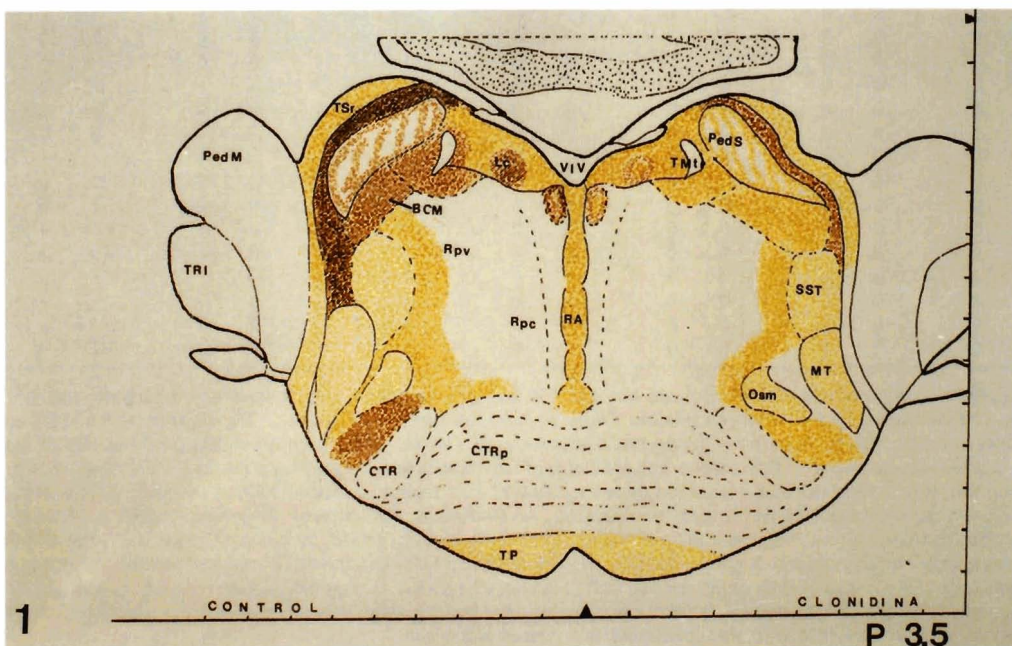
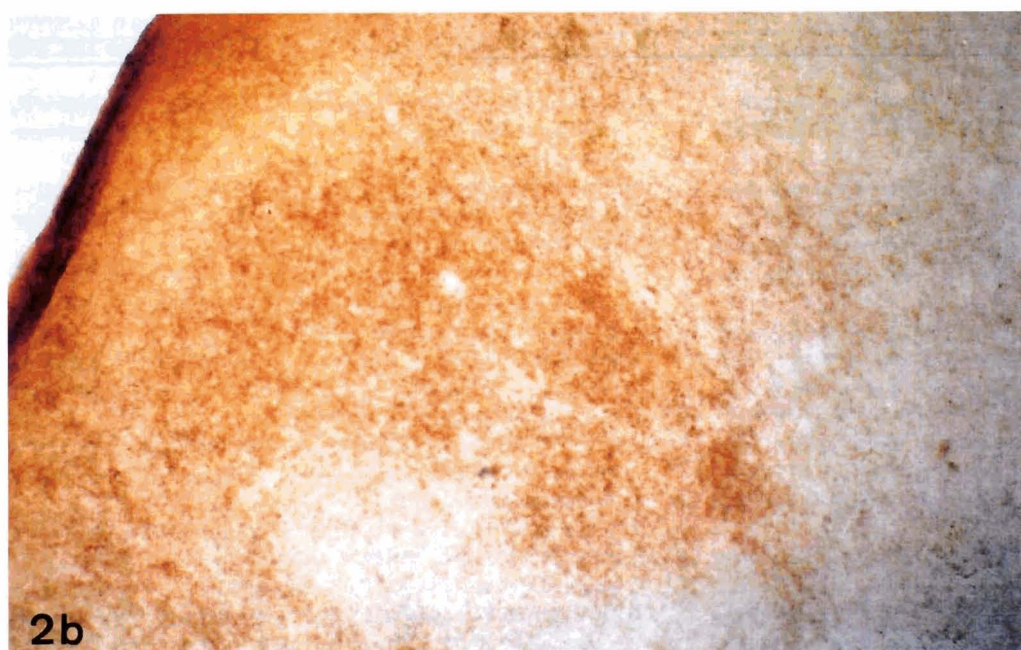
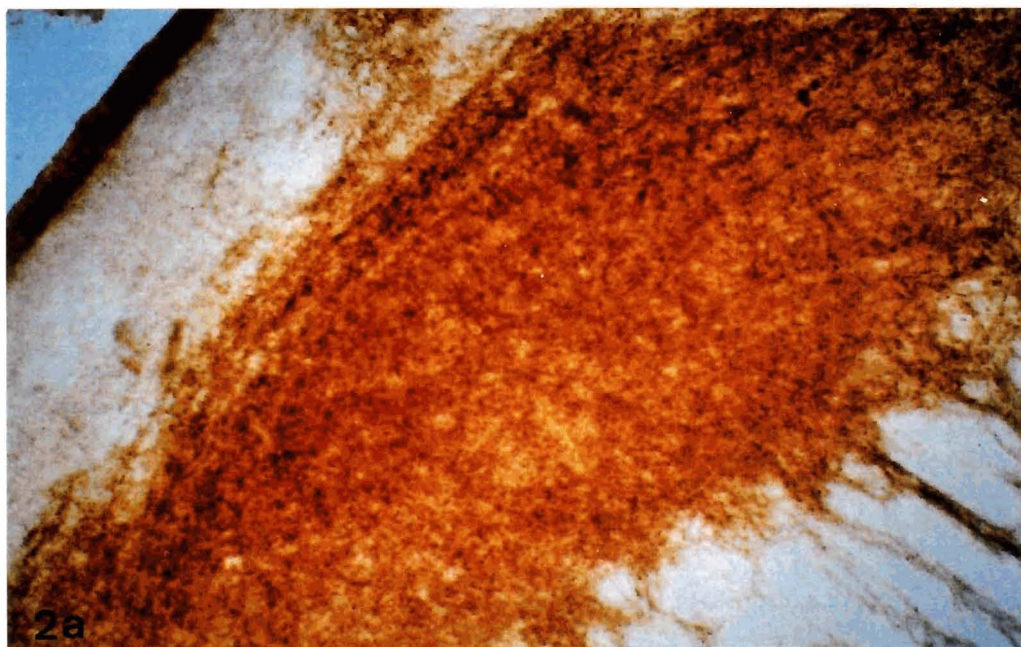


Fig. 1. Representation of met-enkephalin immunoreactivity. The results obtained in the control animals are shown on the right and those in the experimental group on the left. The increasing brown intensity indicates five levels of immunoreactivity: very weak, weak, moderate, intense and very intense. Control: very weak (Mt, Osm, Sst, TP); moderate (Bcm, TMtr, Tsr, PedS); intense (Lc). Experimental: very weak (Mt, Osm, Sst, TP); weak (Bcm, PedS, TMtr, Tsr); moderate (Lc).

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efferent (analgesic) arm of the arc encompassing the anatomy of pain: septal area, with the nucleus lateralis, medialis and acumbens (Cáceres, 1991; Vázquez et al., 1995); pars met-enkephalinergica of medial forebrain bundle (Vázquez et al., 1995); the habenula and pars met-enkephalinergica of habenulointerpeduncular bundle or the retroreflective bundle of Meyner (Muñoz, 1986; Vázquez et al., 1990); the nucleus interpeduncularis (Fernández, 1989; Vázquez et al., 1995); the substantia grisea periaqueductal (Reynolds, 1969; Fernández,

1989); the formatio reticularis (García, 1991); colliculus inferior; nucleus of raphe; nucleus cuneiformis of Olszewski; locus coeruleus; and nucleus and structures dependent on the trigemini activated by the met-enkephalinergic reticulotrigeminal fibres. The rafe-spinal system (Pérez Casas and Bengoechea, 1977; Gonzalo, 1979) that, via the funiculus dorsolateralis connection with the posterior horn of the medulla, activate the met-enkephalinergic interneurons of the I, II and V laminae of Rexel that inhibit, possibly pre-synaptically, the



**Fig. 2. Locus coeruleus.**  
**A.** Control group: intense immunoreactivity. **B.** Clonidine (experimental) group: moderate immunoreactivity. x 100

primary afferent nociceptors by blocking the release of substance P.

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