Serotonin as an Alternative Transmitter in Sympathetic Nerves of Large Cerebral Arteries of the Rabbit

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The distribution of serotonin (5-HT)-like immunoreactive (5-HT-LI) nerves and the potential role of 5-HT as a vasoconstrictor transmitter in large cerebral arteries of the rabbit were examined. 5-HT-LI fibers with weak immunofluorescence were observed in the anterior cerebral, middle cerebral, and basilar arteries when fixed by immersion after dissection from exsanguinated animals. The 5-HT-LI fibers, however, were not detected in these arteries when fixed either in vitro or in situ after first being perfused with Krebs solution in situ to flush the blood component from the lumen prior to dissection. In these arteries, 5-HT-LI nerve fibers with intense immunofluorescence, however, reappeared following incubation with 5-HT in vitro. The intensity of the 5-HT-LI fibers seemed to be proportional to the duration and 5-HT concentration during incubation. Following chronic surgical sympathectomy, 5-HT-LI fibers were not detected in arteries before or after incubation with 5-HT. Transmural nerve stimulation elicited constriction in 50% of the control arterial segments examined. The constriction was not affected by ketanserin but was prevented by guanethidine and chronic surgical sympathectomy. The remaining arterial segments that did not respond to transmural nerve stimulation, however, became constrictive on transmural nerve stimulation following incubation with 5-HT in vitro. The constriction was blocked by ketanserin and clonidine. These results demonstrate that the large cerebral artery of the rabbit brain has extremely sparse or no authentic 5-HT-LI nerves. The intense 5-HT-LI nerves observed following incubation with 5-HT are primarily due to uptake of 5-HT into sympathetic nerves, and on transmural nerve stimulation 5-HT can be released to induce vasoconstriction. It is suggested that 5-HT can act as an alternative sympathetic vasoconstrictor transmitter in cerebral arteries. (Circulation Research 1987;60:220–228)
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Table of Abbreviations

- CSF: cerebrospinal fluid
- 5-HT: serotonin
- 5-HT-LI: serotonin-like immunoreactive
- NE: norepinephrine
- PBS: phosphate-buffered saline
- TNS: transmural nerve stimulation

Male white rabbits (1.5-3.0 kg) were anesthetized with sodium pentobarbital (50 mg/kg, i.v.). In the first series of experiments, 5 rabbits were sacrificed by exsanguination and brains were removed and immersed in 0.01 M phosphate buffered saline (PBS). The cerebral arteries were then dissected and fixed in 4% paraformaldehyde. In the second series of experiments, brains from 4 other rabbits were perfused with PBS followed by 4% paraformaldehyde in situ through the left ventricle before exsanguination. The brain was removed and the cerebral arteries were dissected and further fixed in the same fixative for 2 to 48 hours. In the third series of experiments, brains from 4 rabbits were perfused with Krebs solution through the left ventricle. The animals were then exsanguinated, and cerebral arteries were dissected and incubated in Krebs solution containing various concentrations of 5-HT (0, 10⁻⁸ to 10⁻⁴ M) at 37°C for 30 or 60 minutes. After incubation, the tissues were washed with Krebs solution and fixed in 4% paraformaldehyde to induce 5-HT immunofluorescence in whole mount preparations by an indirect immunofluorescence method. The composition of the Krebs solution was as follows (mM): Na⁺ 144.2; K⁺ 4.9; Ca²⁺ 1.3; Mg²⁺ 1.2; Cl⁻ 126.7; HCO₃⁻ 25.0; SO₄²⁻ 1.2; glucose 11.1; and calcium disodium ethylenediaminetetraacetate (EDTA) 0.023. The Krebs solution was constantly bubbled with a gas mixture of 95% O₂ and 5% CO₂.

Immunohistochemistry

Following fixation in 4% paraformaldehyde, tissues were dehydrated with ethanol, cleared with xylene, and rehydrated. Anti-5-HT antibody (Immunonuclear Corp., Stillwater, Minn.) was applied at a concentration of 1:200 dilution for 16 to 24 hours at 4°C. After incubation, the tissues were washed with PBS and applied with fluorescein isothiocyanate (FITC)-labelled antirabbit IgG (Sigma, St. Louis, Mo.) at a concentration of 1:40 for 1 hour. After a brief wash with PBS, the tissue was mounted in buffered glycerol (glycerol:PBS = 3:1) and examined under a Zeiss fluorescence microscope. Tissues incubated with 5-HT antibodies preadsorbed with 5-HT (3 x 10⁻³ M) served as controls. Since antibodies may cross-react with unknown substances, it is appropriate to use 5-HT-like substances for the immunoreactive product.

In Vitro Tissue Bath Studies

All rabbits were perfused in situ with Krebs solution through the left ventricle under sodium pentobarbital anesthesia (50 mg/kg, i.v.) and exsanguinated. The brain was removed and the basilar artery dissected. Ring segments (4 mm long) of the arteries were carefully cannulated with a stainless steel rod (30-gauge hemispherical section) and a short piece of platinum wire, and mounted in an isolated tissue bath containing 30 ml Krebs solution at room temperature. The platinum wire was bent into an inverted U shape and anchored to a plastic gate, which could be moved up and down by a fine control micrometer (unislide series A1500). The steel rod was connected to a Gould UC-2 strain gauge for isometric recording of changes in force. After 10 minutes, the temperature of the Krebs solution was gradually increased to and maintained at 37°C. Resting tension was then adjusted to 0.5 g and a period of 1 hour was allowed for equilibration. A pair of stimulating electrodes, one on either side of the vessel, was arranged for TNS, which was delivered across the electrodes via a stimulator and a modified coupling device, which provided a low source impedance. Stimulation parameters (trains of 100 biphasic square wave pulses of 0.3 milliseconds, 160 mA across the electrodes) were selected to achieve supra-maximal nerve stimulation. Actual stimulation parameters were monitored with an oscilloscope (Tektronix).

Since the response obtained in this stimulating condition was completely blocked by tetrodotoxin (9 x 10⁻⁷ M), this stimulation was referred to as TNS. After the first response of the tissue on TNS was recorded, the tissue was then incubated with 5-HT of different concentrations for 30 minutes. The tissue was then washed with Krebs solution and the TNS repeated.

Statistical Methods and Drugs

Statistical analyses were made using paired and unpaired t tests. Drugs used were serotonin creatinine sulfate (Calbiochem-Behring Corp., La Jolla, Calif.), guanethidine sulfate (Sigma Chemical Co., St. Louis, Mo.), ketanserin (Janssen), tetrodotoxin (Sigma) and clonidine HCl (Sigma).

Results

Immunohistochemistry

When cerebral arteries were dissected from anesthetized animals that were killed by exsanguination, and were fixed by immersion, weak 5-HT-LI nerves were frequently observed in these arteries. In 5 of 5 animals, blood was observed in the lumen of these arteries at the time of dissection under a dissecting microscope. The intensity of the 5-HT-LI, however, varied among the tissues; in some, immunofluorescence was almost absent.
When the brain arteries were fixed by perfusion in situ before exsanguination, in 4 of 4 animals 5-HT-LI nerves were not observed in the arteries examined (anterior communicating, middle cerebral, internal carotid, basilar, and vertebral arteries) (Figure 1b). In these tissues, no trace of blood remained in the lumen at the time of dissection.

In the third series of experiments, brains of 4 rabbits were perfused in situ with Krebs solution. The cerebral and ear arteries were then dissected and incubated in the presence or absence of 5-HT (10^{-7} - 10^{-4} M) for 30 minutes at 37° C. The tissues fixed immediately after dissection without incubation, or after incubation in Krebs solution in the absence of 5-HT, exhibited no 5-HT-LI nerves (Figure 2a). On the other hand, cerebral arteries (anterior communicating, middle cerebral, and basilar arteries) and central ear arteries incubated with 5-HT for 30 minutes at 37° C exhibited dense distribution of 5-HT-LI nerves (Figure 2b-d). Although the immunofluorescence was weak in tissues incubated with 10^{-8} M 5-HT for 30 minutes, the intensity was enhanced by incubation with a higher concentration of 5-HT or longer (1 hour) incubation time (not shown). Following chronic superior cervical ganglionectomy, 5-HT-LI nerves were not observed in cerebral arteries (3 of 3 sympathectomized animals) before or after incubation with 5-HT (Figure 3). Successful sympathetic denervation was confirmed by the absence of CA-fluorescent nerves (Figure 3) in adjacent segments of the artery.

In Vitro Tissue Bath Studies

TNS induced vasoconstriction in 16 of 33 basilar arterial rings. The vasoconstriction was not affected by ketanserin (Figure 4a), a result consistent with our previous reports. In the remaining arteries that did not respond to TNS, exogenous 5-HT (10^{-6} M) caused constriction (110.4 ± 19.5 mg/mg wt). 5-HT was then kept in the tissue bath for another 30 minutes. After 5-HT was washed out from the bath, TNS was repeated and all arteries responded with constriction (Figures 4b and 5). This TNS-induced vasoconstriction after 5-HT incubation was blocked by ketanserin (10^{-7} M) (Figure 4b) and abolished by guanethidine (Figure 5). The inhibition of the TNS-induced vasoconstriction at 8 and 16 Hz by ketanserin (10^{-7} M) was 40.7 ± 11.0% and 45.6 ± 6.7%, respectively (Figure 6). The remaining vasoconstrictor responses were abolished by tetrodotoxin (TTX, 9 x 10^{-7} M) or guanethidine (10^{-6} - 3 x 10^{-8} M) (Figures 4, 5, and 7).

The effects of clonidine, an α_{2}-adrenergic agonist, on the TNS-induced constriction in basilar arteries following incubation with 5-HT were also examined. Clonidine (10^{-8} - 10^{-6} M), which did not affect the resting tone of the basilar artery (Figure 7), decreased the TNS-induced vasoconstriction in this artery (Figures 7 and 8). The residual vasoconstriction was blocked by ketanserin (10^{-7} M). On the other hand, in arterial segments that failed to respond to TNS, pretreatment with NE (10^{-6} M) for 30 minutes did not render the tissue constrictive on TNS (Figure 5).

Discussion

Reports on the presence of 5-HT-containing nerves in large cerebral arteries have been inconsistent. Using immunohistochemical methods, 5-HT-LI nerves have been demonstrated to be present in large cerebral arteries of the rat and rabbit, but Itakura et al did not find them in the cerebral arteries of the rat. In the present study, large cerebral arteries of the rabbit exhibited 5-HT-LI nerves with weak immunofluorescence when arteries contained blood in the lumen at the time of dissection and immersion fixation. However, no 5-HT-LI nerves were detected in arteries that were...
perfused with physiological solution in situ to remove blood from the lumen before dissection and fixation. Similarly, 5-HT-LI nerves were not observed in the arteries fixed in situ by perfusion with fixative. One explanation for the latter finding may be that perfusion with the physiological solution or fixative may result in depletion of 5-HT from serotonergic nerves. This is unlikely, however, since similar perfusion procedures did not appreciably affect the CA-fluorescence nerves in cerebral arteries. These results indicate that authentic 5-HT-LI nerves are extremely sparse or not present in large cerebral arteries of the rabbit. The presence of dense 5-HT-containing nerves in the isolated cerebral blood vessel wall may therefore be related to procedures used to remove the artery prior to the fixation. It is interesting to note that in the reports by Griffith et al,12 Edvinsson et al,11 and Alafaci et al,13 the cerebral arteries were dissected and fixed by immersion in vitro, while in the work of Itakura et al,14 cerebral arteries were initially fixed by in situ perfusion.

Several studies have demonstrated that sympathetic adrenergic nerves in peripheral arteries can take up and store 5-HT.15,16 Perivascular nerves in cerebral arteries have also been shown to be capable of taking up radiolabelled 5-HT.9,13 In the present study, no 5-HT-LI nerve was found in the cerebral arteries dissected from...
FIGURE 3. The effect of unilateral sympathectomy on the serotonin (5-HT)-like immunoreactive nerves in the middle cerebral artery and the catecholamine (CA)-fluorescence nerves in the internal carotid artery. The brain was perfused with Krebs solution before arteries were dissected. The middle cerebral artery of control (Panel a) and denervated (Panel b) were incubated with 5-HT ($10^{-6}$ M) for 30 minutes at 37°C before fixing for 5-HT-U nerves. The CA-fluorescence nerves are present in control internal carotid artery (Panel c) and are absent in the denervated arteries (Panel d). The distribution patterns of 5-HT-LI nerves and CA-fluorescence nerves are similar (240×).

Brains preperfused with Krebs solution. These cerebral arteries, however, after incubation with 5-HT in vitro exhibited dense mesh-like 5-HT-LI nerves similar to those of sympathetic adrenergic nerves. It is possible that 5-HT is taken up and stored in sympathetic nerves in cerebral arteries. This possibility was confirmed by the observation that both 5-HT-LI and noradrenergic nerves disappeared completely in cerebral arteries after chronic superior cervical ganglionectionomy. Furthermore, the rabbit central ear artery, like the basilar artery, also exclusively receives sympathetic nerves. In these arteries, 5-HT-LI nerves appeared only after incubation with 5-HT. In the sympathetically denervated ear arteries, the 5-HT-LI nerves were not detected even after incubation with 5-HT. These results indicate that sympathetic adrenergic nerves in cerebral and ear arteries indeed can take up and store 5-HT. The exact source of 5-HT is not known. 5-HT may originate primarily from the circulation. During exsanguination or dissection, 5-HT may be released into and/or from the blood. It is known that platelets and mast cells contain 5-HT that can be released during various conditions. It has been demonstrated that TNS-induced vasoconstriction of the rabbit basilar artery is blocked by guanethidine but is partially resistant to α-adrenoceptor antagonists. Accordingly, TNS-induced vasoconstriction was suggested to be due to release of a
second transmitter in addition to NE from the sympathetic nerve. It has been suggested that neuropeptide Y (NPY) may be the transmitter mediating vasoconstriction in cerebral arteries. Positive identification of this conclusion, however, has not been presented. Griffith et al. further suggested that 5-HT was the transmitter substance responsible for neurogenic vasoconstriction in the rabbit vertebral artery since the TNS-induced vasoconstriction in this artery was blocked by ketanserin. This is in conflict with our previous report that the TNS-induced vasoconstriction in the rabbit basilar artery was resistant to ketanserin and methergoline. This latter finding further supports the presence of a nonserotonergic, nonadrenergic vasoconstrictor transmitter in the rabbit basilar artery. In the present study, TNS elicited constriction in 50% of the basilar arteries examined. The neurogenic vasoconstriction, as previously described, was resistant to ketanserin (Figure 4A). On the other hand, the arteries, which failed to constrict on TNS, became responsive.

**FIGURE 4.** The effect of ketanserin on the vasoconstriction induced by transmural nerve stimulation (TNS) at 8 and 16 Hz in the basilar artery before and after incubation with 5-HT (10^{-6} M). Panel A: TNS-induced constriction of the artery without 5-HT preincubation. The constriction was not blocked by ketanserin. Panel B: The basilar artery failed to respond on TNS. Following incubation with 5-HT (10^{-6} M) for 30 minutes, the artery became responsive with constriction on TNS at 8 and 16 Hz. The constrictions on TNS at both 8 and 16 Hz were reduced by ketanserin (10^{-7} M).

**FIGURE 5.** TNS-induced vasoconstriction in arteries pretreated with 5-HT (10^{-6} M) and NE (10^{-6} M). The basilar artery was divided into 3 segments. Each arterial segment is indicated by the heavy bar (arrowhead). All 3 segments failed to respond on TNS prior to incubation with 5-HT. 5-HT constricted the artery while NE at the same concentration did not cause a response. 5-HT and NE remained in the bath for 30 minutes before wash (W). TNS-induced constriction in arteries preincubated with 5-HT but not in those with NE. The TNS-induced constriction was abolished by guanethidine.
with constriction on TNS only after incubation with 5-HT. This neurogenic vasoconstriction was then blocked by ketanserin (Figure 4B), which suggests that vasoconstriction was due to release of stored 5-HT from the sympathetic nerves in the cerebral arterial wall. Taken together, these results demonstrate that 5-HT not only can be taken up by sympathetic nerves, but also can be released from sympathetic nerves to induce vasoconstriction. According to Baldessarini and Fischer, a substance can act as an alternative transmitter if the substance is not normally present but can be accumulated by nerve terminals and released on nerve impulse. 5-HT therefore may be considered as an alternative vasoconstrictor transmitter in the rabbit basilar artery. The nature of the transmitter(s) mediating the ketanserin-resistant vasoconstriction remains to be identified.

On the other hand, dense CA- or NE-fluorescence is always observed in the rabbit basilar arteries, and its appearance in the present study does not seem to be affected by the fixation procedure. The rabbit basilar artery, however, is very insensitive to NE. The arteries that failed to constrict on TNS still did not elicit constriction on TNS following incubation with NE, a result different from that of 5-HT-incubation. This result is consistent with the previous reports that NE is probably not the primary vasoconstrictor in cerebral sympathetic nerves. The transmitter role of NE in the cerebral sympathetic nerve remains unanswered.

The functional significance of 5-HT uptake into, and subsequent release from, sympathetic nerves in vivo is not known. Cerebrospinal fluid (CSF) in normal subjects has been shown to contain less than $10^{-9}$ M 5-HT. This concentration of 5-HT can cause a rise in CSF of patients with ruptured intracranial aneurysm. The enhanced 5-HT concentration in CSF may be the genesis of the initial vasospasm. Therefore, one important role of sympathetic nerves based on the results of the present study may be that these nerves serve as a "sink" for taking up vasoactive substance such as 5-HT and protect cerebral arteries from massive constriction.

On the other hand, the neurorally accumulated 5-HT may be released to act like a vasoconstrictor transmitter, also shown in the present study. It is known that 5-HT is involved in the pathogenesis of several cerebral vascular diseases. For example, it has been suggested that 5-HT is involved in the etiology of vascular headaches associated with the intracranial disorder head sergide and cyproheptadine, and adrenergic agents such as clonidine and propranolol. It should be pointed out that clonidine and propranolol have been shown to impair adrenergic neurotransmission by activating the inhibitory presynaptic α subadrenoceptors and inhibiting the facilitatory presynaptic β subadrenoceptors, respectively. Although the presynaptic α-adrenoceptors in rabbit cerebral arteries have not been positively clarified, the presynaptic α-adrenoceptors in cerebral arteries of the cat have been shown to be of the α subtype. Our results have also demonstrated that neurogenic vasoconstriction in rabbit basilar arteries is potentiated by phentolamine.

**Figure 6.** Histograms depicting the effect of ketanserin ($10^{-7} M$) on the TNS-induced constriction of basilar arteries after incubation with 5-HT. The arteries did not respond to TNS before 5-HT incubation. Vertical bars represent the SEM (n = 5). *Significantly different (p<0.05, paired t-test) between the two values.

**Figure 7.** Effect of clonidine on the TNS-induced constriction in the rabbit basilar artery incubated with 5-HT ($10^{-6} M$) for 30 minutes. Yohimbine, and α-receptor antagonist, potentiates the TNS-induced constriction. Clonidine at $10^{-7} - 10^{-8} M$ did not change the basal tone, but reduced the TNS-induced vasoconstriction at 8 and 16 Hz. The residual vasoconstrictions were further reduced by ketanserin and abolished by tetrodotoxin (TTX). W = wash.
Thus, the pathophysiologic role of 5-HT as an alternative vasoconstrictor transmitter in cerebral arteries, especially in those regions receiving dense sympathetic innervation, becomes evident and deserves further detailed examination.

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