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The Spinal Antinociceptive Effect of Nocistatin in Neuropathic Rats Is Blocked by D-Serine

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Background: **The neuropeptide nocistatin (NST) has been implicated in the modulation of nociceptive responses in the spinal cord. Depending on the dose, both pronociceptive and antinociceptive effects have repeatedly been reported. The pronociceptive effect is most likely attributable to inhibition of synaptic glycine and γ-aminobutyric acid release and a subsequent reduction in the activation of inhibitory glycine and γ-aminobutyric acid receptors, but the mechanisms of its antinociceptive action have hitherto remained elusive. It has recently been demonstrated that synaptically released glycine contributes to *N*-methyl-D-aspartate receptor activation. The authors therefore investigated whether a reduction in glycine release might also account for the antinociceptive action of NST in neuropathic rats.**

Methods: **The authors analyzed the effects of spinally applied NST in the chronic constriction injury model of neuropathic pain. NST was injected intrathecally from nanomolar to picomolar doses and its effects on thermal paw withdrawal latencies were monitored. Furthermore, we tested whether D-serine (100 μg per rat), a full agonist at the glycine binding site of the *N*-methyl-D-aspartate receptor, would interfere with the effects of NST.**

Results: **At high doses (10 nmol/rat), intrathecally injected NST was pronociceptive, whereas lower doses (1 pmol/rat) elicited antinociception. The antinociceptive, but not the pronociceptive, action was occluded by intrathecal pretreatment with D-serine. L-serine, which does not bind to *N*-methyl-D-aspartate receptors, affected neither the pronociceptive nor the antinociceptive effect.**

Conclusions: **These results demonstrate that NST produces a biphasic dose-dependent effect on neuropathic pain. The spinal antinociception by NST is most likely attributable to inhibition of glycine-dependent *N*-methyl-D-aspartate receptor activation.**

THE neuropeptide nocistatin (NST) has originally been described as a functional antagonist of nociceptin induced hyperalgesia and allodynia.[1–4] Meanwhile several lines of evidence indicate that NST is a biologically active peptide *per se*.[5,6] In the spinal cord, NST inhibits the release of the two major fast inhibitory neurotransmitters glycine and γ-aminobutyric acid but does not interfere with the release of the excitatory neurotransmitter L-glutamate.[7] This action is restricted to the dorsal horn, the site of spinal sensory processing. It is absent in the ventral horn, the site of spinal motor control.[8]

NST can modulate nociception in opposite directions after intrathecal application. High nanomolar doses facilitate, whereas significantly lower doses inhibit, nociceptive responses in the formalin test.[5,6] It appears reasonable to assume that the pronociceptive effects of NST originate from a reduction in the synaptic release of glycine (and γ-aminobutyric acid) and a subsequent reduction in the activation of inhibitory strychnine-sensitive glycine (and γ-aminobutyric acid $_A$) receptors.

During recent years increasing evidence has accumulated indicating that glycine not only serves as the primary inhibitory neurotransmitter in the spinal cord and brain stem but also may contribute to neuronal excitation.[8–10] To fully activate *N*-methyl-D-aspartate (NMDA) receptors, the binding of glycine or D-serine at the glycine binding site of the NMDA-receptor is needed.[11–13] The affinity of glycine to NMDA receptors is even two to three orders of magnitude higher than that of glycine to strychnine-sensitive glycine receptors (100–300 nM *versus* 50 μM).[14,15] We recently suggested that glycine released from inhibitory interneurons may thus facilitate the activation of excitatory glutamate receptors of the NMDA subtype through a process called spillover.[8] In the present study, we have addressed the question of whether a reduction in glycine release and a subsequent inhibition of NMDA receptor activation may underlie the antinociceptive action of NST in neuropathic pain.

To address this question we have employed the chronic constriction injury model of neuropathic pain.[16] We show that NST dose-dependently exerts antinociceptive and pronociceptive effects in neuropathic rats. We further demonstrate that the antinociceptive effect is selectively occluded after pretreatment with spinal D-serine, which can fully substitute for the binding of glycine to NMDA receptors.

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Materials and Methods

All behavioral tests were performed with permission of the local government (Bezirksregierung Düsseldorf, Düsseldorf, Germany) and were in accordance with the guidelines of the International Association for the Study of Pain.

The pronociceptive or antinociceptive effects of NST were analyzed in the chronic constriction injury (CCI) model.[16] Wistar rats weighing 350–400 g were anesthetized with intraperitoneal injection of pentobarbital (60 mg/kg). Unilateral constriction injury of the left sciatic nerve just proximal to the trifurcation was performed with four loose ligatures as previously described.[16] In sham-operated animals the nerve was exposed, the connective tissue was freed, and no ligatures were applied. In addition, the rats were implanted with polyethylene catheters (inner diameter, 0.28 mm; outer diameter, 0.61 mm) that were advanced from the cisterna magna to the rostral edge of the lumbar enlargement. Rats with impaired motor function after implantation of the catheter were excluded from the study. Heat hyperalgesia was tested 6–10 days after surgery in an air-conditioned room. The lateral plantar surface of both hind paws was exposed to a defined radiant heat stimulus through a transparent Perspex surface (Ugo Basile, Comerio VA, Italy) and paw withdrawal latencies were recorded.[17] A cutoff time of 20 s was set to avoid tissue damage. Paw withdrawal latencies of both hind paws were determined from three independent measurements for each time point. Only rats that exhibited significantly lowered thermal withdrawal latencies after CCI were included in the study.

Behavioral testing as described above was performed once before CCI, once immediately before application of drugs, and seven times after application of drugs at regular intervals of 5 min. NST, D-serine, L-serine, or vehicle (0.9% NaCl) was applied to the spinal cord *via* intrathecal catheters in a total volume of 10 μl. Rats were randomly assigned to the different treatment groups consisting of seven to 10 rats each. All behavioral observations were performed in a blinded fashion.

After the behavioral tests, rats were sacrificed by a lethal intraperitoneal dose of pentobarbital. Proper position of the catheter tip was verified after laminectomy and methylene blue injection through the catheter.

Peptides and Chemicals

rNST1–17 (rat prepronN/OFQ 116–132) was obtained from Research Genetics (Huntsville, AL). D-serine and L-serine were purchased from Sigma (Deisenhofen, Germany). All chemicals were dissolved in 0.9% NaCl and stored in aliquots at −20°C. Fresh dilutions were made with 0.9% NaCl on every experimental day.

Statistical Analysis

Group data are presented as mean ± SE (SEM). Withdrawal latencies in the different treatment groups were analyzed by repeated measures analysis of variance followed by Bonferroni *post hoc* tests. $P \leq 0.05$ was assumed significant. In detail, in the experiments assessing the effect of the different doses of NST, each of the five doses used was compared with saline at seven time points (5 to 35 min). In the experiments comparing low and high dose NST with and without D-serine (or L-serine) four comparisons were made at seven time points.

Results

Ninety percent of rats with loose ligature of the left sciatic nerve developed thermal hyperalgesia with significantly lowered thermal paw withdrawal latencies on the left side. No statistically significant changes in thermal withdrawal latencies occurred in the right (uninjured) paw or in sham-operated rats (figs. 1 and 2).

When injected intrathecally NST dose-dependently exerted pronociceptive or antinociceptive effects in the CCI model. Low doses were antinociceptive whereas higher doses elicited a profound pronociceptive effect (fig. 1). Five minutes after injection of 1 pmol NST paw withdrawal latencies in the left (injured) paw were 7.7 ± 0.3 s, compared with 5.3 ± 0.2 s after vehicle injection ($P \leq 0.05$, n = 7 and 8). At a dose of 10 nmol, NST decreased paw withdrawal latencies from 5.1 ± 0.1 s to 3.7 ± 0.3 s at 10 min after injection ($P \leq 0.05$, n = 8 each) (fig. 1). Within 25 min after administration of NST thermal withdrawal latencies had returned to preinjection values (fig. 1). NST affected thermal paw withdrawal latencies not only in the injured (left) paw but also in the right (uninjured) paw (fig. 1) (*i.e.*, 5 min after injection of 1 pmol NST *versus* control: 13.4 ± 1.1 *versus* 9.4 ± 0.7; 10 min after injection of 10 nmol NST *versus* control: 6.6 ± 0.3 *versus* 9.4 ± 0.7; $P \leq 0.05$, n = 7 and 8). In contrast, in sham-operated rats neither 10 nmol nor 1 pmol NST changed thermal withdrawal latencies (fig. 2).

In CCI rats, pretreatment with D-serine (100 μg per rat) prevented the antinociceptive action evoked by low doses of NST in both the left (injured) paw and in the right (uninjured) paw. For example, paw withdrawal latencies (1 pmol NST plus 100 μg D-serine *versus* 1 pmol NST) were 5.2 ± 0.3 s *versus* 7.8 ± 0.2 s in the left (injured) paw and 7.4 ± 0.5 s *versus* 13.6 ± 0.6 s in the right paw ($P \leq 0.05$, n = 10 each) 5 min after injection of NST (fig. 3). In contrast, pretreatment with L-serine (100 μg per rat), which does not bind to NMDA receptors, had no effect on the antinociception induced by low-dose NST (*e.g.*, 7.6 ± 0.3 s *versus* 7.8 ± 0.2 s, paw withdrawal latencies in the left injured paw and 12.9 ± 0.7 s *versus* 13.6 ± 0.6 s in the right uninjured paw, not significant, n = 10 each, again 5 min after injection of NST; fig. 3).

Neither D-serine nor L-serine *per se* had any significant effect on the duration of the thermal withdrawal latencies in the CCI model compared with vehicle-

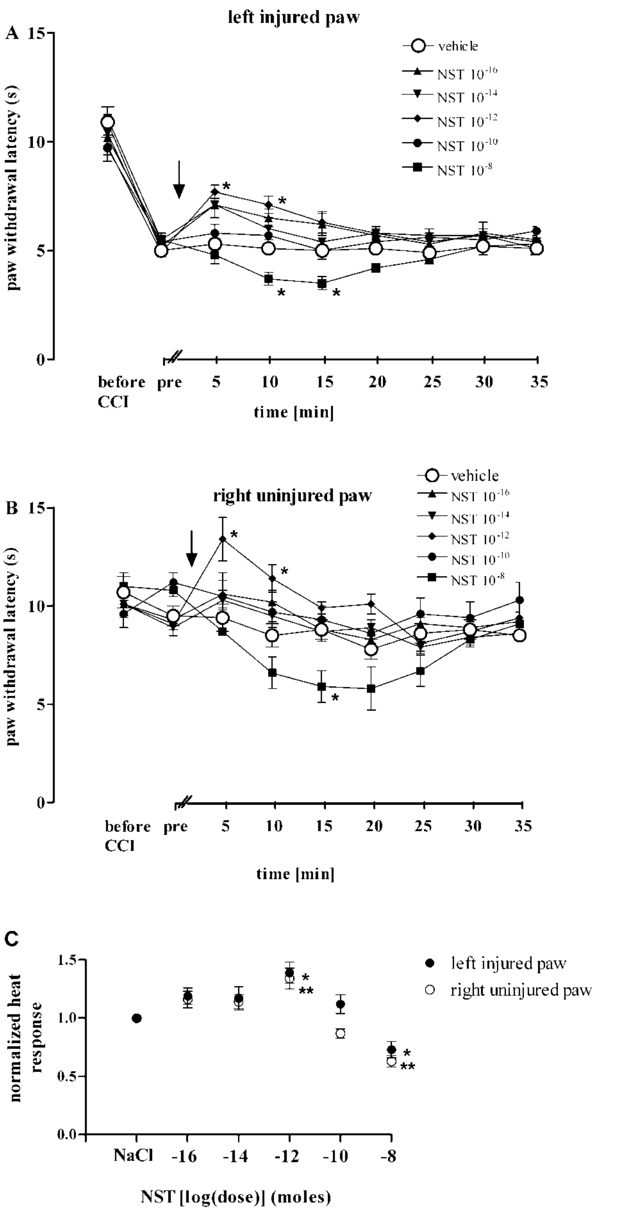

Fig. 1. Effects of nocistatin (NST) on the nociception in the chronic constriction injury model. (*A* and *B*) Dose-dependent effects of rNST1–17 on pain-related behavior in neuropathic rats (chronic constriction injury). Thermal withdrawal latencies (mean ± SEM) (in s) of rats treated with different doses of rNST1–17 injected intrathecally *versus* time. 10^{-8}, 10^{-10}, 10^{-12}, 10^{-14}, 10^{-16} M/rat, and vehicle. Arrow indicates time of rNST1–17 injection. (*C*) Dose-response relationship for rNST1–17 for the left, injured, paw (●) and the right, uninjured, paw (○) 10 min after intrathecal injection of rNST1–17. Values normalized to vehicle treated animals. *$P \leq 0.05$, **$P \leq 0.01$ *versus* vehicle-treated animals, n = 7 to 8 rats per group.

treated animals (*e.g.*, paw withdrawal latencies 5 min after drug injection in D-serine treated animals were 5.2 ± 0.3 s and in L-serine treated animals 5.3 ± 0.4 s *versus* 5.3 ± 0.2 s after vehicle injection in the left injured paw and 10 ± 0.3 s/9.8 ± 0.4 s *versus* 9.4 ± 0.7 s in the right uninjured paw; n = 8–10, not significant; fig. 4).

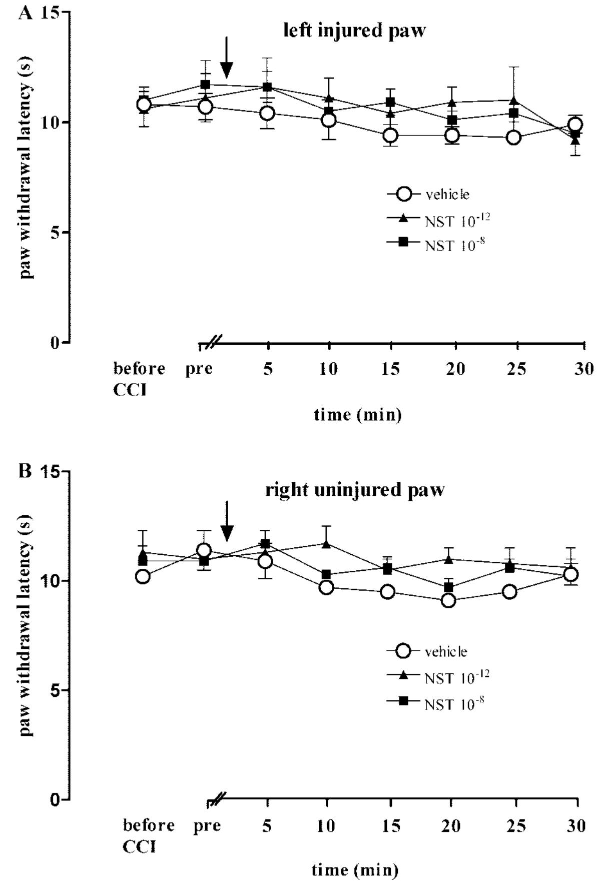

Fig. 2. Nocistatin (NST) in sham-operated rats. Thermal withdrawal latencies (mean ± SEM) (in s) of rats treated with different doses of rNST1–17 injected intrathecally *versus* time. 10^{-8} and 10^{-12} M/rat, vehicle. Arrow indicates time of rNST1–17 injection. n = 6 to 8 rats per group.

Discussion

Pronociception and Antinociception of NST in the Chronic Constriction Injury

Both pronociception and antinociceptive effects of spinally applied NST have repeatedly been described.[5–7,18–20] Our results suggest that these discrepant results might be explained by the fact that different doses were used in the various studies. In our experiments picomolar doses of NST elicited robust antinociception in the chronic constriction injury in both paws, whereas pronociception was elicited when nanomolar doses were employed. This dose-dependent action has already been described in the formalin test,[8] which renders the possibility unlikely that pronociceptive or antinociceptive effects depended on the pain model used. In contrast, same doses of NST did not change thermal withdrawal latencies in either paw in sham-operated rats (fig. 2), indicating that the observed effects of NST are specific for neuropathic pain and are not present in rats without ongoing pain or neuropathic changes.

Seemingly conflicting results have recently been reported by Ma *et al.*[21] They injected 10 μg (*i.e.*, approximately 10^{-9} M) bovine nocistatin intrathecally in rats

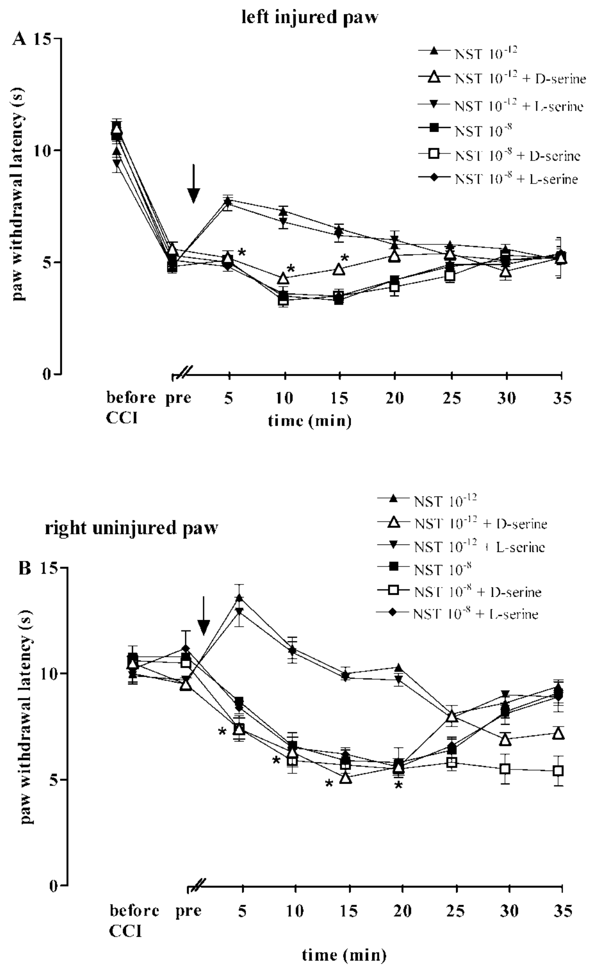

Fig. 3. Effect of D-serine on the pronociceptive and antinociceptive effects of nocistatin (NST) in the chronic constriction injury model (CCI). Thermal withdrawal latencies (mean ± SEM) (in s) in neuropathic rats (CCI) injected intrathecally with: 10^{-12} M/rat rNST1–17, 10^{-12} M/rat rNST1–17 with 100 μg D-serine or L-serine, 10^{-8} M/rat rNST1–17, 10^{-8} M/rat rNST1–17 with 100 μg D- serine or L-serine. Arrow indicates time of drug injection. *$P \leq 0.05$ rNST1–17 *versus* rNST1–17 with D-serine, n = 10 rats per group.

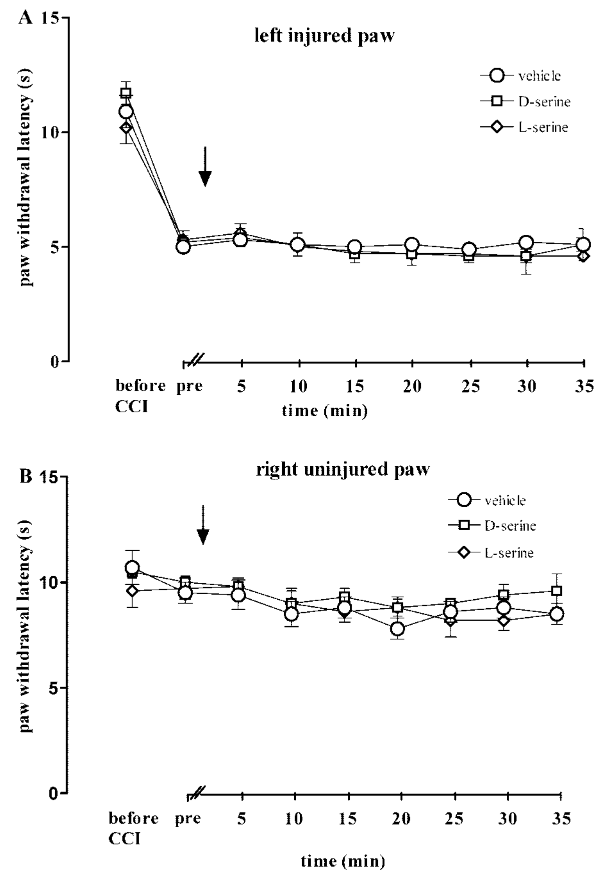

Fig. 4. D- and L-serine in the chronic constriction injury model. Thermal withdrawal latencies (mean ± SEM) (in s) of rats treated with D-serine (100 μg), L-serine (100 μg), or vehicle injected intrathecally *versus* time. Arrow indicates time of drug injection. n = 8 to 10 rats per group.

with chronic constriction injury and thermal withdrawal latencies were not changed *versus* control. Indeed, this dose of NST only blocked the analgesic effect of its functional antagonist nociceptin but had no effect *per se*. Other doses were not tested by Ma *et al.*[21] We obtained similar results by intrathecal injection of rat NST 1–17 in a dose of 10^{-10} M per rat, which had no effect on thermal withdrawal latencies. However, the higher dose of 10^{-8} M NST per rat elicited pronociception and 10^{-12} M NST per rat elicited antinociception (fig. 1).

Unlike many other neuropeptides, NST is not very well conserved among different species. In this context it is noteworthy that similar results were obtained in the present study, which employed a peptide consisting only of the 17 C-terminal amino acids, and in a previous study by our group, which used the complete peptide consisting of 35 amino acids.[8] Previously we had already reported that both peptides exerted almost identical effects on dorsal horn synaptic transmission.[7]

Here, we show that both pronociceptive and antinociceptive action of NST is restricted to the first 20 to 25 min after injection of NST. This may be explained by rapid degrading of the neuropeptide NST by endogenous peptidases.[20]

We tested only thermal hyperalgesia and not mechanical allodynia in neuropathic pain. In other pain behavior tests the results may be different.

Possible Mechanism of Action of Nocistatin

So far the only well documented effect of NST on the cellular level is its inhibitory action on the synaptic release of the spinal inhibitory neurotransmitters glycine and γ-aminobutyric acid. This effect is restricted to the dorsal horn of the spinal cord and is pertussis toxin sensitive, indicating that it most likely occurs through the activation of a yet to be identified transmembrane receptor coupling to Gi/Go proteins.[7]

Antinociception by NST was completely prevented or even converted into a pronociceptive action by pretreatment with D-serine, which is a full agonist at the glycine-binding site of NMDA receptors but inactive at strychnine-sensitive glycine receptors.[22] The specific prevention of NST-mediated antinociception by D-serine indicates the pivotal role of glycine binding to NMDA receptors in this process. A significant contribution of glycine to the activation of NMDA receptors in painful diseases is also evident from the antinociceptive effect of glycine site antagonists (so-called glycineB antagonists) in various pain models.[23] We have recently shown that glycine released from spinal inhibitory interneurons can escape the synaptic cleft and reach nearby NMDA receptors *via* diffusion, a process that is called spillover, and that this process is relevant *in vivo* in tonic pain, *i.e.*, in the formalin test in rats.[8]

The present data now suggest (in accordance with our previous data)[8] that NST inhibition of synaptic glycine release not only reduces the activation of strychnine-sensitive glycine receptors but may also lead to a diminished NMDA receptor activation in chronic neuropathic pain. Thus the effect on NMDA receptors dominates over that on inhibitory glycine receptors at low doses of NST (resulting in antinociception), whereas disinhibition, *i.e.*, reduced activation of strychnine sensitive glycine receptors, dominates at higher doses of NST (resulting in pronociception). In our previous publication[8] we proposed a cellular basis for our present observations. In principle, it is possible that D-serine has a pronociceptive effect by itself and acts through an mechanism independent from nocistatin in neuropathic pain. However, D-serine has no effect when given alone (fig. 4), which argues against this. In addition, at least for the formalin test,[8] D-serine did not antagonize the antinociceptive effect of MK-801, which blocks NMDA receptors independent of the glycine-binding site.

Several factors might contribute to the different dose-dependencies observed for the antinociceptive *versus* pronociceptive effects of NST (fig. 1). First, the affinities of glycine at strychnine-sensitive glycine inhibitory glycine receptors and at NMDA receptors differ by two to three orders of magnitude.[14,15] Furthermore, NMDA receptors are located further away from glycine release sites than strychnine sensitive glycine receptors and glycine transporters located between glycinergic terminals and NMDA receptors may significantly affect the glycine concentration reached at NMDA receptors after synaptic release of glycine. In addition, it is well possible that reduction in NMDA receptor activation and inactivation of strychnine-sensitive glycine receptors responsible for the antinociceptive and pronociceptive effects may occur in different laminae of the spinal cord. Local intrathecal injection most likely will result in a concentration gradient of NST through the spinal cord.

Interestingly, D-serine not only reversed in the right, uninjured, paw (as in the left, injured, paw) the antinociception induced by low-dose NST but actually produced pronociception. One possible explanation for this effect is that the glycine site of the spinal NMDA receptor on the right, uninjured, side is not saturated under the conditions of peripheral mononeuropathy (in contrast to the left, injured, side, where full saturation seems reasonable).

The responsiveness of the contralateral side to NST application confirms previous reports that central changes after unilateral nerve injury are not restricted to the side of the injury. Numerous reports suggest that unilateral nerve injury also leads to morphological changes in the contralateral side similar to those seen on the injured side.[24] In our study thermal hyperalgesia could not be detected in the contralateral side, *i.e.*, thermal withdrawal latencies were not lowered after CCI in the uninjured (right) paw. However, after NST application the contralateral paw showed responses similar to those seen on the injured side, indicating that the contralateral side is also influenced by an increase in glycine acting at the NMDA receptor. These observations resemble those by Vissers *et al.*,[25] who have found CCI-induced cold allodynia only in the injured side. However, both injured and noninjured sides were similarly hypersensitive to formalin-induced nociception. One might therefore speculate that the detection of subtle changes in contralateral pain sensitivity requires more sophisticated interventions.

In summary, our results provide further support for a relevant contribution of glycine to spinal nociceptive processing. They suggest that synaptically released glycine may not only act as an inhibitory neurotransmitter, but may also facilitate NMDA receptor-mediated excitation. Modulation of extracellular glycine concentration may be achieved by targeting glycine transporters that regulate or limit spillover or by activation of a putative NST receptor. One should consider, however, that modulation of extracellular glycine concentration has the potential to exert pronociceptive action in addition to antinociception, probably depending on extracellular glycine concentration and the precise site of action.

Given the pivotal role NMDA receptors serve in plastic changes in nociceptive transmission[26] and in the development of chronic pain states,[27] one might speculate that inhibition of NMDA receptor function *via* modulation of extracellular glycine concentrations may be considered a novel strategy for the prevention or treatment of pathologic pain states.

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