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RESEARCH PAPER

Adrenergic stimulation-released 5-HT stored in adrenergic nerves inhibits CGRPergic nerve-mediated vasodilatation in rat mesenteric resistance arteries

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BACKGROUND AND PURPOSE

5-HT is taken up by and stored in adrenergic nerves and periarterial nerve stimulation (PNS) releases 5-HT to cause vasoconstriction in rat mesenteric arteries. The present study investigated whether PNS-released 5-HT stored in adrenergic nerves affects the function of perivascular calcitonin gene-related peptide-containing (CGRPergic) nerves.

EXPERIMENTAL APPROACH

Rat mesenteric vascular beds without endothelium and with active tone were perfused with Krebs solution. Changes in perfusion pressure in response to PNS and CGRP injection were measured before (control) and after perfusion of Krebs solution containing 5-HT (10 μM) for 20 min. Distributions of 5-HT- and TH-immunopositive fibres in mesenteric arteries were studied using immunohistochemical methods.

KEY RESULTS

PNS (1–4 Hz) frequency dependently caused adrenergic nerve-mediated vasoconstriction followed by CGRPergic nerve-mediated vasodilatation. 5-HT treatment inhibited PNS-induced vasodilatation without affecting exogenous CGRP-induced vasodilatation, while it augmented PNS-induced vasoconstriction. Guanethidine (adrenergic neuron blocker), methysergide (non-selective 5-HT receptor antagonist) and BRL15572 (selective 5-HT1D receptor antagonist) abolished inhibition of PNS-induced vasodilatation in 5-HT-treated preparations. Combined treatment with 5-HT and desipramine (catecholamine transporter inhibitor), but not fluoxetine (selective 5-HT reuptake inhibitor), did not inhibit PNS-induced vasodilatation. Exogenous 5-HT inhibited PNS-induced vasodilatation, which was antagonized by methysergide. In immunohistochemical experiments, 5-HT-immunopositive nerves, colocalized with adrenergic TH-immunopositive nerves, were observed only in 5-HT-treated mesenteric arteries, but not in control preparations or arteries co-treated with desipramine.

CONCLUSIONS AND IMPLICATIONS

These results suggest that 5-HT can be taken up by and released from adrenergic nerves *in vitro* by PNS to inhibit CGRPergic nerve transmission in rat mesenteric arteries.

Abbreviations

BRL15572, 3-(4-(4-chlorophenyl)piperazin-1-yl)-1,1-diphenyl-2-propanol; CGRPergic nerve, calcitonin gene-related peptide-containing nerve; PNS, periarterial nerve stimulation

Introduction

The biogenic amine 5-HT, which is widely distributed in the body, plays a physiological and pathophysiological role in biological functions. Although 5-HT has been shown to act as a neurotransmitter in the CNS, its role in the peripheral nervous system, especially perivascular nerves, has not been fully clarified. Kawasaki and Takasaki (1984) demonstrated that 5-HT is taken up by and accumulated and/or stored in the vascular adrenergic nerves of rat mesenteric arteries and the stored 5-HT is released by nerve stimulation in a calcium-dependent manner. Additionally, the 5-HT released from adrenergic nerve endings produces a vasoconstrictor response, which is mediated by postsynaptic 5-HT_{2A} receptors (Kawasaki and Takasaki, 1984), implying that 5-HT has the ability to act as a neurotransmitter in the mesenteric perivascular nerves.

Rat mesenteric resistance arteries have been shown to be densely innervated by perivascular sympathetic adrenergic nerves, which are vasoconstrictor nerves. In addition to adrenergic nerves, the artery is densely innervated by non-adrenergic non-cholinergic calcitonin gene-related peptide (CGRP)-containing (CGRPergic) nerves, which are vasodilator nerves and sensitive to capsaicin (a CGRP depletor) (Kawasaki *et al.*, 1988). Furthermore, adrenergic and CGRPergic nerves interact to regulate vascular tone. The neurotransmitter noradrenaline released from adrenergic nerves presynaptically inhibits the function of CGRPergic nerves and CGRPergic nerves release the vasodilator CGRP to postsynaptically suppress adrenergic nerve function (Kawasaki *et al.*, 1990; 2009; Shiraki *et al.*, 2000; Eguchi *et al.*, 2004). 5-HT has been shown to have an inhibitory effect on capsaicin-sensitive sensory nerves (Carmichael *et al.*, 2008). Thus, stimulation-released 5-HT, which had accumulated in sympathetic adrenergic nerves, has the ability to exert an effect on CGRPergic nerve function.

Therefore, the present study was designed to investigate the effect of 5-HT released from sympathetic nerve endings on the function of CGRPergic nerves in rat mesenteric vascular beds.

Methods

Animals

Ninety-six male Wistar rats weighing 300–350 g (purchased from Shimizu Laboratory Supplies Co., Ltd, Shizuoka, Japan) were used. All animals were given food and water *ad libitum*, and housed in the Animal Research Center, Okayama University at a controlled ambient temperature of 22 ± 2°C with 50 ± 10% relative humidity and a 12 h light/12 h dark cycle (lights on at 8:00 a.m.). This study was carried out in accordance with the Guidelines for Animal Experiments at Okayama University Advanced Science Research Center, Japanese Government Animal Protection and Management Law /No. 105/, and Japanese Government Notification on Feeding and Safekeeping of Animals /No. 6/. The results of all studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (McGrath *et al.*, 2010). Every effort was made to minimize the number of animals used and their suffering.

Perfusion of mesenteric vascular beds and perfusion pressure measurements

The animals were anaesthetized with pentobarbital-Na (50 $mg \cdot kg^{-1}$, i.p.) and the mesenteric vascular beds were isolated and prepared for perfusion as described previously (Kawasaki *et al.*, 1988). The superior mesenteric artery was cannulated and flushed gently with Krebs-Ringer bicarbonate solution (Krebs solution) to eliminate blood from the vascular bed. After removal of the entire intestine and associated vascular bed, the mesenteric vascular bed was separated from the intestine by cutting close to the intestinal wall. Only four main arterial branches from the superior mesenteric trunk running to the terminal ileum were perfused. All other branches of the superior mesenteric artery were tied off. The isolated mesenteric vascular bed was then placed in a water-jacketed organ bath maintained at 37°C and perfused with a modified (see later) Krebs solution at a constant flow rate of 5 $mL \cdot min^{-1}$ with a peristaltic pump (model AC-2120, ATTO, Tokyo, Japan) and superfused with the same solution at a rate of 0.5 $mL \cdot min^{-1}$ to prevent drying. The Krebs solution was bubbled with a mixture of 95% O_2–5% CO_2 before passage through a warming coil maintained at 37°C. The Krebs solution was of the following composition (mM): NaCl 119.0, KCl 4.7, $CaCl_2$ 2.4, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2, disodium EDTA 0.03 and dextrose 11.1 (pH 7.4). Changes in perfusion pressure were measured with a pressure transducer (model TP-400T, Nihon Kohden, Tokyo, Japan) and recorded on a pen recorder (model U-228, Nippon Denshi Kagaku, Tokyo, Japan).

Periarterial nerve stimulation (PNS)

PNS was applied for 30 s using bipolar platinum ring electrodes placed around the superior mesenteric artery. Rectangular pulses of 1 ms and a supramaximal voltage (50 V) were applied at 1, 2 and 4 Hz using an electronic stimulator (model SEN 3301, Nihon Kohden).

Chemical removal of vascular endothelium

To avoid possible 5-HT-mediated endothelium-dependent vascular action, all experiments were carried out in preparations without an endothelium. To remove the vascular endothelium, preparations with resting tension were perfused with a 1.8 $mg \cdot mL^{-1}$ solution of sodium deoxycholate in saline for 30 s as described previously (Takenaga *et al.*, 1995; Shiraki *et al.*, 2000). This caused a transient increase (20–30 mmHg) in perfusion pressure. Then, the preparation was rinsed with

sodium deoxycholate-free Krebs solution for 60 min. After the preparation was made to contract by perfusion with Krebs solution containing 2 μM methoxamine (α_1-adrenoceptor agonist), chemical removal of the endothelium was assessed by the lack of a vasodilator response to the injection of a bolus of ACh (1 nmol). ACh was injected directly into the perfusate proximal to the arterial cannula with an injection pump (model 975; Harvard Apparatus Inc., Holliston, MA, USA). The volume injected was 100 μL for 12 s.

Experimental protocol for vascular responses

To produce active tone and elevate perfusion pressure, the perfused mesenteric vascular bed was perfused with methoxamine. In several preparations, to observe vascular responses in the preparation with intact endothelium, PNS and injection of ACh and CGRP were carried out. Additionally, to confirm the CGRPergic nerve-mediated vasodilatation, PNS and injection of ACh and CGRP were performed in the preparation treated with capsaicin (5 μM) for 20 min as described by Kawasaki *et al*. (1988).

In denuded preparations, after the elevated perfusion pressure had stabilized, PNS (1, 2 and 4 Hz) was applied for 30 s as a control (S1). In another preparation, a bolus of CGRP (50 pmol) was injected as a control (I1). The CGRP was injected directly into the perfusate proximal to the arterial cannula with an infusion pump. A volume of 100 μL was administered over a period of 12 s. After the pressure had returned to baseline, the next PNS or CGRP injection was carried out. Thereafter, Krebs solution containing methoxamine was switched to methoxamine-free Krebs solution to return to basal perfusion levels. After baseline pressure had stabilized, methoxamine-free Krebs solution was changed to methoxamine-free Krebs solution containing 5-HT (10 μM) and perfused for 20 min followed by normal Krebs solution (methoxamine and 5-HT-free) for 30 min to wash out the preparation. Thereafter, the preparation was reperfused with Krebs solution containing methoxamine to elevate perfusion pressure, and then PNS or another injection of CGRP was performed in the absence of 5-HT (S2 or I2). To estimate the effects of 5-HT and various agents, changes in perfusion pressure in response to PNS or CGRP injection are expressed as % or the ratio between S2 and S1 and I2 and I1.

In experiments using a non-selective 5-HT receptor antagonist, methysergide (0.1 μM), or a selective 5-HT_{1D} receptor antagonist, BRL15572 (0.1 μM), and an adrenergic neuron blocker, guanethidine (5 μM), the perfusion of each agent was started 5 min after the discontinuation of 5-HT perfusion throughout the rest of the experiment. Thereafter, the preparation was perfused with Krebs solution containing methoxamine and each agent to elevate perfusion pressure and then subjected to PNS.

In experiments using a selective catecholamine transporter inhibitor, desipramine (0.1 μM), or a 5-HT reuptake inhibitor, fluoxetine (1 μM), the perfusion of these drugs was begun 20 min before the combined perfusion of 5-HT and each inhibitor for 20 min, and then the perfusion of desipramine or fluoxetine alone was continued for 30 min during the washout period. Thereafter, the preparation was perfused with Krebs solution containing methoxamine and desipramine or fluoxetine to elevate perfusion pressure and then subjected to PNS.

In another series of experiments, the effect of exogenous 5-HT on responses to PNS and CGRP injection was examined. After the responses to PNS (1 to 4 Hz) and CGRP injection were recorded as control, Krebs solution containing methoxamine and guanethidine (5 μM) was switched to Krebs solution containing methoxamine, 5-HT (0.01, 0.1 or 1 μM) and guanethidine or Krebs solution containing methoxamine, 5-HT (0.1 μM), methysergide (1 μM) and guanethidine, and then PNS and CGRP injection were carried out during the perfusion of 5-HT.

At the end of each experiment, the preparation was perfused with 100 μM papaverine to produce complete relaxation. Vasodilator activity was expressed as a percentage of the perfusion pressure at maximum relaxation induced by papaverine. Vasoconstrictor activity was expressed as a percentage of the perfusion pressure before PNS or CGRP injection.

Immunohistochemical analysis

The animals were anaesthetized with pentobarbital-Na (50 mg kg^{-1}, i.p.). The mesenteric vascular bed was removed together with the intestine as described previously (Kawasaki *et al*., 1990). The superior mesenteric artery was cannulated with polyethylene tubing, perfused with Krebs solution containing 5-HT (10 μM) for 20 min, and washed with normal 5-HT-free Krebs solution for 60 min. In the experiments using desipramine, the preparations were perfused with Krebs solution containing desipramine (0.1 μM) for 10 min, then Krebs solution containing 5-HT and desipramine for 20 min, and, finally, Krebs solution containing desipramine alone during a 30 min washing period. Thereafter, the vascular bed was infused and fixed with Zamboni solution (2% paraformaldehyde and 15% picric acid in 0.15 M phosphate buffer). The small mesenteric arteries proximal to the intestine were removed and immersion-fixed in the Zamboni solution for 48 h. Next, the artery was repeatedly rinsed in PBS, immersed in PBS containing 0.5% Triton X-100 overnight and incubated with PBS containing normal goat serum (1:100) for 60 min. The artery was then incubated with a rabbit polyclonal anti-5-HT (Biogenesis Ltd, Poole, UK) antibody at 1:100 or a rabbit polyclonal anti-TH (Phoenix Pharmaceuticals Inc., Belmont, CA, USA) antibody at 1:100 for 72 h at 4°C. The site of the antigen–antibody reaction was revealed by incubation with fluorescein-5-isothiocyanate-labelled goat anti-rabbit IgG (diluted 1:100) (ICN Pharmaceuticals, Inc., Aurora, OH, USA) for 60 min. Thereafter, the artery was mounted on slides, coverslipped with glycerol/PBS (2:1 v v^{-1}) and observed under a confocal laser scanning microscope (CLSM510, Carl Zeiss GmbH, Jena, Germany) in the Okayama University Medical School Central Research Laboratory.

At least three preparations were examined for immunostaining in each group.

Statistical analysis

Experimental results are expressed as the mean ± SEM. Statistical analysis was performed using Student's unpaired *t*-test

for comparisons between two groups and a one-way ANOVA followed by Tukey's test for comparisons among multiple groups. A value of $P < 0.05$ was considered statistically significant.

Drugs

The following drugs were used: ACh chloride (Daiichi-Sankyo Pharmaceutical, Tokyo, Japan), BRL 15572 (Sigma-Aldrich Japan, Tokyo Japan), capsaicin (Sigma-Aldrich), 5-HT hydrochloride (Sigma-Aldrich), guanethidine sulphate (Sigma-Aldrich), methoxamine hydrochloride (Nippon Shinyaku, Kyoto, Japan), sodium deoxycholate (Sigma-Aldrich), methysergide maleate salt (Sigma-Aldrich), desipramine hydrochloride (Sigma-Aldrich), fluoxetine hydrochloride (Sigma-Aldrich) and papaverine hydrochloride (Dainippon-Sumitomo Pharmaceutical, Osaka, Japan). Sodium deoxycholate and capsaicin were dissolved in 0.9% saline and 50% ethanol, respectively. All other drugs were dissolved in distilled water and diluted with Krebs solution containing 2 μM methoxamine, when perfused or injected directly.

Results

Vascular responses to PNS and CGRP injection

As shown in Figure 1A, in the preparation with an intact endothelium and with an active tone produced by methoxamine (7 μM), the injection of ACh (1 nmol) produced a rapid drop in perfusion pressure due to an endothelium-dependent vasodilatation (Figure 1A). In this preparation, PNS at 1, 2 and 4 Hz induced a transient increase in perfusion pressure due to vasoconstriction followed by a long-lasting decrease in perfusion pressure due to vasodilatation in a frequency-dependent manner. Additionally, the bolus of CGRP induced a long-lasting vasodilatation, which mimicked the PNS-induced vasodilatation (Figure 1A). As shown in Figure 1B, in the preparation treated with capsaicin (CGRP depletor), PNS at 1–4 Hz induced a vasoconstrictor response without vasodilatation, while ACh and CGRP injection induced vasodilatation similar to control responses.

In the preparation without an endothelium and with an active tone produced by methoxamine (2 μM), the injection of ACh (1 nmol) did not produce a sharp vasodilatation (Figure 1C), indicating that the endothelium was effectively removed (control, 83.5 ± 3.6%, $n = 5$; endothelium removal, 5.7 ± 1.2%, $n = 6$, $P < 0.01$). As shown in Figure 1C, the first series of PNS (S1) at 1, 2 and 4 Hz caused a transient vasoconstriction followed by a long-lasting vasodilatation in a frequency-dependent manner. As shown in Figure 1D, the second series of PNS (1, 2 and 4 Hz) induced reproducible vasoconstrictor (S2/S1 ratio; 1 Hz, 0.93 ± 0.08; 2 Hz, 1.12 ± 0.15; 4 Hz, 1.10 ± 0.15) and vasodilator (S2/S1 ratio; 1 Hz, 1.12 ± 0.22; 2 Hz, 1.14 ± 0.11; 4 Hz, 1.02 ± 0.05) responses similar to responses to S1-PNS (Figure 1E and F).

In the first series of injections, the bolus of CGRP (I1) induced a long-lasting vasodilatation (Figure 1C). The second series of CGRP injection (I2) reproduced responses similar to the first (I2/I1 ratio; 0.99 ± 0.04) (Figure 1G).

Effect of 5-HT treatment on PNS- or CGRP-induced vascular responses

As shown Figure 2, the perfusion of 5-HT (10 μM) induced a sharp increase in perfusion pressure due to vasoconstriction (138.8 ± 4.8 mmHg, $n = 5$), which reached a maximum at 3 to 4 min and thereafter gradually returned to the baseline level within 20 min. After the 5-HT treatment, initial vasoconstrictor responses to PNS at 2 and 4 Hz were significantly augmented (Figure 2C and D) (S2/S1 ratio; 1 Hz, 1.35 ± 0.40; 2 Hz, 1.52 ± 0.24; 4 Hz, 1.48 ± 0.21), while the PNS-induced vasodilatation was significantly inhibited as shown in Figure 2C and E (S2/S1 ratio; 1 Hz, 0.59 ± 0.10; 2 Hz, 0.45 ± 0.05; 4 Hz, 0.53 ± 0.05).

However, 5-HT treatment did not affect the vasodilator response to exogenously applied CGRP (Figure 2C and F) (I2/I1 ratio; 1.00 ± 0.04).

Effect of guanethidine on vascular responses to PNS after 5-HT treatment

In denuded mesenteric vascular beds treated with 5-HT for 20 min, perfusion of 5 μM guanethidine, an adrenergic neuron blocker, abolished vasoconstrictor responses to PNS, but not vasodilator responses to PNS, which were similar to control responses before the 5-HT treatment (Figure 3C–E) (S2/S1 ratio; 1 Hz, 0.93 ± 0.16; 2 Hz, 0.98 ± 0.11; 4 Hz, 1.08 ± 0.11).

Effects of 5-HT receptor antagonists on vascular responses to PNS after 5-HT treatment

To assess possible mechanisms underlying the altered vascular responses to PNS after the 5-HT treatment, the effects of 5-HT receptor antagonists were examined in preparations without an endothelium but with active tone. As shown in Figure 4, in the preparations treated with 5-HT, PNS in the presence of a non-selective 5-HT receptor antagonist, methysergide (0.1 μM), or a selective 5-HT_{1D} receptor antagonist, BRL15572 (0.1 μM), induced vasodilatation similar to control responses before the 5-HT treatment (Figure 4B and D) (methysergide S2/S1 ratio; 1 Hz, 1.09 ± 0.18; 2 Hz, 1.14 ± 0.09; 4 Hz, 1.03 ± 0.04; BRL15572 S2/S1 ratio; 1 Hz, 0.98 ± 0.25; 2 Hz, 0.85 ± 0.18; 4 Hz, 0.85 ± 0.12). In preparations treated with 5-HT, BRL15572 (0.1 μM) markedly augmented the vasoconstrictor responses to PNS (S2/S1 ratio; 1 Hz, 1.53 ± 0.36; 2 Hz, 1.94 ± 0.30; 4 Hz, 2.16 ± 0.52), while methysergide had no effect (Figure 4A and C) (S2/S1 ratio; 1 Hz, 0.92 ± 0.07; 2 Hz, 1.01 ± 0.11; 4 Hz, 1.15 ± 0.15).

Effects of desipramine and fluoxetine on vascular responses to PNS after 5-HT treatment

In denuded preparations without 5-HT treatment, perfusion of desipramine, a selective catecholamine transporter inhibitor, increased the vasoconstrictor response to PNS (S2/S1 ratio; 1 Hz, 1.28 ± 0.17; 2 Hz, 1.57 ± 0.40; 4 Hz, 2.19 ± 0.64, $n = 3$), while it did not affect the vasodilator response to PNS (S2/S1 ratio; 1 Hz, 1.09 ± 0.19; 2 Hz, 0.95 ± 0.13; 4 Hz, 0.93 ± 0.01, $n = 3$).

In denuded preparations treated with 5-HT in the presence of desipramine (0.1 μM), PNS at 1, 2 and 4 Hz induced

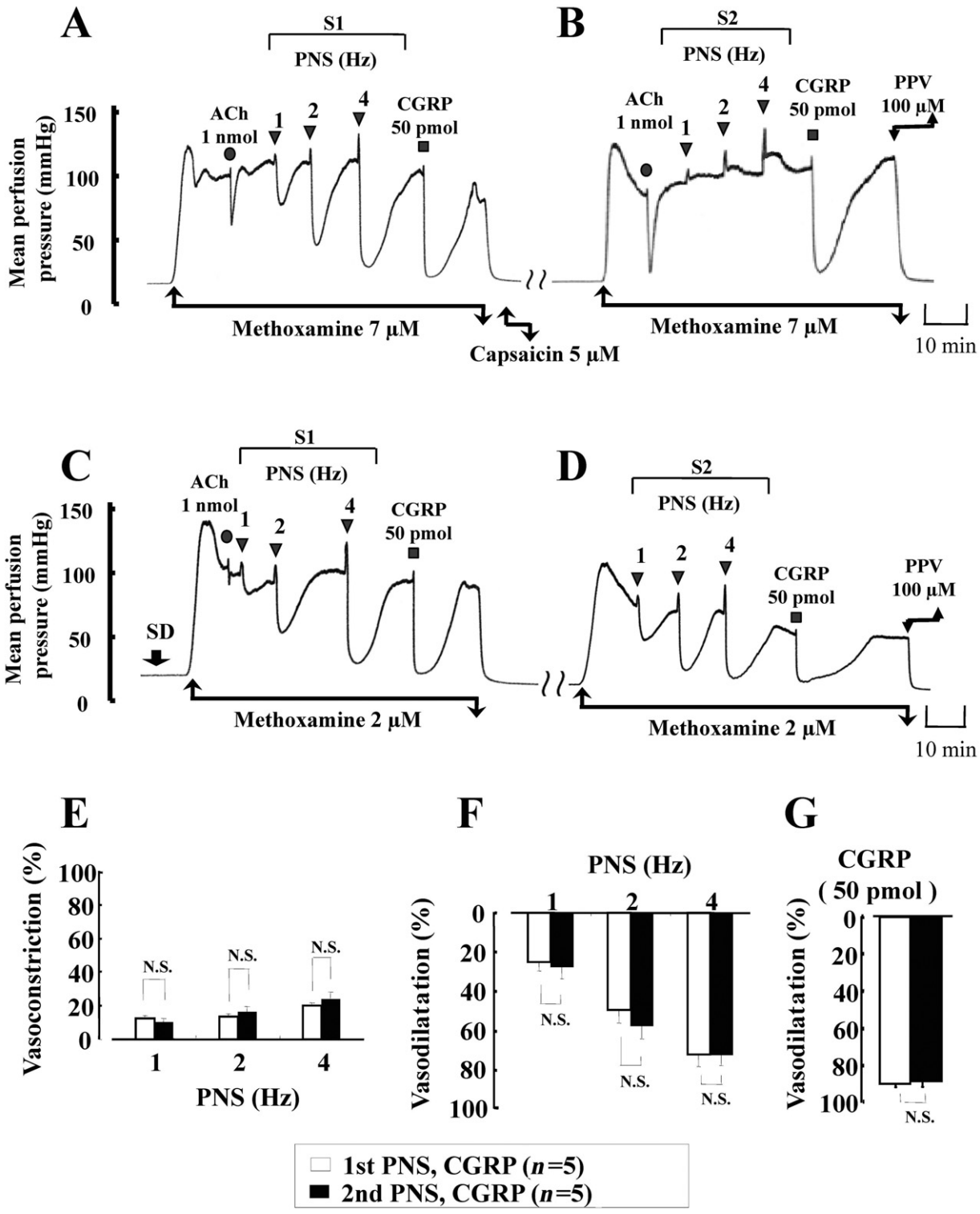

Figure 1

Typical recordings (A, B, C and D) and histograms (D, F and G) showing control vascular responses to periarterial nerve stimulation (PNS; 1, 2 and 4 Hz) and injection of ACh (1 nmol) and CGRP (50 pmol) in rat perfused mesenteric vascular beds. (A and B) Indicate vascular responses in the preparation with an endothelium and treatment with capsaicin (5 μM for 20 min), respectively. (C and D) Vascular responses in the preparation without an endothelium. The active tone was produced by 2 or 7 μM methoxamine. S1 and S2, responses to the first and second PNS, respectively. SD, the perfusion of sodium deoxycholate for 30 s. PPV, the perfusion of papaverine. (E, F and G) Changes in vasoconstrictor and vasodilator responses to PNS and vasodilator responses to CGRP injections without temporary 5-HT treatment, respectively. Data are presented as the mean ± SEM. N.S., not significantly different.

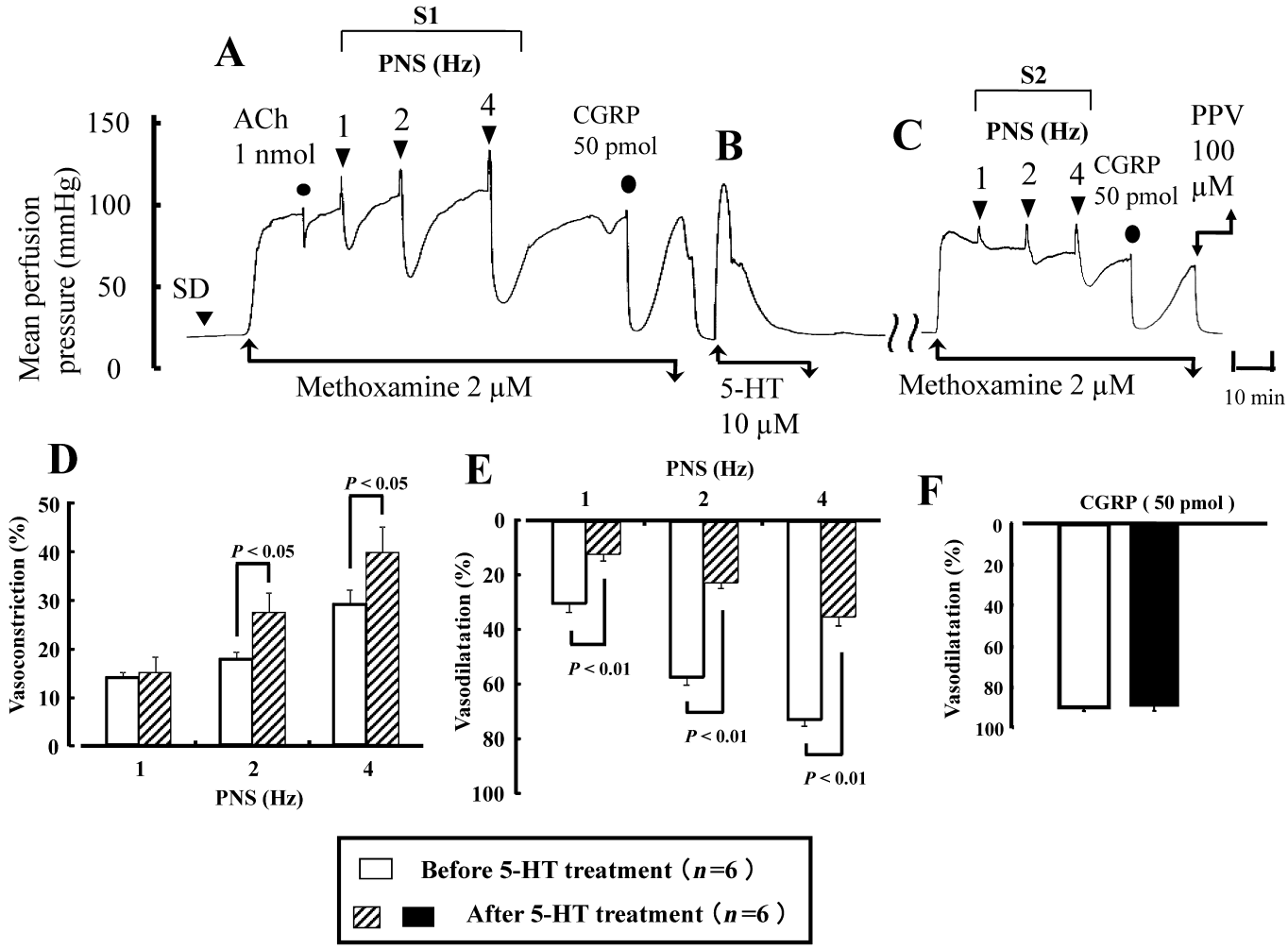

Figure 2

Typical recordings (A, B and C) and histograms (D, E and F) showing effects of temporary 5-HT treatment on vascular responses to PNS (1, 2 and 4 Hz) and CGRP injections (50 pmol) in rat perfused mesenteric vascular beds without an endothelium and with active tone produced by 2 μM methoxamine. (A) Control responses to PNS and CGRP injection. (B) 5-HT (10 μM) perfusion for 20 min. (C) Vascular responses to PNS without 5-HT perfusion. (D, E and F) Changes in vasoconstrictor and vasodilator responses to PNS and vasodilator responses to CGRP injections after temporary 5-HT treatment, respectively. S1 and S2 indicate responses to the first and second PNS, respectively. SD, the perfusion of sodium deoxycholate for 30 s. PPV, the perfusion of papaverine. Data are presented as the mean ± SEM.

vasodilator responses similar to control responses before the combined treatments (S2/S1 ratio; 1 Hz, 0.95 ± 0.15; 2 Hz, 0.89 ± 0.06; 4 Hz, 0.94 ± 0.07). The combined treatments caused further augmentations of vasoconstrictor responses to PNS (S2/S1 ratio; 1 Hz, 1.38 ± 0.29; 2 Hz, 1.63 ± 0.23; 4 Hz, 2.00 ± 0.42), as shown in Figure 5A and B.

In denuded preparations without 5-HT treatment, perfusion of fluoxetine, a selective 5-HT re-uptake inhibitor, did not affect vasoconstrictor responses to PNS (S2/S1 ratio; 1 Hz, 0.99 ± 0.28; 2 Hz, 1.11 ± 0.12; 4 Hz, 1.4 ± 0.25, n = 3) or vasodilator responses to PNS (S2/S1 ratio; 1 Hz, 0.97 ± 0.35; 2 Hz, 1.12 ± 0.14; 4 Hz, 0.98 ± 0.08, n = 3). Additionally, the combination of 5-HT and 1 μM fluoxetine had no effect on the PNS-induced vasoconstrictor (S2/S1 ratio; 1 Hz, 1.14 ± 0.21; 2 Hz, 1.16 ± 0.18; 4 Hz, 1.26 ± 0.24) (Figure 5C) or vasodilator responses (S2/S1 ratio; 1 Hz, 0.60 ± 0.07; 2 Hz, 0.51 ± 0.06; 4 Hz, 0.60 ± 0.05) (Figure 5D).

Effects of exogenous 5-HT on vascular responses to PNS and CGRP injection

As shown in Figure 6A and B, PNS at 1–4 Hz and CGRP (50 pmol) injection induced vasodilatation. Perfusion of 5-HT at concentrations of 0.01 to 1 μM significantly inhibited the PNS-induced vasodilatation, but not CGRP-induced vasodilatation. The inhibitory effect of 5-HT on PNS-induced vasodilatation was abolished by combined perfusion with methysergide (Figure 6A).

Immunohistochemical analysis

As shown in Figure 7A, there was no 5-HT-like immunoreactive (LI) nerves in the control mesenteric artery. However, the

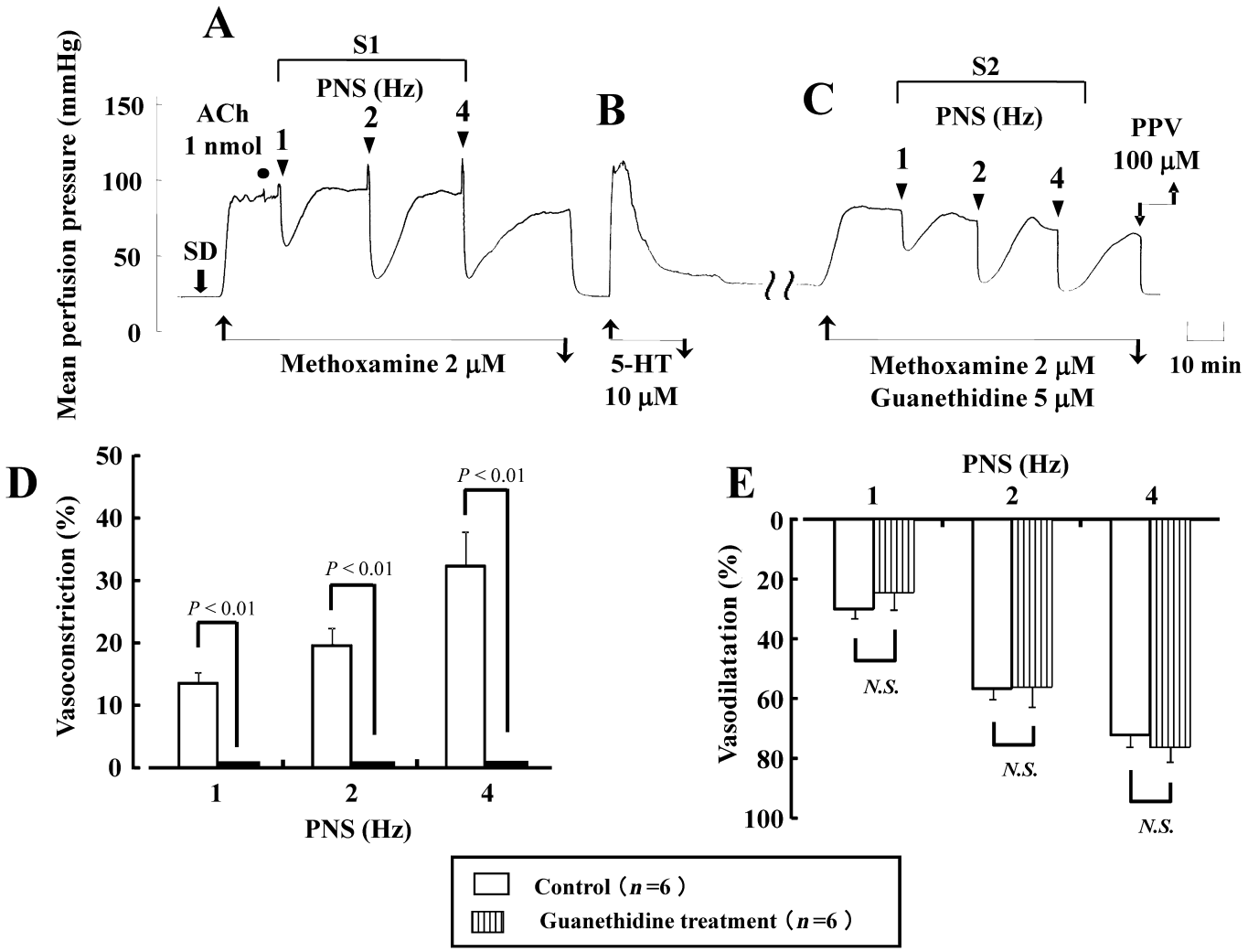

Figure 3

Typical recordings (A, B and C) and histograms (D and E) showing effects of guanethidine on vascular responses to PNS (1, 2 and 4 Hz) after 5-HT treatment in rat perfused mesenteric vascular beds without an endothelium and with active tone produced by 2 μM methoxamine. (A) Control responses. (B) 5-HT (10 μM) perfusion for 20 min. (C) vascular responses to PNS after 5-HT treatment in the presence of guanethidine. (D and E) Effect of guanethidine on vasoconstrictor and vasodilator responses to PNS after temporary 5-HT treatment, respectively. S1 and S2, responses to the first and second PNS, respectively. SD, the perfusion of sodium deoxycholate for 30 s. PPV, the perfusion of papaverine (100 μM). Data are presented as the mean ± SEM.

rat arteries were densely innervated by adrenergic TH-LI nerves (Figure 7B). As shown in Figure 7C, double immunostaining showed only TH-immunopositive nerves in the control artery.

In the artery treated with 5-HT, as shown in Figure 7D, dense innervation by 5-HT-LI nerves was observed in the same area in which dense TH-LI nerves were found (Figure 7E). Double immunostaining demonstrated that 5-HT-immunopositive nerves were colocalized with TH-immunopositive nerves, as indicated by the yellow colour (Figure 7F). In contrast, in the artery treated with 5-HT in the presence of desipramine, there were no 5-HT-LI nerves (Figure 7G), while many TH-LI nerves were observed (Figure 7H). As shown in Figure 7I, double immunostaining showed only TH-immunopositive nerves in the desipramine-treated artery.

Discussion

In the rat perfused mesenteric vascular bed with active tone, PNS produced an initial vasoconstriction followed by a long-lasting vasodilatation. The PNS-induced vasoconstriction was shown to be mediated by perivascular adrenergic nerves, since it was abolished by the adrenergic neuron blocker guanethidine (Figure 3), α_1-adrenoceptor antagonist prazosin and adrenergic neurotoxin 6-hydroxydopamine (Shiraki *et al.*, 2000; Kawasaki *et al.*, 2009). By contrast, the PNS-

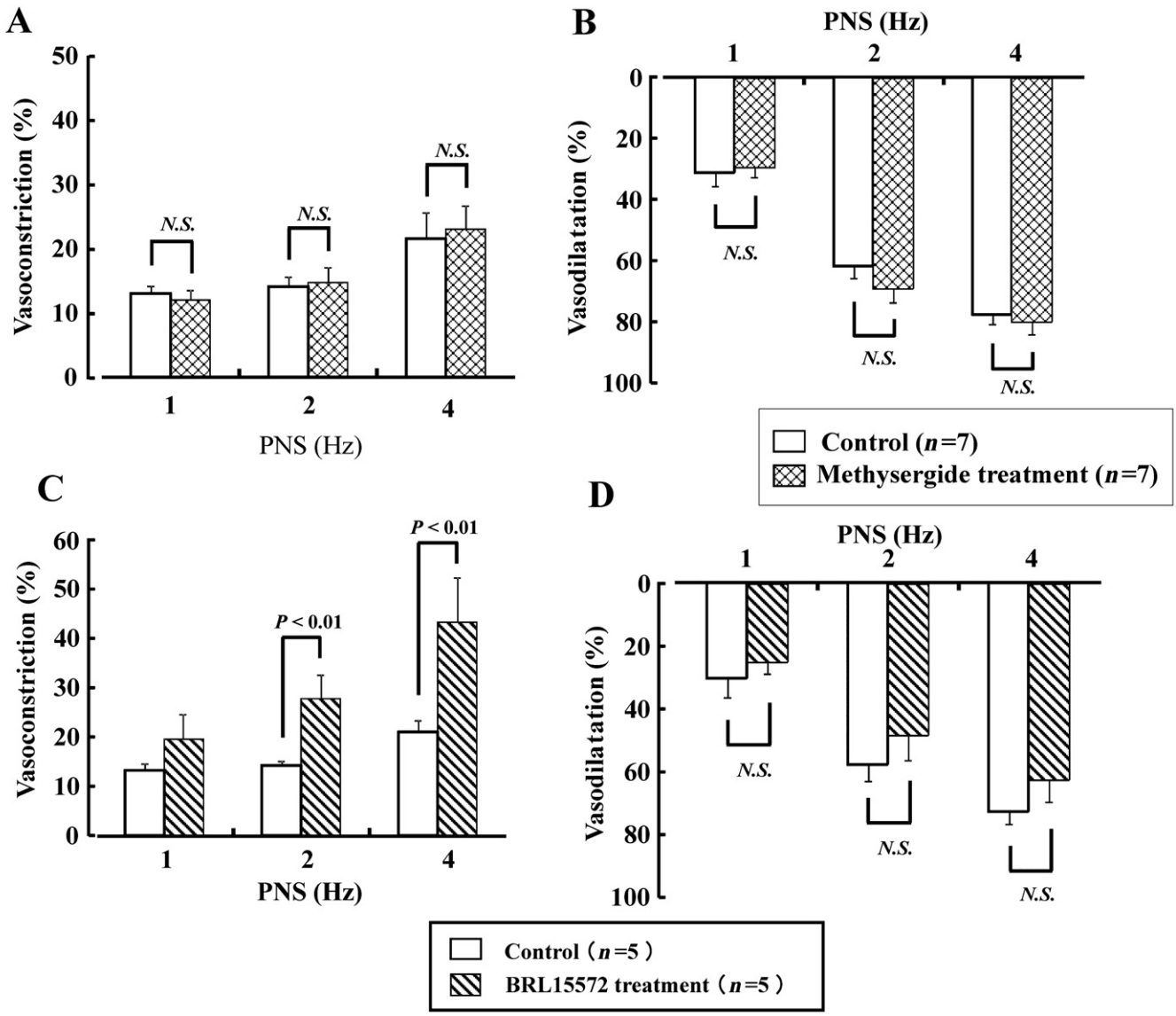

Figure 4

Histograms showing effects of methysergide (A, B) and BRL15572 (C and D) on vascular responses to PNS (1, 2 and 4 Hz) after temporary 5-HT treatment in rat perfused mesenteric vascular beds without an endothelium and with active tone produced by 2 μM methoxamine. (A and C) Changes in vasoconstrictor responses to PNS. (B and D) Changes in vasodilator responses to PNS. Data are presented as the mean ± SEM.

induced vasodilatation has been reported to be mediated by perivascular CGRPergic nerves, since it is abolished by capsaicin (CGRP depletor) and CGRP8-37 (CGRP receptor antagonist) (Kawasaki *et al.*, 1988; 1991), suggesting that perivascular adrenergic and CGRPergic nerves regulate mesenteric vascular tone.

The present study demonstrated that when mesenteric vascular beds without an endothelium were temporarily treated with 5-HT, the vasoconstrictor responses to PNS were significantly augmented and PNS-induced vasodilatations were significantly suppressed even in the absence of 5-HT. Previous studies revealed that exogenously applied 5-HT is taken up by and accumulates in perivascular adrenergic nerves of rat mesenteric arteries (Kawasaki and Takasaki, 1984) and femoral arteries (Urabe *et al.*, 1991). These studies also demonstrated that 5-HT taken up into and accumulated in vascular adrenergic nerves is released by perivascular nerve stimulation to produce a vasoconstrictor response, which is mediated by 5-HT_{2A} receptors (Kawasaki and Takasaki, 1984). Additionally, the release of 5-HT has been shown to be calcium- dependent and blocked by the adrenergic neuron blocker guanethidine (Kawasaki and Takasaki, 1984; 1986; Kawasaki *et al.*, 1989). In the present study, the augmentation of PNS-induced vasoconstrictor responses observed after temporary 5-HT treatment was abolished by guanethidine. Since guanethidine blocks adrenergic neurotransmission, it is suggested that exogenous 5-HT is taken up by and neuronally released from adrenergic nerves to induce vasoconstriction, which helps to augment adrenergic nerve-mediated vasoconstriction. This notion is supported by the present immuno-

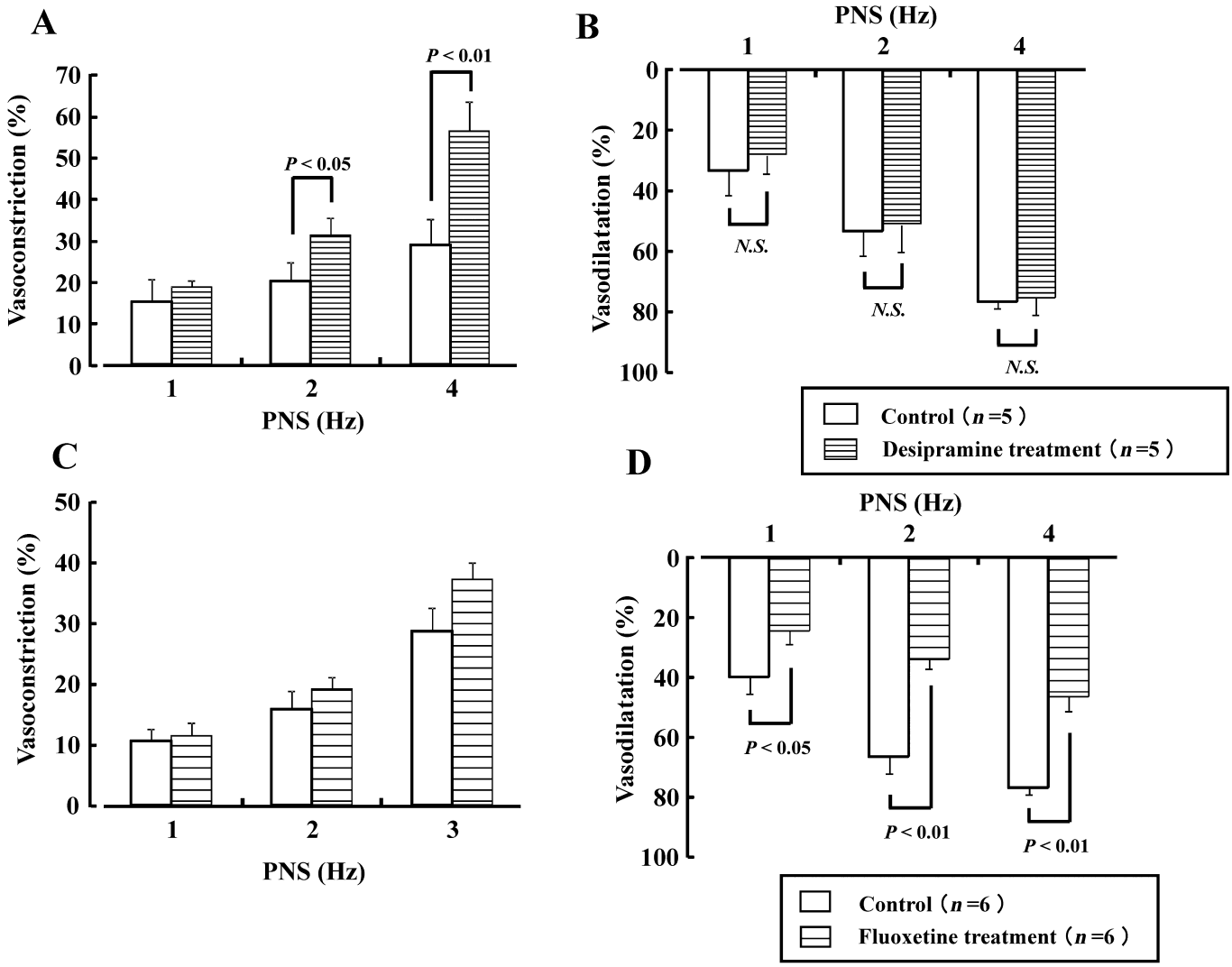

Figure 5

Histograms showing effects of desipramine (A and B) and fluoxetine (C and D) on vascular responses to PNS (1, 2 and 4 Hz) induced after 5-HT treatment in rat perfused mesenteric vascular beds without an endothelium and with active tone produced by 2 μM methoxamine. (A and C) Changes in vasoconstrictor responses to PNS. (B and D) Changes in vasodilator responses to PNS. Data are presented as the mean ± SEM.

histochemical findings that 5-HT-immunopositive nerves were observed only when the mesenteric artery was treated with 5-HT. Furthermore, double immunostaining clearly showed that 5-HT-LI nerves merged with adrenergic TH-LI nerves in the mesenteric artery. Moreover, no 5-HT-immunopositive fibres were observed after combined treatment with the adrenergic uptake inhibitor desipramine and 5-HT, suggesting that rat mesenteric arteries have no 5-HT-containing nerves. Therefore, it is very likely that 5-HT is taken up by and stored in the perivascular adrenergic nerves. Additionally, the non-selective 5-HT receptor antagonist methysergide also abolished the augmentation of PNS-induced vasoconstriction. Therefore, it appears that neuronally released 5-HT causes vasoconstriction via postsynaptic 5-HT receptors located on the vascular smooth muscle. The present findings accord well with the those obtained previously (Kawasaki and Takasaki, 1984).

The present study is the first to demonstrate that temporary treatment with 5-HT results in a significant inhibition of PNS-induced vasodilatation, which is mediated by perivascular CGRPergic nerves (Kawasaki *et al.*, 1988). Since the vasodilator response to exogenous CGRP was not affected after the temporal 5-HT treatment, it is unlikely that the treatment decreases the activity of postsynaptic CGRP receptors. The significant suppression of PNS-induced vasodilatation after 5-HT treatment was almost completely abolished by guanethidine and methysergide, suggesting that neuronally released 5-HT is responsible for the suppression. This is supported by the present findings that exogenous 5-HT inhibited the PNS-induced vasodilatation without affecting the CGRP-induced vasodilatation and that methysergide antagonized the 5-HT-induced inhibition. The degree of inhibition induced by exogenous 5-HT, 0.1 μM, was approximately 50%, which was almost equal to the degree of inhibition evoked by

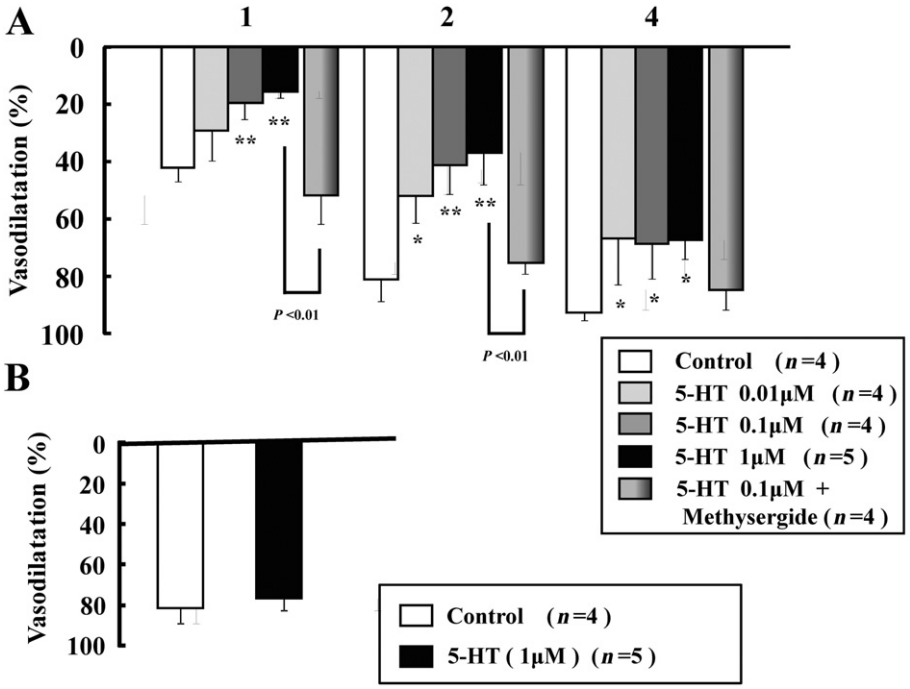

Figure 6

Histograms showing effects of exogenous 5-HT on vascular responses to PNS (1, 2 and 4 Hz) (A) and CGRP (50 pmol) (B) induced in the absence or presence of methysergide in rat perfused mesenteric vascular bed without an endothelium and with active tone produced by 2 μM methoxamine in the presence of guanethidine. Data are presented as the mean ± SEM. *P < 0.05, **P < 0.01, versus control.

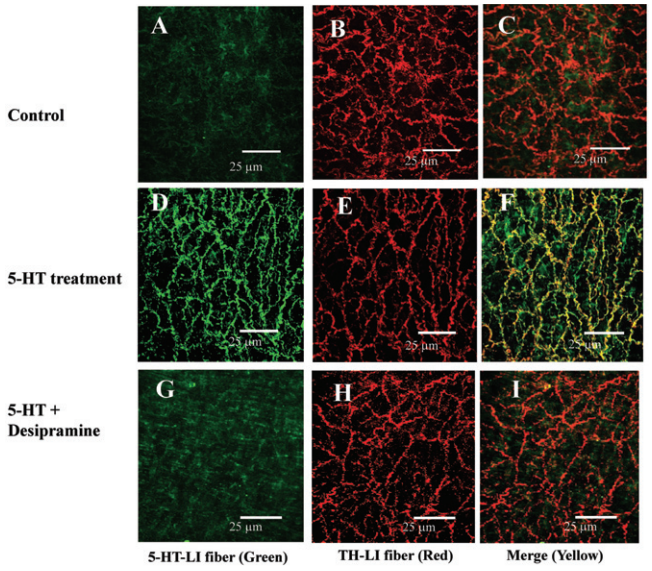

Figure 7

Confocal laser photomicrograph showing 5-HT-like immunopositive (LI)- and TH-LI-containing fibres in the mesenteric artery. Both types of fibres in the control group (A), 5-HT-treated group (D) and 5-HT- and desipramine-treated group (G) were photographed in the same region of whole-mounted arteries. The colocalization of 5-HT-LI and TH-LI is recognized as yellow in many fibres. The scale bar in the right lower corner of each image indicates 25 μm.

neuronally released 5-HT. Therefore, the concentration of neuronally released 5-HT is estimated at 0.1 μM. Additionally, a selective 5-HT_{1D} receptor antagonist, BRL15572, abolished the suppression of PNS-induced vasodilatation. It should be noted that BRL15572 did not affect the augmentation of vasoconstrictor responses to PNS after 5-HT treatment, while methysergide abolished the augmentation, implying that 5-HT_{1D} receptors are located on presynaptic sites of CGRPergic nerves. This notion is supported by the findings that 5-HT_{1D} receptors were colocalized with CGRP-containing nerves (Ma *et al.*, 2001).

Immunohistochemical studies have revealed that CGRPergic nerves accompany adrenergic nerves in mesenteric arteries (Eguchi *et al.*, 2004; Kawasaki *et al.*, 2009). Furthermore, the activation of 5-HT_{1D} receptors has been shown to inhibit sensory CGRPergic nerve-mediated vasodilatation (Carmichael *et al.*, 2008). Taken together, these findings suggest that neuronally released 5-HT from adrenergic nerves acts on $5HT_{1D}$ receptors located on adjacent CGRPergic nerves to suppress the neurotransmission of CGRPergic nerves, resulting in a decrease in CGRPergic nerve-mediated vasodilatation.

Kawasaki and Takasaki (1984) reported that combined perfusion of 5-HT and cocaine, a catecholamine transporter inhibitor, markedly reduced neuronal 5-HT release from sympathetic adrenergic nerves by PNS in 5-HT-treated mesenteric vascular beds of the rat. In the present study, combined perfusion of 5-HT and desipramine, a selective catecholamine transporter inhibitor, almost completely abolished the suppression of PNS-induced vasodilator responses, whereas PNS-

induced vasoconstrictor responses were further augmented. This augmentation seems to have resulted from an increased concentration of noradrenaline in the synaptic cleft due to re-uptake inhibition. Furthermore, double immunostaining of the mesenteric artery treated with 5-HT in the presence of desipramine did not reveal 5-HT-immunopositive nerves. However, fluoxetine, a selective 5-HT re-uptake inhibitor, had no effect on the PNS-induced vasodilatation after 5-HT treatment. These findings strongly suggest that exogenously applied 5-HT is taken up into sympathetic adrenergic nerve endings via catecholamine transporters. It is unlikely that 5-HT transporters exist on sympathetic adrenergic nerve endings innervating rat mesenteric arteries.

In conclusion, the present results suggest that 5-HT is taken up by and accumulates in sympathetic adrenergic nerves via catecholamine transporters in rat mesenteric arteries, and when released by nerve stimulation reduces adjacent CGRPergic nerve-mediated vasodilatation through the activation 5-HT_{1D} receptors, which are located on CGRPergic nerves.

CGRP is released during the headache phase of a migraine and plays a role in migraine through its potent vasodilator activity. Although the actual mechanisms underlying migraine attack remain unknown, changes in plasma 5-HT levels have been hypothesized during and after the attack (Panconesi, 2008). Although more studies are needed to clarify the 5-HT uptake activity in cerebral arteries during migraines, the adrenergic nerve 5-HT uptake system could serve as a novel modality for migraine research.

Conflicts of interest

No competing financial interests exist.

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