Tryptophan Hydroxylase Activity in Rat Brain Base Arteries Related to Innervation Originating From the Dorsal Raphe Nucleus

María Jesús Moreno, PhD; Angel Luis López de Pablo, PhD; Emilio J. Marco, PhD

Background and Purpose Tryptophan hydroxylase activity was assayed in cell-free extracts of rat brain base arteries as marker of a serotonergic innervation.

Methods Estimation of the enzymatic activity was made in untreated male Sprague-Dawley rats (n=53) and in those who underwent destruction of the dorsal and median raphe nuclei (n=10).

Results Tryptophan hydroxylase activity was measured in rat cerebral arteries. The time-dependent 5-hydroxytryptophan production was undetectable in the absence of tryptophan or 6-methyltetrahydropterine and in the presence of 6-fluorotryptophan, and it was significantly reduced in the presence of p-chlorophenylalanine. Destruction of the dorsal raphe nucleus but not the median raphe nucleus brought about a significant reduction in enzyme activity.

Conclusions These results suggest that rat cerebral arteries receive a serotonergic innervation arising from the dorsal raphe nucleus. (Stroke. 1994;25:1046-1049.)

Key Words • cerebral arteries • serotonin • raphe nuclei • rats

See Editorial Comment, page 1049

Serotonin can be found in the cerebral blood vessels of several animal species, including man1-4; its origin, however, remains a matter of controversy.

Biochemical and functional data indicate that the amine is stored in nerves arising from the raphe nuclei. Serotonin levels in cerebral blood vessels increase after the administration of tryptophan or a monoamine oxidase inhibitor and decrease after the animals are injected with p-chlorophenylalanine or the dorsal and medial raphe nuclei are destroyed.1-3,5 This serotonergic innervation seems functionally active, because the lesion of the dorsal raphe nucleus brings about an enhancement in the contractile response to serotonin of isolated cat middle cerebral artery6 and the electrical stimulation of the dorsal raphe nucleus reduces cerebral blood flow in several brain areas.7

The morphological evidence, however, shows that serotonin is bound to sympathetic nerve endings,8,9 although there is not a true serotonergic innervation of the cerebral blood vessels. The serotonin-like immunohistochemistry found in cerebral blood vessels disappears after cervical sympathectomy.10,11 It also disappears when the blood vessels are perfused with a saline solution or the animals are treated with amine uptake blockers.10-12 Thus, the serotonin revealed by histochemistry would be the result of its uptake by the sympathetic nerve terminals during the isolation procedure and not an actual neurotransmitter.

In vivo experiments indicate that tryptophan hydroxylase, the specific enzyme of the serotonin biosynthetic pathway, might be present in cerebral blood vessels. In animals the administration of an inhibitor of the aromatic amino acid decarboxylase brings about an increase in the 5-hydroxytryptophan (5-HTP) content in this kind of vessels.13 Such an increase is reduced when the main serotoninergic pathway is destroyed with 5,7-dihydroxytryptamine.14

However, other attempts to show the presence of tryptophan hydroxylase in the cerebral blood vessels have yielded conflicting results. Thus, Mathieu et al15 were unable to detect 5-HTP accumulation in isolated rat cerebral arteries after inhibiting the decarboxylase using an in vitro assay, whereas Cohen et al16 observed that the tryptophan hydroxylase-like immunoreactivity found in these vessels disappeared after cervical sympathectomy, although they could not demonstrate the same reactivity in the superior cervical ganglia.

The aim of the present work was to assay tryptophan hydroxylase activity in rat cerebral arteries by means of a classical in vitro biochemical method and determine whether this enzymatic activity might be linked to nerve fibers arising from the dorsal and median raphe nuclei.

Materials and Methods

Sixty-three male Sprague-Dawley rats, weighing 130 to 180 g, from the strain ICO:OFA SD (I.O.P.S. Caw) were used in the present study. The animals were housed in the proper facilities, complying with European Community directive 86/609/CEE and Spanish legislation (R.D. 223/1988) regarding the care of animals used in experimentation and other scientific purposes. The experiments reported here were approved by the Biosafety and Animal Care Unit Committee (Comisión de Bioseguridad y Gabinete Veterinario) of the Faculty of Medicine of the Autónoma University of Madrid.

In 10 anesthetized rats (35 mg/kg IP sodium pentobarbital) the dorsal and median raphe nuclei were destroyed by electrocaululation. The electrode was implanted according to the coordinates described by König and Klippel,17 and the rats were unable to detect 5-HTP accumulation in isolated cerebral arteries after inhibiting the decarboxylase using an in vitro assay, whereas Cohen et al16 observed that the tryptophan hydroxylase-like immunoreactivity found in these vessels disappeared after cervical sympathectomy, although they could not demonstrate the same reactivity in the superior cervical ganglia.

The aim of the present work was to assay tryptophan hydroxylase activity in rat cerebral arteries by means of a classical in vitro biochemical method and determine whether this enzymatic activity might be linked to nerve fibers arising from the dorsal and median raphe nuclei.

Received November 1, 1993; accepted January 19, 1994.

From the Departamento de Fisiología, Facultad de Medicina, Universidad Autónoma de Madrid (Spain). Correspondence to Dr Emilio J. Marco, Departamento de Fisiología, Facultad de Medicina, Universidad Autónoma de Madrid, Arzobispo Morcillo, 2, 28029 Madrid, Spain.
were killed 15 days later. The control group rats were subjected to the same procedure, but no current was passed through the electrode. The accuracy of the lesion placement was tested by measurement of tryptophan hydroxylase activity in the hippocampus and striatum.

After the rats were decapitated, the brain was quickly removed. The circle of Willis with some of its branches was dissected out, with the left hippocampus and striatum as well in the lesioned animals. The dissected tissues, and in some cases the whole brain, were frozen on dry ice and stored at −15°C.

The frozen brains were placed on a metal block cooled to −8°C, and frontal slices 0.5 to 1 mm thick were cut freehand with a razor blade. The slices were kept frozen on the block, and the dorsal raphe nucleus was punched out. The nucleus was identified following the description by König and Klippel.23

The assay of tryptophan hydroxylase was similar to that described by Meek and Neckers.18 The tissues were homogenized by sonication in 0.05 mol/L Tris buffer (pH 7.4) containing 10−3 mol/L mercaptoethanol and 0.05% Triton X-100. The volume used for the samples of cerebral arteries and dorsal raphe nuclei was 300 μL; for the hippocampi and striata, 600 μL. The homogenates were centrifuged at 12,000 rpm for 10 minutes in a Beckman microfuge. One hundred μL of the supernatant was added to an Eppendorf microcentrifuge tube with 20 μL of a standard reaction mixture containing, in 0.05 mol/L Tris buffer (pH 7.4), tryptophan (2 mmol/L), 6-methyltetrahydropteridine (16 mmol/mL), mercaptoethanol (1 mmol/L), and catalase (2.5 mg/mL). The tubes were incubated in a Suppelco Blok Heater at 37°C for 1 hour. Some cell-free extracts from cerebral blood vessels were incubated under the same conditions for 2 hours. The reaction was stopped by adding 20 μL of 11.64 mol/L HClO4. Blank or 0-hour tubes were made by adding the perchloric acid to the supernatants and shaking them before adding the reaction mixture. The samples were centrifuged at 12,000 rpm for 5 minutes, and 5-HTP was assayed in 20 μL of the supernatant by high-pressure liquid chromatography (HPLC) with fluorometric detection. Proteins were determined in the precipitates of the first homogenates by the method of Lowry et al,19 with 5-HTP enzymatic activity that was unchanged in hippocampus and striatum and cerebral arteries showed a decreased enzymatic activity that was unchanged in hippocampus.

In some instances, the standard reaction mixture was devoid of tryptophan or 6-methyltetrahydropteridine, or added 6-fluorotryptophan (1 mmol/L) or p-chlorophenylalanine (1 mmol/L).

The statistical analysis of the results was performed with Student's t test.

**Results**

When cell-free extracts of rat cerebral arteries were allowed to remain in contact with the standard reaction mixture, a time-dependent production of 5-HTP was obtained (Fig 1). No 5-HTP was detected when the reaction did not take place or the substrate or the cofactor was absent in the reaction mixture (Fig 1 and Table 1, respectively).

![Graph showing time course of 5-hydroxytryptophan (5-HTP) production by cell-free extracts of rat cerebral arteries. Each point represents mean±SEM values of five animals.](image)

Fig 1. Graph showing time course of 5-hydroxytryptophan (5-HTP) production by cell-free extracts of rat cerebral arteries. Each point represents mean±SEM values of five animals.

When p-chlorophenylalanine or 6-fluorotryptophan was added to the reaction mixture, a significant decrease in the production of 5-HTP was obtained (Table 1).

Two weeks after median raphe nucleus destruction, only tryptophan hydroxylase activity in hippocampus appeared significantly reduced, whereas it remained unaffected in striatum and brain base arteries (Fig 2). If the area affected by the lesion was dorsal raphe nucleus, striatum and cerebral arteries showed a decreased enzymatic activity that was unchanged in hippocampus (Fig 3).

Tryptophan hydroxylase activity measured in samples of dorsal raphe nucleus was 858±160 pmol/mg protein per hour.

**Discussion**

The present results support the existence of a serotonergic innervation impinging on the rat brain base arteries.

Tryptophan hydroxylase can be assayed in these blood vessels by means of a biochemical method. When a standard reaction mixture is in contact with cell-free extracts of rat cerebral arteries, there is a time-dependent production of 5-HTP. This 5-HTP is synthesized from tryptophan by the action of tryptophan hydroxylase. 

**Tryptophan Hydroxylase Activity In Cell-Free Extracts of Rat Cerebral Arteries In the Presence of p-Chlorophenylalanine and 6-Fluorotryptophan and the Absence of Tryptophan and 6-Methyltetrahydropteridine**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>5-HTP (pmol/mg protein/hr)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>35.25±4.28</td>
<td>8</td>
</tr>
<tr>
<td>+p-Chlorophenylalanine (1 mmol/L)</td>
<td>15.83±6.00*</td>
<td>5</td>
</tr>
<tr>
<td>+6-Fluorotryptophan (1 mmol/L)</td>
<td>ND</td>
<td>4</td>
</tr>
<tr>
<td>- Tryptophan</td>
<td>ND</td>
<td>5</td>
</tr>
<tr>
<td>- 6-Methyltetrahydropteridine</td>
<td>ND</td>
<td>5</td>
</tr>
</tbody>
</table>

n indicates number of samples; ND, not detectable. *P<.05 compared with control.
MRN LESION

![Bar graph showing effect of median raphe nucleus (MRN) lesion on tryptophan hydroxylase activity in hippocampus (HIP), striatum (STR), and cerebral blood vessels (CBV) of untreated (sham) and lesioned rats. Numbers in parentheses indicate number of animals used. *Significantly different (P<.05) from sham-operated rats.]

Previous studies have shown that the serotonergic innervation of rat cerebral arteries originates from dorsal and median raphe nuclei. Our present data suggest that only dorsal raphe nucleus innervates rat brain base arteries. The reason for this difference might be that in the present study only the major cerebral arteries were used, whereas previous studies have also included other cerebral microvessels. A similar result is obtained in cats, in which lesion of the dorsal but not the median raphe nucleus induces supersensitivity to serotonin in the middle cerebral artery.

The results of the present study agree with experiments demonstrating tryptophan hydroxylase activity in cerebral blood vessels in vivo. The discrepancy of the findings by Mathiau et al with this evidence might be due to methodological differences. These authors incubated the cerebral blood vessels in a medium devoid of enzyme cofactor that was in addition thoroughly bubbled with oxygen. Because cofactor concentration is critical for the correct activity of tryptophan hydroxylase, it is possible that their assay did not reach saturating conditions, as they assumed. Another explanation might be that they were under the limit of 5-HTP detection in their cerebral blood vessel preparation because according to the findings shown here, tryptophan hydroxylase activity in the nerve terminals of the vessels is 50 times less than that in dorsal raphe nucleus. Their own data also support this explanation: when these authors measure the enzyme activity by giving labeled tryptophan and estimating the formation of 14C]-5-HTP, some incorporation of radioactivity into the reaction product is found.

Regarding the differences between the morphological evidence and the results published here, no explanation can be given. The tryptophan hydroxylase–like immunoreactivity found in cerebral blood vessels seems to belong to the peripheral sympathetic innervation, for cervical gangliectomy dramatically reduces it, whereas lesions of the central serotonergic pathways have no effect. The same technique could not, however, unveil the presence of the enzyme in the cervical ganglia.

This is remarkably similar to the results obtained when a similar technique was used to detect serotonin in the cerebral blood vessels. The serotonin-like immunohistochemical fluorescence disappears after cervical gangliectomy or treatment of the animals with an amine uptake inhibitor or when the blood vessels are perfused with a saline solution before dissection of the tissue. However, biochemical analyses of the serotonin content of the cerebral arteries give the opposite results, with no change in the amine levels after cervical sympathectomy or superfusion of the blood vessels with saline solution.

The present findings, then, support the biochemical evidence and stress once more the divergence between the morphological techniques and the rest of the methodologies used to approach the problem exposed here.

In any case, the morphological evidence is not as homogeneous as it seems. For instance, when horseradish peroxidase (HRP) is applied in the cat middle cerebral artery, HRP-labeled neurons are found in the dorsal raphe nucleus, and when the serotonin-like immunoreactivity is compared with the immunoreactivity to noradrenaline in rabbit cerebral arteries, they do not superimpose.

Acknowledgment

Supported by Fondo de Investigaciones Sanitarias grant 93/0316.
References

Editorial Comment

The preceding article describes biochemical evidence to support the existence of tryptophan hydroxylase within nerve fibers surrounding the rat circle of Willis. Because this enzyme is specific for the biosynthesis of serotonin and because destruction of the dorsal raphe nucleus significantly reduces enzyme activity within large cerebral arteries, the authors conclude that serotonin is contained within nerve fibers projecting from parenchymal brain stem neurons located within the dorsal raphe. This study of Moreno et al is important because it adds data to the controversy over whether or not serotonin is contained within perivascular sympathetic fibers by an uptake-dependent mechanism and/or whether it is contained within ascending projections from dorsal raphe nucleus. Although the data presented support the latter possibility, the existence of an uptake-dependent mechanism within sympathetics has not been excluded by this study.

Michael A. Moskowitz, MD, Guest Editor
Massachusetts General Hospital
Harvard Medical School
Boston, Mass
Tryptophan hydroxylase activity in rat brain base arteries related to innervation originating from the dorsal raphe nucleus.
M J Moreno, A L López de Pablo and E J Marco

Stroke. 1994;25:1046-1049
doi: 10.1161/01.STR.25.5.1046
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1994 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/25/5/1046

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/