Sciatic Chronic Constriction Injury Produces Cell-Type-Specific Changes in the Electrophysiological Properties of Rat Substantia Gelatinosa Neurons

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Balasubramanyan, Sridhar, Patrick L. Stemkowski, Martin J. Stebbing, and Peter A. Smith. Sciatic chronic constriction injury produces cell-type-specific changes in the electrophysiological properties of rat substantia gelatinosa neurons. J Neurophysiol 96: 579–590, 2006. First published 24 April 2006; doi:10.1152/jn.00087.2006. Peripheral nerve injury increases spontaneous action potential discharge in spinal dorsal horn neurons and augments their response to peripheral stimulation. This “central hypersensitivity,” which relates to the onset and persistence of neuropathic pain, reflects spontaneous activity in primary afferent fibers as well as long-term changes in the intrinsic properties of the dorsal horn (centralization). To isolate and investigate cellular mechanisms underlying “centralization,” sciatic nerves of 20-day-old rats were subjected to 13–25 days of chronic constriction injury (CCI; Mosconi-Kruger polyethylene cuff model). Spinal cord slices were then acutely prepared from sham-operated or CCI animals, and whole cell recording was used to compare the properties of five types of substantia gelatinosa neuron. These were defined as tonic, irregular, phasic, transient, or delay according to their discharge pattern in response to depolarizing current. CCI did not affect resting membrane potential, rheobase, or input resistance in any neuron type but increased the amplitude and frequency of spontaneous and miniature excitatory postsynaptic currents (EPSCs) in delay, transient, and irregular cells. These changes involved alterations in the action potential-independent neurotransmitter release machinery and possible increases in the postsynaptic effectiveness of glutamate. By contrast, in tonic cells, CCI reduced the amplitude and frequency of spontaneous and miniature EPSCs. Such changes may relate to the putative role of tonic cells as inhibitory GABAergic interneurons, whereas increased synaptic drive to delay cells may relate to their putative role as the excitatory output neurons of the substantia gelatinosa. Complementary changes in synaptic excitation of inhibitory and excitatory neurons may thus contribute to pain centralization.

INTRODUCTION

Pain is fundamental to survival. It protects an individual from potential or actual tissue injury. However, injury to the nervous system per se can produce intractable pain that persists for months or years after the initial injury has healed. This “neuropathic” pain serves no obvious biological purpose (Iadarola and Caudle 1997). It can be initiated by a variety of perturbations, including peripheral nerve trauma (Kim and Chung 1992; Kim et al. 1997), spinal cord trauma (Hao et al. 2005), neuronal inflammation, infection, or disease, particularly postherpetic neuralgia or diabetes (Chen and Pan 2002).

In experimental animals, peripheral nerve damage induces pain-related behaviors that are widely accepted as a model for human neuropathic pain (Kim et al. 1997; Mosconi and Kruger 1996). These behaviors, which include mechanical and thermal hyperalgesia and allodynia, have been attributed to at least three pathophysiological processes: persistent ectopic activity in both damaged and undamaged peripheral nerves and their dorsal root ganglia, changes in the intrinsic properties of neurons and synaptic networks within the dorsal horn (centralization) (Coull et al. 2003, 2005; Dalal et al. 1999; Moore et al. 2002; Woolf 1983; Woolf and Mannion 1999), and alterations in descending brain stem control of spinal nociceptive pathways (Suzuki et al. 2002). Taken together, these three processes contribute to the phenomenon of “central hypersensitivity,” wherein spontaneous action potential discharge in the dorsal horn is increased (Sotgiu and Biella 2000), and the response of dorsal horn neurons to peripheral stimulation is augmented and prolonged (Coderre and Katz 1997; Dalal et al. 1999; Laird and Bennett 1993). The relative contribution of central and peripheral mechanisms to the generation of central hypersensitivity is frequently debated (Coderre and Katz 1997; Sotgiu and Biella 2000; Yoon et al. 1996). Because the classical studies of the effects of sciatic nerve injury on dorsal horn neurons were done with extracellular unit recording in vivo (Laird and Bennett 1993), it has been difficult to distinguish injury-induced changes in the intrinsic properties of dorsal horn from altered excitation resulting from spontaneous primary afferent activity (but see Sotgiu and Biella 2000). We therefore used whole cell recording to carry out a systematic study of the effect of sciatic nerve injury on the intrinsic electrophysiological properties of neurons in the substantia gelatinosa of young adult rats. Experiments were done on spinal cord slices, acutely prepared from sham-operated animals or those subject to 13- to 25-day sciatic chronic constriction injury (CCI) (Mosconi and Kruger 1996). Because acute rhizotomy occurred during the preparation of the slices, this limited the contribution of CCI-induced ectopic primary afferent activity (Amir et al. 2005; Wall and Devor 1983; Wall and Gutnick 1974) to the observed alterations in substantia gelatinosa activity.

The morphological (Gobel 1978a,b), immunohistochemical (Todd and Spike 1993), electrophysiological (Dougherty et al. 2005; Graham et al. 2004; Grudt and Perl 2002; Lu and Perl 2005; Prescott and de Koninck 2002; Thomson et al. 1989), and functional (Lu and Perl 2005) properties of dorsal horn neurons are far from homogeneous. It was therefore necessary to examine whether CCI affected all cell types in the same way. A simple electrophysiological classification of substantia gelatinosa neurons was used because the exact relationship between various morphological phenotypes and their electrical...
properties is still an area of active investigation (Grudt and Perl 2002; Huntman et al. 2004; Heinke et al. 2004; Lu and Perl 2003, 2005; Ruscheweyh and Sandkühler 2002). Neurons were therefore identified as tonic, delay, irregular, phasic, or transient according to their discharge pattern in response to sustained depolarizing current. A report of some of our findings has appeared in abstract form (Balasubramanyan et al. 2004).

METHODS

All experimental procedures complied with the guidelines of the Canadian Council for Animal Care and the University of Alberta Health Sciences Laboratory Animal Services Welfare Committee.

Surgery and behavioral studies

Sciatic nerve chronic constriction injury (CCI) was induced using the polyethylene cuff method (Mosconi and Kruger 1996). The left sciatic nerve of 20–day-old rats was exposed at mid-thigh level under isoflurane anesthesia. Polyethylene cuffs (2 mm long) were made from PE90 tubing (0.86 mm ID, 1.27 mm OD; Intramedic Clay Adams, Becton Dickinson, Sparks, MD). These were slit open longitudinally and applied to enclose the nerve using fine forceps. Each animal received two cuffs. The wound was closed with silk sutures. For sham surgery, animals were anesthetized with isoflurane, and the sciatic nerve was exposed but not deliberately manipulated. Mechanical hypersensitivity was quantified by measuring the paw withdrawal response to von Frey filament stimulation (Chaplan et al. 1994). Animals were placed in a plexiglass box with a wire grid bottom through which a series of von Frey hairs were applied to the midplantar area of the left paw (injured side). A scoring system was used to evaluate the response of the rats to the stimulation. The responses observed belonged to one of the following categories: 0 = no response; 1 = withdrawal response (where the animal withdrew its paw away from the stimulus); 2 = the animal avoided further contact with the stimulus by passively moving away and/or licking the stimulated paw. Rats were habituated to the test procedure on preoperative days. A basal response was taken on the day before surgery. The rats were then tested for mechanical hypersensitivity for a 2-wk period after nerve injury. The range of von Frey hairs used was adjusted so that they remained within 1–15% of the mean body weight of the rats. Three sets of measurements were obtained with each hair. In each set, three consecutive measurements were made at 5-min intervals, and scores were added for each set. Thus an animal can have a maximum score of 6 for each set of measurements and 18 for each hair. Scores obtained were then converted to percentage of this maximum possible score.

Preparation of spinal cord slices

Electrophysiological analysis of effects of CCI was carried out on animals 13–25 days after surgery. Methods for recording from substantia gelatinosa neurons in 30– to 45-day-old Sprague–Dawley rats were similar to those described previously (Moran and Smith 2002; Moran et al. 2004). Briefly, animals were anesthetized with a large overdose of urethane (1.5 g/kg ip) as required by our local animal welfare committee. The spinal cord was removed and glued with cyanoacrylate glue (Vetbond, WPI, Sarasota, FL) to a trapezoid-shaped block cut from 4% agar. This block, with attached spinal cord, was glued to the bottom of a 60-mm glass petri dish, submerged in ice-cold dissection solution containing (in mM) 118 NaCl, 2.5 KCl, 26 NaHCO3, 1.3 MgSO4, 1.2 NaH2PO4, 1.5 CaCl2, 5 MgCl2, 25 D-glucose, and 1 kynurenic acid, and continuously bubbled with 95% O2-5% CO2. Kynurenic acid is a nonselective acidic amino acid receptor antagonist (Perkins and Stone 1982) that was used to limit acute excitotoxicity during manipulation of the spinal cord. Transverse slices (300 μm) were cut using a vibratome (TPI). All recordings in CCI and sham animals were made from spinal neurons ipsilateral to the sciatic injury. To identify the side of each slice that was ipsilateral to the injury, it was removed from the vibratome with a small piece of agar attached. This piece of agar was cut in a triangular shape using the long side of the triangle to mark the side of the cord ipsilateral to the CCI or sham lesion.

Electrophysiology

For recording, slices were superfused at room temperature (~22°C) with 95% O2–5% CO2 saturated artificial cerebrospinal fluid (ACSF) that contained (in mM) 127 NaCl, 2.5 KCl, 1.2 NaH2PO4, 26 NaHCO3, 1.3 MgSO4, 2.5 CaCl2, and 25 D-glucose, pH 7.4. The substantia gelatinosa appeared as translucent bands under infrared differential-interference optics, and neurons were patched using visual control. Recording pipettes had resistances of 5–10 MΩ when filled with an internal solution containing (in mM) 130 potassium gluconate, 1 MgCl2, 2 CaCl2, 10 HEPES, 10 EGTA, 4 Mg-ATP, and 0.3 Na-GTP, pH 7.2, 290–300 mosM. In a few experiments, neurons were studied using mystatin-perforated patches. Recording pipettes were tip-filled with normal internal solution and back-filled with internal solution containing pluronide acid F127 (0.3%), mystatin (0.05%), and DMSO (0.03%). Cell access was obtained within 20–40 min of attaining a tight seal.

Recordings were made using an NPI SEC-05LX amplifier in discontinuous, single-electrode, current- or voltage-clamp mode. Data were only collected from neurons that exhibited clear overshooting action potentials of >60 mV in amplitude. All neurons were categorized on the basis of their discharge pattern in response to 800-ms square-wave depolarizing current pulses from –60 mV to 0 mV (see Fig. 2).

Current-voltage relationships were determined under voltage-clamp using a series of 800-ms voltage commands. Current was measured just prior to the termination of each voltage pulse. Membrane excitability was quantified by examining discharge rates in response to ramp current commands. These were delivered from a set holding voltage of –60 mV at 33, 67, 10, or 133 pA/S. Cumulative latencies for the first, second, third, and subsequent action potentials were noted (see Fig. 4). Data were acquired and analyzed using pCLAMP 8.0 (Axon Instruments, Burlington, MA). Statistical comparisons were made with unpaired t-test or χ2 tests as specified and appropriate, using GraphPad InStat 3.05 (GraphPad Software, San Diego, CA). Statistical significance was taken as P < 0.05.

Miniature excitatory postynaptic currents (mEPSC), which reflect action-potential-independent release of neurotransmitter (Edwards et al. 1990), were recorded at ~70 mV in the presence of 1 μM TTX (see Fig. 6D). The effectiveness of TTX was confirmed by observing its blockade of action potentials generated by depolarizing current pulses. Spontaneous EPSCs (sEPSC), which reflect both action-potential-dependent and -independent components of release (Edwards et al. 1990), were recorded in the same way but without TTX (see Fig. 6C). Mini Analysis Program (Synaptosoft, Decatur, GA) was used to distinguish sEPSC and mEPSC from baseline noise. Details of this program and its implementation may be found at www.synaptosoft.com. Spontaneous or miniature postynaptic currents were detected automatically by setting appropriate amplitude and area threshold for each neuron. All detected events were then re-examined and visually accepted or rejected based on subjective visual examination. Mini Analysis Program was used to further analyze the data and to provide spreadsheets for the generation of figures. Cumulative probability plots were generated to compare the amplitude and interevent intervals of sEPSCs and mEPSCs in neurons from sham-operated animals and those subject to CCI. Cumulative probability plots ranked individual amplitudes or interevent intervals in order of increasing size and then plotted this rank value against the amplitude or interevent interval. The Kolmogorov–Smirnov (KS) two-sample test was used to compare distributions of amplitudes and interevent
intervals (Prescott and de Koninck 2002). Distributions were considered significantly different if $P < 0.05$. For each neuron, sEPSCs were recorded for a total of 3 min. Neurons that failed to generate a detectable event during a 3-min period were classified as silent and excluded from the analysis. Plots in Fig. 5, A–J, were obtained by analyzing the first 50 events seen after 1 min of recording. Data were pooled from 15 to 34 neurons of each type from sham-operated animals and compared with data from 17 to 26 neurons of each type in CCI animals (see legend to Fig. 5 for additional detail). This approach was not feasible with phasic cells as the frequency of sEPSCs was very low (~1 event every 15s). Data were therefore analyzed from the full 3 min of recording time.

For plots of mEPSC frequency and amplitude, data were pooled from 6 to 11 neurons of each type in sham-operated animals and 6–11 neurons of each type in CCI animals. Because the mEPSC frequency was considerably slower than sEPSC frequency, data were analyzed from the first 30 events in each neuron using the whole 3-min standard acquisition period (Fig. 5, K–R; see legend to Fig. 5 for additional detail). Because the mEPSC frequency in phasic neurons was so slow, insufficient data were available for the construction of cumulative probability plots for this neuron type.

**Histology**

Some of the recorded neurons were filled with biocytin (0.2%) for post hoc identification. At the completion of recording, the slice was transferred to cold (4°C) 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, and stored overnight at 4°C. Slices were rinsed three times with PBS and transferred to a 24-well tissue culture dish for staining. Slices were incubated with 0.3% Triton-X100 and streptavidin–Texas red conjugate (1:50 dilution, Molecular Probes, Eugene, OR) for 50 min on a three dimensional rotator. Slices were thoroughly rinsed with distilled water, transferred to slides, allowed to dry overnight and a coverslipped with Cytoseal-60 (Richard-Allan Scientific, Kalamazoo, MI). A Biorad MRC1024 confocal laser scanning system, installed on a Zeiss Axioplan 2 microscope, was used to examine the tissue. It was equipped with a krypton-argon laser and Texas red filter (wavelength: 568 nm) as well as a transmitted light detector that was used to simultaneously acquire dark field images. All images were acquired using Zeiss LSM image browser software. Darkfield and brightfield microscopy were used to define the position of neurons within outer or inner lamina II or at the border between the outer and inner regions (Grudt and Perl 2002). Figures were converted to black and white to increase contrast and clarity.

**Drugs and chemicals**

All drugs and chemicals were from Sigma, St. Louis, MO, except for tetrodotoxin (TTX), which was provided in citrate buffer by Alomone Laboratories, Jerusalem, Israel. TTX was dissolved in distilled water and capsaicin dissolved in DMSO to make 1 mM stock solutions that were stored at $-20^\circ$C until use. On the day of each experiment, TTX and/or capsaicin were diluted to 1 μM in carbogen-bubbled external solution. Drug solutions were placed in storage reservoirs fashioned from 20-ml syringes and their flow into the recording chamber controlled manually by three-way stop cocks.

**RESULTS**

**Behavioral testing**

Because there is some question as to whether 20-day-old rats, as used in our study, exhibit pain behavior after peripheral nerve injury (Howard et al. 2005), we used standard von Frey hairs to assess mechanical hypersensitivity after CCI. Scores represented in Fig. 1 were obtained from responses of the left paw that was ipsilateral to the nerve injury. The basal scores prior to surgery indicate that the filament diameters used did not generate withdrawal responses in unoperated animals. The difference in sensitivity between CCI and sham-operated animals progressively increased over the 14-day testing period. By this time, almost all animals subject to CCI had developed significant mechanical hypersensitivity.

Rats were also assumed to have attained a threshold for displaying mechanical hypersensitivity when they exhibited a withdrawal score of 4 for a total of nine tests. Because the maximum attainable score was 18, this corresponds to a 22.2% response. This is close to the 20% response used by others (Decosterd and Woolf 2000). Using this criterion, the baseline threshold prior to surgery threshold was $>15$ g. Two weeks after the injury, the operated rats showed a reduction in mechanical threshold to $5.0 \pm 0.5$ g ($n = 46$), whereas threshold in sham-operated animals still exceeded $15$ g ($n = 46$).

Because both methods of assessing mechanical hypersensitivity revealed clear, statistically significant increases at 2 wk, we chose a time frame starting at 13-day postsurgery to examine CCI-induced changes in the electrophysiological properties of substantia gelatinosa neurons.

**Electrophysiological characterization of neuron types**

Tonic neurons were defined as those that exhibited continued discharge of action potentials in response to depolarizing
current commands and an increased frequency of discharge in response to increasing current intensity (see Fig. 2A). Tonic cells exhibited the lowest rheobase of cell types examined (~24 pA, see supplementary data; the online version of this article contains supplemental data). Delay neurons were defined as those that exhibited a sustained delay prior to the onset of action potential discharge in response to a depolarizing current (see Fig. 2B). Phasic neurons were those which discharged three or more action potentials in response to an appropriate depolarizing stimulus followed by accommodation and cessation of firing (see Fig. 2C). Spontaneous excitatory synaptic activity was relatively rare in phasic neurons. Transient or single-spike neurons were those that fired a single action potential and never more than two spikes no matter how much depolarizing current was injected (see Fig. 2D). Transient neurons exhibited the highest rheobase of any group of neurons studied (~75 pA, see supplementary data). Irregular neurons were those in which there was little correspondence between intensity of depolarizing current and discharge pattern or frequency (see Fig. 2E). We also encountered a few cells (4.3% of those studied) that did not readily fit into any of the five defined categories. Such unclassified neurons were excluded from the analysis.

To confirm that the different firing patterns seen in substantia gelatinosa neurons were not an artifact of cellular perfusion during whole cell recording, we also examined the firing properties of 20 neurons by means of nystatin-permeabilized patches. Of these, seven displayed tonic discharge, seven displayed delay discharge, two displayed phasic discharge, and four displayed irregular discharge, but none displayed transient discharge (data not shown). Thus with the possible exception of transient discharge, the discharge patterns observed can be

FIG. 2. A–E: current-clamp recordings to show discharge patterns evoked in substantia gelatinosa neurons in response to 3 different intensities of depolarizing current as indicated at the bottom of each panel of records. Membrane potential was set to ~60 mV prior to injection of current pulses. Typical firing characteristics of a tonic neuron (A); discharge persists throughout application of depolarizing current and discharge rate increases with increasing depolarization (B, a delay neuron); note long delay prior to onset of spike discharge (C, a phasic neuron); spike discharge ceases after 1–4 spikes (D, a transient neuron); only 1 spike is discharged regardless of the applied current intensity (E, an irregular neuron); number of spikes discharged is not directly related to intensity of depolarizing current. F and G: bar graphs to show percentage of each neuron type identified in recordings from sham-operated and CCI animals. n = 149 neurons from sham-operated animals, n = 179 neurons from CCI animals. There is no significant difference in percentage of cells in the 2 groups, P values range from 0.08 to 0.95, χ² test. H: confocal image of biocytin cell fill of presumed islet or central cell. Inset: orientation of section as dorsal, ventral, medial, lateral, rostral, caudal (mediolateral plane is into the page). I: image of cell fill of radial cell, orientation of section in H, scale bar = 20 μm and applies to H–J. J: image of cell fill of vertical cell, inset shows orientation of section (rostrocaudal plane is into page).
ascribed to true variations in cellular physiology rather than to differences in recording conditions of substantia gelatinosa neurons.

All neuronal types were encountered in the medial and lateral portions of the substantia gelatinosa and in inner lamina II (II), in outer lamina II (IIo), and at the inner/outer border (IIi/o). For tonic cells, 7/29 were from II, 7/29 from IIi, and 15/29 from IIo. For delay cells, 6/24 were from II, 9/24 from IIi, and 9/24 from IIo. For phasic cells, 11/18 were from II, 4/18 from IIi, and 3/18 from IIo. For transient cells, 4/17 were from II, 8/17 from IIi, and 5/14 from IIo. For irregular cells, 6/17 were from II, 6/17 from IIi, and 5/17 from IIo. Overall, 32% of the neurons studied were recorded from II, 32% from IIi, and 35% from IIo. Although a detailed morphological analysis was not carried out, cells were encountered with islet/central, vertical, and radial morphologies described by Grudt and Perl (2002). Islet and central cells were grouped together as it was not possible to distinguish them in the transverse sections we used. Examples are shown in Fig. 2, H–J.

Because the substantia gelatinosa is the site of termination of many primary afferent C and Aδ fibers, it is assumed to play an indispensable role in the processing of nociceptive information (Millan 1999). Recent in vivo studies have shown, however, that tonic, phasic, transient, and delay neurons in mouse superficial dorsal horn can participate in the processing of both innocuous and noxious stimulation (Graham et al. 2004). We found that capsaicin (1 μM) increased the frequency or induced sEPSCs in 12/14 tonic cells, 5/6 delay cells, 4/6 phasic cells, 3/7 transient cells, and 8/9 irregular cells in rat substantia gelatinosa. It is probable, therefore, that all cell types we studied participate in nociceptive processing. Similar numbers of cells displayed capsaicin sensitivity after CCI: 12/12 tonic cells, 7/9 delay cells, 6/7 phasic cells, 4/5 transient cells, and 9/10 irregular cells.

Sciatic nerve injury could promote a phenotypic shift such that one or more of the aforementioned cell types disappear completely or become more dominant in the neuronal population of the substantia gelatinosa as a whole. This did not appear to be the case because tonic, irregular, phasic, transient, and delay neurons were found in similar proportions in a sample of 149 neurons from sham operated rats and 179 neurons from CCI rats (Fig. 2, F and G). Numbers of neurons of different type in the two populations were compared with χ² tests and P values ranged from 0.08 to 0.95, indicating no significant change in the contribution of each neuron type to the total population. Analysis of the electrophysiological properties of tonic, delay, phasic, transient, and irregular neurons from sham operated and CCI animals was therefore undertaken.

Effects of CCI on passive membrane properties

CCI did not affect resting membrane potential (rmp), rheobase, or input resistance of any neuron type (see supplementary data and Fig. 3). It also did not produce a global change in membrane excitability or spontaneous excitatory synaptic activity but rather produced a clear pattern of neuron type-specific changes.

![FIG. 3. Lack of effect of CCI on current-voltage relationships. A: tonic cells (n = 15 neurons from sham-operated animals; n = 17 neurons from CCI animals). B: delay cells (n = 11 neurons from sham-operated animals; n = 19 neurons from CCI animals). C: phasic cells (n = 9 neurons from sham-operated animals; n = 10 neurons from CCI animals). D: transient cells (n = 9 neurons from sham-operated animals; n = 13 neurons from CCI animals). E: irregular cells (n = 14 neurons from sham-operated animals; n = 17 neurons from CCI animals; P > 0.1 for difference between currents at −140 and 0 mV). Error bars indicating SE are smaller than data points in some cases.](http://jn.physiology.org/)
effects of CCI on spontaneous excitatory synaptic currents

CCI changed excitatory synaptic transmission in different ways in different cell types. Spontaneous excitatory synaptic activity was reduced in tonic cells but increased in delay, transient, and irregular cells.

The effects of CCI on sEPSC amplitude are illustrated in Fig. 5, A–E, and its effects on interevent interval (reciprocal frequency) are illustrated in F–J. Figure 5, A and F, shows that CCI reduced the amplitude and frequency of sEPSCs in tonic cells. By contrast, CCI increased the frequency and amplitude of sEPSCs in delay, transient, and irregular neurons (Fig. 5, B, D, E, G, I, and J). Although CCI increased sEPSC frequency in phasic cells (Fig. 5H), amplitude was reduced (C). All changes were significant according to the KS test (P values presented in Fig. 5). Despite this, it is possible that the presence of silent cells in the various populations may have biased the data. Neurons were classified as silent if they failed to display one or more events during the 3-min sampling period and were omitted from the analysis. However, if such cells fired one event, say, every 4 min, they should have been included as very slow firing cells. The inadvertent omission of such cells may have altered the nature of cumulative probability plots. It is unlikely that this type of bias was introduced because comparable numbers of silent cells were found in each cell category in both sham and after CCI animals (see supplementary data).

We also used a simpler but more rigorous parametric t-test to analyze the same data set as used in Fig. 5, A–J. This generally identified the same trends as the KS test. Figure 6E shows that sEPSC amplitude was reduced in tonic neurons and increased in delay, transient and irregular neurons. These findings correspond to those shown in Fig. 5, A, B, D, and E. Phasic cell sEPSC amplitude was unchanged according to the t-test (Fig. 6E) but decreased according to the KS test (Fig. 5C). Figure 6F shows that sEPSC interevent intervals for tonic cells were increased (frequency decreased), whereas those for delay, transient and irregular were reduced (frequency increased). Again, the more rigorous t-test generally supports the conclusions from the KS test (Fig. 5, F, G, I, and J).

Fig. 4. Effect of CCI on membrane excitability measured as cumulative latency of action potential discharge in response to depolarizing current ramps from −60 mV. A–E, left: sample records of responses of tonic, delay, phasic, transient, and irregular cells to various depolarizing current ramps. Right, A: decreased excitability seen in tonic cells after CCI (*P < 0.05; ramp rate = 67 pA/S; n = 33 for sham; n = 28–35 for CCI). B: unchanged excitability of delay cells (ramp rate = 67 pA/S; n = 13–19 for sham; n = 17–25 for CCI). C: increased excitability in phasic cells after CCI (P < 0.05; ramp rate = 67 pA/S; n = 9–14 for sham; n = 14–24 for CCI). D: unchanged excitability for transient cells (ramp rate = 100 pA/S; n = 3 for sham; n = 7 for CCI). E: unchanged excitability of irregular cells (ramp rate = 100 pA/S; n = 8–17 for sham; n = 10–22 for CCI). Highest n values are seen for lowest spike numbers with the exception of transient cells, all other cell types discharged ≥3 spikes in response to the current ramp but not every cell discharged 8 or 9 spikes. Current, voltage, and time calibration in A refers to all traces. Solid lines are voltage traces, superimposed dashed lines are corresponding current traces. In some cases, error bars indicating SE are smaller than the symbols used to plot the data.
FIG. 5. Cumulative probability plots for effects of CCI on amplitude and interevent interval of spontaneous and miniature excitatory postsynaptic currents (sEPSCs and mEPSCs) in substantia gelatinosa neurons. The following total numbers of events were acquired by analysis of the 1st 50 events that followed the 1st minute of recording: tonic neurons: 1,700 events from sham-operated animals, 1,300 events from CCI animals; delay neurons: 1,150 (sham), 1,200 (CCI); transient neurons: 750 (sham), 1,000 (CCI); irregular neurons: 750 (sham), 1,000 (CCI). Data for phasic neurons, which exhibited low frequencies of spontaneous activity, were acquired from 3 min of recording: 520 sEPSCs were analyzed from sham-operated animals and 847 from CCI animals.

A–E and F–J: cumulative probability plots for sEPSC amplitude and interevent interval from tonic, delay, transient, and irregular cells. P values derived from KS test indicated on graphs.

K–N and O–R: cumulative probability plots for mEPSC amplitude and interevent interval from tonic, delay, transient, and irregular cells. P values derived from KS test indicated on graphs. Insufficient data were available from phasic cells. The following total numbers of mEPSCs were acquired by analysis of the 1st 30 events at the start of recording: tonic neurons, 325 events from sham-operated animals, 175 events from CCI animals; delay neurons, 238 (sham), 293 (CCI); transient neurons, 112 (sham), 256 (neurons CCI); and irregular neurons, 143 (sham), 166 (CCI).
Effects of CCI on mEPSCs

Spontaneous postsynaptic events reflect action-potential-dependent as well as action-potential-independent release of neurotransmitter. The latter process, which represents ongoing activity of the release process, can be investigated by examining mEPSCs in the presence of TTX (Edwards et al. 1990). Figure 6A compares the mean amplitudes of sEPSCs in tonic, delay, transient, and irregular cells with the mean amplitudes of mEPSCs recorded in 1 μM TTX. Insufficient data were available from mEPSCs in phasic cells. TTX had little effect on event amplitude in tonic, delay, and transient cells but reduced it in irregular cells. It has been argued that each mEPSC represents the release of a single quantum of neurotransmitter (Edwards et al. 1990). If this is so, the lack of effect of TTX on event amplitude likely implies that each sEPSC also represents the release of a single quantum. Thus as is typical for central neurons, each action-potential-dependent sEPSC in tonic, delay, and transient cells has a low quantal content. Alternatively, if the amount of neurotransmitter released in one mEPSC may saturate the postsynaptic (AMPA) binding sites so that any increased transmitter release during a sEPSC would not be detected. Figure 6B shows that TTX increased interevent interval (decreased frequency) by 100% in tonic, 72% in delay, 149% in transient, and 80% in irregular cells. This implies about half of the sEPSCs recorded in the absence of TTX are actually mEPSCs that reflect the action-potential-independent release of neurotransmitter. The latter process, which represents ongoing activity of the release process, can be investigated by examining mEPSCs in the presence of TTX (Edwards et al. 1990). If this is so, the lack of effect of TTX on event amplitude likely implies that each sEPSC also represents the release of a single quantum. Thus as is typical for central neurons, each action-potential-dependent sEPSC in tonic, delay, and transient cells has a low quantal content. Alternatively, if the amount of neurotransmitter released in one mEPSC may saturate the postsynaptic (AMPA) binding sites so that any increased transmitter release during a sEPSC would not be detected. Figure 6B shows that TTX increased interevent interval (decreased frequency) by 100% in tonic, 72% in delay, 149% in transient, and 80% in irregular cells. This implies about half of the sEPSCs recorded in the absence of TTX are actually mEPSCs that reflect the action-potential-independent turn-over of the release process (Edwards et al. 1990) (i.e., 50% of spontaneous events in tonic cells are mEPSCs, 41% in delay cells, 60% in transient cells, and 44% in irregular cells). Sample recordings illustrate that mEPSCs (Fig. 6D) are of comparable amplitude to sEPSCs (Fig. 6C) but occur less frequently.

There was a marked similarity between CCI-induced changes in mEPSC (Fig. 5, K–R) and those seen in sEPSCs (Fig. 5, A, B, D–G, and I). Thus mEPSC amplitude and frequency were decreased in tonic cells (Fig. 5, K and O) and increased in delay and transient cells (Fig. 5, L, M, P, and Q). Because the frequency of mEPSCs in phasic cells was so low, it was not possible to prepare cumulative probability plots from the available data. Also changes in mEPSC interevent interval were not significant for irregular cells (Fig. 5K), whereas statistical significance was attained for changes in interevent interval of sEPSC (Fig. 5J). As with sEPSCs, we had to consider the possibility that the presence of silent cells, (i.e., those that failed to generate a mEPSC during 3-min recording) could bias the data to yield inaccurate cumulative probability plots. This again did not seem to be the case because similar...
proportions of all neuron types exhibited mEPSC activity in both sham-operated and CCI animals (see supplementary data).

As was done for sEPSCs, the pool of mEPSC data analyzed with KS statistics (Fig. 5, K–R) was reanalyzed using a parametric t-test (Fig. 6, G and H), and similar changes were found. Thus CCI significantly reduced mEPSC amplitude of tonic cells but increased that of delay, transient, and irregular cells (Fig. 6G). It also increased interevent interval (reduced the frequency) of mEPSCs in tonic cells but decreased the interevent interval (increased the frequency) of delay and transient cells. CCI failed to affect the mEPSC frequency of irregular cells (Fig. 6H), thus confirming the conclusion reached using the KS test (Fig. 5R).

**Discussion**

CCI produced clear changes in the intrinsic properties of the substantia gelatinosa. The most obvious effects involved alterations in spontaneous, excitatory synaptic activity. We found that tonic cells responded differently than most other cell types. Whereas excitatory synaptic drive to delay, transient, and irregular cells was increased, that to tonic cells was diminished.

CCI thus altered the amplitude and/or the frequency of mEPSC and of sEPSC in all cell types (Figs. 5 and 6). Changes in amplitude were attributed to postsynaptic effects, whereas changes in frequency were assumed to reflect presynaptic effects. Moreover, CCI-induced alterations in the frequency of mEPSCs, which were seen in the presence of TTX, likely involved a direct effect on the neurotransmitter release machinery. Because mEPSCs accounted for 41–60% of the sEPSCs recorded (Fig. 6B), alterations in the release process inevitably contributed to the overall changes in sEPSC frequency. Any additional contribution of alterations in presynaptic action potential frequency to altered sEPSC frequency was difficult to assess. This is because CCI-induced changes in sEPSC frequency (Fig. 5, F–J) almost always paralleled those seen in mEPSC frequency (Fig. 5, O–R).

Although the relationships between various neuronal morphologies, their neurotransmitter phenotypes, cytochemical markers, and electrophysiological properties are starting to be understood, there is, as yet, no universally accepted classification scheme for substantia gelatinosa neurons (Grudt and Perl 2002; Huntman et al. 2004; Heinke et al. 2004; Ruscheweyh et al. 2004; Todd and Spike 1993). We therefore used simple electrophysiological criteria to classify neurons as tonic, delay, phasic, transient, or irregular. These five categories are similar to those used by other workers in the field (Graham et al. 2004; Grudt and Perl 2002; Huntman et al. 2004; Lu and Perl 2003, 2005; Melnick et al. 2004; Safronov 1999). In confirmation of the findings of Grudt and Perl (2002), we found that delay cells had a high level of spontaneous excitatory activity (Fig. 6F). Tonic cells, like those in lamina I (Prescott and de Koninck 2002), had the lowest rheobase (see supplementary data).

**Effects of CCI on tonic cells**

Many GABAergic interneurons in lamina II correspond to islet cells that are defined morphologically by the rostrocaudal orientation of their dendritic tree (Fig. 2H) (Todd and Spike 1993). Because islet cells often display a tonic discharge pattern (Lu and Perl 2003, 2005), many tonic cells are likely to be GABAergic. This possibility is supported by the observation that intracellular stimulation of islet cells in vitro evokes monosynaptic, bicuculline-sensitive IPSCs in central neurons (Lu and Perl 2003).

CCI reduced both the amplitude and frequency of both mEPSCs and sEPSCs in tonic cells (Figs. 5, A, F, K, and O, and 6, E–H). It also reduced their membrane excitability (Fig. 4A). If one accepts the hypothesis that many tonic cells are GABAergic, a CCI-induced decrease in excitatory synaptic drive and membrane excitability fits with the finding that GABAergic tone is reduced in neuropathic pain states (Baba et al. 2003; Laird and Bennett 1992; Moore et al. 2002; Woolf and Mannion 1999). Moreover, depressed synaptic drive to inhibitory neurons may contribute to an overall increase in dorsal horn excitability.

The decrease in sEPSC frequency seen in tonic cells after CCI (Fig. 5F) may have reflected alterations in the transmitter release process because, as already mentioned, mEPSC frequency was also reduced (Fig. 5O) and mEPSCs account for 50% of the events recorded in the absence of TTX (Fig. 6B). Also it has recently been shown that sciatic CCI promotes a transient and selective loss of IB4-positive, nonpeptidergic primary afferent terminals in inner lamina II (Bailey and Ribeiro-da-Silva 2006). If these fibers selectively innervate tonic cells, their loss may provide an alternative or additional explanation for the decrease in both mEPSC and sEPSC frequency.

**Effects of CCI on delay, transient, and irregular cells**

Delay cells may function as excitatory interneurons corresponding to vertical cells with dorsoventral orientation (Fig. 2J) (Grudt and Perl 2002). These are thought to serve as output neurons conveying information to lamina I projection neurons (Lu and Perl 2005). In direct contrast to tonic cells, CCI increased all indices of spontaneous excitatory synaptic activity in delay neurons (Figs. 5, B, G, L, and P, and 6, E–H). This coincides with the possibility that an increase in the synaptic drive to delay cells would increase output to projection neurons of lamina I and thereby promote the transfer of nociceptive information.

Increased mEPSC and sEPSC frequency in delay cells presumably reflects presynaptic changes. These may include upregulation of the transmitter release mechanisms or the possible formation of new synapses (Okamoto et al. 2001). These possibilities are supported by microarray analysis after peripheral nerve injury that revealed increases in message for synaptic vesicle proteins (Xiao et al. 2002). There is also evidence for increased expression of the presynaptic vesicle protein, synaptophysin after chronic constriction injury (Chou et al. 2002).

Uncertainties regarding the contribution of increased frequency of presynaptic action potentials to the overall CCI-induced increase in sEPSC frequency have already been alluded to. If such an effect did occur, might it reflect increased action potential activity in primary afferent terminals? Although dorsal roots are acutely cut during the preparation of slices, all cell types exhibited increased sEPSC frequency in response to capsaicin. This indicates that primary afferent fibers remain viable and capable of transmitter release within the slices. We cannot therefore exclude the possibility that...
ectopic action potentials, which originate in the axons (Amir et al. 2005) or terminals of primary afferent neurons within the spinal cord, contribute to the CCI-induced increase in sEPSC frequency in delay cells. We can, however, exclude any contribution from ectopic activity in dorsal root ganglia (Wall and Devor 1983) or from the site of the injury (Görvin-Lippmann and Devor 1978; Wall et al. 1979).

Although it is possible that presynaptic mechanisms such as altered quantal size or content contribute to the increases in sEPSC and mEPSC amplitude seen in delay cells, postsynaptic mechanisms are more likely to play a major role. One possibility may involve an increased postsynaptic effectiveness of glutamate. This is because GluR2 adapter proteins, which control the function of specific AMPA receptor subtypes as well as GluR3 itself, are upregulated in neuropathic pain (Garry et al. 2003) or peripheral nerve injury (Yang et al. 2004). Interestingly, these subunits may be confined to excitatory neurons (Albuquerque et al. 1999; Kerr et al. 1998). Putative inhibitory neurons such as tonic/islet cells are thought to express the GluR1 subunit (Kerr et al. 1998), and this may not be affected by CCI in the same way as GluR2. It is therefore possible that GluR1 AMPA receptors on putative inhibitory tonic neurons are unchanged or even downregulated by CCI, whereas GluR2 AMPA receptors on putative excitatory, delay cells are upregulated. This possibility remains to be tested.

Because so little is known about the functional role of transient or irregular cells, it is difficult to attach any significance to the altered excitatory synaptic drive that they receive (Figs. 5, D, E, I, J, M, N, Q, and R, and 6, E–H).

Effects of CCI on phasic neurons

Some phasic cells may be excitatory interneurons, some of which may correspond to morphologically defined central cells (Lu and Perl 2005). It should be noted that these authors used the term transient cells to define the population we refer to as phasic cells. In mouse lamina I neurons, however, identified GABAergic neurons display both tonic and phasic discharge patterns that can interconvert depending on the current stimulus parameters (Dougherty et al. 2005). Moreover, also in mouse, but in lamina II, it has been suggested that the majority of GABAergic neurons display a phasic rather than a tonic discharge pattern (Heinke et al. 2004). These apparently conflicting findings preclude the characterization of phasic cells as a purely excitatory or inhibitory population. They may instead represent a mixed population of functional phenotypes. Because spontaneous synaptic activity was so rare in phasic cells, and the statistical analyses of sEPSC amplitude and frequency produced different results depending on the method used (Figs. 5, C and H, and 6, E and F), little can be concluded as to the effect of CCI on synaptic activity in this population. Phasic cells did, however, exhibit a marked increase in membrane excitability after CCI (Fig. 4C), and this may relate to the altered Na+,1,3 channel expression seen in dorsal horn after peripheral nerve injury (Hains et al. 2004).

Given the increase in the excitability of dorsal horn neurons that follows peripheral nerve injury in vivo (Dalal et al. 1999; Sotgiu and Biella 2000) and the presence of mechanical hypersensitivity in our experimental animals (Fig. 1), the observed changes in the properties of substantia gelatinosa neurons at first seem relatively modest. There is no change in rheobase, and only one of the five cell types exhibits a slight increase in membrane excitability (Fig. 4). Although changes in sEPSC and mEPSC amplitude and frequency attain high levels of statistical significance (Figs. 5 and 6), and all neurons, except tonic cells, may become more sensitive to glutamate, the biological significance of these differences is difficult to assess. We did not find, for example, that neurons in CCI animals generated spontaneous action potentials, whereas those from sham-operated animals did not. It should be noted, however, that our experiments were done in thin (300 μM) transverse slices of spinal cord. Thus some of the intrinsic excitatory synaptic connections between individual neurons within the substantia gelatinosa (Lu and Perl 2005) may have been compromised by the slice preparation process. Perhaps a more profound effect of CCI would have been seen had more endogenous circuitry remained intact. Also, the acute rhizotomy, which occurred as the slices were prepared, would have removed much if not all of the contribution of injury-induced spontaneous primary afferent activity to central hypersensitivity (Coderre and Katz 1997; Sotgiu and Biella 2000; Yoon et al. 1996). Had this excitatory drive been preserved, perhaps we would have found spontaneously discharging neurons in the CCI animals. Yet another possibility is that CCI affects descending brain stem control of spinal nociceptive circuits (Suzuki et al. 2002). Such changes, which would not be detected by our methodology, could also contribute to CCI-induced hyperexcitability of the dorsal horn in vivo. Last, it should be emphasized that the present study was confined to changes in excitatory synaptic transmission. Injury-induced changes in inhibitory transmission are also well documented in the superficial laminae (Coull et al. 2003, 2005), and these very likely contribute to pain centralization.

It is therefore concluded that CCI produces bona fide centralization. That is, it alters the intrinsic properties of the dorsal horn by exerting both pre- and postsynaptic effects on excitatory synaptic transmission and by attenuating inhibitory transmission. The relative contribution of intrinsic, peripheral and descending mechanisms to nerve injury-induced central hypersensitivity is yet to be determined.

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References


