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Immunocytochemical distribution of Met-enkephalin-Arg^6-Gly^7-Leu^8 (Met-8) in the auditory system of the rat

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Abstract

Methionine-enkephalin-Arg^6-Gly^7-Leu^8 (Met^8) is known to act as a neurotransmitter or neuromodulator and it has been implicated in pain, cardiovascular and motor mechanisms, but its role in audition is currently unknown. In the present study we have applied an immunocytochemical technique and describe the distribution of cell bodies and fibers containing Met^8 in the auditory pathway of the rat. The main finding is that we found either Met^8-immunoreactive fibers or cell bodies or both in virtually all nuclei of the rat auditory system except for the medial superior olive and the ventral division of the medial geniculate body in which we did not find any immunoreactivity for Met^8. This suggests that the neuropeptide Met^8 is widely distributed throughout the auditory system of the rat. Our results suggest that Met^8 could play at least two roles in hearing. It seems to be involved in the processing of the descending auditory pathway, and it may be implicated in the multisensory integration of auditory information that takes place in the non-lemniscal auditory pathway.
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1. Introduction

It is known that methionine-enkephalin-Arg^6-Gly^7-Leu^8 (Met^8) is exclusively produced from pro-enkephalin and not from the other two precursors (pro-opiomelanocortin and pro-dynorphin) of the opioid peptides. This means that the presence of Met^8 is a selective marker for the pro-enkephalin system (Abe et al., 1987; Murakami et al., 1987). Met^8 acts as a neurotransmitter or neuromodulator and has been implicated in pain, cardiovascular and motor mechanisms (Akil et al., 1984; Basbaum and Fields, 1984; Pasternak, 1987; Douglas and Kitchan, 1992). The distribution of Met^8 has been studied using immunocytochemical, radioim-

munoassay or in situ hybridization techniques in the rat (Pittius et al., 1984; Zamir et al., 1985; Fallon and Leslie, 1986; Merchenthaler et al., 1986; Murakami et al., 1987, 1989; Hoffman et al., 1993; Tanaka et al., 1993; Wynne et al., 1995; Robertson and Mulders, 2000), cat (Belda et al., 2003), monkey (Ibuki et al., 1986) and human (Pittius et al., 1984; Iadarola et al., 1991) central nervous system.

The functional role of Met[8] in the auditory system is currently unknown and except for a few studies (e.g., Merchenthaler et al., 1986; Murakami et al., 1989; Hoffman et al., 1993; Tanaka et al., 1993; Robertson and Mulders, 2000) describing the general distribution of Met[8] in the rat brain there are no detailed accounts of Met[8] in the auditory system of this animal model. The auditory system is made up of a series of relay nuclei from the cochlear nuclei to the cortex, each one having a distinct set of neuronal types. Neurons in the cochlear nucleus give rise to a number of parallel ascending pathways that are involved in the processing of different properties (monaural, binaural, temporal and spectral) of the auditory stimulus (for reviews see Malmierca, 2003; Malmierca and Merchán, 2004). The first report on enkephalin immunostaining in the auditory system by Fex and Altschuler (1981) was made in guinea pig. This study first showed the potential involvement of enkephalin in the modulation of the cochlea through the descending olivocochlear system. Since Met[8] may act as a neurotransmitter or a neuromodulator, a detailed analysis of the cell types in the auditory system that contain Met[8] could provide a deeper understanding of their role in audition.

In the light of the above, we studied the distribution of cell bodies and fibers containing Met[8] in the auditory pathway of the rat using an immunocytochemical technique and described the neuronal types containing the neuropeptide in the auditory nuclei of the rat.

2. Materials and methods

2.1. Tissue preparation

Six female adult Wistar rats (300 g body weight) obtained from commercial sources (Charles River) were used in this study. The animals were kept in cages under standardized conditions of light (lights on at 06.00 h and off at 20.00 h) and temperature (25°C) and had free access to food and water. The animals remained in their cages for 10 days before experiments. The experimental design, protocols, and procedures of this work have been performed under the guidelines of the ethical and legal recommendations of the Spanish and European laws. Under deep anesthesia (1 ml/kg) with a mixture of ketamine (4 mg) with 0.3 ml of 2%

Rompun, two animals were placed in a stereotaxic apparatus and received a unilateral intraventricular (fourth ventricle) injection of colchicine (50 μg of the drug diluted in 5 μl of saline solution) following the stereotaxic coordinates (1.52 mm interaural, 6 mm depth, 0 mm lateral) from the atlas of Paxinos and Watson (1998), in order to enhance the immunoreactivity of cell bodies (the drug acts on microtubule systems and then inhibits axoplasmic transport). One day after administration of the drug, the treated and untreated animals were deeply anesthetized and perfused via the ascending aorta with 100 ml of Ringer's solution (pH 6.9) at 37°C and 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed and postfixed in the latter solution, and placed in increasing sucrose baths (10–30%) until they sank. Using a freezing microtome, 40 μm frontal sections were cut, collected in 10 bins filled with phosphate-buffered saline (PBS) and kept at 4°C. The first section obtained was kept in recipient one, the second in recipient two and so on until the tenth section in recipient 10. The eleventh section was kept in recipient one, the twelfth section in recipient two and so on. Eight of 10 sections were used for immunocytochemistry and the remaining sections (those located in recipients five and 10) were stained for Nissl.

2.2. Immunocytochemistry

Free floating sections were treated with a mixture of methanol and H_2O_2 for 30 min in order to avoid possible interference by endogenous peroxidases. Then, the sections were transferred into a PBS solution containing 1% normal horse serum and 0.3% Triton X-100 for 30 min. Afterwards, the sections were incubated overnight in the same PBS solution containing the primary antiserum Met[8] (diluted 1:3000). After a 30 min wash in PBS, the sections were incubated with biotinylated anti-rabbit IgG diluted 1:200 in the same buffer for 1 h at room temperature, followed by avidin–biotin–peroxidase complex diluted 1:100 for 1 h. After washing in PBS (30 min) and Tris–HCl (pH 7.6, 10 min) the tissue-bound peroxidase was revealed by H_2O_2 using 3,3′-diaminobenzidine as chromogen. Finally, the sections were kept in PBS, placed on gelatinized slides, dehydrated, mounted in Entellan and coverslipped.

2.3. Specificity of antisera

Polyclonal primary antibody was raised in rabbits against immunogens prepared by coupling the whole synthetic peptide Met[8] to a carrier protein (human serum albumin) with glutaraldehyde. Rabbits were initially immunized with immunogens emulsified with Freund's adjuvant and then, at 2 week intervals, were

given booster doses of incomplete Freund's adjuvant. Plasma from rabbits was obtained 10 days after three such booster injections, and periodically thereafter.

The immunological properties of the primary antibody have been previously reported, and the specificity of the immunostaining was controlled in the present study following the protocols described in a previous paper (Belda et al., 2003). In brief, the specificity of the immunostaining was controlled by: (a) treating the first antiserum with an excess (100 μm/ml diluted antiserum) of synthetic Met[8]; (b) omitting the first antiserum in the first incubation bath – in both cases, no residual immunoreactivity was found; (c) no significant reduction in immunolabelling was found when the Met[8] antiserum was preabsorbed with an excess (10^{-7} M) of synthetic methionine-enkephalin, leucine-enkephalin, methionine-enkephalin-Arg[6]-Gly[7], α-, β-, and γ-endorphin, β-lipotropin, adrenocorticotropin hormone, β-endorphin (1–27), α-, β-, and γ-melanocyte-stimulating hormone, dynorphin A, or α-neo-endorphin.

2.4. Mapping

Identification of the different auditory nuclei is based on the general nomenclature used by Paxinos and Watson (1998). More detailed mapping was made following the detailed studies in the superior olivary complex (Osen et al., 1984; Kulesza and Berrebi, 2000), nuclei of the lateral lemniscus (Merchán et al., 1994; Merchán and Berbel, 1996), inferior colliculus (Faye-Lund and Osen, 1985; Malmierca et al., 1993, 1995), medial geniculate body (LeDoux et al., 1985, 1987; Clerici and Coleman, 1990; Clerici et al., 1990; Winer et al., 1999a,b) and auditory cortex (Games and Winer, 1988; Zilles et al., 1980, 1990; Doron et al., 2002) in the rat (for a detailed review see Malmierca, 2003; Malmierca and Merchán, 2004). In order to determine the density of the Met[8]-immunoreactive fibers (high, moderate, low) in the auditory nuclei of the rat, we followed the protocol described by Coveñas et al. (1999). This involved viewing the sections under light illumination at constant magnification with reference to photographs of the defined series of densities. The density of the immunoreactive cell bodies containing Met[8] was considered high (>10 cell bodies/section), middle (5–10 cell bodies/section), and low (<5 cell bodies/section). Cell bodies with the largest diameter below 15 μm were termed small; those with a diameter of 15–25 μm medium-sized, and those with a diameter above 25 μm large.

Photomicrographs were obtained with a CoolSNAP digital camera attached to a Zeiss Axiophot microscope. To improve the visualization of the results, only the brightness and contrast of the images were adjusted without any further manipulation of the photographs.

Finally, the term 'Met[8]-like immunoreactive' (Met[8]-ir) was used to describe the staining observed in our material.

3. Results

In this study we have analyzed the immunoreactivity for Met-8 throughout the entire auditory pathway (see Table 1). In the following we will describe the cell types and the existence or absence of terminals fields in each of the centers that make up the auditory pathway.
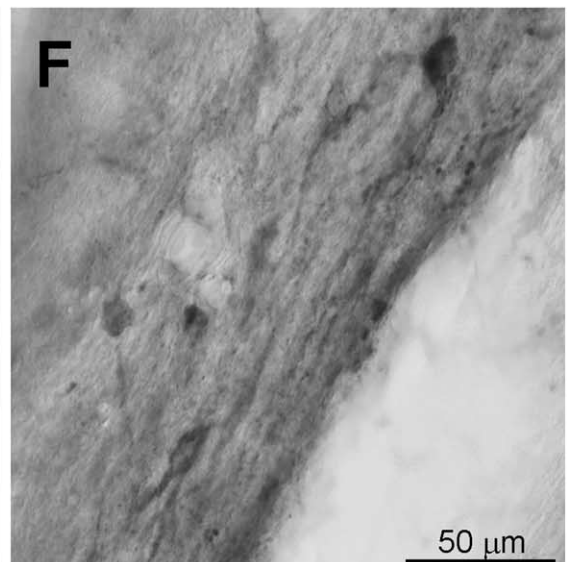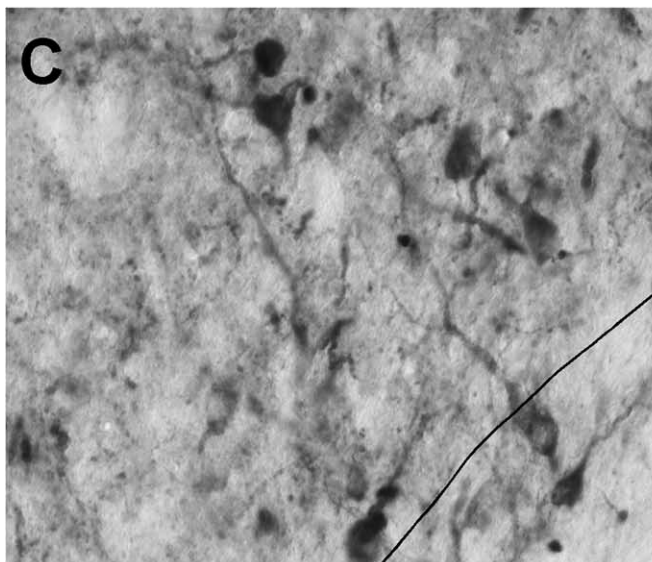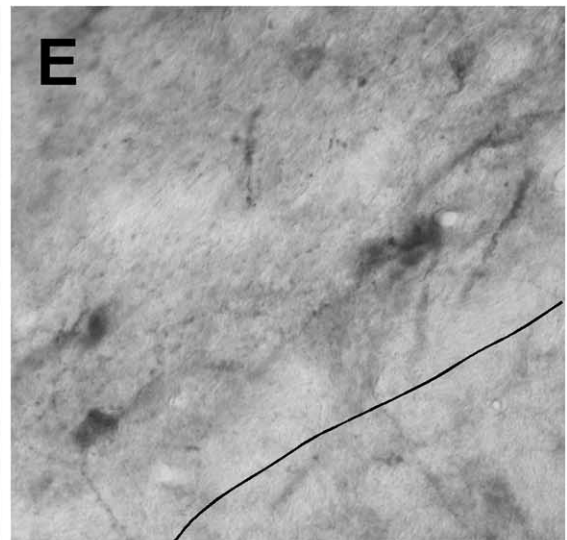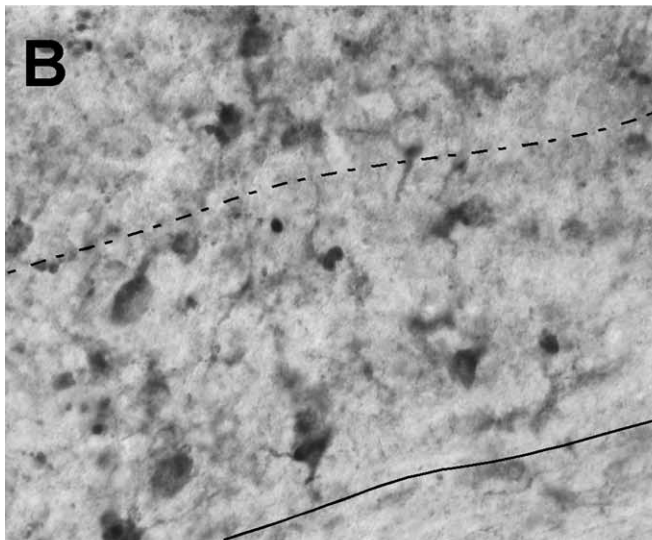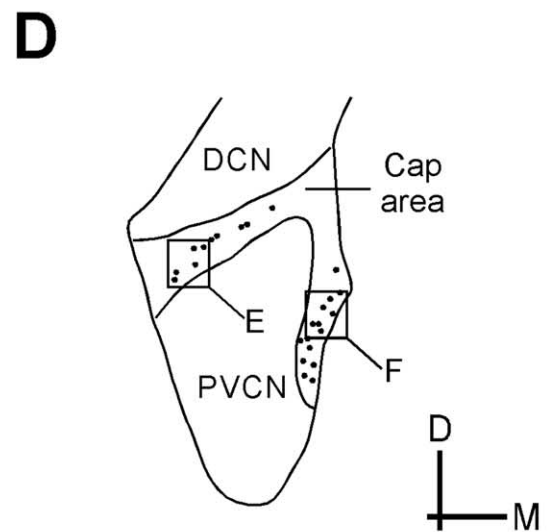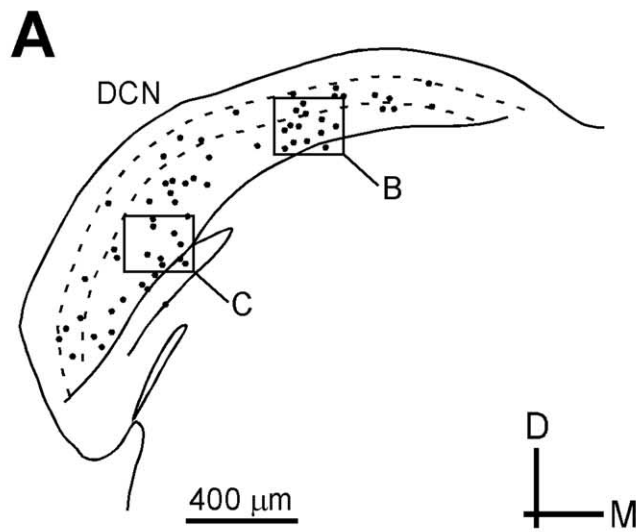
3.1. Cochlear nuclear complex (CNC)

The most remarkable feature observed in sections from animals treated with colchicine is that the CNC contains a distinct and heavy labelling of neuronal somata in the deep layer of the dorsal cochlear nucleus (DCN) (Fig. 1A). These neurons seem to be of the giant type based on their location and soma size (Fig. 1B,C). Also some other smaller cells are present in the deep DCN. A few immunoreactive neurons in the pyramidal layer cell are also present (Fig. 1A–C). In the group of

Table 1
Cell bodies and fibers containing Met[8] in the auditory pathway

Nucleus	Cell bodies	Fibers
DCN	+++	++
VCN (cap area)	+++	−
PVCN	−	++
AVCN	−	+
MNTB	−	+++
LSO	+++	−
MSO	−	+
VNTB	+++	+++
LNTB	−	++
SPON	−	−
VCLL	−	+
DNLL	−	+
CNIC	+++	+
DCIC	+++	+++
ECIC	+++	+
BIC	−	++
nBIC	−	++
MGM	−	++
MGB	−	++
MGV	−	−
PP/PIL	−	+++
ZI	−	++
RTN	−	++
AC	+++	+

The presence of Met[8]-ir structures in the rat auditory central system is based on the results obtained from both control and colchicine-treated animals. Fibers: high density (+++), moderate density (++), low density (+). Cell bodies: high density >10 cell bodies/section; moderate density: 5–10 cell bodies/section; low density ≤ 5 cell bodies/section. −: absence of Met[8]-ir cell bodies or fibers. For nomenclature see list of abbreviations.
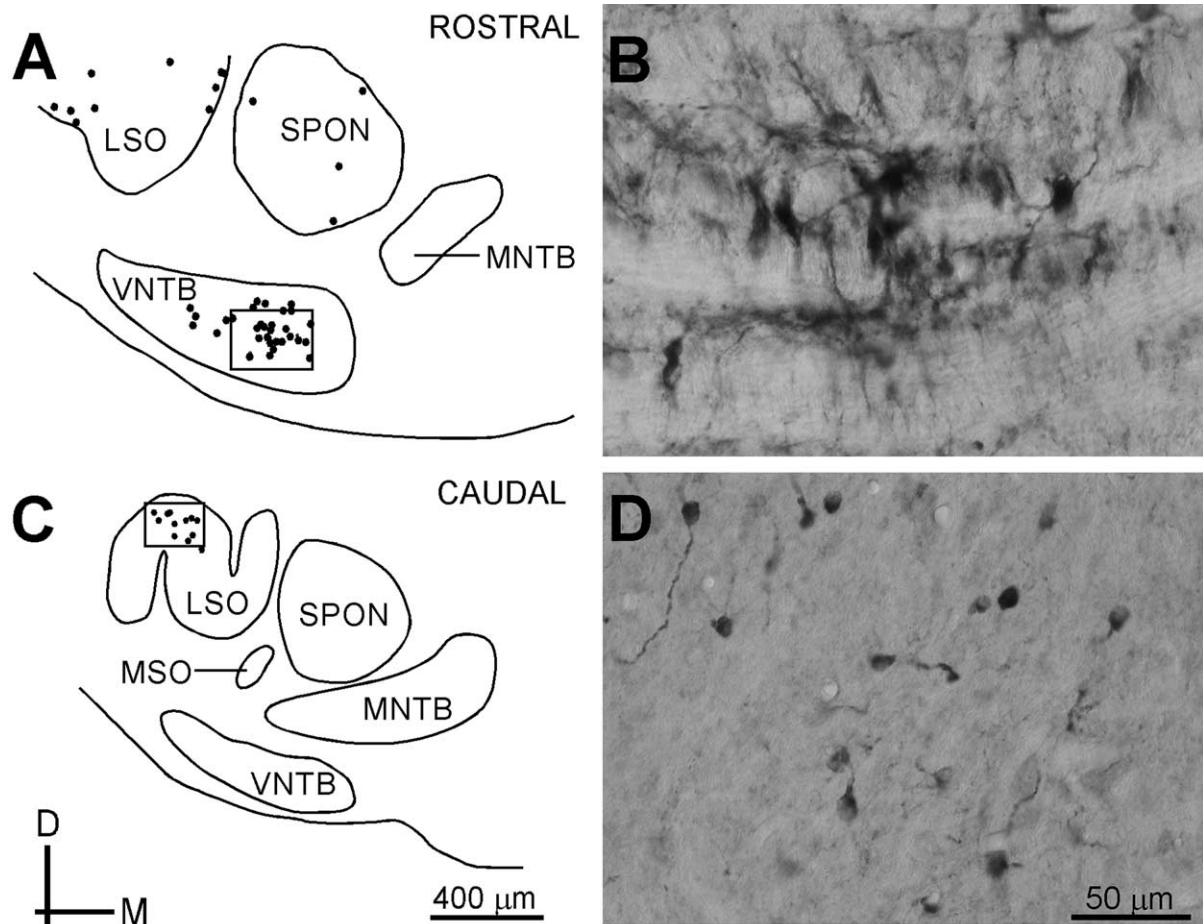
Fig. 2. Labelling of Met[8]-ir somata and fibers in the superior olivary complex. Note labelling of neuronal somata in the LSO, VNTB and SPON and terminal fibers in VNTB only (B). B and D, high magnification of frames in A (from VNTB) and B (from LSO), respectively.

animals not treated with colchicine, the neuronal types are similar, but the degree of immunoreactivity seems to be higher in the pyramidal cell layer than that observed in the same region in rats not treated with the drug.

The ventral cochlear nucleus (VCN) was devoid of immunoreactive cells except for the small cells of the marginal shell of cells (Fig. 1D–F), also referred to as the cap area by Osen (1969). Although this area is smaller in rat than in cat (Cant, 1993), the cells are clearly labelled. These cells were not observed in the group of animals treated with colchicine.

Immunoreactive terminal fields with a moderate density were found in the DCN and with a low density in the VCN in an animal treated with colchicine (Fig. 1B,C,E,F). By contrast, axonal terminal fields in the DCN were absent in the animals untreated with colchicine.

3.2. Superior olivary complex (SOC)

We have found immunoreactive neurons to Met[8] primarily in the ventral nucleus of the trapezoid body (VNTB)(Fig. 2A,B) in animals treated with colchicine. Neurons in the center and in the lateral and dorsal edges of the lateral superior olive (LSO) were present with a low level of immunoreactivity (Fig. 2A–D). These neurons seem to correspond to the shell and intrinsic types that belong to the lateral olivocochlear system (LOC) as judged by their morphological features (Vetter et al., 1991). Likewise, a few immunoreactive neurons were seen in the superior paraolivary nucleus (SPON) (Fig. 2A). No labelling was apparent in untreated animals. Immunoreactive fibers were mostly found in the VNTB of the animals treated with colchicine (Fig. 2B).

←

Fig. 1. Camera lucida drawings of the distribution of Met[8]-ir somata and fibers in the cochlear nuclear complex. (A,D) Each dot indicates one immunoreactive neuron. (A) DCN; (B,C) high magnification details of the insets highlighted in A. (D) VCN showing Met[8]-ir fibers and somata in the marginal cap area; (E,F) high magnification details of the insets highlighted in D. For nomenclature in this figure and successive ones see list of abbreviations.
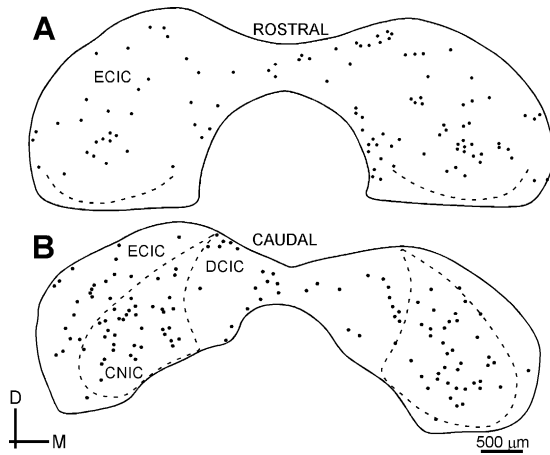
Fig. 3. Distribution of labelling of Met[8]-ir somata in the inferior colliculus.

3.3. Nuclei of the lateral lemniscus (NLL)

Virtually no immunoreactive neurons were observed in the ventral complex of the lateral lemniscus (VCLL) and in the dorsal nucleus (DNLL) of the animals treated with colchicine. Immunoreactive neuronal somata were lacking in both nuclei in the non-treated group of animals.

Terminal fields were absent in both the VCLL and DNLL in both groups of treated and untreated animals. Most of the labelled fibers were observed in the medial edge of the LL.

3.4. Inferior colliculus (IC)

Immunoreactive neurons were observed in the central nucleus (CNIC), dorsal (DCIC) and external cortices of the IC (ECIC) in the group of animals treated with colchicine (Figs. 3A,B and 4A–C). The neuronal types labelled in the CNIC seems to be of both flat (Fig. 4A, arrows) and less flat type. In the cortical areas, they are multipolar (Fig. 4B,C). By contrast, in the group of non-treated animals, only the DCIC and ECIC possess immunoreactive neurons. The density of immunoreactive terminal fields is low in both the treated and untreated groups of animals (see Table 1 and Fig. 4A–C). It should be noted also that there were immunoreactive fibers in the brachium of the inferior colliculus.

3.5. Thalamus and cortex

There were no immunoreactive neurons in any of the different subnuclei of the auditory thalamus (medial geniculate body, MGB) in either the treated or the untreated animals. By contrast, the animals treated with colchicine showed a distinct labelling of terminal fields in the medial divisions of the MGB (MGM; Fig. 5C,D)

and in some very large terminals in the dorsal division of the MGB (MGD) (Fig. 5A,B, arrows). Neither immunoreactive neurons nor terminal fields were observed in the ventral division of the MGB. Fig. 5A,C shows a large number of terminal fields and fibers labelled in the posterior intralaminar nucleus (PIL) and in the peripeduncular nucleus (PP). In the auditory sector of the
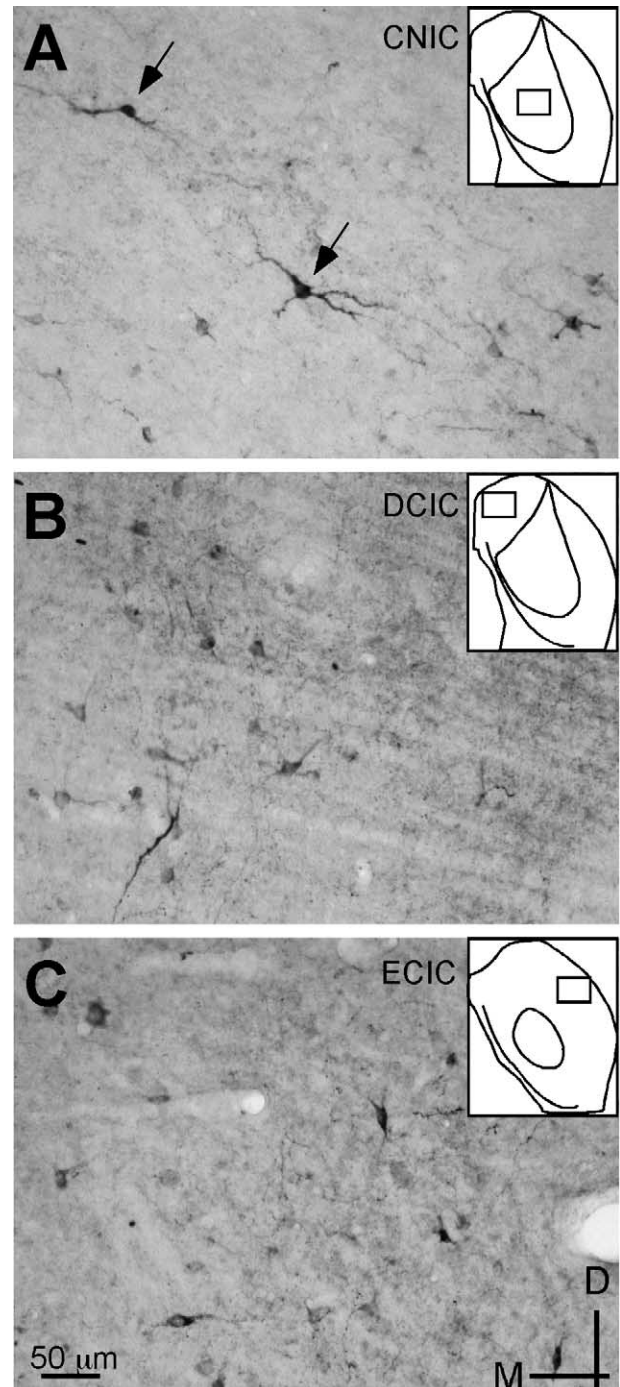

Fig. 4. High magnification photomicrographs showing the labelling of Met[8]-ir somata and fibers in the CNIC (A, flat neurons, arrows), DCIC (B) and ECIC (C).
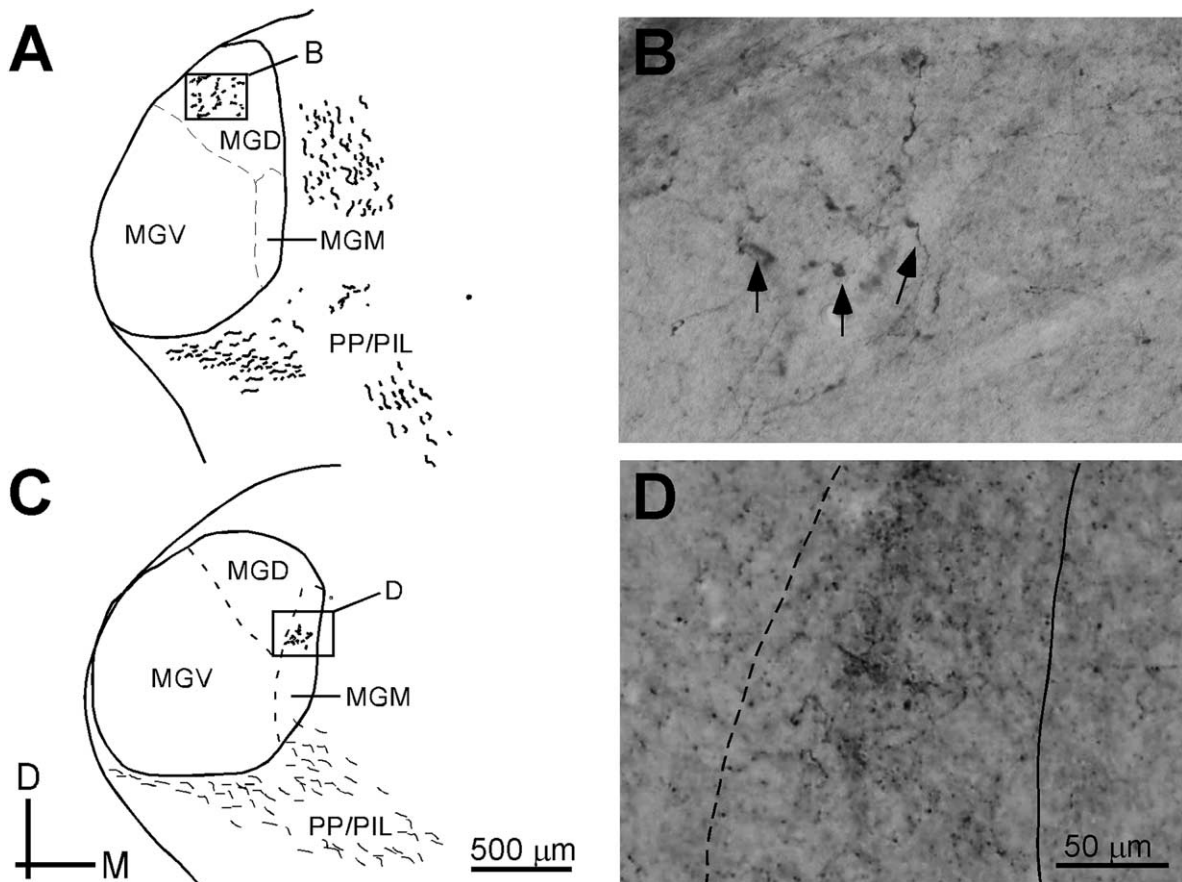
Fig. 5. Labelling of Met[8]-ir fibers in the medial geniculate body. Note the lack of labelling in the MGV and the presence of terminal fields in the MGD (large terminals, arrows in B) and MGM (C,D) as well as in the PP and PIL.

reticular thalamic nucleus a moderate density of immunoreactive terminal fields was apparent (see Table 1).

In the auditory cortex, a few scattered and small neurons were observed in layers I–V throughout parts Te1. However, neurons were more abundant and densely packed in the secondary Te3 of the auditory cortex of both treated and non-treated animals, but no terminal fields were present (Fig. 6A–C).

Outside the main auditory pathway, it should be mentioned that we have also observed some terminal fibers in the zona incerta (ZI). We make special mention of this nucleus, which does not belong to the auditory pathway, because in a recent study Mitrofanis (2002) showed an auditory region in this nucleus.

4. Discussion

This study shows a detailed account of the distribution of Met[8]-ir fibers and cell bodies in the mammalian auditory system. The main finding is that we found either Met[8]-ir fibers or cell bodies or both in virtually all nuclei of the rat auditory system except for the medial superior olive and the ventral division of the MGB.

These two regions did not show any immunoreactivity for Met[8]. The main limitation of our study is the lack of quantitative data. Although it would have been interesting to analyze the density of labelled neurons, our material unambiguously demonstrates that the neuropeptide Met[8] is widely distributed throughout the auditory system of the rat.

4.1. Comparison with previous studies

In general, our results are in agreement and further complement those previous studies on the distribution of structures containing Met[8] in the auditory system. Thus, for example, some authors described neurons in the CNC (Hoffman et al., 1993; Murakami et al., 1987), SOC (Abou-Madi et al., 1987; Murakami et al., 1987; Reuss et al., 1999; Robertson and Mulders, 2000) and IC (Tanaka et al., 1993; Murakami et al., 1987) but to the best of our knowledge, there are no studies that show labelled cells in the auditory cortex. Similarly, terminal fibers have been described in several studies (Fallon and Leslie, 1986; Merchenthaler et al., 1986; Palkovits, 1988; Abou-Madi et al., 1987; Murakami et al., 1987; Hoffman et al., 1993; Tanaka et al.,
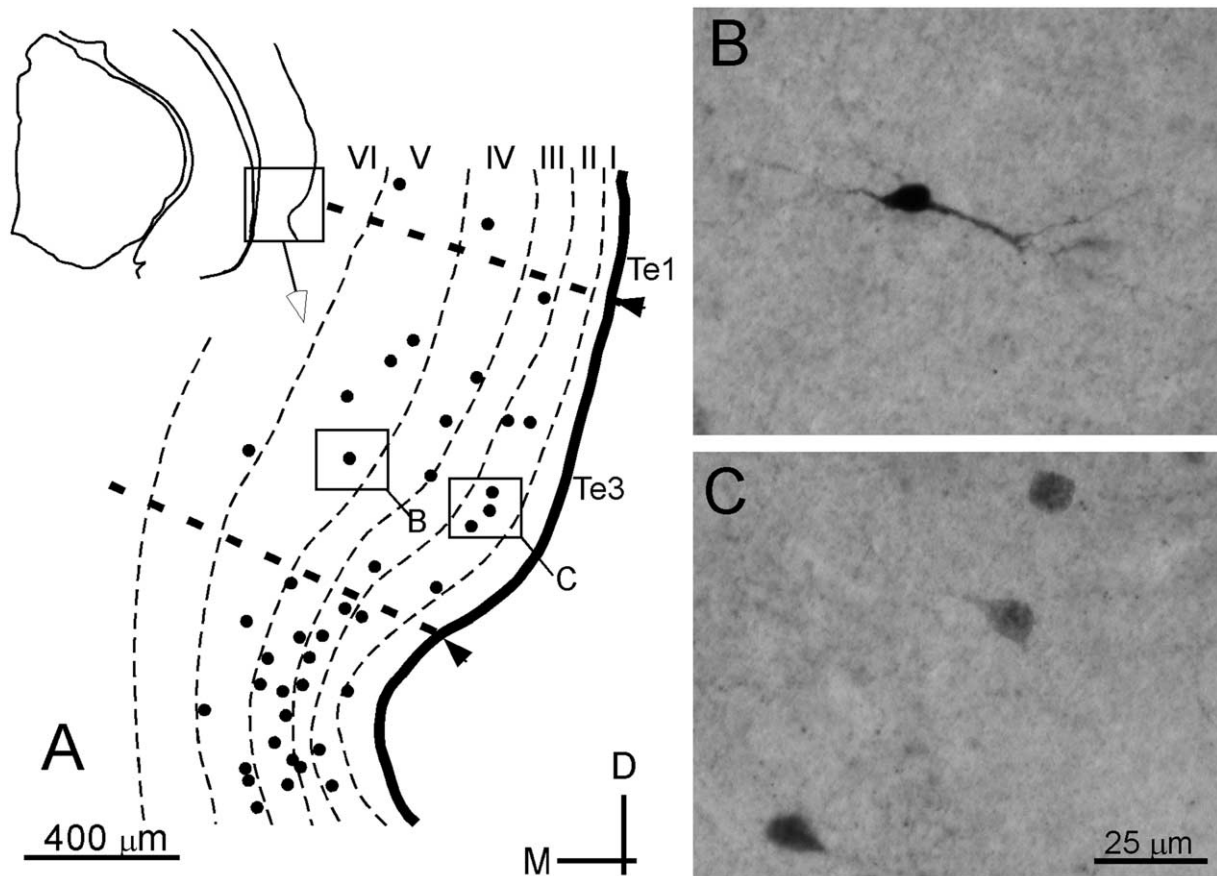
Fig. 6. Labelling of Met[8]-ir neuronal somata in the auditory cortex (area Te3).

1993; Reuss et al., 1999; Robertson and Mulders, 2000). However, terminal fields in the MGB and in the ZI are first described in the present study.

It is now well established that Met[8] is concerned with cardiovascular, pain and motor mechanisms (Akil et al., 1984; Basbaum and Fields, 1984; Pasternak, 1987; Douglas and Kitchan, 1992) and can function as a neurotransmitter or neuromodulator, but its functional role in the auditory system has not been assigned. Our results suggest that Met[8] could play, at least, two roles in hearing. It seems to be involved in the processing of the descending auditory pathway and it may be implicated in the multisensory integration of auditory information that takes place in the non-lemniscal auditory pathway. In the following these two potential roles are dealt with. However, these suggested roles need to be demonstrated physiologically in future studies.

4.2. Speculations of the functional roles of Met[8] in the auditory system

The first indirect evidence that suggested an association of enkephalin with the olivocochlear system was made using guinea pig as an experimental animal (Fex and Altschuler, 1981). These authors already suggested that enkephalin could be involved in both the LOC and the medial olivocochlear system (MOC). Since then some other studies have implicated Met[8] in the olivocochlear system, as a modulator of the cochlear activity (for review see Robertson and Mulders, 2000). This is because both the neurons from the VNTB (related to the MOC) and LSO (related to the LOC) are labelled. Our results support this notion because the neurons labelled in the LSO resemble the morphological features of both intrinsic and shell neurons from the MOC and the neurons of the VNTB (Vetter et al., 1991; Vetter and Mugnaini, 1992; Thompson and Thompson, 1993; Reuss et al., 1999). Furthermore, neuropharmacological studies that used microiontophoretic application of enkephalin have demonstrated an abolition of the excitatory effect of glutamate receptor agonists upon primary afferent neurons in the guinea pig cochlea (Burki et al., 1993). The same authors demonstrated that the mentioned effect is blocked by naloxone, an opiate antagonist. These neuropharmacological experiments support the notion that the LOC fibers release enkephalins on their terminals upon different dendrites beneath the inner hair cells and have an overall inhibitory effect upon

the cochlear output (for review see Robertson and Mulders, 2000). We do not have a direct demonstration that the CNIC neurons labelled in our study project to the VNTB, but we strongly suggest so because the main target of the IC is to the MGV and in our material, no terminal fields were observed in this subdivision of the MGB. Thus, the axons of the labelled neurons in the IC may be those observed in the VNTB. It is well known that VNTB neurons receive descending input from the IC neurons (Caicedo and Herbert, 1993; Vetter et al., 1993; Malmierca et al., 1995; Mulders and Robertson, 2002). Another reason to suggest a role of Met[8] in the processing or modulation of the descending information can be derived from the finding that there are very large terminals (>2 μm, Fig. 5B, arrows) labelled in the MGD in our material. These terminals have been described previously to arise from the pyramidal cells in layer V in the rat (Rouiller and Welker, 1991; Bartlett et al., 2000) and in the cat (Ojima, 1994; Bajo et al., 1995; Winer et al., 1999a, 2001) auditory cortex. In summary, we provide some baseline data to suggest that Met[8] is implicated in the processing or modulation of the descending auditory pathway.

A second role for Met[8] may be related to the integration of multisensory information. In a recent study Malmierca et al. (2002) have described a new pathway from the CNC to the MGB, namely to the MGM with a small component to the suprageniculate nucleus. This is a non-lemniscal pathway that has been suggested to transfer multimodal information to the cortex more rapidly than the lemniscal pathway. The cellular types that give rise to this projection were identified as the small cells from the cap area of the VCN and the pyramidal and giant cells of the DCN. Interestingly, we have shown here that these cell types are immunoreactive for Met[8] and that the only immunoreactive fibers seen in the MGB are in the medial division and in the suprageniculate body. Furthermore, it is known that axons of DCN neurons travel in the medial edge of the lateral lemniscus (Osen, 1972), where we have seen immunoreactive fibers. The conventional lemniscal pathway is clearly important for the perception of sound. In contrast, the direct CNC projection to the MGM may be important for rapidly alerting the animal to biologically significant sounds. The MGM and the PIL together with the PP (also labelled in our material) provide the key auditory input to the lateral amygdala and striatum (LeDoux et al., 1985, 1990; Doron and LeDoux, 1999; Linke et al., 2000), and are required for the associative conditioning of auditory stimuli and emotional responses (LeDoux et al., 1983). Thus, the present results are consistent with a potential role for Met[8] in associative conditioning and the establishment and maintenance of auditory emotional memories.

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