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Materials and Methods

Slice preparation and electrophysiological recordings

Transverse slices (250 μm thick) of the lumbar spinal cord were prepared from 14- to 21-day-old Sprague-Dawley rats as described previously (*1*). Whole-cell patch-clamp recordings were performed from neurons identified under visual control by using the infrared gradient contrast technique coupled to a video microscopy system. Slices were continuously superfused with external solution, which contained (in mM) 125 NaCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, and 10 glucose (pH 7.30, 315 mosmol/l) and was bubbled with 95% O_2, 5% CO_2. Patch pipettes (4to 5 MΩ) were filled with internal solution containing (in mM) 130 K-gluconate, 20 KCl, 2 $MgCl_2$, 0.05 EGTA, 3 Na-ATP, 0.1 Na-GTP, and 10 Na-Hepes (pH 7.30). QX-314 (5 mM) was added to the internal solution to block voltage-activated sodium currents. Postsynaptic current responses were elicited at a frequency of 1/15 s by extracellular electrical stimulation (100 μs, 3 to 10 V) by using a glass electrode placed about 50 μm from the recorded neuron. Unless otherwise stated, all recordings were made at a near physiological temperature of 35°C except for those shown in Fig. 1A, which were recorded at room temperature (20°C). EPSCs and IPSCs were isolated using appropriate combinations of NBQX (10 μM), APV (50 μM), bicuculline (10 μM), and strychnine (0.3 μM). The holding potential was –80 mV, unless otherwise stated. NMDA-EPSCs were recorded at –30 mV to partially remove the Mg^{2+} block. Short hyperpolarizing voltage steps to –90 mV were applied every minute to monitor input and access resistance. Drug-containing solutions were applied by bath perfusion at a rate of 1 to 2 ml/min. To study membrane currents elicited by exogenous glycine or NMDA, both transmitters (at a concentration of 100 μM) were applied from glass pipettes to the soma of the recorded neuron by short (10-ms) electronically controlled pressure pulses. Percent inhibition of current responses was determined from the average amplitude of 10 consecutive currents evoked immediately before application of the NST and when a steady state of inhibition was reached, usually about 3 min after application.

Behavioral testing

The pro- and/or antinociceptive effects of NST were analyzed in the rat formalin test (*2*) as described previously (*3*). Briefly, experiments were performed at room temperature. Sprague-Dawley rats weighing 350 to 400 g were anesthetized with ketamine (100 mg/kg i.p.) and xylazine (5 mg/kg i.p.) and implanted with polyethylene catheters (i.d. 0.28 mm, o.d. 0.61 mm), which were extended from the cisterna to the rostral edge of the lumbar enlargement. Only rats with unimpaired motor function were used. Formalin tests were performed 6 to 10 days after implantation. Rats were randomly assigned to the different treatment groups consisting of at least 6 rats each. NST or vehicle (ACSF) were delivered to the spinal cord through the catheters in a total volume of 10 μl. In the formalin tests, formalin (50 μl, 5%) was injected subcutaneously into the dorsal surface of the left hind paw 10 min after intrathecal injection of NST or vehicle. Flinches of the injected paw were counted at 1-min intervals for 60 min starting 10 min after intrathecal injection. Motor performance was tested in the rotarod test (*4*). Rats were trained on two consecutive days, and the maximum speed at which the rats could stay on the apparatus for more than 2 min was determined. On the experimental day the test was performed 10 min after intrathecal injection. After the tests, rats were killed by CO_2 inhalation, and proper position of the catheter tip was visually verified after laminectomy and methylene blue injection. All

behavioral tests and the killing of the animals were performed in accordance with the institutional guidelines of the University of Erlangen-Nürnberg and of the Society for Neuroscience.

Biocytin labeling

To document the location and their gross morphology, neurons were filled during whole-cell recording (≥15 min) with standard internal solution containing biocytin (5 mg/ml). After removal of the pipette slices were kept in the recording chamber for an other 30 min to allow diffusion and transport of biocytin into fine neuronal processes (*5*). Slices were then cryoprotected in buffered sucrose and serially cryostat sectioned at 40 µm. Biocytin was detected by incubating the sections on slide in a 1:1000 solution of Cy3-tagged streptavidin in tris-buffered saline (TBS) at pH 7.4 for 2 hours at room temperature. After mounting on coverslips in glycerol-containing TBS, we performed confocal laser scanning microscopy. Images were generated by superposing 3 to 5 confocal optical sections taken at *z* intervals of 1 µm in the extended focus mode.

Peptides and chemicals

The rat homolog of NST (rat prepronociceptin/orphanin FQ 98–132 with the following sequence H-MPRVRSVVQARDAEPEADAEPVADEADEV EQKQLQ-OH) was obtained from Phoenix Pharmaceuticals and from R. Frank, ZMBH, Heidelberg, Germany (>95% purity). NST was dissolved in external recording solution and stored in aliquots (1 mM) at –20°C. Fresh dilutions were made with standard external solution on every experimental day.

Supporting Figures

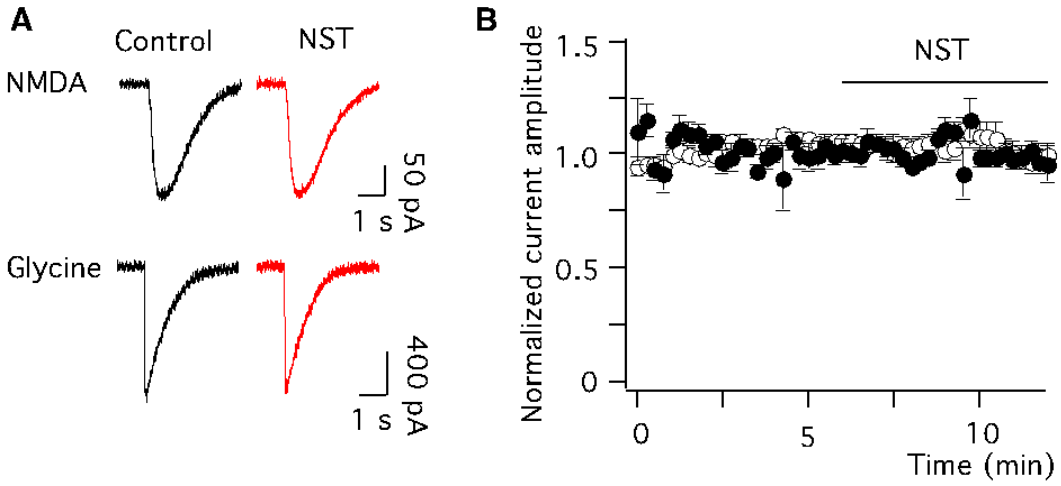

Fig. S1. NST does not directly interfere with NMDA or glycine receptors. NST (10 µM) does not affect membrane currents elicited by exogenous application NMDA (○) or glycine (●) (100 µM, each). (A) Individual current responses. (B) Time course of the experiments. $n = 10$ (NMDA), $n = 6$ (glycine).

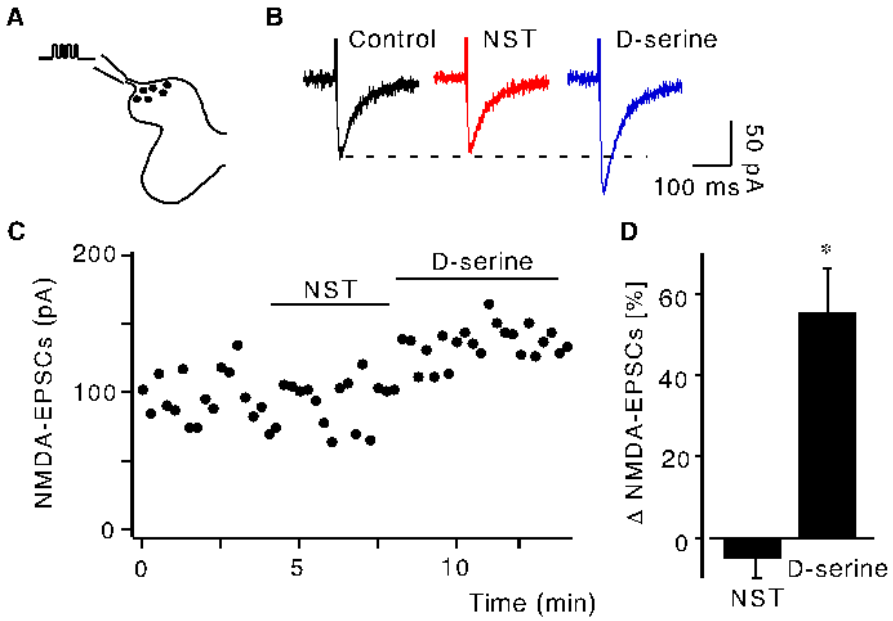

Fig. S2. Effects of NST (10 μM) and D-serine (100 μM) on NMDA-EPSCs evoked by activation of a pure glutamatergic pathway. (**A**) Short dorsal rootlets were stimulated by four extracellular electrical stimuli (at 500 Hz) and at 35°C. (**B**) Averaged current traces. (**C**) Time course of an individual experiment, where NST and D-serine were applied to the same cell consecutively. (**D**) Statistical analysis. *$P < 0.05$. NST was without effect ($P = 0.36$, $n = 5$ and 6, for NST and D–serine, respectively).

Supporting References

S1. S. Ahmadi, S. Lippross, W. L. Neuhuber, H. U. Zeilhofer, *Nature Neurosci.* **5**, 34 (2002).
S2. D. Dubuisson, S. G. Dennis, *Pain* **4**, 161 (1977).
S3. H. U. Zeilhofer, U. Muth-Selbach, H. Gühring, K. Erb, S. Ahmadi, *J. Neurosci*. **20**, 4922 (2000).
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S5. K. Ceranik *et al., J. Neurosci.* **17**, 5380 (1997).