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Dynorphin is contained within hippocampal mossy fibers: Immunochemical alterations after kainic acid administration and colchicine-induced neurotoxicity

(opioid peptide neurons/immunocytochemistry/radioimmunoassay/perforant pathway/enkephalin)

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ABSTRACT **Antisera raised against synthetic dynorphin or [Leu^5]enkephalin demonstrate immunostaining in hippocampal mossy fibers and in dentate granule cells. However, dynorphin immunoreactivity (ir) appears to be denser in immunocytochemical preparations and is quantitatively greater by radioimmunoassay than enkephalin-ir. Immunostaining with dynorphin antisera is eliminated by adsorption with 1–100 μM dynorphin-17 whereas immunostaining with enkephalin antisera is eliminated by adsorption with 1–100 μM [Leu^5]enkephalin, dynorphin-17, dynorphin-(1–13), or α-neo-endorphin. Intrahippocampal colchicine injections, which selectively destroy dentate granule cells, significantly decrease the dynorphin-ir and enkephalin-ir levels in rat hippocampus. Intraventricularly administered kainic acid, which selectively destroys CA3-4 pyramidal cells, results in an increase of enkephalin immunostaining in mossy fibers and a significant increase in enkephalin-ir by radioimmunoassay in whole hippocampus. The enkephalin-ir cells and fibers in entorhinal/perirhinal cortex, which innervate rat hippocampus and dentate gyrus, do not contain dynorphin-ir.**

Iontophoretically applied opioid peptides elicit potent excitatory responses from hippocampal CA1 pyramidal cells (1, 2). However, initial immunocytochemical (ICC) studies of endogenous opioid peptides revealed only a sparse distribution of enkephalin-immunoreactive (ir) cells and fibers in the hippocampus (3, 4). More recent examinations have revealed that the highest concentration of enkephalin-ir is located in the dentate gyrus and in the CA3-4 fields of rat hippocampus (5, 6).

Enkephalin-ir is present in two major hippocampal systems (7–9): the intrinsic dentate granule cell–mossy fiber path which innervates CA3-4 pyramidal cells (10) and the extrinsic lateral perforant/tempero-ammonic path which arises in entorhinal cortex and innervates both granule and pyramidal cells (11). However, mossy fibers exhibit weak enkephalin-ir, suggesting that crossreacting opioid peptides may be at least partially responsible for the enkephalin immunostaining in this pathway (9, 12). Likely candidates are the COOH-terminal-extended [Leu^5]enkephalin peptides dynorphin and α-neo-endorphin which have been detected in the hippocampus (13–15).

We report here the results of radioimmunoassay (RIA) and ICC experiments to distinguish the dynorphin-ir and enkephalin-ir in the rat hippocampus. Intrahippocampal colchicine injections were used to produce selective degeneration of dentate granule cells and mossy fibers (16), and intracerebroventricular infusions of kainic acid were used to increase mossy fiber activity (17), resulting in destruction of CA3-4 pyramidal cells (18). These selective neurotoxic actions altered the apparent opioid peptide content of the hippocampus accordingly. Our data are compatible with the view that the two major enkephalin-ir hippocampal pathways contain different opioid peptide families.

MATERIALS AND METHODS

Thirty Sprague–Dawley (Charles River Breeding Laboratories) rats (200–350 g) were used. All surgical manipulations and perfusions were performed under chloral hydrate anesthesia (350 mg/kg, intraperitoneally). For intracerebroventricular infusions of kainic acid or phosphate-buffered saline (P_i/NaCl) infusions, cannulae were implanted in the left lateral ventricle of 16 rats. Seven days after implantation, the rats were anesthetized with chloral hydrate. Kainic acid (1 μg in 1 μl of P_i/NaCl) or 1 μl of P_i/NaCl alone was infused over 1 min through a microliter syringe connected to an inner cannula by a short length of polyethylene tubing. Colchicine (3 μg in 0.6 μl of P_i/NaCl) was injected into the dorsal and ventral hilus of the left hippocampus, and P_i/NaCl alone was injected into identical sites in the right hippocampus of eight rats as described (16). In order to visualize immunostained cell bodies by blocking axoplasmic transport (20), four additional rats were given colchicine (1 μg in 1 μl of saline) by intracerebroventricular injection 24–48 hr prior to perfusion.

Antiserum Specificity for ICC Studies. Antiserum 84C (provided by L.T.) against dynorphin-17 conjugated to thyroglobulin by the carbodiimide method was used for ICC studies. By RIA (data not shown) this antiserum has an apparent K_d of 5×10^{-10} M. Its antigenic specificity is directed toward the COOH-terminal region with no detectable crossreactivity to α-neo-endorphin, [Leu^5]- or [Met^5]enkephalin, β-endorphin, or dynorphin-(1–8), and with 2% crossreactivity with dynorphin-(1–13) (unpublished data). Antiserum A206 (provided by R. J. Miller, University of Chicago) raised against [Leu^5]enkephalin was also used for ICC studies. As described (21), this antiserum crossreacts with [Met^5]enkephalin but has not been evaluated for dynorphin crossreactivity in RIA. For ICC, the specificities of these antisera were evaluated by blocking experiments in which the antisera diluted for tissue reactions were exposed overnight to solutions of dynorphin-17, dynorphin-(1–13), α-

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Abbreviations: ir, immunoreactivity; ICC, immunocytochemical; P_i/NaCl, phosphate-buffered saline; RIA, radioimmunoassay.

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neo-endorphin, or [Leu^5]enkephalin at concentrations of 1, 10, or 100 μM. Diluted antisera stored under the same conditions without added peptide served as controls for the adsorption procedure.

Tissue Preparation. Three days after intracerebroventricular kainic acid or P_i/NaCl infusions and 7 days after colchicine injections, the rats were decapitated for RIA or perfused with 5% paraformaldehyde for ICC as described (22).

RIA Processing. Hippocampi were dissected within 2 min of decapitation, immediately frozen in liquid nitrogen, and stored in dry ice. Frozen samples were coded and shipped to the Addiction Research Foundation (Palo Alto, CA) for RIA of dynorphin [antiserum "Lucia" raised against dynorphin-(1–13); crossreactivity with [Leu^5]enkephalin, 1:400 × 10^6] or [Leu^5]-enkephalin [antiserum NZB, donated by Iris Lindberg and June Dahl; crossreactivity, with [Met^5]enkephalin, 2.6%; with dynorphin-(1–13), 1.7%]. RIAs were conducted as described (23) in a blind fashion.

ICC Processing. Perfused brains were dissected and post-fixed for 2–3 hr. Samples were then transferred to 16% sucrose and stored at 4°C overnight. Adjacent, frozen, coronal sections (50 μm) were cut on a freezing microtome, every 250 μm through the rostro-caudal extent of the hippocampus. Adjacent free-floating sections were collected in 10 mM P_i/NaCl and then incubated with anti-[Leu^5]enkephalin or anti-dynorphin-17 at dilutions of 1:1,000 to 1:2,000 at 4°C for 18 hr. The sections were rinsed with two or three changes of P_i/NaCl and then incubated in goat anti-rabbit IgG horseradish peroxidase for 2 hr or in biotinylated goat anti-rabbit IgG (Vectastain, Vector, Burlingame, CA) for 1 hr. After two P_i/NaCl rinses, the sections incubated with biotinylated IgG were placed in a solution of avidin–biotin–horseradish peroxidase complex (Vectastain) for 1 hr and then rinsed twice in P_i/NaCl. All sections were incubated in 0.05% 3′,3′-diaminobenzidine tetrahydrochloride (Electron Microscopy Sciences, Fort Washington, PA) in 0.05 M phosphate buffer with 0.003% H_2O_2. The sections were mounted on gelatin-coated slides and coverslipped in paraffin oil or counterstained, dehydrated, and coverslipped in Permount.

Statistical Analysis. RIA data were analyzed by a two-way analysis of variance followed by individual comparisons by *t* test for independent means.

RESULTS

Dynorphin immunostaining was blocked when dynorphin antiserum was preadsorbed with 1–100 μM dynorphin-17 but was not affected by adsorption with 1–100 μM dynorphin-(1–13), α-neo-endorphin, or [Leu^5]enkephalin (Table 1). Enkephalin immunostaining was abolished when enkephalin antiserum was preadsorbed with 1–100 μM [Leu^5]enkephalin, dynorphin-17, dynorphin-(1–13), or α-neo-endorphin.

Unilateral intrahippocampal colchicine injections (16), aimed at the left dorsal and ventral dentate gyrus, selectively destroyed the ipsilateral dentate granule cells without affecting other cell types of the hippocampus. Seven days after colchicine injections, ipsilateral granule cell degeneration was advanced and microglia predominated in the granule cell layer (compare Fig. 1 *A* and *B*). Kainic acid treatment destroyed the ipsilateral CA3 and CA4 pyramidal neurons (Fig. 1*C*) which are the post-synaptic target cells of the mossy fibers (10). The dentate granule cells and the CA1 pyramidal cells remained intact.

Table 1. Immunocytochemical specificity tests

Antiserum absorbed with 1–100 μM	Immunoreactivity	
	Dynorphin	[Leu^5]Enkephalin
None	++++	++
Dynorphin-17	0	0
Dynorphin-(1–13)	++++	0
[Leu^5]Enkephalin	++++	0
α-Neo-endorphin	++++	0

In untreated adult rats, enkephalin-ir was observed in the mossy fibers and in the entorhinal cortical projection to the hippocampus but dynorphin-ir was detected only in the mossy fiber system. Enkephalin-ir observed in the mossy fibers appeared less intense than dynorphin-ir (compare Fig. 1 *D* and *E*). Similarly, RIA demonstrated that dynorphin-ir was 3 times the concentration of [Leu^5]enkephalin-ir in the hippocampus (Table 2).

Within 3 days of kainic acid treatment, the intensity of enkephalin immunostaining in mossy fibers and in lateral perforant/tempero-ammonic path fibers was dramatically increased both ipsilateral as well as contralateral to the infusion sites (Fig. 1*F*). In kainic acid-treated rats, RIA revealed a significant increase in enkephalin-ir (179%) on the contralateral, nonlesion side (Table 2). Increases in enkephalin-ir on the lesion side (143% of control) and dynorphin-ir on the lesioned (122%) and contralateral side (133%) were not statistically significant (0.05 level). Intradentate colchicine injections markedly diminished the enkephalin-ir and dynorphin-ir in the hippocampus. ICC preparations indicated that the decrease in mossy fiber immunostaining with dynorphin and enkephalin antisera correlated with the degree of granule cell destruction (Fig. 1*G*). Similarly, RIA measurements demonstrated that intrahippocampal colchicine injections significantly reduced ipsilateral enkephalin-ir to 58% and dynorphin-ir to 34% of control values (Table 2).

Although mossy fibers arise from dentate granule cells, few granule cells (Fig. 2*A*) showed either enkephalin-ir or dynorphin-ir unless the animal had been treated with colchicine (intracerebroventricular injection). Injection of 50 μg of colchicine blocked axoplasmic transport but was not cytotoxic. The types and distribution of dentate cells which then demonstrated dynorphin-ir were similar to previous findings for enkephalin-ir cells (7). Dentate granule cells were seen at different locations within the granule cell layer (Fig. 2 *A–C*). Not all dynorphin-ir cells in the dentate gyrus were granule cells; scattered, isolated, multipolar cells in the dentate molecular layer also exhibited dynorphin immunostaining (Fig. 2*D*).

DISCUSSION

We have demonstrated by ICC and RIA studies in untreated and pharmacologically manipulated rats that: (*i*) enkephalin antiserum A206 in ICC preparations crossreacts with the larger peptides dynorphin and α-neo-endorphin which contain the [Leu^5]enkephalin sequence, whereas dynorphin antiserum 84C does not crossreact with [Leu^5]enkephalin or α-neo-endorphin; (*ii*) dynorphin-ir is found in greater concentration in the hippocampal formation than is enkephalin-ir; (*iii*) enkephalin-ir increases in the hippocampal formation after kainic acid treatment; (*iv*) dynorphin-ir and enkephalin-ir significantly decrease after colchicine-induced dentate granule cell destruction; (*v*) the lateral perforant/tempero-ammonic pathway which contains enkephalin-ir does not contain dynorphin-ir. These relationships are summarized in Fig. 3.

The distribution of dynorphin-ir intrinsic to the hippocampal formation is similar to that which has been described for enkephalin-ir with antiserum A206 (7). The crossreactivity of this antiserum with dynorphin and α-neo-endorphin in ICC preparations suggests that the majority, if not all, of the enkephalin-

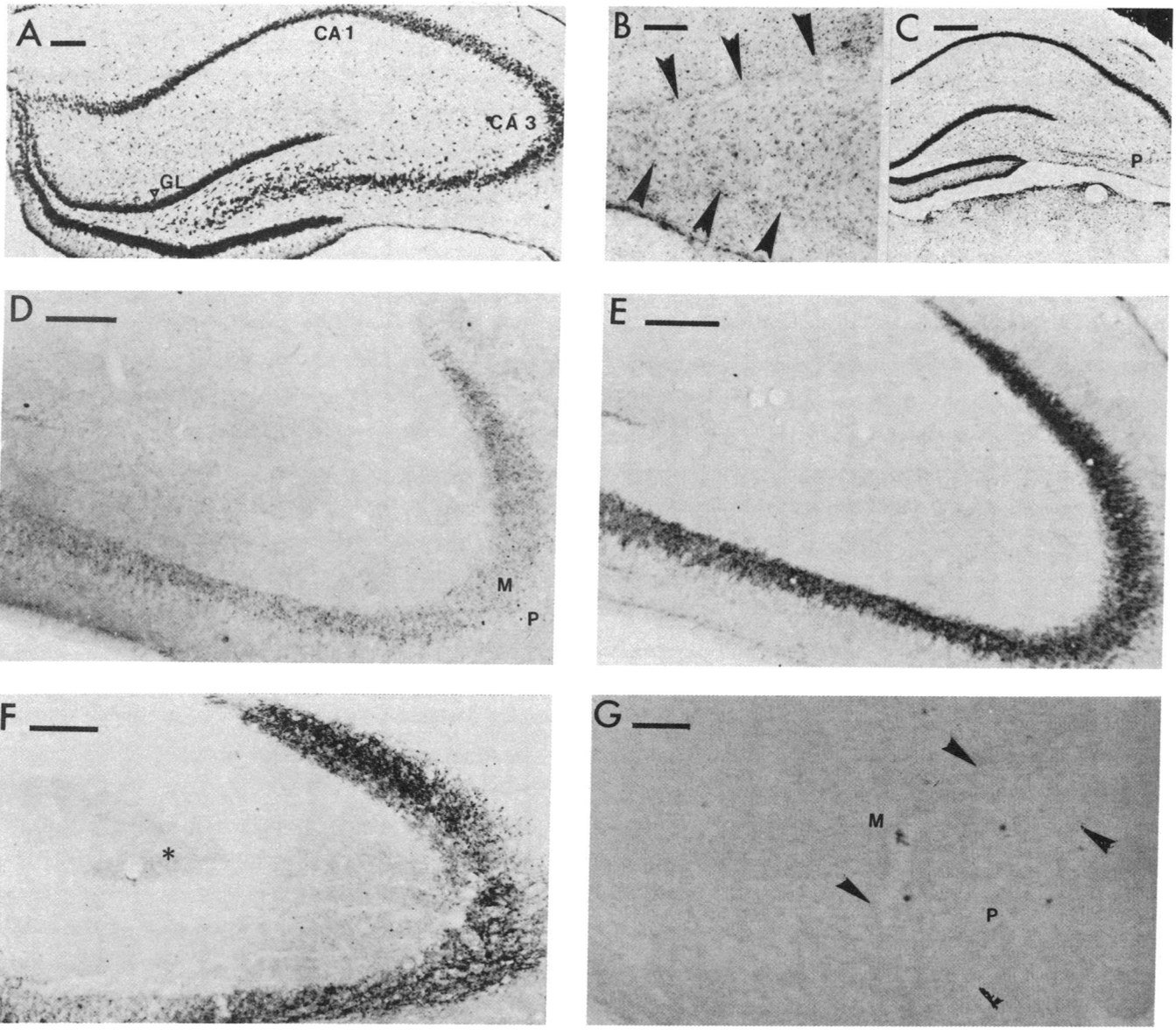

FIG. 1. Coronal sections of dorsal hippocampus. (*A*) Cresyl violet stained section of normal hippocampus illustrating the granule cell layer (GL) and the pyramidal cell layer of the CA1 and CA3 regions. (Bar = 250 μm.) (*B*) Nissl-stained dentate hilus, 7 days after an intrahippocampal injection of 3 μg of colchicine in 0.6 μl of P_i/NaCl. Granule cells are replaced by microglia in granule cell layer (arrowheads). (Bar = 50 μm.) (*C*) Nissl-stained section of hippocampus ipsilateral to site of kainic acid injection (1 μg in 1 μl of P_i/NaCl). Note presence of granule cells in dentate gyrus and pyramidal cells in CA1 but absence of pyramidal cells in CA3 (P). (Bar = 1 mm.) (*D*) Mossy fibers (M) in CA3 region stained with anti-[Leu^5]enkephalin. P, pyramidal cell layer. (Bar = 150 μm.) (*E*) Mossy fibers in CA3 region stained with anti-dynorphin-17. (Bar = 150 μm.) (*F*) Enkephalin-ir in mossy fibers of hippocampus contralateral to site of kainic acid injection. Note increase in staining intensity compared with *D*. *, Enkephalin-ir tempero-ammonic path originating in entorhinal cortex. (Bar = 150 μm.) (*G*) Absence of dynorphin-ir in mossy fiber layer (M) of CA3 region after ipsilateral intrahippocampal colchicine injection. P, pyramidal cell layer outlined by arrowheads. (Bar = 50 μm.)

ir visualized in the granule cell/mossy fiber system and in intrinsic hippocampal cells is due to the presence of the larger opioid peptides which contain the [Leu^5]enkephalin sequence. This would explain the difficulty various investigators have had in visualizing enkephalin-ir in the mossy fibers (3, 4).

However, our RIA data indicate the presence of additional enkephalin-ir in the hippocampus which is not due to cross-reactivity with dynorphin. This enkephalin-ir may represent [Leu^5]enkephalin, [Met^5]enkephalin, or several other larger enkephalin-containing peptides, or a combination of these. [Leu^5]Enkephalin may be cleaved from dynorphin or α-neo-endorphin or from an additional COOH-terminal-extended [Leu^5]enkephalin present in the dynorphin/α-neo-endorphin prohormone (24, 25). [Met^5]Enkephalin-containing peptides may be cleaved from a precursor similar or identical to the adrenal pro-enkephalin (26, 27). Antisera raised against fragments of pro-enkephalin such as BAM22 (28) or [Met^{5}Arg^{6}Phe^7]-enkephalin (P. Panula, personal communication) do not stain mossy fibers but do stain fibers in the entorhinal cortex. The distribution pattern of these [Met^5]enkephalin peptides in the hippocampal formation is opposite to what we have observed with dynorphin antiserum and is consistent with RIA data (5) which have demonstrated more [Met^5]enkephalin-ir in the caudal–ventral rather than in the dorsal hippocampal formation. Thus, the two major enkephalin-ir hippocampal pathways may contain different opioid peptides from two distinct prohormone sources.

In our neurotoxic studies, the greater decrease in ipsilateral

Table 2. Effects of colchicine or kainic acid on dynorphin and enkephalin in rat hippocampus

Side	Peptide, fmol/mg protein		
	Control	Kainic acid	Colchicine
	Dynorphin-ir		
Right	240 ± 40	320 ± 70	240 ± 40
Left	250 ± 20	300 ± 60	80 ± 10*
	Enkephalin-ir		
Right	80 ± 10	150 ± 30†	70 ± 10
Left	90 ± 10	120 ± 20	50 ± 10†

Kainic acid (1 $\mu g/\mu l$) was infused in the left lateral cerebral ventricle 3 days before decapitation. Colchicine (3 $\mu g/0.6$ μl) was infused in the left hippocampus 7 days before decapitation. Values represent mean ± SEM; $n = 4$ in each group. Right and left hemisphere control values were pooled for statistical analysis.
* For difference from control value, $P < 0.001$.
† For difference from control value, $P < 0.025$.

hippocampal dynorphin-ir (66%) than in enkephalin-ir (42%) after colchicine-induced granule cell destruction may be due to the remaining enkephalin-ir input from entorhinal cortex to the hippocampal formation. Colchicine destruction of granule cells and mossy fibers does not eliminate enkephalin-ir from lateral perforant/tempero-ammonic path fibers although enkephalin-ir content in the terminals of these fibers may be diminished as a consequence of partial axoplasmic transport blockade. Similarly, colchicine is not lethal to the enkephalin/dynorphin-ir CA1 neuronal population (unpublished data). ICC preparations demonstrating tubulin-ir indicate that the CA1 cells maintain their integrity in the presence of colchicine although axoplasmic transport in these neurons may be affected (ref. 9; unpublished data).

In kainic acid-treated rats, opioid peptide content increased

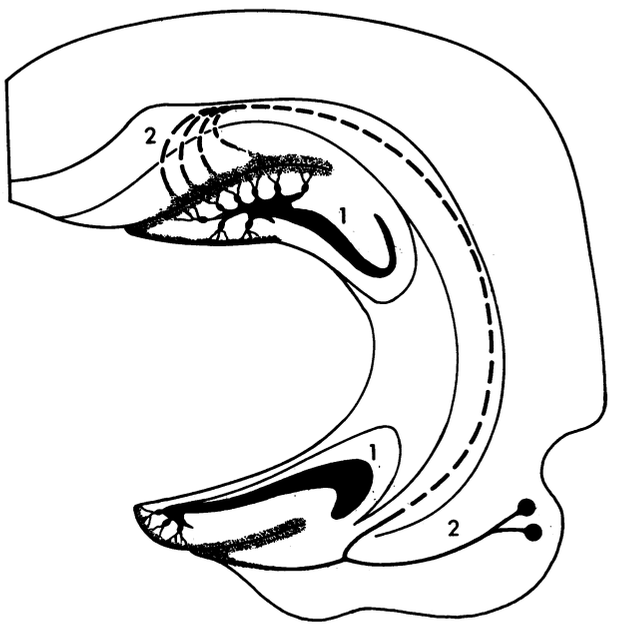

FIG. 3. Schematic illustration of the major dynorphin-ir and enkephalin-ir hippocampal pathways. 1, Dynorphin/enkephalin-ir mossy fibers originating from granule cells in both the dorsal and ventral dentate gyrus; 2, enkephalin-ir lateral perforant/tempero-ammonic path originating in the lateral entorhinal cortex and innervating the outer molecular layer of the dentate gyrus and the stratum lacunosum-moleculare of hippocampal fields CA1-3. Enkephalin-ir is densest in the caudal–ventral divisions of this entorhinal pathway. The precise route enkephalin-ir fibers take to the rostral–dorsal hippocampus from the entorhinal cortex has not been determined.

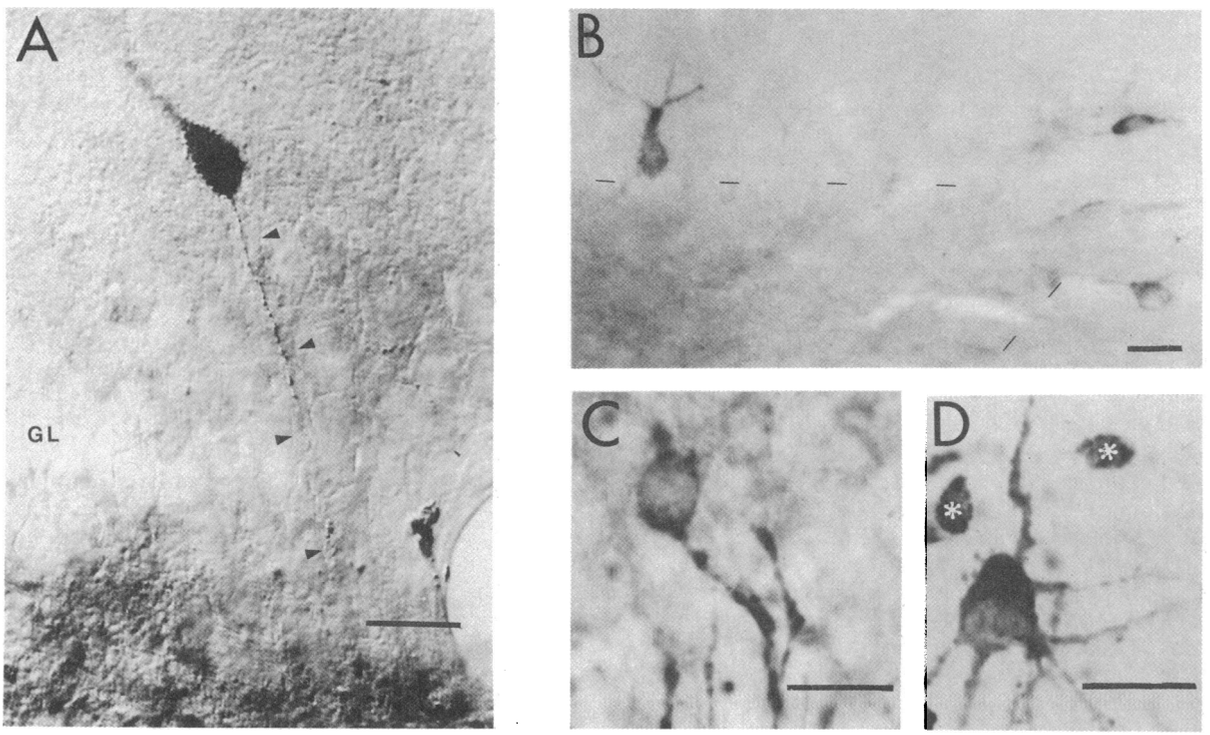

FIG. 2. Dynophin-ir cells in dentate gyrus. (Bar = 25 μm in all panels.) (*A*) "Displaced" granule cell located superficially to granule cell layer (GL) with axon (arrowheads) joining mossy fibers in the hilus. (Nomarski optics.) (*B*) Granule cells at tip of dentate hilus after colchicine treatment. Short lines outline borders of mossy fiber distribution. (*C*) Granule cells with swollen axons after colchicine treatment. (*D*) Multipolar cell in dentate molecular layer after colchicine treatment. *, Endogenous peroxidase in histiocytes near colchicine injection site.

more in the intact (contralateral) hippocampus than in the hippocampus with the lesion (Table 2). This effect may represent a transient neurotoxic effect of kainic acid on ipsilateral mossy fibers presynaptic to degenerating pyramidal cells (29). The mechanism by which kainic acid causes a bilateral increase in hippocampal opioid peptide content most likely is related to the development of granule cell epileptiform activity which spreads from the ipsilateral hippocampus to the contralateral hippocampus and limbic cortex (30). The excitatory discharge of mossy fibers presumably causes an acute depletion of opioid peptides (19) followed by a postdischarge depression of release, resulting in a sustained increase in opioid peptide content. The greater increase in hippocampal enkephalin-ir than in dynorphin-ir may reflect the spread of excitatory discharge to the entorhinal cortex or an increased cleavage of [Met^5]- and [Leu^5]enkephalin peptides from their precursors, or both, under these conditions.

Our results indicate that dynorphin (and possibly other peptides containing the [Leu^5]enkephalin sequence), rather than enkephalin itself, predominates in the hippocampal mossy fiber system. However, the dense immunostaining of mossy fibers with dynorphin antiserum 84C, which is directed to the COOH terminus, raises the possibility that 84C may be reading a COOH-terminal fragment of dynorphin-17. Dynorphin fragments 1–8 and 9–17 have been found in regional concentrations which are equimolar with α-neo-endorphin and up to 10 times greater than dynorphin-17 (31, 32).

In light of our results, investigations of the role of opioid peptides in the hippocampal formation must now be expanded. Detection and comparison of the electrophysiological actions of COOH-terminal-extended [Leu^5]enkephalin peptides at the mossy fiber–pyramidal cell synapse (33) and of [Met^5]enkephalin peptides at the lateral perforant path–dentate granule cell synapse are necessary. Furthermore, the endogenous opioid peptides and possible co-transmitters (12) released at these synapses must be identified and the receptor subtype(s) that mediates the actions of these opioid peptides in the hippocampus must be characterized.

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