Uptake and Release of Serotonin in Rat Cerebrovascular Nerves After Subarachnoid Hemorrhage

Csaba Szabò, MD; Kjell Emilsson, MD; Jan Erik Hardebo, MD; Sverker Nystedt, MD; and Christer Owman, MD

Background and Purpose: Serotonin released from platelets has been suggested as one substance causing the vasospasm following subarachnoid hemorrhage. We studied whether such serotonin is able to constrict pial vessels.

Methods: We studied the uptake of serotonin in pial perivascular nerves by immunohistochemistry. We measured the contractile response in rat basilar artery after in vitro incubation with serotonin and during electrical field stimulation of perivascular nerves following experimental subarachnoid hemorrhage.

Results: After incubation with serotonin, electrical field stimulation caused a tetrodotoxin- and ketanserin-blockable contractile response. We observed no such response in vessels from rats treated with 6-hydroxydopamine or after blockade of serotonin uptake. After subarachnoid hemorrhage, a pronounced network of serotonin-immunoreactive nerve fibers was demonstrated in the vessel wall. In vessels from control rats, no serotonin fibers were seen, and in vessels from 6-hydroxydopamine-treated animals with subarachnoid hemorrhage only a few such fibers were seen. Electrical field stimulation of the basilar artery from rats tested 2 or 16 hours (but not 10 minutes or 24 hours) after subarachnoid hemorrhage showed contractile responses that were prevented by tetrodotoxin, ketanserin, and prior 6-hydroxydopamine treatment.

Conclusions: Our study demonstrates a capacity of the perivascular sympathetic nerves to take up serotonin both in vitro and during the early phase of subarachnoid hemorrhage. Such uptake may help to remove excess serotonin from the subarachnoid space. Only if serotonin is subsequently released upon nerve activation may minor smooth muscle contraction develop.

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Vasospasm in large cerebral arteries frequently develops following aneurysmal subarachnoid hemorrhage (SAH) and is the main cause of subsequent death and disability. The mechanism underlying the spasm is still poorly understood. One possible spasmogenic substance is serotonin (5-hydroxytryptamine, 5-HT) released from aggregating platelets in the subarachnoid space; 5-HT is a potent constrictor of large cerebral vessels both in vivo and in vitro. It has been shown that cerebrospinal fluid from patients with SAH causes a pronounced constriction of isolated vessels that may be blocked by the 5-HT antagonist ketanserin. Further, the vasoconstrictor action of fresh blood can be partially counteracted by methysergide. Measurements of cerebrospinal fluid have revealed high levels of 5-HT during the acute, but not the chronic, spasm phase of SAH, but prolonged exposure of vessels to 5-HT causes a desensitization. Thus, it is possible that 5-HT may contribute to the acute, but not the chronic, spasm phase.

Both sympathetic and nonsympathetic perivascular nerves have been proposed to be involved in the genesis of cerebral vasospasm. Recent studies have shown that 5-HT can be taken up into the sympathetic nerves in various vessels. Electrical field stimulation can release such perivascular 5-HT, and some studies have demonstrated a vasoconstrictor capacity of the released 5-HT. Thus, after incubation in the presence of 5-HT an enhancement of the sympathetic contractions was observed in rat
FIGURE 1. Dose–response curve for contractions induced by serotonin (5-HT) in rat basilar artery. Number of vessels = 8.

FIGURE 2. Typical tracings of amplifying effect of incubation in presence of serotonin (5-HT) for 30 minutes on contractions induced by electrical field stimulation (12 V) in untreated rats (a) and absence of amplifying effect after treatment with 6-hydroxydopamine (6-OHDA) (b). Note change in time scale in middle of each tracing. w, washout.

Materials and Methods

We used 38 male Sprague-Dawley rats weighing 300–400 g that were anesthetized with diethyl ether, perfused through the ascending aorta with 20 ml Krebs-Ringer buffer solution (millimolar composition: NaCl 118, KCl 4.5, CaCl₂·2H₂O 1.5, MgSO₄·7H₂O 1.2, NaHCO₃ 25, KH₂PO₄ 1.0, and glucose 6.0) at room temperature for 1 minute, and then decapitated. The brains were quickly removed and immersed in Krebs-Ringer buffer solution. The basilar arteries were dissected out under a surgical microscope and divided into three ring segments.

For measurement of isometric circular muscle contractions following exposure to various agents and electrical field stimulation, within 10 minutes after dissection the vessel segments were mounted on two L-shaped metal holders between platinum electrodes in an organ bath with the buffer solution at pH 7.35 and 37°C. After mounting, the vessels were subjected to a passive load of 2.5 mN and then allowed to stabilize for 60 minutes. The passive load applied was of a magnitude that would give maximum contraction of the vessels as tested in separate experiments. Agents used to enhance or block the responses to electrical field stimulation were added to the bath 15 minutes before stimulation, except for 10⁻⁸ M 5-HT, which was added 2 minutes before. Incubations for 30 minutes in the presence of 5 × 10⁻⁷ M 5-HT (in some experiments together with 5 × 10⁻⁸ M paroxetine) were also performed.

Electrical field stimulation was delivered as 10-second trains of monophasic square-wave impulses by a Grass S44 stimulator (Quincy, Mass.) with an additional power supply to overcome the limited output of commercial stimulators. The waves were perfectly square. A voltage of 12 or 15 V was used. With the platinum wire electrodes used this voltage corresponded to currents of 96 and 120 mA, respectively. The frequency was set at 6 Hz, and the duration of individual impulses was 0.1 msec.

Sympathetic denervation was achieved by the intraperitoneal injection of 50 mg/kg 6-OHDA (dissolved in saline containing 0.1% ascorbic acid) 2 days before and of 100 mg/kg 6-OHDA 1 day before sacrifice.
Table 1. Contraction of Rat Basilar Artery Segments Induced by Electrical Field Stimulation Before and After Incubation With 5-HT

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Before 5-HT incubation</th>
<th>Induced by 5-HT</th>
<th>Induced by stimulation after incubation</th>
<th>Amplification after incubation</th>
<th>Contraction induced by stimulation after incubation in presence of</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated controls</td>
<td>12</td>
<td>0.69±0.15</td>
<td>1.98±0.32</td>
<td>1.63±0.27*</td>
<td>0.94±0.19</td>
<td>0.62±0.14</td>
</tr>
<tr>
<td>6-Hydroxydopamine in vivo</td>
<td>9</td>
<td>0.67±0.15</td>
<td>2.87±0.53</td>
<td>0.54±0.8†</td>
<td>0.13±0.09†</td>
<td>...</td>
</tr>
<tr>
<td>Paroxetine in vitro</td>
<td>8</td>
<td>0.63±0.22</td>
<td>1.68±0.37</td>
<td>0.79±0.25†</td>
<td>0.17±0.18†</td>
<td>...</td>
</tr>
</tbody>
</table>

5-HT, serotonin. Values are mean±SEM mN. n, Number of experiments.
* p<0.001 different from before incubation.
† p<0.01, ‡ p<0.001, § 0.01<p<0.001 respectively, different from untreated.

For immunohistochemical examination, the basilar arteries were fixed for 24 hours in a solution containing 2% formaldehyde and 15% saturated aqueous picric acid in 0.2 M phosphate buffer (pH 7.2). After fixation the arteries were thoroughly rinsed for 48 hours in Tyrode’s solution and then stretched on chrome alum-coated slides as whole-mount preparations. The indirect immunohistochemical procedure of Coons et al29 was used. The vessel preparations were incubated with antiserum against 5-HT raised in rabbits (gift from H.W.M. Steinbusch, Department of Pharmacology, Free University, Amsterdam, The Netherlands) at a dilution of 1:250. After repeated rinsing in 0.1 M phosphate buffer solution containing 0.3% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) for 30 minutes, the vessels were incubated with swine anti-rabbit IgG conjugated with fluorescein isothiocyanate (Dakopatts, Glostrup, Denmark) at a dilution of 1:20 for 45 minutes at room temperature, rinsed, and mounted in a 1:1 mixture of the buffer and glycerin.

Experimental SAH was simulated according to the previously described method of Delgado et al.26 The rats were anesthetized with 0.3 ml/100 g body wt i.p. Equithesin (mixture of 40.5 ml [60 mg/ml] pentobarbital, 25 ml absolute alcohol, 99 ml propanediol, 10.6 g chloral hydrate, 5.3 mg magnesium sulfate, and 85.5 ml distilled water). A radiopaque catheter was inserted into the cisterna magna for subsequent injection of blood. The proximal part of the catheter was blunted and attached to the atlantooccipital membrane with a suture. The distal tip of the catheter was sealed and sutured to an external skull muscle. Three to 6 days after implantation of the cisternal catheter, the rats were prepared for SAH. During brief diethyl ether anesthesia, 0.3 ml autologous blood was injected into the cisterna magna via the catheter over about 30 seconds. Ten minutes or 2, 16, or 24 hours after the injection the rats were killed under ether anesthesia by perfusion via the ascending aorta with 20 ml Krebs-Ringer solution and the basilar arteries were removed for immunohistochemical examination or isometric tension measurements as previously described. In some of the rats killed 2 hours after SAH, chemical sympathectomy was performed by 6-OHDA treatment 1 and 2 days before the injection of blood. Control rats were injected with 0.3 ml saline instead of blood.

The following drugs were used: ascorbic acid (Sigma), 6-OHDA hydrochloride (Sigma), paroxetine (Ferrosan, Soborg, Denmark), prazosin hydrochloride (Pfizer Inc., New York, N.Y.), 5-HT creatinine sulfate (Sigma), and tetrodotoxin (Sigma). For statistical analysis, Student’s t test for paired or unpaired data was applied. The indicated values are mean±SEM.

Results

Characterization of Contractions Induced by Electrical Field Stimulation

The electrically induced contractions of basilar arteries from control rats were slow in onset, continued to rise for several seconds after the end of the stimulation, and slowly returned to baseline within about 60–90 seconds. The stimulus intensities used resulted in contractions corresponding to about 15–25% of those induced by a maximum-depolarizing effect of 5-HT on electrically induced contractions. Correlation was significant (p<0.001).

![FIGURE 3. Scatterplot of correlation between stable contractions induced by serotonin (5-HT) during 30 minutes of incubation and absolute values of amplifying effect of 5-HT on electrically induced contractions. Correlation was significant (p<0.001).](http://stroke.ahajournals.org/DownloadedFrom)
potassium solution. This contractile response remained unchanged after the application of $3 \times 10^{-7}$ M tetrodotoxin, $3 \times 10^{-8}$ M ketanserin, or $5 \times 10^{-8}$ M prazosin. This indicates that the contractions are nonneurogenic and represent direct electrical activation of the smooth muscle (see also References 28 and 30). A low concentration of 5-HT ($10^{-8}$ M), which by itself caused only minor contraction (Figure 1), failed to potentiate this electrically induced response. This stimulation intensity was, however, necessary to get a 5-HT-induced contractile component in subsequent experiments.

The same vessel segments were exposed to $5 \times 10^{-7}$ M 5-HT for 30 minutes. The amine caused a marked contractile response (about 40–60% of the maximum potassium-induced contraction) that remained stable during the entire incubation period. After 30 minutes the incubation was interrupted by repeated washouts, whereby the vascular tone returned to baseline. Upon electrical field stimulation with the same intensity as before incubation, an enhanced contractile response, more rapid in onset, was obtained (Figure 2a). The additional contractile component seen after incubation was abolished following the addition of $3 \times 10^{-7}$ M tetrodotoxin or $10^{-8}$ M ketanserin. In the presence of these agents, the contractile response was not significantly different from that before incubation (Table 1). The absolute value of the amplified response corresponded to the contractile action of $8 \times 10^{-8}$ M exogenous 5-HT as estimated from sepa-

![Figure 4](http://stroke.ahajournals.org/)

**Figure 4.** Photomicrographs ($\times 300$) of rat basilar arteries prepared to show serotonin (5-HT) immunofluorescence. Panel a: Control animals have no visible fibers. Panel b: 10 minutes after experimental subarachnoid hemorrhage dense network of 5-HT-positive fibers appears.
rate concentration–response curves for 5-HT in rat basilar artery (Figure 1).

There was a significant correlation between the contractions induced by 5-HT during incubation and the amplified electrogenic response obtained after incubation (Figure 3). As expected, no significant correlation was found between the contractions induced by electrical field stimulation before incubation and the amplifying effect of the incubation.

Electrical field stimulation of vessels from 6-OHDA-treated rats induced contractions that were similar in magnitude to those observed in vessels from control animals, further indicating a nonneural origin of this contractile component (Table 1). In vessels from 6-OHDA-treated rats incubation with 5-HT did not enhance the response (Figure 2b, Table 1). By comparison, no significant amplification was observed when vessels from control rats were incubated with 5-HT in the presence of paroxetine (Table 1).

Effect of Subarachnoid Hemorrhage on Immunofluorescence in Perivascular Nerves

In the control rats no 5-HT-immunoreactive fibers were seen in the basilar artery (Figure 4a). Following experimental SAH, many 5-HT-immunoreactive fibers were seen to form a network in the adventitia of vessels from animals killed 10 minutes or 2 hours after blood injection (Figure 4b). The number of positive fibers had declined markedly by 24 hours after the injection. Almost no fibers were seen in the adventitia of 6-OHDA-treated rats 2 hours after experimental SAH.

In the animals receiving cisternal blood, a marked increase in the number of 5-HT–immunoreactive mast cells was seen at all intervals after the injection.

Responses of Basilar Artery to Electrical Field Stimulation After Subarachnoid Hemorrhage

In contrast to the findings in control rats, the electrogenic contractile response after experimental SAH included components blockable by $3 \times 10^{-7} \text{M tetrodotoxin as well as by } 10^{-8} \text{M ketanserin (Figure 5, Table 2). Both agents significantly reduced the vascular contractions at 2 hours and 16 hours, but not those at 10 minutes and 24 hours, after SAH (Table 2). The absolute values of the tetrodotoxin- and ketanserin-blockable components, after subtraction of the nonneurogenic component at different times after experimental SAH, are given in Figure 6. There was no significant difference between the capacities of the two agents to block this component. Its size corresponded to the contractile activity of $1 - 5 \times 10^{-8} \text{M exogenous 5-HT. A minor tetrodotoxin- and ketanserin-sensitive component was observed in vessels from the 6-OHDA-denervated rats killed 2 hours after experimental SAH; this component was, however, significantly less (p<0.05) than that observed in vessels from nondenervated animals killed 2 hours after the blood injection (Figure 6).}

Discussion

Electrical field stimulation induced a contractile response in the basilar artery of control rats that was not blockable by tetrodotoxin, ketanserin, or prazosin.

**TABLE 2. Contractions of Rat Basilar Artery Induced by Electrical Field Stimulation After Experimental Subarachnoid Hemorrhage**

<table>
<thead>
<tr>
<th>Time after subarachnoid hemorrhage</th>
<th>n</th>
<th>Tetrodotoxin</th>
<th>Ketanserin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>10 min</td>
<td>13</td>
<td>1.02±0.23</td>
<td>0.89±0.17</td>
</tr>
<tr>
<td>2 hr</td>
<td>14</td>
<td>0.98±0.20</td>
<td>0.51±0.14*</td>
</tr>
<tr>
<td>2 hr (treated with 6-hydroxydopamine)</td>
<td>9</td>
<td>1.37±0.39</td>
<td>1.23±0.33</td>
</tr>
<tr>
<td>16 hr</td>
<td>9</td>
<td>1.18±0.17</td>
<td>0.54±0.11*</td>
</tr>
<tr>
<td>24 hr</td>
<td>9</td>
<td>1.14±0.34</td>
<td>0.93±0.29</td>
</tr>
</tbody>
</table>

Values are mean±SEM mN. n, Number of experiments. *p<0.001 different from before.
or by prior in vivo sympathectomy and was not augmented by acute exposure to exogenous 5-HT. These findings are in accordance with previous studies of this artery showing that such contraction is not of neural origin and thus represents direct smooth muscle activation by the electrical field. The lack of an α-adrenergic electrogenic contraction agrees with the absence of postsynaptic α-adrenoceptors in this artery. We used this fact to obtain a vascular contraction that was due solely to activation of 5-HT receptors.

The uptake and release of radiolabeled 5-HT from perivascular nerves has been demonstrated in several vascular beds including rat pial vessels. Several studies have also shown that 5-HT, after uptake into the perivascular nerves during incubation, is released by electrical field stimulation and causes vascular smooth muscle contraction. Our results indicate that the perivascular sympathetic nerves in rat basilar artery are capable of taking up exogenous or neuronal 5-HT and of releasing the indole in concentrations high enough to cause constriction in addition to the nonneurogenic response. This enhanced response does not represent mere potentiation of the nonneurogenic response since there was no correlation between the contraction induced by electrical field stimulation before incubation and the serotoninergic amplification. Further, acute exposure to 5-HT failed to enhance the nonneurogenic contractions induced by electrical field stimulation. The fact that treatment with tetrodotoxin, 6-OHDA, or ketanserin blocked the contraction obtained after in vitro incubation with 5-HT or in vivo exposure to blood (SAH) indicates that the nerves taking up 5-HT are sympathetic. By comparison, the uptake and release of radioactive 5-HT from rat pial arteries is abolished after superior cervical ganglionectomy. We found a highly significant correlation between the 5-HT-induced contractions and the amplified contractile response after incubation with 5-HT.

After induction of experimental SAH, but not in control rats, a network of 5-HT-immunoreactive nerve fibers was seen in the pial vessel walls. In 6-OHDA-treated rats, only a few fibers were seen 2 hours after the deposition of blood. These findings agree with studies by Jackowski et al, who showed that 5-HT can be demonstrated in pial perivascular nerves only after prior exposure to blood.

The presence of an enhanced number of 5-HT-containing mast cells throughout the entire post-SAH period might indicate fast uptake and slow elimination of 5-HT by these cells. The cells could subserve a protective function, helping to eliminate 5-HT from the subarachnoid space.

The time course of spasm in this experimental SAH model has been determined in a previous study. In that study it was found that the acute spasm phase peaked 10 minutes after blood injection, whereas the chronic phase peaked 2 days later. In our study, 10 minutes after the blood injection there was only a small, nonsignificant serotoninergic vascular contraction, and 1 day after SAH, when 5-HT immunoreactivity of the perivascular nerves had decreased (see also Reference 25), again only a very small, nonsignificant 5-HT-induced contractile component was observed, despite the possibility that postsynaptic 5-HT receptors may have become hypersensitive. These findings suggest that neuronal accumulation and subsequent release of 5-HT may not be principally responsible for the acute or chronic spasm phases. The SAH-induced electrogenic contractile component corresponded to a relatively low concentration of exogenous 5-HT (at most 5×10^-8 M), which induced only about 10% of the maximum contraction possible for this vessel and 15% of the maximum 5-HT-induced contractile response. It is therefore conceivable that neurogenic 5-HT release may play an only minor role, if any, in the development of vasospasm. Previous experiments with neonatal 6-OHDA treatment using the same SAH model have shown an acute spasm of the same degree as in rats with intact sympathetic nerves.

The uptake of 5-HT into perivascular nerves may function as a protective mechanism to eliminate 5-HT of platelet origin from the cerebrospinal fluid, and mast


**KEY WORDS**  • serotonin  • subarachnoid hemorrhage  • rats
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