MOLECULAR PHARMACOLOGY, 12, 1070-1081

Dopaminergic Neurons: Effects of Electrical Stimulation on Tyrosine Hydroxylase

L. CHARLES MURRIN,[1] VICTOR H. MORGENROTH, III,[2] AND ROBERT H. ROTH

Departments of Pharmacology and Psychiatry, Yale University School of Medicine, New Haven, Connecticut 06510

(Received February 3, 1976)

(Accepted July 27, 1976)

SUMMARY

MURRIN, L. CHARLES, MORGENROTH, VICTOR H., III & ROTH, ROBERT H. (1976) Dopaminergic neurons: effects of electrical stimulation on tyrosine hydroxylase. *Mol. Pharmacol.*, 12, 1070-1081.

Electrical stimulation of the nigro-neostriatal and mesolimbic dopaminergic pathways was employed in order to examine the effects of increased impulse flow on the properties of tyrosine hydroxylase prepared from the neostriatum and olfactory tubercles. Stimulation at a frequency of 15 Hz for 10 min resulted in an increase in the activity of tyrosine hydroxylase prepared from the neostriatum on the stimulated side and assayed in the presence of subsaturating concentrations of substrate and pterin cofactor. Stimulation periods of 15 and 20 min did not produce a further significant increase in tyrosine hydroxylase activity. This activation of the enzyme persisted unchanged for 10 min after cessation of stimulation and was partially present 15 min after stimulation. Further analysis of this activation showed that it was characterized by decreases in K_m of tyrosine hydroxylase for substrate, tyrosine, and cofactor, tetrahydrobiopterin, and an increase in K_i for the feedback inhibitor, dopamine. Similar kinetic changes due to electrical stimulation of the mesolimbic pathway were found in the tyrosine hydroxylase isolated from the olfactory tubercles. Addition of adenosine cyclic 3',5'-monophosphate to high-speed supernatants prepared from the neostriatum produced kinetic changes in neostriatal tyrosine hydroxylase similar to those produced by increased impulse flow due to electrical stimulation. However, addition of cyclic AMP produced no further change in the properties of the enzyme already activated by electrical stimulation. These results are discussed in terms of possible mechanisms by which impulse flow may control dopamine synthesis.

INTRODUCTION

Increased impulse flow produced by electrical stimulation of both peripheral and central catecholamine-containing neurons has been shown to increase the synthesis of the transmitter associated with these neurons (1-7). There is a great deal of evidence that in the peripheral sympathetic nervous system the acceleration of norepinephrine synthesis occurs at the tyrosine hydroxylase step (2, 4, 5, 8). Moreover, it has recently been reported that the

[1] This work is derived in part from a dissertation presented to the Yale University Graduate School in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Present address, Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205.

[2] Present address, Department of Pharmacology, School of Medicine-Dentistry, Georgetown University, Washington, D. C.

mechanism responsible for the increase in tyrosine hydroxylase activity is an alteration in the kinetic properties of this enzyme (9). Thus there is an increased affinity of tyrosine hydroxylase for cofactor, $DMPH_4$,[3] and substrate, tyrosine, and a decreased affinity for the endogenous feedback inhibitor, norepinephrine.

Electrical stimulation of the nigro-neostriatal and mesolimbic dopaminergic neurons leads to an increase in dopamine synthesis (6, 7). Moreover, if the apparent activity of neostriatal tyrosine hydroxylase is determined *in vivo* by measuring the accumulation of endogenous dopa after inhibition of central dopa decarboxylase, electrical stimulation results in a dramatic increase in tyrosine hydroxylase activity. Further studies showed that this increase in apparent tyrosine hydroxylase activity persisted for some time following cessation of stimulation, suggesting that it would be feasible to investigate further the mechanism responsible for this stimulus-induced activation of tyrosine hydroxylase.

This paper presents a study of the effects of electrical stimulation of dopaminergic neurons on the kinetic properties of tyrosine hydroxylase isolated from brain regions containing the terminals of the stimulated neurons. Part of this work has been presented previously in abstract form (10).

METHODS

Electrical stimulation experiments. Electrical stimulations were carried out as previously described (7). In brief, male Sprague-Dawley rats (Charles River, Inc., Wilmington, Mass.) were placed in a stereotaxic holder (David Kopf Instruments) under chloral hydrate anesthesia (400 mg/kg, intraperitoneally). The nigro-neostriatal and mesolimbic dopaminergic pathways were stimulated using a coaxial electrode (NE-100, David Kopf Instruments) placed near the medial forebrain bundle [approximately A 2800 μm, L 1.4 mm, V 3.0 mm in the König and Klippel rat atlas (11)]. Square-wave pulses were delivered from a BBS-1 electrical stimulator (Electronic Instruments, Inc., Northford, Ct.). Pulses were 200 μamp, 1.5-msec-duration monophasic, or 400 μamp, 3.0-msec-duration biphasic, delivered at a frequency of 15 Hz. Stimulations were carried out for 20 min unless indicated otherwise. At the end of the stimulation period the brain sections needed for assay were dissected and frozen on Dry Ice. Tissues were homogenized in 10 volumes of ice-cold 0.05 M Tris-acetate buffer, pH 6.0, and centrifuged at 104,000 $\times$ g (average) for 90 min at 2–3°. In some experiments neostriata or olfactory tubercles were pooled for assay. Stimulated tissues were always compared with the contralateral unstimulated tissues. Brain stems were examined histologically to verify placement of the stimulating electrode.

Tyrosine hydroxylase assay and kinetic calculations. Tyrosine hydroxylase was assayed by a modification of the methods of Shiman *et al.* (12) and of Coyle (13), as described in detail by Morgenroth *et al.* (9). The high-speed supernatant (104,000 $\times$ g) served as the enzyme source in all experiments.

In experiments involving cyclic AMP, this nucleotide (50 μM) was added to the enzyme assay mixture 10 min prior to the addition of labeled tyrosine, so that a 10-min instead of a 5-min preliminary incubation was used. The reaction was stopped after 45 min by the addition of 50 μl of glacial acetic acid. The formation of ^3H_2O was followed as a measure of dopa formation. The rate of dopa formation was linear with time for at least 1 hr, and with protein concentration from 25 to 600 μg/ml.

K_m was determined by the method of Lineweaver and Burk (14), and K_i, by the method of Dixon (15). Protein concentrations were measured by the method of Lowry *et al.* (16).

Liquid scintillation counting. Liquid scintillation counting was carried out in a scintillation fluid made up of 1 liter of dioxane (Mallinckrodt Scintillar grade), 1 liter of toluene (Packard scintillation grade), and 1 liter of absolute ethanol (Gold Shield) to which were added 240 g of naphthalene, 15 g of 2,5-diphenyloxazole, and 0.3 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (all Packard scintillation grade reagents). Counting was carried

[3] The abbreviations used are: $DMPH_4$, 6,7-dimethyl-2-amino-4-hydroxy-5,6,7,8-tetrahydropteridine; BH_4, tetrahydrobiopterin.

out in a Packard Tri-Carb liquid scintillation counter. Corrections for counting efficiency were made using both internal and external standards. Significance was determined by use of paired *t*-test on a Hewlett-Packard programmable calculator, model 9810A.

Materials. Catalase, NADPH, and ultrapure Tris were purchased from Schwarz/Mann. L-[3,5-^{3}H]Tyrosine (30 Ci/mmole) was obtained from New England Nuclear Corporation. BH_4 was a gift from Dr. K. J. M. Andrews, Roche Products, Ltd. Chloral hydrate, $DMPH_4$, cyclic AMP, and dopamine were obtained from Aldrich, Calbiochem, Regis, and Sigma, respectively.

RESULTS

Effect of electrical stimulation on neostriatal tyrosine hydroxylase activity. Electrical stimulation of the nigro-neostriatal pathway at 15 Hz for 20 min was found to increase the activity of neostriatal tyrosine hydroxylase significantly (10, 17) (Table 1). Chloral hydrate, which has been shown to increase the firing rate of the nigro-neostriatal neurons (18), causes a partial activation of neostriatal tyrosine hydroxylase. About 30 pmoles of dopa are formed per milligram of protein per minute by the enzyme isolated from neostriata of chloral hydrate-treated animals, compared to about 23 pmoles of dopa per milligram of protein per minute for enzyme from control animals. Stimulation boosts the level of enzyme activity to 47 pmoles of dopa formed per milligram of protein per minute. Similar results were obtained using either monophasic or biophasic pulses during stimulation. These results confirm the data *in vivo* suggesting that electrical stimulation of nigro-neostriatal dopaminergic neurons causes an increase in neostriatal tyrosine hydroxylase activity (7). In view of these results, we investigated the time course of the activation of tyrosine hydroxylase by electrical stimulation.

As shown in Table 1, stimulation for 5 min results in no appreciable change in neostriatal tyrosine hydroxylase activity compared to the enzyme activity in the contralateral neostriata. However, continuous stimulation for 10 min produces an increased enzyme activity that is significantly different from the level of activity in control neostriata and contralateral neostriata (chloral hydrate treatment). The level of enzyme activity following 15- and 20-min periods of stimulation is not significantly different from the levels of enzyme activity obtained after 10 min of continuous stimulation.

Decrease in tyrosine hydroxylase activ-

TABLE 1

Time course of neostriatal tyrosine hydroxylase activity after electrical stimulation

The nigro-neostriatal pathway was stimulated as described in METHODS for various time periods at 15 Hz with 400-μamp biphasic electrical pulses of 3.0-msec duration. The animals were immediately decapitated, and the stimulated and contralateral, unstimulated neostriata were assayed for tyrosine hydroxylase activity. The assay was carried out as described in METHODS in the presence of subsaturating concentrations of tyrosine (10 μM) and $DMPH_4$ (100 μM). Values are the means ± standard errors of four experiments.

Duration of stimulation	Treatment	Tyrosine hydroxylase activity	
		Unstimulated contralateral	Stimulated
min		*pmoles dopa/mg protein/min*	
0	None	23.1 ± 2.1	
0	Chloral hydrate[a]	30.5 ± 1.6[b]	
5	Chloral hydrate	28.7 ± 1.5[b]	32.3 ± 2.8
10	Chloral hydrate	32.6 ± 1.5[b]	43.4 ± 3.5[c]
15	Chloral hydrate	31.9 ± 2.1[b]	45.8 ± 2.8[c]
20	Chloral hydrate	30.3 ± 1.8[b]	49.5 ± 3.1[c]

[a] Chloral hydrate was administered at a dose of 400 mg/kg intraperitoneally.

[b] Significantly different from non-chloral hydrate-treated preparations ($p < 0.01$).

[c] Significantly different from the contralateral, unstimulated side ($p < 0.01$).

ity after stimulation. In a similar series of experiments, animals were stimulated for 20 min and decapitated at various times thereafter. At each time point the stimulated neostriata were compared with their contralateral neostriata in order to control for varying effects of chloral hydrate, which was injected 10–15 min before the beginning of stimulation and which had begun to lose effect at the longer time points.

As can be seen in Fig. 1, the increased tyrosine hydroxylase activity is virtually unchanged for 10 min after the end of stimulation. Beyond 10 min the enzyme activity begins to decrease. At 15 min after stimulation the enzyme is still 50% activated compared to control preparations (23.1 ± 2.1 pmoles of dopa per milligram of protein per minute) and is still significantly more active than the enzyme prepared from the contralateral, nonstimulated sides. By 20 min after the end of stimulation the activity of tyrosine hydroxylase in the stimulated neostriata is not significantly different from the activity in the contralateral neostriata. These results suggested that it would be feasible to study the properties of this activated enzyme further, since the activation persisted under conditions of freezing, thawing, and homogenization. With this in mind, the following experiments were carried out.

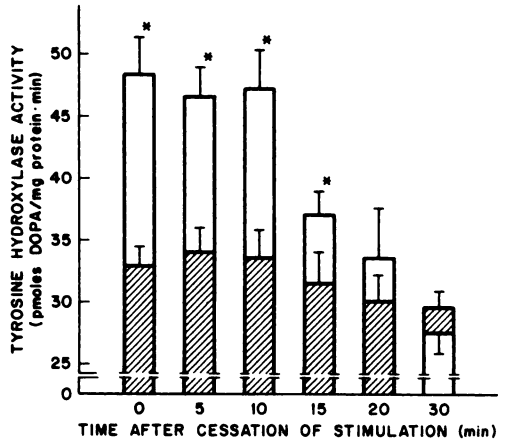

FIG. 1. *Time course for decay of poststimulation activation of neostriatal tyrosine hydroxylase*

The nigro-neostriatal pathway was stimulated as described in METHODS for 20 min with 15-Hz, 400-μamp, biphasic, 3.0-msec electrical pulses. The animals were killed at various time intervals after termination of stimulation, and the neostriata were dissected for assay of tyrosine hydroxylase. Enzyme activity was measured in the presence of subsaturating concentrations of tyrosine (10 μM) and $DMPH_4$ (100 μM). Hatched areas of the graph represent tyrosine hydroxylase activity of enzyme isolated from neostriata contralateral to the stimulation. Unshaded areas represent tyrosine hydroxylase activity of enzyme isolated from neostriata ipsilateral to the stimulation. Values are the means determined from four separate experiments. Vertical bars represent standard errors of the mean.

* Significantly different from contralateral, nonstimulated neostriata ($p < 0.01$).

Effect of electrical stimulation on neostriatal tyrosine hydroxylase kinetics. A kinetic analysis of tyrosine hydroxylase was carried out in order to gain some insight into the mechanism responsible for the observed increase in tyrosine hydroxylase activity. In determination of the K_m for the substrate, tyrosine, $DMPH_4$ was used as cofactor at a concentration of 1.0 mM and tyrosine was used at seven concentrations ranging from 0.1 to 100 μM. The K_m for tyrosine in control preparations was 54.7 μM, while the K_m for enzyme from chloral hydrate-treated animals was 41.5 μM. Electrical stimulation caused a marked decrease in K_m for tyrosine, to 17.1 μM (Fig. 2 and Table 2). No significant change in V_{max} was observed (Fig. 2).

In a similar group of experiments the effect of stimulation on the K_m for BH_4, believed to be the natural cofactor for tyrosine hydroxylase in the neostriatum (19, 20), was studied. In these studies tyrosine was used at a concentration of 0.1 mM and the BH_4 concentrations ranged from 0.05 to 2.0 mM. The K_m for BH_4 of the tyrosine hydroxylase prepared from the neostriatum obtained from control rats was 0.42 mM, while the K_m for BH_4 of the enzyme prepared from the neostriata obtained from chloral hydrate-treated rats was 0.36 mM (Fig. 3 and Table 2). Electrical stimulation caused a decrease in K_m for BH_4 to 0.08 mM. Again, no significant change in V_{max} was observed in these experiments (Fig. 3).

When $DMPH_4$ was used as cofactor, results similar to those found with BH_4 were obtained (Table 2). The K_m of tyrosine hy-

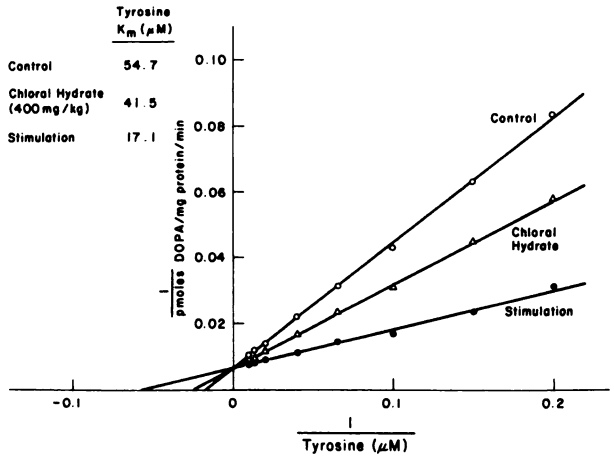

FIG. 2. *Effect of electrical stimulation on K_m for tyrosine of neostriatal tyrosine hydroxylase*

Control enzyme was isolated from neostriata of untreated rats. Chloral hydrate enzyme was isolated from neostriata contralateral to the stimulation. The nigro-neostriatal pathway was stimulated as described in METHODS for 20 min with 15-Hz, 400-μamp, biphasic, 3.0-msec electrical pulses. The animals were killed immediately, and the K_m for tyrosine was determined at a $DMPH_4$ concentration of 1 mM and at eight tyrosine concentrations ranging from 5 to 100 μM. Each value for K_m is the mean of the intercepts generated from six separate lines.

TABLE 2

Effect of electrical stimulation on activity and kinetic parameters of neostriatal tyrosine hydroxylase

Control data are for tyrosine hydroxylase prepared from neostriata of untreated rats; chloral hydrate data represent enzyme from neostriata contralateral to stimulation. The nigro-neostriatal pathway was stimulated as described in METHODS for 20 min with 15-Hz electrical pulses of 400 μamp and 3.0-msec duration (biphasic) or 200 μamp and 1.5-msec duration (monophasic). The animals were killed immediately after stimulation, and tyrosine hydroxylase was isolated and assayed as described in METHODS. The K_m for tyrosine was determined at a $DMPH_4$ concentration of 1 mM and eight tyrosine concentrations ranging from 5 to 100 μM. The K_m for BH_4 was determined at 100 μM tyrosine and seven BH_4 concentrations ranging from 50 μM to 2 mM. K_m values are the means ± standard errors of values generated from six separate lines. The K_i for dopamine was determined at 100 μM tyrosine, at 1, 10, and 100 μM $DMPH_4$, and at seven dopamine concentrations (1 μM–1 mM). K_i values are the means ± standard errors of six determinations.

Treatment	Tyrosine hydroxylase activity	K_m for tyrosine	K_m for BH_4	K_m for $DMPH_4$	K_i for dopamine
	pmoles dopa/mg protein/min	*μM*	*mM*	*mM*	*mM*
Control	22.5 ± 1.6	54.7 ± 5.1	0.42 ± 0.08	0.87 ± 0.04	0.05 ± 0.02
Chloral hydrate (400 mg/kg)	31.5 ± 2.6	41.5 ± 3.6	0.36 ± 0.09	0.54 ± 0.07	0.06 ± 0.03
Electrical stimulation (biphasic)	47.2 ± 5.1	17.1 ± 1.6	0.08 ± 0.01	0.23 ± 0.02	0.29 ± 0.04
Chloral hydrate (400 mg/kg)	30.6 ± 1.9	42.8 ± 2.7		0.59 ± 0.09	0.08 ± 0.01
Electrical stimulation (monophasic)	48.3 ± 3.6	16.7 ± 1.8		0.19 ± 0.01	0.21 ± 0.02

droxylase for $DMPH_4$ was 0.87 mM in control preparations, 0.54 mM in chloral hydrate-treated preparations, and 0.23 mM in stimulated preparations.

A further study was carried out to see whether stimulation would cause the tyrosine hydroxylase isolated from the neostriatum to have an altered affinity for dopamine. In these experiments the K_i of tyrosine hydroxylase for dopamine was determined at three concentrations of $DMPH_4$ and at six concentrations of dopamine. The tyrosine concentration in these experiments was 0.1 mM.

As shown in Fig. 4 and Table 2, the K_i for dopamine of the enzyme prepared from

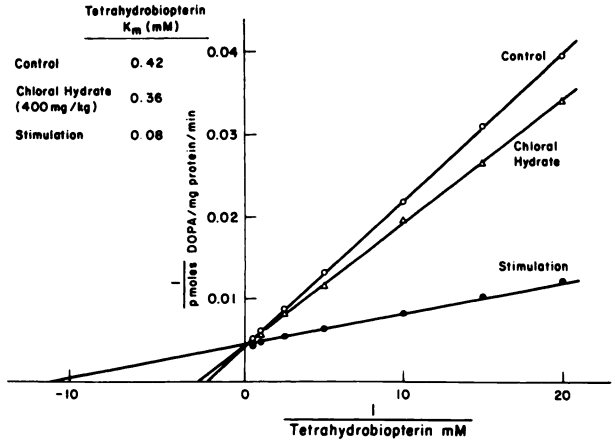

FIG. 3. *Effect of electrical stimulation on K_m for tetrahydrobiopterin of neostriatal tyrosine hydroxylase*
Control enzyme was isolated from neostriata of untreated rats. Chloral hydrate enzyme was isolated from neostriata contralateral to the stimulation. The nigro-neostriatal pathway was stimulated as described in METHODS for 20 min with 15-Hz, 400-μamp, biphasic, 3.0-msec electrical pulses. The animals were killed immediately, and the K_m for BH_4 was determined at a tyrosine concentration of 100 μM and seven BH_4 concentrations ranging from 50 μM to 2 mM. Each value for K_m is the mean of the intercepts generated from six separate lines.

chloral hydrate-anesthetized rats (0.06 mM) was not significantly different from the K_i for dopamine of the enzyme prepared from untreated control animals (0.05 mM). However, biphasic electrical stimulation of the nigro-neostriatal pathway produced a large increase in the K_i of neostriatal tyrosine hydroxylase for dopamine, to 0.29 mM. Similar changes in kinetic parameters were found in tyrosine hydroxylase isolated from neostriata stimulated with monophasic pulses (Table 2).

Effect of electrical stimulation on olfactory tubercle tyrosine hydroxylase. Analysis of tyrosine hydroxylase isolated from olfactory tubercles indicated that electrical stimulation of the mesolimbic neurons causes changes in tyrosine hydroxylase in olfactory tubercles similar to changes found in neostriatal tyrosine hydroxylase following stimulation of the nigro-neostriatal pathway (Table 3). Stimulation caused a 57% increase in enzyme activity. In view of this observation, we also investigated the kinetic properties of tyrosine hydroxylase prepared from the olfactory tubercles following stimulation of the mesolimbic pathway. A 53% decrease in the K_m for $DMPH_4$ was found, along with a 44% increase in the K_i for dopamine, when compared with enzyme from contralateral olfactory tubercles.

Effect of cyclic AMP on neostriatal tyrosine hydroxylase. Recent reports that neostriatal tyrosine hydroxylase can be activated by cyclic AMP (21-28) led us to examine the effect of this nucleotide on the enzyme prepared from neostriata obtained from nonstimulated and stimulated preparations. It was found that addition of cyclic AMP (50 μM) to the assay mixture 10 min before addition of labeled tyrosine caused an activation of the enzyme prepared from control and chloral hydrate-treated animals similar to the activation produced by electrical stimulation (Table 4). Thus, addition of cyclic AMP produced an increase in total activity of the enzyme, a decrease in the K_m of tyrosine hydroxylase for tyrosine and BH_4, and an increase in the K_i of the enzyme for dopamine.

Addition of cyclic AMP to the assay solution containing the enzyme isolated from stimulated neostriata, however, had no significant effect on the total activity of tyrosine hydroxylase or on the kinetic parameters of the enzyme beyond the effects of stimulation alone.

DISCUSSION

It has been shown that stimulation of central dopaminergic neurons, whether by addition of high potassium concentrations to the incubation medium (29) or by elec-

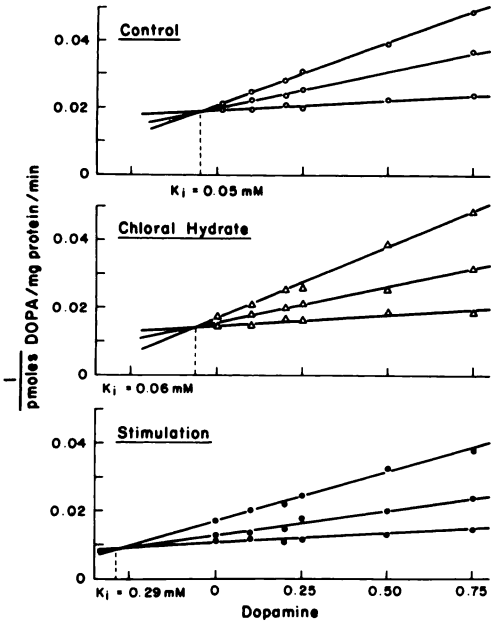

FIG. 4. *Effect of electrical stimulation on K_i for dopamine of neostriatal tyrosine hydroxylase*

Control enzyme was isolated from neostriata of untreated rats. Chloral hydrate enzyme was isolated from neostriata contralateral to the stimulation. The nigro-neostriatal pathway was stimulated as described in METHODS for 20 min with 400-μamp biphasic electrical pulses of 15 Hz and 3.0-msec duration. The animals were killed immediately, and the neostriata were dissected. The K_i for dopamine was determined at a tyrosine concentration of 100 μM at 1, 10, and 100 μM $DMPH_4$, and at seven dopamine concentrations (six shown) from 1 μM to 1 mM. The values for K_i are the means of six determinations. Dopamine values are plotted as millimolar concentrations.

trical stimulation (7), causes an increase in the synthesis of dopamine in the terminal regions of these neurons. Harris and Roth (29) found no increase in synthesis when labeled dopa was used as precursor, indicating that the increase in dopamine synthesis occurred at the tyrosine hydroxylase step. In recent experiments we followed dopa accumulation after inhibition of central dopa decarboxylase as an indication of tyrosine hydroxylase activity *in vivo*, and confirmed that stimulation of the nigro-neostriatal pathway produces an increase in neostriatal tyrosine hydroxylase activity *in vivo* (7). Studies on the poststimulation accumulation of dopa and poststimulation increases in the conversion of labeled tyrosine to dopamine suggested that this stimulation-induced increase in tyrosine hydroxylase activity persisted for some time after the cessation of stimulation and that it would be feasible to study the poststimulation activation of tyrosine hydroxylase more closely under isolated conditions.

Initial experiments using a tyrosine hydroxylase assay *in vitro* confirmed that tyrosine hydroxylase was activated by an increase in impulse flow in the nigro-neostriatal neurons, produced by electrical stimulation (10, 17) (Table 2). The enzyme from stimulated neostriata was about 50% more active than enzyme from contralateral neostriata when compared with control enzyme. Subsaturating levels of tyrosine and $DMPH_4$ were used in these experiments so that a change in enzyme activity could readily be demonstrated. In the presence of saturating concentrations of substrate and cofactor no change in enzyme activity was observed, since the activated enzyme showed no significant change in V_{max} when compared with the enzyme prepared from unstimulated tissue.

Tyrosine hydroxylase was found to be almost fully activated by 10 min of continuous electrical stimulation at a frequency of 15 Hz (Table 1). The observation that there was no significant increase in activity after 5 min of stimulation and no further significant increase in activity following 15 and 20 min of stimulation suggests that the increase in tyrosine hydroxylase activity produced by stimulation may be dependent both on the frequency and on the number of impulses delivered. This idea is also suggested by data on dopa accumulation following decarboxylase inhibition as measure of tyrosine hydroxylase activity *in vivo* (7).

Tyrosine hydroxylase was found to remain fully activated for at least 10 min and partially activated for 15 min following the termination of stimulation (Fig. 1). These data provide an explanation for the poststimulation increases in dopamine specific activity following administration of labeled tyrosine and for the increase in the accumulation of dopa in neostriata which we reported earlier (7). They also indicate that the period of time necessary

TABLE 3

Effect of electrical stimulation on activity and kinetic parameters of olfactory tubercle tyrosine hydroxylase

Chloral hydrate data were obtained for enzyme from olfactory tubercles contralateral to stimulation. The median forebrain bundle was stimulated as described in METHODS for 20 min with 15-Hz biphasic electrical pulses of 400 μamp and 3.0-msec duration. The animals were killed immediately after stimulation, and tyrosine hydroxylase was isolated and assayed as described in METHODS. K_m values were determined by the method of Lineweaver and Burk (13), and K_i values, by the method of Dixon (14). Total activity was determined in the presence of subsaturating concentrations of tyrosine (10 μM) and $DMPH_4$ (100 μM). The K_m for tyrosine was determined at 1 mM $DMPH_4$ and with six tyrosine concentrations ranging from 1 to 100 μM. The K_m for $DMPH_4$ was determined at 100 μM tyrosine and at seven $DMPH_4$ concentrations ranging from 100 μM to 5 mM for control enzyme and from 50 μM to 1 mM for chloral hydrate-treated and stimulated enzyme. The K_i for dopamine was determined at 100 μM tyrosine, at 1, 10, and 100 μM $DMPH_4$, and at six dopamine concentrations (25–750 μM). Results are means ± standard errors of three determinations.

Treatment	Tyrosine hydroxylase activity	K_m for tyrosine	K_m for $DMPH_4$	K_i for dopamine
	pmoles dopa/mg protein/min	*μM*	*mM*	*mM*
None	8.4 ± 0.8	55.7 ± 5.1	0.96 ± 0.13	0.19 ± 0.02
Chloral hydrate (400 mg/kg)	10.1 ± 0.9		0.58 ± 0.02	0.25 ± 0.01
Chloral hydrate + electrical stimulation	15.9 ± 5.8		0.27 ± 0.09	0.36 ± 0.07

TABLE 4

Effect of cyclic AMP on neostriatal tyrosine hydroxylase

Cyclic AMP data represent values for enzyme incubated with cyclic AMP as described in METHODS. Chloral hydrate data are for enzyme from neostriata contralateral to stimulation. The nigro-neostriatal pathway was stimulated as described in METHODS for 20 min with 15-Hz, 400-μamp, 3.0-msec, biphasic electrical pulses. The animals were killed immediately after stimulation, and tyrosine hydroxylase was isolated and assayed as described in METHODS. Cyclic AMP (50 μM) was added 10 min prior to tyrosine addition. Total activity was determined in the presence of subsaturating concentrations of tyrosine (10 μM) and $DMPH_4$ (100 μM). The K_m for tyrosine was determined at 1 mM $DMPH_4$ and seven tyrosine concentrations ranging from 0.5 to 100 μM. The K_m for BH_4 was determined at 100 μM tyrosine and seven BH_4 concentrations ranging from 50 μM to 2 mM. The K_i for dopamine was determined at 100 μM tyrosine, at 1, 10, and 100 μM BH_4, and at seven dopamine concentrations (10 μM–1 ma5m). Results are the means ± standard errors of six determinations.

Additions to assay	Treatment	Tyrosine hydroxylase activity	K_m for tyrosine	K_m for BH_4	K_i for dopamine
		pmoles dopa/mg protein/min	*μM*	*mM*	*mM*
None	None	22.6 ± 2.8	54.7 ± 5.1	0.42 ± 0.08	0.05 ± 0.02
Chloral hydrate (400 mg/kg)	None	31.5 ± 2.6	41.5 ± 3.6	0.36 ± 0.09	0.06 ± 0.03
None	Cyclic AMP (50 μM)	40.1 ± 3.2	24.9 ± 1.7	0.05 ± 0.01	0.38 ± 0.06
Chloral hydrate	Cyclic AMP (50 μM)	36.7 ± 2.8	26.1 ± 1.3	0.07 ± 0.02	0.34 ± 0.08
Chloral hydrate + electrical stimulation	None	47.2 ± 5.1	17.1 ± 1.6	0.08 ± 0.01	0.29 ± 0.04
Chloral hydrate + electrical stimulation	Cyclic AMP (50 μM)	48.1 ± 1.7	20.2 ± 1.1	0.06 ± 0.03	0.39 ± 0.05

for dissection and freezing of the neostriata after the death of the animal (about 3 min) is insufficient for any significant decay in the activation of the enzyme.

The fact that it was possible to demonstrate this poststimulation increase in tyrosine hydroxylase activity in tissue that had been subjected to freezing, thawing, and homogenization made it feasible to study the properties of this activated en-

zyme in more detail by examining the kinetic properties of the isolated enzyme.

These studies revealed that the affinity of tyrosine hydroxylase for substrate tyrosine was increased during periods of increased impulse flow in the nigro-neostriatal neurons (Table 2 and Fig. 2). Chloral hydrate, which has also been shown to cause an increase in firing rate of nigro-neostriatal dopaminergic neurons (18), caused a small decrease in K_m, from 54.7 to 41.5 μM, in controls. Electrical stimulation caused a dramatic further decrease in K_m, to 17.1 μM. This represents a 3-fold change from control neostriata and a 2.5-fold change from the contralateral, nonstimulated neostriata. No significant change in V_{max} due to electrical stimulation was observed (Fig. 2). The control value for K_m is similar to that reported by Kuczenski and Mandell (30) and by Zivkovic *et al.* (31) for tyrosine hydroxylase from rat neostriatum. Alterations in K_m for tyrosine were not seen by others following treatment with neuroleptic drugs (26, 31) which are known to increase firing in dopaminergic neurons (18). The reason for this discrepancy is probably related to small differences in assay conditions. In our own laboratory, when tyrosine hydroxylase assays were carried out in a volume of 0.3 ml and on low-speed rather than high-speed striatal supernatants, we have not observed any significant change in V_{max} or K_m of the enzyme for tyrosine after treatment of rats with haloperidol (1 mg/kg), although the decrease in K_m for pterin cofactor was still demonstrable.[4]

The physiological importance of this change in affinity for tyrosine observed in the experiments reported above is unknown, but from data presently available it seems unlikely that this change is responsible for the increase in tyrosine hydroxylase activity observed *in vivo*. Tyrosine levels in the rat brain are about 100 μm (32), and the concentration of tyrosine found in the rat neostriatum is also in the range of 100 μM (7). This level of tyrosine is roughly twice the K_m value when K_m is measured with $DMPH_4$ as cofactor, and about 7 times the K_m measured using BH_4 as cofactor (19). Since BH_4 or a close derivative appears to be the natural cofactor, the enzyme is probably saturated with tyrosine *in vivo*. Moreover, Kaufman (19) has reported that maximal tyrosine hydroxylase activity in brain is attained with tyrosine at 100 μM. Higher concentrations of tyrosine began to inhibit the enzyme. Thus the change in K_m of tyrosine hydroxylase for tyrosine is probably not of great physiological importance.

Changes in the affinity of neostriatal tyrosine hydroxylase for the cofactor, BH_4, were similar to the changes seen in the affinity for tyrosine (Table 2 and Fig. 3). Chloral hydrate treatment caused a small, statistically insignificant increase in the enzyme affinity for BH_4. Electrical stimulation produced a large increase in affinity for BH_4, with the K_m decreasing to 0.08 mM from 0.42 mM in control preparations. This represents a 5-fold and 4-fold decrease from control and chloral hydrate-treated preparations, respectively.

The physiological importance of this change is uncertain. BH_4 is thought to be the natural cofactor for tyrosine hydroxylase, and there is evidence which suggests that the concentrations of BH_4 in the neuron may be rate-limiting with respect to tyrosine hydroxylase activity (20, 33, 34). Thus, the increased affinity of neostriatal tyrosine hydroxylase for BH_4 seen with electrical stimulation would bring the concentration of BH_4 *in vivo* closer to levels necessary for saturation of the enzyme and thus effectively increase the rate of tyrosine hydroxylation. Stimulation of the nigro-neostriatal pathway also increased the affinity for the artificial cofactor, $DMPH_4$ (Table 2). The control value for K_m for $DMPH_4$ is similar to that reported by others (30, 31). Similar alterations in the tyrosine hydroxylase K_m for cofactor due to treatment with neuroleptic drugs have been reported (10, 17, 26, 31).

No significant changes in V_{max} were seen with tyrosine or BH_4 (Figs. 3 and 4). This indicates that, over the time periods studied, no new enzyme was being synthesized, nor was enzyme being mobilized from an inactive to an active form.

Electrical stimulation at a frequency of

[4] J. R. Simon, and R. H. Roth, unpublished observations.

15 Hz also caused a dramatic increase in K_i of neostriatal tyrosine hydroxylase for dopamine, reflecting a large decrease in the ability of dopamine to inhibit the enzyme. The K_i for control enzyme was 0.05 mM dopamine; the K_i for the enzyme prepared from neostriata of chloral hydrate-treated animals was 0.06 mM dopamine, and the K_i for enzyme prepared from the neostriata on the stimulated side was 0.29 mM dopamine. This represents a 6-fold increase over control preparations and a 5-fold increase over contralateral chloral hydrate-treated neostriata. The K_i for control tyrosine hydroxylase is similar to that reported by Kuczenski and Mandell (30) for soluble tyrosine hydroxylase treated with heparin.

Again, the physiological significance of this stimulus-dependent alteration in the properties of tyrosine hydroxylase is not known with certainty, since the concentration of dopamine in the vicinity of the enzyme in the nerve terminal is unknown. As with the change in K_m for BH_4, there is a strong possibility that change in the K_i of neostriatal tyrosine hydroxylase for dopamine plays an important role in the increased activity of tyrosine hydroxylase observed *in vivo* during periods of increased impulse flow. Feedback inhibition of tyrosine hydroxylase by its end products has been considered an important factor in the regulation of this enzyme for some time. Nagatsu and co-workers (35) first introduced this idea when they reported that tyrosine hydroxylase was inhibited by norepinehrine. That end product feedback inhibition might be a physiological factor was suggested by Alousi and Weiner (1) when they found that incubation of the guinea pig hypogastric nerve–vas deferens preparation in the presence of norepinephrine reduced norepinephrine synthesis and inhibited the increase in synthesis due to stimulation. This concept was supported by work from a number of laboratories (8, 36–38).

The mechanism generally proposed for increased tyrosine hydroxylase activity due to stimulation was that release of norepinephrine from the nerve terminal depleted a small, strategic pool of norepinephrine which regulated tyrosine hydroxylase activity by feedback inhibition. There was evidence, however, that this might not be the complete answer (39–41). The data presented here and other data from this laboratory (9) suggest that equally or perhaps more important is a dramatic decrease in the ability of end product to inhibit tyrosine hydroxylase. This decrease in the ability of dopamine to inhibit tyrosine hydroxylase in dopaminergic neurons, coupled with an increase in enzyme affinity for BH_4, offers an alternative explanation for the large increases in tyrosine hydroxylase activity which occur during periods of increased impulse flow.

Kinetic changes similar to those reported above were seen in tyrosine hydroxylase isolated from olfactory tubercles following stimulation of the median forebrain bundle (Table 3). Stimulation produced about a 53% decrease in K_m for $DMPH_4$ and a 44% increase in the K_i for dopamine. Thus the mesolimbic dopaminergic system reacts to increased impulse flow in a manner similar to that of the nigro-neostriatal dopaminergic system.

Results similar to those described above have also been reported in the central noradrenergic neurons with cell bodies in the locus ceruleus and terminals in the hippocampus (42, 43).

Evidence that cyclic AMP and its derivatives are able to activate neostriatal tyrosine hydroxylase (21–28) and results of this study demonstrating that cyclic AMP produces this activation by a kinetic alteration of tyrosine hydroxylase which is quite similar to that produced by electrical stimulation (Table 4) suggest that these two activations may occur by a similar mechanism. In our studies we have found that cyclic AMP addition alone is able to activate the tyrosine hydroxylase present in the high-speed supernatant obtained from the striatum or hippocampus, and this activation is prevented if the enzyme preparation is purified on a Sephadex G-25 column (24, 25, 43, 44). This differs from the results reported by Lovenberg *et al.* (26) and Goldstein *et al.* (27), who observed activation only when cyclic AMP was added together with ATP and Mg^{++}. This discrepancy is perhaps explained by use of

Triton X-100 or some other variable in the assay procedure. In the absence of a detergent, or perhaps because of more rapid dissection of the tissue, the brain supernatant may contain sufficient quantities of ATP (also protein kinase and Mg^{++}) to produce some degree of enzyme activation without further additions.

Since it is known that during neuronal depolarization there is a significant increase in the accumulation of endogenous cyclic AMP (45, 46), it seems likely that this event could mediate the observed activation of tyrosine hydroxylase produced during electrical stimulation of the nigro-neostriatal pathway. However, numerous other mechanisms are plausible, and more direct studies are necessary before it can be concluded that cyclic nucleotides play a role in this activation of tyrosine hydroxylase *in vivo*.

In conclusion, these experiments provide evidence that the nigro-neostriatal and mesolimbic dopaminergic neurons respond to electrical stimulation in a manner similar to the response of peripheral and central noradrenergic neurons to electrical stimulation. Thus the dopaminergic neurons increase synthesis of dopamine by an increase in activity of tyrosine hydroxylase that is characterized by kinetic alterations of the enzyme. This activation takes a finite period of time to manifest itself and decays within about 15 min following the termination of the stimulation period. Experiments are in progress in an attempt to define further the mechanisms involved in this stimulus-induced activation of neostriatal tyrosine hydroxylase.

REFERENCES

1. Alousi, A. & Weiner, N. (1966) *Proc. Natl. Acad. Sci. U. S. A.* **56**, 1491–1496.
2. Gordon, R., Reid, J. V. O., Sjoerdsma, A. & Udenfriend, S. (1966) *Mol. Pharmacol.*, **2**, 610–613.
3. Roth, R. H., Stjärne, L. & von Euler, U. S. (1966) *Life Sci.*, **5**, 1071–1075.
4. Roth, R. H., Stjärne, L. & von Euler, U. S. (1967) *J. Pharmacol. Exp. Ther.*, **158**, 373–377.
5. Sedvall, G. C. & Kopin, I. J. (1967) *Life Sci.*, **6**, 45–51.
6. Murrin, L. C. & Roth, R. H. (1973) *Pharmacologist*, **15**, 247.
7. Murrin, L. C. & Roth, R. H. (1976) *Mol. Pharmacol.*, **12**, 463–475.
8. Weiner, N. & Rabadjija, M. (1968) *J. Pharmacol. Exp. Ther.*, **160**, 61–71.
9. Morgenroth, V. H., III, Boadle-Biber, M. C. & Roth, R. H. (1974) *Proc. Natl. Acad. Sci. U. S. A.*, **71**, 4283–4287.
10. Murrin, L. C., Morgenroth, V. H., III & Roth, R. H. (1974) *Pharmacologist*, **16**, 213.
11. König, J. F. R. & Klippel, R. A. (1970) *The Rat Brain: a Stereotaxic Atlas of the Forebrain and Lower Parts of the Brain Stem*, Krieger, New York.
12. Shiman, R., Akino, M. & Kaufman, S. (1971) *J. Biol. Chem.*, **246**, 1330–1340.
13. Coyle, J. T. (1972) *Biochem. Pharmacol.*, **21**, 1935 -1944 .
14. Lineweaver, H. & Burk, D. (1934) *J. Am. Chem. Soc.*, **56**, 658–666.
15. Dixon, M. (1953) *Biochem. J.*, **55**, 170–171.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265–275.
17. Roth, R. H., Walters, J. R., Murrin, L. C. & Morgenroth, V. H., III (1975) in *Pre- and Postsynaptic Receptors* (Usdin, E. & Bunney, W. E., Jr., eds.), pp. 5–48, Marcel Dekker, New York.
18. Bunney, B. S., Walters, J. R., Roth, R. H. & Aghajanian, G. K. (1973) *J. Pharmacol. Exp. Ther.*, **185**, 560–571.
19. Kaufman, S. (1974) in *Aromatic Amino Acids in the Brain*, pp. 85–108, Elsevier, Amsterdam.
20. Kettler, R., Bartholini, G. & Pletscher, A. (1974) *Nature*, **249**, 476–478.
21. Goldstein, M., Anagnoste, B. & Shirron, C. (1973) *J. Pharm. Pharmacol.*, **25**, 340–351.
22. Richelson, E. (1973) *Nat. New Biol.*, **242**, 175–177.
23. Anagnoste, B., Shirron, C., Friedman, E. & Goldstein, M. (1974) *J. Pharmacol. Exp. Ther.*, **191**, 370–376.
24. Harris, J. E., Morgenroth, V. H., III, Roth, R. H. & Baldessarini, R. J. (1974) *Nature*, **252**, 175–177.
25. Harris, J. E., Baldessarini, R. J., Morgenroth, V. H. & Roth, R. H. (1975) *Proc. Natl. Acad. Sci. U. S. A.*, **72**, 789–793.
26. Lovenburg, W., Bruckwick, E. A. & Hanbauer, I. (1975) *Proc. Natl. Acad. Sci. U. S. A.*, **72**, 2955–2958.
27. Goldstein, M., Ebstein, B., Bronaugh, R. L. & Roberge, C. (1975) in *Chemical Tools in Catecholamine Research II* (O. Almgren, A. Carlsson & J. Engel, eds.), pp. 257–265, American Elsevier, New York.
28. Lloyd, T. & Kaufman, S. (1975) *Biochem. Biophys. Res. Commun.*, **66**, 907–913.

29. Harris, J. E. & Roth, R. H. (1971) *Mol. Pharmacol.*, 7, 593–604.
30. Kuczenski, R. T. & Mandell, A. J. (1972) *J. Biol. Chem.*, 247, 3114–3122.
31. Zivkovic, B., Guidotti, A. & Costa, E. (1974) *Mol. Pharmacol.*, 10, 727–735.
32. Tallan, H. H. (1962) in *Amino Acid Pools* (Holden, J. T., ed.), pp. 471–485, Elsevier, New York.
33. Musacchio, J. M., D'Angelo, G. L. & McQueen, C. A. (1971) *Proc. Natl. Acad. Sci. U. S. A.* 68, 2087–2091.
34. Lloyd, T. & Weiner, N. (1971) *Mol. Pharmacol.*, 7, 569–580.
35. Nagatsu, T., Levitt, M. & Udenfriend, S. (1964) *J. Biol. Chem.*, 239, 2910–2917.
36. Spector, S., Gordon, R., Sjoerdsma, A. & Udenfriend, S. (1967) *Mol. Pharmacol.*, 3, 549–555.
37. Stjärne, L., Lishajko, F. & Roth, R. H. (1967) *Nature*, 215, 770–772.
38. Kupferman, A., Gillis, C. N. & Roth, R. H. (1970) *J. Pharmacol. Exp. Ther.*, 171, 214–222.
39. Boadle-Biber, M. C., Hughes, J. & Roth, R. H. (1970) *Br. J. Pharmacol.*, 40, 702–720.
40. Carlsson, A., Kehr, M., Lindqvist, M., Magnusson, T. & Atack, C. V. (1972) *Pharmacol. Rev.*, 24, 371–384.
41. Cloutier, G. & Weiner, N. (1973) *J. Pharmacol. Exp. Ther.*, 186, 75–85.
42. Roth, R. H., Salzman, P. M. & Morgenroth, V. H., III (1974) *Biochem. Pharmacol.*, 23, 2779–2784.
43. Roth, R. H., Morgenroth, V. H., III & Salzman, P. M. (1975) *Naunyn-Schmiedebergs Arch. Pharmacol.*, 289, 327–343.
44. Morgenroth, V. H., III, Hegstrand, L. R., Roth, R. H. & Greengard, P. (1975) *J. Biol. Chem.*, 250, 1946–1948.
45. Kakiuchi, S., Rall, T. W. & McIlwain, H. (1969) *J. Neurochem.*, 16, 485–491.
46. Shimizu, H., Creveling, C. R. & Daly, J. W. (1970) *Adv. Biochem. Psychopharmacol.*, 3, 135–154.