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Use of mRNA Hybridization and Radioimmunoassay to Study Mechanisms of Drug-Induced Accumulation of Enkephalins in Rat Brain Structures

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SUMMARY

The repeated administration of haloperidol or fenfluramine for several days led to an increase of enkephalin content in specific brain areas. In order to characterize the nature of the dynamic changes underlying this increase, we measured the content of proenkephalin mRNA (PE-mRNA), of high molecular weight (HMW) enkephalin precursors, and of low molecular weight enkephalin peptides (LMW) in various brain areas. To measure PE-mRNA, we hybridized the specific mRNA with a [^{32}P]cDNA probe for human pheochromocytoma PE. HMW and LMW enkephalin content was measured by radioimmunoassay after separation of the immunoreactive peaks by Bio-Gel P-2 column chromatography and enzymatic digestion of the precursors. Haloperidol treatment increased enkephalins, the precursor, and PE-mRNA content in the striatum, suggesting that this drug might increase enkephalin steady state by increasing transcription, translation, or both processes. In contrast, fenfluramine increased hypothalamic and striatal enkephalin content by preferentially reducing neuropeptide utilization or decreasing its catabolism without changing its synthesis.

INTRODUCTION

Various neuropeptides that are synthesized and stored in neurons modulate or mediate neuronal communication at brain synapses. The participation of a neuropeptide in the action of specific centrally acting drugs and/or in the expression of specific behavioral patterns could be monitored by studying how the precursor, specific mRNA, and peptide content change. At least theoretically, it is possible to estimate the dynamic state of brain neuropeptides using isotope methods (1). However, these efforts so far have not yielded the needed methodology, probably because neuropeptides are not a direct product of mRNA translation but are synthesized as part of HMW[1] polyproteins containing one or more copies of a given neuropeptide together with other neuropeptides (2–4). These polyproteins are processed into the final product by multiple post-translational enzymatic modifications, and the extent to which processing occurs is tissue-specific (5). Since the molecular mechanisms operative in this processing are still poorly understood and the sequence of events in proenkephalin processing is unclear, one cannot measure neuropeptide turnover by classical isotopic methods involving precursor-product relationships at steady state. In the absence of a more precise method, we propose that modifications in the dynamic state of neuropeptides can be estimated by monitoring *in vivo*, simultaneously, the products of DNA transcription, mRNA translation, and precursor processing. We have measured the specific PE-mRNA content of various brain areas by mRNA hybridization with a radioactive cDNA probe and the content of HMW and LMW enkephalins by RIA. We show that haloperidol, a dopamine receptor antagonist, increases the content of the specific mRNA and the precursor as well as the enkephalin content in striatum whereas fenfluramine, a drug which decreases serotonin content, increases enkephalin content of both striatum and hypothalamus without changing either the mRNA or the precursor content. These results suggest that, despite the apparent similarity in the increase in the enkephalin content, the synthesis of enkephalin is specifically increased by haloperidol but not by fenfluramine.

MATERIALS AND METHODS

Treatment of animals. Sprague-Dawley male rats (Zivic Miller, Allison Park, PA) weighing 200–250 g were maintained at constant temperature (23–25°), five per cage, in a room illuminated with alternating light (14 hr) and dark (10 hr) cycles. Haloperidol (McNeil Pharmaceutical, Spring House, PA) was dissolved in a few drops of glacial acetic acid; the solution was adjusted to pH 6 with sodium

[1] The abbreviations used are: HMW, high molecular weight; LMW, low molecular weight; PE, proenkephalin; RIA, radioimmunoassay; ME, Met^5-enkephalin; MEAP, Met^5-enkephalin-Arg^6-Phe^7; MEAGL, Met^5-enkephalin-Arg^6-Gly^7-Leu^8.

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acetate (pH 6) and diluted with water. Fenfluramine (A. H. Robins, Richmond, VA) was dissolved in saline. Both drugs were given intraperitoneally. Control animals received the vehicle of the respective drugs also intraperitoneally. Various brain regions were dissected according to the method of Glowinski and Iversen (6). Tissues for mRNA hybridization were kept frozen in liquid nitrogen until assayed at a later time.

Proenkephalin mRNA hybridization analysis. For mRNA hybridization, tissues were homogenized and processed as previously described (7). The hybridization with the cDNA probe prepared from the plasmid pHPE-9 (8), a gift from Drs. Michael Comb and Edward Herbert (University of Oregon, Eugene, OR), the purification of tissue poly(A)-RNA, and the RNA blot analysis were carried out as described previously (7). In order to determine the amount of PE-mRNA relative to total RNA present in a given tissue sample, two separate densitometric scans were carried out. Examples of such scans are presented in Ref. 7, Fig. 2. To provide a relative quantitation of the total amount of RNA applied to each lane of the gel, a photograhic negative of the gel (Fig. 1A) was scanned across the 28 S band, the area between the 28 S and 18 S bands, the 18 S band, and the area below it. The sum of these areas provided a relative amount of RNA which was comparable to that obtained by optical density readings at 260 nm (see legend to Fig. 1A); occasionally, however, a discrepancy is observed between these two measurements and the scan then represents a better estimate of the amount of RNA actually in a given gel lane. The PE-mRNA bands, indicated by the arrow on the autoradiogram (Fig. 1B), were quantitated in the same way. Poly(A)-RNA from liver was included as a negative control and poly(A)-RNA from bovine adrenal medulla was a positive control (7). All data on mRNA content are presented in units, defined by the area under the peak for the PE-specific band from the autoradiogram, divided by the area under the peak for total RNA applied to the gel. Control striatum has been arbitrarily set equal to 1 and all other values were calculated relative to it. Within certain limits, a linear relationship exists between the autoradiogram signal and the area scanned (7) as well as between the amount of ethidium bromide-stained RNA and the area scanned: all measurements have been made within the linear parts of these relationships.

Radioimmunoassays. RIA of ME, MEAP, and MEAGL were carried out with antibodies and procedures described (9–11). Recovery of enkephalin peptides ranged from 90–95%. Proteins were assayed according to Lowry *et al.* (12).

Gel filtration. Tissues were homogenized in approximately 10 volumes of ice-cold 1 M acetic acid containing 20 mM HCl and 0.1% β-mercaptoethanol. Aliquots were removed for protein determination and the samples were then centrifuged at 20,000 × *g* for 30 min. The clear supernatant, after lyophilization, was dissolved in 0.5 ml of 1 M acetic acid and chromatographed on a Bio-Gel P-2 column (0.9 × 60 cm) equilibrated and eluted with 1 M acetic acid. The flow rate was 0.1 ml/min. The fractions (1 ml) were lyophilized and resuspended in RIA buffer (0.1 M Tris-HCl, pH 7.4, containing 0.1% bovine serum albumin, 0.05 M NaCl, 0.1% β-mercaptoethanol) and then assayed for MEAP and ME.

Fractions containing the peak of HMW (fractions 13–18) and LMW (fractions 38–50) enkephalins, respectively, were combined, lyophilized, and then resuspended in 1 ml of 0.1 M Tris-HCl, pH 7.4. The sample was divided into two aliquots of 0.5 ml which were incubated with or without TPCK-trypsin (Sigma) (10 μg/ml for 2.5 hr at 37°) followed by a second incubation with or without carboxypeptidase B (Worthington) (1 μg/ml for 30 min at 37°), and then boiled for 5 min as described (11). All the incubation mixtures were then assayed for ME. The recovery of ME from MEAP standards subjected to identical enzymatic digestion ranged between 60 and 70%.

RESULTS

An analysis of the quantity of PE-mRNA relative to total poly(A)-RNA in several brain regions of rats receiving daily doses of the dopamine receptor blocker haloperidol or vehicle is shown in Fig. 1. Fig. 1A shows the total RNA content, separated on a 1.1% agarose-formaldehyde gel, present in each sample. Fig. 1B shows an autoradiogram obtained following transfer of the mRNA to nitrocellulose paper and hybridization with the [^{32}P]cDNA probe. A single species of mRNA hybridizable to the proenkephalin probe was detected on the blot, with a mobility compatible with a size of approximately 1400 bases, in agreement with previous results (7); therefore, only this portion of the blot is presented in the figure. Following a 3-week haloperidol treatment (1 mg/kg), the amount of PE-mRNA was increased in striatum. Table 1 presents the content of PE-mRNA in various brain regions in units as calculated by densitometry scans of Fig. 1, A and B (see Materials

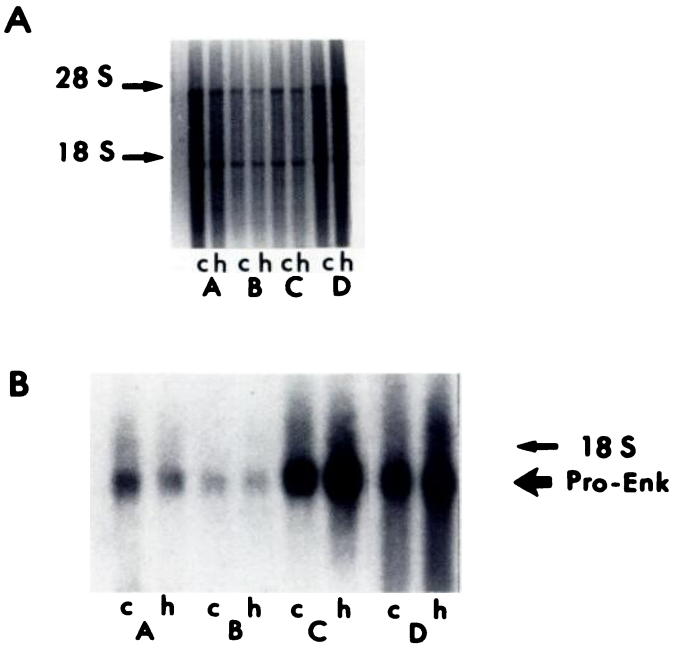

FIG. 1. *RNA blot analysis of brain regions from rats receiving haloperidol or vehicle*

Rats received 1 mg/kg haloperidol intraperitoneally daily for 3 weeks and were sacrificed 24 hr after the last injection. Brain regions were dissected according to the method of Glowinski and Iversen (6) and stored in liquid nitrogen until analyzed. A, photographic negative of the RNA gel. Poly(A)-RNA was prepared and size-fractionated on a 1.1% agarose-formaldehyde gel (7). The RNA was stained with ethidium bromide and the photograph was taken under UV light. Each letter refers to two samples from the indicated brain region: *c* is from vehicle-treated rats and *h* is from haloperidol-treated rats. Lane *A*, cortex (*c*, 45 μg of RNA; *h*, 39 μg); *B*, hypothalamus (*c*, 11 μg; *h*, 11 μg); *C*, striatum (*c*, 15 μg; *h*, 16 μg); *D*, brain stem (*c*, 47 μg; *h*, 58 μg). Size markers are 18 S and 28 S ribosomal RNA. B, autoradiogram of the nitrocellulose blot of the RNA gel shown in A. The RNA in the gel was transferred to nitrocellulose paper by blotting. The nitrocellulose was hybridized at 42° overnight with [^{32}P]PE-cDNA (300 cpm/pg) in 40% formamide, washed 3 × 20 min in 0.3 M NaCl, 30 mM Na citrate, and 0.1% sodium dodecyl sulfate at 25° and 2 × 20 min in 15 mM NaCl, 1.5 mM Na citrate, 0.1% sodium dodecyl sulfate at 42°, and exposed to X-ray film for 15 days at −70° using DuPont Cronex intensifying screens. The small amount of hybridization seen above the PE band in samples C and D represents nonspecific binding to 18 S RNA, detected because the blot was overexposed to show the PE bands in hypothalamus (B). Other details were as previously described (7).

TABLE 1

Proenkephalin mRNA content of various brain regions: effect of haloperidol

Rats were treated daily for 3 weeks with vehicle or haloperidol (1 mg/kg intraperitoneally). The samples are the same as those shown in Fig. 1. The units of PE-mRNA were obtained by scanning the negative shown in Fig. 1A to determine total RNA/sample and the specific PE-mRNA bands in Fig. 1B to determine PE-mRNA; the area for the band for PE-mRNA was then divided by the area for total RNA to produce a unit of PE-mRNA (see Materials and Methods for further details). The content in striatum of rats receiving vehicle was set equal to 1.0 and all others are calculated relative to it. The values are the mean ± standard error ($n = 3$).

Brain region	PE-mRNA	
	Control	Haloperidol
	units	
Striatum	1.0	3.0 ± 0.1
Hypothalamus	0.37 ± 0.02	0.36 ± 0.04
Brain stem	0.20 ± 0.02	0.20 ± 0.01
Cortex	0.11 ± 0.01	0.10 ± 0.01

TABLE 2

Enkephalin content in striatum of rats receiving haloperidol

Rats were treated daily with vehicle or haloperidol (1 mg/kg intraperitoneally) for 2 weeks. Values are expressed as mean ± standard error ($n = 12$). We found no change in the content of either MEAP or MEAGL in hypothalamus: others have reported no change of Met^5-enkephalin (13).

	Met^5-Enkephalin	MEAP	MEAGL
	ng/mg protein		
Control	11.0 ± 1.0	3.8 ± 0.3	4.1 ± 0.3
Haloperidol	20.9 ± 0.9[a]	7.9 ± 0.2[b]	8.3 ± 0.5[b]

[a] $p < 0.02$.
[b] $p < 0.01$.

and Methods). While the PE-mRNA content increased 3.0-fold in striatum, there was no change in the other brain regions examined when the results were corrected for total RNA. Thus, for striatum (lanes C), equal amounts of RNA were applied to the gel (15 and 16 μg, Fig. 1A) but the PE band on the autoradiogram is clearly darker for the haloperidol (*h*)-treated sample and the calculated results (Table 1) show this difference (control (*c*), 1.0 *vs.* haloperidol (*h*), 3.0 units). In contrast, for brain stem (lanes D), more RNA was applied to the gel (control, 47 μg; haloperidol, 58 μg); therefore, since the densities of the PE bands on the autoradiogram are lower than those for striatum, the content of PE-mRNA in brain stem is correspondingly less (0.20 unit) and not changed by haloperidol. In agreement with previously published data (13, 14), the striatal content of enkephalins in rats receiving haloperidol also increased (Table 2), whereas there were no changes in the enkephalin content of other brain regions.

The increases in PE-mRNA and enkephalin content caused by haloperidol had a specific time dependency. Striatal PE-mRNA and MEAGL content failed to change 1 (data not shown) or 24 hr after a single haloperidol injection (Fig. 2). The amount of striatal PE-mRNA was increased after four daily doses of haloperidol and reached peak values following 2 to 3 weeks of treatment. The content of striatal MEAGL increased with a comparable time course: a 25% increase after 4 days and a 2-fold increase by 3 weeks (Fig. 2).

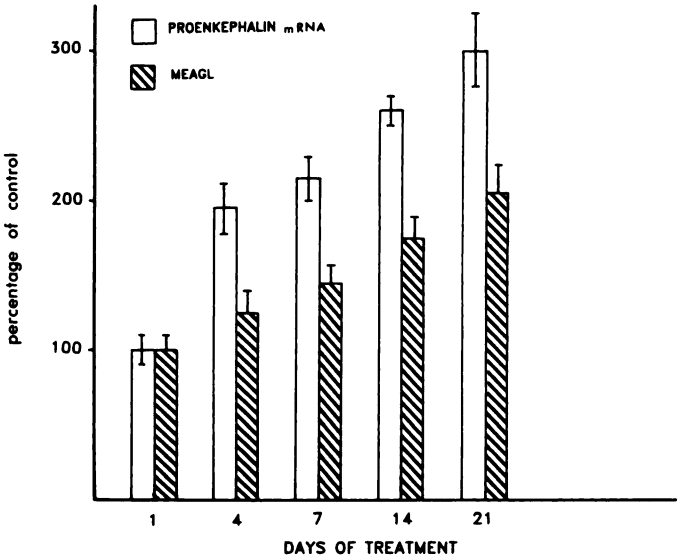

FIG. 2. *Time course of effect of haloperidol on PE-mRNA and MEAGL content of rat striatum*

Rats received 1 mg/kg intraperitoneally of haloperidol daily for the days indicated. Poly(A)-RNA was prepared from striata, and analyzed as in Fig. 1. Data for PE-mRNA are the mean of two experiments and the bars indicate the two values obtained, each calculated relative to its own control. MEAGL was measured by radioimmunoassay (9) and data are the mean ± standard error of five determinations. Values are expressed as percentage of control. The MEAGL content of haloperidol-treated samples is significantly increased on days 7 ($p < 0.005$), 14 ($p < 0.01$), and 21 ($p < 0.001$) relative to control.

TABLE 3

PE-mRNA and MEAGL content in striatum and hypothalamus of rats receiving fenfluramine

Rats were treated with 15 mg/kg fenfluramine intraperitoneally for 5 days and sacrificed 24 hr later. This treatment resulted in a greater than 50% decrease of both serotonin and 5-hydroxyindoleacetic acid. PE-mRNA values are expressed as units (mean ± standard error of three different experiments). MEAGL is expressed as nanograms per mg of protein (mean ± standard error for $n = 10$). The increase in ME and MEAP levels was comparable to that in MEAGL.

Treatment	Striatum		Hypothalamus	
	PE-mRNA	MEAGL	PE-mRNA	MEAGL
Control	1.0	4.0 ± 0.2	0.35 ± 0.06	1.8 ± 0.2
Fenfluramine	1.1 ± 0.1	8.0 ± 0.6[a]	0.39 ± 0.02	4.2 ± 0.1[b]

[a] $p < 0.05$.
[b] $p < 0.005$.

Fenfluramine, a drug which reduces brain serotonin content (15, 16), has been shown to elevate the hypothalamic and striatal content of ME (17–19). In the present experiments, 5 days of treatment with fenfluramine (15 mg/kg) (which depleted both serotonin and 5-hydroxyindoleacetic acid more than 50%) led to an increase of MEAGL, as well as MEAP and ME (data not shown), in both striatum and hypothalamus without changing the content of PE-mRNA in either brain area (Table 3). Thus, unlike haloperidol, fenfluramine increased the enkephalin content of hypothalamus and

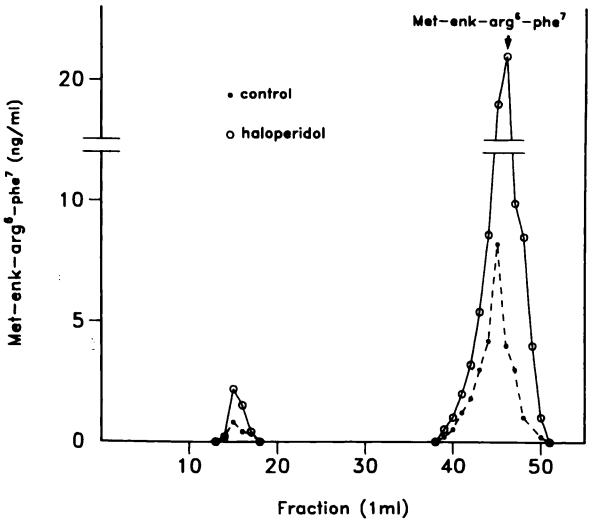

FIG. 3. *Bio-Gel P-2 column chromatography of extracts from striata of rats receiving vehicle or haloperidol*

Extracts corresponding to a pool of striata from two rats treated with haloperidol (1 mg/kg intraperitoneally for 2 weeks) (○) or with vehicle (●) were applied to a 0.9 × 60 cm column of Bio-Gel P-2, equilibrated in 1 N acetic acid. The protein content of the samples was: control, 20.2 mg; haloperidol, 20 mg. Each fraction (1 ml) was lyophilized, redissolved in RIA buffer, and assayed for MEAP. Results are expressed as nanograms of MEAP/fraction. The void volume is fractions 13–16. The LMW peak eluted in the same position as authentic MEAP, MEAGL, and ME. All fractions were assayed but only those containing immunoactivity are shown with symbols.

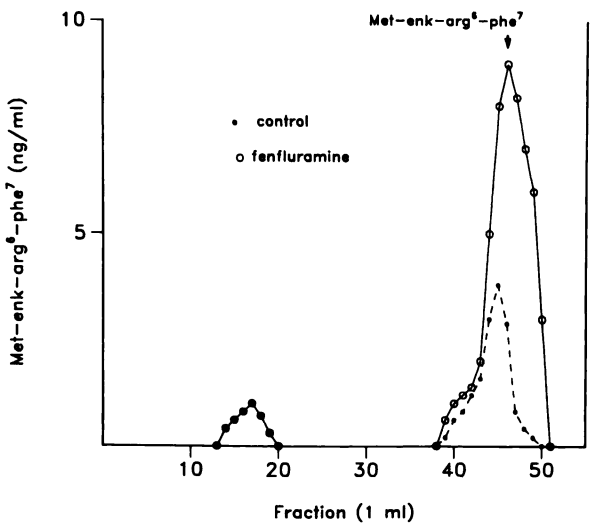

FIG. 4. *Bio-Gel P-2 column chromatography of extracts from hypothalami of rats receiving vehicle or fenfluramine*

Extracts corresponding to a pool of hypothalami from two rats receiving fenfluramine (15 mg/kg intraperitoneally for 5 days) (○) or vehicle (●) were analyzed as described in the legend to Fig. 3. The protein content of the samples was: control, 6.8 mg; fenfluramine, 6.2 mg. All fractions were assayed but only those containing immunoactivity are shown with symbols.

TABLE 4

Effect of trypsin and carboxypeptidase B treatment on Met⁵-enkephalin content of the high molecular weight immunoreactive peak

Rats were treated daily with vehicle, haloperidol (1 mg/kg intraperitoneally for 2 weeks), or fenfluramine (15 mg/kg intraperitoneally for 5 days). The HMW peaks from the Bio-Gel P-2 fractionations (Fig. 3, fractions 13–18; Fig. 4, fractions 13–20) were incubated with or without trypsin and carboxypeptidase B and then assayed for ME as described in Materials and Methods. The values have been corrected for the recovery (70%) of standard MEAP carried through the same digestion.

Treatment	Met⁵-Enkephalin	
	No digestion	Trypsin + carboxypeptidase B
	total ng	
Control	5	40
Haloperidol	10	76
Control	4	18
Fenfluramine	5	20

striatum without a concomitant change of PE-mRNA content.

These results suggest that haloperidol increases enkephalin content through an increase of the proenkephalin synthesis rate by modifying transcription whereas fenfluramine changes the enkephalin content without changing the rate of synthesis, presumably by decreasing its utilization. This difference in mechanism should be reflected in a difference in the proenkephalin content. In order to study this question, tissue extracts were chromatographed on Bio-Gel P-2 to separate a HMW fraction which will contain proenkephalin and other larger MW fragments derived from it from a LMW fraction which contains small molecular weight enkephalins such as ME, Leu⁵-enkephalin, MEAP, and MEAGL. The results presented in Fig. 3 show the relative amounts of HMW (fractions 13–18) and LMW (fractions 38–51) enkephalin-like immunoreactivity present in striatal extracts. Haloperidol treatment increased the immunoreactivity present in both peaks; but in both control and haloperidol extracts, the relative immunoactivity of the LMW fraction is 10 times greater than that of the HMW fraction.

The immunoreactivity of the HMW peaks was assayed following digestion with trypsin and carboxypeptidase B, which should increase the total enkephalin content due to the presence of seven copies of enkephalin or carboxy terminus-extended enkephalin-like peptides in the structure of proenkephalin. The actual amount of the increase is tissue-specific, related to the degree of processing which occurs in a given tissue (5). As shown in Table 4, the enzymatic digestion increased the ME immunoactivity of the HMW peaks in striatal extracts from rats receiving either saline or haloperidol, and the 2-fold increase in the HMW peak immunoactivity after haloperidol was maintained.

These findings with haloperidol contrast with the findings presented in Fig. 4 showing that whereas fenfluramine treatment increases the LMW enkephalin-immunoactivity of hypothalamus by about 3-fold, there is no increase in the HMW component in hypothalamus (Fig. 4) or striatum (results not shown). In addition, trypsin-carboxypeptidase treatment of these HMW peaks (Table 4) shows that fenfluramine treatment did not increase the content of HMW enkephalins in hypothalamus, as expected from the unchanged PE-mRNA content of tissues from rats receiving fenfluramine.

DISCUSSION

Theoretically, one could postulate a number of different mechanisms whereby the tissue content of enkephalins could increase. This might result from an increase in the synthesis rate of the enkephalin precursor, an increase in processing, or a decrease in the rate of enkephalin utilization and/or degradation. At steady state, when biosynthesis is increased, the HMW and LMW peptide content might rise as a result of an increase of transcription and/or translation. In the present report, such a response is typified by the action of haloperidol, which preferentially increased striatal PE-mRNA and the HMW and LMW content of enkephalin-like material. *In vitro* nuclear runoff transcription experiments with striatal nuclei prepared according to Eberwine and Roberts (20) from rats receiving vehicle or haloperidol show that haloperidol increases the rate of PE-mRNA transcription (preliminary results). This cannot exclude the possibility that part of the increase of PE-mRNA is also the result of mRNA stabilization.

It is possible that the content of the LMW enkephalin peptides might not increase even though synthesis is increased, if the release rate of this peptide were increased by an extent equal to the increase of the biosynthesis rate. In such an instance, one would find an increase of PE-mRNA and HMW precursor content without changes in LMW enkephalin content. Conversely, if the release rate of the peptide were decreased without a change in the rate of synthesis or processing of the HMW precursor, one might find accumulation of the LMW enkephalin without changes in the PE-mRNA or HMW precursors. An alternative possibility is that an increased rate of translation of existing PE-mRNA would produce more HMW enkephalin, which in turn would be processed more quickly into LMW enkephalin peptides; with no change in the rate of release, one would see increased LMW enkephalin with no apparent change in PE-mRNA or HMW precursor. Fenfluramine increased LMW enkephalin content in both striatum and hypothalamus without a concomitant increase in PE-mRNA or HMW enkephalin content, but until methods become available to assay the rates of processing and of release, we cannot distinguish between the alternative mechanisms.

Thus, the data presented (taken together with the preliminary results on nuclear transcription rates) are consistent with the view that haloperidol specifically enhances the transcription rate of the proenkephalin gene while fenfluramine may reduce the utilization of LMW enkephalin peptides. The procedure presented in this paper allows for a preliminary characterization of different mechanisms operative in causing changes in the steady state content of brain neuropeptides. This procedure utilizes a combination of [^{32}P]cDNA probe hybridization analysis of the PE-mRNA for proenkephalin, coupled with RIA of the content of both HMW (precursor) and LMW enkephalin-like immunoreactivity. Measurement of the actual turnover rate for neuropeptides will not be possible until the rates of release and degradation can also be determined.

Using this approach, we have differentiated two mechanisms underlying the drug-induced increase in hypothalamic and striatal enkephalin contents. The mechanism whereby haloperidol increases enkephalin content is compatible with an increase of enkephalin synthesis. Presumably haloperidol, a dopamine receptor blocker, stimulates synthesis by relieving a tonic transsynaptic inhibition mediated by dopamine. In contrast, the reduction of the serotonin tone caused by fenfluramine may decrease enkephalin utilization. Further experiments will be required to confirm the general applicability of the procedure herein described. However, this procedure has already allowed us to study interactions between classical neurotransmitter systems and neuropeptides and has thereby allowed us to begin to study the dynamics of neuropeptides as they are affected by transsynaptic influences.

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