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Increase of proenkephalin mRNA and enkephalin content of rat striatum after daily injection of haloperidol for 2 to 3 weeks

(brain distribution of mRNA/[Met^5]enkephalin/[Leu^5]enkephalin/[Met^5]enkephalin-Arg^6-Phe^7/cDNA probe)

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ABSTRACT **Proenkephalin mRNA has been detected in striatum, hypothalamus, cortex, cerebellum, hippocampus, midbrain, and brain stem of rat by RNA ("Northern") blot analysis using a 918-base-pair DNA hybridization probe complementary to proenkephalin mRNA [Comb, M., Seeburg, P. H., Adelman, J., Eiden, L. & Herbert, E. (1982) *Nature (London)* 295, 663–666]. The size of the mRNA species in all brain regions is approximately 1,400 bases, and it was found to be comparable with that of bovine adrenal medulla and human pheochromocytoma. The data were quantified by densitometric scanning of the autoradiograms: the area under the peak is proportional to the amount of standard proenkephalin mRNA from bovine adrenal medulla. The relative content of proenkephalin mRNA in the various brain regions correlates generally with the content of [Met^5]enkephalin-like immunoactivity of these regions. Rats receiving daily intraperitoneal injection of haloperidol (1 or 2 mg/kg) show a fourfold increase of proenkephalin mRNA content in striatum but not other brain regions. In agreement with previous reports, [Met^5]enkephalin-like immunoactivity increased twofold in striatum.**

Enkephalins have been proposed to function as putative neurotransmitters or neuromodulators in brain. Although their distribution and location in various brain structures is known (1, 2), there is no way to determine their rate of utilization. Hence, the changes in enkephalin content of selected brain structures that are elicited by drug treatment cannot be easily interpreted in terms of inhibition or increase of rates of enkephalin utilization. One reason for such difficulty is lack of understanding of the enzymatic processes that regulate the formation of various molecular forms of enkephalins from proenkephalin, the high molecular weight precursor of enkephalin that contains one or more replicas of at least four molecular forms of enkephalin peptides [referred to as proenkephalin A by Kakidani *et al.* (3)]. The successful cloning and sequence analysis of the mRNA for proenkephalin from bovine adrenal medulla (4, 5) and human pheochromocytoma (6) provide a method to determine whether an increase in the content of a given enkephalin in a brain structure is associated with an increase in the amount of the specific mRNA for proenkephalin. We have reasoned that, when an increase in enkephalin content is associated with an increase in proenkephalin mRNA content, one could infer that the increase in enkephalin content might reflect an increase in availability of the peptide.

We report the use of the cDNA probe for human pheochromocytoma proenkephalin (6) to determine whether repeated daily injections of haloperidol in doses that cause a selective increase of striatal enkephalin content (7) also cause a selective increase in proenkephalin mRNA content in striatum. In a previous paper, indirect evidence was presented suggesting that repeated daily injection of haloperidol increases enkephalin biosynthesis in striatum (8): we now report that haloperidol increases proenkephalin mRNA in striatum but not in other brain areas in which the enkephalin content fails to increase.

MATERIALS AND METHODS

Treatment of Animals. Sprague–Dawley male rats (Zivic Miller, Allison Park, PA) weighing 200–300 g were maintained at constant temperature (23–25°C), six per cage, in a room illuminated with alternating light and dark cycles (14 and 10 hr, respectively). Haloperidol (McNeil Pharmaceutical, Spring House, PA) was dissolved in a few drops of glacial acetic acid, and the solution was adjusted to pH 6 with 10 N NaOH and diluted with saline. Animals were injected intraperitoneally with 1 to 2 mg/kg of body weight daily for 2 to 3 wk and sacrificed 24 hr after the last injection. The animals were decapitated and the brain regions were dissected according to the method of Glowinski and Iverson (9). Tissues were immediately frozen and stored in liquid nitrogen for analysis at a later time.

Preparation of the cDNA Probe. The plasmid pHPE-9, a gift from Michael Comb and Edward Herbert (6), contains ≈1,300 bases of the proenkephalin mRNA sequence cloned into the *Pst* I site of pBR322. The plasmid was grown in *Escherichia coli* (MC 1061), harvested, and banded twice on a cesium chloride gradient. The 918-base-pair probe used was cut out by digestion with *Hin*cII (Bethesda Research Laboratories) and purified on a low-melting agarose (Bethesda Research Laboratories) gel and passage over a DEAE-cellulose column. The probe was nick-translated using [α-^{32}P]dCTP (Amersham; 2,000–3,000 Ci/mmol; 1 Ci = 37 GBq), DNase (Worthington), and DNA polymerase I (Boehringer Mannheim).

Preparation of Poly(A)-RNA. Tissues frozen in liquid nitrogen were pulverized and extracted with 8 vol of guanidinium thiocyanate (Kodak) (10). The 8,000 × *g* supernatant was layered onto 4 ml of 5.7 M cesium chloride (Bethesda Research Laboratories)/25 mM sodium citrate, pH 7, and centrifuged for 14–16 hr at 150,000 × *g*. The RNA pellet was dissolved in 0.5 M NaOAc (pH 6) and poly(A)-RNA was prepared by two passages over an oligo-dT cellulose (P-L Biochemicals) column.

RNA Blot Analysis. Poly(A)-RNA tissue samples were denatured by heating at 70°C for 3 min in 5 mM NaOAc/1 mM EDTA/20 mM morpholinopropanesulfonic acid, pH 7.0/6% formaldehyde/50% formamide (11). Electrophoretic separation was carried out on a 1.1% agarose (Bethesda Research Laboratories)/6% formaldehyde gel at 30–40 V for 12–14 hr. Transfer of the RNA to nitrocellulose paper (Schleicher & Schuell) was carried out according to Thomas (12). Nick-translated probe

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(150–300 cpm/pg) was used in the hybridization, which was carried out overnight at 42°C in 50% formamide/0.75 M NaCl/75 mM Na citrate/7 mM Tris·HCl, pH 7.5/0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone/herring sperm DNA (25 μg/ml)/10% dextran sulfate (12). The nitrocellulose blots were washed, dried, and exposed to Kodak X-Omat film for 2–7 days at −70°C using DuPont Cronex intensifying screens.

Radioimmunoassay of [Met^5]enkephalin-Like Immunoactivity and [Met^5]enkephalin-Arg^6-Phe^7-Like Immunoactivity. Brain samples were homogenized in 1 ml of ice-cold 1 M acetic acid, boiled for 15 min, and centrifuged at 12,000 × *g* for 30 min, and the supernatants were lyophilized. Some of the radioimmunoassays were carried out by J. Tang using a previously described [Met^5]enkephalin antibody (1).

RESULTS

When nitrocellulose blots containing poly(A)-RNA from various brain regions were hybridized with a ^{32}P-labeled probe, a single mRNA band was detected on the autoradiogram in each brain region (Fig. 1). This band had a mobility corresponding to a RNA of ≈1,400 bases, similar to the human pheochromocytoma (6) and bovine adrenal medulla (4, 5) proenkephalin mRNAs. The relative abundance of proenkephalin mRNA per μg of poly(A)-RNA varied from one brain region to another, with striatum the highest.

Densitometric scanning of the autoradiograms was used to provide a relative quantitation of these differences. The suitability of the method was first tested with an autoradiogram from a RNA blot of a gel in which three concentrations of poly(A)-RNA from bovine adrenal medulla, as well as rat liver poly(A)-RNA, had been run (Fig. 2*a*). Each lane of the gel on the autoradiogram was scanned (Fig. 2*b*) and the area under the peaks corresponding to the position of the proenkephalin mRNA was measured. The results showed a linear relationship between the density of the band on the autoradiogram (expressed as the area under the peak) and the amount of poly(A)-RNA applied to the gel, a relationship that was true for autoradiograms from two different exposure times of the same blot (3 days and 14 days) (Fig. 2*c*). Liver poly(A)-RNA never gave a positive signal for proenkephalin mRNA and was therefore used as a negative control on all blots, while bovine adrenal medulla poly(A)-RNA was used as the positive control.

The amounts of proenkephalin mRNA detected in the various rat brain regions, quantitated by densitometry as described, and the content of three enkephalin peptides, ([Met^5]-enkephalin, [Leu^5]enkephalin, and [Met^5]enkephalin-Arg^6-Phe^7), as determined by radioimmunoassay (1, 13) are given in Table 1. In general, the peptide content/mRNA content ratio was fairly constant, although the hypothalamus showed a relatively higher ratio while the cerebellum had a lower ratio.

The striatum of rats receiving daily injections of haloperidol, a dopamine receptor antagonist, for 3 wk had larger amounts

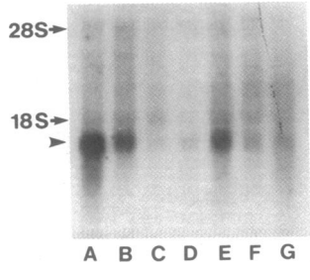

FIG. 1. Detection of proenkephalin mRNA in rat brain regions. Poly(A)-RNAs were prepared from 1 g of tissue and various amounts were applied to a gel. Lanes: A, striatum (3.80 μg); B, hypothalamus (4.75 μg); C, hippocampus (3.90 μg); D, cortex (3.73 μg); E, cerebellum (5.19 μg); F, midbrain (4.58 μg); G, brain stem (3.97 μg). Arrowheads indicate proenkephalin mRNA.

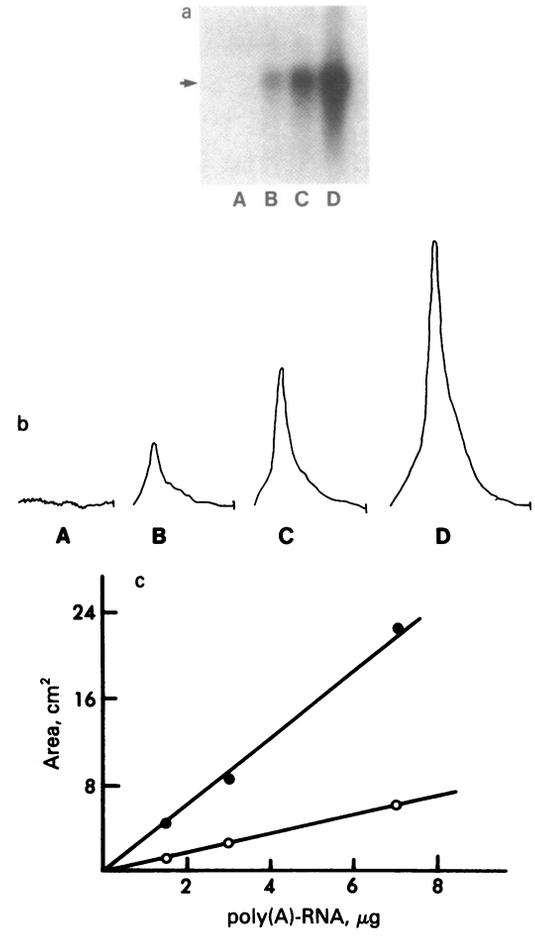

FIG. 2. Quantitation of mRNA by densitometric scanning of the autoradiogram. (*a*) Position of proenkephalin mRNA on the RNA blot. Lanes: A, liver [15 μg of poly(A)-RNA]; B, bovine adrenal medulla (1.5 μg); C, bovine adrenal medulla (3.0 μg); D, bovine adrenal medulla (7.0 μg). (*b*) Densitometric scans of the bands on the autoradiogram shown in *a*. (*c*) Correlation between amounts of bovine adrenal medulla poly(A)-RNA applied to the gel and areas under the peaks on the scan of the blot shown in *a*. Autoradiography was for 3 (○) or 14 (●) days.

Table 1. Proenkephalin mRNA and opiate peptides in various regions of rat brain

Brain region	Pro-EK mRNA	[Met]EK	[Leu]EK	MEAP	Total peptides
Striatum	4.45	16.0	2.3	2.5	20.8
Hypothalamus	1.53	8.4	1.2	1.6	11.2
Midbrain	0.50	1.9	0.23	0.42	2.6
Cortex	0.42	1.7	0.31	0.26	2.3
Hippocampus	0.44	1.2	0.11	0.15	1.5
Cerebellum	1.24	0.63	0.23	0.11	0.97

Proenkephalin mRNA (Pro-EK mRNA) is expressed as U (area under the peak measured by densitometric scanning) per μg of poly(A)-RNA applied to the gel. Values are derived from the RNA blot in Fig. 1. The experiment was repeated twice. [Met^5]Enkephalin ([Met]EK) and [Leu^5]enkephalin ([Leu]EK) results are expressed as pmol/mg of protein and are from ref. 1; [Met^5]enkephalin-Arg^6-Phe^7 (MEAP) results are from ref. 13. Total peptides represent the sum of the results for [Met]EK, [Leu]EK, and MEAP.

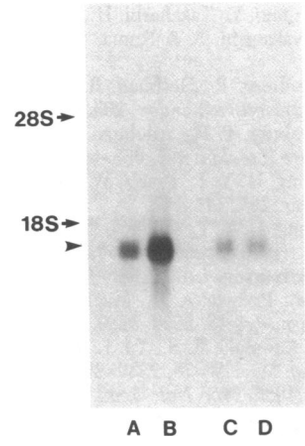

FIG. 3. Effect of chronic haloperidol treatment on proenkephalin mRNA content of rat striatum and hypothalamus. Rats were treated for 3 wk with haloperidol at 2 mg/kg, the brain regions were dissected, and poly(A)-RNA was prepared. Arrowheads indicate proenkephalin mRNA. Lanes: A, control striatum [4.3 μg of poly(A)-RNA]; B, haloperidol-treated striatum (4.3 μg); C, control hypothalamus (7.2 μg); D, haloperidol-treated hypothalamus (7.2 μg).

of proenkephalin mRNA than the striatum of rats receiving saline (Fig. 3). There was no significant change in total poly(A)-RNA content. The increase was specific to striatum; no change was detected in hypothalamus (Fig. 3) or in cortex, cerebellum, brain stem, or hippocampus (data not shown). A comparable increase of striatal proenkephalin mRNA was found after 2 wk of treatment (a 3.5-fold increase vs. a 3.6-fold increase at 3 wk). The changes in mRNA content are compared with the changes in [Met^5]enkephalin content of striatum from animals treated for 3 wk with haloperidol in Table 2. Whereas the proenkephalin mRNA content was increased 3.6-fold, the [Met^5]enkephalin content increased only 88%, in good agreement with the twofold change in [Met^5]enkephalin immunoactivity reported by Hong *et al.* (7).

DISCUSSION

We have used a cDNA probe for human pheochromocytoma proenkephalin to answer three questions: can proenkephalin mRNA be measured in various brain parts; is this content related to the [Met^5]enkephalin content; is the increase in immunoreactive [Met^5]enkephalin content elicited by haloperidol associated with an increase in proenkephalin mRNA? Analysis of RNA blots of poly(A)-RNA from seven rat brain regions (striatum, hypothalamus, cortex, cerebellum, midbrain, brain stem, and hippocampus) showed the presence of a single band of mRNA, with an apparent molecular weight corresponding to $\approx$1,400 bases, in all of the brain regions. The results thus show that transcription of the proenkephalin gene occurs in all of these brain regions. *In situ* hybridization in tissue slices will allow more specific examination of the cell types involved in the synthesis of proenkephalin. Detection of brain mRNA by using a probe specific to the adrenal medullary proenkephalin suggests that the brain proenkephalin must be similar in amino acid sequence to the adrenal medulla precursor. Since this precursor contains six copies of [Met^5]enkephalin (two extended at the carboxyl terminal) and one of [Leu^5]enkephalin, the results provide an explanation for the finding in most brain regions of a constant ratio (5 to 7) of [Met^5]enkephalin to [Leu^5]enkephalin and for a constant ratio of [Met^5]enkephalin to [Met^5]enkephalin-Arg^6-Phe^7.

Table 2. Effect of chronic haloperidol treatment on striatal proenkephalin mRNA and opiate peptide content

Treatment	Pro-EK mRNA	[Met]EK
None (control)	3.4	6.2 ± 1.0
Haloperidol	12	12.0 ± 1.8*

Proenkephalin (Pro-EK) mRNA is expressed as U (area under peak) per μg of poly(A)-RNA applied to the gel and [Met^5]enkephalin ([Met]-EK) is expressed as pmol/mg of protein (mean ± SEM). The experiment was repeated twice. The corresponding results for proenkephalin mRNA in hypothalamus were control, 0.72 U/μg of poly(A)-RNA; haloperidol-treated, 0.69 U/μg.
*$P < 0.01$ (n = 8–10).

Densitometric scanning of the autoradiograms provided a relative quantitation of the proenkephalin mRNA content of the various brain regions. The striatum has the highest amount, 3- to 10-fold more than other regions. This relative distribution is in reasonable agreement with the peptide distributions obtained by radioimmunoassay (1, 13) (Table 1). However, at least two factors could explain a differential distribution of mRNA versus peptide. If the cell bodies (containing mRNA and presumably also peptides) are localized in a different site from the terminals (containing only peptides), one would expect a high mRNA/peptide ratio at cell body sites and the converse at terminal sites. Alternatively, differences in the rate or pattern of precursor processing or peptide metabolism could result in a differential distribution. Further work will be needed to explore these possibilities.

Certain drugs, including the antipsychotics, require days to weeks of administration before they become pharmacologically effective. Haloperidol, a cataleptogenic antipsychotic, falls in this class of drugs and previous studies had suggested that one of its delayed effects was to increase the [Met^5]enkephalin and [Leu^5]enkephalin contents of striatum after daily injection of the drug for 3 wk (7), possibly through increased biosynthesis (8). We have used the proenkephalin cDNA probe to study whether haloperidol treatment results in increased transcription of the proenkephalin gene, with a specificity restricted to striatum. Chronic haloperidol treatment resulted in a fourfold increase of proenkephalin mRNA and a corresponding twofold increase in [Met^5]enkephalin content of striatum. The increase in mRNA could be due to either increased synthesis or decreased degradation; further experiments will be required to answer this question. The disparity between the magnitude of the changes in mRNA and peptide content could reflect several factors: mRNA is not translated rapidly into proenkephalin; the precursor is not processed rapidly into the peptide products; or much of the peptide is rapidly released and metabolized. Although we do not have all the necessary information to support this inference, we suggest that the increase in striatal enkephalins elicited by haloperidol is associated with an increased utilization of enkephalins. From this, one might infer that the beneficial effects of haloperidol in schizophrenia are associated with increased production of enkephalins in striatum and perhaps in nucleus accumbens, two areas in which activity of enkephalinergic neurons may be inhibited by dopamine. Haloperidol acts as a dopamine receptor blocker; thus, dopamine may exert a tonic inhibition of the firing of some or all enkephalin neurons in the striatum. Removal of this inhibition leads to increased release of enkephalin, which would necessitate increased synthesis of precursor. The availability of the cloned DNA has allowed analysis of the mechanism of drug action on putative neuromodulators at the level of gene transcription. Measurement of mRNA for proenkephalin as well as for other peptide precursors can be used as a tool to interpret neuropeptide accumulation in a selected brain region in functional terms. On the basis of this study, we suggest that, if the ac-

cumulation of a neuropeptide is not associated with an increase of the pertinent mRNA, the accumulation of the peptide may not reflect an increase in its rate of formation and release but rather that the accumulation of the peptide results from a decrease in peptide utilization.

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