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REGULATION OF METHIONINE-ENKEPHALIN PRECURSOR MESSENGER RNA IN RAT STRIATUM BY HALOPERIDOL AND LITHIUM

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SUMMARY: Daily injections of rats with haloperidol for 21 days or lithium chloride for 5 days elevated the content of Met-enkephalin in the striatum by 86% or 66%, respectively. Poly(A)+ RNA from striata of drug-treated and control rats was translated in vitro, and the amount of synthesized enkephalin precursor [^{35}S]preproenkephalin A was determined by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. Haloperidol or lithium treatment increased the relative amount of preproenkephalin A synthesized by 67-98% or 25-29%, respectively. These results suggest that haloperidol elevates the Met-enkephalin content primarily by increasing the precursor mRNA content or activity, while lithium exerts its effect only in part by this mechanism.

The opioid pentapeptides methionine^5-enkephalin (ME, Tyr-Gly-Gly-Phe-Met) and leucine^5-enkephalin (Tyr-Gly-Gly-Phe-Leu) are believed to function as neurotransmitters or modulators in the nervous system (1). The striatum of the rat brain contains relatively high concentrations of the enkephalins localized to intrinsic interneurons (2,3). Little is known about the factors that regulate enkephalin biosynthesis or metabolism in the striatum.

Hong et al. (4,5) reported that daily injections of rats for 2-3 weeks with haloperidol, a dopamine receptor blocker, increased by 90-100% the level of ME in the striatum and nucleus accumbens, two regions richly innervated by dopamine-containing neurons. Hong et al. (6) found that in cycloheximide-treated rats the rate of decline of striatal ME content is steeper in haloperidol-treated rats than in controls. Furthermore, the rate of release of ME from isolated striata of haloperidol-treated rats is greater than that of controls (7). These results support the proposal (6) that enkephalin biosynthesis in striatal neurons is under a tonic trans-synaptic inhibition by

Abbreviations: ME, Met^5-enkephalin; ME-Arg^6-Phe^7, Met^5-enkephalin-Arg^6-Phe^7; poly(A)+ RNA, polyadenylated RNA; SDS, sodium dodecyl sulfate.

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dopaminergic neurons; thus prolonged blockade of dopamine receptors by haloperidol may accelerate the biosynthesis of the enkephalins.

Gillin et al. (8) reported that daily injections of rats for 5-7 days with lithium chloride increased the levels of ME in the striatum. However, unlike haloperidol, which produces a persistent elevation of ME content, lithium produces a transitory elevation that subsides during the second week of treatment.

The investigation of the regulation of enkephalin biosynthesis in the brain requires a means of quantitating the mRNA coding for the Met- and Leu-enkephalin common precursor preproenkephalin A (9-12). Recently Sabol et al. (13,14) demonstrated the in vitro biosynthesis of [^{35}S]preproenkephalin A by translation of brain and adrenal mRNA. In the present study the effects of repeated injections of haloperidol or lithium on rat striatal functional preproenkephalin mRNA levels are examined by the cell-free translation method.

MATERIALS AND METHODS

Materials: Male Fischer-344 strain rats (240-280 g, Harlan Laboratories, Indianapolis, IN) were used in this study. They were housed four to a cage with a 12-hr light-dark cycle, 21 ± 2° C and 50 ± 10% relative humidity.

Haloperidol was a gift from McNeil Laboratories (Fort Washington, PA). Lithium chloride was reagent grade (Fisher). The RB-13 antiserum against ME-Arg^6-Phe^7 has been described previously (13). The IgG fraction was purified on a column of Protein A-Sepharose CL-4B (Pharmacia). The recovery of [^{125}I]ME-Arg^6-Phe^7 binding activity was quantitative. The RB-4 antiserum against ME has also been described previously (15); it recognizes the free carboxyl end of ME and has little or no affinity for proenkephalin.

Drug treatments: Rats received daily injections of haloperidol (2 mg base/kg/day, s.c.) for 21 days, or lithium chloride (5 meq/kg/day, i.p.) for 5 days. They were killed by decapitation 24 hr after the last injection. Control rats received 0.9% NaCl solution (1 ml/kg/day).

Determination of Met-enkephalin content: ME content was determined by radioimmunoassay as previously described (4).

Preparation of mRNA: Striata were excised immediately after death and stored over liquid N_2. RNA was extracted according to the procedure of Chirgwin et al. (16). Poly(A)+ RNA was partially purified by a single cycle of chromatography on Poly(U)-Sepharose 4B (Pharmacia) as described (15) except that LiCl was substituted for NaCl in the RNA binding buffer. The fraction eluted with 80% formamide (termed poly(A)+ RNA) was 4-6% of the applied RNA.

Cell-free translation and immunoprecipitation of preproenkephalin A: Poly(A)+ RNA was translated in the rabbit reticulocyte system supplemented with [^{35}S]L-methionine (1000 Ci/mmol, New England Nuclear) under the conditions specified previously (13). Aliquots (2 µl) were withdrawn for determination of total protein synthesis by the precipitation of [^{35}S]protein with 5% trichloroacetic acid at 90°C. [^{35}S]Preproenkephalin A was purified by immunoprecipitation as described (13) with the following modification: Each reaction mixture contained 32 µg IgG protein from RB-13 antiserum and up to 0.14 ml cell-free translation reaction mixture in a final volume of 0.3 ml.

Electrophoresis and quantitation of synthesized preproenkephalin A: Immunoprecipitated ^{35}S-labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis in 0.75 mm-thick discontinuous slab gels (17). Unless otherwise indicated, the separating gel contained 10% acrylamide-0.27% methylene-bis-acrylamide. After electrophoresis at 300 V for 5 hr, each gel

was impregnated with fluorographic enhancer (ENHANCE, New England Nuclear), dried, and autoradiographed with Kodak XAR-5 film at -120°C.

To extract proteins contained in specific bands, each dried gel was marked with radioactive ink and autoradiographed again to produce a guide film. Regions to be excised were drawn on the guide film, which was then used to guide the excision of bands of the dried gel. Gel slices were incubated in 2 ml 90% NCS (Amersham) at 65°C for 24 hr. The resultant suspensions were neutralized and counted. Radioactivity was corrected by subtraction of the radioactivity of the minus-mRNA blank of each experiment, as well as for isotopic decay of ^{35}S during the interval between the translation reaction and the final radioactivity determination.

RESULTS

Because the strain and size of rats employed in this study differ from those used previously (4,8), the effects of haloperidol and lithium on the striatal ME content were reinvestigated in animals similar to those used for analysis of mRNA. As shown in Table I, the ME content was increased 86% or 66% by treatment with haloperidol or lithium, respectively.

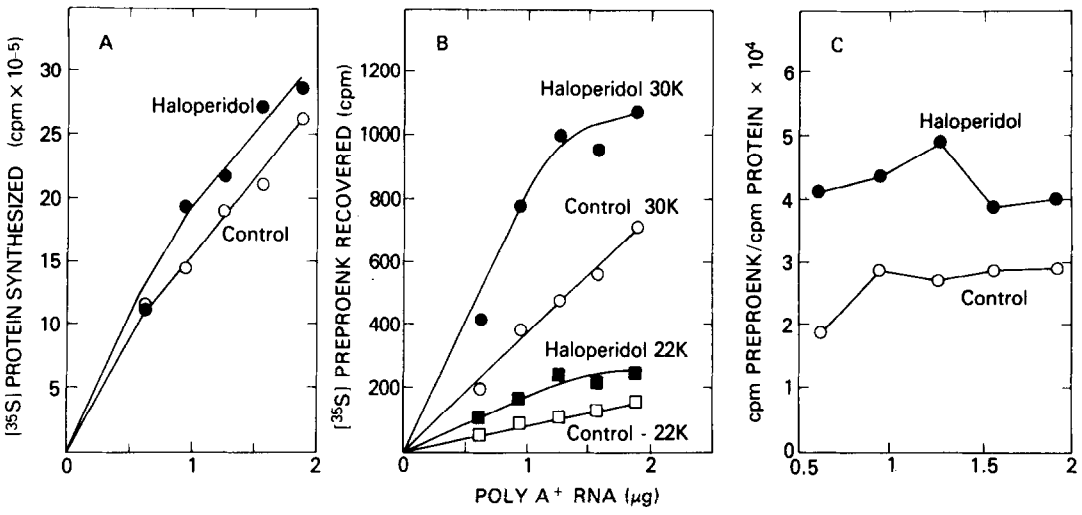
Changes in the relative abundance or activity of functional preproenkephalin A mRNA in rat striatum were measured by cell-free translation of rate-limiting quantities of striatal mRNA. Synthesized [^{35}S]preproenkephalin A was immunoprecipitated with antibodies recognizing the carboxyl end (Tyr-Gly-Gly-Phe-Met-Arg-Phe), then subjected to electrophoresis and quantitation.

The effect of haloperidol treatment on functional preproenkephalin A mRNA levels is shown in Fig. 1. Incorporation of [^{35}S]Met into protein was slightly greater with poly(A)+ RNA from haloperidol-treated animals than with equal amounts of poly(A)+ RNA from control animals (panel A). Electrophoretic analysis of the immunoprecipitates (panel D) revealed four proteins, having apparent M_r 31,000, 30,000, 22,000, and 20,000, whose immunoprecipitation was blocked by excess unlabeled ME-Arg^6-Phe^7. These proteins were not immunoprecipitated by an antiserum directed to the carboxyl terminus of ME. The major M_r 30,000 is most likely preproenkephalin A, while the M_r 22,000 and 20,000 proteins may be fragments of preproenkephalin A (13). The M_r 31,000 immunoreactive protein has not been previously detected. As shown in panel B, translation of poly(A)+ RNA from haloperidol-treated rats at the lowest three con-

Table 1. Effects of daily injections of haloperidol or lithium on the Met-enkephalin content of rat striatum

Treatment	ng ME/g tissue ± SE	n	% of control
Control	2200 ± 140	4	100
Haloperidol	4090 ± 210*	4	186
Control	2230 ± 74	6	100
Lithium chloride	3700 ± 60*	6	166

Treatment schedules are given in Materials and Methods. *Significantly different from control, $P < 0.001$



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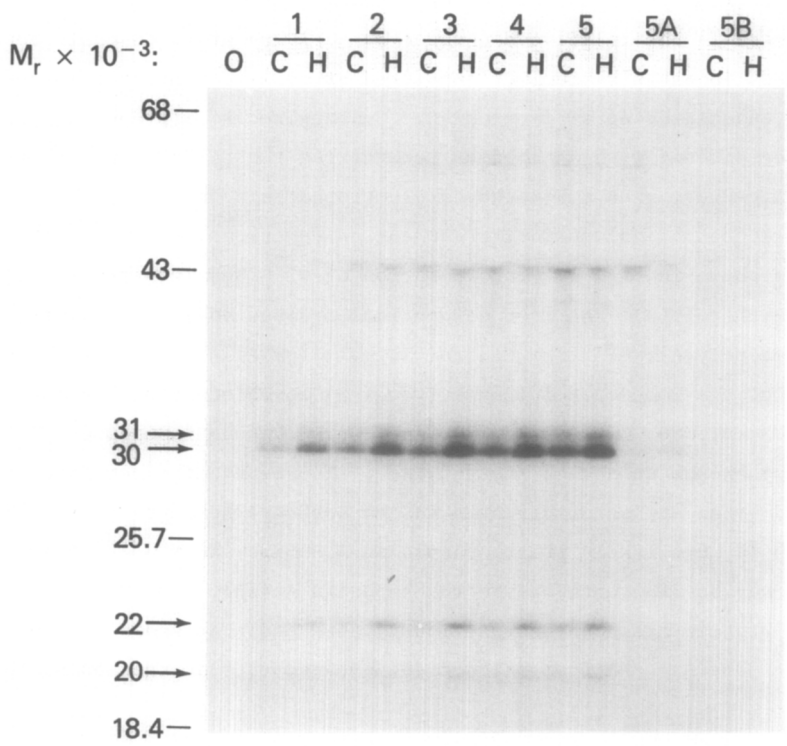

Figure 1. Effect of haloperidol on preproenkephalin A mRNA levels in rat striatum, as determined by cell-free translation of poly(A)+ RNA. Striata from control and treated rats (1.58 and 1.74 g tissue, respectively) yielded 1.34 and 1.26 mg cellular RNA, respectively, and 66 μg and 53 μg poly(A)+ RNA, respectively. Panel A: Incorporation of [^{35}S]Met into protein as a function of added poly(A)+ RNA. Panel B: Amount of [^{35}S]preproenkephalin A recovered from cell-free translation mixtures (0.14 ml) after immunoprecipitation and gel electrophoresis (shown in panel D). Radioactivity in M_r 30,000+31,000 bands (circles) and M_r 22,000 bands (squares) are plotted. Experimentally, equal ^{35}S radioactivity rather than equal volumes of paired samples were subjected

centrations tested (linear portion of curves) resulted in 106% more synthesis of the M_r 30,000+31,000 proteins and 108% more synthesis of the 22,000 protein than that obtained with equal amounts of poly(A)+ RNA from control rats. Radioactivity in the M_r 20,000 band was found to be too low for accurate quantitation. As shown in panel C, the average ratio of M_r 30,000+31,000 protein synthesized to total protein synthesized was 85% higher for the lowest three concentrations of poly(A)+ RNA from haloperidol-treated rats than for corresponding amounts of control poly(A)+ RNA. From data of two experiments of this type, the averages ($\pm$ SE, n=6) of haloperidol-elicited increases were 98 $\pm$ 7% per µg poly(A)+ RNA and 67 $\pm$ 12% per unit of total protein synthesis.

Translation of rat hypothalamic poly(A)+ RNA resulted in a low but detectable level of [^{35}S]preproenkephalin A synthesis that was not affected by haloperidol treatment (not shown). Little or no [^{35}S]preproenkephalin A was synthesized by translation of poly(A)+ RNA from frontal cortex or cerebellum.

Preproenkephalin A synthesis was also examined by translation of unfractionated RNA to reduce potential variation in the degree of purification of compared mRNA preparations (Fig. 2). However, unfractionated RNA contains inhibitor(s) that result in a low maximal rate of translation (panel A). Translation of RNA from haloperidol-treated rats resulted in the synthesis of an average of 35% more M_r 30,000+31,000 preproenkephalin than that obtained with equal amounts of RNA from control rats (panel B). The average ratio of preproenkephalin synthesized to total protein synthesized was 44% higher due to haloperidol treatment. From data of three experiments, the average ($\pm$ SE, n=10) increase was 52 $\pm$ 5% per unit of total protein synthesized.

The effect of repeated injections of lithium on striatal preproenkephalin mRNA levels is shown in Fig. 3. Poly(A)+ RNA from control and lithium-treated rats possessed identical translational activity (panel A). Translation of poly(A)+ RNA from lithium-treated rats resulted in the synthesis of an

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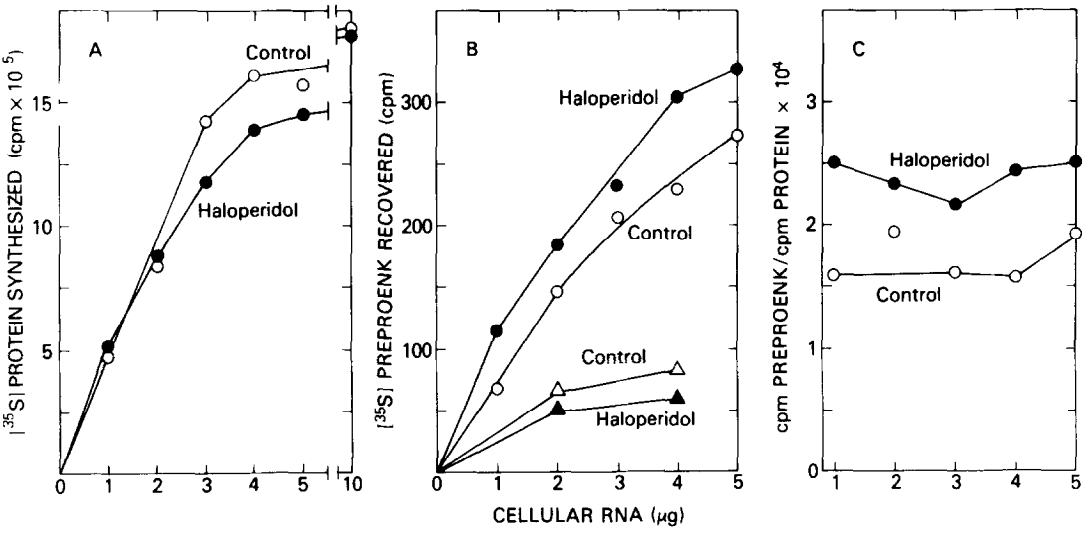
Fig. 1 (Continued)
to immunoprecipitation/electrophoresis to correct for the slightly greater activity of haloperidol poly(A)+ RNA; however, preproenkephalin recovery data were corrected so that equal amounts of poly(A)+ RNA are compared. Panel C: The ratios of the radioactivity of M_r 30,000+31,000 [^{35}S]preproenkephalin (PREPROENK) to that of striatal mRNA-dependent [^{35}S]protein. Panel D: Autoradiogram (9 day exposure) of electrophoresed immunoprecipitated proteins. Lanes are labeled as follows: 0, no added RNA; C and H, poly(A)+ RNA from control and haloperidol-treated rats, respectively. Translation mixtures used for pairs of lanes 1,2,3,4, and 5 contained 0.61, 0.94, 1.25, 1.56, and 1.88 µg, respectively. For lanes marked 5A and 5B, 1.88 µg poly(A)+ RNA was employed; for 5A, immunoprecipitation was in the presence of 0.1 mM ME-Arg^6-Phe^7; for 5B, immunoprecipitation was with 3 µl RB-4 anti-ME serum. At left, numbers indicate positions and M_r values of labeled protein standards (lines) and immunoreactive proteins (arrows).



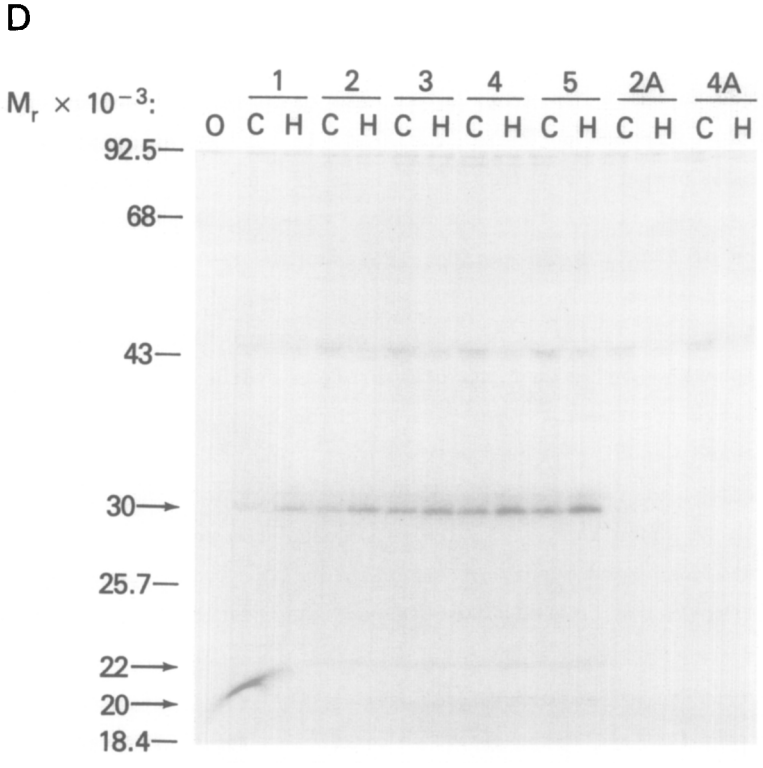


Figure 2. Effect of haloperidol on preproenkephalin A mRNA levels in rat striatum, as determined by cell-free translation of unfractionated RNA. Striata from control and treated rats (1.58 g tissue for each group) yielded 0.97 and 1.16 mg RNA, respectively. The RNA was used for cell-free translation (reaction volume 0.15 ml) without poly(A) selection. Panel A: Incorporation of [^{35}S]Met into protein as a function of added RNA. Panel B: Amount of M_r 30,000+31,000 [^{35}S]preproenkephalin A recovered from translation mixtures (0.135 ml) after immunoprecipitation and gel electrophoresis (shown in panel D). Immunoprecipitation was performed in the absence (circles) or presence (triangles) of 0.1 mM ME-Arg^6-Phe^7. Panel C: The ratios of the radioactivity of [^{35}S]preproenkephalin (PREPROENK) to the radioactivity of striatal mRNA-dependent [^{35}S]protein. Panel D: Autoradiogram (12 day exposure) of electro-

average of 26 ± 3% more M_r 30,000+31,000 preproenkephalin (panel B). The average ratio of preproenkephalin to total protein synthesized was increased 25 ± 1% by lithium treatment (panel C). From data of two experiments the average (± SE, n=10) increase was 29 ± 4% per unit of total protein synthesized.

DISCUSSION

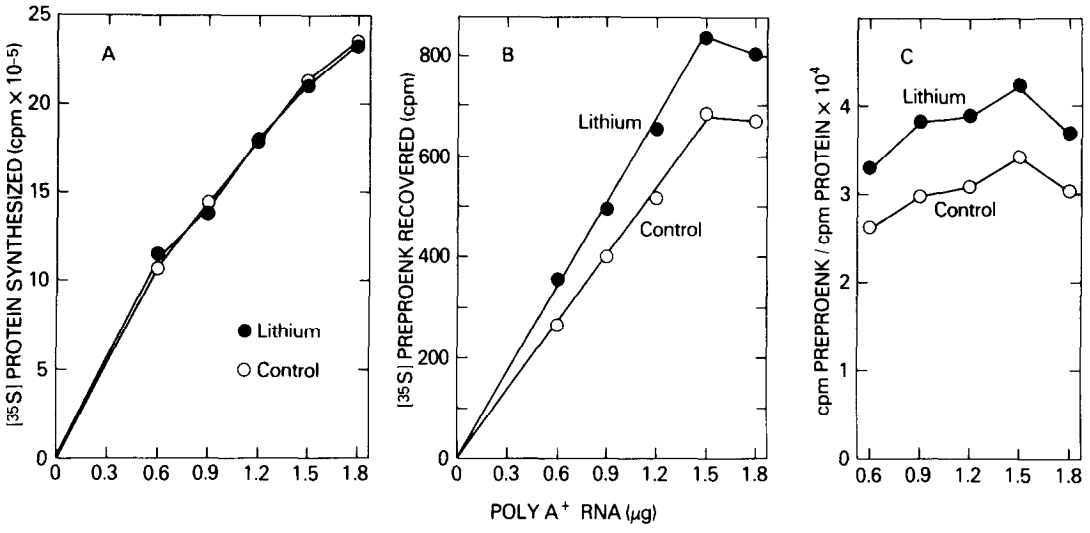
This study demonstrates that *in vitro* translation of purified or unpurified poly(A)+ RNA is a useful technique for evaluating changes in the levels of functional preproenkephalin mRNA in the striatum. Quantitation of mRNA concentration should also be evaluated by solution or blot hybridization with cloned cDNA probes, which are not yet available for rat preproenkephalin A.

This study demonstrates that preproenkephalin A mRNA is elevated by haloperidol treatment to an extent (67-98%) consistent with the concomitant elevation (86%) of the striatal ME content. We propose that haloperidol elevates striatal ME content primarily by increasing the content or translational activity of preproenkephalin A mRNA. Thus, antischizophrenic dopamine-receptor blocking drugs such as haloperidol may block an endogenous dopaminergic inhibition of the expression of enkephalin precursor gene(s) in the striatum. This delayed activation of the enkephalin system may participate in delayed motor disturbances, *e.g.* tardive dyskinesia elicited by the drugs.

In contrast, treatment of rats with lithium elicits a relatively small increase (25-29%) in striatal preproenkephalin A mRNA content while eliciting a larger (66%) increase in the ME content. This discrepancy suggests that an elevation of preproenkephalin A biosynthesis may not be the sole mechanism for the elevated ME content. Other possible mechanisms, such as a reduction in the degradation or neuronal release of ME apparently contribute to the response.

An unexpected finding of this study is a previously undetected protein of apparent M_r 31,000 that reacts with antibodies against ME-Arg^6-Phe^7. This protein is probably related to the M_r 30,000 preproenkephalin A, because its synthesis is similarly increased by haloperidol treatment (Fig. 1, panel D). Whether the M_r 31,000 and 30,000 proteins are encoded by the same or different genes remains to be investigated.

Fig. 2 (Continued)
phoresed immunoprecipitated proteins. Lanes are labeled as follows: 0, no added RNA; C and H, RNA from control and haloperidol-treated rats, respectively. Numbers at top indicate the µg RNA added. For lanes 2A and 4A, 2 and 4 µg RNA, respectively, were translated; immunoprecipitation was in the presence of excess ME-Arg^6-Phe^7. The streak at lower left is a film artifact.



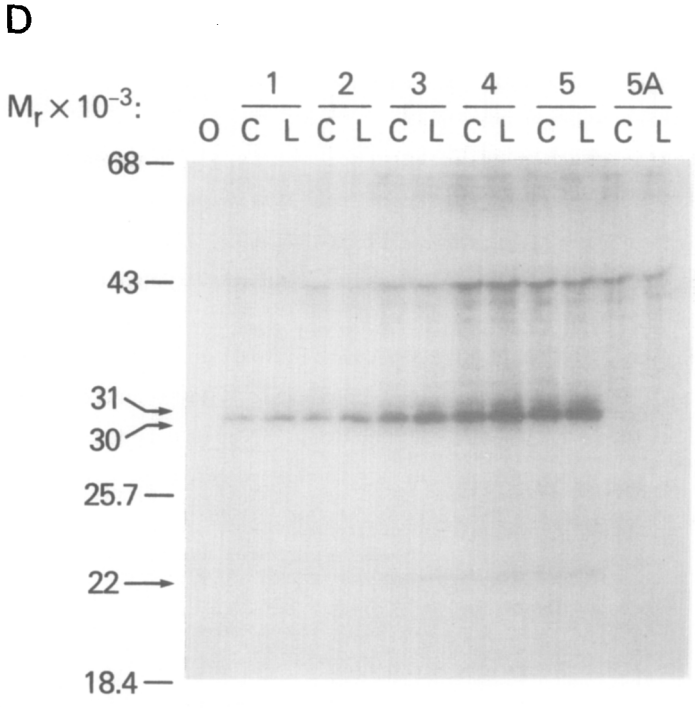


Figure 3. Effect of lithium treatment on preproenkephalin A mRNA levels in rat striatum. Striata from control and treated rats (1.54 and 1.53 g tissue, respectively) yielded 0.94 and 1.09 mg cellular RNA, respectively, and 51 and 53 μg poly(A)+ RNA, respectively. Panel A: Incorporation of [^{35}S]Met into protein as a function of added poly(A)+ RNA. Panel B: Amount of M_r 30,000+31,000 [^{35}S]preproenkephalin A recovered from translation mixtures (0.14 ml) after immunoprecipitation and gel electrophoresis (shown in panel D). Panel C: The ratios of radioactivity of [^{35}S]preproenkephalin to that of striatal mRNA-dependent [^{35}S]protein. Panel D: Autoradiogram (8 day exposure) of immunoprecipitated proteins electrophoresed in an 11% gel. Lanes are labeled as follows: O, no added mRNA; C and L, RNA from control and lithium-treated rats, respectively. Amounts of poly(A)+ RNA for pairs of lanes 1,2,3,4, and 5 were 0.6, 0.9, 1.2, 1.5, and 1.8 μg, respectively. For lanes 5A, 1.8 μg RNA was translated, and immunoprecipitation was in the presence of 0.1 mM ME-Arg^6-Phe^7.

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