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EFFECTS OF METHIONYL-TYROSYL-LYSINE ON NEURONES IN THE RAT CENTRAL NERVOUS SYSTEM

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1 The effects of microiontophoretically applied methionyl-tyrosyl-lysine (Met-Tyr-Lys) were studied on single neurones in several brain regions of rats anaesthetized with urethane.

2 Met-Tyr-Lys inhibited 13.5%–25% of neurones in the spinal cord, cerebellar cortex, thalamus and hippocampal formation. No significant inhibitory effects were seen in the cerebral cortex.

3 Additionally, Met-Tyr-Lys excited some cells in the Purkinje cell body layer of the cerebellar cortex (11%) and in the pyramidal cell body layer of the hippocampus and granule cell body layer of the dentate gyrus within the hippocampal formation (17.5%).

4 Both excitatory and inhibitory effects of Met-Tyr-Lys were dose-dependent, of similar rapid time course and were observed both on spontaneously active cells and cells induced to fire by continuous iontophoretic application of DL-homocysteic acid.

5 The possibility that Met-Tyr-Lys might be a novel inhibitory neurotransmitter in both spinal and supraspinal regions of the mammalian CNS is discussed.

Introduction

Methionyl-tyrosyl-lysine (Met-Tyr-Lys) was originally extracted from spinal cord and dorsal root ganglia of sheep (Logan & Wolstencroft, 1980; Logan, Lote, Wolstencroft, Gent, Fox, Hudson & Szelke, 1980a) where it represented one of a number of small molecular weight peptides. Analysis of the actions of this tripeptide in the cat spinal cord using microiontophoretic techniques showed that it had only inhibitory actions: these were found predominantly on neurones in Laminae V and VI which responded to some form of proprioceptive input from the periphery (Logan, Lovick, West & Wolstencroft, 1980b). Further investigations in the cat spinal cord confirmed this action and showed that Met-Tyr-Lys-induced inhibitions were not antagonized by bicuculline or strychnine applied at doses that abolished responses to microiontophoretically applied γ-aminobutyric acid (GABA) or glycine (Logan, Lovick, West & Wolstencroft, 1980c; Logan, Lovick, West & Wolstencroft, 1982). Similarly, inhibition of dorsal horn cells responsive to Met-Tyr-Lys, evoked by some forms of peripheral nerve stimulation, was also unaffected by bicuculline or strychnine (Logan *et al.*, 1980c).

This raised the possibility that Met-Tyr-Lys might be a neurotransmitter mediating certain types of inhibition in the dorsal horn. In an attempt to investigate whether Met-Tyr-Lys has activity in other regions of the mammalian CNS and to study its actions in another species we applied Met-Tyr-Lys to single neurones in several regions of the brain and spinal cord in the rat.

Methods

Animal preparation

Experiments were performed on adult Sprague-Dawley rats (290–320 g) anaesthetized with urethane (approximately 1.5 g/kg i.v.) injected via a tail vein. A tracheal cannula was inserted and animals were allowed to breathe spontaneously throughout the course of the experiment. Surgical procedures required for access to the thalamus, hippocampal formation and cerebral cortex involved exposure of the skull by removal of overlying skin and connective tissue to reveal the bregmoidal and lambdoidal suture junctions. A hole (approximately 5 mm diam.) was then made in the skull at the required position with a dental drill. Exposed bone edges were sealed with bone wax and the meninges cut and reflected over these edges. The area was covered with warm liquid paraffin. For access to the cerebellum and brainstem, an area of skin from between the ears to 3–4 cm caudal was removed. A midline incision was made in the splenius capitis muscle, its points of

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attachment to the occipital crest were severed and the muscle was retracted laterally. The underlying semispinalis capitis muscle (pars biventer cervicis and pars complexus) was likewise detached from the occipital crest to reveal the rectus capitis posterior major and rectus capitis posterior minor muscles. These were then detached from the skull, with a bone scraper, to expose the occipital bone. Miniature rongeurs were then carefully inserted into the foramen magnum and the occipital bone and interparietal bone were gradually removed to a distance of 1 mm rostral to the occipital crest. All exposed bone edges were sealed with bone wax and the meninges overlying the cerebellum were cut and reflected over these edges. For experiments in the cerebellum the area was then kept moist with warm liquid paraffin. For experiments in the brainstem, the cerebellum was removed by careful insertion of a spatula between the lower plane of the cerebellum and the upper surface of the brainstem followed by lateral movement to sever the cerebellar peduncles. The exposed upper surface of the brainstem was then covered with warm liquid paraffin. Access to the spinal cord was achieved following dorsal laminectomy to reveal segments T12–L1. The area was then covered by a liquid paraffin pool, retained by supported skin flaps, and was maintained at 37–38°C by a radiant heat source controlled by negative feedback from a thermocouple immersed in the pool.

Animals were mounted in a stereotaxic frame and brain penetrations were made according to the atlas of Pellegrino, Pellegrino & Cushman (1979). Rat body temperature was maintained at 37.5°C by an electric heater blanket controlled by a negative feedback system incorporating a rectal temperature probe.

Equipment

Single units were recorded extracellularly and drugs were applied by microiontophoresis from five- or seven-barrelled glass micropipettes (tip diam. 5–8 μm). Iontophoretic currents (± 0–200 nA) were supplied by a custom-built six-channel microiontophoresis unit. Spontaneous efflux of substances between ejections was automatically checked by the use of small retaining currents (5–10 nA) and ejections were repeated at regular intervals. Drug ejections were routinely compared with current controls to check for current artefacts and were occasionally current compensated. Single unit recordings were made through a Digitimer Neurolog system headstage (NL 100), preamplifier (NL 103) and filter stage (NL 125). The resultant signal was then passed into a spike processer (Digitimer D.130) which converted action potentials of chosen amplitude into standard pulses which were then counted in successive epochs (5 s). Care was taken to ensure that only action potentials of constant amplitude were counted by continuous monitoring of input and output signals from the spike processer on an oscilloscope (Tektronix 502A). An output was also made to a chart pen recorder (Texas Instruments Servo/Riter II).

Iontophoretic solutions

Substances used for iontophoresis were made up as follows (all in distilled water unless indicated): L-methionyl-L-tyrosyl-L-lysine monoacetate salt (Met-Tyr-Lys), (a gift from Dr M. Szelke), 7.0 or 14.0 mM, pH 5.0–6.0, ejected as a cation; DL-homocysteic acid (DLH), (Koch-Light Labs), 100 mM, pH 7.5–8.0, ejected as an anion; Pontamine Sky Blue 6BX, (G.T. Gurr, Ltd.), 2% w/v in 0.5 M sodium acetate, pH 7.7, ejected as an anion; sodium chloride (NaCl), 4 M, pH 7, for extracellular recording; and NaCl, 150 mM or 1 M, pH 7, for current controls and/or current compensation.

Histology

The locations of cells which responded to Met-Tyr-Lys, or at least one recording site per brain penetration, were marked by ejection of Pontamine Sky Blue dye (100 μA min). The positions of such dye spots were subsequently checked and recorded in 50 μm thickness frozen brain sections following aortic perfusion with formal saline (10% v/v formalin in isotonic saline) of animals that had been killed.

Results

The effects of Met-Tyr-Lys, applied with currents of 0–60 nA, were investigated on 292 neurones in the spinal cord, medullary reticular formation, cerebellar cortex, thalamus, hippocampal formation and cerebral cortex (Table 1). In all regions studied the effects of Met-Tyr-Lys were assessed both on spontaneously active cells and on cells activated by continuous application of DLH. Met-Tyr-Lys influenced the activity of between 19.5% and 35% of neurones in each of the brain regions studied with the exception of the cerebral cortex. The most frequent finding was inhibition of between 13.5% and 25% of cells. These responses were rapid, with onset times of the order of 30 s and the return to baseline firing rate, following termination of the ejection current, occurred over a similar period (Figures 1a, 2a, 3a, 5a and c). A significant number of excitatory responses to Met-Tyr-Lys were only encountered in the cerebellar cortex and hippocampal formation. The time course of these effects (Figures 3b and 5b) was essentially the same as for their inhibitory counterparts.

Table 1 Summary of the effects of methionyl-tyrosyl-lysine on single neurones in the rat CNS

	Spinal cord	*Medullary reticular formation*	*Cerebellar cortex*	*Thalamus*	*Hippocampal formation*	*Cerebral cortex*
Excitation	0	0	4 (11%)	1 (1.5%)	6 (17.5%)	1 (2%)
No effect	49 (78%)	33 (75%)	28 (75.5%)	54 (80.5%)	22 (65%)	45 (96%)
Inhibition	14 (22%)	11 (25%)	5 (13.5%)	12 (18%)	6 (17.5%)	1 (2%)
Total	63	44	37	67	34	47

The figures refer to the number of cells tested and percentages indicate the proportion of cells showing each category of response in each region.

Responses to Met-Tyr-Lys were dose-dependent in all regions (e.g. Figures 1a, 2a and 5b) with typical effective ejection currents being of the order of 20–40 nA irrespective of whether the responses were inhibitory or excitatory. On no occasion were both inhibitory and excitatory responses observed on the same cell during repeated applications and similarly none of the responses, either inhibitory or excitatory, showed any significant biphasic character. Both excitatory and inhibitory responses were observed on successive cells sampled using the same micropipette and sometimes during the same brain penetration. Desensitization to successive applications of Met-Tyr-Lys on the same cell was only observed in a small proportion of cells responsive to Met-Tyr-Lys where the effect was minimal and it was easily reversed by a relatively small elongation of the interval between successive ejections.

Spinal cord

Cells were sampled in both the dorsal and ventral horn in upper lumbar segments. None was excited by Met-Tyr-Lys but 22% were inhibited, all of which were situated dorsally (Figure 1b). Although inhibitions were seen on cells in several laminae in the dorsal horn many of these cells, and particularly those in or near Laminae V and VII, were either excited or inhibited by flexion or extension of the hind limbs or feet or by pressure applied to the leg muscles (Figure 1a).

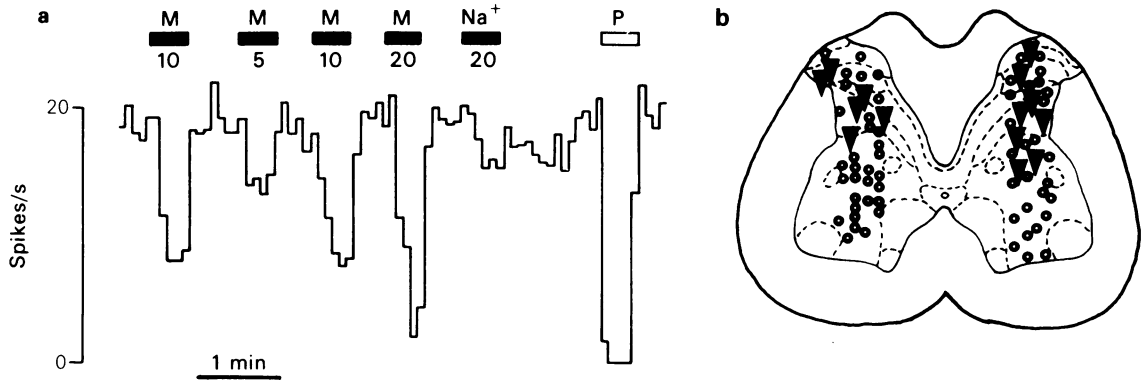

Figure 1 (a) Firing rate of a neurone in the dorsal horn of the spinal cord inhibited by microiontophoretic application (solid bars) of Met-Tyr-Lys (M) and by pressure (P) applied to muscles of the ipsilateral hind limb (open bar). The effect of Met-Tyr-Lys was dose-dependent at up to 20 nA while control ejection of sodium ions (Na^+) was without effect. Numbers below the bars indicate iontophoretic current in nA and the ordinate represents mean firing rate of the neurone in spikes per s in successive 5 s epochs. (b) Diagrammatic representation of a section through the spinal cord at L1 showing the recording sites of cells studied: (▼) represents cells inhibited by iontophoretic application of Met-Tyr-Lys and (○) represents cells unaffected by Met-Tyr-Lys. The recording sites of all cells affected by Met-Tyr-Lys were marked by ejection of Pontamine Sky Blue. Dotted lines indicate the approximate position of Rexed's laminae (according to Steiner & Turner, 1972).

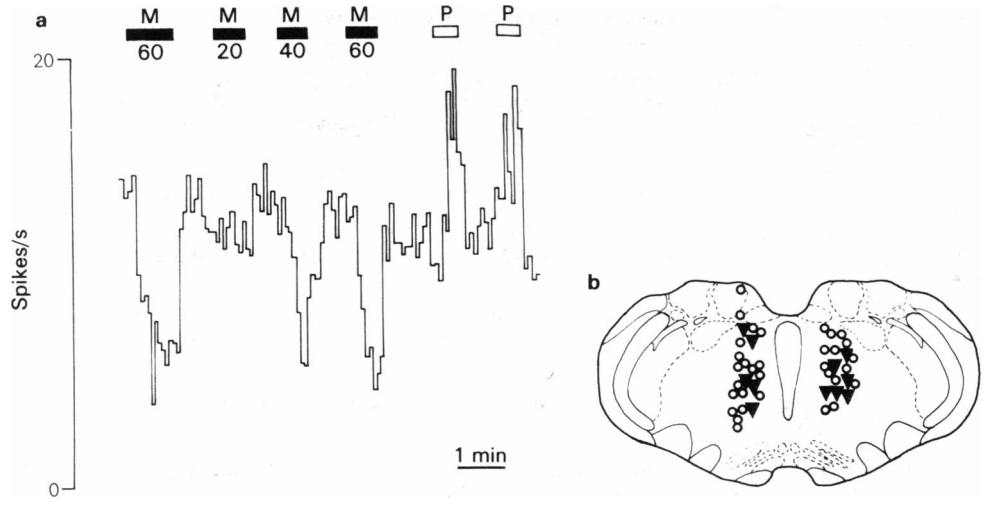

Figure 2 (a) Firing rate of a neurone in the nucleus reticularis gigantocellularis of the brain stem inhibited by iontophoretic application of Met-Tyr-Lys (M) (solid bars) and excited by pressure (P) applied to muscles of the ipsilateral hind limb (open bars). The effects of Met-Tyr-Lys were dose-dependent at up to 60 nA Details as in Figure 1a. (b) Diagrammatic representation of a coronal section through the brain stem (at −4.6 mm from the interaural line) showing the recording sites of cells studied: (▼) represents cells inhibited by iontophoretic application of Met-Tyr-Lys and (○) represents cells unaffected by Met-Tyr-Lys. All cells affected by Met-Tyr-Lys were marked by ejection of Pontamine Sky Blue. Regional subdivisions are in accordance with Pellegrino *et al.* (1979).

Medullary reticular formation

Most of the cells studied in this region were located in the nucleus reticularis gigantocellularis (Figure 2b); 25% of the neurones encountered were inhibited by Met-Tyr-Lys (Figure 2a) and none was excited. Most of the neurones inhibited by Met-Tyr-Lys were excited by pressure applied to limb muscles or by flexion or extension of the limbs and by other stimuli, such as pinching over large areas of the body surface (Figure 2a).

Cerebellar cortex

The cells studied in the cerebellum regularly fired in rapid bursts of 3–5 large spikes and the dye spots which marked the position of the micropipette tip were almost always found in, or very close to, the Purkinje cell body layer (Figures 3c, 6d and 6e). Thus the cells studied were most probably Purkinje cells. As indicated in Table 1, 11% of the cells were excited (Figure 3b) and 13.5% were inhibited (Figure 3a) by Met-Tyr-Lys. These cells did not show any consistent pattern in their responsiveness to either proprioceptive or other forms of peripheral stimulation.

Thalamus

Of the cells studied in various thalamic nuclei, 18% were inhibited by Met-Tyr-Lys (Figure 5c) and one cell (1.5%) was excited. However, the single excitatory response was of small amplitude with prolonged onset and was thus considered to be of questionable significance. Cells inhibited by Met-Tyr-Lys in this region showed no evidence of being localized to a particular nucleus or group of nuclei (Figure 4). Furthermore, these cells failed to show any consistency in their responsiveness to various forms of peripheral stimulation, though a few showed clear excitations or inhibitions in response to flexion or extension of limbs (Figure 5c).

Hippocampal formation

As judged from the position of dye spots marking micropipette tip positions, some cells studied were probably granule cells of the dentate gyrus while others were pyramidal cells of regions CA1 and CA4 of the hippocampus proper (Figures 4, 6a, b and c). Of the latter, many were observed to fire in rapid bursts of between 2–6 spikes of progressively decreasing amplitude. Of the total population of cells studied throughout the hippocampal formation 17.5% were inhibited by Met-Tyr-Lys (Figure 5a) and 17.5% were excited (Figure 5b). Both types of response were found in both sites within the hippocampal formation.

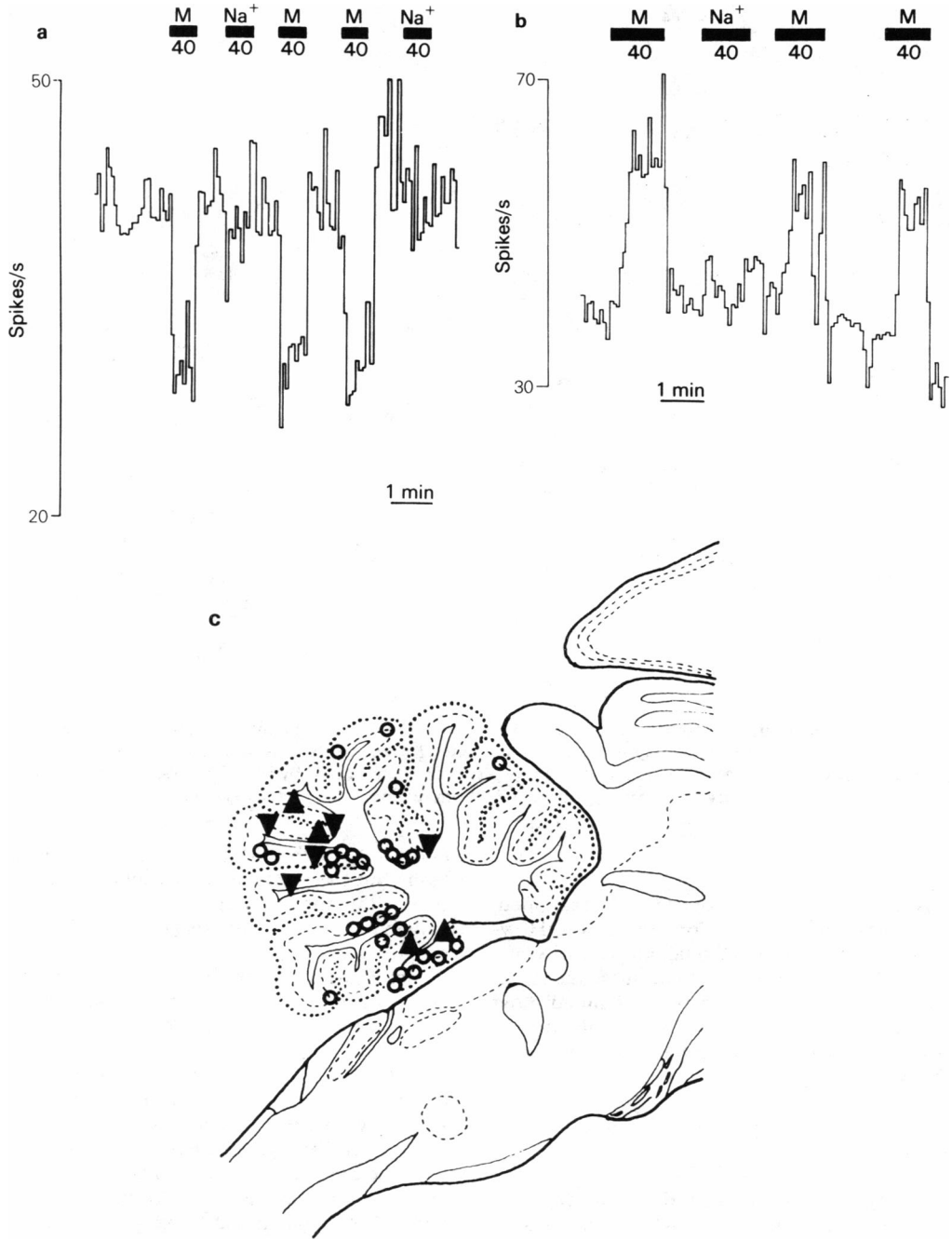

Figure 3 (a) Activity of a neurone in the cerebellar cortex inhibited by iontophoretic application of Met-Tyr-Lys (M). Control ejections of sodium ions (Na^+) were without effect. Details as in Figure 1a. (b) Activity of a neurone in the cerebellar cortex excited by iontophoretic application of Met-Tyr-Lys (M). Control ejection of sodium ions (Na^+) was without effect. Details as in Figure 1a. (c) Diagrammatic representation of a longitudinal section through the cerebellum (at 1.1 mm lateral to the midline) showing the recording sites of cells studied: (▼) represents cells in hibited by Met-Tyr-Lys; (▲) represents cells excited by Met-Tyr-Lys and (○) represents cells unaffected by Met-Tyr-Lys. Other details as in Figure 2b.

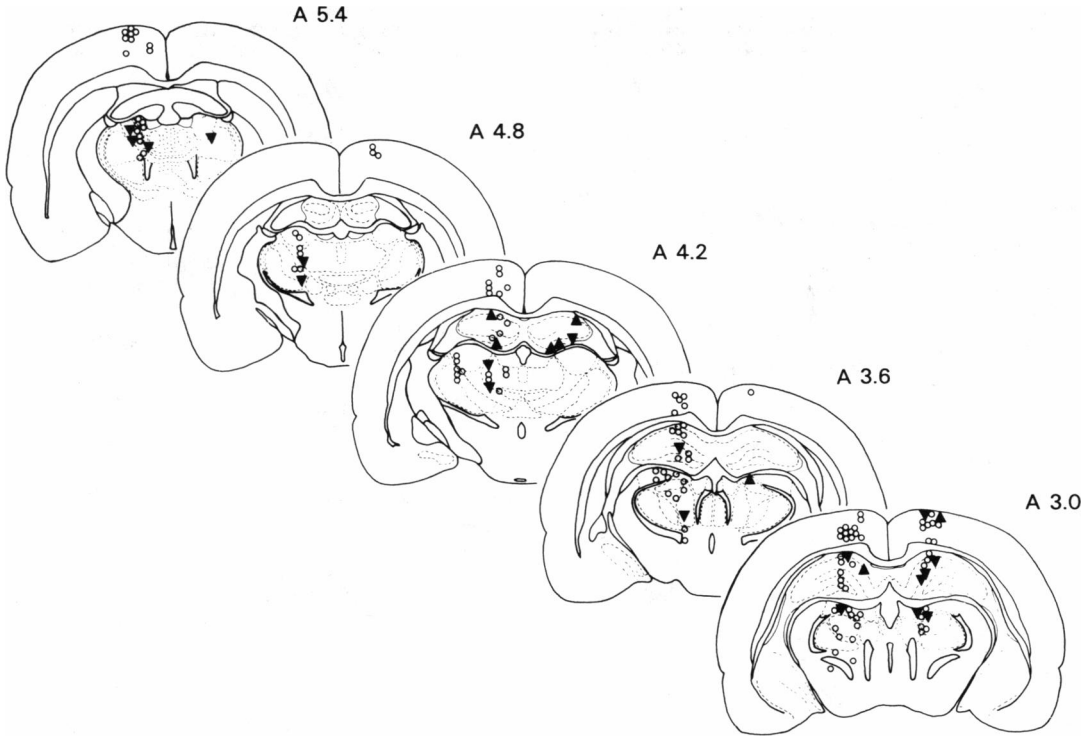

Figure 4 Diagrammatic representation of coronal sagittal sections through the thalamus, hippocampus and cerebral cortex showing the recording sites of cells studied: (▼) represents cells inhibited by Met-Tyr-Lys, (▲) represents cells excited by Met-Tyr-Lys and (○) represents cells unaffected by Met-Tyr-Lys. Values adjacent to each section represent distances (in mm) anterior to the interaural line. Other details as in Figure 2b.

Cerebral cortex

Met-Tyr-Lys had no effect on 47 cells examined, except for one inhibition and one excitation. However, both responses were of small amplitude with a long onset time and thus of doubtful significance. Cells were sampled during penetrations aimed at the underlying hippocampal formation and thalamus and were situated in several different layers of the cortex (Figure 4).

Discussion

Many biologically active peptides isolated from mammalian brain, such as TRH, somatostatin, neurotensin and others, have been found to be active when tested on neurones in the spinal cord (Nicoll, 1977; Randić & Miletić, 1978; Miletić & Randić, 1979). Met-Tyr-Lys is the first example, to our knowledge, of a peptide isolated and characterized from the spinal cord (Logan *et al.*, 1980a; Logan & Wolstencroft, 1980) which conversely shows activity when tested on neurones in the brain. We do not know yet whether Met-Tyr-Lys is present in brain tissue but the widespread distribution of many peptides within the CNS suggests that this is not unlikely and an investigation of this possibility is currently in progress.

Met-Tyr-Lys was tested by microiontophoresis on neurones in the spinal cord, medullary reticular formation, cerebellar cortex, thalamus, hippocampal formation and cerebral cortex of the rat. Inhibitory effects were observed on a similar proportion of neurones (13.5%–25%) in each of the regions studied with the exception of the cerebral cortex where Met-Tyr-Lys appeared to be inactive.

In the lumbar spinal cord of the rat, Met-Tyr-Lys evoked inhibitory responses similar to those previously observed in the cat (Logan *et al.*, 1980b; Logan *et al.*, 1982). Inhibitions were confined to neurones in the dorsal horn and intermediate region and many of the neurones inhibited had proprioceptive inputs as judged by their responsiveness to flexing and/or extending the ipsilateral leg. Also, the time course of these responses to Met-Tyr-Lys was indistinguishable from that seen in the cat (Logan *et al.*, 1980a). Thus Met-Tyr-Lys had similar actions in a corres-

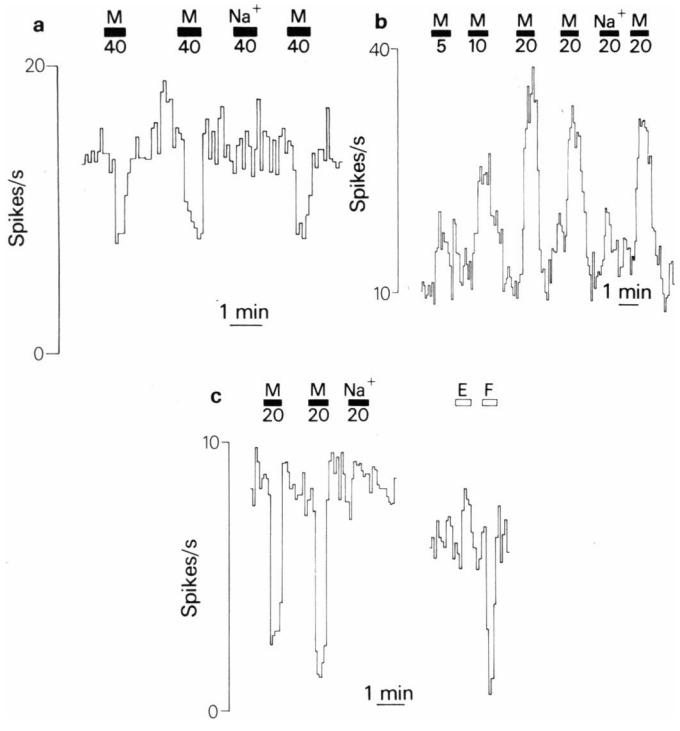

Figure 5 (a) Activity of a neurone in the hippocampus inhibited by iontophoretic application of Met-Tyr-Lys (M). Control ejection of sodium ions (Na^+) was without effect. Details as in Figure 1a. (b) Activity of a neurone in the hippocampus excited by iontophoretic application of Met-Tyr-Lys (M). The effect of Met-Tyr-Lys was dose-dependent at up to 20 nA while control ejection of sodium ions (Na^+) was without effect. (c) Activity of a neurone in the ventral nucleus of the thalamus inhibited by iontophoretic application (solid bars) of Met-Tyr-Lys (M) and by flexion (F) of the contralateral forelimb (open bar). Extension of the same limb (E) evoked a small but reproducible increase in neuronal firing rate. Control ejection of sodium ions (Na^+) was without effect. Details as in Figure 1a.

ponding region of the CNS in two different species, and this together with the fact that Met-Tyr-Lys was originally extracted from sheep spinal cord suggests that it is likely to be a peptide common to mammals.

Inhibitions seen to Met-Tyr-Lys elsewhere in the rat CNS were similar to those encountered in the spinal cord with responses in all regions being rapid in time course and dose-dependent. Additionally, many of the neurones inhibited by Met-Tyr-Lys in the brain stem and the occasional cell in the thalamus had proprioceptive inputs similar to those found in the spinal cord. However, no evidence was found for such inputs on cells inhibited by Met-Tyr-Lys elsewhere in the brain. Thus it is probable that Met-Tyr-Lys is involved in processes unrelated to proprioception in these regions.

Excitatory responses to Met-Tyr-Lys were only encountered in significant proportions in the hippocampal formation and cerebellar cortex (17.5% and 11% respectively). Met-Tyr-Lys is not the only inhibitory peptide with excitatory actions in the hippocampus and cerebellum. This has also been observed with enkephalin (Nicoll, Siggins, Ling, Bloom & Guillemin, 1977; Zieglgänsedrger, French, Siggins & Bloom, 1979) and in the hippocampus it has been suggested that the excitatory effect might be due to inhibition of neighbouring inhibitory interneurones, producing disinhibition (Zieglgänsberger *et al.*, 1979; Nicoll, Alger & Jahr, 1980). However, there is evidence for an alternative explanation in terms of increased release of excitatory transmitter by a presynaptic opioid action (Haas & Ryall, 1980). Therefore, similar detailed experiments for Met-Tyr-Lys will be needed before the precise mechanism of action of the excitations described here can be stated.

The widespread actions of Met-Tyr-Lys in the rat CNS suggest that this peptide may be a novel inhibitory transmitter with both spinal and supraspinal actions. Studies are currently in progress to investigate this possibility and to assess the relationship between such actions and a role in the processing of proprioceptive information.

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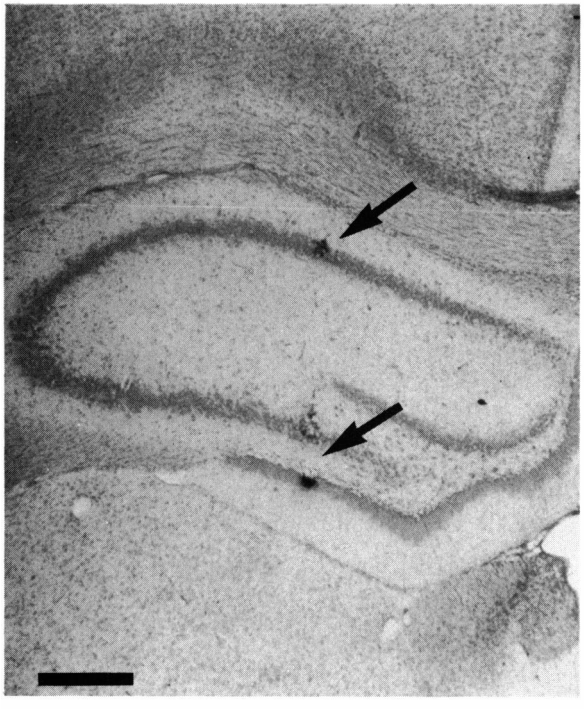
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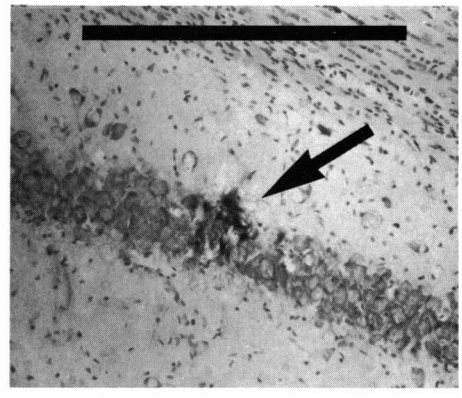
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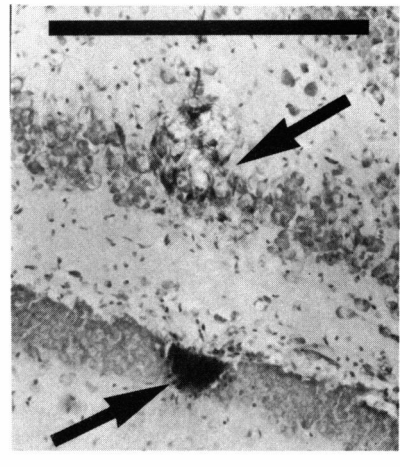
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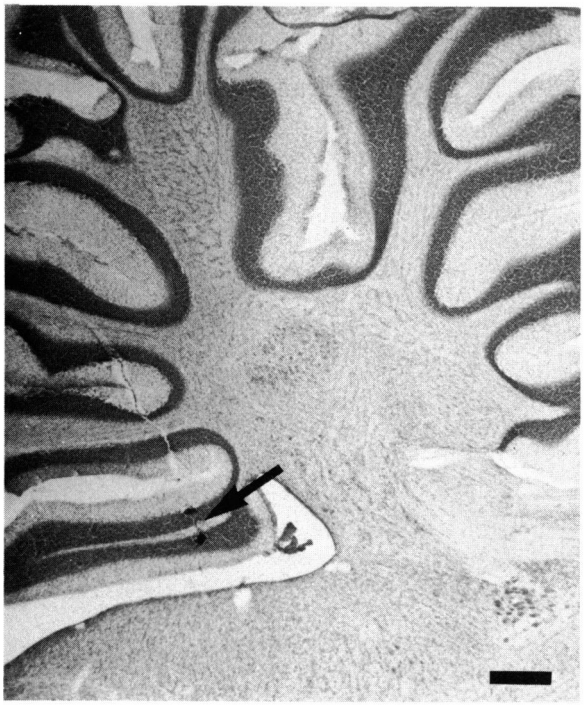
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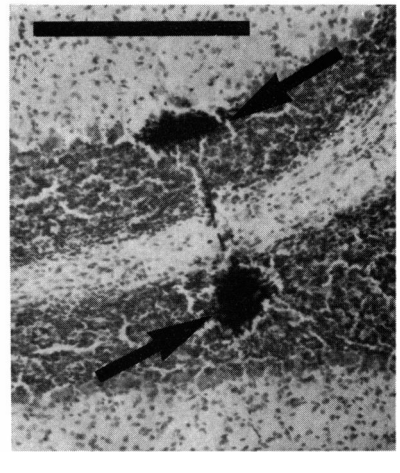
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Figure 6 Photomicrographs of the position of Pontamine Sky Blue dye spots (arrowed) marking the position of recording sites in the hippocampal formation and cerebellar cortex. (a) Low power photomicrograph showing the typical positions of dye spots in the hippocampus and dentate gyrus. (b) High power photomicrograph of the uppermost cell in (a) showing the precise location of the dye spot to be in the pyramidal cell body layer of the CA1 region of the hippocampus. This cell was excited by iontophoretic application of Met-Tyr-Lys. (c) High power photomicrograph of the lower two dye spots in (a) showing their precise locations to be in the pyramidal cell body layer of the CA4 region of the hippocampus (upper arrow) and granule cell layer of the dentate gyrus (lower arrow). These particular cells were unaffected and excited, respectively, by iontophoretic application of Met-Tyr-Lys. (d) Low power photomicrograph showing the position of dye spots in the cerebellar cortex. (e) High power photomicrograph showing the precise location of the dye spots in (d). The uppermost spot was in the Purkinje cell body layer and was typical of most of the cells sampled in this region. This cell was excited by iontophoretic application of Met-Tyr-Lys. The lower dye spot was in the granular cell layer and this cell was unaffected by Met-Tyr-Lys. Scale bars (0.5 mm) indicate the relative magnification of each photomicrograph.

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