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Mechanisms of noradrenergic modulation of synaptic transmission and neuronal excitability in ventral horn neurons of the rat spinal cord

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Abstract—Noradrenaline (NA) modulates the spinal motor networks for locomotion and facilitates neuroplasticity, possibly assisting neuronal network activation and neuroplasticity in the recovery phase of spinal cord injuries. However, neither the effects nor the mechanisms of NA on synaptic transmission and neuronal excitability in spinal ventral horn (VH) neurons are well characterized, especially in rats aged 7 postnatal days or older. To gain insight into NA regulation of VH neuronal activity, we used a whole-cell patch-clamp approach in late neonatal rats (postnatal day 7–15). In voltage-clamp recordings at −70 mV, NA increased the frequency and amplitude of excitatory postsynaptic currents via the activation of somatic α_1- and β-adrenoceptors of presynaptic neurons. Moreover, NA induced an inward current through the activation of postsynapticα_1- and β-adrenoceptors. At a holding potential of 0 mV, NA also increased frequency and amplitude of both GABAergic and glycinergic inhibitory postsynaptic currents via the activation of somatic adrenoceptors in presynaptic neurons. In current-clamp recordings, NA depolarized resting membrane potentials and increased the firing frequency of action potentials in VH neurons, indicating that it enhances the excitability of these neurons. Our findings provide new insights that establish NA-based pharmacological therapy as an effective method to activate neuronal networks of the spinal VH in the recovery phase of spinal cord injuries.

Keywords: monoamine, adrenoceptor, ventral horn neuron, spinal cord injury, patch clamp.

INTRODUCTION

Noradrenaline (NA) directly modulates the spinal motor networks for locomotion and, similar to other monoamines, plays a role in locomotion coordination. (Kiehn et al., 1999; Sqalli-Houssaini and Cazalets, 2000; Machacek and Hochman, 2006; Beliez et al., 2014). Descending noradrenergic fibers, originating from the locus coeruleus in the brainstem, control the excitability of spinal neurons (Jankowska et al., 2000; Jordan et al., 2008; Noga et al., 2011). Although the neuromodulatory roles and reaction pathway of NA in the locomotor system have been previously studied, in the majority of those studies, the cellular mechanisms of NA in synaptic transmission and the excitability of spinal ventral horn (VH) neurons remain unclear (Rekling et al., 2000). The only study that investigated the roles of NA on synaptic transmission of spinal VH neurons used early neonatal rats (postnatal days 1–5 [P1–5]) (Tartas et al., 2010); thus, no studies have investigated this relationship in more mature rats. Generally, it has been reported that the descending pathways from the brain to the lumbar spinal cord develop and arrive by the end of the first postnatal week (Vinay et al., 2005). Moreover, inhibitory transmitters are developmentally depolarizing but become hyperpolarizing to motoneurons after P7 (Singer et al., 1998).

Monoamines, such as NA, have been reported to facilitate neuroplasticity, hence enabling adaptive changes in spared neuronal circuitries and contributing to neuronal recovery after spinal cord injury (SCI) (Rossignol et al., 1996; Chau et al., 1998; Musienko et al., 2011). Currently, the clinically established method to facilitate neuronal network activation and neuroplasticity is aggressive neurorehabilitation (Beamount

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Abbreviations: ACSF, artificial cerebrospinal fluid; AP, action potential; EPSC, excitatory postsynaptic current; GABA, γ-aminobutyric acid; IPSC, inhibitory postsynaptic current; IR-DIC, infrared-differential interference contrast; NA, noradrenaline; mEPSC, miniature EPSC; mIPSC, miniature IPSC; RMP, resting membrane potential; SCI, spinal cord injury; sEPSC, spontaneous EPSC; sIPSC, spontaneous IPSC; TEA, tetraethylammonium; TTX, tetrodotoxin; VH, ventral horn.

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et al., 2008), but several authors have commented on the possibility of monoaminergic agents, including NA, to promote neuroplasticity (Dietz and Fouad, 2014; van den Brand et al., 2015). Therefore, the mechanisms of NA action in spinal motor neurons need to be fully elucidated to advance the development of NA-based pharmacological therapies.

The goal of the present study was to investigate how NA modulates synaptic transmission and neuronal excitability in spinal VH neurons using electrophysiological techniques, to gain mechanistic insight into NA action on VH neurons and to characterize the functional distribution of adrenoceptors on the synapse or somatic membrane.

METHODS

Study approval

All experimental procedures involving animals were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and approved by the Animal Care and Use Committee of Niigata University Graduate School of Medical and Dental Sciences (Niigata, Japan; certification number SA00123).

Spinal cord slice preparation

Slices of rat spinal cords were prepared as described previously (Honda et al., 2011; Ohashi et al., 2016a). Neonatal Wistar rats of either sex at P7–15 were anesthetized with urethane injected intraperitoneally (1.5 g/kg). The number of postnatal days for these rats was 10.2 ± 0.2 (mean ± standard error of the mean[SEM]): P7, n = 13 (11.8%); P8, n = 14 (12.7%); P9, n = 16 (14.5%); P10, n = 18 (16.4%); P11, n = 17 (15.5%); P12, n = 14 (12.7%); P13, n = 8 (7.3%); P14, n = 9 (8.2%); P15, n = 1 (0.9%). A dorsal laminectomy was performed, and spinal cords including a lumbosacral enlargement were removed (Honda et al., 2011) and stored in pre-oxygenated, ice-cold (2–4 °C) artificial cerebrospinal fluid (ACSF). ACSF contained (in mM) 117 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 1.2$MgCl_2$, 1.2NaH_2PO_4, 25 $NaHCO_3$, and 11.5 D-glucose. Spinal cord specimens were cut into 500-μm thick transverse slices on a microslicer (Linear Slicer PRO 7; Dosaka, Kyoto, Japan) at the level of L4–5, which was identified using L5 roots as a reference. Slices were then transferred to a recording chamber and placed on the stage of an upright microscope featuring an infrared-differential interference contrast (IR-DIC) system (E600FN; Nikon, Tokyo, Japan). Each slice was fixed with an anchor and superfused with ACSF, equilibrated in a gas mixture of 95% O_2 and 5% CO_2 (resulting in a pH of 7.4), at 5–6 mL/min. The superfused slices were maintained at 36 ± 0.5 °C using a temperature control system (TC-324B; Warner Instruments, Hamden, CT, USA). Potentials were recorded after a recovery period (over 40 min).

Patch-clamp recordings from ventral horn neurons

Whole-cell patch-clamp recordings were performed from visually identified large VH neurons in Rexed lamina IX using an IR-DIC microscope combined with a charge-coupled device camera (C2400-79H; Hamamatsu Photonics, Hamamatsu, Japan) (Honda et al., 2011; Yamamoto et al., 2012; Ohashi et al., 2016a). Recordings were made from the large VH neurons in Rexed lamina IX (size: >25 μm), which were most frequently seen in the ventrolateral and ventromedial areas. A soma size >20 μm was used as the cut-off in previous studies of postnatal rat lumbar motor neurons (Takahashi, 1990); however, large interneurons (> 20 μm) have been detected in the ventral half of the rat lumbar spinal cord (Thurbon et al., 1998). Therefore, putative motor neurons were identified as the largest cells in the VH (soma diameter > 25 μm) (Ohashi et al., 2016a, 2016b). Borosilicate glass capillaries were used to construct whole-cell patch-clamp pipettes (1.5 mm diameter; World Precision Instruments, Sarasota, FL, USA). Pipettes typically had a resistance of 4–8 MΩ when filled with an internal solution. Signals were amplified using an Axopatch 200B amplifier (Molecular Devices, Union City, CA, USA), filtered, and digitized at 2 kHz and 5 kHz. Data were collected and analyzed using the pCLAMP 10.3 data acquisition program (Molecular Devices).

Voltage-clamp protocols

A cesium-sulfate (Cs_2SO_4)-based solution containing (in mM) 110 Cs_2SO_4, 5 tetraethylammonium (TEA), 0.5 $CaCl_2$, 2 $MgCl_2$, 5 EGTA, 5 HEPES, and 5 ATP-Mg was used as an internal solution in pipettes. Because Cs^{2+} and TEA inhibit the postsynaptic effects of potassium channels, thereby interfering with α_2-adrenoceptor function (Kawasaki et al., 2003), a potassium-gluconate-based solution containing (in mM) 135 potassium gluconate, 5 KCl, 0.5 $CaCl_2$, 2 $MgCl_2$, 5 EGTA, 5 HEPES, and 5 ATP-Mg (pH 7.2) was used in tests involving an α_2-adrenoceptor agonist or antagonist. After the whole-cell configuration was established, neurons were held in voltage-clamp at −70 mV to record excitatory postsynaptic currents (EPSCs), or at 0 mV to record inhibitory postsynaptic currents (IPSCs). In spinal VH neurons, the reversal potentials of EPSCs are 0 mV, and those of IPSCs are −70 mV (Aoyama et al., 2010; Honda et al., 2012). Hence, in the membrane current trace, EPSCs appeared as downward deflections at −70 mV, and IPSCs as upward deflections at 0 mV.

Current-clamp protocol

The aforementioned potassium-gluconate-based pipette solution was used in current-clamp recordings to study neuronal excitability and membrane properties. Resting membrane potentials (RMPs) were measured under $I = 0$ conditions, and neurons with RMPs above −50 mV were excluded from the analysis (Ohashi et al., 2016b). In order to evaluate the firing of action potentials (APs), a depolarizing current of 100 pA for 1 s was injected after establishing of stable access.

Drug application

The following pharmacological agents were purchased from Wako (Osaka, Japan): tetrodotoxin (TTX), bicuculline, strychnine, phenylephrine, clonidine, yohimbine, and

propranolol. NA, isoproterenol, and prazosin were purchased from Toronto Research Chemicals (Toronto, Canada). Bicuculline and NA were dissolved in dimethyl sulfoxide, and the other compounds were stored as solutions in distilled water. Chemicals were diluted to their working concentrations in ACSF, and the spinal cord slices were superfused with these solutions at a constant perfusion speed. The volume of the recording chamber was approximately 1.0 mL. The reagents reached the recording chamber within 15 s after opening the stopcock, and were completely washed out within 90 s after closing the cock.

Data analysis

The effects of the drugs on postsynaptic currents were analyzed by comparing the postsynaptic current frequencies and amplitudes before and after drug administration using Mini Analysis program 6.0.7 (Synaptosoft, Decatur, GA, USA). The threshold for the detection of postsynaptic currents was set at twice the root mean square of the background noise and each automatically identified event was visually confirmed to eliminate artifacts. The amplitude of a drug-induced current was calculated as the maximum difference between the holding currents in the presence and absence of a drug. The relative amplitude of a current was calculated as the amplitude of the NA-induced current in the presence of a drug divided by the amplitude of the current induced by NA alone.

Statistical analysis

All numerical data were presented as mean ± SEM. Student's paired t-tests for a single comparison and repeated measures analysis of variance (ANOVA) with Bonferroni post hoc test for multiple comparisons were used to determine statistical significance. The Kolmogorov–Smirnov test was used to compare cumulative distributions of postsynaptic current parameters in the presence and absence of NA. Significance was set at $P < 0.05$ in all tests. In the electrophysiological data, the number of samples "n" refers to the number of neurons recorded. Continuous concentration-response curves for NA were drawn according to the Hill equation:

$$Y = Y_{min} + (Y_{max} - Y_{min}) / \left(1 + 10^{(\log EC_{50} - X) n_H}\right).$$

where Y is the relative frequency of EPSCs (%) increased by bath-applied NA or the amplitude (pA) of the inward current induced by NA, X represents the log [NA concentration], Y_{min} is the minimum Y value, Y_{max} is the maximum Y value, EC_{50} is the half maximum effective concentration, and n_H is the Hill slope.

RESULTS

Effects of noradrenaline on spontaneous EPSCs and holding currents in ventral horn neurons

To investigate how NA modulates excitatory synaptic transmission and neuronal activity, responses of VH motor neurons were recorded in a whole-cell voltage-clamp configuration at a holding potential of −70 mV. In the absence of NA, sEPSCs had a frequency of 32.9 ± 6.9 Hz and an amplitude of 15.4 ± 1.9 pA, as a control values (n = 11). The frequencies and amplitudes of spontaneous EPSCs (sEPSCs) before, during, and after superfusion with NA were compared as control, NA, and washout, respectively, showing significant differences among groups (frequency, $F_{(1.08,\ 10.81)} = 19.88$, $P < 0.01$; amplitude, $F_{(1.05,\ 10.45)} = 13.12$, $P < 0.01$; repeated-measures ANOVA). Superfusion with NA (40 μM) for 2 min significantly increased the frequency of sEPSCs (214.1 ± 26.5% of the control, $P < 0.001$, Bonferroni post hoc test, n = 11; Figs. 1A and B); the increased frequency returned to the control levels after at least 10 min of washout following the end of the NA application (89.8 ± 6.1% of the control, P = 1.0, Bonferroni post hoc test, n = 11). The amplitude of sEPSCs was significantly increased by NA (184.1 ± 24.3% of the control, $P < 0.01$, Bonferroni post hoc test, n = 11; Fig. 1A and C), returning to the no-NA baseline level after 10 min of washout (98.1 ± 4.6% of the control, P = 1.0, Bonferroni post hoc test, n = 11). The cumulative distributions of inter-event intervals and sEPSC amplitudes are shown in Fig. 1D and E. The proportion of sEPSCs with shorter event intervals than that in the control was significantly increased by NA ($P < 0.001$, Kolmogorov–Smirnov test) and returned to near-control levels after washout ($P > 0.09$, Kolmogorov–Smirnov test). The cumulative distribution of amplitudes revealed a NA-induced rightward shift ($P < 0.001$, Kolmogorov–Smirnov test) that disappeared after washout ($P > 0.13$, Kolmogorov–Smirnov test). Holding current measurement showed that bath application of NA (40 μM) induced an inward current (>5 pA) in all VH neurons recorded (n = 11), with an amplitude of 55.4 ± 6.4 pA (Fig. 1A).

When NA (40 μM) was applied a second time, after a more than 10-min interval from the initial application, it increased the frequency and amplitude of sEPSCs relative to the no-NA baseline, similarly to the initial NA application (Fig. 2A). The increases in frequency by the first and second NA applications were 265.4 ± 59.8% and 218.0 ± 46.7%, respectively ($T = 1.83$, $P > 0.12$, Student's paired t-test, n = 7; Fig. 2A and B), and those in amplitude were 169.3 ± 24.1% and 158.8 ± 17.9%, respectively ($T = 0.39$, $P > 0.71$, Student's paired t-test; Figs. 2A and C). The amplitudes of inward currents induced by the secondary application of NA were not significantly different from those induced by the first application (93.56 ± 5.36% of the 1st NA application, $T = 1.20$, $P > 0.28$, Student's paired t-test, n = 7; Fig. 2D).

The sEPSC frequency and inward current amplitude increased with increasing NA concentrations (Figs. 3A–C). The concentration–response curve for the relative frequency observed with bath-applied NA, fitted using the Hill equation, is presented in Fig. 3B. The EC_{50} for NA was 35.3 ± 2.1 μM with a Hill slope coefficient of 2.6 ± 0.57. The concentration–response curve for the NA-induced inward current amplitude was shown in Fig. 3C. The EC_{50} for NA was 21.2 ± 1.1 μM with a Hill coefficient of 1.9 ± 0.13.

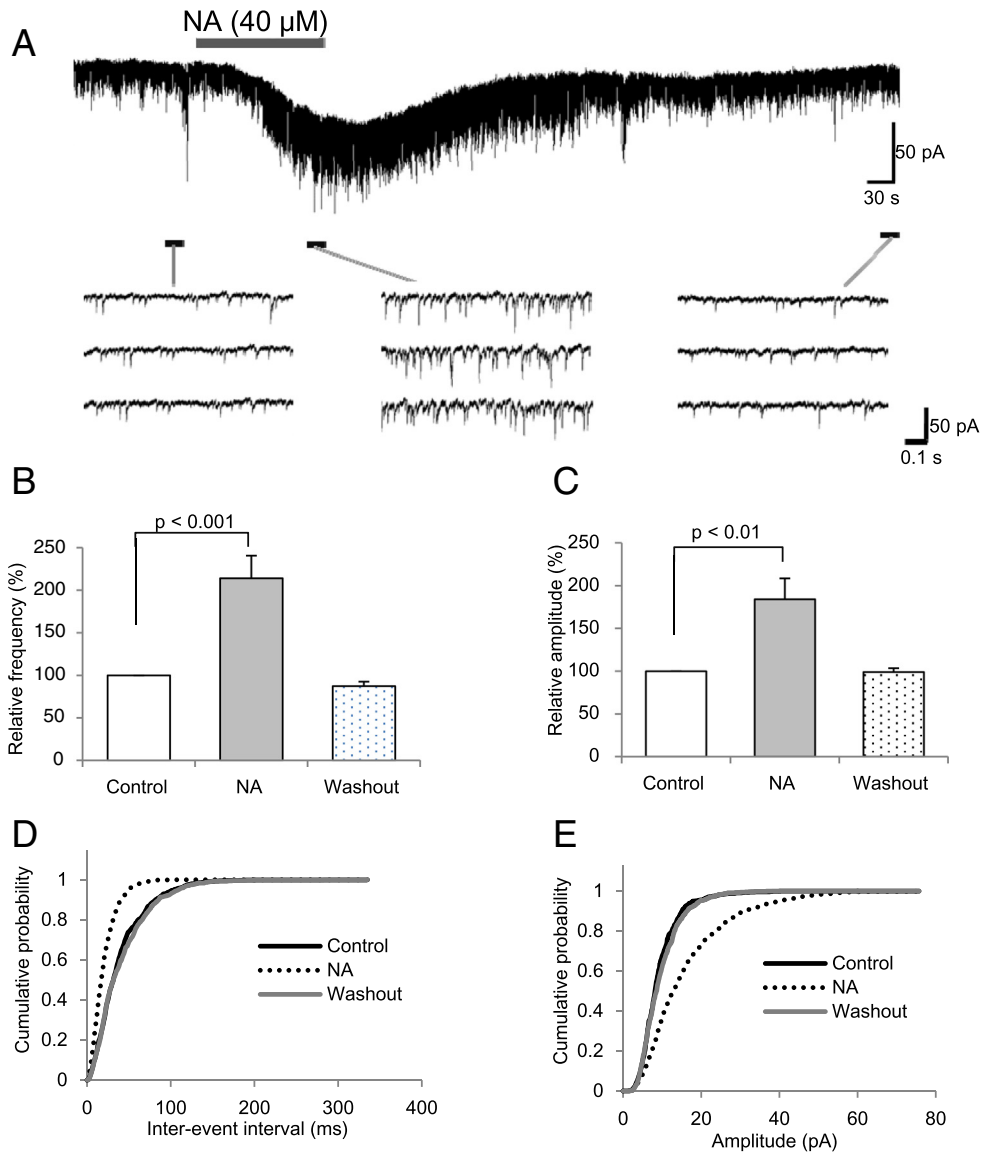


Fig. 1. Noradrenaline (NA) increases the frequency and amplitude of spontaneous excitatory postsynaptic currents (sEPSCs) in spinal ventral horn neurons. (A) A continuous chart recording of sEPSCs before, during, and after an NA (40 μM) superfusion. The horizontal bar above the recordings shows the timing of the NA superfusion. Selected portions of the trace, indicated with the small horizontal bars, are shown below the main recording on a different time scale. Three representative traces are shown. Representative traces were obtained from a rat aged 10 postnatal days. (B and C) Percentage change in frequency and amplitude relative to the control (before NA application). Error bars indicate standard error of the mean. P values were determined by Bonferroni post hoc test. The frequency (B) and amplitude (C) of sEPSCs were increased by NA application, and returned to control levels after washout (cells, n = 11). (D and E) An example of cumulative distributions of inter-event intervals (D) and amplitudes (E) of sEPSC before (black line), during (dotted line), and after (gray line) the NA superfusion. Values were compared to the no-NA control with the Kolmogorov–Smirnov test. The inter-event interval during NA superfusion was shorter than that of the control ($P < 0.001$). The cumulative distribution of amplitudes showed a rightward shift during the NA superfusion ($P < 0.001$). (A), (D), and (E) show data from the same neuron. The holding potential was −70 mV for all recordings. Washout values were recorded at least 10 min after the end of the NA application.

Effects of tetrodotoxin on noradrenaline-induced changes in EPSCs and holding current

We next investigated the effects of NA on excitatory synaptic transmission and holding current in the presence of the Na^+ channel blocker, TTX. Since the conduction of APs is blocked by TTX, the effects of NA on the presynaptic terminals could be isolated. In the presence of TTX, postsynaptic responses to spontaneously released neurotransmitters were detected as miniature (m)EPSCs.

In the presence of TTX (1 μM), NA did not induce any significant changes in mEPSC frequency or amplitude (frequency 111.8 ± 13.0% of the control, $T = 0.90$, $P > 0.40$; amplitude 116.5 ± 8.9% of the control, $T = 1.85$, $P > 0.11$, Student's paired t-test; $n = 8$; Figs. 4A–C).

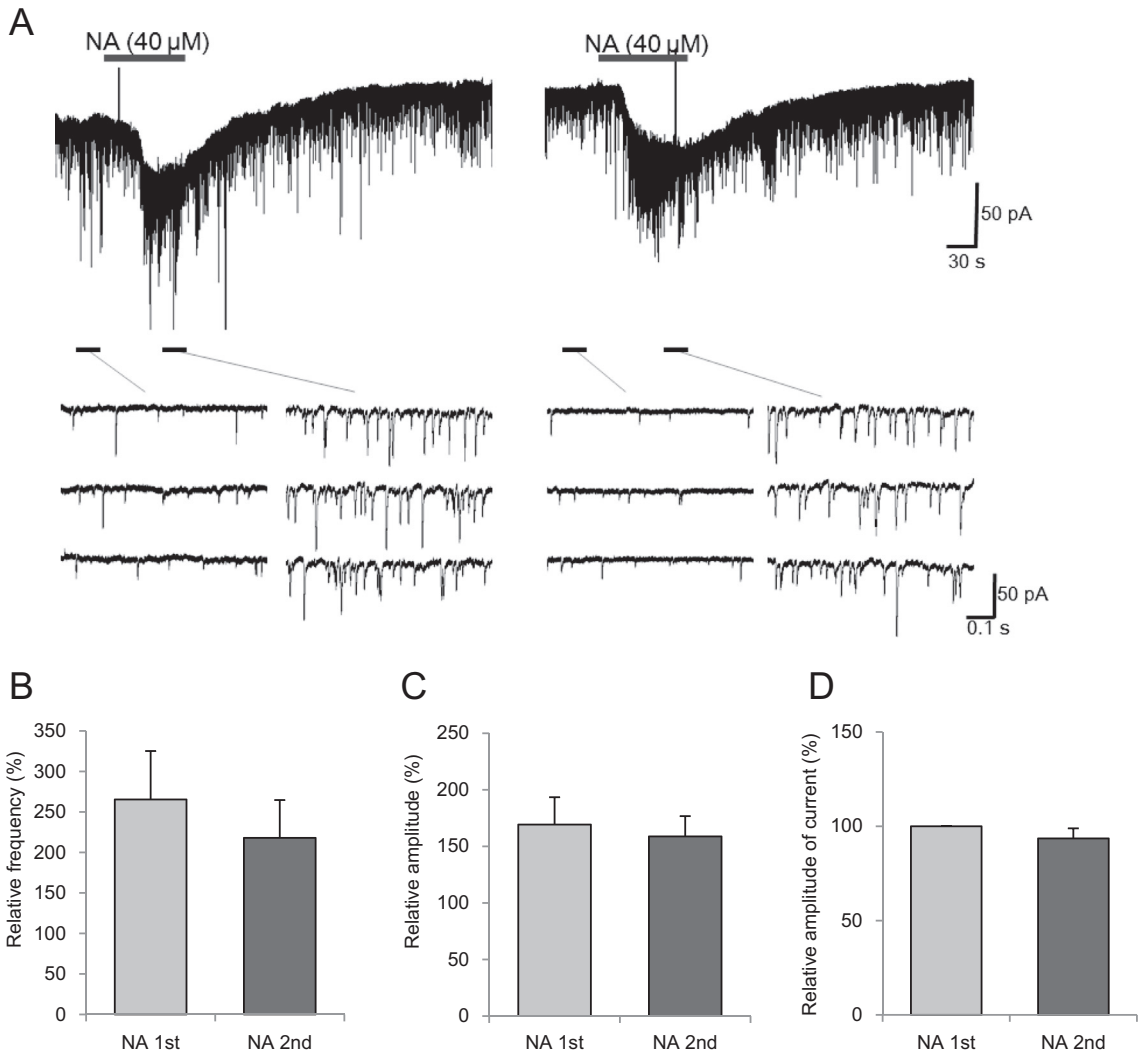


Fig. 2. Re-application of noradrenaline (NA) induces increases in the frequency and amplitude of spontaneous excitatory postsynaptic currents (sEPSCs), and an inward current to the same extents as the initial application. (A) After a sufficiently long washout period following the initial NA application (left trace), additional NA was superfused to the same cell (right trace). Below each trace are its two portions, indicated with the small horizontal bars, on a different time scale. Three representative traces are shown. Representative traces were obtained from a rat aged 8 postnatal days. (B and C) The increases in the frequency and amplitude of sEPSC induced by the second NA administration were similar to those induced by the first NA application (frequency, $P > 0.12$; amplitude, $P > 0.71$; paired Student's t-test; $n = 7$). (D) The amplitude of the inward current induced by the second NA administration was similar to that observed after the first NA application ($P > 0.28$; paired Student's t-test; $n = 7$). Error bars indicate standard error of the mean.

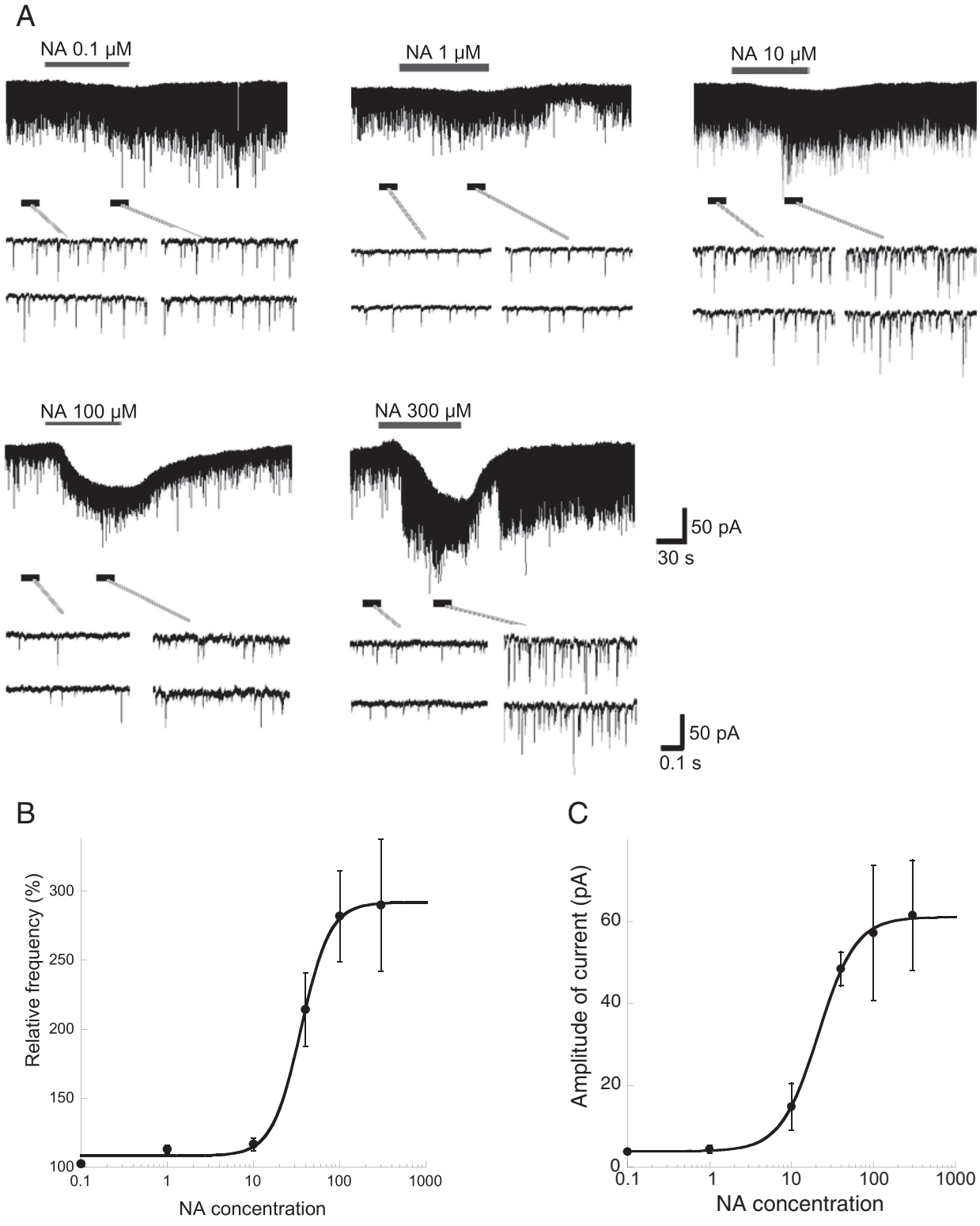
However, TTX did not decrease the amplitude of NA-induced inward currents (93.1 ± 16.3% of NA alone, $T = 0.42$, $P > 0.69$, Student's paired t-test, $n = 8$; Figs. 4A and D).

Antagonists of α1- and β-adrenoceptors inhibit noradrenaline-induced changes in spontaneous EPSCs and holding current

To determine which adrenoceptors are involved in the mechanisms of NA-induced changes in sEPSCs and inward currents, we investigated the effects of adrenoceptor antagonists. The concentration of bath-applied NA was set to 40 μM, which is higher than the estimated EC_{50} values for the effects of NA on both sEPSC frequency and holding current. The bath-application of antagonists was started after more than 10 min of washout to remove the bath-applied NA. At least 3 min after the onset of the antagonist application, NA was added to the bath with the antagonist.

As shown in Fig. 5A, the α_1-adrenoceptor antagonist prazosin (1 μM) inhibited the NA-induced increases in sEPSC frequency (87.8 ± 14.6% of the control [prazosin only], $T = 0.84$, $P > 0.44$, Student's paired t-test, $n = 6$; Fig. 5E) and amplitude (98.0 ± 7.6% of the control [prazosin only], $T = 0.26$, $P > 0.81$, Student's paired t-test, $n = 6$; Fig. 5F). NA application in the presence of the nonselective β-adrenoceptor antagonist propranolol (1 μM) significantly increased the sEPSC frequency (173.5 ± 23.9% of the control [propranolol only], $T = 3.08$, $P < 0.05$, Student's paired t-test, $n = 7$; Fig. 5B and E) and amplitude (139.7 ± 16.0% of the control [propranolol only], $T = 2.45$, $P < 0.05$, Student's paired t-test, $n = 7$; Fig. 5F). However, the increase in sEPSC frequency induced by NA in the presence of

propranolol was smaller than that induced by NA alone in the same cells (NA alone, 256.5 ± 44.8% of the control, T = 3.39, P < 0.05, Student's paired t-test, n = 7), indicating that propranolol had a partial inhibitory effect. The NA-induced inward current was also suppressed in the presence of prazosin (14.7 ± 6.7% of the control [NA only], T = 12.65, P < 0.001, Student's paired t-test, n = 6; Fig. 5A and G) and propranolol (53.4 ± 7.5% of the control [NA only], T = 6.20, P < 0.01, Student's paired t-test, n = 7; Fig. 5B and G). In addition, prazosin and propranolol showed an additive inhibitory effect on the NA-induced inward current (6.4 ± 1.4% of the control [NA only], T = 65.20, P < 0.001, Student's



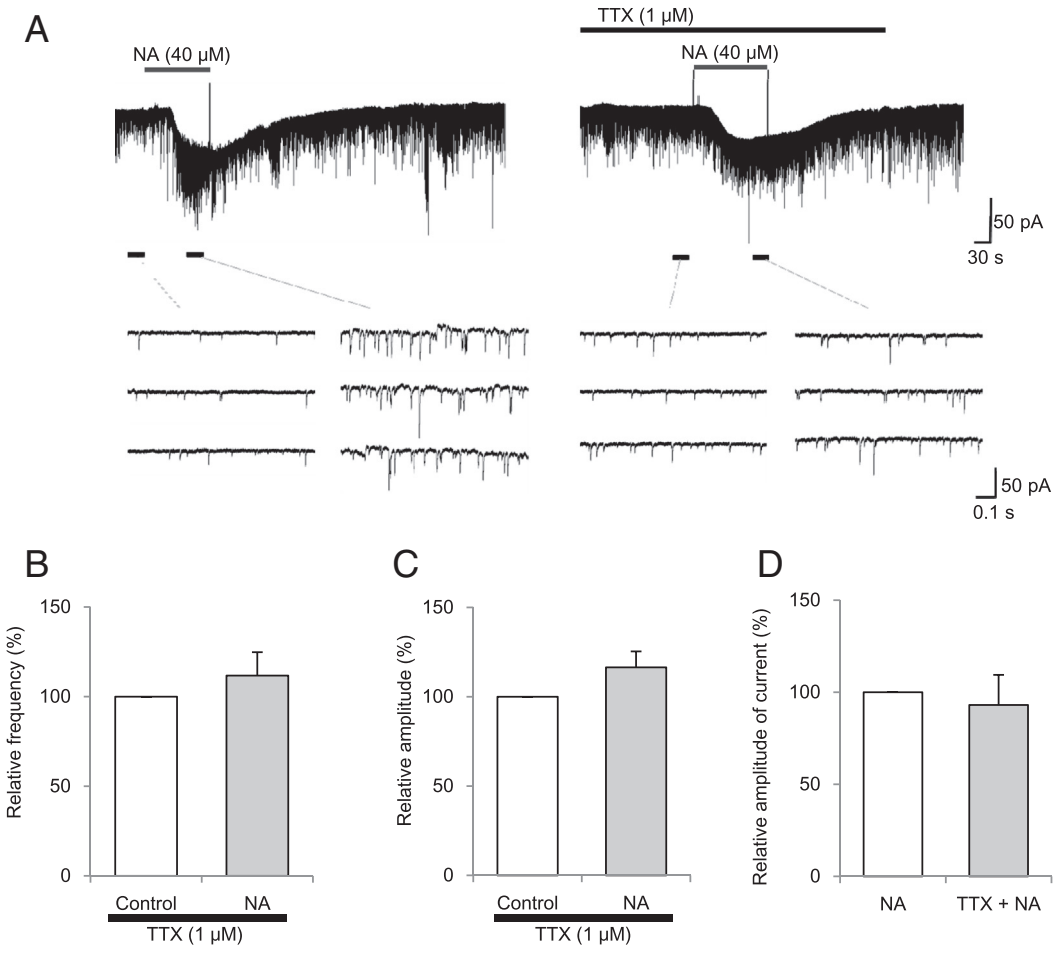


Fig. 4. Effects of tetrodotoxin (TTX) on noradrenaline (NA)-induced changes of excitatory postsynaptic currents (EPSCs) and holding current. (A) After an initial application of NA alone (left trace) followed by a washout, NA was reapplied in the presence of TTX (right trace), which blocked the conduction of action potentials and isolated action potentials on presynaptic terminals. Horizontal bars above the traces show the timing of the NA/TTX application. Selected portions of the traces, indicated with small horizontal bars, are shown below the main traces on a different time scale. Three representative traces are shown. Representative traces were obtained from a rat aged 8 postnatal day. (B and C) The relative frequency (B) and amplitude (C) of miniature (m)EPSCs in the presence of TTX were not affected by bath-applied NA (frequency, $P > 0.40$; amplitude, $P > 0.11$; paired Student's t-test; n = 8). (D) NA-induced inward current was not significantly different in the presence or absence of TTX ($P > 0.69$; paired Student's t-test; n = 8). Error bars indicate standard error of the mean.

paired t-test, n = 6; Fig. 5C and G). Surprisingly, when both prazosin and propranolol were present, the frequency and the amplitude of sEPSC were significantly decreased during the bath application of NA (frequency, 68.5 ± 11.4% of the control [prazosin and propranolol], Student's paired t-test, $T = 2.76$, $P < 0.05$, Fig. 5E; amplitude, 87.1 ± 4.9% of the control [prazosin and propranolol], $T = 2.62$, $P < 0.05$; Student's paired t-test, Fig. 5F).

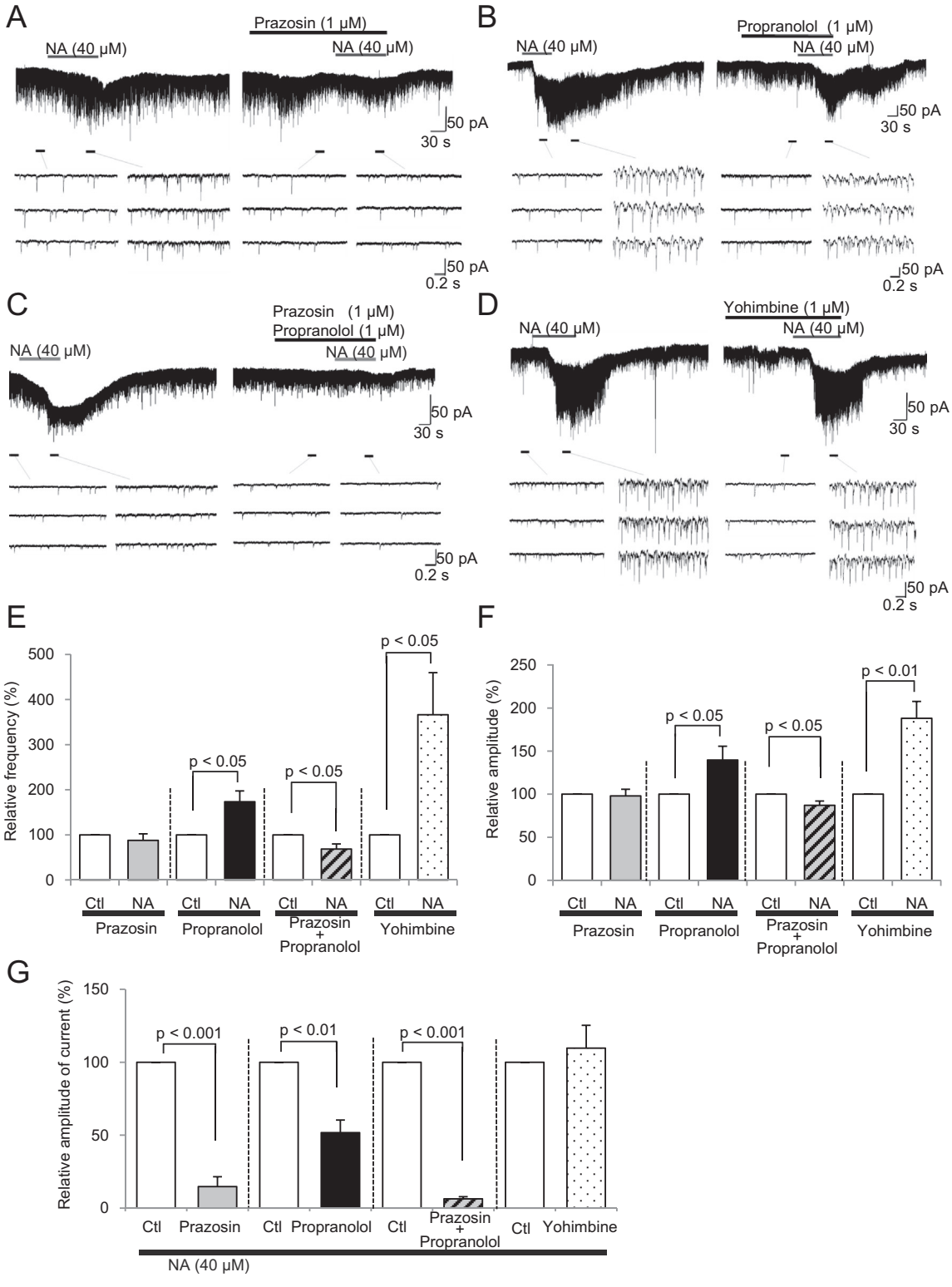
By contrast, yohimbine (1 μM), an α_2-adrenoceptor antagonist, did not have inhibitory effects on the NA-induced

Fig. 3. Concentration response characteristics of the relative frequency of noradrenaline (NA)-induced spontaneous excitatory postsynaptic currents (sEPSCs) and inward current amplitude. (A) The relative frequency of sEPSCs and the amplitude of the NA-induced inward current increased with increasing NA concentrations. Representative traces are shown for five different NA concentrations. Horizontal bars above the traces show the timing of NA superfusion at each concentration. Representative traces were obtained from postnatal day 9 (P9) rats for the NA concentrations of 0.1–10 μM, a P14 rat for the NA concentration of 100 μM, and a P10 rat for the NA concentration of 300 μM. (B) The concentration–response curve for the relative frequency of NA-induced sEPSCs. The relative frequencies induced by increasing concentrations of bath-applied NA were plotted on a log-linear scale (0.1 μM NA, n = 5; 1 μM, n = 11; 10 μM, n = 5; 40 μM, n = 11; 100 μM, n = 5; 300 μM, n = 5). The continuous curve was based on the Hill plot, with a half maximum effective concentration (EC_{50}) of 35.3 ± 2.1 μM and a Hill coefficient of 2.6 ± 0.57 [mean ± standard error of the mean (SEM)]. (C) The concentration–response curve for the NA-induced inward current amplitude. The amplitudes at varying NA concentrations were plotted on a log-linear scale (the numbers of recorded cells were the same as in B). The continuous curve was based on the Hill plot, with an EC_{50} of 21.2 ± 1.1 μM and a Hill coefficient of 1.9 ± 0.13 (mean ± SEM). Error bars indicate SEM.

changes in either sEPSCs (frequency, 366.4 ± 93.2% of the control [yohimbine only], T = 2.86, P < 0.05; amplitude, 188.1 ± 19.6% of the control [yohimbine only], T = 4.50, P < 0.01, Student's paired t-test; Figs. 5D, E, and F) or inward current (109.7 ± 15.6% of the control [NA only], T = 0.62, P > 0.56, n = 6; Fig. 5D and G).

Effects of adrenoceptor agonists on spontaneous EPSCs frequency and amplitude, and holding current

Next, we investigated the inhibitory effects of α_1-, α_2-, and β-adrenoceptor antagonists on excitatory synaptic transmission and holding current. The concentrations of



bath-applied agonists were selected according to those previously used in spinal VH neurons (Tartas et al., 2010).

As shown in Fig. 6A, the α_1-adrenoceptor agonist, phenylephrine (50 μM) significantly increased sEPSC frequency (260.5 ± 39.7% of the control, T = 4.05, P < 0.01, Student's paired t-test, n = 8; Figs. 6A and D) and amplitude (213.9 ± 37.9% of the control, T = 3.01, P < 0.05, Student's paired t-test, n = 8; Fig. 6E). Nonselective β-adrenoceptor agonist, isoproterenol (50 μM) also showed a significant increase in sEPSC frequency (139.2 ± 11.0% of the control, T = 3.56, P < 0.01, Student's paired t-test, n = 8; Figs. 6B, D) and amplitude (107.2 ± 2.4% of the control, T = 3.03, P < 0.05, Student's paired t-test, n = 8; Fig. 6E). Phenylephrine and isoproterenol induced inward currents (>5 pA) in all cells recorded, the amplitudes of which were 35.4 ± 6.9 pA and 30.8 ± 6.4 pA, respectively (Figs. 6A and B). In contrast, 1 mM of clonidine elicited outward currents in all cells (28.9 ± 10.4 pA, n = 6; Fig. 6C), and significantly decreased the sEPSC frequency and amplitude (frequency 71.9 ± 5.2% of the control, T = 5.37, P < 0.01; amplitude 93.9 ± 2.2% of the control, T = 2.81, P < 0.05; Student's paired-t test, n = 6, Figs. 6D and E).

Effects of noradrenaline on spontaneous and miniature IPSCs

To evaluate the effects of NA on inhibitory synaptic transmission, the responses of VH neurons were recorded in a whole-cell, voltage-clamp configuration at a holding potential of 0 mV. The frequency and amplitude of spontaneous IPSCs (sIPSCs) were reversibly increased by bath-applied NA (40 μM) (frequency 237.8 ± 21.6% of the control, T = 6.39, P < 0.001, Student's paired t-test, n = 9, Figs. 7A and B; amplitude 266.0 ± 63.2% of the control, T = 2.63, P < 0.05, Student's paired t-test, n = 9, Fig. 7A and C). Fig. 7 D and E show that the NA application shortened the average inter-event interval with a significant change in amplitude relative to the control (NA superfusion: frequency, P < 0.0001; amplitude, P < 0.0001; Kolmogorov–Smirnov test). By contrast, in the presence of TTX, NA administration did not significantly increase the frequency or amplitude of mIPSCs (frequency, 95.0 ± 9.5% of the control [TTX only], T = 0.53, P > 0.69, Student's paired t-test, n = 7; amplitude, 99.0 ± 5.7% of the control [TTX only], T = 0.18, P > 0.87, Student's paired t-test, n = 7; Fig. 7B and C).

Since GABA and glycine are both released from axon terminals that synapse onto spinal motor neurons (Ornung et al., 1994; Taal and Holstege, 1994; Jonas et al., 1998; Baer et al., 2003), we evaluated the types of sIPSCs facilitated by NA. In the presence of the $GABA_A$ receptor subtype antagonist bicuculline (20 μM), glycinergic sIPSCs were recorded at a holding potential of 0 mV (Fig. 8A). Application of NA resulted in remarkable and reversible increases in the frequency (609.9 ± 128.0% of the control [bicuculline only], T = 3.99, P < 0.05, Student's paired t-test, n = 4; Fig. 8B) and amplitude (311.3 ± 31.4% of the control [bicuculline only], T = 6.73, P < 0.01, Student's paired t-test, n = 4; Fig. 8C) of glycinergic sIPSCs. However, these increases were abrogated in the presence of TTX (frequency 87.9 ± 9.6% of the control [bicuculline and TTX], T = 1.27, P > 0.29; amplitude 111.4 ± 14.3% of the control [bicuculline and TTX], T = 0.80, P > 0.48; n = 4; Figs. 8 A–C).

Similarly, in the presence of the glycine receptor antagonist strychnine (2 μM), GABAergic sIPSCs were recorded at a holding potential of 0 mV. Bath-applied NA reversibly increased the frequency (279.6 ± 66.1% of the control [strychnine only], T = 2.72, P < 0.05, Student's paired t-test, n = 8; Fig. 8E) and amplitude (165.0 ± 27.1% of the control [strychnine only], T = 2.40, P < 0.05, Student's paired t-test, n = 8; Fig. 8F) of GABAergic sIPSCs. These increases were inhibited by TTX (frequency 93.3 ± 8.0% of the control [strychnine and TTX], T = 0.64, P > 0.43, Student's paired t-test, n = 8; amplitude 111.9 ± 4.4% of the control [strychnine and TTX], T = 1.20, P > 0.29, n = 8; Fig. 8D-F).

Noradrenaline enhances neuronal excitability in ventral horn neurons

Our finding that NA activated both excitatory and inhibitory synaptic transmission prompted us to examine the effects of NA on the membrane excitability of spinal VH neurons. The excitability of VH neurons was measured by recording RMPs in a whole-cell current-clamp configuration at a holding current of 0 pA. The RMP of VH neurons in the absence of NA was −63.0 ± 1.3 mV. Following NA administration, the membrane was depolarized to −54.5 ± 1.9 mV (T = 7.07, P < 0.001, Student's paired t-test, n = 7; Fig. 9A

Fig. 5. Effects of noradrenergic antagonists on synaptic transmission and noradrenaline (NA)-induced inward current. (A–D) Horizontal bars above the traces show the timing of the NA/antagonist application. Selected portions of the traces, indicated with small horizontal bars, are shown below the main traces on a different time scale. Three representative traces are shown. The NA-induced changes of spontaneous excitatory postsynaptic currents (sEPSCs) and inward current are inhibited by the application of the α_1-adrenoceptor antagonist prazosin (1 μM; A), or the β-adrenoceptor antagonist propranolol (1 μM; B), but not by the application of the α_2-adrenoceptor antagonist yohimbine (1 μM; D). (C) Prazosin (1 μM) and propranolol (1 μM) showed an additive effect when applied concurrently. (E and F) The effects of adrenoceptor antagonists on the frequency (E) and the amplitude (F) of sEPSCs. The relative frequency in the presence of prazosin (1 μM, n = 6), propranolol (1 μM, n = 7), prazosin and propranolol combined (1 μM each, n = 6), or yohimbine (1 μM, n = 6) was compared with the respective control (Student's paired t-test). The NA-induced increases in sEPSC frequency and amplitude were inhibited by prazosin (frequency, P > 0.44; amplitude P > 0.81), but not by yohimbine. NA application under β-adrenoceptor antagonist propranolol (1 μM) increased the sEPSC frequency; however, the increase was smaller than that induced by NA alone in the same cells (not shown in the figure, P < 0.05, Student's paired t-test), indicating that propranolol had a partial inhibitory effect. The frequency and the amplitude of sEPSCs in the presence of both prazosin and propranolol were decreased by NA application. (G) NA-induced inward currents were inhibited by prazosin, propranolol, or both prazosin and propranolol, but not by yohimbine (Student's paired t-test). The amplitudes of currents recorded with NA in the absence of the adrenoceptor antagonist were used as control. Error bars indicate standard error of the mean. Representative traces in the (A), (B), (C), and (D) were obtained from the rats at postnatal day 14 (P14), P8, P8, and P8, respectively.

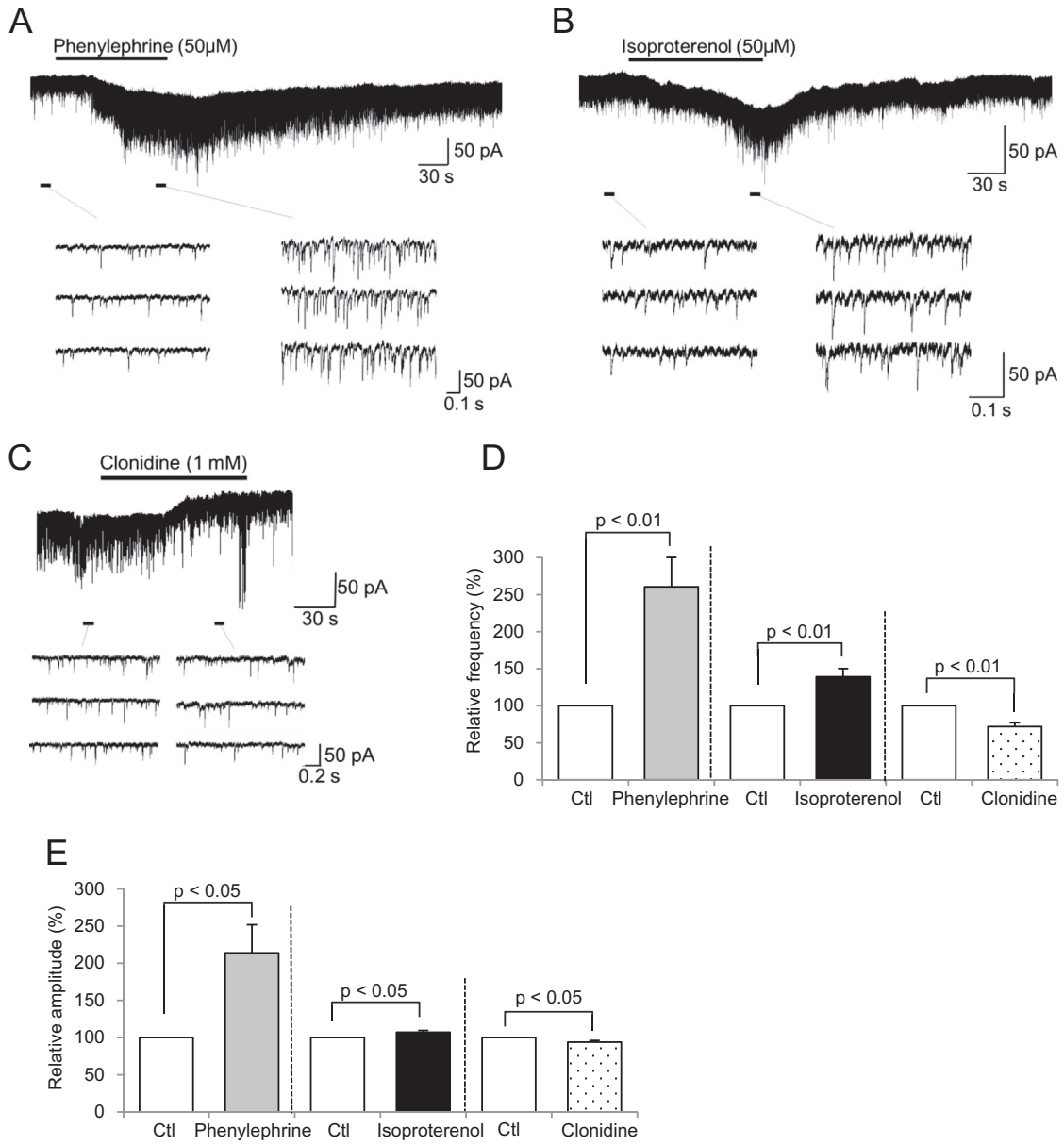


Fig. 6. Effects of adrenoceptor agonists on spontaneous excitatory postsynaptic currents (sEPSCs) and holding current. (A–C) Horizontal bars above the main trace show the timing of the agonist application. Selected portions of the traces, indicated with small horizontal bars, are shown below the main trace on a different time scale. Three representative traces are shown. (A) α_1-adrenoceptor agonist, phenylephrine (50 μM) induced inward currents (> 5pA) in all cells with the amplitudes of 35.4 ± 6.9 pA (mean ± standard error of the mean [SEM]; n = 8). The frequency (D) and the amplitude (E) of sEPSCs were significantly increased higher after the application of phenylephrine than those before the agonist application (Student's paired t-test). Error bars indicate SEM. (B) β-adrenoceptor agonist, isoproterenol (50 μM) induced inward currents (> 5pA) in all cells recorded, with the amplitudes of 30.8 ± 6.4 pA (n = 8). Isoproterenol significantly increased the frequency (D) and the amplitude (E) of sEPSCs, (Student's paired t-test). (C) Superfusion of the α_2-agonist, clonidine (1 mM), induced outward currents in all cells with an amplitude of 28.9 ± 10.4 pA (n = 6). The frequency (D) and amplitude (E) of sEPSCs were significantly reduced by clonidine (Student's paired t-test). Representative traces in the (A), (B), and (C) were obtained from the rats at postnatal day 7 (P7), P9, and P8, respectively.

and B), and returned to control levels after an ACSF wash in all neurons recorded.

We next investigated AP discharge activity by the injection of a depolarizing current. NA significantly increased firing frequency from 23.1 ± 3.2 to 34.3 ± 2.6 Hz ($P < 0.01$, Bonferroni post hoc test, n = 10; Figs. 9C and D), which was decreased to control levels after NA washout (25.3 ± 3.4 Hz, $P = 1.0$, Bonferroni post hoc test, n = 10).

DISCUSSION

The present study investigated the cellular mechanisms underlying the effects of NA on synaptic transmission and neuronal excitability in spinal VH neurons. Machacek and Hochman examined NA dependent ventral-root-evoked synaptic actions in VH neurons using a whole-cell patch-clamp technique; however, the authors used hemisected spinal cords and did

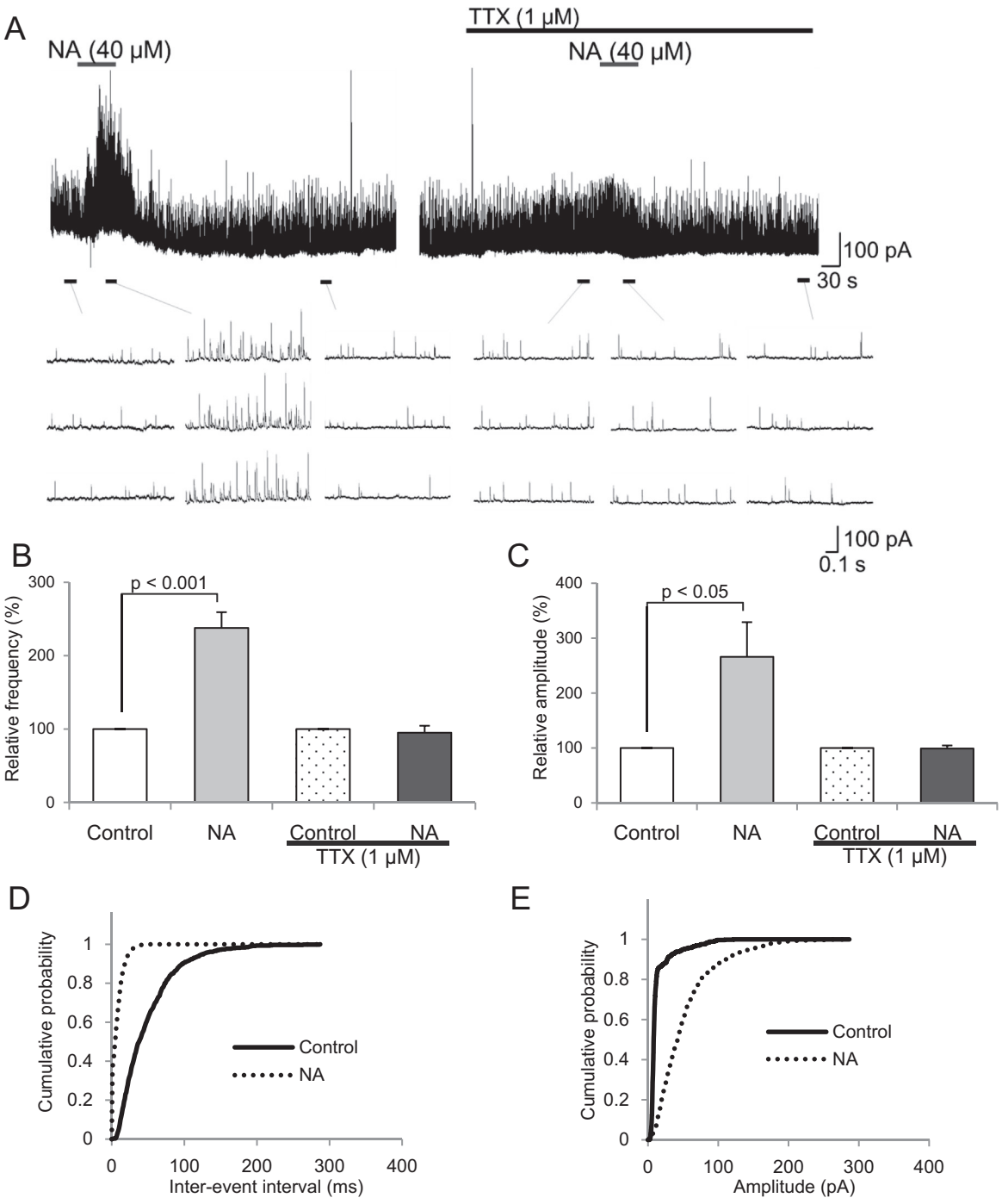


Fig. 7. Effects of noradrenaline (NA) on spontaneous inhibitory postsynaptic current (sIPSCs) and miniature (m)IPSCs. (A) Chart recordings of sIPSCs and mIPSCs in the presence of tetrodotoxin (TTX), before, during, and after NA superfusion. Representative traces were obtained from a rat with the age of postnatal day 12. Bath-applied NA remarkably increased the frequency and amplitude of sIPSCs; however, these effects were blocked by TTX. The horizontal bars above the main traces show the timing of NA/TTX application. Selected portions of each trace, indicated with small horizontal bars, are shown below the main trace on a different time scale. (B and C) Effects of TTX on the frequency (B) and amplitude (C) of sIPSCs and mIPSCs. P values were determined by Student's paired t-test between the no-NA control and NA superfusion. The frequency and amplitude of sIPSCs were increased by NA, while those of mIPSCs in the presence of TTX were unaffected by NA (frequency, $P > 0.69$; amplitude, $P > 0.87$; $n = 9$). Error bars indicate the standard error of the mean. (D and E) Cumulative distributions of inter-event intervals (D) and amplitudes (E) of sIPSCs before (black line) and during (dotted line) NA superfusion. (A), (D), and (E) show data from the same neuron. The event interval of sIPSCs was significantly shortened by NA ($P < 0.0001$, Kolmogorov–Smirnov test). The cumulative distribution of amplitudes was shifted rightward by the application of NA ($P < 0.0001$, Kolmogorov–Smirnov test).

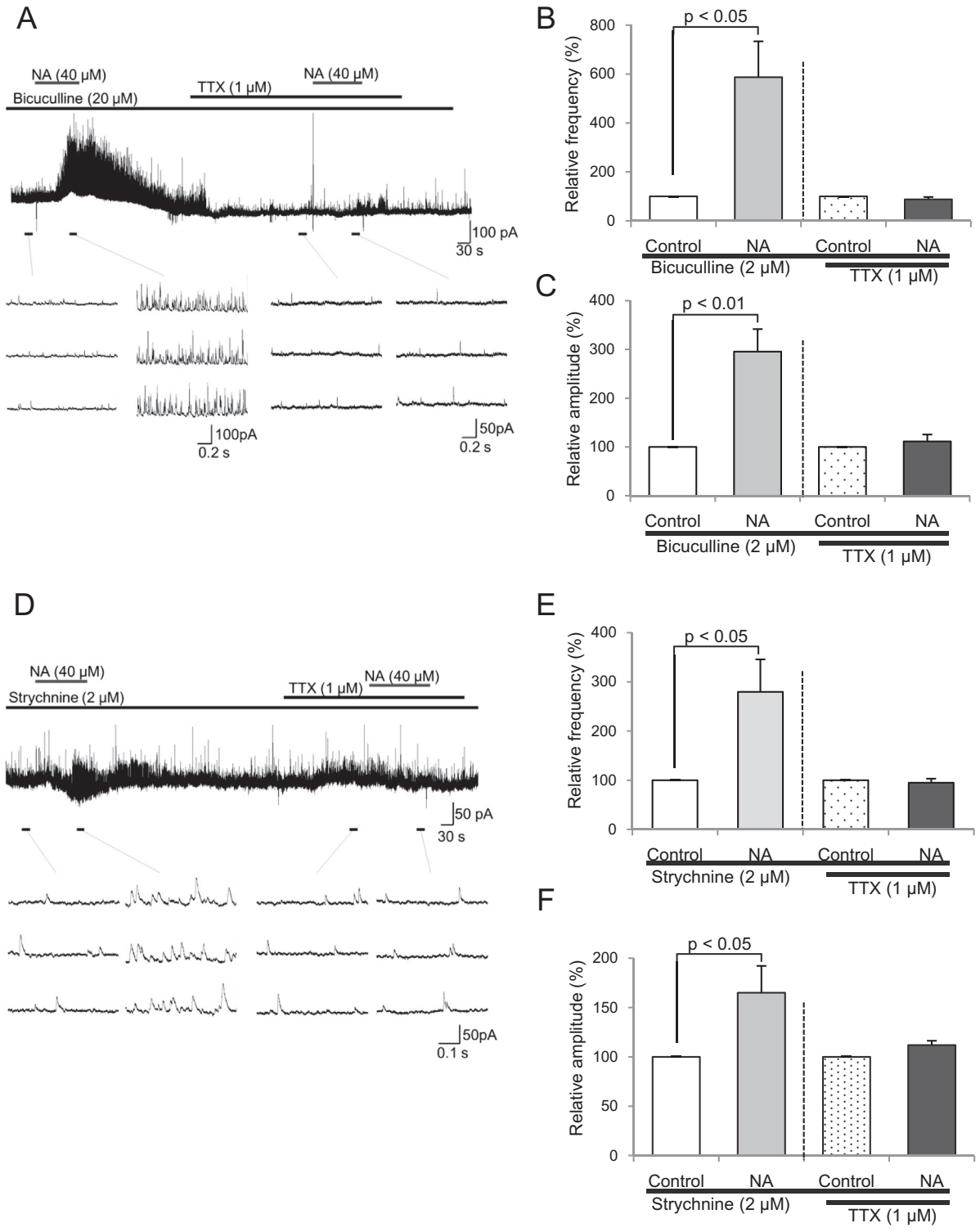


Fig. 8. Noradrenaline (NA)-induced changes in glycinergic and γ-aminobutyric acid (GABA)ergic spontaneous inhibitory postsynaptic currents (sIPSCs) and miniature (m)IPSCs. (A) A chart recording of glycinergic IPSCs before and after NA superfusion. Representative traces were obtained from a rat with the aged 8 postnatal days. The $GABA_A$ receptor antagonist bicuculline was used to isolate glycinergic potentials in ventral horn neurons. (B and C) Effects of TTX on the frequency (B) and amplitude (C) of glycinergic IPSCs. The frequency and amplitude of glycinergic sIPSCs were significantly increased by NA, but those of glycinergic mIPSCs in the presence of TTX were unaffected by NA (frequency, $P > 0.29$; amplitude $P > 0.48$; paired t-test, $n = 4$). (D) A chart recording of GABAergic IPSCs before and after NA superfusion. Representative traces were obtained from a rat aged of 11 postnatal day. The glycine receptor antagonist strychnine was used to isolate GABAergic potentials in ventral horn neurons. (E and F) Effects of TTX on the frequency (E) and amplitude (F) of GABAergic IPSCs. NA induced increases in the frequency and amplitude of GABAergic sIPSCs, but not in mIPSCs in the presence of TTX (frequency, $P > 0.43$; amplitude $P > 0.29$, Student's paired t-test, $n = 8$). Error bars indicate the standard error of the mean.

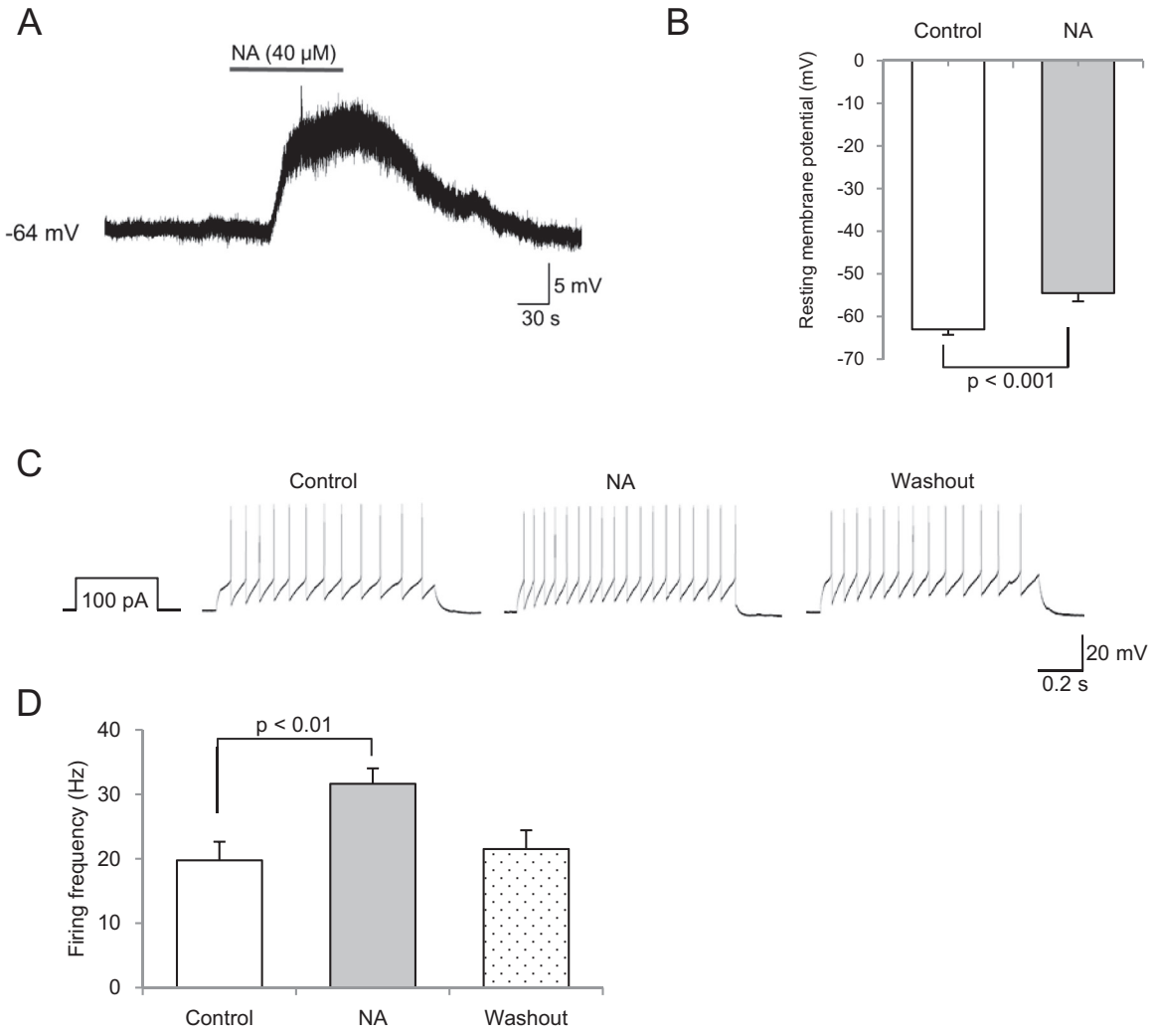


Fig. 9. Noradrenaline (NA) depolarizes resting membrane potentials (RMPs) and enhances neuronal excitability under a current-clamp configuration. (A) RMPs in ventral horn (VH) neurons were measured under *I* = 0 conditions. Representative traces were obtained from a rat aged 10 postnatal days. The horizontal bar above the trace shows the timing of NA application. (B) The RMPs of VH neurons were depolarized from −63.0 ± 1.3 mV (mean ± standard error of the mean [SEM]) to −54.5 ± 1.9 mV following a bath application of NA in all recorded cells ($P < 0.001$, Student's paired-t test, n = 7). (C and D) Action potentials (APs) were evoked by 100 pA depolarizing current injection in VH neurons. Representative traces were obtained from a rat aged 12 postnatal days. The Firing frequency of APs was increased by the NA application and decreased to control levels after the NA washout ($P = 1.0$, Bonferroni post hoc test, n = 10).

not examine the effects of NA on spontaneous synaptic transmission and neuronal excitability (Machacek and Hochman, 2006). Furthermore, Tartas et al. reported the cellular mechanisms of NA in the early neonatal rats aged P1–5 (Tartas et al., 2010). Nevertheless, it has been reported that the descending pathways from the brain to the lumbar spinal cord develop and arrive by the end of the first postnatal week (Vinay et al., 2005). In addition, inhibitory transmitters are developmentally depolarizing but become hyperpolarizing to motoneurons after P7 (Singer et al., 1998). Therefore, we used late neonatal rats (P7–15) to assess the effects of NA and demonstrated that NA administration facilitates both spontaneous excitatory and inhibitory transmissions. In addition, NA induces an inward current as previously reported (Heckman et al., 2009; Tartas et al., 2010; Rank et al., 2011) suggesting that the excitability of VH neurons is enhanced, consistent with our results showing that NA depolarizes the RMPs of VH neurons.

The mechanism of noradrenaline-induced facilitation of spontaneous EPSCs involves the activation of α_1- and β-adrenoceptors in excitatory interneurons

NA increased both the frequency and amplitude of sEPSCs in VH neurons. In general, the strength of synaptic transmission can be altered through modulation of both the probability of transmitter release and postsynaptic responsiveness. The NA-induced increase in sEPSC frequency indicates an increase in glutamate release from presynaptic terminals. Moreover, this effect of NA was completely blocked by an α_1-adrenoceptor antagonist (prazosin), and partially blocked by a β-adrenoceptor antagonist (propranolol), but not by an α_2-adrenoceptor antagonist (yohimbine), suggesting that

the NA-induced facilitation of sEPSCs is mediated by the activation of α_1- and β-adrenoceptors. This was corroborated by the results showing that an α_1-adrenoceptor agonist (phenylephrine) and a β-adrenoceptor agonist (isoproterenol) increased sEPSC frequency and amplitude. On the other hand, bath-applied NA in the presence of both α_1- and β-adrenoceptor antagonists reduced sEPSC frequency and amplitude. In general, the activation of α_1- or β-adrenoceptors can induce depolarization of neurons, while α_2-adrenoceptor activation induces hyperpolarization (Marzo et al., 2009). Therefore, our results suggest that the activation of α_2-adrenoceptor is involved in the reduction of sEPSC frequency and amplitude by NA in the presence of both α_1- and β-adrenoceptor antagonists. Indeed, the α_2-adrenoceptor agonist (clonidine) reduced sEPSC frequency and amplitude, and induced an outward current.

In contrast, in the presence of TTX, which blocks conduction of APs and isolates the effects of NA on presynaptic terminals, NA did not increase the frequency and amplitude of mEPSCs. These data indicate that NA does not facilitate the quantal release of glutamate from the presynaptic terminals of excitatory interneurons. In other words, neither α_1- nor β-adrenoceptors, at least electrophysiologically, function in the excitatory presynaptic terminals in the spinal VH. Moreover, the finding that the mEPSC amplitude was not affected by the application of NA indicates that NA does not enhance the responsiveness of postsynaptic glutamate receptors. Therefore, in the spinal VH, NA likely activates excitatory interneurons via their somatic α_1- and β-adrenoceptors, stimulating glutamate release from the excitatory presynaptic terminals.

Our results are in an apparent contradiction to those of Tartas et al., who found that the mEPSC frequency is presynaptically inhibited via the activation of α_2- and β-adrenoceptors and presynaptically enhanced via the activation of α_1-adrenoceptors in spinal VH neurons of rats aged 1–5 days (mean 2.4 days) (Tartas et al., 2010). This discrepancy might be caused by the differential distribution of adrenoceptors during rat development. For example, α_{2A}-adrenoceptors are transiently expressed in rat motor neurons at high levels during embryonic and early postnatal periods, reaching sustained low levels by approximately postnatal day 14 (Unnerstall et al., 1984; Winzer-Serhan et al., 1997; Selvaratnam et al., 1998). Moreover, β-adrenoceptor sites are concentrated on the cell bodies of α-motor neurons in the spinal cords of adult chickens (Bondok et al., 1988). Taken together, these reports suggest that, in the spinal VH of neonatal rats aged 7–15 days that were used in our study, the function of adrenoceptors may be negligible in the presynaptic terminals, thus explaining the lack of NA effects on mEPSCs in our experiments. A more detailed anatomical analysis will be required to determine the distribution of the receptors in VH neurons and should be a future studies.

The mechanisms of noradrenaline-induced inward currents involve the activation of α_1- and β-adrenoceptors

Bath application of NA produced inward currents, generally associated with increased neuronal excitability, at −70 mV in all VH neurons recorded. These currents were not affected by TTX, and NA did not have any effects on mEPSC frequency or amplitude. These results suggest that the generation of the NA-induced inward current is a postsynaptic effect in VH neurons. Moreover, the NA-induced inward currents were inhibited by the application of α_1- or β- but not of α_2-adrenoceptor antagonists, indicating that both α_1- and β-adrenoceptors are expressed in the postsynaptic membranes of VH neurons. These results are consistent with those of a previous report (Tartas et al., 2010). In contrast, α_2-antagonists did not affect the NA-induced currents, while an α_2-agonist at a high concentration (1 mM) produced an outward current. These observations may be explained by the low-level expression of α_2-adrenoceptors in rat spinal VH neurons during postnatal days 7–15 (Winzer-Serhan et al., 1997; Rekling et al., 2000; Venugopalan et al., 2006).

Noradrenaline enhances GABAergic and glycinergic spontaneous IPSCs via activation of inhibitory interneurons

NA enhanced both GABAergic and glycinergic sIPSCs, and these effects were completely abolished in the presence of TTX. These data indicate that NA does not facilitate the quantal release of GABA or glycine from the presynaptic terminals of inhibitory interneurons, which in turn suggests that adrenoceptors are electrophysiologically absent or negligible in inhibitory presynaptic terminals in the spinal VH. Moreover, our finding that mIPSC amplitude was not affected by the application of NA indicates that NA does not increase the responsiveness of postsynaptic GABA and glycine receptors. Therefore, similar to its effect on excitatory synaptic transmission, NA stimulates inhibitory interneurons via the activation of their somatic adrenoceptors, leading to an increased release of GABA and glycine from inhibitory presynaptic terminals.

Noradrenaline-induced alteration of membrane properties and neuronal excitability in ventral horn neurons

Since NA application activated both excitatory and inhibitory synaptic transmission, we investigated whether the excitability of VH neurons was enhanced by NA. Our current clamp recordings demonstrated that bath-applied NA depolarized RMPs and increased firing frequency in VH neuron, suggesting that NA enhanced the VH neuronal excitability. According to our results and previous reports, α_1-, α_2-, and β-adrenoceptors are expressed and function on the postsynaptic membrane in VH neurons. In general, the activation of α_1-adrenoceptors decreases the leak potassium current (McCormick and Wang, 1991), and that of β-adrenoceptors reduces the potassium conductance through a block of calcium dependent potassium channels (Haas and Rose, 1987), which induce depolarization of postsynaptic neurons. By contrast, the activation of α_2-adrenoceptors induces postsynaptic membrane hyperpolarization through the opening of ATP-dependent potassium channels by $G_{i/o}$ protein (Zhao et al., 2008). Thus, we consider that bath-application of NA increased VH neuronal

excitability in our study because the expression or function of α_2-adrenoceptors was at lower levels than those of α_1- and β-adrenoceptors in VH neurons.

Clinical significance and future perspectives

Our results suggest that α_1- and β- agonists enhance excitatory synaptic transmission and neuronal excitability, indicating that they could be effective in activating neuronal networks during recovery from SCI. However, α_1-adrenergic stimulation of neurons after SCI contributes to muscle spasms and spasticity (Harvey et al., 2006; Rank et al., 2011; D'Amico et al., 2014). Because muscle spasms and spasticity can interrupt smooth motion, α_1-agonists may fail to promote sufficient functional recovery. In contrast, no studies to date have reported that β-adrenergic stimulation is associated with muscle spasms or spasticity. We demonstrated that the activation of β-adrenoceptors was also involved in the mechanism of the NA-induced simulation of excitatory synaptic transmission and neuronal excitability in VH neurons. Therefore, our results may provide the foundation of a novel strategy for activating neuronal networks after the subacute phase of SCI, which impinges on the activation of β-adrenoceptors without muscle spasms or spasticity. Moreover, the activation of β-adrenoceptors is expected to facilitate neuronal plasticity in spinal cord because NA modulates brain neuroplasticity via the activation of β-adrenoceptors (Marzo et al., 2009; Connor et al., 2011; Laing and Bashir, 2014). Further studies are required to elucidate the mechanisms of NA-mediated neuroplasticity in the spinal cord and evaluate the feasibility of β-adrenergic pharmacotherapies in SCI.

AUTHOR CONTRIBUTIONS

This work was performed at Division of Anesthesiology and Orthopedic Surgery in Niigata University Graduate School of Medical Dental Sciences. H.S. and M.O. conducted the experiments, and analyzed the data, and wrote the manuscript. H.S., M.O., T.H., K.W., N.E., H.B., and T.K. conceived and designed the experimental plan. T.H., K.W., N.E., H.B., and T.K. revised the manuscript critically for important intellectual content. All authors approved the final version.

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DECLARATION OF INTERESTS

None.

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