

Electrophysiologic Evidence for Increased Endogenous GABAergic but Not Glycinergic Inhibitory Tone in the Rat Spinal Nerve Ligation Model of Neuropathy

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Background: **Changes in the inhibitory activity mediated by γ-aminobutyric acid (GABA) and glycine, acting at spinal $GABA_A$ receptors and strychnine-sensitive glycine receptors, are of interest in the development of neuropathic pain. There is anatomic evidence for changes in these transmitter systems after nerve injuries, and blocking either $GABA_A$ or glycine receptors has been shown to produce allodynia-like behavior in awake normal animals.**

Methods: **In this study, the possible changes in GABAergic and glycinergic inhibitory activity in the spinal nerve ligation model of neuropathic pain were studied by comparing the effects of the $GABA_A$-receptor antagonist bicuculline and the glycine-receptor antagonist strychnine in neuropathic rats to their effects in sham-operated and nonoperated control rats.**

Results: **Bicuculline produced a dose-related facilitation of the Aδ-fiber–evoked activity in all study groups and increased C-fiber–mediated activity in the spinal nerve ligation group but not in either of the control groups. There were no differences in the effect of bicuculline on low threshold responses between the study groups. The glycine receptor antagonist strychnine did not have a statistically significant effect on any of the parameters studied in any of the control groups.**

Conclusions: **These results support the idea of an increased GABAergic inhibitory tone in the spinal cord of neuropathic rats, possibly as compensation for increased excitability after nerve injury.**

γ-AMINOBUTYRIC acid (GABA) is the most abundant inhibitory neurotransmitter in the central nervous system and plays roles in the control of the pathways that transmit sensory events, including nociception.[1] GABA acts as a transmitter in spinal interneurons[2] and in descending inhibitory tracts terminating in the dorsal horn of the spinal cord.[3] Glycine acts as an inhibitory transmitter at the strychnine-sensitive glycine receptors, which are often colocalized with $GABA_A$ receptors.[4–6] There is evidence that both GABA and glycinergic interneurons are preferentially associated with the control of low-threshold afferent input to the spinal cord.[6–9] Consistent with this, intrathecal administration of the $GABA_A$-receptor antagonist bicuculline[10] or the glycine receptor antagonist strychnine[10,11] produces segmentally localized tactile allodynia-like behavior in conscious rats and increased responses to mechanical stimulation in anesthetized cats.[12] Intrathecal strychnine has also been shown to produce in anesthetized rats reflex responses similar to those produced by nociceptive stimuli[13,14] and enhanced neuronal responses to low-threshold mechanical stimulation.[15] These studies indicate a tonic GABAergic and glycinergic inhibition of low-threshold afferents innervating mechanoceptors in normal rats. Thus, reduced activity in these spinal inhibitory systems could result in mechanical allodynia.

Changes in the inhibitory activity mediated by these two transmitters are therefore of interest in the development of neuropathic pain. GABA immunoreactivity in the dorsal horn of the spinal cord has been reported to decrease after partial injury of a sciatic nerve[16,17] and transection of the sciatic nerve.[18] Similar changes occur in a model of spinal injury–induced pain,[19] suggesting that a decrease in GABAergic tone in the spinal cord may underlie the allodynia observed in these models. However, these decreases in content could be interpreted as resulting from increased release of the amino acid; indeed, increased concentrations of GABA have been measured in the dorsal quadrant of the spinal cord ipsilateral to the nerve injury.[20] Both $GABA_A$- and $GABA_B$-receptor binding in the spinal cord have been shown to change in a complicated manner after neurectomy.[21] After tight ligation of the L5 spinal nerve, the mRNA for the γ2 subunit of the $GABA_A$ receptor has been shown to be significantly downregulated in the medium- to large-sized L5 dorsal root ganglion neurons ipsilateral to the ligation as compared with the contralateral side, suggesting loss of presynaptic inhibition of the ipsilateral L5 primary afferent terminals.[22] However, the $GABA_A$ receptor expression in the noninjured L4 dorsal root ganglions was unchanged.[22] Thus, it is presently unclear how these complex anatomic changes relate to the functional role of GABA in the spinal cord after nerve injury.

Much less is known about changes in the glycinergic system after nerve injuries. Glycine receptor density in the grey matter of the spinal cord dorsal horn has been

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reported to decrease bilaterally after a unilateral sciatic nerve constriction injury.[23] In addition, the uptake of glycine in the spinal cord is enhanced after peripheral crush injury of sciatic nerve but returns to control levels 9–12 days after the injury.[24] These results suggest that nerve injury can induce changes in the spinal glycinergic system, but the time course and bilateral nature of some changes do not appear to correlate well with the development of symptoms of allodynia.

The aim of the present study was to assess whether the role of the spinal GABAergic and glycinergic systems in spinal sensory processing is altered in a model of neuropathic pain compared with that in normal animals.

Methods

Model of Neuropathic Pain

Male Sprague-Dawley rats (Central Biological Services, University College London) weighing 120–160 g at the time of the surgery were used. Rats were housed in groups of five in plastic cages in artificial lighting with a fixed 12-h light–dark cycle. Laboratory chow and water were available *ad libitum*. Guidelines for animal research by the United Kingdom Home Office and the Council of the American Physiologic Society were followed. The spinal nerve ligation (SNL) model of neuropathic pain was used.[25] In brief, the animals were anesthetized with halothane (Fluothane; Zeneca, Macclesfield, United Kingdom) in nitrous oxide and oxygen (50:50). The left L5 and L6 spinal nerves were exposed by removing a small piece of the paravertebral muscle and a part of the left transverse process of the L5 lumbar vertebra. The L5 and L6 spinal nerves were then carefully isolated and tightly ligated with 6-0 silk. A sham operation was performed by exposing but not ligating the spinal nerves. After checking for hemostasis, the muscle, the adjacent fascia, and the skin were closed with sutures.

Mechanical and Cold Sensitivity

The behavioral signs of mechanical and cold allodynia were measured with a series of von Frey filaments and by application of a drop of acetone as previously described.[26] In brief, the rats stood on a metal mesh platform, and the plantar surface of the paw was touched with different von Frey filaments (Semmes-Weinstein monofilaments; Stoelting, Wood Dale, IL) with a bending force of 0.2–15 g. Each filament was applied five times at 5-s intervals until the weakest filament that induced paw withdrawal on more than half the occasions it was presented was found. Cold allodynia was measured as the number of brisk foot withdrawal responses after five consecutive applications of a drop of acetone to the plantar surface of the paw. The assessment of mechanical and cold sensitivity was performed in the SNL and sham-operated rats 7 and 14 days after the operation to verify that the animals entered to the electrophysiologic study exhibited mechanical and cold allodynia.

Table 1. Characteristics of the Neurons used in the Bicuculline Experiment

	SNL	Sham	Nonoperated
No. of neurons	15	16	9
Depth (μm)	696 ± 34	681 ± 32	813 ± 143
C-fiber threshold (mA)	2.53 ± 0.2	2.34 ± 0.2	2.7 ± 0.4
No. of baseline action potentials when stimulated at 3× the C-fiber threshold:			
Aβ	83 ± 7.1	108 ± 5.8	127 ± 16*
Aδ	51 ± 6.9	46 ± 8.7	63 ± 11
C	179 ± 26	234 ± 35	339 ± 63*
After discharge	148 ± 22	141 ± 37	201 ± 40

Means (± SEM) of the parameters are given for the spinal nerve ligation (SNL), sham-operated, and nonoperated control groups.

* Statistically significant difference between the SNL and the nonoperated control group.

Electrophysiology

The electrophysiology was performed 15–18 days after the surgery and on nonoperated control animals of similar size, as described previously.[27,28] The rat was anesthetized with halothane (3% during induction, 1.5% during the recordings) in nitrous oxide and oxygen (66%:33%), and the trachea was cannulated. No neuromuscular blockers were used. The rat was secured in a stereotaxic frame, and a laminectomy was performed over lumbar segments L1–L3. The spine was clamped rostral and caudal to the laminectomy, and the dura covering the exposed part of the spinal cord was removed. Parylene-coated tungsten electrodes were used to make single-unit extracellular recordings of dorsal horn neurons receiving afferent input from the hind paw. The electrode was lowered into the spinal cord using a SCAT microdrive (Digitlmer, Welwyn, UK), which allowed measurement of the depth of the neuron relative to the surface of

Table 2. Characteristics of the Neurons Used in the Strychnine Experiment

	SNL	Nonoperated
No. of neurons	7	10
Depth (μm)	746 ± 82	920 ± 36
C-fiber threshold (mA)	2.2 ± 0.4	2.0 ± 0.3
No. of baseline action potentials when stimulated at 3× the C-fiber threshold:		
Aβ	109 ± 39	123 ± 49
Aδ	100 ± 18	141 ± 19
C	207 ± 44	355 ± 67
After discharge	132 ± 40	240 ± 52

Means (± SEM) of the parameters are given for the spinal nerve ligation (SNL), and the nonoperated control group. No statistically significant differences between the study groups were found in these parameters.

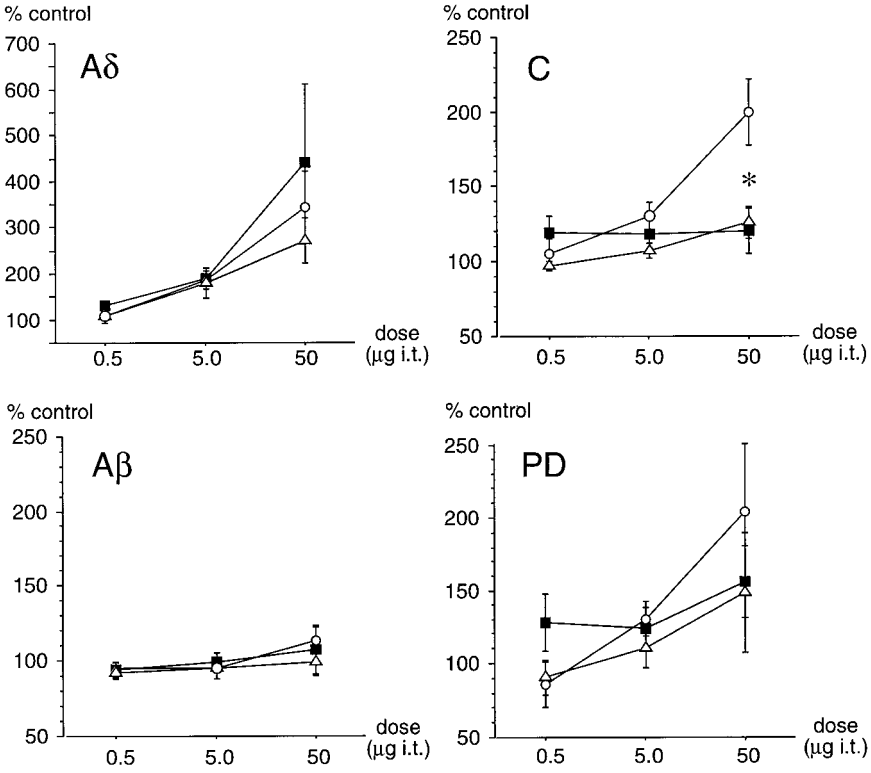

Fig. 1. The effect of bicuculline on Aβ-, Aδ-, and C-fiber–evoked activity (16 stimuli of 3 × C-fiber threshold, 0.5 Hz, 2-ms-wide pulse) and postdischarge (PD) of a population of dorsal horn neurons in rats with spinal nerve ligation–induced neuropathy (circles) and sham-operated (triangles) and nonoperated control animals (squares). Mean of the effect achieved 20 min after intrathecal administration of bicuculline (± SEM) is plotted against the respective dose. In the spinal nerve ligation group, 7 cells were recorded after 0.5 μg bicuculline, 10 after 5 μg bicuculline, and 10 after 50 μg bicuculline. In the sham-operated control group, n = 9, 12, and 10, respectively, and in the nonoperated control group, n = 7, 9, and 8, respectively. Asterisk indicates statistically significant difference between the study groups.

the dorsal horn. Gentle tapping of the plantar surface of the hind paw with the fingers was used as the search stimulus for finding wide-dynamic-range neurons.

Transcutaneous electrical stimulation of the receptive field was applied at three times the C-fiber threshold, and poststimulus histograms to a train of 16 stimuli at 0.5 Hz with a 2-ms pulse width were constructed (Spike 2 software, C.E.D. 1401 interface; Cambridge Electronic Design, Cambridge, UK). The resulting Aβ-, Aδ-, and C-fiber–evoked responses were separated by latency (0–20, 20–90, and 90–300 ms, respectively) and quantified. In addition, after discharge, the activity after the main C-fiber–evoked band between 300 and 800 ms was recorded and quantified. Poststimulus histograms were constructed at 10-min intervals before administration of the drug until a stable (< 10% change between tests) response was obtained, and thereafter for 40 min after each drug dose. For analyzing wind-up, the initial response of the neuron to the first stimulus in the train and the final response of the neuron after the repeated 16 stimuli in the train were measured over the C-fiber and postdischarge latencies (90–800 ms).

Drugs

Bicuculline methobromide was obtained from Tocris Cookson Ltd. (Bristol, United Kingdom), and strychnine-HCl was obtained from RBI (Poole, United Kingdom). Both drugs were dissolved in 0.9% NaCl. The vials containing strychnine solutions were kept wrapped in aluminium foil to prevent exposure to light. All drugs were stored at 4°C. The doses of bicuculline and strychnine were administered on the spinal cord in volume of 50 μl using a glass Hamilton microsyringe in a cumulative manner, with a 1-h interval between each dose. The number of neurons recorded was 5–12 for each drug dose in each study group. The exact numbers are given in the figure legends.

Statistical Analysis

Normally distributed continuous variables, such as the properties of the recorded neurons, were analyzed using analysis of variance followed by the Dunn *post hoc* test when appropriate. Discrete variables, such as the measures of mechanical and cold sensitivity, were analyzed using the Mann–Whitney U test for paired comparisons and the Wilcoxon signed rank and Kruskal-Wallis tests were used for comparisons between the groups, as appropriate. Significance was set at P less than 0.05.

Results

Mechanical and Cold Sensitivity

The SNL group rats developed mechanical and cold allodynia in the operated paw. The von Frey filament force that induced paw withdrawal in SNL rats 7 days after the operation was 7.0 ± 1.5 g (median and median absolute deviation) and 8.5 ± 3.2 g 14 days after the operation, whereas applying the strongest filament used (15 g) did not cause paw withdrawal in any of the rats in

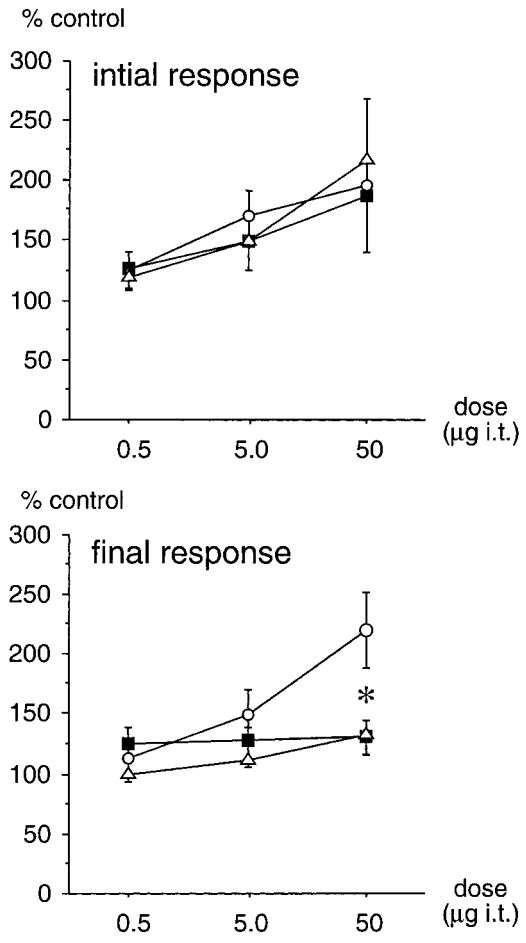

Fig. 2. The effect of bicuculline on the initial response after the first electrical stimulus of the train and the final response after 16 consecutive electrical stimuli (0.5 Hz) measured in the C-fiber and postdischarge latencies (90–800 ms) in rats with spinal nerve ligation–induced neuropathy (circles) and sham-operated (triangles) and nonoperated control animals (squares). Mean of the effect achieved 20 min after intrathecal (i.t.) administration of bicuculline (± SEM) is plotted against the respective dose. Numbers of cells recorded after each bicuculline dose are the same as in figure 1. Asterisk indicates statistically significant difference between the study groups.

the sham-operated group at either 7 ($P < 0.001$) or 14 days after the operation ($P < 0.001$). No paw withdrawal responses to mechanical stimulation occurred in the paw contralateral to the operation in either the SNL or the sham-operated rats. In the acetone drop test 7 days after the operation, the median number of responses was 1.5 in the operated paw (range, 0–4) and 0 in the sham-operated group ($P < 0.001$), and 14 days after the operation was 1.0 (range, 0–4) in the operated paw and 0 in the sham-operated group ($P < 0.001$). No paw withdrawal responses to the acetone stimulation occurred in the paw contralateral to the operation in either the SNL or the sham-operated rats. These behavioral manifestations of nerve injury did not differ between the rats used in the bicuculline and strychnine studies (data not shown).

Neurons Recorded

All neurons recorded were wide-dynamic-range type and had receptive fields located on the plantar surface of the hind paw. The baseline characteristics of the neurons recorded are described in tables 1 and 2. Neurons that showed constant spontaneous activity during the baseline recordings were not used in this experiment.

Effects of Intrathecal Bicuculline

Bicuculline significantly increased the Aδ-fiber–evoked activity in a dose-related manner in all three experimental groups (fig. 1). The peak of this effect was observed 20 min after each dose. There were no significant differences between the SNL rats and either of the control groups with respect to the effect of bicuculline. The C-fiber–evoked activity was significantly increased with bicuculline in the SNL animals but not in the control groups ($P = 0.008$ for SNL *vs.* nonoperated, and $P = 0.004$ for SNL *vs.* sham with 50 μg bicuculline; fig. 1). Bicuculline did not significantly alter the Aβ-fiber–mediated activity or the postdischarge in any of the experimental groups (fig. 1).

The initial neuronal response was increased by bicuculline in a dose-related manner in all three study groups (fig. 2). There were no statistically significant differences among the study groups (fig. 2). The final response after 16 consecutive electrical stimuli (0.5 Hz) was significantly increased after bicuculline in the group with SNI-induced neuropathy but not in the sham-operated or nonoperated control animals (fig. 2; $P = 0.05$ for SNL *vs.* sham and 0.05 for SNL *vs.* normal). In the SNL group, 4 of the 15 neurons started to fire spontaneously after bicuculline administration, with a mean peak firing rate of 12 ± 4.7 Hz with 0.5 μg bicuculline (n = 3), 14 ± 7.0 Hz with 5 μg bicuculline (n = 3), and 17 ± 10 Hz with 50 μg bicuculline (n = 4). In the sham-operated control group, bicuculline administration caused 2 of 16 neurons to fire spontaneously, with a mean peak firing rate of 1.2 Hz with 0.5 μg bicuculline (n = 1), 14 Hz with 5 μg bicuculline (n = 2), and 18 Hz with 50 μg bicuculline (n = 2).

Effects of Intrathecal Strychnine

Because intrathecal strychnine did not produce any effect in the pilot experiments in normal animals, only SNL rats and nonoperated control animals were studied. Strychnine did not significantly alter the activity mediated by any of the fiber types (fig. 3). The initial response to the first electrical stimulus of the train and the final response after all 16 stimuli (see above) were not changed by strychnine in either group of animals. However, in a number of animals, segmental motor responses, such as spontaneous tail flicks or muscle twitches in the body occurred, although there were no changes in the activity of the dorsal horn neurons.

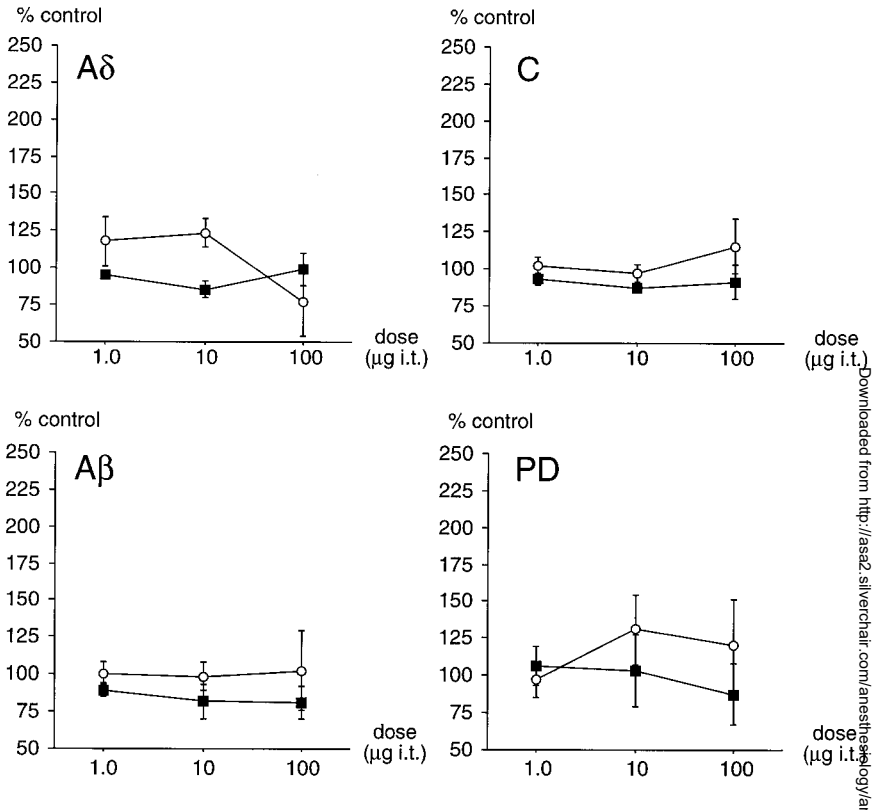

Fig. 3. The effect of strychnine on Aβ-, Aδ-, and C-fiber–evoked activity (16 stimuli of 3 × C-fiber threshold, 0.5 Hz, 2-ms-wide pulse) and postdischarge (PD) of a population of dorsal horn neurons in rats with spinal nerve ligation–induced neuropathy (circles) and nonoperated control animals (squares). Mean of the effect (± SEM) achieved 20 min after intrathecal administration of strychnine is plotted against the respective dose. In the spinal nerve ligation group, 7 cells were recorded after 1 μg strychnine, 6 after 10 μg strychnine, and 6 after 100 μg strychnine. In the nonoperated control group, n = 9, 9, and 8, respectively.

Discussion

The mechanical allodynia that occurred in the SNL group after the nerve injury was comparable to the results reported previously.[25,26,28,29] In the electrophysiologic recordings, the $GABA_A$ antagonist bicuculline produced statistically significant increases in the Aδ-fiber–evoked activity in SNL and sham-operated and nonoperated control rats, and increased C-fiber–mediated activity in the SNL group but not in either of the control groups. The glycine-receptor antagonist strychnine did not have any statistically significant effect on any of the parameters studied in either SNL rats or nonoperated control animals.

In an earlier electrophysiologic study in normal rats, bicuculline facilitated significantly Aδ- and C-fiber–evoked responses in a dose-dependent manner. The facilitation of the C-fiber–evoked response was statistically significant but small compared with the profound potentiation observed on the Aδ-fiber–evoked response.[30] This would agree with findings reported in normal rats with spinal transection, where the benzodiazepine midazolam, which potentiates $GABA_A$ receptor function, caused only a weak depression of C-fiber–evoked responses but had a marked dose-dependent depressive effect on the Aδ-fiber–evoked response of the spinal cord wide-dynamic-range neurons.[31] This $GABA_A$ effect on Aδ-fiber–evoked responses may have a morphologic basis: GABAergic terminals contact many more Aδ-fiber terminals than nonglomerular C-fiber terminals and form no contacts at all with glomerular C-fiber terminals.[32] In a recent study, the benzodiazepine agonist midazolam, acting spinally, reduced Aδ-evoked responses in a dose-dependent manner in both SNL and control animals but inhibited C-fiber–mediated activity significantly only in the SNL-injured animals.[33] Overall, these results perfectly mirror the present findings in that the profile of midazolam, which enhances GABA transmission, was the exact opposite of the effects of bicuculline, which blocks the actions of GABA at the $GABA_A$ receptor. Thus it can be surmised that GABAergic neurons are subject to plasticity after nerve injury. In the present study, the profound facilitation of the Aδ-fiber–evoked responses by bicuculline was unchanged in all study groups. In contrast, an enhancement of the C-fiber responses and the final response, a measure of the enhanced activity in a neuron produced by wind-up, was observed only after nerve injury. The overall evoked response after C-fiber stimulation was increased in these animals, although the initial response, a measure of the ability of the first C-fiber volley to evoke a response, was unaltered (fig. 2). Thus, it appears likely that the enhanced C-fiber responses and final responses produced by the drug after nerve injury result from postsynaptic mechanisms, possibly increased $GABA_A$-mediated controls acting on interneurons in polysynaptic nociceptive C-fiber pathways. Figure 4 is a schematic diagram illustrating the proposed changes in the spinal GABAergic systems in neuropathy. The marked GABAergic inhibitory tone on the Aδ-fiber–evoked responses is not altered in neuropathy, whereas the less pronounced GABA control of C-fiber responses

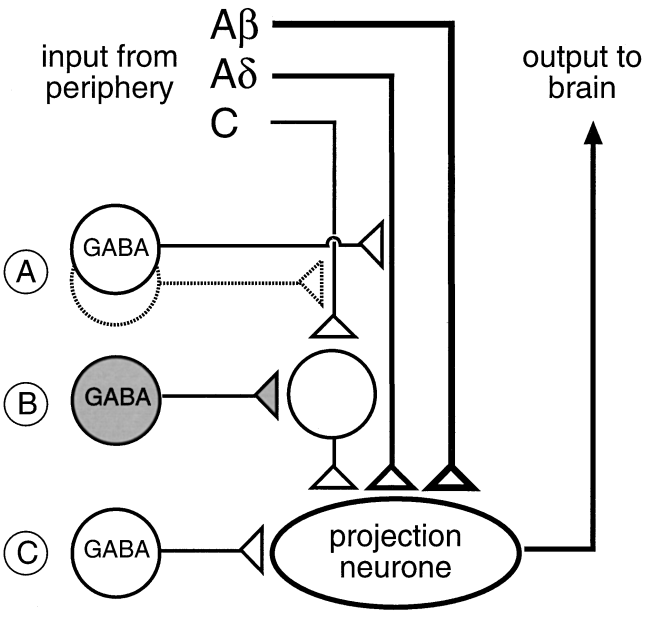

Fig. 4. Possible mechanisms by which spinal nerve ligation alters GABAergic modulations of Aδ- and C-fiber–mediated nociceptive input. (*A*) In normal unijured state, there is a profound, most likely presynaptic GABAergic inhibitory tone on the Aδ-fiber–evoked responses and a less pronounced presynaptic inhibition also on the C-fiber–mediated responses. These are not altered in neuropathy. (*B*) On the basis of the present findings, we propose enhanced GABAergic control of the polysynaptic C-fiber pathway after nerve injury. (*C*) There are also likely to be GABAergic inhibitory interneurons synapsing with the projection neuron. The possible afferent drive on to the GABA neurons is omitted for clarity.

is enhanced, which may reflect changes in the polysynaptic C-fiber pathways after nerve injury.

In behavioral experiments, intrathecal administration of both bicuculline and strychnine produce allodynia-like behavior, facilitation of nocifensive flexor reflexes, and cardiovascular responses similar to those produced by nociceptive stimuli.[10–14] In the present study, administration of strychnine (1.0–100 μg intrathecally) did not significantly change the responses of the wide-dynamic-range neurons in the dorsal horn of the spinal cord in the electrophysiologic recordings. This discrepancy between the previous behavioral studies and the electrophysiologic findings in the present study could be a result of several factors, including the type of stimulation used, the species, the population of cells studied, and the depth of anesthesia. However, bicuculline was effective under the same conditions.

Strychnine-induced allodynia has been shown to be selective for nonnoxious light tactile stimulation[34,35] and initiated by primary afferents not normally involved in nociception, possibly Aβ βfibers[13,34]. The fact that intrathecal strychnine does not produce hyperalgesia to mechanical, thermal, or chemical noxious stimuli further indicates the independence of nociceptive pathways and strychnine-sensitive afferent inputs to the spinal cord.[13,34] In the present study, suprathreshold electrical stimulation at three times the C-fiber threshold was used to study changes in both the C- and the Aδ-fiber–mediated effects. Strychnine applied near the dorsal horn neurons in α-chloralose–anesthetized cats through a dialysis fiber causes a significantly enhanced response to hair follicle activation only.[15] Similar treatment with bicuculline results in increased responses to hair deflection, low- and high-threshold tonic mechanical stimulation of the receptive field, and increased background discharge in nociceptive dorsal horn neurons.[12]

The behavioral allodynia produced by spinal strychnine is segmentally localized and can be prevented with intrathecally administered glycine,[13] which would indicate a spinal effect. However, the cardiovascular responses produced by intrathecal administration of strychnine can be observed only in lightly anesthetized animals, but not in animals during surgical anesthesia.[15] We required anesthesia depth at surgical levels for electrophysiologic recordings. Despite this, some animals showed segmental spontaneous and, above all, evoked motor responses to low-threshold stimuli, such as touching the animal's tail. It is conceivable that a large part of the agitation in the behaving animals could result from blockade of the recurrent inhibitory glycinergic interneurons in the motor response arc, the Renshaw neuron, rather than a selective effect on sensory neurons.

In conclusion, bicuculline produced dose-related facilitations of the Aδ-fiber–evoked activity in all study groups and significantly increased C-fiber–mediated activity in the SNL group. This suggests a modality-dependent shift in the inhibitory controls exerted by $GABA_A$ after nerve injury, possibly as compensation for increased C-fiber–evoked excitability. This would indicate that the extent of hyperalgesia in neuropathy is modulated by endogenous spinal GABA neurons. There were no differences in the effect of bicuculline on low-threshold responses among the study groups, indicating that $GABA_A$-mediated control of low-threshold inputs is not altered by nerve injury, at least under these conditions. These findings would suggest that the purported loss of GABA controls on low threshold inputs in neuropathy, proposed to be a basis for allodynia, is unlikely. The morphologic evidence for reduced level of GABA in neuropathy may, in fact, result from increased release of the transmitter rather than a downregulation of these inhibitory controls. This, in turn, may partly be responsible for the reduced baseline responses of the spinal neurons in the SNL model of neuropathic pain. These results and our previous findings with midazolam would suggest that a reassessment of GABAergic mechanisms in neuropathic pain is needed.

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