Local Treatments of Dorsal Raphe Nucleus Induce Changes in Serotonergic Activity in Rat Major Cerebral Arteries

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Background and Purpose—Rat major cerebral arteries seem to receive serotonergic fibers originating from the dorsal raphe nucleus (DRN), but little is known about their function. The aim of our present work was to establish a functional relationship between this brain stem nucleus and the cerebral blood vessels by studying the effects of several treatments in the DRN on cerebrovascular serotonergic activity.

Methods—Serotonin, clomipramine, 8-OH-DPAT, and WAY-100635 were administered in DRN. A stereotaxically localized electrode allowed the electrical stimulation of this brain stem nucleus. Serotonergic activity was appraised in major cerebral arteries, striatum, and hippocampus from 5-hydroxytryptophan accumulation after aromatic L-amino acid decarboxylase inhibition with NSD-1015.

Results—Serotonin significantly decreased serotonergic activity in major cerebral arteries and striatum without affecting it in hippocampus. This reduction was blocked by previous injection of WAY-100635 in DRN. Local administration of 8-OH-DPAT or clomipramine elicited an effect similar to that of serotonin, whereas that of WAY-100635 did not modify serotonergic activity in either of the tissues. Electrical stimulation of DRN significantly increased serotonergic activity in major cerebral arteries and striatum but not in hippocampus.

Conclusions—These results confirm the presence of a serotonergic innervation in rat major cerebral arteries functionally related to DRN. 5-HT1A receptor activation partly mediates the action of serotonin in DRN. A serotonergic tone acting on these somatodendritic receptors was not clearly found. (Stroke. 1999;30:1695-1701.)

Key Words: brain stem & cerebral arteries & serotonin & rats

The existence of serotonergic nerve endings in cerebral blood vessels was first demonstrated in rat cerebral microcirculation.¹ These serotonergic fibers were of central origin, because the serotonin (5-HT) levels in rat intraparenchymal microvessels appeared reduced after dorsal raphe nuclei (DRN) and median raphe nuclei (MRN) were destroyed but not after superior cervical sympathectomy was performed.¹ The presence of serotonergic fibers was later confirmed in pial cerebral vessels and major cerebral arteries from other animal species by use of different approaches.²–⁷ In pial cerebral vessels, the origin of this serotonergic innervation was found to be the DRN and MRN and not the superior cervical ganglia.⁴ In contrast, the attempts to show serotonergic fibers in major cerebral arteries did not give results as clear cut as those obtained in cerebral microvessels or pial arteries.

Although serotonin was shown by immunohistochemistry in the major cerebral arteries of several animal species,²,³,⁸,⁹ the presence of 5-HT was attributed to its uptake by sympathetic nerve endings. Such a conclusion was reached after observing that immunohistofluorescence to serotonin was greatly reduced when the animals were pretreated with amine reuptake inhibitors or submitted to superfusion with saline or buffer solutions before dissection of the blood vessels.¹⁰–¹⁴ On the contrary, other morphological findings indicate the existence of a true serotonergic innervation impinging on brain base arteries. Thus, tryptophan hydroxylase-immunoreactive fibers were found in rat major cerebral arteries. The origin of these fibers seemed to be the superior cervical ganglia, because they disappeared after cervical sympathectomy but remained virtually unaffected by intracerebral injections of 5,7-dihydroxytryptamine.¹⁵ However, the same experiments could not demonstrate the presence of the enzyme in superior cervical ganglia with the same technique, which made elucidation of the fibers’ origin difficult.¹⁵ In this regard, other data seem to confirm that rodent major cerebral arteries do not receive a serotonergic innervation originating from superior cervical ganglia. When the serotonin-like immunoreactivity was compared with the immunoreactivity to noradrenaline in rabbit major cerebral arteries, they did not superimpose.¹⁶ A similar result was obtained when distribution of dopamine–β-hydroxylase–immunoreactive fibers in rat major cerebral arteries was compared with that of tryptophan hydroxylase–immunoreactive fibers.¹⁵ Moreover,
5-hydroxytryptophan (5-HTP) uptake could be detected by histofluorescence in rat major cerebral arteries after bilateral cervical gangliectomy. On the other hand, horseradish peroxidase-labeled neurons were found in the DRN after application of the retrograde tracer in cat middle cerebral artery, which suggests a central origin for the serotonergic fibers.

Data obtained with biochemical or pharmacological approaches seem to be more conclusive in this regard. They indicate the presence of a structure of neural nature in major cerebral arteries containing serotonin and the machinery needed for its synthesis and with the ability to release and take up the amine. The origin of this serotonergic innervation depends on the animal species.

Cat major cerebral arteries seem to receive a serotonergic innervation of central and peripheral origins. They possess serotonin that appears to be stored in nerve endings because its levels are reduced after lesioning DRN and MRN or removing both superior cervical ganglia. These nerve terminals can release serotonin, as suggested by the reduction found in the contractile response to tyramine of isolated segments of cat middle cerebral artery in the presence of ketanserine, even when the animals are previously submitted to cervical sympathectomy.

Cat cerebral arteries also possesstryptophan hydroxylase activity that is reduced by DRN or MRN lesion or by bilateral cervical ganglionectomy. In contrast, rat brain base arteries are innervated by serotonergic fibers of central origin. Serotonin has been measured by HPLC in these blood vessels and appears to be stored in nerve fibers, as revealed by immunohistochemistry. The specific serotonin uptake present in these blood vessels was diminished by intracebral administration of 5,7-dihydroxytryptamine but not by bilateral cervical ganglionectomy. On the other hand, only DRN lesions decreased tryptophan hydroxylase activity in rat cerebral arteries, whereas it remained unaffected after lesioning of the MRN or excision of both superior cervical ganglia. The lack of projections from MRN to rat major cerebral arteries differs from results found in microvessels and pial arteries, probably due to regional differences in the distribution of the serotonergic innervation, as suggested elsewhere.

The function of this serotonergic innervation is unknown. Because serotonin is a powerful constrictor of major cerebral arteries, one can assume that it might participate in the control of the cerebral blood flow. Thus, the neurogenic contraction elicited by electrical stimulation of isolated rat basilar artery segments was reduced in the presence of serotonergic antagonists. DNR lesions induced the development of supersensitivity in the contraction evoked by serotonin in isolated cylinders of cat middle cerebral artery or altered their contractile response to tyramine.

The present work was intended to confirm the existence of a functional relationship between DRN and major cerebral arteries by studying whether treatments applied locally in the brain stem nucleus might affect serotonergic activity in rat brain base arteries. Serotonergic activity was appraised from 5-HTP accumulation after inhibition of aromatic L-amino acid decarboxylase. The treatments consisted of electrical stimulation of DRN and local injections in DRN of serotonin, the 5-HT_1A agonist 8-OH-DPAT, the 5-HT uptake blocker clomipramine, and the 5-HT_1A antagonist WAY-100635. Preliminary results have shown that systemically administered clomipramine reduces serotonin turnover in rat major cerebral arteries as well as in DRN, which suggests the existence of a direct link between the brain stem nucleus and the brain base blood vessels.

Materials and Methods
Male Sprague-Dawley rats, weighing 130 to 180 g, of the strain ICO:OF/S (L.P.S. Saw) were used. The animals were housed in the proper facilities, complying with the 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.

Drug Administration
Serotonin, 8-OH-DPAT, and clomipramine were prepared by dissolving 5 mg of each drug in 5 mL of 0.067 mol/L phosphate buffer, pH 7.3, with 2 mg ascorbic acid. In some experiments, 0.5 mg of serotonin was dissolved in 5 mL of the vehicle. The drugs were locally injected by means of a microinjection unit (model 500, Kopf Instruments) placed in a stereotaxic apparatus (Kopf Instruments). The tip of the syringe was implanted in DRN (A, 0 mm; L, 0 mm; H, −1.3 mm) according to the coordinates of König and Klippel. One microliter of the drug solution was delivered during a period of 8 minutes. Thereafter, 5-HTP accumulation was induced in the animals by injecting them with m-hydroxybenzylhydrazine (NSD-1015, 125 mg/kg IP), an inhibitor of the aromatic L-amino acid decarboxylase, and killing them 60 minutes later. The control animals were submitted to the same procedure but received 1 μL of the vehicle in DRN instead of the drugs.

Because serotonin was injected 60 minutes before the animals were killed, the amount of amine remaining in its injection site after this time was measured. 5-HT (1 μg) was locally administered to a group of rats killed 2 minutes or 60 minutes after ending the injection of serotonin. The control group was injected only with vehicle in DRN and killed 2 minutes thereafter.

To study the effect of WAY-100635 on the action of serotonin, 5 mg of WAY-100635 was prepared in 5 mL of 0.067 mol/L phosphate buffer, pH 7.3, with 2 mg ascorbic acid and locally injected in DRN. One minute thereafter, 1 μg serotonin was delivered into DRN. At the end of 5-HT administration, NSD-1015 (125 mg/kg IP) was given to the rats killed by decapitation 60 minutes later.

Electrical Stimulation of DRN
In some anesthetized animals (40% chloral hydrate in saline solution, 1 mL/kg IP), a bipolar electrode (Rhodes NEX-200, 0.2-mm contact diameter and 0.5-mm exposed contact separation) was implanted in the DRN as described above. Afterward, NSD-1015 (125 mg/kg IP) was administered to the rats, and 29 minutes later an electrical current supplied by a stimulator (CIBERTEC CS 20) was applied during 30 minutes as square pulses of 3-ms duration and 50-Hz frequency. The intensity of the current (150 μA, 300 μA, and 900 μA) was measured on a 10-kΩ resistor placed in serial between the stimulator and the brain stem nucleus and monitored on an oscillograph (HAMEG Instruments). One minute after interruption of the current delivery, the animals were killed by decapitation. The control group was submitted to the same procedure, but no current was passed through the electrode.

Sample Preparation
After the rats were decapitated, the brain was rapidly removed and the circle of Willis with some of its branches, as well as the right
hippocampus and striatum, were dissected out. The dissected tissues were frozen on dry ice and stored at −15°C. The accuracy of the drug injection or electrode location in DRN was tested by measuring their effects on serotonergic activity in hippocampus and striatum, which are mainly if not exclusively innervated by MRN and DRN, respectively.34,35 A similar approach has been previously used for this purpose.4,23,24,36

In the case of the animals used to test the serotonin removal from DRN, the brain was quickly dissected out, frozen, and stored. DRN was dissected from the frozen brain by placing this on a metal block cooled down to −8°C and cutting 0.5- to 1-mm-thick frontal slices freehand with a razor blade. The slices were kept frozen on the block, and DRN samples were punched out and stored at −15°C. The brain stem nucleus was identified by following the description of König and Klippel.33

The tissue samples from each animal were homogenized separately by sonication in 0.4 mol/L HClO4 with 0.002% (wt/vol) ascorbic acid (those of cerebral arteries and DRN in 300 µL and those of striatum and hippocampus in 500 µL) and centrifuged at 6000g for 5 minutes. 5-HTP was measured in the supernatants of the samples of cerebral arteries, striatum, and hippocampus, whereas 5-HT was assayed in the supernatants of those of DRN.

Assay of 5-HTP and 5-HT
5-HTP or 5-HT was assayed in 50-µL aliquots of supernatants by reverse-phase HPLC with electrochemical detection. Proteins were determined in the precipitates by the method of Lowry et al.37 with 5-HTP and 5-HT final concentrations being referred to them. The HPLC system consisted of a sample injector (Gilson, model 231) with a dilutor (Gilson, model 401) and a Rhodyne injection valve with a 50-µL loop, a pump (Gilson, model 305) with a manometric module (Gilson, model 805) giving a flow rate of 1.200 mL/min, and a reverse-phase column (μBondapak C18, Waters) with a guard column (Bondapak C8/Corasil, Waters). 5-HT and its precursor were detected with an electrochemical detector (LC-4A, Bioanalytical Systems) with a glassy carbon electrode set at +0.5 V versus an Ag/AgCl reference electrode. The mobile phase was prepared according to Lackovic et al.38

Statistical Analysis
Statistical analysis of the results was performed with the Student t test. When 2 experimental groups were compared with the same control, a Kruskal-Wallis test was used.99

Chemicals
5-Hydroxy-L-tryptophan, 5-hydroxytryptamine creatinine sulfate, (±)-5-hydroxy-2-di-p-propyl-amino)tetralin hydrobromide (8-OH-DPAT), and m-hydroxybenzylhydrazine (NSD-1015) were purchased from Sigma and N-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-N-(2-pyrindinyl)cyclohexanecarboxamide maleate (WAY-100635) from RBI. Clomipramine hydrochloride was a kind gift from Ciba (Madrid, Spain). The rest of the reagents were of HPLC grade.

Results
Local injections of 0.1 µg 5-HT in DRN did not significantly alter 5-HTP accumulation in rat major cerebral arteries, striatum, and hippocampus. When the amount of the serotonin administered was increased to 1 µg, a significant reduction of 5-HTP accumulation in cerebral arteries and striatum was observed, whereas it was unaffected in hippocampus when compared with animals receiving only the vehicle (Figure 1).

Sixty minutes after serotonin was injected (1 µg) into DRN, 5-HT levels in the brain stem nucleus were approximately 90% lower than 2 minutes thereafter. Nevertheless, they were still significantly higher than those found in DRN samples of animals that received only the vehicle (Table).

The reduction of serotonergic activity in cerebral arteries and striatum obtained with the injection of 1 µg 5-HT in DRN was not observed when 1 µg WAY-100635 was administered into the brain stem nucleus before the amine (Figure 2). On the other hand, local injection of 1 µg 8-OH-DPAT in DRN induced a significant decrease of 5-HTP accumulation in cerebral arteries and striatum without affecting it in hippocampus (Figure 2).

Serotonergic activity in cerebral arteries, striatum, or hippocampus was not altered by local administration of WAY-100635 (1 µg) into DRN (Figure 3). However, 1 µg clomipramine applied to DRN evoked significant decrease of 5-HTP accumulation in brain base arteries and striatum without changing it in hippocampus when compared with control situation (Figure 3).

Electrical stimulation of DRN with a current intensity of 300 µA for 30 minutes induced a significant increase in

![Figure 1](http://stroke.ahajournals.org/)

Figure 1. Top graph, Effect of local injection of 0.1 µg 5-HT in DRN on 5-HTP accumulation in rat major cerebral arteries (CBV), striatum (STR), and hippocampus (HIP) compared with the local administration of vehicle alone (Control). Bottom, Effect of 1 µg 5-HT in DRN on 5-HTP accumulation in rat major cerebral arteries, striatum, and hippocampus compared with the local administration of vehicle alone (Control). Results are expressed as mean±SEM. Figures in parentheses indicate number of animals used. *P<0.05.
5-HTP accumulation in rat major cerebral arteries and striatum without affecting it in hippocampus (Figure 4). When the current intensity was lowered to 150 μA or augmented to 900 μA, no change in serotonergic activity was observed in the tissues (results not shown). A lesion of the brain stem nucleus could be seen with the naked eye when the greatest intensity was used.

**Discussion**

5-HTP accumulation in major cerebral arteries and striatum after inhibition of the aromatic-L-amino acid decarboxylase has been used as a tool to appraise 5-HT turnover or serotonergic activity in the central nervous system. If a treatment increases it, this means an activation of the serotonergic nerve fibers present in the structure studied, and conversely, a decrease would reflect their inhibition. Previous work has shown that rat major cerebral arteries receive nerve fibers originating from DRN which possess tryptophan hydroxylase and, therefore, the ability to synthesize 5-HTP as well as to accumulate it after decarboxylation inhibition. Any local treatment in DRN that affects 5-HTP accumulation in major cerebral arteries would indicate a variation in the activity of the serotonergic fibers impinging on them and at the same time reinforce the existence of this kind of innervation.

In the present work, local injection of 1 μg 5-HT in DRN induces a reduction of 5-HTP accumulation in rat base brain
arteries in the same way it does in striatum, which indicates that serotonin is inhibiting serotonergic nerve fibers activity by its action on cell bodies located in the brain stem nucleus. This amount of serotonin may seem high, but no effect was observed when 5-HT was administered at a 10-fold lesser dose. The need to use 1 \( \mu \)g of the amine is probably due to the fact that only 10% of the initial injected amount of serotonin can be found at the time of an animal’s death. On the other hand, the small and nonsignificant changes in serotonergic activity found in hippocampus can be explained on the basis that some nerve fibers can project from DRN to hippocampus.\(^5\) Another possibility might be that some of the injected 5-HT reached the MRN. This would not affect, however, the previous conclusion, because rat major cerebral arteries do not receive serotonergic fibers originating from MRN. Indeed, neither tryptophan hydroxylase activity nor 5-HT accumulation are altered in these blood vessels after the electrolytic lesion of MRN.\(^20,22\) The lack of projections from MRN to rat major cerebral arteries differs from results found in microvessels and pial arteries, which receive fibers from both brain stem nuclei.\(^1,4,5\) The difference may lie in the cerebral arteries harvested for the experiments. Nevertheless, experimental evidence suggests that major cerebral arteries are not uniformly innervated by the brain stem nucleus. Thus, lesion of the DRN or MRN induces a reduction of TPOH activity measured in cell-free extracts of cat arteries from the circle of Willis and its branches,\(^20\) whereas only destruction of the dorsal raphe evokes supersensitivity to 5-HT in the isolated middle cerebral artery of the cat.\(^28\) This means that there may be regional differences regarding how cerebral arteries are innervated by these fibers, not only between pial and major cerebral arteries but also among the major cerebral arteries themselves.

It has been shown that a decreased serotonergic activity in striatum can be obtained by the activation of 5-HT\(_{1A}\) receptors in DRN.\(^41,42\) Therefore, we explored the possibility that the decreased activity measured after 5-HT injection in serotonergic nerve endings present in major cerebral arteries was due to the activation of the same serotonergic receptor subtype. This seems to be the case, because local delivery of a 5-HT\(_{1A}\) antagonist, WAY-100635, prevented the reduction in serotonergic activity elicited by serotonin in cerebral arteries and striatum. This conclusion is reinforced by the fact that 8-OH-DPAT, a 5-HT\(_{1A}\) agonist, also decreased 5-HTP accumulation both in major cerebral arteries and in striatum when injected in DRN.

The existence of a basal serotonergic tone acting on these receptors was also studied, but the results obtained are inconclusive. Local administration of WAY-100635 alone did not modify the serotonergic activity in cerebral arteries or striatum, which would indicate the absence of a serotonergic tone acting on 5-HT\(_{1A}\) somatodendritic receptors. However, local injection of a serotonin reuptake blocker such as clomipramine in DRN lowered 5-HTP accumulation in brain base arteries and striatum. Since systemic or local administration of serotonin uptake blockers increases 5-HT levels in DRN,\(^43-45\) it can be assumed that the effect of clomipramine on serotonergic activity is due to an enhanced local concentration of the released 5-HT in the corresponding synaptic cleft. This would support the presence of a serotonergic tone acting on the cell bodies of the fibers innervating cerebral arteries and striatum. It has been suggested that the failure to demonstrate a serotonergic tone in DRN using 5-HT\(_{1A}\) receptor blockers might be due to the fact that most of the experiments are performed in anesthetized animals, when the serotonergic activity is depressed and cannot be shown.\(^46\) If this is the reason, clomipramine might be acting as an amplifying factor of the reduced serotonergic tone by increasing local 5-HT concentration in DRN.

Electrical stimulation of DRN increased 5-HTP accumulation after inhibiting the aromatic-L-amino acid decarboxylase with NSD-1015 in rat major cerebral arteries as well as in striatum, which indicates a serotonergic activity enhancement in both tissues due to the increased activity in the cell bodies. Because serotonergic activity in hippocampus was not altered, it can be concluded that MRN was not affected by the electrical stimulus applied. The present results seems to disagree with data published previously by Bonvento et al,\(^47\) which indicate that electrical stimulation of DRN had no effect on 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) levels of rat major cerebral arteries, whereas it enhanced 5-HIAA concentration without varying 5-HT levels in striatum. The explanation may lie in the different approach used to measure serotonergic activity. Bonvento et al\(^47\) employed the 5-HT/5-HIAA ratio as an indication of change in turnover rate, which seems to be a less sensitive method for detecting changes in serotonergic activity than 5-HTP accumulation after decarboxylase inhibition. For instance, systemically administered clomipramine decreases 5-HT turnover in striatum, hippocampus, cortex, septum, and hypothalamus when it is appraised by measuring 5-HTP accumulation after decarboxylase inhibition or 5-HIAA accumulation after acid transport blockade with probenecid.\(^48\) However, clomipramine does not change 5-HIAA steady-state levels in these brain areas, and diminishes 5-HT basal levels only in striatum, hypothalamus, and cortex.\(^47\) This means that the effect of clomipramine on serotonergic activity in septum and
hippocampus had been passed unnoticed when the 5-HT/5-HIAA ratio was used for appraising it. One can only speculate about the functional relevance that changes in serotonergic activity in rat major cerebral arteries may have on the cerebral circulation. Because 5-HT is a potent constrictor agent of these blood vessels,25–27 it might be expected to cause a change in cerebral blood flow according to the variations of the serotonergic tone. It has been reported that chemical or electrical stimulation of DRN induces a decrease of regional flow in several brain areas,49–51 whereas systemic administration of 8-OH-DPAT augments cerebral blood flow in different brain regions.52,53 Although these works refer to actions of serotonin on cerebral microvessels, it can be assumed that similar effects may be expected in major cerebral arteries. In this regard, the finding that DRN lesion brings about the development of supersensitivity to 5-HT in isolated cat middle cerebral artery28 suggests the removal of a serotonergic tone exerted on this blood vessel by a serotonergic innervation of central origin.

In summary, the present results confirm the existence of a serotonergic innervation acting on rat brain base arteries whose activity is subjected to influences on its cell bodies located in DRN, and open new possibilities to explain its functional role.

Acknowledgments

The authors wish to thank Mercedes del Río and Pedro Fernández for their technical assistance. This work was supported by grant 95/0508 from the Fondo de Investigaciones Sanitarias.

References

Changes in activation in one area of the brain may effect vascular changes in another remote area of the brain by 2 mechanisms: Neuronal activation may lead to changes in metabolism that have secondary vascular effects mediated by local mechanisms. A second and more interesting mechanism is the activation of vasomotor nerves. The first mechanism is well recognized. There is also considerable evidence in the literature for the second mechanism, but the existence of vasomotor nerves, particularly from the brain stem to the rest of the brain, has remained controversial. In the article above, Marco and colleagues report evidence supporting the presence of serotonergic nerves originating in the dorsal raphe nucleus, presumably innervating major cerebral arteries. The authors showed that either electrical or chemical stimulation of this nucleus induced changes in the concentration of 5-HTP in the vessels. This evidence, coupled with evidence from other investigators showing that changes in the activity of the dorsal raphe nucleus can induce changes in cerebral blood flow, makes a strong case for the presence of serotonergic vasomotor innervation originating in this nucleus and influencing major cerebral vessels. It remains to be seen whether this pathway can be activated by physiological or pathophysiological stimuli. Clearly, additional work would be necessary to show the functional significance of this pathway.

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Stroke. 1999;30:1695-1701
doi: 10.1161/01.STR.30.8.1695

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