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Biosynthesis of Tyrosine Hydroxylase in Rat Adrenal Medulla after Exposure to Cold

(radioimmunoassay/tyrosine hydroxylase degradation/sympathetic ganglia)

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ABSTRACT **Exposure of rats to cold increases the content of tyrosine hydroxylase [EC 1.14.16.2; L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating)] in adrenal medulla, causing a long-lasting enhancement of the enzymatic activity. We have used an antibody specific to tyrosine hydroxylase to study the molecular mechanisms involved in the trans-synaptic induction of adrenal tyrosine hydroxylase. The rate of [^{3}H]-leucine incorporation into adrenal tyrosine hydroxylase was measured by specific immunoprecipitation at various times after exposure to cold (4 hr). This enhanced rate of incorporation was evident between 11 and 30 hr after the beginning of exposure to cold, but not at 7 and 50 hr. The increase of ^{3}H incorporation preceded the maximal enhancement of adrenal tyrosine hydroxylase activity, which occurred about 30 hr after stimulation. Neither the activity of tyrosine hydroxylase nor the rate of ^{3}H incorporation into tyrosine hydroxylase in cervical sympathetic ganglia was changed by 4 hr of exposure to cold. The rate of degradation of tyrosine hydroxylase was estimated at 26 and 50 hr after the beginning of cold stress, as determined by the technique of double-isotope labeling. The data indicate that the tyrosine hydroxylase degradation rate was not reduced by exposure to cold. Thus, the induction of adrenal tyrosine hydroxylase appears to be due to an increased rate of its synthesis.**

In the adrenal medulla of the rat the activity of tyrosine hydroxylase [EC 1.14.16.2; L-tyrosine, tetrahydropterine: oxygen oxidoreductase (3-hydroxylating)], is increased after exposure to cold (1), immobilization stress (2), or the administration of reserpine (3), 6-OH-dopamine (4), and aminophylline (5). In these experimental conditions, the presence of intact afferent nerves is an essential prerequisite for the delayed increase of tyrosine hydroxylase activity (4–7) to occur; hence, it may be inferred that a common mechanism is operative. Recently, immunochemical studies have indicated that the number of tyrosine hydroxylase molecules in medulla increases after reserpine treatment (8), cold exposure, immobilization stress, and administration of 6-OH-dopamine (9). These results rule out the possibility that an activation of preexistent enzyme molecules explains the rise in tyrosine hydroxylase activity. Earlier studies have shown that the rise in tyrosine hydroxylase after reserpine injections (10) or swimming stress (11) is abolished by treatment with actinomycin D or cycloheximide, suggesting that RNA and protein synthesis are involved in the trans-synaptic induction of adrenal tyrosine hydroxylase. However, these reports fail to show whether the various stimuli that increase the number of tyrosine hydroxylase molecules in adrenal medulla enhance the synthesis rate of the enzymes, decrease its degradation rate, or both. In an attempt to investigate which of these processes mediates the trans-synaptic induction of tyrosine hydroxylase, we have prepared antibody specific to bovine adrenal tyrosine hydroxylase and used immunoprecipitation together with pulse labeling with [^{3}H]- and [^{14}C]aminoacids to estimate the rate of synthesis and degradation of tyrosine hydroxylase molecules. This report shows that the increased number of tyrosine hydroxylase molecules elicited by exposure to cold in the adrenal gland is associated with an enhanced rate of tyrosine hydroxylase synthesis. Furthermore, the degradation rate of tyrosine hydroxylase does not seem to be decreased after exposure to cold.

MATERIALS AND METHODS

Purification of Tyrosine Hydroxylase from Bovine Adrenals and Preparation of Antiserum Specific to Tyrosine Hydroxylase. Tyrosine hydroxylase from bovine adrenal medulla was solubilized with chymotrypsin and was purified according to the procedures described by Kaufman and his coworkers (12, 13). As a result of this purification, at least 85% of the proteins present moved in a single band with a R_F value of 0.70 when subjected to electrophoresis on a polyacrylamide gel. Two minor protein bands were also detected with R_F values of 0.60 and 0.55. However, the tyrosine hydroxylase activity was associated with the band moving with an R_F value of 0.70. The tyrosine hydroxylase activity was assayed by the method of Waymire *et al.* (14), which measures $^{14}CO_2$ evolved by the decarboxylation of the 3,4-dihydroxyphenylalanine (dopa) formed from carboxyl-labeled tyrosine (specific activity, 10 μCi/μmole) used as a substrate.

Fractions containing the peak of the major band were used as the source of antigen for the antibody production. Sufficient amounts of control serum were obtained from rabbits, which were then injected intravenously with 100 μg of the antigen protein in an emulsion containing complete Freund's adjuvant. The intravenous injections were repeated five times at 7-to-10-day intervals. After the fifth injection, when the antibody to tyrosine hydroxylase was detected, the antiserum to tyrosine hydroxylase was obtained by cardiac puncture. Both control serum and tyrosine hydroxylase antiserum were concentrated with ammonium sulfate precipitation (0–50%). The precipitate was collected by centrifugation and then resuspended in one-third of the original volume in a solution containing 0.9% NaCl and 1 mM potassium phosphate, pH 7.4. The resuspended precipitate was then extensively dialyzed against the same buffer.

Double immunodiffusion was run on agarose gel as described by Kabat and Meyer (15). Immunochemical titration of tyrosine hydroxylase and the precipitation of the antigen–antibody complex were performed in a volume of 400 μl containing 20 mM potassium phosphate (pH 7.4), 140 mM KCl, 3 mM $MgCl_2$, 0.2% Triton X-100 (medium A), various amounts of purified tyrosine hydroxylase, and the concentrated rabbit antiserum. The mixtures were incubated at 37° for 1 hr and then at 4° for 16 hr. The precipitates were removed by centrifugation at 10,000 × *g*. The enzymatic activity remaining in the supernatant was then determined. The precipitates were washed with 1 ml of medium A containing 0.2% bovine serum albumin and again centrifuged. The pellets were washed three more times, and finally the protein content in the pellets was determined according to Lowry *et al.* (16).

Measurements of the Rate of Synthesis and Degradation of Tyrosine Hydroxylase in Adrenal Medulla. Sprague–Dawley male rats (Zivic Miller Laboratory, Pa.) (about 125 g body weight) were forced to swim in water at 25° for 1 min and then placed in the cold (4 ± 2°) for 4 hr. At various times thereafter, these rats and control rats kept at 25° received 700 μCi of [^{3}H]leucine (46 Ci/mmole, Schwarz/Mann, Rockville, Md.) intraperitoneally and were decapitated 90 min later. Each pair of adrenal glands or superior cervical sympathetic ganglia was homogenized in 0.5 ml of medium A and centrifuged at 10,000 × *g* for 20 min. The supernatant was further centrifuged at 100,000 × *g* for 1 hr. Aliquots of 175 μl of the supernatant were incubated with 100 μl of either antiserum or normal rabbit serum. Purified tyrosine hydroxylase (15 μg) was added to serve as a carrier. The reaction mixtures in a final volume of 400 μl containing medium A were incubated, centrifuged, and processed according to the immunoprecipitation assay described in the previous section. The incorporation of radioactive amino acids into precipitated tyrosine hydroxylase was measured by liquid scintillation spectrometry. The precipitate was solubilized in 1 ml of 0.5 M NaOH; radioactivity was determined in 10 ml of Aquasol scintillator mixture (New England Nuclear, Boston, Mass.). The radioactivity obtained with normal rabbit serum processed as described above was used as a background; it was never greater than 20% of the radioactivity obtained with antiserum. Aliquots were also taken from the 100,000 × *g* supernatant to measure the incorporation of radioactive amino acids into soluble proteins. The proteins of the supernatant were precipitated with 5% hot trichloroacetic acid and the precipitates were filtered through a Millipore filter (HA 025, 0.45 μm). The filter was washed four times with trichloroacetic acid at room temperature; radioactivity was determined in Aquasol.

The double-isotope labeling technique (17–19) was used to estimate the rate of tyrosine hydroxylase degradation. Thirty hours before exposure to cold, rats received 120 μCi of [^{14}C]-leucine (320 mCi/mmole, Schwarz/Mann) intraperitoneally and were divided into two groups. They received 700 μCi of [^{3}H]leucine either 24 or 48 hr after the beginning of exposure to cold; they were then killed 90 min after the injection of [^{3}H]leucine. The incorporation of ^{3}H and ^{14}C into tyrosine hydroxylase and into the soluble proteins of adrenals was determined as described above. Rats kept at 25° received the two injections of the labeled amino acid and were used as a control.

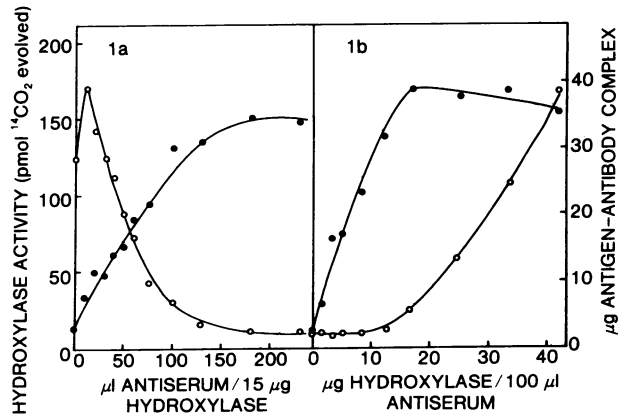

FIG. 1. Immunochemical titration of purified tyrosine hydroxylase with rabbit antiserum. (a) Increased amounts of antiserum were added to a constant amount of purified tyrosine hydroxylase. (b) Increased amounts of purified tyrosine hydroxylase were added to a constant amount of antiserum. Procedures for immunotitration were described in *Materials and Methods*. After immunoprecipitates were removed by centrifugation, 50-μl aliquots of the supernatant were used for the assay of tyrosine hydroxylase activity in a final reaction mixture of 100 μl. O, Hydroxylase activity: ●, precipitate of antigen–antibody complex.

RESULTS

The final specific activity of the bovine tyrosine hydroxylase used as the antigen was 1200- to 1500-fold greater than the starting homogenate and represented about 1% of the tyrosine hydroxylase activity present in the original beef adrenal medulla homogenate. When this antigen was subjected to disc gel electrophoresis, only one protein band could be detected with an R_F of about 0.7. Moreover, the tyrosine hydroxylase activity was exclusively present in this protein peak. The specificity of the rabbit antibody against tyrosine hydroxylase was first examined by Ouchterlony double-diffusion technique (15). The antiserum was run against highly purified adrenal tyrosine hydroxylase or crude adrenal extracts from either beef or rat. In all cases, a single precipitin line was observed. Fig. 1a shows that addition of increased amounts of antiserum to a constant amount of purified tyrosine hydroxylase resulted in increasing inhibition of the enzymatic activity. The extent of inhibition correlated well with the amounts of protein precipitated. The converse experiment, i.e., the addition of increasing amounts of enzyme to a constant amount of antiserum is shown in Fig. 1b. The equivalence point (beyond which enzyme activity becomes detectable) occurred when the precipitation of the protein was complete. It should be stated that the serum of normal rabbits that was obtained prior to immunization neither inhibited the activity of tyrosine hydroxylase nor precipitated the enzyme molecules.

The technique of immunoprecipitation was then used to measure amino-acid incorporation into the tyrosine hydroxylase of adrenal and sympathetic ganglia. The rats were injected with [^{3}H]leucine 90 min prior to decapitation and the tissue homogenate was centrifuged to remove the ribosomes. To the supernatant was added purified bovine tyrosine hydroxylase as a carrier and the antibody to this tyrosine hydroxylase in the optimal proportion established by immunotitration experiments (see Fig. 1a and b). Since the indirect approximation of the turnover rate of adrenal tyrosine

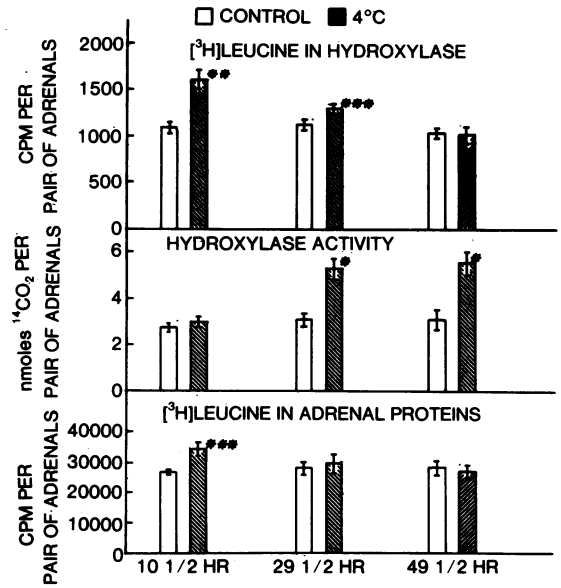

FIG. 2. Effect of cold exposure of [^{3}H]leucine incorporation and tyrosine hydroxylase enzyme activity. Each column represents the mean ±SE (brackets) of five to seven pairs of adrenals. The rats were killed at 10.5, 29.5, and 49.5 hr, which represent the time (hours) after the beginning of exposure to cold. Ninety minutes before they were killed, the animals received [^{3}H]leucine. Tyrosine hydroxylase activity was assayed at 37° for 30 min. $*P < 0.001$; $**P < 0.01$; $*** P < 0.05$ when compared with control animals.

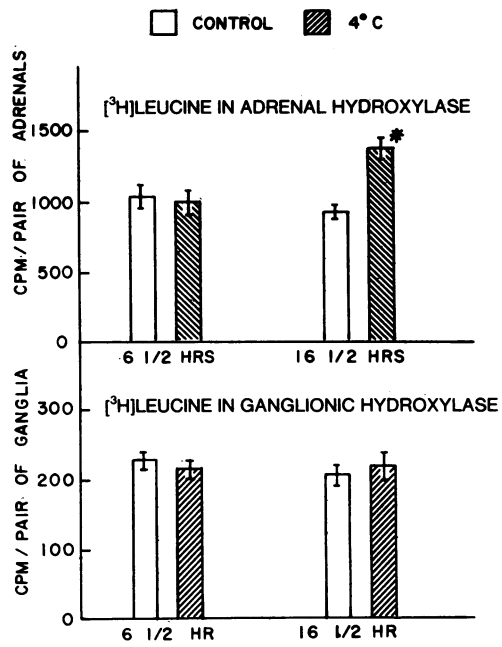

FIG. 3. Incorporation of [^{3}H]leucine into tyrosine hydroxylase in adrenals and ganglia after exposure to cold. Each column represents the mean ±SE (brackets) of five to seven experiments. The animals were decapitated at 6.5 and 16.5 hr, which represent the times (hours) after the beginning of exposure to cold. Ninety minutes before they were killed, the rats were injected with [^{3}H]-leucine. $*P < 0.001$ when compared with control animals.

hydroxylase indicates that this enzyme has a $t_{1/2}$ of several days (2,10), the ^{3}H incorporation into tyrosine hydroxylase during 90 min after a pulse of [^{3}H]leucine may be related to the rate of tyrosine hydroxylase synthesis. The data in Fig. 2 indicate that the ^{3}H incorporated into adrenal tyrosine hydroxylase during 90 min was greater in rats that were killed at 10.5 hr after the beginning of the exposure to cold than in rats kept at 25°. However, at this time the tyrosine hydroxylase activity of adrenals was equal in the two groups of rats (Fig. 2). When the animals were killed at 29.5 hr after exposure to cold, the ^{3}H incorporation into adrenal tyrosine hydroxylase was still greater than that of rats kept at 25°. However, at the 49.5 hr ^{3}H incorporation into adrenal tyrosine hydroxylase was no longer increased. In the adrenals of rats that were decapitated at 29.5 and 49.5 hr after the beginning of exposure to cold, the tyrosine hydroxylase activity was increased by 80%. The ^{3}H incorporation in the soluble proteins of adrenals in rats decapitated at 10.5 hr after exposure to cold was greater than that of normal rats, but the ^{3}H in adrenal soluble proteins in rats killed at 29.5 and 49.5 hr was similar to that of normal rats.

The tyrosine hydroxylase of cervical sympathetic ganglia is not increased by an exposure to cold for 4 hr (20, 21). The rates of the [^{3}H]leucine incorporation in the tyrosine hydroxylase of adrenal and sympathetic ganglia were measured at 6.5 and 16.5 hr after the beginning of exposure to cold (Fig. 3). At 6.5 hr, neither the ^{3}H incorporation in adrenal tyrosine hydroxylase nor that in ganglia tyrosine hydroxylase was changed. At 16.5 hr, the ^{3}H incorporation in adrenal tyrosine hydroxylase was elevated by about 50%, while that in ganglionic tyrosine hydroxylase was unchanged. In this group of rats the activity of adrenal tyrosine hydroxylase was increased but that of ganglia was not.

Since an increase of ^{3}H incorporation in adrenal tyrosine hydroxylase might also reflect a reduction of the degradation rate of tyrosine hydroxylase, we have investigated the rate of tyrosine hydroxylase degradation using a double-isotope labeling technique (17–19). Rats were injected with [^{14}C]leucine 30 hr before exposure to the cold. At 24 and 48 hr after the beginning of exposure to cold, they received [^{3}H]leucine and were decapitated 90 min later. The ratio of ^{3}H/^{14}C incorporated into tyrosine hydroxylase and that incorporated into adrenal soluble proteins was used to estimate possible changes in amino-acid precursor pools. The results of this experiment are shown in Table 1. When the radioactivity in adrenal soluble proteins of rats exposed to cold was compared to that of rats kept at 25°, the ^{3}H/^{14}C ratio and the amount of ^{3}H or ^{14}C present at 26 and 50 hr after the beginning of the stress were similar. In contrast, the amount of ^{3}H incorporated into tyrosine hydroxylase was greater in rats killed at 26 hr after the beginning of the stress than in rats that were not stressed. Such a difference between stressed and normal rats was no longer detectable when the rats were killed 50 hr after the beginning of exposure to cold. The amount of ^{14}C remaining in tyrosine hydroxylase was similar in normal rats and in the rats exposed to cold when they were killed at 26 and 50 hr after the beginning of the stress. The activity of tyrosine hydroxylase was elevated by 75 and 78% at 26 and 50 hr, respectively, after the beginning of the stress (data not shown).

DISCUSSION

The results reported demonstrate that the delayed elevation of adrenal tyrosine hydroxylase activity elicited by 4 hr of

TABLE 1. *Double isotope incorporation into soluble adrenal protein and adrenal tyrosine hydroxylase*

Hr after beginning of cold exposure	Soluble adrenal proteins			Adrenal hydroxylase			
	cpm/pair of adrenals			cpm/pair of adrenals			
	^3H	^{14}C	$^3H/^{14}C$	^3H	^{14}C	$^3H/^{14}C$	k_d hr^{-1}
26							
Control	32380 ± 2120	11440 ± 460	2.83 ± 0.13	1242 ± 72	459 ± 29	2.73 ± 0.16	—
4°	36064 ± 2528	12320 ± 748	2.89 ± 0.13	1461 ± 74*	537 ± 28	2.72 ± 0.05	—
50							
Control	28928 ± 1964	6632 ± 388	4.35 ± 0.12	1030 ± 48	200 ± 21	5.22 ± 0.25	0.033
4°	27980 ± 1368	7024 ± 480	4.02 ± 0.15	1012 ± 85	236 ± 18	4.37 ± 0.19†	0.033

Each value represents the mean ±SE of five to seven experiments. Experimental conditions and the measurements for the incorporation into soluble adrenal protein and adrenal tyrosine hydroxylase were as described in *Materials and Methods*.

* $P < 0.05$ when compared with control animals.

† $P < 0.02$ when compared with control animals.

—, not done.

exposure to cold is associated with an increased incorporation rate of [^{3}H]leucine into tyrosine hydroxylase. This enhanced incorporation rate was evident between 10.5 and 29.5 hr after the beginning of the stress but not at 6.5 hr. The increase of [^{3}H]leucine incorporation into tyrosine hydroxylase preceded the increase of the tyrosine hydroxylase activity (see Fig. 2), which became detectable in about 12–15 hr after the beginning of the cold stress and reached its maximum at 24 hr, persisting for several days thereafter. It has been suggested that the turnover rate of adrenal tyrosine hydroxylase is rather slow (2, 10). Hence, it seems appropriate to interpret the enhanced incorporation of ^{3}H into tyrosine hydroxylase during 90 min after a pulse of [^{3}H]leucine as an increased rate of tyrosine hydroxylase synthesis. However, the stabilization of preexisting enzyme molecules cannot be excluded from these experiments on [^{3}H]leucine incorporation nor from earlier observations that long-term induction of adrenal tyrosine hydroxylase can be prevented by treatment with cycloheximide or actinomycin D (10, 11).

The half-time of adrenal tyrosine hydroxylase has been estimated from the decay of the reserpine-elicited increase of tyrosine hydroxylase activity (10). Assuming that nerve activity is necessary to maintain the induction of tyrosine hydroxylase, Mueller *et al.* (10) severed the splanchnic nerve 3 days after reserpine injection and measured the decline of adrenal tyrosine hydroxylase. However, it is presently believed that nerve activity is not the essential stimulus that maintains the induction of adrenal tyrosine hydroxylase. Moreover, the present experiments indicate that the increase of nerve impulse rates elicited by cold is temporally unrelated to an increase rate of leucine incorporation into tyrosine hydroxylase. The increased incorporation begins 8–9 hr after the stimulus initiation and persists for the next 24 hr when the activity of neurons impinging upon the adrenal should be back to normal. During the enzyme induction, the time course whereby a new steady state of tyrosine hydroxylase is approached solely depends on the rate constant of enzyme degradation (19). Hence, we performed the double-isotope pulse labeling experiments reported in Table 1 to detect whether the tyrosine hydroxylase degradation rate was reduced during induction. The ratio of $^3H/^{14}C$ incorporated into total soluble proteins of adrenal was identical to that incorporated into the adrenal tyrosine hydroxylase of normal and stressed rats. This identity indicates that the kinetics of the amino-acid precursor pools involved in the synthesis of tyrosine hydroxylase and of the soluble adrenal proteins are similar and that the stress does not change the pools of these precursors. Since in stressed rats the tyrosine hydroxylase is in steady state between 26 and 50 hr after the stimulus application, the loss of radioactivity incorporated into tyrosine hydroxylase during this time is exponential. This decay allows for calculation of the first-order rate constant of enzyme degradation (k_d) from the difference of the ^{14}C incorporated into tyrosine hydroxylase at 26 and 50 hr. From these data (Table 1) the k_d for adrenal tyrosine hydroxylase can be estimated at 0.033 hr^{-1} for both stressed and normal rats. From the steady-state relationship, $k_s = k_dE$, where k_s is the zero-order rate constant of synthesis (unit time^{-1}) and E is the enzyme content (units/gland), k_s can now be calculated. The data of Fig. 2 show that a pair of normal adrenals produces 6 nmol of dopa per hr; this enzyme activity increases at 26 and 50 hr after stress. At this time a pair of adrenals can synthesize 10.7 nmol of dopa per hr. The kinetic properties of the induced tyrosine hydroxylase have been shown to be identical to those of the control enzyme (9). Thus, from our results it can be estimated that before stimulus a pair of adrenals synthesizes in 1 hr a number of enzyme molecules that can form 0.198 nmol of dopa per hr. In contrast, during the process of induction a pair of adrenals synthesizes in 1 hr a number of tyrosine hydroxylase molecules that can form 0.353 nmol of dopa per hr.

Obviously, this is an approximation based on the assumption that between 2 and 3 days after pulse labeling the interferences caused by the amount of isotopic amino-acid reutilization for the synthesis of tyrosine hydroxylase is insignificant. However, should this assumption not be valid, our estimation of rate of synthesis would be less than the actual rate.

It was shown by Thoenen and his coworkers that the induction of adrenal tyrosine hydroxylase was completely abolished if actinomycin D was given immediately before or after swimming stress, but was only slightly reduced if given 16 hr later (11). Our data, taken with their results, strongly suggest that the induction of adrenal tyrosine hydroxylase involves a new transcription of DNA and that the production of new RNA probably occurs shortly after the stimulus application. The

induction of tyrosine hydroxylase in adrenal medulla elicited by exposure to cold or drugs is preceded by an increase of medullary 3′,5′-cyclic AMP concentration (22). Moreover, it was shown (5–7, 22) that there is a good correlation between the duration of the increase in cyclic AMP concentration and the subsequent induction of tyrosine hydroxylase. We feel that this cyclic nucleotide is the most likely candidate as the effector that promotes the synthesis of specific messenger RNA. Cyclic AMP perhaps acts through a cyclic-AMP dependent protein kinase which phosphorylates specific nuclear proteins or other cytoplasmic proteins that are involved in the mediation of the increase of DNA transcription which is initiated by neuronal activity.

We have not characterized the molecular nature of the newly synthesized tyrosine hydroxylase molecules, nor do we know whether the newly synthesized tyrosine hydroxylase represents a precursor molecule.

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