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Afferent and efferent enkephalinergic systems of the tegmental nuclei of Gudden in the rat: an immunocytochemical study

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Key words: L-Enkephalin; Dorsal tegmental nucleus; Ventral tegmental nucleus; Fiber connection; Mammillary body; Interpeduncular nucleus; Immunocytochemistry; Rat

We studied the afferent source of L-enkephalin-like immunoreactive (L-ENKLI) fibers in the ventral tegmental nucleus (VT) of Gudden, and efferent and afferent connections of L-ENKLI structures in the dorsal part of the dorsal tegmental nucleus (DDT) of Gudden in the rat, using immunocytochemistry combined with knife-cut and lesion experiments. The VT had a dense plexus of L-ENKLI fibers but no L-ENKLI cells. Destruction of the dorsal premammillary nucleus and medial mammillary nucleus pars medialis, which contained a large group of L-ENKLI neurons, markedly reduced L-ENKLI fibers in the ipsilateral VT, suggesting that most of these fibers originate in these nuclei. The DDT contained a large collection of L-ENKLI neurons together with dense L-ENKLI fibers. Destruction of the DDT caused a contralateral marked reduction of L-ENKLI fibers in the dorsolateral part of the interpeduncular nucleus (DL), suggesting that L-ENKLI neurons in the DDT project contralaterally to the DL. L-ENKLI fibers in the DDT may be of intrinsic origin.

INTRODUCTION

Since the discovery of the enkephalins, which are two naturally occurring pentapeptides with a high affinity for the opiate receptor[13], the widespread presence of enkephalins in the central nervous system has been detected by radioimmunoasay and immunocytochemistry[7,12,16,22,23,29,30,33,35]. The dorsal (DT) and ventral (VT) tegmental nuclei of Gudden are related to the limbic function as a relay center of the upper brainstem, which is connected to the posterior hypothalamus and the interpeduncular nucleus[1,2,4,5,8–11,15,24,25,27,31,34,36]. Immunocytochemical studies have shown the presence of L-enkephalin-like immunoreactive (L-ENKLI) structures in the DT and VT[21,28]. These findings suggest that L-ENKLI here relates closely to limbic function. To elucidate the function of L-ENKLI structures here, the fiber connections of L-ENKLI structures in the DT and VT must be first explored. Accordingly, we here studied enkephalinergic projections in the DT and VT in the rat using immunocytochemistry combined with knife-cut and lesion experiments.

MATERIALS AND METHODS

Experimental animals

We used 39 albino rats (body weight, about 70 g). Seven were used in the analysis of the normal distribution of L-ENKLI structures. Of these, 3 received an injection of 6 μl of colchicine (3 μg/μl) into the third or fourth ventricle stereotaxically. These rats were killed 24-48 h later. Colchicine inhibits axonal transport and has been used to increase the cell-body levels of neurotransmitters and neuromodulators[6,19]. Here it was injected to make visible L-ENKLI neurons that would otherwise be undetectable. The remaining rats were divided into two groups for a knife-

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cut study (17 animals) and a lesion study (15 animals).

Knife-cut study

In the brains of 17 rats, 4 kinds of transection and two kinds of knife-cuts were made. The transections were just caudal to the tegmental nuclei (Fig. 1a), just rostral to the tegmental nuclei, just caudal to the interpeduncular nucleus (IP) (Fig. 1b), just caudal to the mammillary body (MB) (Fig. 1c) or just rostral to the MB (Fig. 1d). The sagittal cuts were just lateral to the MB or just lateral to the tegmental nuclei (Fig. 1e). The rats were kept alive for 5–7 days after the operation and then processed for immunocytochemistry.

Lesion study

The above study suggested (1) that L-ENKLI fibers in the VT generally arise on the area enclosed in the cut shown in Fig. 1c–e and (2) that L-ENKLI neurons in the DDT project to the DL. Therefore, various areas located in the area enclosed by 3 kinds of knife-cuts and DDT were unilaterally destroyed in other rats using stereotaxic equipment by passage of a DC current of 1 mA for 10 s through a monopolar electrode. Damage was assessed using a bright-field microscope after Cresyl violet staining. These animals were kept alive for 5–7 days after the operation and processed for immunocytochemistry.

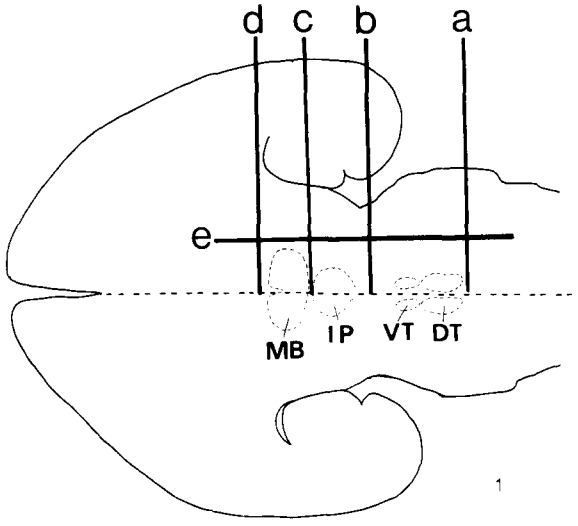

Fig. 1. Schematic drawing showing knife-cut sites in the brain. a: transection just caudal to the dorsal tegmental nucleus of Gudden (DT). b: transection just rostral to the ventral tegmental nucleus of Gudden (VT). c: transection just rostral to the interpeduncular nucleus (IP). d: transection just rostral to the mammillary body (MB). e: sagittal section lateral to the MB. Horizontal plane.

Preparation of tissue

All rats were perfused intracardially with 50 ml of ice-cold saline followed by 200 ml of cold Zamboni's fixative[37]. The brain was removed rapidly, immersed in the same fixative for 12 h, and then rinsed for 24 h in phosphate buffer containing 30% sucrose at 4 °C. Serial frontal sections 10 μm thick were cut on a cryostat. Half of the sections was immediately incubated in cold phosphate-buffered saline (PBS) for 30 min, then processed for indirect immunofluorescence[3]. Rabbit antiserum diluted 1:1000 with PBS against L-enkephalin (L-ENK) was applied to the sections, which were then incubated for 12 h at room temperature (17 °C) in a humid atmosphere. The sections were rinsed in cold PBS containing 1% Triton X-100 for 10 min and in PBS for 30 min, followed by 12 h of incubation at room temperature with goat antirabbit IgG conjugated with fluorescein isothiocyanate (FITC) and diluted 1:1000 with PBS containing 1% Triton X-100 for 10 min and in cold PBS for 30 min, and were then mounted in a PBS-glycerine mixture (1:1). After observations, the sections were stained with Cresyl violet for exact identification of the brain regions. The remaining sections were rinsed in PBS and stained with Cresyl violet. The brain region and the extent of the damage caused by the knife-cut or lesion were assessed on these sections.

Specificity of antiserum

Antiserum against L-ENK was produced in response to a conjugate of 1-ethyl-*o*-(3,3'-dimethylaminopropyl)carbodiimide, synthetic L-ENK (Japanese Peptide Lab.), and bovine thyroglobulin (Sigma). The specificity of the L-ENK antiserum was evaluated by radioimmunoassay. Cross-reactivity was less than 1% with methionine-enkephalin (M-ENK), less than 0.01% with dynorphin, and less than 0.01% with adrenocorticotropic hormone. There was no cross-reactivity with α, β or γ-endorphins, or with substance P (SP), somatostatin (SOM), neurotensin (NT), luteinizing hormone-releasing hormone (LH-RH), cholecystokinin-8 (CCK), or thyrotropic-releasing hormone (TRH). The specificity of the antiserum was also tested histochemically, by (1) re-

placement of the specific serum with normal rabbit serum, (2) omission of the specific serum, and (3) adsorption of anti-L-ENK serum to L-ENK, M-ENK, SP, NT, CCK, SOM, and LH-RH. Adsorption was at 4 °C with each peptide at a final concentration of 10^{-5} M, followed by centrifugation at 3000 *g* for 30 min. The supernatant was used for the later tests. Structures that satisfied all the following criteria were taken to be positive for L-ENK: there was no immunostaining (1) when the specific serum was replaced by normal serum or omitted or (2) when anti-L-ENK serum was absorbed with L-ENK, but (3) there was no decrease in immunostaining when sections were incubated with antiserum against L-ENK adsorbed by any of the other peptides tested.

RESULTS

Distribution of L-ENKLI structures in the DT and VT

In the VT, a dense plexus of L-ENKLI fibers was seen (Fig. 2a–c). L-ENKLI fibers were evenly distributed in this nucleus in its rostral and caudal one-third (Fig. 2a, c). However, at the middle level of this nucleus, the fibers tended to be concentrated in the dorsolateral portion (Fig. 2b); in the ventromedial portion, fewer L-ENKLI fibers were detected. No immunoreactive neurons were identified in this nucleus, even in the rats treated with colchicine.

The DT is divided into the pars dorsalis (DDT) and pars ventralis (VDT) based upon the cytoarchitecture[11]. In the DDT, a large collection of L-ENKLI neurons and fibers was detected. Immunoreactive neurons were distributed evenly throughout the DDT (Fig. 2c–e). The VDT was devoid of L-ENKLI structures except for a small region in its rostral pole (Fig. 2c). There, a small group of L-ENKLI neurons together with immunoreactive fibers was found in its medioventral border (Fig. 2c). A group of fibers left this nucleus ventrolaterally and intermingled with a dense plexus of L-ENKLI fibers in the VT (Fig. 2c).

Origin of the L-ENKLI fibers in the VT

Knife-cut study. Unilateral transection of the brain just caudal to the VT (Fig. 1a) did not decrease L-

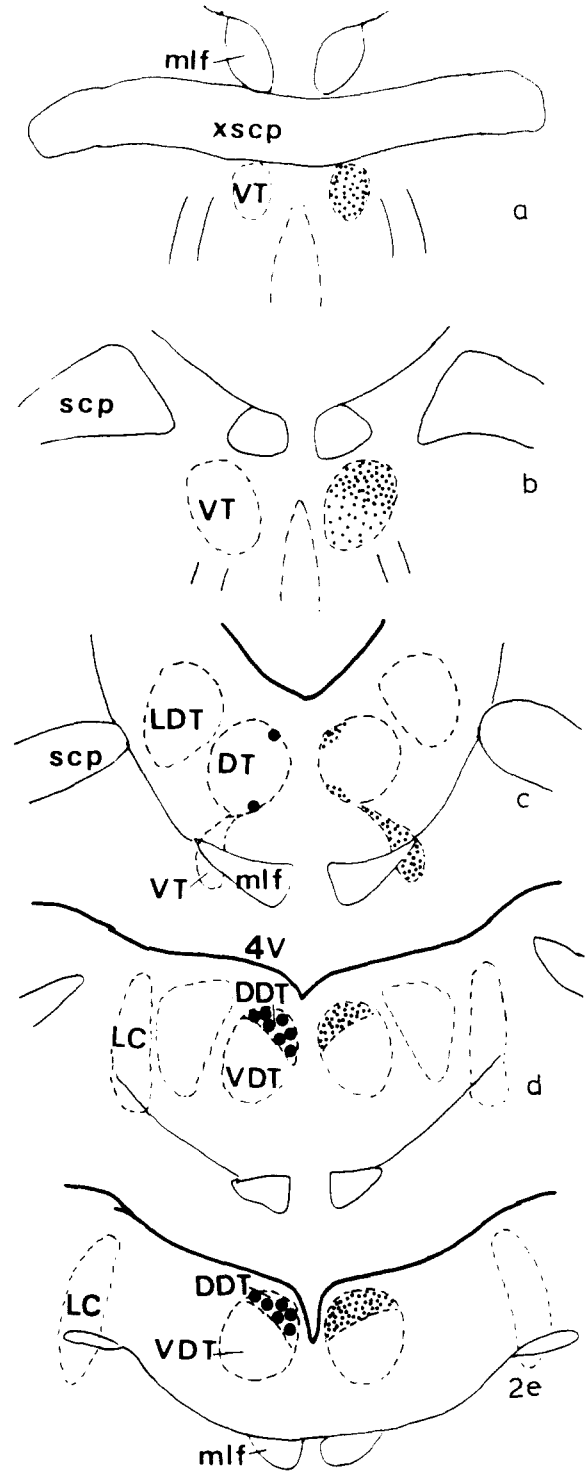

Fig. 2. Schematic drawing of L-ENKLI structures in the tegmental nuclei of Gudden. Large dots indicate the L-ENKLI cells seen in colchicine-treated rats. Small dots indicate L-ENKLI fibers in rats without colchicine treatment. Note the concentration of L-ENKLI fibers in the VT and dorsal part of the DT (DDT), and that of L-ENKLI neurons in the DDT. Frontal plane. Arranged from rostral to caudal. LC, locus coeruleus; LDT, laterodorsal tegmental nucleus; mlf, medial longitudinal fasciculus; scp, superior cerebellar peduncle; VDT, ventral part of the DT, xscp, decussation of the scp; 4V, fourth ventricle.

ENKLI fibers in the VT. Unilateral transection just rostral to the VT (Fig. 1b) or just caudal to the MB (Fig. 1c) resulted in the ipsilateral disappearance of the dense collection of L-ENKLI fibers in the VT. L-ENKLI fibers in the VT were unchanged after transection of the brain just caudal to the ventromedial hypothalamic nucleus (vmh) (Fig. 1d), or after a sagittal cut in the brain at the level lateral to the MB (Fig. 1e). These results suggest that L-ENKLI cells in the area surrounded by the 3 kinds of knife-cuts shown in Fig. 1c–e are the main origin of L-ENKLI fibers in the VT. This area is tentatively called the mammillary area in this study.

Lesion study. Fig. 3 shows the location of L-ENKLI cells in the mammillary area in the colchicine-treated animals. Sites that contained L-ENKLI cells were the dorsal mammillary nucleus (PMD) (Figs. 3, 4a), medial mammillary nucleus pars medialis (MM) (Fig. 3, 4a), arcuate nucleus (Ar), and ventral premammillary nucleus (PMV). Density was highest in the PMD and MM; there were fewer immunoreactive cells in the Ar, and very few in the PMV. In addition, scattered L-ENKLI cells were seen in the neuropil adjacent to these nuclei. L-ENKLI cells found in the PMD fused mediocaudally with those found in the MM (Fig. 3b), and no definite border between the PMD and MM could be seen in the distribution of L-ENKLI cells. Accordingly, in this study, these two nuclei are called the medial mammillary complex (MMC). To decide which of the regions mentioned above is the main source of L-ENKLI fibers in the VT, these regions were destroyed, separately.

When the lesions were centered in the rostral MMC and when other regions of the mammillary area remained intact (Fig. 4b), numbers of L-ENKLI fibers decreased in the caudal part of the ipsilateral VT (Fig. 4c), but L-ENKLI fibers in the rostral part of the VT were unchanged. When the lesion was centered in the caudal part of the MMC and when other regions of the mammillary area remained intact, L-ENKLI fibers in the rostral part of the VT decreased markedly on the operated side, while no large decrease of L-ENKLI fibers was seen in the caudal part of the VT. In addition, when the lesion was large and involved the entire MMC, L-ENKLI fibers disappeared in the entire VT on the operated side. When the lesion involved the mammillotegmental tract (mtg), L-ENKLI fibers disappeared ipsilaterally in

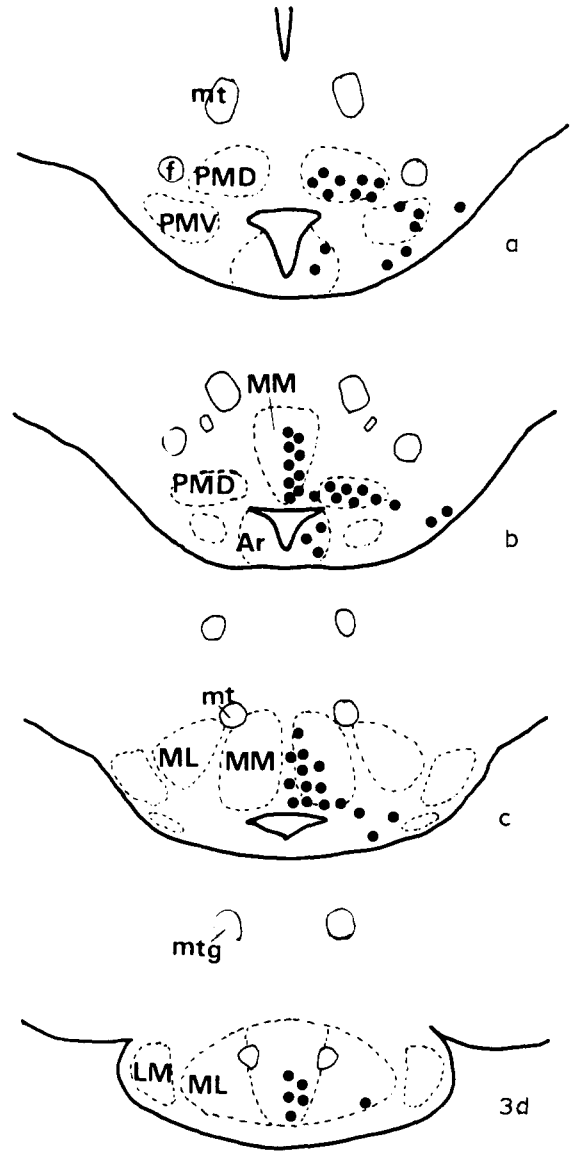

Fig. 3. Schematic drawing of L-ENKLI cells in the area surrounded by the 3 kinds of knife-cuts shown in Fig. 1c, d and e in colchicine-treated rats. The location of these cells is represented on the right side by filled circles. Note a large group of L-ENKLI cells in the medial mammillary nucleus pars medialis (MM) and dorsal premammillary nucleus (PMD). Ar, arcuate nucleus; f, fornix; LM, lateral mammillary nucleus; ML, medial mammillary nucleus pars lateralis; mt, mammillothalamic tract; mtg, mammillotegmental tract; PMV, ventral premammillary nucleus. Frontal plane. Arranged from rostral to caudal.

the VT. In this case, in the mtg rostral to the lesion, fibers that accumulated L-ENKLI structures appeared. These fibers could be traced to the MMC where L-ENKLI neurons had much stronger fluores-

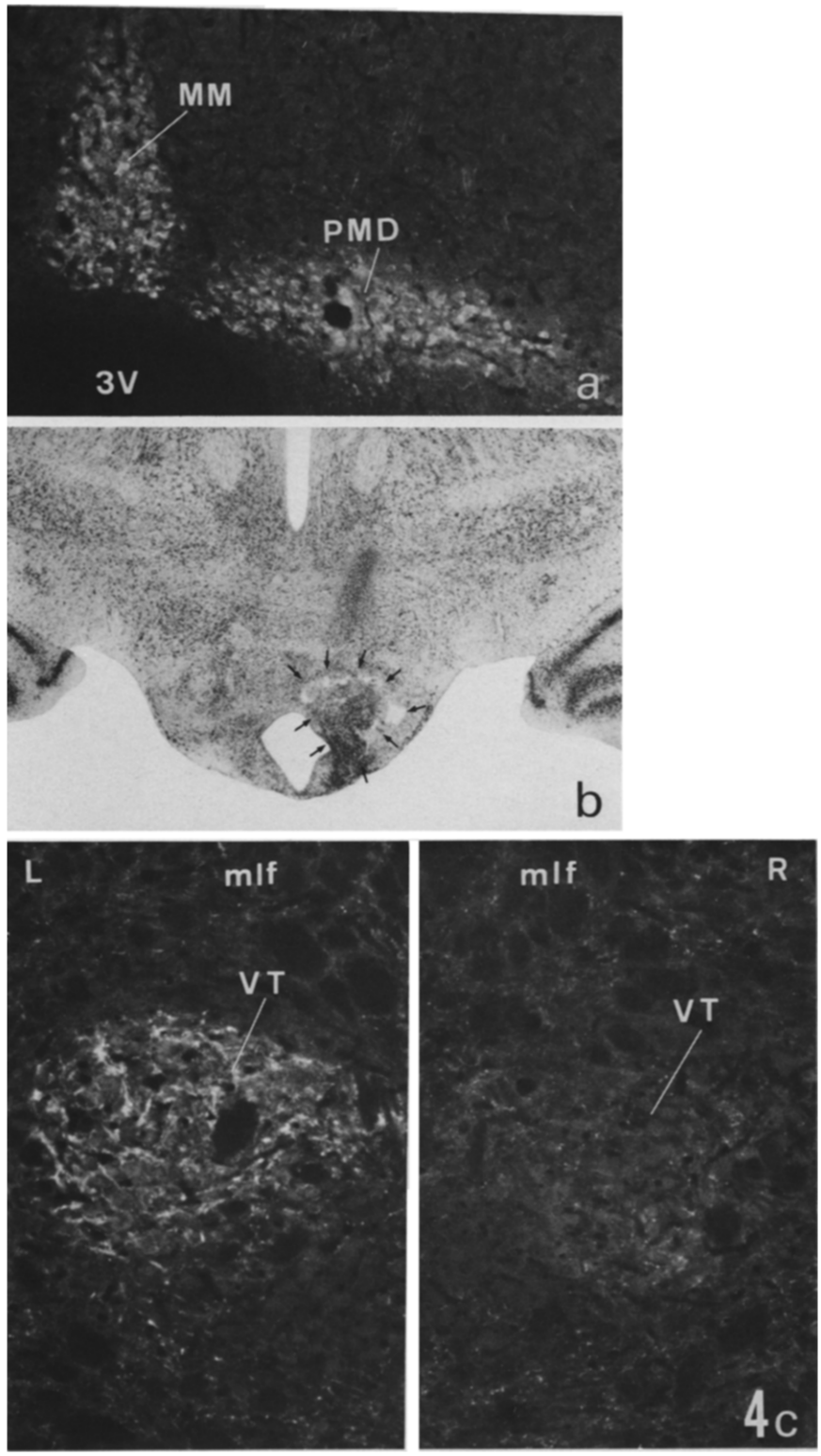

Fig. 4. a: fluorescence micrograph showing large groups of L-ENKLI neurons in the MM and PMD (medial mammillary complex, MMC). Frontal section. 3V, third ventricle. ×158. b: bright-field photomicrograph showing the electrical lesion in the MMC (arrows). Frontal section stained with Cresyl violet. ×16. c: destruction of the MMC caused a marked decrease of L-ENKLI fibers in the ipsilateral VT. Frontal sections. Fluorescence photomicrographs. Right side (R) is the operated side; the left, the control side. ×158.

cent intensity than on the normal side. When the lesions were made in other parts of the mammillary area and the MMC remained intact, L-ENKLI fibers decreased little in the VT.

L-ENKLI projection from DDT to the IP

Hemitransection of the brain rostral to the VT and DT resulted in a contralateral disappearance of L-ENKLI fibers in the dorsolateral part of the IP (DL), while total transection of the brain just caudal to the DT (Fig. 1a), or sagittal cut lateral to the tegmental nuclei (Fig. 1e), did not decrease L-ENKLI fibers in the IP, including the DL. These findings suggest the possibility that L-ENKLI neurons in the DDT project to the contralateral DL, because DDT had many L-ENKLI neurons (Fig. 5a) and was between the two sections mentioned above. To examine this possibility, we destroyed the DT and observed subsequent changes in L-ENKLI fibers in the IP. Following destruction of the DT (Fig. 5b), a marked decrease of L-ENKLI fibers occurred in the contralateral side of the DL (Fig. 5c).

Origins of L-ENKLI fibers in the DDT

Transection of the brain rostral to the DT and caudal to the DT failed to decrease L-ENKLI fibers in the DDT. A sagittal cut lateral to the DT caused no effects on L-ENKLI fibers in the DDT. These findings indicate that L-ENKLI neurons located in the area surrounded by these 3 kinds of knife-cut are the main origin of L-ENKLI fibers in the DDT.

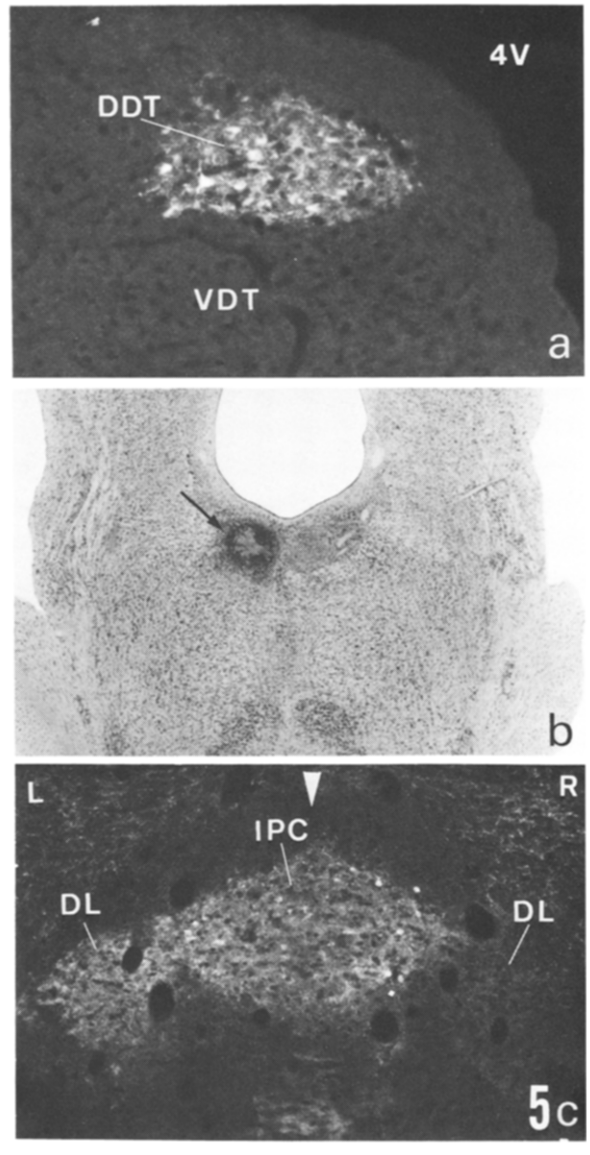

Fig. 5. a: fluorescence photomicrograph showing a large group of L-ENKLI neurons in the DDT. Frontal section. ×138. b: bright-field photomicrograph showing the electrical lesion in the DT (arrow). Frontal section stained with Cresyl violet. ×12. c: destruction of the DT caused a marked decrease of L-ENKLI fibers in the dorsolateral portion of IP (DL) on the contralateral side. Arrow indicates the midline. Left side (L) is the operated side; the right (R), the control side. Frontal section. IPC, central part of the IP. ×69.

DISCUSSION

Afferent source of L-ENKLI fibers in the VT

This study showed that the MMC (PMD and MM) is the major source of L-ENKLI fibers in the VT, and that their projection was mostly ipsilateral. The connection from the MMC to the VT has already established[5,8,10,27,36]. These results mean that L-ENK is one of the major components of this system.

The lesion study here revealed the topographic projection of this L-ENK system. Based upon its results, we concluded that the rostral part of the MMC projects mainly to the caudal VT, while caudal MMC projects mainly to the rostral VT. Since the PMD begins slightly more rostrally than MM and since the MM ends slightly more caudally than the PDM, it is likely that the PMD projects mainly to the caudal part of the VT, while the MM mainly projects to the rostral part of the VT.

The destruction of the mtg markedly reduced the L-ENKLI fibers in the VT, as seen when the MMC was destroyed. Fibers accumulating L-ENKLI struc-

tures that could be traced to the MMC of the operated side were seen in the mtg rostral to the lesion. In addition, L-ENKLI neurons found in the MMC of the operated side were much more intense than those on the normal side. This may be due to the retrograde accumulation of L-ENKLI structures in the soma of the L-ENKLI cells, caused by the transection of the mtg. These findings indicated that the axons from the L-ENKLI neurons in the MMC reached the VT via the mtg.

In the VT, a large collection of CCK-like immunoreactive (CCKLI) fibers was present[17,20]. These fibers arise from CCKLI neurons in the supramammillary region and reach the VT via the mtg[18]. Thus, the origins and their fiber trajectories are very close. However, their function in the VT seem to be different, because CCKLI structures in this system are seen only during the very early postnatal stage[17], while L-ENKLI structures could be identified even in adult rats[21,30]. L-ENK in this system may be a neurotransmitter or neuromodulator, while CCK may relate to the development of the VT.

Efferent projection from L-ENKLI neurons in the DDT

Projections from the DT to the IP have been identified[32,34]. In addition, Groenewegen and Van Dijk[9] have recently shown, by using anterograde transport technique of the lectin, that the axons arising from the DT terminate in the contralateral dorsolateral portion of the IP (DL). The study here showed that this projection arises from the DDT and one of the major components of this system is L-ENK. It is not clear where the axons from L-ENKLI cells in the DDT cross the midline. However, decussation seems to occur at the proximal portion of these axons near the soma, because hemitransections just rostral to the VT, which is slightly rostral to the L-ENKLI neuron group in the DDT, caused a contralateral reduction of L-ENKLI fibers in the DL. In support of this, the study using anterograde tracer[9] has shown the presence of labeled fibers, which could be traced to the IP, in the paramedian raphe nucleus on the contralateral side just rostral to the DT.

Origin of L-ENKLI fibers in the DDT

In the DDT, together with many L-ENKLI neurons, a dense plexus of L-ENKLI fibers was also detected. L-ENKLI neurons in the area surrounded by the 3 kinds of knife-cuts shown in Fig. 1a, b, e mainly originate there. In this area, there were groups of L-ENKLI neurons in the DDT and laterodorsal tegmental nucleus. L-ENKLI neurons are in the DDT itself and the laterodorsal tegmental nucleus is adjacent to the DDT, so it was impossible to observe effects in the DDT after the destruction of these areas. Therefore, at present no direct information is available to help us decide which portion is the major origin of L-ENKLI fibers in the DDT. However, the L-ENKLI neurons in the laterodorsal tegmental nucleus are much fewer than those of the DDT, and L-ENKLI fibers in the DDT can sometimes be traced to L-ENKLI neurons in the DDT, so it is likely that L-ENKLI fibers in the DDT are of intrinsic origin.

Previously, Huitinga et al.[14] suggested, by means of a combination of fluorescent retrograde tracing and immunohistochemical staining for L-ENK, that L-ENKLI fibers in the DT originate from L-ENKLI

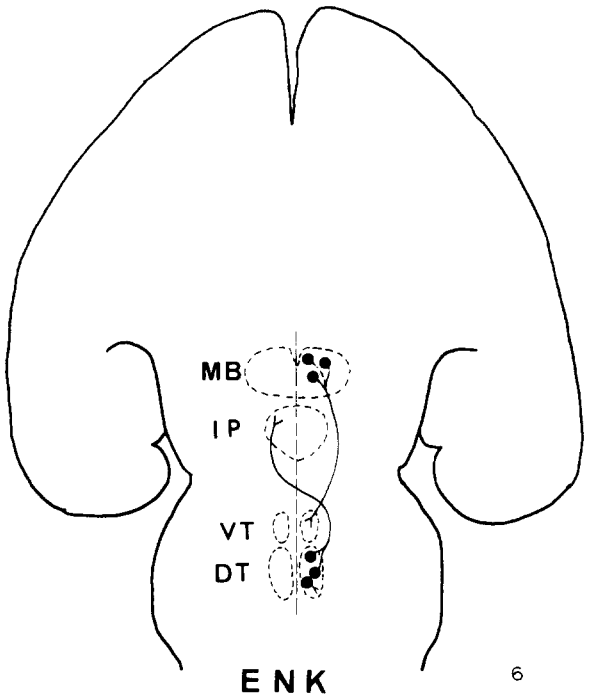

Fig. 6. Diagrammatic representation of L-ENKLI innervation in the tegmental nuclei. The majority of L-ENKLI fibers in the VT are supplied from the MB, particularly from the PMD and MM. L-ENKLI neurons in the dorsal part of the DT (DDT) project to the contralateral dorsolateral portion of the IP (DL). The intrinsic L-ENKLI neuron system also exists in the DDT Horizontal plane.

neurons in the IP. However, this possibility seems to be unlikely, because in our additional experiments, destruction of IP failed to decrease L-ENKLI fibers in the DDT. Thus, the discrepancy between our and their studies could be explained as follows: injection of the fluorescein dye in their study was made into the dorsal tegmental area. Therefore, their injection site involved various adjacent structures in addition to the DT..

In the DDT, in addition to L-ENKLI structures, a dense plexus of substance P-like immunoreactive (SPLI) fibers was present[21,26,28]. Both SPLI and L-ENKLI structures were seen in the DDT, but not in the ventral part of the DT (VDT), so the function of the DDT and VDT is different. The fiber connections of these two subnuclei are different: L-ENKLI fibers are of intrinsic origin, while SPLI fibers are supplied by SPLI neurons outside the DT.

The fiber connections of the L-ENKLI system elucidated in this study are shown schematically in Fig. 6.

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