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Tetrahydrobiopterin in Striatum: Localization in Dopamine Nerve Terminals and Role in Catecholamine Synthesis

Abstract. *The hydroxylase cofactor, tetrahydrobiopterin, and its biosynthetic system are localized in dopaminergic nerve terminals in the striatum. This conclusion is based on the nearly equivalent loss of tyrosine hydroxylase and tetrahydrobiopterin and its initial biosynthetic enzyme, guanosine triphosphate cyclohydrolase, after injection of 6-hydroxydopamine into the substantia nigra. The role of the hydroxylase cofactor in the regulation of dopamine synthesis is reassessed.*

The initial and rate-limiting reaction in the biosynthesis of catecholamine neurotransmitters (dopamine, norepinephrine, and epinephrine) is catalyzed by tyrosine hydroxylase. This enzyme requires molecular oxygen and the reduced form of the hydroxylase cofactor, tetrahydrobiopterin (BH_4), for the conversion of tyrosine to L-dopa, which is then decarboxylated to form dopamine in dopaminergic neurons of the central nervous system (CNS). Tetrahydrobiopterin is also required for tryptophan hydroxylase activity in the neurons that produce the other major biogenic amine neurotransmitter, serotonin. We previously reported that the distribution of hydroxylase cofactor across areas of the rat brain was positively correlated with the total (tyrosine plus tryptophan) hydroxylase enzyme activity (*1*). Since these enzymes are markers for aminergic neurons, our results suggested that BH_4 was at least highly concentrated if not exclusively localized in aminergic neurons in certain areas of the brain. We also reported that the hydroxylase cofactor content in cerebrospinal fluid (CSF) from patients with Parkinson's disease was reduced to 50 percent compared to control (*2*). Since Parkinson's disease is characterized by a degeneration of nigrostriatal dopaminergic neurons, a considerable portion of CSF cofactor appears to be derived from these cells.

In the brain BH_4 is synthesized de novo from guanosine triphosphate (GTP). Although the enzymatic steps in BH_4 biosynthesis have not been completely elucidated, it is accepted that the initial step is the conversion of GTP to dihydroneopterin triphosphate. This reaction is catalyzed by the enzyme GTP cyclohydrolase (*3*). Just as tyrosine hydroxylase serves as a marker enzyme for catecholaminergic neurons, GTP cyclohydrolase should serve as a marker for cells with BH_4 synthesizing capability.

Tyrosine hydroxylase can be activated by such processes as direct protein phosphorylation (*4*) and limited tryptic digestion (*5*) as well as by anions (*6*) and phospholipids (*7*). Activation by protein phosphorylation is expressed in kinetic terms by a lower Michaelis constant (K_m) of tyrosine hydroxylase for its cofactor BH_4 (*4*), and consequently the enzyme is more active in the presence of suboptimal BH_4 concentrations. The decreased K_m for BH_4 is important because of evidence suggesting that intraneuronal concentrations of BH_4 are subsaturating and thus limit the activity of tyrosine hydroxylase in the dopaminergic neurons of the nigrostriatal system (*8, 9*). To develop greater understanding of the cellular localization and site of biosynthesis of BH_4 in the CNS, we studied the nigrostriatal system of the rat as a model aminergic area because of the high concentration of dopaminergic neurons and the functional importance of this area in certain neurological disorders, notably Parkinson's disease.

The specific neurotoxin 6-hydroxydopamine (6-OHDA) was injected (*10*) into the left substantia nigra of male Sprague-Dawley rats to selectively destroy the dopamine neurons whose cell bodies, which are located in the nigra, project to and terminate in the corpus striatum. The contralateral striatum serves as a control since neurotoxic damage is limited to the nigra and striatum ipsilateral to the lesion. The success of the 6-OHDA lesion was tested by monitoring apomorphine-induced rotational behavior (*11*); only animals making three or more clockwise turns per minute were used in biochemical analyses. Striatal tyrosine hydroxylase activity was measured as described in (*12*). Reduced and oxidized forms of biopterin in the striatum were measured by native fluorescence after high-pressure liquid chromatography (*13*). Striatal GTP cyclohydrolase activity was measured by monitoring the conversion of GTP to dihydroneopterin triphosphate as described by Nixon *et al.* (*14*).

The results of the experiments (Table 1) show that tyrosine hydroxylase was depleted in the striatum on the lesioned side by 93 percent in comparison with the control activity in the contralateral striatum. This decrease in striatal tyrosine hydroxylase activity together with the clockwise turning exhibited by the same animals in response to apomorphine indicated almost complete destruction of nigrostriatal dopaminergic neurons. The 6-OHDA lesion also caused a 73 percent loss of total biopterin in the ipsilateral striatum as well as a 68 percent loss of striatal GTP cyclohydrolase activity (Table 1). Bullard *et al.* (*15*), who administered 6-OHDA intraventricularly, suggested that there was some association of reduced pterins with dopaminergic neurons, although the intraven-

Table 1. Effects of unilateral 6-hydroxydopamine injection in the substantia nigra on biopterin and related enzymes in rat striatum. Values are mean ± the standard error of the mean.

Treatment	GTP-Cyclohydrolase (pmole/hour per milligram of protein)	Biopterin (μg/g, wet weight)	Tyrosine hydroxylase (nmole/min per milligram of protein)
Control ($N = 6$)	3.85 ± 0.92	0.33 ± 0.01	2.50 ± 0.04
6-Hydroxydopamine ($N = 6$)	1.22 ± 0.43*	0.09 ± 0.01†	0.17 ± 0.09†
Percent decrease	68	73	93

*$P < .01$ and †$P < .001$; significantly different from control values as determined by Student's paired t-test.

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tricular injection was not totally effective in depleting cofactor.

Our studies with both lesioned and control striata confirm reports (*13, 16*) that over 90 percent of biopterin is in the reduced (tetrahydro) form. Our results also indicate that in the striatum the majority of BH_4 and the capacity to synthesize the cofactor are specifically located in dopamine neurons. The fact that tyrosine hydroxylase activity was depleted slightly more than either biopterin or GTP cyclohydrolase would be expected because of a small percentage of serotonergic innervation of the striatum. Since tryptophan hydroxylase in serotonergic neurons also requires BH_4 for activity, it is likely that the GTP cyclohydrolase and BH_4 remaining in the striatum after the nigral lesion reside in serotonergic nerve terminals whose cell bodies are located in other regions.

These observations support the suggestion that BH_4 is exclusively localized within aminergic neurons throughout the brain (*1, 17*). Alternatively BH_4 may be present in nondopaminergic postsynaptic neurons, which are depleted of cofactor when the dopamine innervation is removed. This explanation would require that most of the BH_4 be present in nondopaminergic structures of the striatum. This seems unlikely in view of our observation (*18*) that BH_4 and tyrosine hydroxylase subunits are present in the striatum in nearly equimolar amounts. Since tyrosine hydroxylase is contained only within dopaminergic terminals, most of the tyrosine hydroxylase subunits would have no immediate access to cofactor molecules.

From calculations based on the gross wet weight of striatal tissue, it was reported that the intracellular concentration of BH_4 within striatal dopamine terminals was approximately 1 μM (*1, 15*). Our results, which demonstrate a high degree of cofactor localization in striatal dopamine nerve terminals, provide the opportunity to more accurately assess the intracellular BH_4 concentration. It is difficult to estimate the small volume contributed by dopamine nerve terminals to the total volume of the striatum, since there is no apparent change in tissue mass after the 6-OHDA lesions. Only biochemical assays of specific aminergic markers or the lack of dopamine histochemical fluorescence (*19*) after 6-OHDA treatment reveals the loss of striatal terminals. However, if one assumes that 1 percent of the striatal volume is occupied by dopamine terminals, then the concentration of BH_4 in the terminal would be approximately 100 μM or possibly higher, depending on the degree of intraneuronal compartmentalization of BH_4. Our observations have important implications for the in vivo regulation of tyrosine hydroxylase activity by BH_4 concentrations and protein phosphorylation.

Recent studies (*20*) show that the K_m of nonphosphorylated striatal tyrosine hydroxylase for cofactor is *p*H-dependent; K_m values increase dramatically with *p*H above 5.8. The K_m of the phosphorylated form of the enzyme for BH_4 is considerably lower and appears to be independent of *p*H. As a result, the difference in K_m values between the activated and control enzyme at *p*H 6.8 could be large. Similarly the K_m values at *p*H 6.8 of adrenal tyrosine hydroxylase for cofactor appear to be between 10 and 30 μM for the phosphorylated form and 500 to 600 μM for the nonphosphorylated form (*18*). On the basis of these values and our estimate of 100 μM as the intraneuronal BH_4 concentration, it appears that the phosphorylated form of tyrosine hydroxylase would be saturated with cofactor and therefore optimally active. Conversely, the nonphosphorylated enzyme would be relatively inactive and would not contribute significantly to the endogenous synthesis of catecholamines. Thus, phosphorylation would represent a mechanism for rapidly increasing the rate of transmitter synthesis.

These findings have implications for the treatment of Parkinson's disease and other disorders in which a lack of sufficient biogenic amine production is suspected. Since most of the BH_4 and its biosynthetic machinery in the nigrostriatal system are localized in dopaminergic neurons, it is probable that the major if not exclusive physiologic role of BH_4 in this area is to serve as cofactor for tyrosine and tryptophan hydroxylases. Therapeutic administration of BH_4 or synthetic analogs should increase neurotransmitter synthesis specifically within aminergic neurons. This appears feasible since both BH_4 and a more lipophilic synthetic analog, 6-methyltetrahydropterin, have been shown to cross the blood-brain barrier and elevate whole brain and striatal cofactor concentrations (*16*). The kinetic data on tyrosine hydroxylase suggest that the intracellular concentration of BH_4 severely limits the activity of the nonphosphorylated enzyme and support other observations (*8, 9*) indicating that the intraneuronal concentration of BH_4 influences dopamine production. We have found that about 80 percent of tyrosine hydroxylase in striata from control animals is in the high K_m form; thus, exogenous administration of pharmacologic doses of cofactor could elevate the activity of this pool of tyrosine hydroxylase molecules and, thereby, accelerate dopamine synthesis in functional nigrostriatal dopaminergic neurons.

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11. Apomorphine was dissolved in saline (1.0 mg/ml) containing 0.1 mg/ml ascorbic acid and kept at 4°C while protected from light; 1.0 mg/kg was injected subcutaneously at the nape of the neck and animals were placed in a round bowl. After 5 minutes, an observer monitored animals for turning behavior for 30 minutes.
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Renin and Angiotensin: The Complete System Within the Neuroblastoma × Glioma Cell

Abstract. *Cells of the homogeneous hybrid line neuroblastoma × glioma (NG108-15) have many neuronal properties. Immunocytochemical tests show that they contain both immunoreactive renin and angiotensin; direct radioimmunoassays show that they are positive for renin, angiotensin I, and angiotensin II; enzymatic assays show that they contain angiotensinogen and converting enzyme as well. The renin appears to be present in an enzymatically inactive form that can be activated by trypsin and then blocked by antiserum to purified mouse submaxillary renin. Renin concentration and activity are increased by enhancing cellular differentiation with dibutyryl cyclic adenosine monophosphate or by serum withdrawal. These findings demonstrate a complete renin-angiotensin system within these neuron-like cells, and suggest that activation of intracellular renin could generate angiotensin II.*

The peripheral renin-angiotensin system is integral to blood pressure control and extracellular fluid volume homeostasis (*1*). The active hormone angiotensin II is generated within the circulation by sequential cleavage by renal renin of liver-derived angiotensinogen to form angiotensin I, and subsequent conversion of angiotensin I to angiotensin II by converting enzyme. Several lines of evidence suggest the possibility of a separate renin-angiotensin system intrinsic to the central nervous system (*2–5*). Exogenous administration of renin, angiotensin I, or angiotensin II into the brain ventricles raises blood pressure, stimulates drinking, and releases antidiuretic hormone and adrenocorticotropin (*2, 6*). Renin enzymatic activity, and renin and angiotensin immunoreactivity have been demonstrated in several regions of the brain (*4, 5*). It is not known whether neural tissue generates renin and angiotensin, or rather utilizes the components synthesized within kidney and liver. Also unknown is whether the renin-angiotensin cascade is completed within the circulation of the brain and then the products absorbed by neural tissue or whether some of the steps in angiotensin II generation can be completed within the nerve cells.

We have investigated a homogeneous cell line in tissue culture, the neuroblastoma × glioma (NG108-15) cell, as a possible model for understanding the renin-angiotensin system in neurons. Because of their nervelike properties these cells have proved helpful in investigations of neurotransmitter synthesis, synapse formation, and membrane excitability (*7*). We report here that they contain antigenic renin that must be activated in order to allow angiotensin I to be generated. They also contain a renin substrate, converting enzyme, angiotensin I, and angiotensin II, suggesting that the complete renin-angiotensin cascade may be completed within a single cell.

Cells of the NG108-15 line of mouse neuroblastoma × rat glioma were grown and passaged in Dulbecco's modification of Eagle's minimum essential medium, supplemented with 5 percent fetal bovine serum, $1 \times 10^{-4}M$ hypoxanthine, $1 \times 10^{-6}M$ aminopterin, and $1.6 \times 10^{-5}M$ thymidine. For radioimmunoassay, cells were washed three times with phosphate-buffered saline, sonicated for 9 seconds on ice, and immediately frozen in phosphate-buffered saline in a bath containing acetone and dry ice. The radioimmunoassay and immunocytochemistry for renin were performed with rabbit antibody to pure renin from mouse submaxillary gland. This renin was purified by the method of Cohen *et al.* (*8*) and appeared as a single band on both sodium dodecyl sulfate (SDS) and polyacrylamide gels. Its biochemical similarity to renal renin has been established (*9*). The direct renin radioimmunoassay was developed according to the method of Michelakis *et al.* (*10*) with iodination by the chloramine-T method. Sensitivity was 50 pg. Angiotensin I and angiotensin II radioimmunoassays were performed by the methods of Poulsen and Jorgensen (*11*) and Nussberger *et al.* (*12*), respectively.

Table 1. All components of the renin-angiotensin system are located in the NG108-15 cell. Only trace amounts of angiotensin I were generated before the cells were exposed to trypsin; the levels reported are those obtained after the cells were trypsinized by the method of Cooper *et al.* (*21*). Trypsinization was terminated after 20 minutes by soybean trypsin inhibitor. Control cells exposed to trypsin that had previously been mixed with inhibitor showed no activity. The values shown here were obtained without the addition of exogenous substrate; values were comparable with its addition. Antibody inhibition experiments were performed with the antiserum diluted 1:1000. The radioassay for converting enzyme was performed with [^{3}H]Hip-Gly-Gly used as substrate and ethyl acetate used for extraction at *p*H 8.0. In each case the converting enzyme inhibitor SQ 14,225 ($10^{-3}M$) blocked the converting enzyme activity of the cells. Cells were exposed to dibutyryl cyclic AMP (1 m*M*) in serum-containing medium for 4 days. Each assay was performed in duplicate and is reported as ± 1 standard deviation; *N* is the number of independent assays. The symbol (+) indicates that angiotensin I could be generated without exogenous substrate.

Group	Renin			Substrate (N = 4)	Angiotensin II (N = 2) (fmole/mg protein)	Converting enzyme	
	Concentration (N = 2) (pg/mg protein)	Activity* (N = 4)	Inhibition by antibody (%)			Activity (N = 3) (U/mg)	Inhibition by 10^{-3} *M* SQ 14,225 (%)
Control	34 ± 10	200 ± 30	87	+	62 ± 5	5.5 ± 0.1	91
Serum-free	132 ± 25	1000 ± 30	90	+	32 ± 3	5.5 ± 0.1	88
Dibutyryl cyclic AMP	Not tested	300 ± 20	92	+	99 ± 2	5.8 ± 0.1	96

*Measured as picograms of angiotensin I per milligram of protein per hour.

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