Noradrenergic effects in rat sacral autonomic nucleus using in vitro slice patch-clamp recordings

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ABSTRACT

Noradrenergic modulation has been frequently discussed in the context of neural activities that are related to pelvic organs. The sacral preganglionic nucleus (SPN) is a spinal nucleus containing parasympathetic preganglionic neurons that send fibers to pelvic nerves. In spite of the abundant presence of noradrenergic fibers around the SPN, the effects of noradrenaline (NA) remain obscure. To explore this issue, NA (50 μ M) was applied to parasympathetic preganglionic neurons in the SPN during whole-cell patch clamp recording. The SPN was labeled with the retrograde tracer, DiI. These neurons demonstrated two classes of firing patterns (delayed and regular) in terms of initiation of firing. Independent of these firing patterns, NA induced inward (56%) or outward (32%) currents in labeled SPN neurons. Phenylephrine, an α 1 receptor agonist, induced an inward current, and clonidine, an α 2 receptor agonist, induced an outward current, indicating the existence of both α 1 and α 2 adrenoreceptors in DiI-labeled SPN neurons. NA also modulated synaptic currents according to the firing patterns. In delayed firing neurons, NA inhibited both spontaneous excitatory post-synaptic currents (sEPSCs) and spontaneous inhibitory post-synaptic currents (sIPSCs). Hence, NA facilitated sEPSCs and sIPSCs in about a half of regular firing neurons. Bath application of phenylephrine facilitated sEPSCs and sIPSCs, and clonidine inhibited them. These results support the hypothesis of multiple effects of NA in the SPN, and may suggest functional differences among SPN neurons.

The sacral parasympathetic nerve controls the functions of pelvic organs such as the urinary bladder, distal end of the colon, and penis. Preganglionic neurons in the sacral parasympathetic nucleus (SPN), which constitutes the pelvic nerve, are located in the lateral edge and/or medial part of laminae V–VII of L6–S1 in the spinal cord of the rat. These neurons receive input from several brainstem nuclei and spinal interneurons. Retrograde tracers such as fast blue and fast DiI are frequently used to distinguish parasympathetic preganglionic neurons (PGNs) with-

in the SPN, because the SPN also contains interneurons, which show similar membrane properties to the PGNs (6). Induced firing of retrogradely labeled SPN neurons in the rat shows several distinct properties (4, 19). One type of neurons show delayed firing in response to injected current pulse, and the other type of neurons show regular firing as a response. Various neurotransmitters and chemicals modulate SPN neuronal activities. However, differences in neurochemical modulation of these two types of neurons have not been fully studied.

Noradrenaline (NA) is a well-known neurotransmitter in the central nervous system (CNS) and is present in descending pathways from brainstem nuclei A5 and A6 (locus coeruleus) to the spinal cord (27). NAergic modulation of spinal regions has been extensively studied. NA directly depolarizes motor

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neurons (29, 33) and some populations of interneurons (10), whereas it directly hyperpolarizes the majority of spinal dorsal horn neurons (24, 26). A number of experiments have shown the involvement of NA in pelvic organ function and have indicated its multiple actions. For example, intrathecal administration of NA elicits two different effects on the micturition reflex during different bladder conditions. NA initiates the reflex when the bladder condition is subthreshold for the reflex but facilitates the reflex when the bladder contracts rhythmically (28). Therefore, we investigated the direct effect of NA on SPN neurons.

Patch-clamp recordings from retrogradely labeled PGNs in the SPN *in vitro* slices were performed. We used Fast-DiI labeling from the pelvic viscera and fluorescence microscopy to identify the PGNs within the SPN (18, 23). In this study, NA induced both inward and outward currents through $\alpha 1$ and $\alpha 2$ receptors. These results were similar to those in sympathetic preganglionic neurons (12). NA also altered the spontaneous synaptic current subordinate to their firing pattern.

In this paper, we provide direct evidence for modulation of SPN neurons by NA. Consistent with previous studies, NAergic modulation was bidirectional, suggesting direct and indirect (mediated by interneurons that have synaptic connection with DiI-labeled SPN neurons) effects on DiI-labeled SPN neurons. Furthermore, our report suggests the possibility of functional differences in the two types of PGNs.

MATERIALS AND METHODS

Animals. Sprague-Dawley rats (30 pups of either sexes) were purchased with their mothers (one mother per 10 pups) at 9–10 days of age and were used at 14–21 days of age. The animals were purchased from Japan SLC (Hamamatsu, Japan) and housed in a temperature-controlled room ($21 \pm 1^\circ\text{C}$) with a 12-hour light/dark cycle with their mother, who was given free access to food and water. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of the National Institutes of Natural Sciences, and were performed in accordance with the institutional guidelines for animal experiments. All efforts were made to reduce the number of animals for this study.

Preparation of acute spinal cord slices. The methods used for obtaining spinal cord slice preparations are identical to those described elsewhere (32, 34). Rats were anesthetized with urethane (1.2–1.5 g/kg,

i.p.), and a thoracolumbar laminectomy was performed. The lumbosacral segments of the spinal cord (L2–S2) were placed in ice-cold Krebs solution equilibrated with 95% O_2 /5% CO_2 . The Krebs solution contained (in mM) 117 NaCl, 3.6 KCl, 2.5 CaCl_2 , 1.2 MgCl_2 , 1.2 NaH_2PO_4 , 25 NaHCO_3 , and 11 glucose. Immediately after removal of the spinal cord, the rats were exsanguinated under the urethane anesthesia. The pia-arachnoid membrane was removed after cutting all of the ventral and dorsal roots. The spinal cord was mounted on a vibratome, and 250 to 300 μm -thick transverse or horizontal slices were cut. Each slice was placed in the recording chamber in a volume of 0.5 mL and was completely submerged and perfused with Krebs solution saturated with 95% O_2 and 5% CO_2 at $36 \pm 1^\circ\text{C}$ at a flow rate of 10–15 mL/min.

Whole-cell patch-clamp of DiI-labeled PGNs. Also as described previously (23, 25), we recorded DiI-labeled SPN neurons from rats that had been injected with the retrograde tracer fast DiI (1,1'-dilino-lyl-3,3,3',3'-tetramethylidocarbocyanine, 4-chlorobenzenesulfonate+; Molecular Probes, Eugene, OR, USA; 10 μL , 20 $\mu\text{g}/\mu\text{L}$ dissolved in DMSO) into the peritoneal cavity at 9–11 days after birth. Animals were used 3–10 days after the injection. Transverse or horizontal sections of the L6–S1 spinal cord were made. DiI-labeled neurons were visualized in the SPN using an upright microscope (BX51WI; Olympus Optical, Tokyo, Japan) equipped with infrared differential interference contrast optics with a fluorescence filter (U-MWIGA3, Olympus). DiI-labeled neurons were present at the lateral edge and/or medial part of laminae V–VII of the L6–S1 spinal cord. DiI was mostly localized in the cell body (Fig. 1A, B).

Whole-cell patch-clamp recordings were performed with K-gluconate pipette solution containing (in mM): 136 K-gluconate, 5 KCl, 0.5 CaCl_2 , 2 MgCl_2 , 5 EGTA, 5 HEPES, and 5 ATP-Mg (pH 7.2 adjusted with KOH). Signals were acquired with a patch-clamp amplifier (Axopatch 700A; Molecular Devices, Union City, CA, USA). The data were digitized with an analog-to-digital converter (Digidata 1321A, Molecular Devices).

Firing properties of neurons in response to current injections from the recording pipettes were examined under the current-clamp condition. Any delays of first firing to current injections were considered as a delayed firing types of neurons, and other cells were considered as regular firing types. In some cases, the recorded neurons were anatomically identified

by intracellular injection of neurobiotin (0.2% in the pipette solution; Vector Laboratories, Burlingame, CA, USA). After electrophysiological recording, the spinal cord slices were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C and then rinsed in phosphate buffer. One or two neurons were recorded in each slice using the neurobiotin-containing pipette solution. Neurobiotin-filled neurons were visualized using fluorescence immunohistochemistry. Free-floating sections were incubated overnight at 4°C in phosphate-buffered saline with 0.3% Triton X-100 containing AlexaFluor555-labeled streptavidin. After further washes in phosphate-buffered saline, the sections were mounted in glycerol-based mounting medium (Vectashield, Vector). Labeled neurons were viewed with a fluorescent microscope and photographed with a Leica SP5 laser scanning microscope. Each neuron was imaged with the following settings: 1024 × 1024 pixels, Z-step 1 μm, line average 2, stacks 100 μm. Post-hoc fluorescence immunohistochemical identification of recorded neurons shows the morphological characteristics of the SPN neurons (21).

Data analysis. Drugs dissolved in Krebs solution were applied to the spinal cord slice by exchanging solutions via a three-way stopcock. The time necessary for the solution to flow from the stopcock to the surface of the spinal cord was approximately 5 s, and the solution in the recording chamber was completely exchanged with the drug-containing solution within 15 s. The effects of the drug were analyzed with software 30 s before the drug application as a control, and after or during 30 s as the drug-affected period. pCLAMP software (10.4 version, Molecular Devices) was used for data analysis for calculation of inward and outward currents, and for creating the I–V curve. Holding currents changed more than 3 pA were considered to be drug-induced changes. The frequencies and amplitudes of spontaneous post-synaptic currents (sPSCs) were analyzed with MiniAnalysis software (Version 6.0.3; Synaptosoft, Decatur, GA, USA). Parameters were chosen manually to analyze each cell.

Statistical analysis of data was performed using Graph Pad prism (version 6.00; Graph Pad Software Inc., San Diego, CA, USA). All data were expressed as the mean ± SEM. Statistical significance was determined as $P < 0.05$ using paired or unpaired *t*-test, Wilcoxon rank analysis, or one-way ANOVA followed by a post-hoc Tukey test. In all cases, *n* refers to the number of neurons studied.

Drugs. The drugs used were L-(–)-norepinephrine (+)-bitartrate salt monohydrate (NA) (Sigma) at 50 μM, tetrodotoxin (TTX) (Wako, Osaka, Japan) at 0.5–1 μM, strychnine (Sigma) at 2 μM, bicuculline (Sigma) at 10–20 μM, phenylephrine (Sigma) at 30 μM, A61603 hydrate (Sigma) at 10 μM, and clonidine hydrochloride (Sigma) at 10 μM. All drugs were stored at –20°C as stock solution (1000 times higher concentration) and used within the day.

RESULTS

Direct effects of noradrenaline

Recordings were obtained from 96 SPN neurons labeled by retrograde axonal transport of a fluorescent dye and established under differential interference contrast optics as described previously (19, 23). After obtaining the whole-cell recording (Fig. 1B, C), we tested the firing properties of neurons under the current-clamp mode. From the responses to injected current through the pipette, neurons were classified into two types, (see methods). Both delayed (52) and regular (44) firing types of voltage responses were used to discriminate neurons (Fig. 1D–H). First, we examined NA-induced effects of holding currents under the voltage-clamp mode. After perfusion of NA (50 μM) for 90 s, changes in the holding current were observed both inwardly and outwardly when the voltage was held at –70 mV in both firing types of DiI-labeled SPN neurons (Fig. 2A–1, 2). These NA-induced currents were also observed when holding levels were changed to –40 mV.

In delayed type neurons, the observed inward current was -6.4 ± 1.2 pA ($n = 10$), and the outward current was 8.4 ± 3.0 pA ($n = 6$). In regular type neurons, the observed inward current was -11.3 ± 2.6 pA ($n = 9$), and the outward current was 8.8 ± 1.6 pA ($n = 5$). The averaged inward currents of both firing patterns were -10.0 ± 1.5 pA at V_h –70 mV, and -15.0 ± 1.7 pA at V_h –40 mV. The observed outward currents were 10.4 ± 1.5 pA in V_h –70 mV, and 16.7 ± 3.1 pA in V_h –40 mV. There were significant increase in outward current between holding potential –70 mV and –40 mV ($P < 0.005$ for inward, $P < 0.05$ for outward, unpaired *t*-test).

Summary graphs of responses were shown in Fig. 2B, and C. The α1-specific receptor agonist phenylephrine produced an inward current response (delayed, $n = 8$, -2.4 ± 0.9 pA; regular, $n = 8$, -5.5 ± 1.9 pA. Fig. 2D), and the α2-specific agonist clonidine produced an outward current response (delayed, $n = 3$, 8.6 ± 3.5 pA; regular, $n = 5$, 6.5 ± 3.6 pA). A61603, an α1A receptor-specific agonist, produced

Fig. 1 Photomicroscopic images demonstrate fluorescent Dil-labeled neurons (A, B), and electrophysiological traces show representative delayed (D) or regular (F) firing patterns of the neurons. Photomicroscopic images show neurobiotin-injected delayed (E) and regular neurons (G, H). Notes: The delayed or regular pattern of firing properties in response to 1000-ms step pulses (delayed, -120 , 160 pA; regular, -120 , 200 pA) from a membrane potential of -80 mV. A representative picture during recording from a Dil-labeled SPN neuron (same as in Fig. B) is shown in C. CC; central canal, DH; dorsal horn, LF; lateral funiculus, VH; ventral horn.

both inward (delayed, 3 of 7, -4.0 ± 0.8 pA; regular, 2 of 6, -10.7 ± 3.2 pA) and outward (delayed, 4 of 7, 7.0 ± 3.4 pA; regular, 4 of 6, 6.9 ± 2.1 pA) current responses in the holding current. These responses in the holding current were also elicited in the presence of TTX (Fig. 3), and thus, we surmise that these effects of various agonists were direct action on recorded neurons.

Neurons that showed inward or outward currents were tested with a voltage step protocol (500-ms steps from -120 mV to $+40$ mV in 20-mV increments) while holding current responses in the presence of TTX (0.5 μ M) and NA (50 μ M) to verify the effect of NA on holding currents in Dil-labeled SPN neurons (Fig. 3A, B). Cells that showed large inward ($n = 3$) or outward ($n = 3$) currents were used in this experiment. NA-induced changes in the holding currents were calculated by subtracting the control current from the currents during action of NA (Fig. 3A-3, 4, B-3, 4). The NA-induced inward cur-

rent remained inward across the entire voltage range, and a large enhancement at voltages more positive than -40 mV. These currents were with non-linear characteristics, indicating the voltage dependence of the inward currents (Fig. 3A-4). NA-induced outward current was linear within the voltage range of -120 to -20 mV and reversed at voltages between -100 and -80 mV. These currents shifted inward when the voltage were above 0 mV (Fig. 3B-4). In all recordings, the I-V relationships in control were outwardly rectifying. We found no differences in the characteristics of NA-induced current between delayed and regular firing types neurons (data not shown).

NAergic modulation of spontaneous excitatory synaptic transmission (sEPSC) and spontaneous inhibitory post-synaptic current (sIPSC)

Next, we examined NAergic modulation of synaptic responses. Facilitation of sEPSC occurs through

Fig. 2 Bath application of NA (50 μ M) influences whole-cell current and firing activities induced by current injection in DiI-labeled SPN neurons. Both inward and outward currents are observed in both types of firing pattern cells. Phenylephrine (Phe, 30 μ M) induced inward currents, and clonidine (Clo, 10 μ M) induced outward current. **A-1, 2**, Voltage-clamp recordings from DiI-labeled SPN neurons at a holding potential (V_h) of -70 mV represent the effect of NA on holding current. **B**, Changes in the holding current in the presence of NA (50 μ M) were plotted as black circles for each cell (delayed, $n=30$; regular, $n=29$). **C-1, 2**, Pie charts show the percentage of each response. Changes between 3 and -3 pA were considered as no responses and are represented as white areas. Black areas represent percentages of the inward current response. Gray areas represent percentages of the outward current response. **D**, Mean normalized effects of NA and selective agonists (phenylephrine (α_1), clonidine (α_2), A61603 (α_1A)) on changes in holding current. Changes in the holding current in the presence of drug were plotted for each drug. Averages of these changes were plotted as bars (Mean $\pm$ SEM).

α_1 -adrenoreceptors, and suppression is mediated by α_2 -adrenoreceptors in other CNS regions (3, 5, 8, 17, 20). Regarding the micturition reflex, it was proposed the involvement of interneurons, which may be reflected as NAergic effects of sE/IPSC on DiI-labeled SPN neurons (5).

To investigate the modulatory effects of NA on spontaneous excitatory synaptic transmission (sEPSC), we applied NA (50 μ M) under voltage-clamp recording (-70 mV) after firing pattern separation (Fig. 4). sEPSC had a frequency of 5.6 ± 0.9 (delayed, $n=16$) and 4.1 ± 0.5 (regular, $n=13$) Hz, and a mean amplitude of 14.2 ± 0.9 and 9.8 ± 0.8 pA, respectively (Fig. 4B, C, G, H). sEPSC had significantly larger amplitudes in delayed type neurons than in regular type neurons ($P < 0.001$, Mann-Whitney test). In delayed type neurons, application of NA (50 μ M) had an inhibitory effect in 81% of neurons (13 of 16) and reduced the frequency ($-56 \pm 9\%$, $n=13$) and amplitude ($-16 \pm 3\%$, $n=13$; Fig. 4A-C). How-

ever, in a few neurons, NA increased the frequency ($60 \pm 22\%$, $n=3$) and amplitude ($30 \pm 23\%$, $n=3$). In regular type neurons, application of NA caused two opposite effects on sEPSC (Fig. 4F-H). Some neurons showed an increased frequency ($129 \pm 61\%$, 6 of 13) and amplitude ($32 \pm 23\%$, 5 of 13). The remaining neurons showed an inhibition of frequency ($-53 \pm 8\%$, 7 of 13) and amplitude ($-13 \pm 5\%$, 8 of 13). Phenylephrine (30 μ M) and A61603 (10 μ M) increased the frequency ($128 \pm 66\%$, $n=6$; $324 \pm 210\%$, $n=6$, respectively; Fig. 4I, J) in regular type neurons, but not in delayed type neurons ($2 \pm 14\%$, $n=10$; $12 \pm 30\%$, $n=7$, respectively; Fig. 4D, E). Clonidine (10 μ M) reduced the frequency in almost recorded neurons, both delayed ($-18 \pm 16\%$, $n=6$) and regular ($-38 \pm 13\%$, $n=4$) types of neurons (Fig. 4D, I). These agonists did not change the amplitudes significantly (delayed; regular, phenylephrine, $-3 \pm 3\%$; $+9 \pm 8\%$, A61603, $-9 \pm 5\%$; $-4 \pm 7\%$, clonidine, $-11 \pm 7\%$; $-13 \pm 12\%$).

Fig. 3 NA differentially influenced the whole-cell current in labeled SPN neurons. **A, B-1** Representative traces of voltage-clamp recording illustrating the NA-induced (50 μ M) inward (A-1) and outward (B-1) currents in the presence of TTX (0.5 μ M). **A, B-2** Current traces recorded in response to a series of voltage step commands (-120 to $+40$ mV, 20-mV increments) before NA application and during NA application. **A, B-3, 4** The steady-state I-V relationship obtained before (black circles) and during the application of NA (50 μ M, red circles) in the inward (A-3) and outward (B-3) current response. Black triangles show different currents induced by NA. Inward different currents were entirely inward across the tested voltage range in this experiment (A-3, 4). Outward different currents were reversed at voltages between -100 and -80 mV (B-3, 4). Values are presented as the mean $\pm$ SEM. The boxed region is enlarged and shown above the main I-V graphs.

Spontaneous inhibitory post-synaptic current (sIPSC) was differently modulated by NA, depending on the firing properties. sIPSC showed a frequency 4.0 ± 0.9 (delayed, $n = 17$) and 4.1 ± 0.7 (regular, $n = 18$) Hz and mean amplitudes of 13.3 ± 0.9 and 16.5 ± 1.1 pA, respectively (Fig. 5). The amplitude of sIPSC was larger in regular type neurons than in delayed type neurons (their values are indicated above, $P < 0.05$, Mann-Whitney test). To distinguish the two types of neurons according to their firing properties, we could not clearly separate IPSC from EPSC responses. However, we abolished sIPSC by application of bicuculline (20 μ M) and strychnine (2 μ M) in some recordings (Fig. S1). In delayed type neurons, NA inhibited both the frequency ($-51 \pm 5\%$, 12 of 17) and amplitude ($-9 \pm 1\%$, 9 of 17; Fig. 5A–C). In regular type neurons, NA increased the frequency ($168 \pm 46\%$, 9 of 18) and amplitude ($39 \pm 9\%$, 12 of 18), while decreased the frequency ($-33 \pm 8\%$, 9 of 18) and amplitude ($-91 \pm 3\%$, 6 of

18; Fig. 5F–H). Phenylephrine increased ($167 \pm 63\%$, $n = 8$) the frequency in regular type neurons, but had no effects on delayed type neurons ($0 \pm 6\%$, $n = 6$). A61603 had no effects on either type of DiI-labeled SPN neurons (delayed, $n = 8$, $21 \pm 16\%$; regular, $n = 6$, $21 \pm 28\%$; Fig. 5D, I). Clonidine (10 μ M) reduced the frequency of sIPSC in almost recorded neurons, both delayed ($-26 \pm 11\%$, $n = 6$) and regular ($-37 \pm 17\%$, $n = 3$) types of neurons (Fig. 5D, I). These agonists did not alter the amplitudes significantly (delayed; regular, phenylephrine, $2 \pm 2\%$; $+35 \pm 26\%$, A61603, $4 \pm 2\%$; $9 \pm 8\%$, clonidine, $-4 \pm 5\%$; $-2 \pm 4\%$).

DISCUSSION

Application of NA led to bidirectional changes in holding potential through $\alpha 1$ or $\alpha 2$ receptors without an association with their firing patterns. At the same time, NA altered synaptic events and modulated

Fig. 4 NA inhibited sEPSC in most (13/16) delayed type neurons, whereas NA both increased (6/13) and inhibited (7/13) sEPSC in regular type neurons. In recording from both types of neurons, α_2 -adrenoreceptors decreased the frequency of sEPSC. α_1 A-adrenoreceptors agonist increased the frequency of sEPSC in regular type neurons. **A, B, C** Spontaneous EPSC in delayed type neurons. NA application inhibited (13/16) the spontaneous EPSC rate and amplitude. Top (A), Representative trace showing NAergic effects on sEPSC in delayed type neurons. Left (B), sEPSC rate (pre = 5.6 ± 0.9 , post = 2.9 ± 0.7 ; $n = 16$, $P = 0.019$). Right (C), sEPSC amplitude (pre = 14.2 ± 0.9 pA, post = 13.1 ± 1.0 pA; $n = 16$, $P = 0.2133$). **D, E** The bar graph shows mean normalized effects of NA and the selective agonists Phenylephrine (α_1), clonidine (α_2), and A61603 (α_1 A) on sEPSC frequency and amplitude of sEPSCs compared with pre-application levels in delayed type neurons. The number of cells tested is given above the bars. **F, G, H** Spontaneous EPSC in regular type neurons. NA application increased (6/13) and inhibited (7/13) the spontaneous EPSC rate and amplitude. Top (D), Representative trace showing NAergic effects on sEPSC in regular type neurons. Left (E), EPSC rate (pre = 4.0 ± 0.5 , post = 4.2 ± 1.0 Hz; $n = 13$, $P = 0.8848$). Right (F), amplitude (pre = 9.8 ± 0.8 , post = 9.9 ± 1.0 pA; $n = 13$, $P = 0.9129$). **I, J** The bar graph shows mean normalized effects of NA and the selective agonists Phenylephrine (α_1), clonidine (α_2), and A61603 (α_1 A) on sEPSC frequency and amplitude of sEPSCs compared with pre-application levels in regular type neurons. The number of cells tested is given above the bars. All comparisons were done with the Student's paired t -test.

Fig. 5 NA inhibited sIPSC in delayed type neurons (12/17) and increased sIPSC in some regular type neurons (9/18). **A**, **B**, **C** Spontaneous IPSC in delayed type neurons. NA application decreased (12/17) the spontaneous IPSC rate and amplitude. Top (**A**), Representative trace showing NAergic effects on sIPSC in delayed type neurons. Left (**B**), IPSC rate (pre = 4.0 ± 0.9 , post = 2.6 ± 0.5 Hz; $n = 17$, $P = 0.0379$). Right (**C**), amplitude (pre = 13.3 ± 0.9 , post = 14.4 ± 1.7 pA; $n = 17$, $P = 0.3413$). **D**, **E** The bar graph shows mean normalized effects of NA and the selective agonists phenylephrine (α_1), clonidine (α_2), and A61603 (α_1A) on sIPSC frequency and amplitude of sEPSCs compared with pre-application levels in delayed type neurons. The number of cells tested is given above the bars. **F**, **G**, **H** Spontaneous IPSC in regular type neurons. Top (**D**), Representative trace showing NAergic effects on sIPSC in regular type neurons. NA application significantly increased the frequency and amplitude. Left (**E**), IPSC rate (pre = 4.1 ± 0.7 , post = 4.6 ± 0.9 Hz; $n = 18$, $P = 0.5722$). Right (**F**), amplitude (pre = 16.5 ± 1.1 , post = 20.4 ± 2.1 pA; $n = 18$, $P = 0.0242$). **I**, **J** The bar graph shows mean normalized effects of NA and the selective agonists phenylephrine (α_1), clonidine (α_2), and A61603 (α_1A) on sIPSC frequency and amplitude of sEPSCs compared with pre-application levels in regular type neurons. The number of cells tested is given above the bars. All comparisons were done with the Student's paired *t*-test.

their activities. The relationships between NAergic effects and firing patterns were observed in the modulation of synaptic events. In regular type neurons, NA increased the frequency of sEPSC and sIPSC through $\alpha 1$ receptors and decreased sEPSC through $\alpha 2$ receptors. In delayed type neurons, NA only decreased sEPSC and sIPSC. An $\alpha 1A$ receptor agonist (A61603) strongly increased sEPSC frequency but did not change sIPSC in regular type neurons. These data indicated that both delayed and regular type neurons in the SPN are directly affected by $\alpha 1$ and $\alpha 2$ receptors. Indirect excitation in regular type neurons may be mediated through $\alpha 1A$ receptors, and indirect inhibition may be through other $\alpha 1$ receptors (presumably $\alpha 1B$ and/or $\alpha 1D$ receptors).

Direct effects of NA in DiI-labeled SPN neurons

In our experiment, NA not only depolarized neurons through $\alpha 1$ receptors, but also hyperpolarized these cells through $\alpha 2$ receptors, and thus, NA changed the activities of DiI-labeled SPN neurons. NAergic modulation of neural activities has been studied in other spinal regions (9, 12, 31). As described above, depolarization through $\alpha 1$ receptors was observed in motor neurons (29) and interneurons located in the dorsal horn (10). Hyperpolarization through $\alpha 2$ receptors was observed in the major populations of spinal dorsal horn neurons (24, 26).

These NAergic effects were much the same as in previous reports of sympathetic preganglionic neurons (12). Bidirectional effect seems to be determined by cell characteristics such as the firing pattern (9, 30). However, we found no correlations between NAergic effects and firing patterns. Our *in vitro* study has a limitation regarding the need to clarify other cellular characteristics. Also, NAergic actions including *in vivo* states are required in further experiments.

We showed the I–V characteristics of inward and outward currents. We found no direct evidence for involvement of specific channels in this experiment. However, we observed two features about inward current: large enhancement of currents may occur at the excited voltage range, and loss of reverse potential may occur at the tested voltage range. From these characteristics of the inward I–V relationships, we speculate the closing of potassium channels or involvement of L-type Ca^{2+} channels. L-type Ca^{2+} channels open following $\alpha 1$ receptor signaling, which is reported in cardiac ventricular cells (15, 16). Outward currents may be influenced by G protein coupled K^+ channels. The reverse potential of K^+ in this experiment was around -90 mV, which

exactly matched the reverse potential of the observed outward current. $\alpha 2$ receptor-mediated activation of G-protein-mediated activation of K^+ channels may be involved, as reported in other spinal regions (26, 35). Characteristics of the I–V relationship suggest that the outward currents were mixed with inward currents, because the shift in the current response was observed at a voltage range of -20 to $+40$ mV. If currents are mixed as described above, we could explain the characteristics of the currents. Further studies are needed because of the lack of direct proof demonstrating the role of channels as discussed above.

Synaptic current modulation of NA

Our results show that the applications of NA differentially modulated synaptic events, depending on the firing patterns. NA inhibited sEPSC in delayed type of neurons. In addition, we found that NA increased sEPSC through $\alpha 1$ receptors in some populations of regular type neurons. Several studies have addressed the relevance of NAergic modulation of synaptic events in the spinal cord (1, 2, 5, 20, 29). In this study, $\alpha 2$ receptor-mediated synaptic inhibition of sEPSC was observed in both types of neurons, consistent with previous reports. Further, we showed $\alpha 1$ receptor-mediated increase of sEPSC. This has also been reported in spinal motor neurons (29) and other supraspinal regions such as the nucleus tractus solitaries (14), paraventricular hypothalamic nucleus (3), and cerebral cortex (17). Comparable to the modulation of sEPSC, inhibition of sIPSC was observed mainly in delayed type neurons, and $\alpha 1$ receptor-mediated enhancement of sIPSC was observed in regular type neurons. Because of the absence of a specific agonist for $\alpha 1$ receptors except the $\alpha 1A$ receptor, we could not determine which subtypes of the $\alpha 1$ receptors are involved in synaptic modulation of sIPSC. However, we report that A61603, an $\alpha 1A$ agonist, increased sEPSC but did not alter the frequency of sIPSC. Such an excitation effect through $\alpha 1A$ receptors has been reported in other CNS regions (11, 31).

Functional and pharmacological implications

The involvement of NAergic modulations of SPN neural activities has been widely discussed and the effect is not so simple (5, 7, 13, 23). Early experiments (25, 34) showed the importance of brainstem-spinal NAergic modulation of micturition reflex. At least in the micturition system, inhibition of adrenoreceptors by $\alpha 1$ receptor blockers inhibits the micturition reflex that is induced by stimulation of

the pontine micturition center, and administration of NA initiates the micturition reflex in some bladder conditions (28). Also, $\alpha 1$ receptor-mediated facilitations of colonic mortalities in the spinal cord were reported in defecation systems (22, 23). Our findings showing excitation through $\alpha 1$ receptor of labeled-PGNs and enhancement of sEPSC may explain the mechanisms of those reports.

At the spinal level, NAergic modulation of the pelvic organ activities has been discussed widely. The effects of NA are not only enhancement or inhibition. Here we report how NA modulates the activity of SPN neurons. Application of NA not only directly excited or inhibited DiI-labeled SPN neurons, but also changed synaptic inputs. NA is a well-known transmitter that has multiple effects, even in the simple circuit of the spinal cord. Revealing such a complexity of the modulation of SPN neuronal activity may help resolve the complex actions of neurotransmitters in the spinal cord and lead to treatments for pelvic organ dysfunction, including not only bladder dysfunction but also colonic motility (23) and penile erection problems (13).

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Fig. S1 Representative trace showing NAergic effects on sIPSC and the responses were abolished in the presence of strychnine (2 μ M) and bicuculline (20 μ M).