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# Neuronal localization of the neutral endopeptidase 'enkephalinase' in rat brain revealed by lesions and autoradiography

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**The cellular localization of rat brain enkephalinase was studied after induction of selective unilateral lesions using *in vitro* quantitative autoradiography of the specific binding of the enzyme inhibitor [$^3$H]-*N*-[(2RS)-3-hydroxyaminocarbonyl-2-benzyl-1-oxopropyl]glycine ([$^3$H]HACBO-Gly). Twenty-one days following injection of kainic acid in the caudate-putamen (CP) [$^3$H]HACBO-Gly binding was locally decreased by 52% with a concomitant reduction of 67 and 78% in the ipsilateral substantia nigra (SN) and globus pallidus (GP), respectively. Inhibition of axonal transport in the CP by unilateral stereotaxic injection of colchicine induced a large (30-60%) and progressive decrease in enkephalinase labelling within the ipsilateral GP and SN. Taken together these results strongly suggest that in the CP a large fraction of enkephalinase is localized on intrinsic striatal neurones, and that the enzyme present both in the GP and the SN is partly localized on nerve terminals originating from neurones in the CP. No change in [$^3$H]HACBO-Gly binding was observed in the CP following injection of 6-hydroxydopamine in the nigrostriatal bundle, contrasting with the 30% depletion in opioid receptors. This would indicate that enkephalinase is present in only very low amounts, if at all, on striatal dopaminergic nerve terminals.**

*Key words:* colchicine/enkephalins/[$^3$H]HACBO-Gly/6-hydroxydopamine/kainic acid

## Introduction

In accordance with their well-admitted neurotransmitter role in the central nervous system (Hughes, 1983), the action of the opioid peptides Leu or Met-enkephalin is switched off *in vivo* by several peptidases: aminopeptidase(s) (Hambrook *et al.*, 1976; Marks *et al.*, 1977), dipeptidylaminopeptidase (Gorenstein and Snyder, 1979) and the neutral endopeptidase (EC 3.4.24.11) (Kerr and Kenny, 1974) also designated enkephalinase (Malfroy *et al.*, 1978).

The biological relevance of these enzymatic processes is supported by the large increase in the recovery of endogenous enkephalins released from rat striatal slices (Waksman *et al.*, 1985a) or spinal cord (Bourgoin *et al.*, 1986), after superfusion with peptidases inhibitors such as thiorphan, a potent inhibitor of enkephalinase (Roques *et al.*, 1980) or kelatorphan, an efficient blocker of the three enkephalin-degrading enzymes (Fournié-Zaluski *et al.*, 1984). Moreover, all these compounds induce a significant naloxone-reversible analgesia (Roques *et al.*, 1980; Fournié-Zaluski *et al.*, 1984) when intracerebrally injected in rodents.

Enkephalinase has been found in high levels in the caudate-putamen (CP), substantia nigra (SN), nucleus accumbens, olfactory tubercles and in the substantia gelatinosa of the spinal cord (Waksman *et al.*, 1986). A good correspondence between the distribution of enkephalinase and that of $\mu$ and $\delta$-opioid receptors (Waksman *et al.*, 1986) reinforces the assumption that this enzyme is involved in terminating the enkephalinergic signal.

Nevertheless, the cellular localization of enkephalinase in the brain has yet to be clearly established. Indeed, the neutral endopeptidase has been found in cultured glial cells (Lentzen and Palenker, 1983), whereas cultured neurones did not exhibit any enkephalinase activity though they possessed both a bestatin-sensitive aminopeptidase and a dipeptidylaminopeptidase activities (Horsthemke *et al.*, 1983). However, it is generally accepted that the structural and functional integrity of brain cell interactions is not completely preserved in this type of culture.

In the present study, we attemped to determine the possible neuronal and/or glial localization of enkephalinase using specific lesion techniques and quantitative autoradiography of the selective tritiated enkephalinase inhibitor [$^3$H]-*N*-[(2RS)-3-hydroxyaminocarbonyl-2-benzyl-1-oxopropyl]-glycine ([$^3$H]HACBO-Gly) (Waksman *et al.*, 1985b). Kainic acid, which is known to induce neuronal degeneration in the immediate surroundings of the injection site (Coyle and Schwarcz, 1976), was stereotaxically injected in the CP and its effects on the level of enkephalinase were evaluated in the CP and in the SN and globus pallidus (GP), two brain areas known to receive a direct input from striatal neurones (Cuello and Paxinos, 1978). Likewise, colchicine, a toxin which blocks axonal transport, was injected in the CP and the levels of [$^3$H]HACBO-Gly binding were deter-

**Table I.** Effects of unilateral injection of kainic acid in the CP on [$^3$H]HACBO-Gly binding

| Region | Day | Control side (fmol/mg protein) | Injected side (fmol/mg protein) | % Decrease |
|---|---|---|---|---|
| Caudate-putamen | 2 | 490 ± 48 | 513 ± 30 | NS |
| | 7 | 429 ± 40 | 416 ± 39 | NS |
| | 21 | 491 ± 33 | 234 ± 45[b] | 52 |
| Substantia nigra | 2 | 867 ± 57 | 772 ± 69 | NS |
| | 7 | 711 ± 29 | 503 ± 37[c] | 29 |
| | 21 | 795 ± 55 | 261 ± 106[c] | 67 |
| Globus pallidus | 2 | 521 ± 31 | 631 ± 48 | NS |
| | 7 | 478 ± 72 | 398 ± 74 | NS |
| | 21 | 457 ± 58 | 99 ± 26[c] | 78 |

The stereotaxic injection of kainic acid was performed as described in Materials and methods and the rats were killed at the indicated time. Coronal sections were incubated with 3 nM [$^3$H]HACBO-Gly. Non-specific binding was determined in the presence of 1 $\mu$M thiorphan and was subtracted from all density readings. Results are means ± SD of determinations obtained from five different rats. Statistical analysis was performed using the Student's *t*-test.
[a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$.

mined in the same two striatal output structures. Finally, in order to test whether enkephalinase is located on a neuronal nigrostriatal pathway, 6-hydroxydopamine (6-OHDA) was injected in the medial forebrain bundle and the peptidase levels were evaluated in the striatum where dopamine terminals have been shown to be associated with opioid receptors (Pollard *et al.*, 1977; Chesselet *et al.*, 1982).

## Results

For each experiment, [$^3$H]HACBO-Gly binding was measured in various areas ipsilateral to the injected side and compared to that of the corresponding contralateral regions. The values of [$^3$H]HACBO-Gly binding in the latter were not different to those of the sham-operated animals.

*Effects of lesioning the CP with kainic acid on [$^3$H]HACBO-Gly binding*

Kainic acid lesions are known to cause neuronal degeneration followed by glial proliferation. In the present study, injection of kainic acid into the left CP induced a reduction of [$^3$H]HACBO-Gly binding sites, not only at the site of injection but also in the ipsilateral GP and SN.

In the CP, there was a lag period followed by a large decrease in the enkephalinase level. Thus no significant change in [$^3$H]HACBO-Gly binding was observed 2 and 7 days after the injection whilst it had decreased markedly (52%), at 21 days post-injection (Table I). The depletion of enkephalinase content in the CP was restricted to the side of injection (left CP) as shown by the lack of significant alteration in [$^3$H]HACBO-Gly binding sites in the contralateral structure (Figure 1A).

In the ipsilateral GP and SN, a reduction of binding sites also occurred but the time course was different. In the SN no change of enkephalinase labelling was observed 2 days after kainate injection, whereas 7 and 21 days post-lesion the labelling had decreased by 29 and 67%, respectively, as compared to that in the contralateral SN (Table I; Figure 1B). On the other hand, in the GP the peptidase level was significantly decreased (78%) 21 days after kainic acid injection, no significant changes in [$^3$H]HACBO-Gly binding sites were observed at earlier lesion times. The labelling in the contralateral GP was unchanged.

*Effects of colchicine injection in the CP on [$^3$H]HACBO-Gly binding*

Colchicine, which is known to block axonal transport, was stereotaxically injected in the CP. Seven and 14 days after the injection, a marked depletion of [$^3$H]HACBO-Gly-associated grains

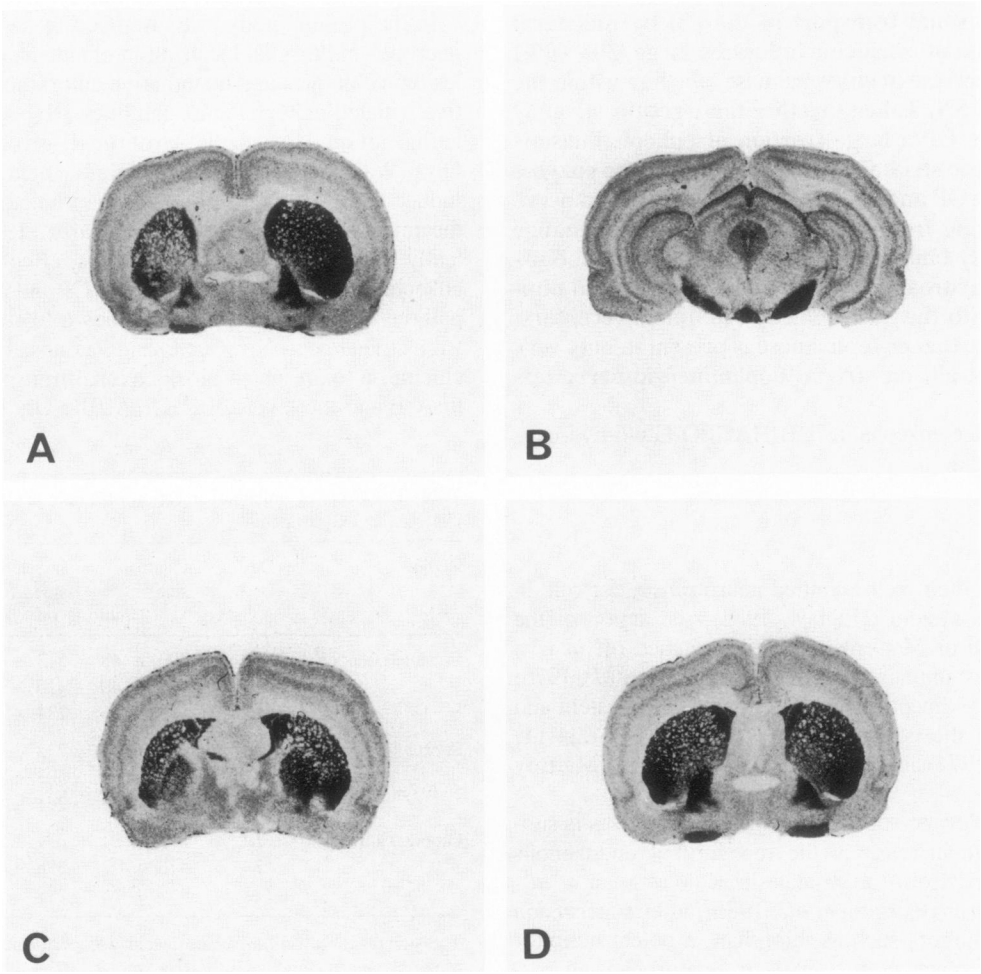


**Fig. 1.** Autoradiographic visualization of enkephalinase in rat brain with 3 nM [$^3$H]HACBO-Gly after different lesions. Autoradiograms **A** and **B** show the effect of lesioning the CP by local injection of kainic acid (**A**: CP level; **B**: SN level). Autoradiogram **C** corresponds to [$^3$H]HACBO-Gly binding after injection of colchicine in the CP (**C**: GP level). Autoradiogram **D** shows enkephalinase labelling in the CP after lesioning the medial forebrain bundle with 6-OHDA (**D**: CP level). Optimal photographic representation of individual autoradiograms is shown in this figure. For a real comparison of the [$^3$H]HACBO-Gly binding see Tables I, II and III.

was apparent in the CP at the site of injection (Table II). Likewise, grain density in the ipsilateral SN was progressively decreased by 24 and 59%, 7 and 14 days after the injection, respectively. In both cases, there was no significant change in the contralateral regions. Furthermore, the labelling of enkephalinase in the GP underwent a progressive decrease corresponding to 32 and 60% of the control content at days 7 and 14 post-injection (Figure 1C). The [$^3$H]HACBO-Gly binding in the unlesioned contralateral GP remained unchanged.

*Lesion of the dopaminergic nigrostriatal pathway: effect on [$^3$H]HACBO-Gly binding*

The effect of lesioning of the nigrostriatal dopaminergic pathway on enkephalinase levels was investigated by unilateral injection of 6-OHDA into the medial forebrain bundle (Table III). No significant change of [$^3$H]HACBO-Gly binding was oberved in the ipsilateral CP (Figure 1D). In contrast, after 14 days this lesion caused, as previously described (Malfroy *et al.*, 1979), a 27% reduction of opioid binding sites (data not shown) in the ipsilateral striatum. The efficiency of the 6-OHDA lesion, however, was demonstrated by the drastic decrease (98%) in dopamine levels in the ipsilateral striatum (7.7 ± 0.2 pg/striatum) as compared to the control side (417 ± 23 pg/striatum).

## Discussion

The good correspondence between the regional distributions of enkephalinase and opioid receptors in rat brain (Waksman *et al.*, 1986) is consistent with a physiological role for this peptidase in the metabolism of endogenous enkephalins and raises the question of its cellular localization. In this study, rat brain lesions widely used to determine the neuronal or glial localization of various transmitters, enzymes and receptors, were coupled to quantitative autoradiographic determinations of enkephalinase levels using the potent and selective inhibitor [$^3$H]HACBO-Gly. This method allowed the simultaneous visualization in different regions of lesion-induced changes in enzyme content. Moreover, the direct evaluation of the enzyme concentration has several advantages over kinetic determinations. With the latter method, ambiguous results could arise from possible modification in turn-over rate of the peptidase or from increased participation of other enkephalin-degrading enzymes in lesioned structures.

The marked (52%) decrease in [$^3$H]HACBO-Gly binding in the lesioned CP, which occurred 21 days after local injection of kainic acid, seems to indicate that a large part of enkephalinase is associated with neuronal structures. Nevertheless, the extent and time course of the reduction do not exclude the presence of additional extraneuronal enkephalinase. Such a dual localization of brain enzyme has already been reported for monoamine oxidases, which were found on both neurones and glial cells (Westlund *et al.*, 1985). Likewise, angiotensin converting enzyme (ACE) has been shown to be preferentially (Strittmatter *et al.*, 1984) but not totally localized on neurones (Koshiya *et al.*, 1984). In the case of enkephalinase, the lack of significant change in its CP concentration, 1 week after kainate injection, could be explained in terms of glial proliferation as a considerable level of enkephalinase activity has previously been detected in cultured glial cells (Horsthemke *et al.*, 1983; Lentzen and Palenker, 1983).

Nevertheless, enkephalinase seems to be more concentrated in neurones than in glia as shown by the selective and concomitant large reduction in silver grains within the GP and the SN after kainate or colchicine injection in the ipsilateral CP. This change in [$^3$H]HACBO-Gly binding was very similar to that found for various well-known presynaptic markers of striatal efferent neurones such as GABA (Nagy *et al.*, 1978; Jessel *et al.*, 1978) substance P (Brownstein *et al.*, 1977) and enkephalins (Cuello, 1983), suggesting that a large part of pallidal and nigral enkephalinase is present on terminals of axons originating from the ipsilateral CP.

There is some evidence to suggest that the GP receives not only enkephalinergic inputs (Cuello and Paxinos, 1978) but also fibres containing prodynorphin-derived peptides, both originating from the striatum (Zamir *et al.*, 1984). On the other hand the striatal efferents which project to the SN have been shown to contain prodynorphin-derived peptides (Palkovits *et al.*, 1984) and tachykinins (Vincent *et al.*, 1982). The results shown here suggest the presence of enkephalinase on long striatal efferents and therefore the enzyme may play an important role in the regulation of enkephalinergic and probably other peptidergic transmissions.

It is interesting to note that from lesion studies other brain peptidases have been localized on neuronal pathways: ACE has been shown to be associated with the striato-nigral pathway (Strittmatter *et al.*, 1984). Since enkephalinase (Matsas *et al.*, 1983) and ACE (Cascieri *et al.*, 1984) can cleave substance P *in vitro*, the possibility that they are associated with the well-defined substance P fibres (Brownstein *et al.*, 1977) connecting the CP and the SN cannot be excluded. Precise immunohistochemical measurements of lesionings effects on Met-enkephalin, dynorphin and substance P levels could allow a definite conclusion to be made.

Exogenous and endogenous opioids have been shown to stimulate the release of striatal dopamine through interaction with δ opioid receptors (Chesselet *et al.*, 1982; Petit *et al.*, 1986).

**Table II.** Effects of unilateral injection of colchicine in the CP on [$^3$H]HACBO-Gly binding

| Region | Day | Control side (fmol/mg protein) | Injected side (fmol/mg protein) | % Decrease |
|---|---|---|---|---|
| Caudate-putamen | 7 | 451 ± 103 | 242 ± 30[a] | 46 |
| | 14 | 357 ± 58 | 253 ± 55[a] | 29 |
| Substantia nigra | 7 | 748 ± 33 | 569 ± 73[b] | 24 |
| | 14 | 805 ± 35 | 334 ± 38[c] | 59 |
| Globus pallidus | 7 | 353 ± 20 | 239 ± 10[a] | 32 |
| | 14 | 414 ± 56 | 166 ± 47[c] | 60 |

Coronal sections were incubated as described in Materials and methods. The values are the mean ± SD of determinations obtained from five different animals and statistical analysis was performed using the Student's *t*-test.
[a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$.

**Table III.** Effects of unilateral injection of OHDA in the medial forebrain bundle on [$^3$H]HACBO-Gly binding

| Region | Day | Control side (fmol/mg protein) | Injected side (fmol/mg protein) | % Decrease |
|---|---|---|---|---|
| Caudate-putamen | 14 | 551 ± 68 | 529 ± 71 | NS |
| Substantia nigra | 14 | 886 ± 152 | 789 ± 153 | NS |

The stereotaxic injection of 6-OHDA in the medial forebrain bundle was performed at the co-ordinates indicated in Materials and methods. The mean values and statistical analysis were obtained as described above (see Table I).
[a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$.

This suggested the presence of an enkephalinergic synapse on dopaminergic nerve terminals (Pollard *et al.*, 1977); an hypothesis supported by a striatal decrease in both opioid receptors (Pollard *et al.*, 1977) and enkephalinase activity (Malfroy *et al.*, 1979) after lesion of the nigrostriatal bundle by 6-OHDA. Although significant reductions in striatal dopamine levels (98%) and in opioid receptors (27%) were found after lesion of the nigrostriatal pathway (G.Waksman *et al.*, in preparation) no change in enkephalinase content could be detected in this study (Table III). This implies that the enzyme present in the CP is mainly located on intrinsic neurones, a result compatible with the changes induced by kainic acid lesion. In conclusion, the present results, reinforced by lesion-induced parallel changes in opioid receptors (Abou-Khalil *et al.*, 1984; G.Waksman *et al.*, in preparation) confirm the occurrence of opioid peptide-associated pathways connecting the CP with GP and SN.

## Materials and methods

*Chemicals*

[$^3$H]-*N*-[(2RS)-3-hydroxyaminocarbonyl-2-benzyl-1-oxopropyl]glycine ([$^3$H]-HACBO-Gly, 1.1 TBq/mmol) was synthesized in our laboratory and tritiated by reduction, with [$^3$H]$_2$, of the benzilidene precursor (Waksman *et al.*, 1985a) at the Commissariat à l'Energie Atomique (CEA Saclay, France). Thiorphan was from our laboratory (Roques *et al.*, 1980). Kainic acid, colchicine, 6-hydroxydopamine and L-ascorbic acid were purchased from Sigma.

*Lesion procedures*

Male adult Sprague–Dawley rats, weighing 250–320 g were anaesthetized with ketamine hydrochloride (Ketalar 100® ) (180 mg/kg ketamine base i.p.) and fixed in a stereotaxic apparatus (David Kopf Instruments). Each animal received one stereotaxic unilateral injection of either kainic acid, colchicine or 6-OHDA. For each experiment four or five rats were used. The location of the needle tip was confirmed by dissection after the injection of a dye in age-matched rats.

Kainic acid (1.5 μg in 1 μl), or colchicine (4 μg in 1 μl) dissolved in 50 mM sodium phosphate buffer (pH 7.2) were injected through a 26-gauge needle into the left CP at interaural co-ordinates: A 9.5, L −3.0, V 3.8 according to the atlas of Paxinos and Watson (Paxinos and Watson, 1982). 6-OHDA (8 μg in 4 μl saline containing 2 μg/μl ascorbic acid) was injected into the left medial forebrain bundle at interaural co-ordinates: A 4.7, L −1.4, V 1.6. The injection rate was 1 μl/min and after the injection, the needle was left in position for 2 min before being slowly withdrawn. Sham-operated animals received 1 μl of 50 mM sodium phosphate buffer or saline (controls) in a similar way. The rats were decapitated at the indicated time after the injection and the brains were quickly removed and frozen in isopentane at −45°C, then stored at −80°C. The efficiency of the lesion was verified by measuring the levels of dopamine in the ipsilateral and contralateral striata by reversed-phase h.p.l.c. with electrochemical detection as described (Semerdjian-Rouquier *et al.*, 1981).

*Binding procedures*

Coronal sections (20 μm thick) were cut on a cryostat at −17°C, thaw-mounted onto gelatin coated slides, and stored at −80°C until used. All sections were warmed to room temperature just prior to incubation for binding as described below.

The neutral endopeptidase was labelled with 3 nM [$^3$H]HACBO-Gly for 60 min at room temperature in 50 mM Tris–HCl (pH 7.4). At the end of the incubation, the sections were washed twice in ice-cold buffer for 5 min, rapidly rinsed in ice-cold distilled water, and immediately dried. Non-specific binding was determined in sections incubated as above but in the presence of 1 μM thiorphan and accounted for $< 15\%$ of the total binding.

*Autoradiography and quantitation of binding sites by densitometry*

After being carefully dried, all labelled sections were mounted with eight calibrated tritium standards and closely apposed to sheets of tritium-sensitive Ultrofilm (LKB, Fisher) inside X-ray cassettes. The films were exposed for 16 weeks at 4°C and then developed as previously described (Waksman *et al.*, 1986). All films were analysed by spot densitometry using a Quantimet 720 (Cambridge Instruments Ltd, Cambridge, UK). The optical densities observed in the different regions were compared to those of the calibrated tritium standards and to film background. Using standard curves, the total amount of [$^3$H]HACBO-Gly bound to each region was calculated from the specific activity of the ligand. The specific binding in each area was calculated by subtracting from the total binding the amount of ligand bound to the corresponding region in sections incubated for non-specific labeling (see above). For each experiment the values of [$^3$H]HACBO-Gly bound are the means of 12 readings/region from five rats. The values were compared to controls using the Student's *t*-test.

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