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Modifications in the distribution of met-enkephalin in the limbic system of the cat brain after electroacupuncture. An immunocytochemical study

J. Vázquez[1], M. Muñoz[2] and J.L. Cáceres[2]

[1]Department of Morphological Sciences, School of Medicine, University of Sevilla and

[2]Department of Pediatry, Hospital Infantil, Sevilla, Spain

Summary. The distribution of met-enkephalin in the limbic system of the cat brain and its modification after low frequency electroacupuncture (EA) stimulation have been studied experimentally using the indirect immunocytochemistry technique. A marked increase of post-stimulation met-enkephalin immunoreactivity was observed in the tractus habenulo-penduncularis, tractus mamilo-thalamicus, and medial forebrain bundle, and a decrease at the level of the nucleus interpeduncularis, medialis dorsalis, stria terminals, septalis lateralis, septalis medialis, accumbens septi, supraopticus, and amygdaloideus centralis. The experimental results link the changes in immunoreactivity (and therefore the structures in which they take place) with the action of low frequency EA, and permit the conclusion that the met-enkephalinergic portion of the limbic system studied is directly related morpho-functionally with analgesia and the anatomic pathways of pain.

Key words: Met-enkephalin, Electroacupuncture, Limbic system, Immunocytochemistry, Cat

Introduction

Pain is the most important human clinical symptom, and despite its being older than man himself, its defeat by medical science is still a long way off. This work aims at contributing to understanding the anatomy of pain and the neurohistochemical basis of the analgesic action of electroacupuncture (EA). We can assume, in the absence of contrary evidence, that from the point of view of the nociceptive mechanism, met-enkephalin (Hughes et al., 1975) equals analgesia and substance P (Euler and Gaddum, 1931) pain (Lembeck, 1953; Jessell and Iverson, 1977; Luque, 1988; Vázquez et al., 1993). The complete arc of the pain pathway comprises an afferent (nociceptive) branch, an efferent (analgesic) branch, and a key or centre, possibly situated in the limbic system (Payne, 1987). It has been shown that there is functional antagonism between substance P and met-enkephalin (Vacca-Galloway et al., 1985; Luque, 1988; Morales, 1990), and an endogenous opiate-like analgesic action of low frequency EA (Pomeranz and Chiv, 1976; Mayer et al., 1977; Peets and Pomeranz, 1978; Gonzalo, 1979; Pomeranz and Cheng, 1979; Vázquez and Muñoz, 1989).

Indirect immunocytochemistry (Coons et al., 1942) can be considered, from the applicative point of view, an anatomical technique that uses biochemical methodology (Sternberger, 1979). Its use has enabled us to study the main neurochemical systems of the CNS, both to identify neuronal groups having a determined neurotransmitter or neuromodulator, and their projections, and to localize and typify the specific receptors of such a transmitter (Florez et al., 1987). The limbic system is made up of a group of archaic brain structures, partly cortical and partly subcortical, situated in the internal surface of the cerebral hemispheres (Ramón y Cajal, 1899; Brodal, 1981). Its essential function is the maintenance of instinctive-affective behaviour and homeostatic regulation (Isaacson, 1974) and of the emotional mechanisms (Pérez Casas and Bengochea, 1977).

Analgesic EA modifies the met-enkephalin content of part of the structures constituting the limbic system of the cat (Cáceres, 1991).

Materials and methods

Experimental procedure

Twelve adult cats (3-5 kg) were divided into two groups: control (5 cats) and experimental (7 cats). All the animals were anaesthetized with ketamine (40 mg/kg) (Conrath-Verrier et al., 1983). Low frequency EA was applied at peripheral stimulation at the Hegu and Kumlun points for 20 min at a frequency of 4 Hz and intensity of 8 mA. Correct stimulation was

Offprint requests to: Prof. Dr. José Vázquez, Avda. Luis Montoto, 30, 2º B, 41018 Sevilla, Spain

confirmed by observation of rhythmic contractions (myoclonus) of the animals' limbs.

Tissue processing

Animals of both groups were perfused first with buffer (500 ml) and then with 4% paraformaldehyde diluted in Sörensen 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 Sörensen buffer. After washing, the encephalon was frozen in liquid nitrogen and 80 µm frontal 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_2O_2 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 visualized with 3,3'-diaminobenzidine (DAB). The following controls were used: a) pre-absorption of the first antibody with met-enkephalin; b) omission of the different antibodies; c) exclusive treatment with DAB. Mapping was carried out according to the stereotaxic atlas of Jasper and Ajmone-Marsan (1966).

Results

The results obtained are summarized in Table 1 and partially illustrated in Fig. 1 (section A7).

In the control group, the nucleus interpendicularis presented very intense immunoreactivity. In the hippocampal complex, immunoreactivity decreased gradually from the fascia dentata (very intense) to the cornu ammonis and subiculum (moderate), and was negative in the presubiculum, fimbria, and fornix. At the level of the epithalamus, immunoreactivity of the nucleus habenularis lateralis and the tractus habenulo-peduncularis was negative and that of the nucleus habenularis medialis weak. In the thalamus, immuno-reactivity of the nucleus medialis, dorsalis, the periventricularis anterior and the stria medularis was moderate, the nucleus anterior medialis weak, and the parataenialis and anterior dorsalis negative. In the septal area, immunoreactivity of the nucleus accumbes septi and banda diagonalis of Broca was moderate, the nucleus septalis medialis and lateralis weak, and the nucleus of the stria terminalis negative. In the hypothalamus, immunoreactivity of the medial forebrain bundle was very intense, the nucleus supraopticus intense, the periventricularis hypothalami and pedunculus mamillaris moderate, the corpus mamillaris medialis and lateralis very weak, and the immuno-reactivity of the nucleus amygdaloideus centralis and corticalis was intense, the nucleus amygdaloideus medialis moderate, and the nucleus amygdaloideus lateralis and basalis and stria terminalis negative.

In the experimental group, immunoreactivity of the nucleus interpendicularis was intense. The hippocampal complex showed no modifications. Immunoreactivity of

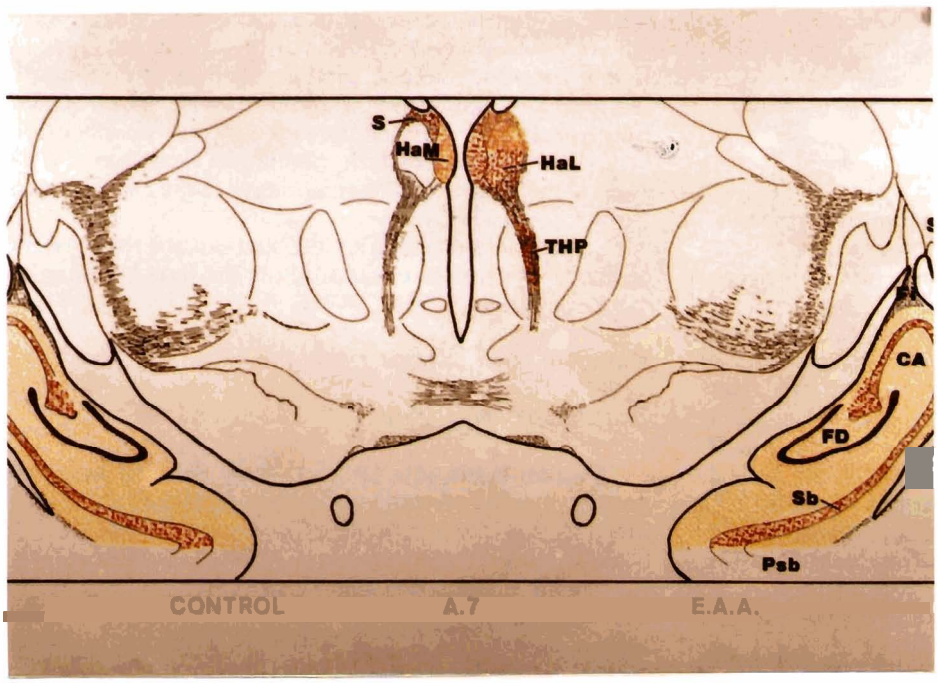

Fig. 1. Representation of met-enkephalin immunoreactivity. The results obtained in the control animals are shown on the right, and those in the experimental group on the left. The increasingly brown intensity indicates five levels of immuno-reactivity: very weak, weak, moderate, intense and very intense. Control: weak (HaM), moderate (S, CA, Sb), very intense (FD). EA: weak (S), moderate (HaL, HaM, CA, Sb), intense (THP) and very intense (FD).

Table 1. Summary of met-enkephalin immunoreactivity.

	A3		A7		A8		A9		A10		A12		A13		A15	
	Control	EA	Control	EA	Control	EA	Control	EA	Control	EA	Control	EA	Control	EA	Control	EA
iP	+++++	++++	0	0	0	0	0	0	0	0	0	0	0	0	0	0
FD	0	+++++	+++++	+++++	++++	+++++	+++++	+++++	0	0	0	0	0	0	0	0
CA	0	0	+++	+++	+++	+++	+++	+++	0	0	0	0	0	0	0	0
Sb	0	0	+++	+++	+++	+++	+++	+++	++	++	0	0	0	0	0	0
Psb	0	0	-	-	-	-	-	-	-	-	0	0	0	0	0	0
Fi	0	0	-	-	-	-	-	-	0	0	0	0	0	0	0	0
Fco	0	0	0	0	0	0	0	0	-	-	-	-	-	-	0	0
Fx	0	0	0	0	0	0	0	0	0	0	0	0	-	-	0	0
HaL	0	0	-	+++	0	0	0	0	0	0	0	0	0	0	0	0
HaM	0	0	++	+++	0	0	0	0	0	0	0	0	0	0	0	0
THP	0	0	-	++++	0	0	0	0	0	0	0	0	0	0	0	0
MD	0	0	0	0	+++	-	+++	-	++	-	0	0	0	0	0	0
PVA	0	0	0	0	0	0	0	0	0	0	++	++++	+	++++	0	0
Am	0	0	0	0	0	0	0	0	0	0	+	++	0	0	0	0
Pt	0	0	0	0	0	0	0	0	0	0	0	0	-	++	0	0
AD	0	0	0	0	0	0	0	0	0	0	0	0	-	++	0	0
S	0	0	+++	++	0	0	0	0	0	0	0	0	-	++	0	0
Nste	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	+
SpL	0	0	0	0	0	0	0	0	0	0	0	0	0	0	++	+
SpM	0	0	0	0	0	0	0	0	0	0	0	0	0	0	++	+
Acc	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+++	++
BDB	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+++	+++
Mm	0	0	0	0	-	-	+	++	0	0	0	0	0	0	0	0
PMm	0	0	0	0	+++	++	0	0	0	0	0	0	0	0	0	0
ML	0	0	0	0	0	0	+	++	-	-	0	0	0	0	0	0
TMt	0	0	0	0	0	0	-	++	0	0	0	0	0	0	0	0
SO	0	0	0	0	0	0	0	0	0	0	++++	++	++++	++	++++	+++
PVH	0	0	0	0	0	0	0	0	0	0	+++	++++	+++	++++	0	0
MFB	0	0	0	0	0	0	0	0	0	0	+	+++	+++	+++	++++	+++
AcL	0	0	0	0	0	0	0	0	+++	+++	0	0	0	0	0	0
AcM	0	0	0	0	0	0	0	0	0	0	++++	+++	++++	++	0	0
AL	0	0	0	0	0	0	0	0	-	-	-	-	-	-	0	0
Abm	0	0	0	0	0	0	0	0	-	-	-	-	-	-	0	0
Abp	0	0	0	0	0	0	0	0	-	-	-	-	-	-	0	0
AM	0	0	0	0	0	0	0	0	0	0	+++	+++	+++	+++	0	0
ACO	0	0	0	0	0	0	0	0	0	0	+++	++++	+++	++++	0	0
St	0	0	-	-	-	-	-	-	-	-	-	-	-	-	0	0

iP: nucleus interpeduncularis; FD: fascia dentata; CA: cornu ammonis; Sb: subiculum; Psb: presubiculum; Fi: fimbria; Fco: columna del fornix; HaM: nucleus habenularis medialis; HaL: nucleus habenularis lateralis; THP: tractus habenulo-peduncularis; MD: nucleus medialis dorsalis; PVA: nucleus periventricularis anterior; Am: nucleus anterior medialis; Pt: nucleus parataenialis; AD: nucleus anterior dorsalis; S: stria medularis; Nste: nucleus of the stria terminalis SpL: nucleus septalis lateralis; SpM: nucleus septalis medialis; Acc: nucleus accumbens septi; BDB: banda diagonalis of Broca; Mm: corpus mamillaris medialis; PMm: pedunculus mamilo-thalamicus; ML: corpus mamillaris lateralis; TMt: tracto mamilo-thalamicus; SO: nucleus supraopticus; PVH: nucleus periventricularis hypothalami; MFB: medial forebrain bundle; AcL: nucleus amygdaloideus centralis pars lateralis; AcM: nucleus amygdaloideus centralis pars medialis; AL: nucleus amygdaloideus lateralis; Abm: nucleus amygdaloideus basalis pars magnocellularis; Abp: nucleus amygdaloideus basalis pars parvocellularis; AM: nucleus amygdaloideus medialis; ACO: nucleus amygdaloideus corticalis; St: stria terminlis. Intensity of staining: -, negative; +, very low; ++, low; +++, moderate; ++++, intense; +++++, very intense; 0, this structure is not present at this level.

the nucleus habenularis lateralis and medialis was moderate, the tractus habenulo-peduncularis intense, and the nucleus medialis dorsalis negative. Immunoreactivity of the nucleus periventricularis anterior was intense, and the stria medularis and nucleus anterior medialis, parataenialis and anterior dorsalis weak. In the septal area, immunoreactivity of the nucleus stria terminalis was negative, the nucleus septalis lateralis and medialis very weak, and the nucleus accumbens septi and banda diagonalis of Broca moderate. Immunoreactivity of the corpus mamillaris medialis and lateralis and pedunculus mamillaris was weak. The tractus mamilo-thalamicus was weak. The nucleus supraopticus was moderate, and the periventricularis hypothalami was intense. Immunoreactivity of the medial forebrain bundle was moderate. In the amygdaloid complex, immunoreactivity of the nucleus amygdaloideus centralis and medialis was moderate, the nucleus amygdaloideus corticalis intense, and the nucleus amygdaloideus lateralis and basalis and stria terminalis negative.

In summary, the most significant findings of our study are that after EA stimulation, met-enkephalin immunoreactivity decreased in the nucleus interpeduncularis, medialis dorsalis, septalis lateralis and medialis, accumbens septi, supraopticus, amygdaloideus centralis (pars medialis) and stria medularis, and

increased in the tractus habenulo-peduncularis (Fig. 2), tractus mamilo-thalamicus, and medial forebrain bundle.

Discussion

The existence of met-enkephalin in the limbic system (Atwe and Kuhar, 1977; Uhl et al., 1979) has been reported in the thalamus (Conrath-Verrier et al., 1986), hypothalamus (Coveñas et al., 1988), mesencephalon (Fernández, 1989), and septal area, amygdala and hippocampus (Cáceres, 1991). Our study confirms these hippocampal complex, where we found very intense immunoreactivity in the fascia dentata and intense immunoreactivity in the cornu ammonis and subiculum. There was no modification after EA stimulation. We consider that these structures at the limit of neurochemical mechanisms of nociception. In the amygdaloid complex, immunoreactivity was moderate to intense in the nucleus centralis, medialis and corticalis, decreasing to weak in the centralis (in both the pars lateralis and pars medialis) after stimulation. This leads us to link the amygdaloid complex with the pain pathway. In the diencephalon, the most significant findings after EA stimulation were absence of immunoreactivity in the nucleus dorso medialis, and a marked increase in the tractus habenulo-interpeduncularis, mamilo-thalamicus and medial forebrain bundle. There was a decrease in the nucleus supraopticus. At the level of the septal area, the diminished immunoreactivity in the nucleus lateralis and medialis, and in the accumbens septi, and the unmodified banda diagonal of Broca, were indicative. Lastly, immunoreactivity decreased in the nucleus interpeduncularis after EA stimulation.

In view of these findings, we consider that immunoreactivity decreases in the nuclei due to the liberation of met-enkephalin resulting from EA stimulation, and increases in the fibrillar tracts with the increased met-enkephalinergic flow (axonal transport). thus we can link the met-enkephalinergic portion of the pain pathway with the following nuclei and tracts: septalis, amygdaloideus centralis (pars medialis, supraopticus, dorso-medialis, interpeduncularis, habenulo-peduncularis, mamilo-thalamicus and medial forebrain bundle. It appears that all these structures are directly related anatomo-functionally with EA analgesia and that the tractus mamilo-thalamicus component of the

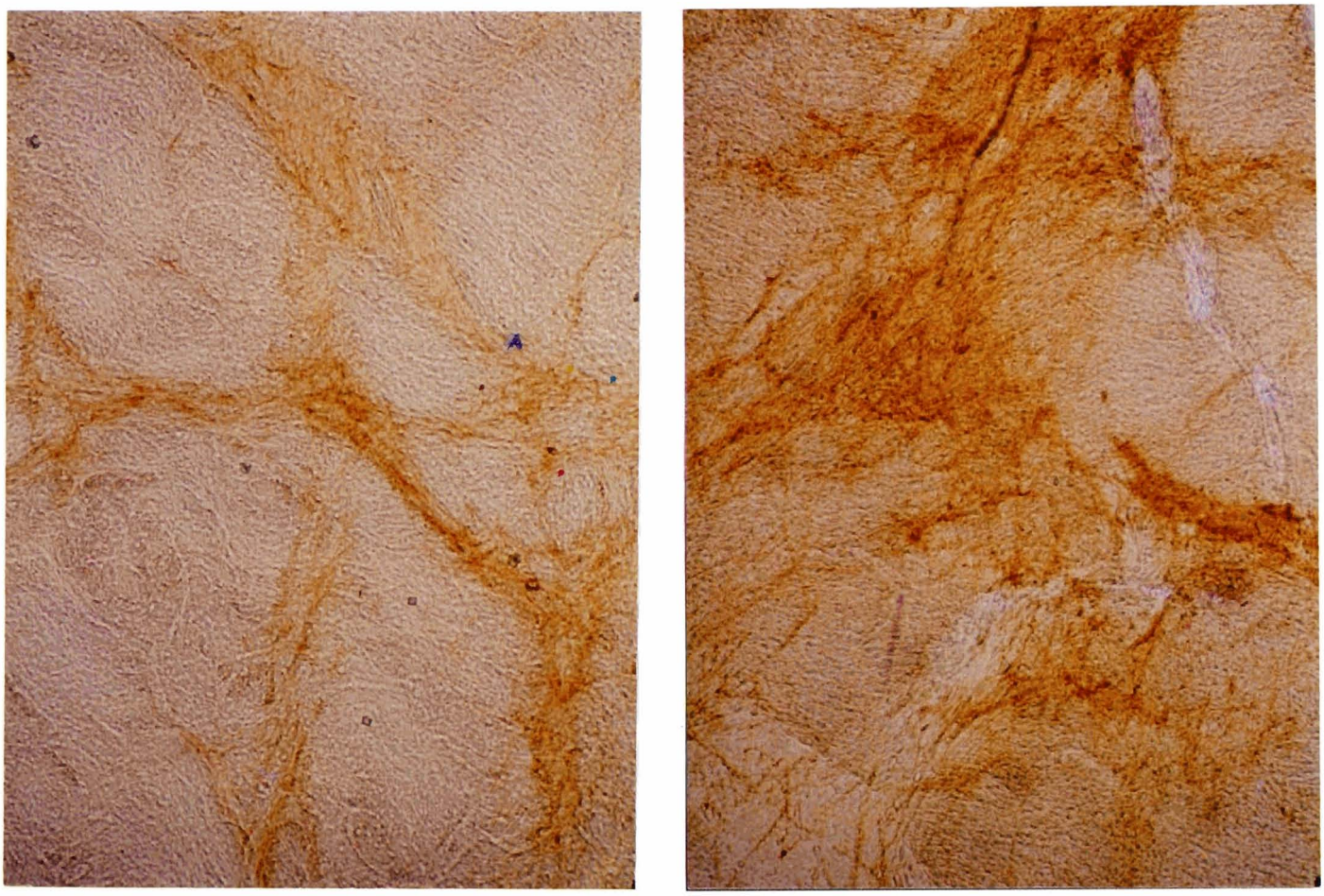

Fig. 2. Tractus habenulo-peduncularis. **a.** Control group. Negative immunoreactivity. **b.** Experimental group. Intense immunoreactivity. x 250

emotional circuit of Papez (Papez, 1937) has a met-enkephalinergic portion dependent of the limbic system and the nociceptive pathway. In the absence of contrary evidence, we believe that EA modifies the subcortical met-enkephalinergic portion of the limbic system, exerting its analgesic action at subcortical level.

All the met-enkephalinergic structures not modified after EA stimulation are unrelated with analgesia, but are related with other pentapeptide functions (Frederickson and Geary, 1982; Sitaram and Guillin, 1982; Akil et al., 1984; Arilla et al., 1986). The structural changes produced by EA stimulation in the met-enkephalinergic portion of the cat limbic system unquestionably link the limbic system with the anatomic pathways of pain. The analgesic action of low frequency EA at the level of the limbic system is endogenous opiate-like, as at the level of the diencephalon (Vázquez et al., 1990). We fully agree with the hypothesis that the opiate receptors of the limbic system are the target of met-enkephalins (Hökfelt et al., 1977; Eccles, 1984, 1992).

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