Norepinephrine Facilitates Inhibitory Transmission in Substantia Gelatinosa of Adult Rat Spinal Cord (Part 1)

Effects on Axon Terminals of GABAergic and Glycinergic Neurons

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Background: The activation of descending norepinephrine-containing fibers from the brain stem inhibits nociceptive transmission at the spinal level. How these descending noradrenergic pathways exert the analgesic effect is not understood fully. Membrane hyperpolarization of substantia gelatinosa (Rexed lamina II) neurons by the activation of α_2 receptors may account for depression of pain transmission. In addition, it is possible that norepinephrine affects transmitter release in the substantia gelatinosa.

Methods: Adult male Sprague-Dawley rats (9–10 weeks of age, 250–300 g) were used in this study. Transverse spinal cord slices were cut from the isolated lumbar cord. The blind whole-cell patch-clamp technique was used to record from neurons. The effects of norepinephrine on the frequency and amplitude of miniature excitatory and inhibitory postsynaptic currents were evaluated.

Results: In the majority of substantia gelatinosa neurons tested, norepinephrine (10–100 μM) dose-dependently increased the frequency of γ-aminobutyric acid (GABA)–ergic and glycinergic miniature inhibitory postsynaptic currents; miniature excitatory postsynaptic currents were unaffected. This augmentation was mimicked by an α_1-receptor agonist, phenylephrine (10–60 μM), and inhibited by α_1-receptor antagonists prazosin (0.5 μM) and 2-(2,6-dimethoxyphenoxyethyl) aminomethyl-1,4-benzodioxane (0.5 μM). Neither postsynaptic responsiveness to exogenously applied GABA and glycine nor the kinetics of GABAergic and glycinergic inhibitory postsynaptic currents were affected by norepinephrine.

Conclusion: These results suggest that norepinephrine enhances inhibitory synaptic transmission in the substantia gelatinosa through activation of presynaptic α_1 receptors, thus providing a mechanism underlying the clinical use of α_1 agonists with local anesthetics in spinal anesthesia. (Key words: Antinociception; blind patch-clamp recording; descending pain control system; inhibitory transmission; *in vitro*; transmitter release.)

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THE sensation of pain is carried to the central nervous system by fine, myelinated (Aδ) and unmyelinated (C) fibers. These fibers terminate in the superficial layers of the spinal cord, particularly the substantia gelatinosa (SG, Rexed lamina II), a region critical for modulating nociceptive information and controlling the activity of projection neurones.[1,2] Anatomic studies[3,4] show a high concentration of norepinephrine-containing terminals in the superficial laminae of the spinal cord. Activation of these noradrenergic fibers, which originate in the brain stem, can inhibit the transmission of nociceptive signals.

Epinephrine and phenylephrine are administered commonly in combination with local anesthetics in spinal anesthesia and have been shown to prolong the duration of analgesia.[5,6] Conventional wisdom has suggested that the beneficial effects of epinephrine and phenylephrine result from local vasoconstriction and a consequent reduction in drug clearance from the subarachnoid space. However, these vasoconstrictors produce analgesia even if administered intrathecally in the absence of local anesthetics,[7,8] and they do not appear to significantly alter the clearance of local anesthetics from the subarachnoid

A

SG

Patch electrode

B

voltage clamp
(-70 mV or
0 mV)

GDP-β-s

Inhibitory

GABA

TTX

SG neuron
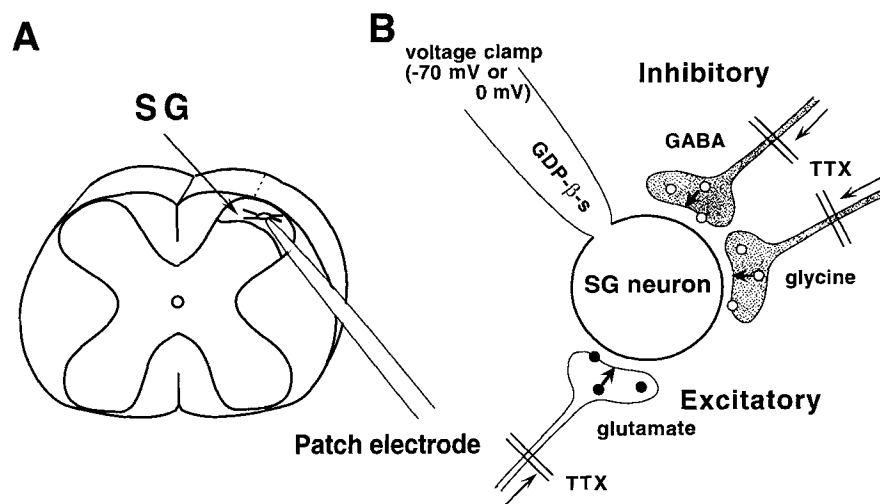
glycine

glutamate

Excitatory

TTX

Fig. 1. (*A*) Schematic diagram of whole-cell patch-clamp recording from a substantia gelatinosa (SG) neuron in an adult rat spinal cord transverse slice. The thickness of the slice was 450–500 μm. Gigaohm sealing was performed blindly. (*B*) Schematic arrangement for voltage-clamp recording of spontaneous miniature excitatory and inhibitory postsynaptic currents (mEPSCs and mIPSCs) from an SG neuron. mEPSCs are mediated by glutamate; mIPSCs are mediated by GABA and glycine. GDP-β-s was added to the pipette solution to block postsynaptic effects of norepinephrine mediated through G proteins. Conduction of action potentials is blocked by tetrodotoxin, so that the effects of chemicals on the presynaptic terminal can be analyzed.

space.[9,10] In addition, clonidine, an α_2 agonist without vasoconstrictive effects, prolongs the duration of analgesia in spinal anesthesia.[11] Thus, it is likely that intrathecal epinephrine and phenylephrine enhance spinal anesthesia *via* direct actions within the spinal dorsal horn, possibly mimicking the action of descending noradrenergic pathways.

However, how descending noradrenergic pathways, and intrathecally administered epinephrine and phenylephrine, inhibit pain transmission at the cellular level is not fully understood. It has been reported that α_2 receptors are concentrated in the SG.[12] Membrane hyperpolarization of SG neurons by norepinephrine acting on α_2-receptors[13] may account for depression of pain transmission, but it is also possible that norepinephrine affects transmitter release in the SG. The purpose of this study was to determine whether spinally administered norepinephrine acts presynaptically to alter excitatory and inhibitory synaptic transmission and, if so, to identify which receptor subtype is involved. To address this question, we used the blind patch-clamp technique to study the action of norepinephrine on miniature excitatory and inhibitory postsynaptic currents (mEPSCs and mIPSCs) in SG neurons from adult rat spinal cord slices.

Materials and Methods

Spinal Cord Slice Preparation

This study was approved by the Animal Care and Use Committee at Niigata University School of Medicine. A portion of the lumbosacral spinal cord (2.0–2.5 cm) was removed from an adult rat (9–10 weeks of age, 250–300 g)

during urethane anesthesia (1.5–2.0 g/kg, intraperitoneal). The isolated spinal cord was then placed in preoxygenated ice-cold Krebs solution (2–4°C). After removal of the dura mater, all ventral and dorsal roots were cut and the pia-arachnoid membrane was removed. The spinal cord was placed in a shallow groove formed in an agar block and glued to the bottom of the microslicer stage with cyanoacrylate adhesive. After immersion in ice-cold Krebs solution, a 450- to 500-μm thick transverse slice (L3–L5 level, fig. 1A) was cut on a vibrating microslicer (DTK1500; Dosaka, Kyoto, Japan). The spinal cord slice was then placed on nylon mesh in the recording chamber and perfused with Krebs solution (10 ml/min) saturated with 95% oxygen and 5% carbon dioxide at 36 to 37°C. The Krebs solution contained NaCl, 117 mM; KCl, 3.6 mM; $CaCl_2$, 2.5 mM; $MgCl_2$, 1.2 mM; NaH_2PO_4, 1.2 mM; $NaHCO_3$, 25 mM; and glucose, 11 mM.

Blind Patch-clamp Recording from Substantia Gelatinosa Neurons

Under a dissecting microscope with transmitted illumination, the SG was clearly discernible as a relatively translucent band across the dorsal horn. However, the contours of individual SG neurons cannot be visualized under these conditions; therefore, gigaohm sealing (attaching electrode to the cell with a resistance of at least 1 GΩ) was performed blindly.[14] Patch pipettes were fabricated from thin-walled, borosilicate, glass-capillary tubing (1.5 mm OD; World Precision Instruments, Sarasota, FL). After establishing the whole-cell configuration, voltage-clamped neurons were held at either −70 or 0 mV for recording mEPSCs and mIPSCs, respectively (fig. 1B). The reversal potentials of EPSCs and IPSCs in SG neurons are 0 and −70 mV, respec-

Fig. 2. Miniature postsynaptic currents recorded from substantia gelatinosa neurons. (A) At a holding potential of −70 mV, only mEPSCs were recorded as downward deflections in the membrane current trace. (B) At a holding potential of −30 mV, both mEPSCs and mIPSCs were recorded as downward and upward deflections, respectively. mIPSCs are indicated by arrowheads. (C) At a holding potential of 0 mV, only mIPSCs were recorded as upward deflections. Two distinct types of mIPSCs (GABAergic and glycinergic) could be distinguished according to their decay time courses. Note that GABAergic mIPSCs (indicated by arrowheads) have longer duration than glycinergic IPSCs. (D) Norepinephrine (NE, 10μM) was applied just after establishing the whole-cell configuration. Norepinephrine did not increase the frequency of mEPSCs recorded at −70 mV (top), but significantly increased the frequency of mIPSCs (bottom). Baseline currents of both traces were elevated by the activation of α_2 receptors. Top and bottom traces were obtained from two different neurons. (E) The effect of norepinephrine (20 and 100 μM) on the frequency of mEPSCs. Norepinephrine did not change or slightly decreased the frequency of mEPSCs ($P = 0.08$ for norepinephrine 20 μM, n = 17; $P = 0.09$ for norepinephrine 100 μM, n = 8, paired t test). (F) The effect of norepinephrine (20 μM) on the frequency of mIPSCs. norepinephrine markedly increased the frequency of mIPSCs ($P < 0.0001$, n = 18, paired t test).
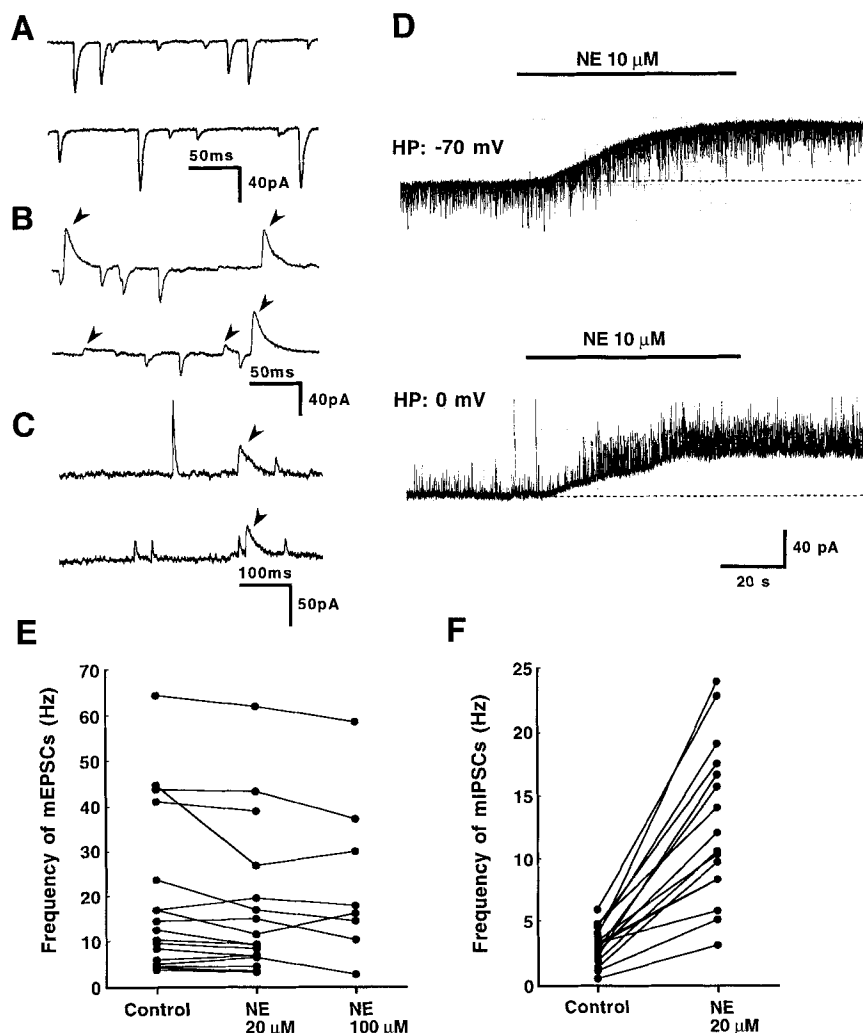
tively[15-17]; therefore, only EPSCs are recorded at −70 mV and only IPSCs at 0 mV. Under these conditions, EPSCs are recorded as downward deflections in the membrane current trace, and IPSCs are recorded as upward deflections (fig. 2). The pipette solution contained Cs-sulfate (Cs_2SO_4), 110 mM; $CaCl_2$, 0.5 mM; $MgCl_2$, 2 mM; EGTA, 5 mM; HEPES, 5 mM; tetraethyl ammonium chloride (TEA), 5 mM; adenosine triphosphate–magnesium salt, 5 mM; and guanosine-5′-O-(2-thiodiphosphate) (GDP-β-S), 1 mM. The resistance of a typical patch pipette was 5–10 MΩ. In all experiments, GDP-β-S (a G-protein blocker) was added to the pipette solution to block postsynaptic norepinephrine effects mediated through G proteins. In the current study, norepinephrine and other adrenergic agonists were superfused at least 10 min after establishing the whole-cell configuration (unless otherwise stated) to avoid possible postsynaptic

effects of norepinephrine. Membrane currents were amplified using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) in voltage-clamp mode. Signals were filtered at 2 kHz and digitized at 5 kHz. Data were stored with a personal computer using pCLAMP 6 software (Axon Instruments). Frequencies and amplitudes of miniature postsynaptic currents were measured using Axo-Graph 3 software (Axon Instruments). The amplitude of each postsynaptic current was measured from the initial inflection point (not from the baseline) to the peak, to avoid the effects of temporal summation on the amplitude distribution.

Drug Application

Drugs were applied by exchanging the perfusion solution with one containing a known drug concentration,

without altering the perfusion rate and the temperature. The time necessary for the drug-containing solution to flow from the three-way stopcock to the recording chamber was approximately 3 s. Drugs used were nor-epinephrine (WAKO, Osaka, Japan), 6-cyano-7-nitroqui-noxaline-2,3-dione (Tocris Cookson, Ballwin, MO), strychnine (Sigma, St. Louis, MO), bicuculline (Sigma), tetrodotoxin (WAKO), GDP-β-S (Sigma), chloroethyl-clonidine (RBI, Natick, MA), prazosin (Sigma), clonidine (Sigma), phenylephrine (Sigma), isoproterenol (Sigma), yohimbine (WAKO), 2-(2,6-dimethoxyphenoxyethyl) aminomethyl-1,4-benzodioxane (WB-4101; Sigma), γ-ami-nobutyric acid (GABA; WAKO), glycine (WAKO), and pro-pranolol (Sigma).

Analysis of Frequency and Amplitude of Miniature Postsynaptic Currents

The elementary unit of neurotransmitter release is the content of a single synaptic vesicle. The amplitude of an evoked EPSC or IPSC is some multiple of the postsynap-tic current in response to the transmitter content of a single vesicle. The notion of quantal transmission origi-nally was derived from synaptic release at the neuromus-cular junction,[18] and considerable evidence has accumu-lated that also supports the quantal hypothesis at central synapses.[19-21] At many synapses, exocytosis of synaptic vesicles occurs spontaneously at a low rate, even in the absence of presynaptic stimulation. In the presence of tetrodotoxin, postsynaptic responses to spontaneously released transmitter can be detected as relatively small amplitude miniature postsynaptic currents (mPSCs) (fig. 1B).

The strength (efficacy) of synaptic transmission can be altered through modulation of both "transmitter release probability" and "postsynaptic responsiveness." Analysis of frequency and amplitude distributions of mPSCs has been used to distinguish between pre- and postsynaptic loci of experimental manipulations,[22,23] including vola-tile anesthetics.[24] From the quantal hypothesis, only pre-synaptic actions can affect the probability of release. Thus, changes in the frequency of mPSCs indicate a presynaptic effect, if recruitment of latent (silent) recep-tors can be ruled out.[23] In this study, we used GDP-β-S in the intrapipette solution to eliminate possible postsyn-aptic effects (changes in postsynaptic responsiveness or recruitment of silent synapses) by norepinephrine; therefore, changes in the frequency of mPSCs can be attributed only to a presynaptic effect of norepineph-rine. Alterations in mPSC peak amplitude can be ex-plained only by changes in postsynaptic responsiveness.

The mean (or median) amplitude can also be altered by changes in postsynaptic responsiveness. However, the shape of the amplitude distribution can be skewed by the existence of synapses that are unaffected by the treatment, resulting only in an *apparent* change in the mean (median) amplitude.

Statistical Analysis

Numeric data are presented as the mean $\pm$ SD (unless otherwise stated). Analyses of the modulation of the frequency of mPSCs were performed using a paired t test. The effects of selective agonists and antagonists were analyzed using the one-way analysis of variance, and statistical significance was further evaluated using the Scheffé test for *post hoc* comparison. The Kolmo-gorov-Smirnov test was used to compare the effect nor-epinephrine on the amplitude distribution of postsynap-tic currents. Differences for which $P < 0.05$ were considered significant and are indicated by asterisks in the figures. Dose-response data were fitted using logistic equations. Curve fitting was accomplished using Origin 4.1 software (Microcal Software, Northampton, MA).

Results

Whole-cell patch-clamp recordings were made from 109 SG neurons. In the presence of tetrodotoxin (1 μM), all SG neurons tested exhibited mEPSCs and mIPSCs. At holding potentials of -70 mV, only mEPSCs were ob-served (figs. 2A and D; top).[14,25] At holding potentials more positive than -60 mV, mEPSCs and mIPSCs both were observed (fig. 2B). However, at 0 mV, only mIPSCs could be observed (fig. 2C).[25,26] mEPSCs observed at -70 mV were completely blocked by 6-cyano-7-nitroqui-noxaline-2,3-dione, suggesting that they were mediated by non-NMDA (AMPA/kainate) receptors. Two distinct types of mIPSCs could be distinguished based on the decay time course (fig. 2C).[14,16,17] One type of mIPSC had a short duration (10-20 ms) and was antagonized by a glycine receptor antagonist: strychnine (1-2 μM). The other had a relatively long duration (50-100 ms) and was antagonized by a GABA$_A$ receptor antagonist, bicu-culline (10 μM), suggesting that the two types of mIPSCs were mediated by glycine and GABA$_A$ receptors, respec-tively. Shortly after establishing the whole-cell patch-clamp configuration, norepinephrine elicited outward currents at -70 mV in the majority of SG neurons (18 of 24 cells; fig. 2D), in agreement with previous reports.[13] This effect was mimicked by clonidine (10 μM) and

Fig. 3. The effect of norepinephrine on mIPSC frequency was mediated by α_1 receptors. (A) Clonidine (10 μM) and isoproterenol (40 μM) failed to increase the frequency of mIPSCs; norepinephrine (NE, 10 μM) increased mIPSC frequency. The data were obtained from the same neuron as in figure 2D (bottom), but 15 min after establishing whole-cell configuration. Note that α_2-receptor–mediated baseline elevation (outward current) could no longer be elicited by clonidine or norepinephrine when diffusion of GDP-β-s was complete. (B) The effects of α_1, α_2, and β agonists on the frequency of mIPSCs. The frequency of mIPSCs was significantly increased by phenylephrine (10 μM, n = 5) but not by clonidine (10 μM, n = 6) or isoproterenol (40 μM, n = 6). *P < 0.01 compared with clonidine and isoproterenol group, one-way analysis of variance. (C) The effects of α_1, α_2, and β antagonists on the facilitatory effect of norepinephrine. The effect of norepinephrine (20 μM) was significantly decreased by the α_1-receptor antagonists prazosin (0.5 μM, n = 5) and WB-4101 (0.5 μM, n = 6). An α_2 antagonist, yohimbine (1 μM, n = 6), and a β antagonist, propranolol (1 μM, n = 5), failed to block the norepinephrine effect. The α_{1B}- and α_{1D}-receptor antagonist chloroethylclonidine (10 μM, n = 6) was also without effect. *P < 0.005 compared with control group (norepinephrine 20 μM, n = 18); one-way analysis of variance.
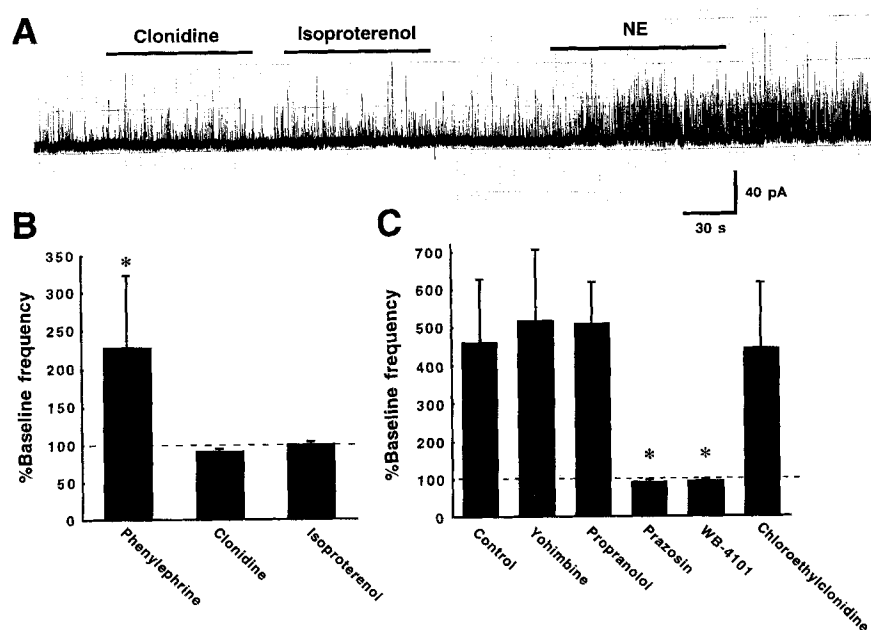
blocked by yohimbine (0.5 μM) (data not shown), indicating that the outward currents were mediated by activation of α_2 receptors. These currents disappeared when diffusion of GDP-β-S was complete (a few minutes after rupturing the patch of membrane; fig. 3A), suggesting that the outward currents were mediated by G-protein-coupled potassium (K$^+$) currents, as reported previously.[27]

Norepinephrine Increases the Frequency of mIPSCs through Activation of α_1 Receptors

In 26 SG neurons, membrane potentials were clamped to −70 mV, and the effect of norepinephrine on the frequency of mEPSCs was evaluated. The baseline frequency of mEPSCs was 20.9 ± 15.2 Hz (n = 26; range, 3.7–64.5 Hz). The frequency of mEPSCs was not significantly affected by norepinephrine (10–100 μM, 92 ± 19% of control for norepinephrine 20 μM; P = 0.08, n = 17, paired t test; figs. 2D and E). In sharp contrast, norepinephrine markedly increased the frequency of mIPSCs (figs. 2D and F). The baseline frequency of mIP-SCs was 2.9 ± 1.5 Hz (n = 59; range, 0.5–6.2 Hz). Norepinephrine (10–100 μM) increased the frequency of IPSCs in 55 of 59 SG neurons tested (459 ± 167% of control for norepinephrine 20 μM; P < 0.0001, n = 18; paired t test; fig. 2F). In 8 of 18 cells in which norepi-

nephrine elicited outward currents at −70 mV, the membrane potentials were also clamped at 0 mV, and the effect of norepinephrine on mIPSCs was evaluated. In all eight cells, norepinephrine elicited an increase in the frequency of mIPSCs. The effect of norepinephrine on mIPSC frequency reached a steady state within 2 min and showed no evidence of desensitization over at least 30 min. After norepinephrine washout, IPSC frequency slowly returned to baseline (recovery time was usually > 5 min).

The effect of norepinephrine was mimicked by the α_1 agonist phenylephrine (10–60 μM; n = 11), but not by the α_2 agonist clonidine (10–40 μM; n = 10) or the β agonist isoproterenol (40 μM; n = 6) (fig. 3B). The α_1-receptor antagonists prazosin (0.5 μM; n = 5) and WB-4101 (0.5 μM; n = 6) reversibly antagonized the norepinephrine effect; the α_{1B} and α_{1D} antagonist chloroethylclonidine (10 μM; n = 6) had no significant effect (fig. 3C). In addition, the α_2- and β-receptor antagonists yohimbine (1 μM; n = 6) and propranolol (1 μM; n = 5), respectively, were without effect (fig. 3C).

Norepinephrine Facilitates Glycinergic and GABAergic mIPSCs

Because mIPSCs consist of glycinergic and GABAergic components, we evaluated which type of mIPSCs were
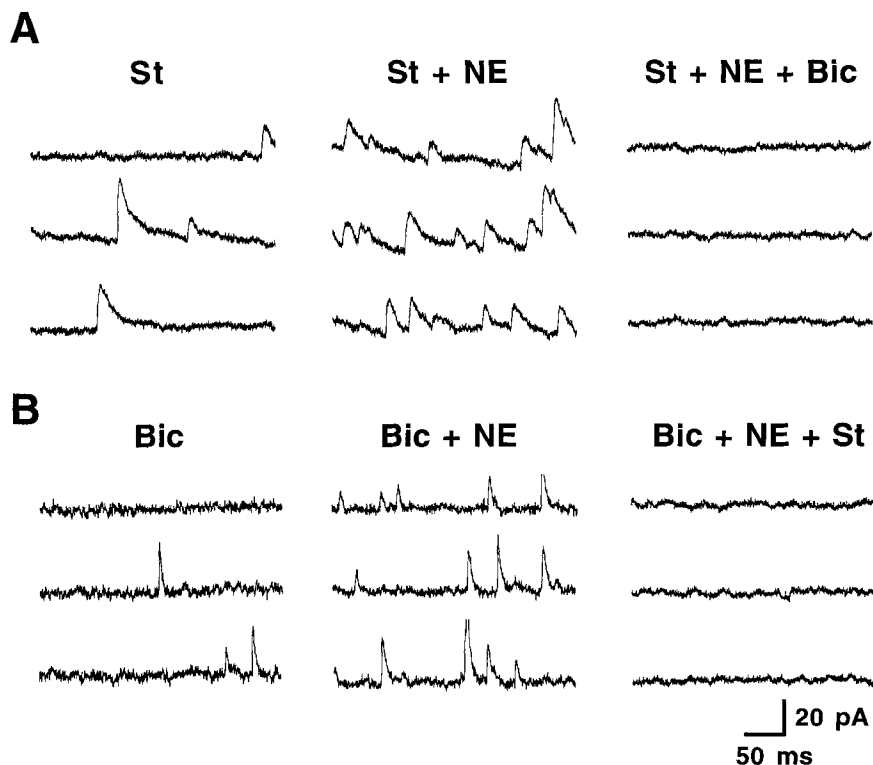
A

St   **St + NE**   **St + NE + Bic**

B

Bic   **Bic + NE**   **Bic + NE + St**

20 pA
50 ms

Fig. 4. Norepinephrine increases the frequency of both GABAergic and glycinergic mIPSCs. (*A*) Norepinephrine increased the frequency of GABAergic mIPSCs. In the presence of strychnine (St; 2 μM), norepinephrine (NE, 60 μM) increased the frequency of remaining mIPSCs. Subsequent application of bicuculline (Bic; 10 μM) completely eliminated the remaining mIPSCs. (*B*) Norepinephrine also increased the frequency of glycinergic mIPSCs. The frequency of mIPSCs recorded in the presence of bicuculline (10 μM) was increased by norepinephrine (60 μM). The facilitated mIPSCs were eliminated by the application of strychnine (2 μM).

facilitated by norepinephrine. In the presence of strychnine (2 μM), norepinephrine increased the remaining mIPSCs (n = 12). These mIPSCs were completely abolished by the simultaneous application of strychnine (2 μM) and bicuculline (10 μM), confirming that facilitated mIPSCs were GABAergic (fig. 4A). Conversely, mIPSCs recorded in the presence of bicuculline were facilitated by norepinephrine (n = 10; fig. 4B). Thus, norepinephrine increased the frequency of both GABAergic and glycinergic mIPSCs.

Facilitation of GABAergic and glycinergic mIPSC frequency by norepinephrine was concentration dependent (fig. 5). Concentration–response curves for GABAergic (n = 7) and glycinergic (n = 8) mIPSCs were well-fitted by logistic equations. The estimated EC_{50} values for the effect of norepinephrine on GABAergic (29.5 ± 12.2 μM) and glycinergic (38.8 ± 37.3 μM) IPSCs were similar, as were the Hill coefficients (GABAergic: 2.8 ± 1.4; glycinergic: 2.8 ± 1.5). Norepinephrine facilitates GABAergic mIPSCs with an approximately twofold greater efficacy than for glycinergic mIPSCs (GABAergic: 1,094 ± 342%; glycinergic: 541 ± 403%).

Amplitude histograms were constructed for each set of GABAergic and glycinergic mIPSCs. In all cells tested, amplitude distribution analyses showed a slight but statistically significant norepinephrine-induced increase in the median amplitude of GABAergic (n = 7) and glycinergic (n = 5) mIPSCs (fig. 6). However, the peak amplitude of both types of mIPSCs was not increased by norepinephrine. The effect of norepinephrine was particularly prominent between 10 and 15 pA for GABAergic and glycinergic mIPSCs. This change in the amplitude distribution is clearly shown in the cumulative histogram (figs. 6B and D). In the presence of norepinephrine, the relative frequency curves were significantly shifted to the right in all cells tested.

Norepinephrine Does Not Affect Postsynaptic Responsiveness to GABA and Glycine

The norepinephrine-induced changes in amplitude distributions might be attributable to an increase in postsynaptic sensitivity to GABA and glycine under conditions in which GDP-β-S diffusion was incomplete. Alternatively, an unknown mechanism not mediated by G protein might mediate a postsynaptic effect of norepinephrine. To rule out these possibilities, we evaluated whether norepinephrine affected postsynaptic sensitivity to exogenously applied GABA and glycine under our recording conditions (*i.e.*, with an internal solution con-
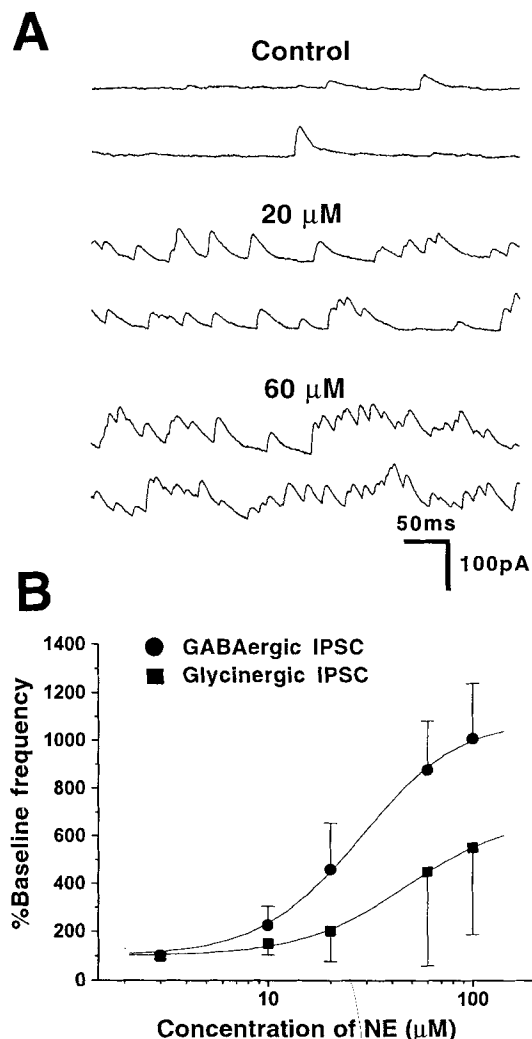
A

Control

20 μM

60 μM

50ms

100pA

B

● GABAergic IPSC
■ Glycinergic IPSC

Concentration of NE (μM)

%Baseline frequency

Fig. 5. Norepinephrine increased the frequency of mIPSCs in a concentration-dependent manner. (*A*) An example of GABAergic mIPSCs and the effects of increasing concentrations of norepinephrine. (*B*) The concentration–response curves of GABAergic (n = 7) and glycinergic (n = 8) mIPSCs. Concentration–response data were fit by logistic equations. GABAergic and glycinergic curves had similar EC_{50} values (GABAergic: 29.5 ± 12.2 μM; glycinergic: 38.8 ± 37.3 μM) and Hill coefficients (GABAergic: 2.8 ± 1.4; glycinergic: 2.8 ± 1.5). The efficacy of the norepinephrine effect was greater for GABAergic (1,094 ± 342%) than glycinergic (541 ± 403%) mIPSCs. $P < 0.05$, unpaired *t* test.

taining GDP-β-S). Bath-applied GABA and glycine elicited outward currents at a holding potential of 0 mV (figs. 7A and B). Although the frequency of GABAergic or glycinergic mIPSCs was increased by norepinephrine, the amplitude of GABA- or glycine-evoked (n = 6 for both) outward currents was not significantly affected by norepinephrine (fig. 7C). We also tested whether norepi-

nephrine affected the kinetics of mIPSCs. The kinetics of GABAergic (n = 3) and glycinergic (n = 3) mIPSCs before and during norepinephrine application were identical, as shown by the superimposed averaged records (fig. 7D). Therefore, norepinephrine does not appear to alter the postsynaptic responsiveness of SG neurons to GABA and glycine.

Discussion

Increase in the Frequency of mIPSCs

We showed that norepinephrine increases the frequency of mIPSCs in the majority (> 90%) of SG neurons tested, without any significant effect on postsynaptic responsiveness. In contrast, norepinephrine did not change or slightly decreased the frequency of mEPSCs. These data indicate that norepinephrine selectively facilitates the quantal release of inhibitory transmitters from presynaptic terminals of inhibitory interneurons. This study shows that norepinephrine increases the frequency of both GABAergic and glycinergic mIPSCs. This may not be surprising in light of the observation that GABA and glycine can be colocalized, at least in a subpopulation of dorsal horn neurons, and cotransmission of GABA and glycine can occur.[28] However, norepinephrine facilitated GABAergic mIPSCs with greater efficacy than glycinergic mIPSCs. The reason for this difference has yet to be determined, but it may result from a differential distribution of α_1 receptors on GABAergic and glycinergic terminals.

The facilitatory effect of norepinephrine was mediated *via* adrenergic α_1 receptors, because phenylephrine (an α_1-receptor agonist) mimicked norepinephrine, and prazosin and WB-4101 (α_1-receptor antagonists) inhibited the norepinephrine effect. Thus, GABA- and glycine-containing neurons are endowed with α_1 receptors on the axon terminals, and activation of these receptors enhances GABAergic and glycinergic inhibitory transmission in the SG. Currently, at least three native α_1-adrenergic receptor subtypes (α_{1A}, α_{1B}, and α_{1D}) have been identified pharmacologically (α_{1C} is lacking).[29] Both α_{1B} and α_{1D} receptors are characterized by a high sensitivity to chloroethylclonidine. The action of norepinephrine on mIPSC frequency was highly sensitive to WB-4101 (an α_{1A}-receptor antagonist), but resistant to a relatively high concentration (10 μM) of chloroethylclonidine (an α_{1B}- and α_{1D}-receptor antagonist). Therefore, the most likely α_1-adrenergic receptor responsible for norepinephrine action is the α_{1A} subtype.
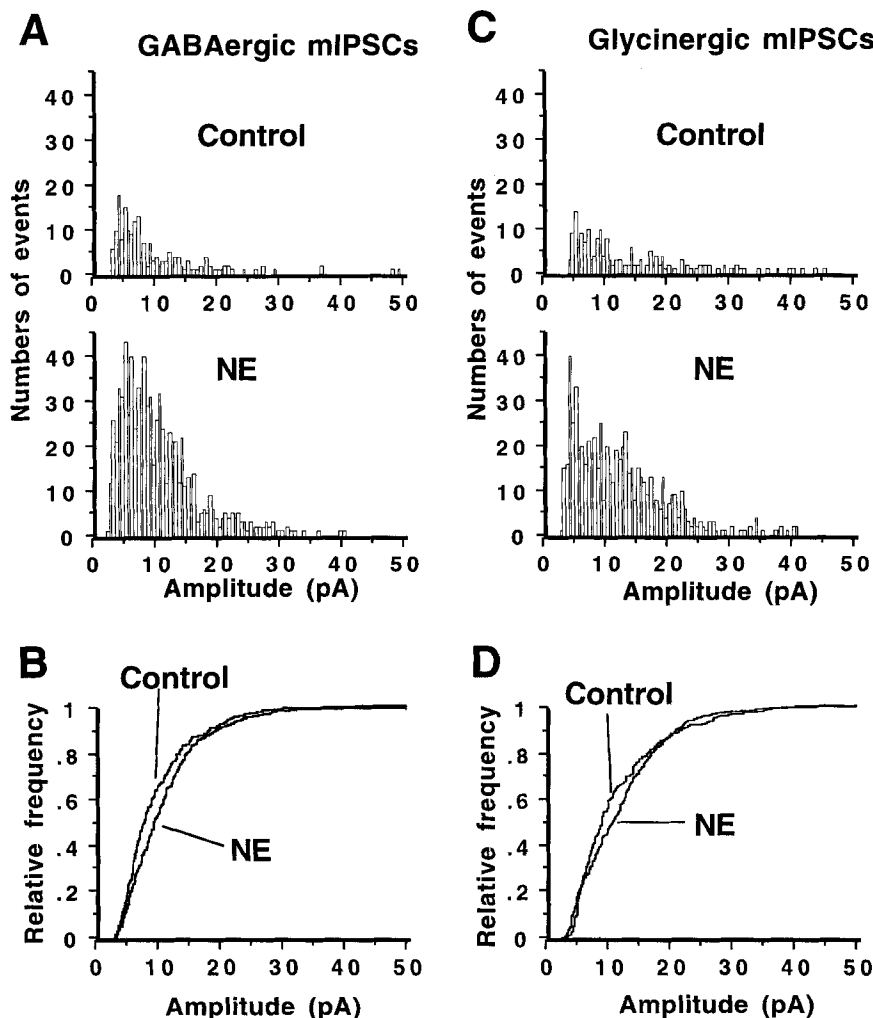
Fig. 6. The effect of norepinephrine on the amplitude distributions of mIPSCs. Amplitude histograms of GABAergic (*A*) and glycinergic (*C*) mIPSCs before and during application of norepinephrine (NE). Each histogram was constructed from continuous recording for 120 s. (*A*) There were 183 and 761 events analyzed before and during application of norepinephrine (10 μM). Norepinephrine increased the frequency from 1.53 Hz to 6.34 Hz. Median amplitudes before and during norepinephrine application were 7.4 pA and 9.0 pA, respectively. (*C*) There were 178 and 645 events analyzed before and during application of norepinephrine (60 μM). Norepinephrine increased the frequency from 1.48 to 5.38 Hz. Median amplitudes before and during norepinephrine application were 8.9 and 10.8 pA, respectively. (*B*, *D*) Cumulative histograms of GABAergic (*B*) and glycinergic (*D*) mIPSCs. Norepinephrine significantly shifted the curves to the right for both GABAergic and glycinergic mIPSCs (Kolmogorov–Smirnov test, $P < 0.005$ for *B*, $P < 0.01$ for *D*).

The norepinephrine-induced increase in mIPSC frequency could be secondary to changes in the postsynaptic neuron. If norepinephrine produces an increase in the sensitivity of postsynaptic GABA$_A$ and glycine receptors, it could potentiate a population of subliminal mIPSCs, such that they would become visible, yielding an apparent increase in mIPSC frequency. Furthermore, if norepinephrine results in the recruitment of "silent" inhibitory synapses in a manner analogous to that described for excitatory synapses,[23,30,31] the apparent mIPSC frequency would increase. It is unlikely that these types of postsynaptic changes mediate the facilitatory effects of norepinephrine because all known adrenergic receptors are coupled to ion channels *via* G proteins, and G proteins were inhibited in postsynaptic (recorded) neurons by GDP-β-S. In addition, the amplitude of outward currents elicited by exogenously applied GABA

and glycine was not altered by norepinephrine, nor were kinetics of mIPSCs. Together, these observations indicate that norepinephrine does not alter postsynaptic responsiveness to GABA and glycine. Therefore, it is surprising that the amplitude-distribution analysis showed a significant norepinephrine-induced increase in the median amplitude of GABAergic and glycinergic mIPSCs and a shift in the cumulative histogram curves to the right. This most likely results, however, from a relatively selective increase in a subpopulation of mIPSCs with amplitudes between 10 and 15 pA (figs. 6A and C). SG neurons receive numerous types of inhibitory inputs from interneurons in the SG and the surrounding laminae (laminae I, III, IV, and so forth).[32] The effect of norepinephrine may be selective for a subpopulation of these inputs. If norepinephrine facilitates quantal release, especially in a population of inhibitory synapses
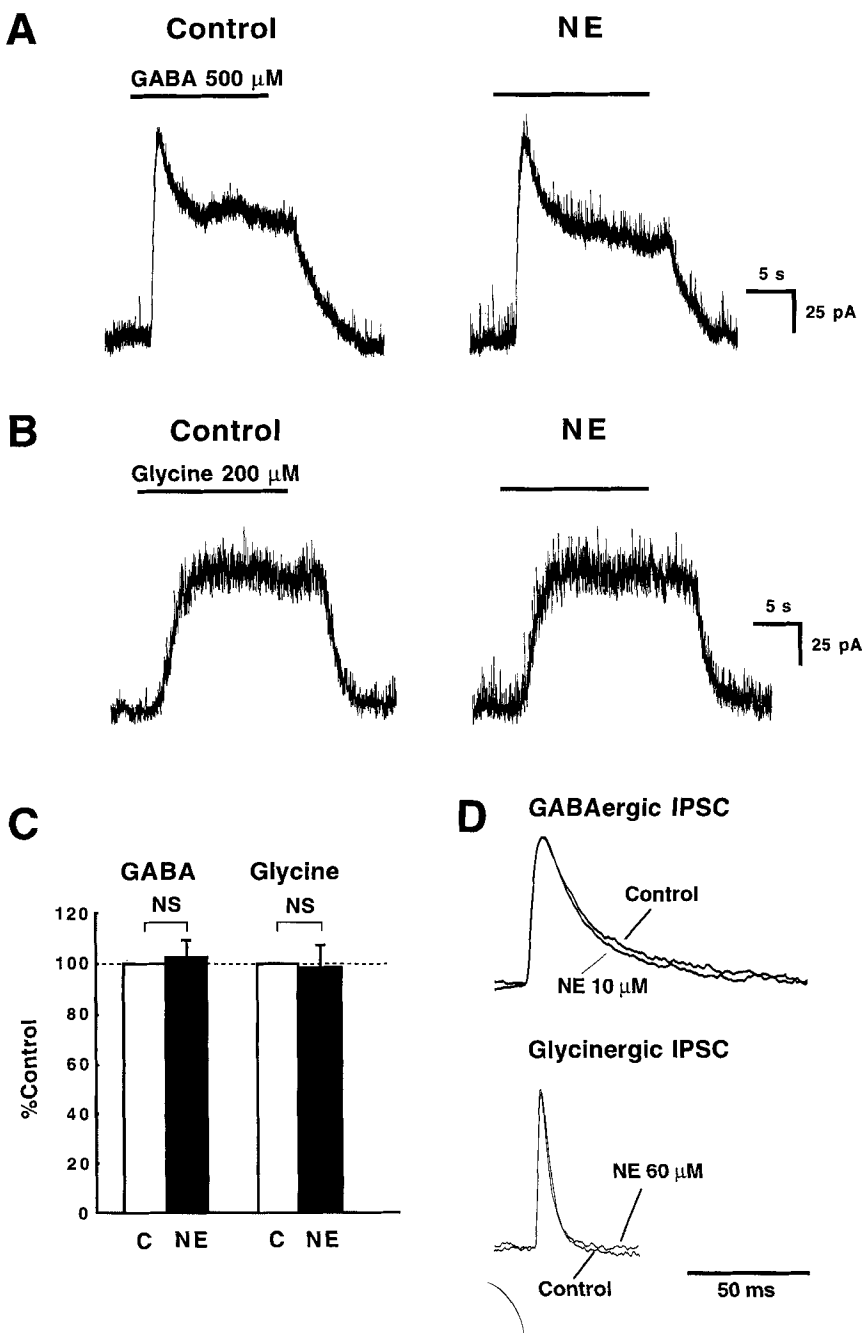
A Control   NE

GABA 500 μM

5 s

25 pA

Fig. 7. Norepinephrine does not affect postsynaptic responsiveness to GABA and glycine. (A, B) Exogenously applied GABA (500 μM) and glycine (200 μM) elicited outward currents. The peak amplitude of the currents evoked by GABA or glycine were virtually identical before and during application of norepinephrine (NE, 20 μM). (C) Norepinephrine did not significantly affect the peak outward currents elicited by GABA (500 μM, n = 6) or glycine (200 μM, n = 6). Open bars and solid bars represent control (C) and norepinephrine (20 μM), respectively. (D) The kinetics of GABAergic (top) and glycinergic (bottom) mIPSCs before and during norepinephrine application were nearly identical, as shown by superimposed records. mIPSCs from each group were averaged and scaled to equal amplitudes. mIPSCs were averaged from the same cells as shown in figure 6.

B Control   NE

Glycine 200 μM

5 s

25 pA

C

GABA   Glycine

NS   NS

120

100

80

%Control

60

40

20

0

C NE   C NE

D GABAergic IPSC

Control

NE 10 μM

Glycinergic IPSC

NE 60 μM

Control   50 ms

that generate mIPSCs with an amplitude of 10–15 pA, the amplitude distribution could be skewed. Although norepinephrine facilitates inhibitory transmitter release via presynaptic α_1 receptors, it may not influence all presynaptic terminals of inhibitory interneurons in the SG in a homogeneous fashion. Alternatively, temporal summation of mIPSCs might result in an increase in the median amplitude of mIPSCs. We measured the ampli-

tude of mIPSCs from the initial inflection point (not from the baseline) to avoid effects of temporal summation on the amplitude distribution. However, in cases in which two (or more) quantal events occur at exactly the same moment in time, they cannot be distinguished; therefore, they may have been measured as a single mIPSC. This type of error is more likely to occur during conditions in which the frequency of mIPSCs is increased.

Nonetheless, we believe this to be rare because carbachol, a muscarinic agonist, also increased the frequency of mIPSCs but did not affect the amplitude distribution.[26]

Functional Consideration

Almost all norepinephrine-containing terminals in the dorsal horn of the spinal cord are supraspinal in origin.[33] Currently, our knowledge of how these descending pain-control pathways inhibit nociceptive transmission at the spinal level is not defined clearly, but there are several possible mechanisms. First, activation of noradrenergic descending systems releases norepinephrine, which can directly hyperpolarize a proportion of SG neurons that may be excitatory interneurons in the pain pathway (postsynaptic inhibition).[13] Second, norepinephrine can inhibit excitatory transmitter (glutamate, substance P, calcitonin gene-related peptide [CGRP], and so forth) release from primary afferent terminals or presynaptic terminals of excitatory interneurons.[34,35] However, our data do not support this type of mechanism in the SG. Third, norepinephrine could depolarize inhibitory interneurons that contain GABA, glycine, or other inhibitory peptides. Iontophoretic application of norepinephrine near nociceptive dorsal horn neurons generally inhibits background activity of these cells and the responsiveness to excitatory amino acids.[36-38] This inhibition most likely results from α_2-receptor activation, which increases K^+ conductance, thereby evoking a membrane hyperpolarization.[13] However, norepinephrine (and brain stem stimulation) also has been reported to produce excitatory effects.[38-40] The neurons excited by iontophoretically applied norepinephrine and electrical stimulation of the periaqueductal gray were low-threshold cells, possibly inhibitory interneurons that synapse onto high-threshold and wide-dynamic-range neurons.[40] In the accompanying article, we directly address whether norepinephrine depolarizes inhibitory interneurons that synapse onto SG neurons.

Here, we describe a distinct type of mechanism: norepinephrine acts at presynaptic axon terminals of GABAergic and glycinergic interneurons to facilitate inhibitory transmitter release. We also demonstrated that some SG neurons display both an α_2-receptor-mediated outward current and an α_1-receptor-mediated increase in mIPSC frequency (fig. 2D). Because 80% of SG neurons are hyperpolarized (or display an outward current) *via* α_2-receptor activation,[13] and more than 90% show an increase in mIPSC frequency (current study), it is likely that the excitability of most SG neurons is inhibited by

exogeneously applied norepinephrine (and probably by epinephrine) through α_2- and α_1-receptor-mediated mechanisms. Finally, it should be noted that norepinephrine may change postsynaptic sensitivity to GABA and glycine. In the current study, we blocked postsynaptic effects of norepinephrine using GDP-β-S to evaluate the presynaptic action. However, in intact SG neurons, norepinephrine may increase postsynaptic responsiveness to inhibitory neurotransmitters by G protein-coupled mechanisms.[41]

γ-Aminobutyric acid and glycine have been shown to be present in cell bodies and terminals in the superficial dorsal horn[42] and are thought to be involved in spinal antinociception.[14,16,17] Electrical stimulation of primary afferent fibers evokes mono- and polysynaptic excitatory postsynaptic potentials (EPSPs) that are then augmented in both amplitude and duration by perfusion with $GABA_A$ and glycine receptor antagonists.[16,17] In addition, intrathecal administration of $GABA_A$ agonists or glycine is antinociceptive,[43,44] and antagonists of $GABA_A$ and glycine receptors produce allodynia and hyperalgesia in animals.[45,46] These observations suggest that GABA- and glycine-containing

interneurons are responsible for discontinuing evoked excitatory responses. The facilitatory effect of norepinephrine on inhibitory interneurons may decrease excitability of dorsal horn neurons, which in turn would increase the threshold for transmission of noxious information.

As reviewed by Willis and Coggsehall,[32] SG neurons receive substantial primary afferent input from nociceptive Aδ and C fibers (these fibers can, however, also form synapses directly onto projection neurons). In addition to nociceptive inputs, the SG receives descending adrenergic input from a number of pontine nuclei.[47] With respect to output from this region, the majority of SG neurons are local interneurons and do not project to the thalamus,[32] although there are a few exceptions.[48] The main projections of SG neurons are to lamina I and to deep dorsal horn neurons, with cell bodies in laminae IV and V, where the projection neurons to the thalamus are located.[32] Unfortunately, in the current study, the phenotype of the SG neurons from which we recorded is unknown; some SG neurons might produce excitatory, and others inhibitory, effects on projection neurons in laminae IV and V. In the companion article, we address whether α_1-receptor activation inhibits transmission of nociceptive inputs to deep dorsal horn neurons.

Although the vasoconstrictive effects of epinephrine and phenylephrine in the spinal cord have not been

established, these drugs are sometimes added to local anesthetics in spinal anesthesia to prolong the duration of analgesia.[5,6] It has been reported that subarachnoid epinephrine and phenylephrine do not affect spinal cord blood flow.[49,50] Another study suggested that at a relatively high dose, phenylephrine decreased blood flow.[51] Furthermore, it has been reported that epinephrine and phenylephrine do not significantly affect the clearance of local anesthetics from the subarachnoid space.[9,10] Thus, it remains controversial whether the vasoconstrictive actions of epinephrine or phenylephrine contribute to the prolongation of analgesia in spinal anesthesia. The mechanism of epinephrine action in spinal anesthesia can be accounted for, at least in part, by activation of α_2 receptors.[7,8,11] As discussed previously, exogenously applied epinephrine (a mixed α_1 and α_2 agonist) can decrease the excitability of SG neurons *via* α_1 and α_2 receptors. Interestingly, phenylephrine (a pure α_1 agonist) can also enhance spinal anesthesia. Considering the evidence that phenylephrine does not affect the clearance of local anesthetics, the action of phenylephrine in spinal anesthesia must be caused by a direct action on spinal neurons *via* α_1 receptors. The presynaptic α_1 action reported here may account for the prolongation of analgesia by α_1 agonists in spinal anesthesia.

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