Effects of Sciatic Nerve Axotomy on Excitatory Synaptic Transmission in Rat Substantia Gelatinosa

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Chen Y, Balasubramanyan S, Lai AY, Todd KG, Smith PA. Effects of sciatic nerve axotomy on excitatory synaptic transmission in rat substantia gelatinosa. J Neurophysiol 102: 3203–3215, 2009. First published September 30, 2009; doi:10.1152/jn.00296.2009. Injury or section of a peripheral nerve can promote chronic neuropathic pain. This is initiated by the appearance and persistence of ectopic spontaneous activity in primary afferent neurons that promotes a secondary, enduring increase in excitability of sensory circuits in the spinal dorsal horn (“central sensitization”). We have previously shown that 10–20 days of chronic constriction injury (CCI) of rat sciatic nerve promotes a characteristic “electrophysiological signature” of pattern of changes in synaptic excitation of five different electrophysiologically defined neuronal phenotypes in the substantia gelatinosa of the dorsal horn. Although axotomy and CCI send different signals to the dorsal horn, we now find, using whole cell recording, that the “electrophysiological signature” produced 12–22 days after sciatic axotomy is quite similar to that seen with CCI. Axotomy thus has little effect on resting membrane potential, rheobase, current–voltage characteristics, or excitability of most neuron types; however, it does decrease excitatory synaptic drive to tonic firing neurons, while increasing that of inhibitory neurons. Further analysis of spontaneous and miniature (tetrodotoxin-resistant) excitatory postsynaptic currents is consistent with the possibility that decreased excitation of tonic neurons reflects loss of presynaptic contacts. By contrast, increased excitation of “delay” neurons may reflect increased frequency of discharge of presynaptic action potentials. This would explain how synaptic excitation of tonic cells decreases despite the fact that axotomy increases spontaneous activity in primary afferent neurons.

INTRODUCTION

Peripheral nerve injury can promote chronic neuropathic pain. This involves an enduring increase in excitability of sensory circuits in the spinal dorsal horn that leads to the phenomenon of “central sensitization” (Millan 1999; Sandkühler 2009; Woolf 1983). Clinically, a variety of types of traumatic nerve injury are observed. These range from complete severance of a nerve, as might be seen in amputation of a limb, to the more commonplace constriction injury that accompanies a traumatic insult and/or a disease state. These two situations are modeled in experimental animals by complete section of the sciatic nerve (axonotmesis) or by various types of chronic constriction injury (CCI) (Kim et al. 1997; Mosconi and Kruger 1996). Although both types of injury can produce chronic pain in humans, axotomy and CCI send quite different signals to the CNS and may therefore promote different types of changes in the spinal dorsal horn (Moore et al. 2002). CCI is thought to affect primary Aβ and Aδ fibers while leaving C-fibers relatively unscathed (Basbaum et al. 1991; Kajander and Bennett 1992). As well, axons close to the edge of a nerve trunk are likely to be severed or to receive some degree of crush injury. Their distal portions undergo Wallerian degeneration as a result of their disconnection from cell bodies in the dorsal root ganglia (DRG). By contrast, axons in the center of the nerve may escape injury altogether. It has been suggested that the release of inflammatory mediators from degenerating axons promotes changes in surviving axons (Shamash et al. 2002). These surviving axons, their terminals, and cell bodies become hyperexcitable and generate ectopic discharges (Amir et al. 2005; Ma et al. 2003). Although there is some debate as to the number and type of fibers that need to be active (Devor 2006; Sandkühler 2009), it is generally held that the arrival of aberrant and sustained sensory activity in the dorsal horn is a primary trigger for the initiation of central sensitization and pain (Sheen and Chung 1993). This process involves the release of various mediators, including adenosine 5’-triphosphate (ATP), from primary afferent terminals and the activation of spinal microglia (Trang et al. 2009; Tsuda et al. 2003, 2005; Xie et al. 2009). These, in turn, release additional mediators such as brain-derived neurotrophic factor (BDNF), which promotes an enduring increase in dorsal horn excitability (Coulil et al. 2005; Lu et al. 2007, 2009).

Because virtually all axons in the sciatic nerve are severed by axotomy, L4–L5 spinal cord neurons no longer receive input from their normal peripheral receptive field (Devor and Wall 1981). Despite this, the proximal segments of the severed axons, which remain attached to their cell bodies in the DRG, remain viable. Although these proximal axons “die back” from the site of transection, degeneration within the first few weeks of injury is minimal (Tandrup et al. 2000). Thus severed axons are not exposed to an environment in which active Wallerian degeneration is taking place. It might thus be argued that axotomy is a weaker stimulus for central sensitization than CCI. Moreover, because ion channel expression in peripheral neurons is dependent on target contact and neurotrophic support (Lai et al. 1997; Petrov et al. 2001), it is possible that axotomy, by downregulating Ca2+ channels, may impede the release of neurotransmitters from primary afferent terminals in the dorsal horn (Baccei and Kocsis 2000). This contrasts with the effects of CCI, reported to increase the expression of N-type Ca2+ channels in the dorsal horn (Cizkova et al. 2002). Despite these differences, it is well established that axotomy, like CCI, increases spontaneous activity in both primary afferent fibers (Govrin-Lippmann and Devor 1978; Wall and Devor...
1983; Zhang et al. 1997) and in dorsal horn neurons (Dalal et al. 1999).

We have shown that 10–20 days of sciatic nerve CCI produces a characteristic “electrophysiological signature” or pattern of changes in synaptic excitation of five different electrophysiologically defined neuronal phenotypes in the rat substantia gelatinosa. Although CCI increases excitatory synaptic drive to four of the five neuronal types, excitation of a large population of neurons—those that exhibit a tonic discharge pattern—is diminished (Balasubramanyan et al. 2006). Because the onset of this “electrophysiological signature” coincides with the appearance of mechanical allodynia and hyperalgesia (Balasubramanyan et al. 2006), it is likely the basis for the “central sensitization” that accompanies CCI.

The present study explores the general applicability of the nerve-injury–induced “electrophysiological signature” to the phenomenon of central sensitization. It was especially instructive to examine the effect of sciatic axotomy because it is impossible to determine, using behavioral tests, whether axotomy produces “central sensitization.” This is because tests of mechanical allodynia or thermal hyperalgesia are precluded when all relevant primary afferent fibers are severed by axotomy. Thus from a clinical perspective, the present work explores the etiology of total nerve section that may produce “phantom limb pain,” as opposed to nerve-trauma–induced neuropathic pain. A preliminary report of some of this work was previously published (Chen and Smith 2008).

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

For axotomy, the left sciatic nerve of 19-day-old rats was exposed at midthigh level under isoflurane anesthesia and a 5-mm segment of sciatic nerve was removed. The wound was closed with silk sutures (Abdulla and Smith 2001a). Visual examination of sciatic nerves 12–22 days after surgery failed to reveal any obvious evidence that regeneration had occurred and that severed axons had grown into the peripheral nerve stumps. For sham surgery, animals were anesthetized with isoflurane and the sciatic nerve was exposed, but not deliberately manipulated. Some of the immunohistochemical data (Fig. 2) were obtained from animals after 10–20 days CCI. These animals were subject to sciatic constriction using polyethylene cuffs (Mosconi and Kruger 1996). Detailed methods for the surgical procedures were previously published (Balasubramanyan et al. 2006).

Acute slice preparation

Electrophysiological analysis of the effects of axotomy was carried out on animals 12–22 days after surgery. Rats were anesthetized with an overdose of intraperitoneally (ip) administered urethane (1.5 g/kg). Laminectomy was performed after the cessation of respiration and complete loss of ocular and nociceptive reflexes. The entire spinal cord, from the sacral to cervical region, was removed. Cardiac ventricles were punctured with scissors prior to animal disposal. The lumbar enlargement of the spinal cord (from L2 or L3 to L6 or S1) was removed and glued with cyanoacrylate glue (“Vetbond”; WPI, Sarasota, FL) to a trapezoid-shaped block cut from 4% agar. This block, with the attached piece of 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, 1.3 MgSO4, 1.2 NaH2PO4, 1.5 CaCl2, 5 MgCl2, 25 t-glucose, and 1 kynurenic acid, continuously bubbled with 95% O2-5% CO2. Transverse slices (300 μm) were cut using a vibrating microtome (Vibratome; TPI, St. Louis, MO). The first few slices from the L2 and/or L3 region were discarded and slices for study were taken from the L4–L5 region. All recordings in axotomized 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 axotomy or sham lesion.

Electrophysiology

For recording, slices were superfused at room temperature (~22°C) with 95% O2-5% CO2 saturated artificial cerebrospinal fluid, which contained (in mM): 127 NaCl, 2.5 KCl, 1.2 NaH2PO4, 26 NaHCO3, 1.3 MgSO4, 2.5 CaCl2, and 25 t-glucose (pH 7.4). The substantia gelatinosa appeared as translucent bands under infrared differential-interference optics and neurons were patched under 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).

Recordings were made using an SEC-05LX amplifier (NPI Electronics, Tamm, Germany) in discontinuous, single-electrode, current-clamp, or voltage-clamp mode. Data were collected only from neurons that exhibited clear overshooting action potentials (APs) 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 (see Fig. 3).

Current–voltage (I–V) relationships were determined under voltage-clamp using a series of 200-ms voltage commands. Current and voltage were measured just prior to the termination of each voltage pulse. Recorded voltage rather than command voltage was used to construct I–V plots. 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 100 or 133 pA/s. Cumulative latencies for the first, second, third, and subsequent APs were noted (see Fig. 4). Data were acquired and analyzed using pCLAMP 9.0 (Axon Instruments/Molecular Devices, Sunnyvale, CA). Statistical comparisons were made with unpaired t-tests and 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 postsynaptic currents (mEPSCs), which reflect AP-independent release of neurotransmitter (Edwards et al. 1990; Fredj and Burrone 2009; Sara et al. 2005), were recorded at −70 mV in the presence of 1 μM tetrodotoxin (TTX). The effectiveness of TTX was confirmed by observing its blockade of APs generated by depolarizing current pulses. Spontaneous excitatory postsynaptic currents (sEPSCs), which reflect both AP-dependent and AP-independent release of neurotransmitter (Edwards et al. 1990), were recorded in the same way but without TTX. Figure 1A shows a typical recording of sEPSC data. Mini Analysis Program (Synaptosoft, Decatur, GA) was used to distinguish sEPSCs and mEPSCs from baseline noise. A typical sEPSC at high gain and high sweep speed is illustrated in Fig. 1B. Spontaneous or miniature postsynaptic currents were detected automatically by setting appropriate amplitude and area thresholds for each neuron. All detected events were then reexamined and visually accepted or rejected based on visual examination. Acceptable events such as that illustrated in Fig. 1B had a sharp onset and exponential offset, a total duration of <50 ms, and an amplitude at least double the baseline noise. The Mini Analysis Program was used to further analyze the data and to provide spreadsheets for the generation of cumulative probability plots. These compared the amplitude and
interevent intervals (IEIs) of sEPSCs and mEPSCs in neurons from sham-operated animals and those subject to sciatic nerve axotomy. The Kolmogorov–Smirnov (K-S) two-sample test was used to compare distributions of amplitudes and IEIs (Prescott and de Koninck 2002). Distributions were considered significantly different if $P < 0.05$. For each neuron, sEPSCs or mEPSCs 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. In most cases, analysis was carried out on the first 100 events observed in each recording session regardless of the time taken for these events to occur (Fig. 1A). For neurons that failed to generate 100 events in 3 min, data were analyzed from all events observed. Because they generally had less spontaneous activity than that of other cell types, 50 events were analyzed in phasic and irregular neurons. Details of the numbers of neurons and events analyzed are shown in the legends to Figs. 5 and 6.

Iba1 immunohistochemistry

As was done for electrophysiological studies, rats were anesthetized with an overdose of urethane (1.5 g/kg, ip) and laminectomy was performed after the cessation of respiration and loss of ocular and nociceptive reflexes. The entire spinal cord, from the sacral to cervical region, was removed and slices were freshly isolated from the lumbar enlargement. Thus the slices used for immunohistochemical analysis and for electrophysiological studies were obtained from the same lumbar segments. A few additional slices were prepared from the thoracic region of the cords. All slices were fixed in 10% formalin solution for 5 min. Fixed slices were then incubated for 30 min in a blocking solution containing 10% horse serum, 0.03% hydrogen peroxide, and 2.5% Triton in phosphate buffered saline (PBS). After blocking, the slices were incubated in a primary antibody solution containing 1:1,000 anti–ionized calcium binding adaptor molecule 1 (Iba1, #01-1974; Wako), which labels microglia, 1% horse serum, and 2.5% Triton in PBS at 4°C overnight. The slices were then washed and incubated with the secondary antibody biotinylated immunoglobulin G (1:200, Serotec) for 30 min followed by further washings and incubation with horseradish peroxidase–conjugated streptavidin (1:200, Vector Laboratories, Burlingame, CA) for 30 min (both were diluted in PBS containing 1% horse serum). The labeling was visualized using diaminobenzidine–hydrogen peroxide (Sigma). Incubation with secondary antibody alone failed to generate any noticeable labeling.

RESULTS

Microglial activation

Activation of spinal microglia is one of the primary consequences of sciatic nerve injury (Coull et al. 2005; Tsuda et al. 2003, 2005; Zhang and de Koninck 2006). To compare the effects of axotomy and CCI, we used the microglial-specific calcium binding protein Iba1 as a microglial marker. Although this protein is present in resting microglia, it is upregulated
when they are activated (Ito et al. 1998; Lai and Todd 2008). The Iba1 antibody used in our experiments exclusively labels microglia and does not colocalize with microtubule-associated protein 2 in neurons, glial fibrillary acidic protein in astrocytes, or 2',3'-cyclic nucleotide 3'-phosphohydrolase in oligodendrocytes (see Supplemental Figs. S1, S2, and S3). Photomicrographs of the dorsal horn ipsilateral or contralateral to the CCI, axotomy, or sham lesion are shown in Fig. 2. A–F. The pattern of Iba1 staining for microglia produced by axotomy is compared with that produced by CCI. Iba1 staining is especially dense in ipsilateral superficial laminae after CCI compared with sham surgery (Fig. 2, A and C) or to the contralateral side (Fig. 2, D and F); however, axotomy produces a different pattern because the ipsilateral staining is spread more deeply and is associated with the central canal (Fig. 2B). The contralateral control is shown in Fig. 2E.

These trends of microglial activation were confirmed by a more quantitative analysis. Data were collected from four sham-operated animals (14–21 days postsurgery), four subject to sciatic CCI (15–30 days postsurgery), and four subject to sciatic axotomy (21 days postsurgery). The dorsal horn was divided into a standard set of six 0.5 × 0.5-mm squares (Fig. 2G) and Iba1-positive profiles, such as those illustrated in Fig. 2H, counted for each square. Four sections were analyzed per animal for sham, axotomy, and CCI conditions and cell counts varied <5% between two independent evaluators. Results are plotted in Fig. 2I. This confirms that Iba1 staining was increased for both CCI and axotomy in ipsilateral quadrant 2 (ipsi 2), which corresponds to the superficial dorsal horn. Even sham-operated animals exhibited increased staining in this quadrant but not in other parts of the superficial dorsal horn. By contrast, axotomy had a more pronounced effect in ipsilateral quadrants 4 and 5 (ipsi 4 and 5), corresponding to deeper laminae and to the midline portion of the cord. There was no clear effect of CCI or axotomy on Iba1-positive profiles in the ipsilateral ventral horn (ventral) or on the contralateral quadrant of the dorsal horn (contra) that mirrored ipsi 4. Although no detailed analysis was made, it was noticed that the nerve-injury–induced pattern of Iba1 staining was also seen in sections from the lumbar spinal cord above the level of the lesion. This may reflect the considerable rostral and caudal projections of primary afferent fibers (Wall and Werman 1976).

**Behavioral consequences of axotomy**

Sciatic nerve axotomy frequently induces a self-mutilatory behavior known as autotomy, which may relate to the onset of neuropathic pain (Coderre et al. 1986; Wall et al. 1979). In a previous study using 32- to 40-day-old rats, we found that a modest amount of autotomy was present in 45% of animals at 2 wk after axotomy (Abdulla and Smith 2001a). The present study, however, was constrained by the need to use young animals (19-day-old) for electrophysiological analysis of spinal cord neurons and we found no evidence of autotomy in any of the animals studied. We did find, however, that 2 wk of CCI produce mechanical hyperalgesia and allodynia in animals that are 20 days old at the time of surgery (Balasubramanyan et al. 2006).

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**FIG. 2.** Ionized calcium binding adaptor molecule 1 (Iba1) staining of microglial cells in rat spinal cord slices. A–C: staining of dorsal quadrant of slice ipsilateral to chronic constriction injury (CCI, A), axotomy (B), or sham surgery (C). D–F: staining of dorsal quadrant of slice contralateral to CCI (D), axotomy (E), or sham surgery (F). The 100-µM scale bar in E applies to A–F of the figure. * Denotes fasciculus gracilus. Dotted line represents gracilus/dorsal horn border. G: photomicrograph of whole spinal cord slice showing 6 standard locations of 500-µM squares used for cell counting (V, ventral). H: high-power image showing Iba1-positive profiles. I: graphical representation of distribution of Iba1-positive cells in the various quadrants defined in G. Points represent mean counts from a total of 16 slices (4 slices from 4 animals) under each condition. Error bars are SE. Contra 4 is the contralateral mirror image of quadrant ipsi 4.

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1 The online version of this article contains supplemental data.
Neuron types and effects of axotomy

In our previous studies of the effects of CCI on substantia gelatinosa, neurons were classified into five different types, depending on their firing pattern in response to depolarizing current as tonic, delay, phasic, transient, or irregular (Balasubramanyan et al. 2006). More recently, it was noted that the delay category included two subgroups of neurons: delay irregular neurons and delay tonic neurons (Lu et al. 2009). Firing patterns of tonic, delay irregular, delay tonic, phasic, transient, and irregular neurons are illustrated in Fig. 3, A–F. The three rows of traces illustrate the firing patterns evoked by increasing current intensities, as indicated in the superimposed current traces (Fig. 3, bottom).

Figure 4A illustrates the percentage contribution of each of the six neuronal types to the whole population of neurons studied from sham animals (n = 148) and from animals subject to sciatic nerve axotomy (n = 74). The percentage contribution of each neuron type to the population was unaffected by axotomy ($\chi^2$ test, $P > 0.4$ for all comparisons). With the exception of delay tonic neurons, that were slightly depolarized, axotomy had no statistically significant effect on the resting membrane potential (RMP) of substantia gelatinosa neurons (Fig. 4B). Axotomy also failed to significantly affect the rheobase (Fig. 4C).

Excitability, as measured from the cumulative latency of APs in response to a depolarizing current ramp command (Fig. 4D), was slightly reduced in phasic neurons but unchanged in all other neuron types. Data for tonic, delay irregular, delay tonic, phasic, and irregular neurons are shown in Fig. 4, E–I. Excitability of transient neurons was not examined because they did not always fire in response to a depolarizing ramp command.

Figure 4, J–N illustrates the effect of axotomy on current–voltage relationships of tonic, delay irregular, transient, phasic, and irregular neurons. Although there appears to be a small reduction in outward current in irregular neurons (Fig. 4N), I–V relationships of the other four neuron types are scarcely affected. Insufficient data were available to present I–V plots for delay tonic neurons.

Effects of axotomy on spontaneous synaptic activity

It was neither necessary nor desirable to pharmacologically isolate the sEPSCs or mEPSCs by using $\gamma$-aminobutyric acid (GABA)/glycine antagonists. This is because spontaneous and miniature inhibitory postsynaptic currents (sIPSCs and mIPSCs, respectively) would be expected to appear as small outward currents at $-70$ mV because the estimated $E_{Cl}$ for the Cl$^-$ concentrations used in our study was $-80$ mV. This is illustrated in Fig. 1C. As well as sEPSCs, occasional small outward currents, assumed to be sIPSCs, occur at $-70$ mV. This recording was derived from a phasic neuron after axotomy. As illustrated in Fig. 1, D and E, we also found that 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 5 $\mu$m) eliminated all spontaneous inward current activity at $-70$ mV. These recordings were made from a sham delay neuron. When the holding potential was changed to 0 mV in the presence of CNQX, occasional spontaneous outward currents were detected (Fig. 1F). These were sIPSCs because they were attenuated by the GABA antagonist 4-[6-imino-3-(4-methoxyphenyl)pyridazin-1-yl]butanoic acid (SR95531 [gabazene]; 10 $\mu$m, Fig. 1G). To further demonstrate that the sEPSCs recorded at $-70$ mV were

![Fig. 3. A–F: firing patterns of tonic, delay irregular, delay tonic, phasic, transient, and irregular neurons illustrated by their response to a series of 3 depolarizing current commands from a preset membrane potential of $-60$ mV. Superimposed current records are shown in bottom row of traces and resulting action potential (AP) discharge for 3 different current intensities shown in the top 3 rows. All recordings were from neurons in sham-operated animals. Current, voltage, and time calibrations in F apply to all current and voltage records.](http://jn.physiology.org/)

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not contaminated with sIPSC activity, we examined the effect of strychnine (1 μM) plus SR95531 (10 μM). Data are presented as cumulative probability plots for sIPSCs in a delay neuron from a sham-operated animal in Fig. 1, H and I. Blockade of GABA and glycine receptors failed to affect the amplitude distribution or IEI of sEPSCs recorded at −70 mV. In some neurons, however, the antagonist mixture actually increased sEPSC frequency. This may perhaps reflect impairment of presynaptic inhibition or disinhibition of presynaptic excitatory neurons. Since their effects on the frequency of sEPSCs were quite variable, GABA/glycine antagonists were not used in our study.

Effects of axotomy on sIPSCs were not considered because under our experimental conditions, using acutely isolated transverse slices, sIPSCs were infrequent and of low amplitude. Moreover, some substantia gelatinosa neurons failed to exhibit recordable sIPSCs. For example, only 16 of 35 tonic neurons from sham-operated animals exhibited sIPSCs at −40 mV. The paucity of sIPSCs can be appreciated by comparing the recordings from a sham-operated delay neuron at −70 mV (Fig. 1D) with those from the same neuron at 0 mV (Fig. 1F); although numerous sEPSCs were seen at −70 mV, only six sIPSCs were seen at 0 mV.

**Effects of axotomy on sEPSCs**

As we saw with CCI (Balasubramanyan et al. 2006; Lu et al. 2009), the predominant effect of axotomy was to alter excitatory spontaneous synaptic activity. As in our previous studies, we analyzed changes in sEPSCs by comparisons of average data values using t-tests and by cumulative probability plots and K-S statistics. For this analysis, we reverted to the classification scheme used by Balasubramanyan et al. (2006). Thus data from “delay irregular” (Fig. 3B) and “delay tonic” (Fig.
neurons were combined into the delay neuron category. This enabled more meaningful comparison between the present results with axotomy and our previous studies of the effects of CCI (Balasubramanyan et al. 2006).

The majority of neurons exhibited sEPSCs and there was no significant change in the fraction of "silent" neurons in slices from axotomized animals. Thus for the tonic category, 3/57 neurons were silent in shams and 4/34 were silent in the axotomy group (P > 0.4, χ2 test); all delay neurons, in both the sham (n = 20) and axotomy (n = 14) groups, exhibited spontaneous activity; all sham phasic neurons exhibited sEPSCs (n = 29) and only 1/14 was silent in the axotomy group (P > 0.7, χ2 test); for the transient category, 1/12 neurons was silent in shams, and 1/33 was silent in the axotomy group (P > 0.4, χ2 test).

A clear pattern of axotomy-induced changes emerged with regard to the IEI (Fig. 5, A and C–G). The IEI for sEPSCs was increased in tonic neurons, unchanged in irregular cells, and decreased in all other neuron types. These effects were significant according to both t-test (Fig. 5A) and K-S statistics (Fig. 5, C–F). Increased IEI in tonic neurons meant that sEPSC frequency decreased (Fig. 5C), whereas the decrease in IEI in delay, phasic, and transient neurons meant that frequency increased (Fig. 5, D–G).

Effects of axotomy on sEPSC amplitude were less obvious and more complex. Although the mean amplitude of sEPSCs in tonic neurons was unchanged (Fig. 5B), data from the cumulative probability plot (Fig. 5H) suggested that the proportion of large events increased, whereas the small events decreased, yielding a significant difference on the K-S test. The decrease in sEPSC amplitude seen in delay cells was significant according to both a t-test (Fig. 5B) and a K-S test (Fig. 5I). The K-S test also yielded significant increases in sEPSC amplitude for phasic and transient neurons (Fig. 5, J and K) and a significant decrease for irregular neurons (Fig. 5L). However, none of these changes was significant according to a t-test (Fig. 5B).

Effects of axotomy on TTX-resistant mEPSCs

The sEPSC population includes both mEPSCs, which are AP independent and reflect the turnover of the release process, and spontaneous events resulting from presynaptic AP activity (Edwards et al. 1990; Fredj and Burrone 2009; Sara et al. 2005). We therefore examined the effects of axotomy on mEPSCs to test whether it exerted an action that was independent of presynaptic AP activity. This analysis was restricted to tonic, delay, phasic, and transient neurons because insufficient data were available from irregular neurons. Despite the addi-

FIG. 5. Effects of axotomy on spontaneous synaptic activity (sEPSC) in substantia gelatinosa neurons. A: effects on mean IEI of 5 defined neuron types; holding potential was −70 mV. B: effects on mean sEPSC amplitude of same neuron groups (*P < 0.05, #P < 0.001, Student’s two-tail t-test). C–L: cumulative probability plots of sEPSC IEI and amplitude data from tonic, delay, phasic, and irregular neurons as indicated. P values from K-S tests indicated on graphs. Data in bar graphs (A and B) obtained from same data sets as cumulative probability plots. For tonic sham neurons, a total of 3,732 events were analyzed from 54 neurons (20–100 events/neuron); for tonic axotomy neurons, 1,770 events were analyzed from 30 neurons (20–100 events/neuron); for sham delay neurons, 1,516 events were analyzed from 18 neurons (30–100 events/neuron); for axotomized delay neurons, 1,495 events were analyzed from 14 neurons (30–100 events/neuron); for sham phasic neurons, 1,001 events were analyzed from 29 neurons (10–50 events/neuron); for axotomized phasic neurons, 667 events were analyzed from 14 neurons (15–50 events/neuron); for sham transient neurons, 2,146 events were analyzed from 33 neurons (10–100 events/neuron); for axotomized transient neurons, 940 events were analyzed from 12 neurons (10–100 events/neuron); for sham irregular neurons, 438 events were analyzed from 14 neurons (12–50 events/neuron); for axotomized irregular neurons, 142 events were analyzed from 4 neurons (20–50 events/neuron).
tion of TTX, the mean amplitudes of mEPSCs were similar to those of sEPSCs (compare Figs. 5B and 6B; see Supplemental Fig. S4). This similarity has been described in several types of central neurons, including hippocampal CA3 pyramidal cells (McQuiston and Colmers 1996) and in supraoptic neuroendocrine cells of the rat hypothalamus (Wuarin and Dudek 1993). It likely implies a small quantal content and low release probability of AP-evoked events at central synapses.

Axotomy decreased mEPSC frequency (increased IEI) in tonic neurons (Fig. 5, A and C). This effect—which was significant according to both a t-test (Fig. 6A) and a K-S test (Fig. 6C)—contrasted with effects seen in all other cell types. Although data from cumulative probability plots and a K-S test for delay neurons (Fig. 6D) were consistent with an increase in mEPSC frequency, analysis of mean frequency using t-test (Fig. 6A) failed to reveal a significant difference. We therefore suggest that axotomy produced only a weak tendency for mEPSC frequency to increase. Increases in mEPSC frequency for phasic and tonic neurons were significant according to both K-S tests (Fig. 6, E and F) and t-test (Fig. 6A).

Axotomy decreased mEPSC amplitude in tonic and phasic neurons (Fig. 6, B, G, and I), increased it in transient neurons (Fig. 6, B and J), but did not affect mEPSC amplitude in delay neurons (Fig. 6, B and I). Differences that were significant according to a K-S test (Fig. 6, G, I, and J) were also significant according to a t-test (Fig. 6B).

Comparison of axotomy effects on sEPSC and mEPSC frequencies

To distinguish possible effects of axotomy on the frequency of presynaptic APs from other effects on presynaptic terminals, we compared its actions on mEPSC frequency with its effect on sEPSC frequency. In tonic cells, axotomy decreased both sEPSC and mEPSC frequencies (increased IEI; Fig. 7). By contrast, in delay neurons, axotomy increased sEPSC frequency (decreased IEI; Fig. 7) but had little or no effect on mEPSC frequency. This suggests that increases in the frequency of presynaptic APs may explain the axotomy-induced reduction of IEI of sEPSCs in delay neurons. Axotomy reduced the IEI of sEPSCs and mEPSCs in phasic and transient neurons by about the same amount (Fig. 7).

DISCUSSION

The essential finding of this study is that sciatic nerve axotomy produces perturbations in excitatory synaptic transmission in the substantia gelatinosa that resemble those seen following sciatic CCI (Balasubramanyan et al. 2006). In other words, when changes in tonic, delay, phasic, transient, and irregular neurons are considered, the “electrophysiological signature” of axotomy resembles that of CCI. Further analysis of the data is consistent with the possibility that axotomy increases excitatory synaptic drive to excitatory neurons yet...
decreases that to inhibitory neurons. Both types of change, which are also seen with CCI (Balasubramanyan et al. 2006), could contribute to the overall increase in dorsal horn excitability that follows peripheral nerve injury (Dalal et al. 1999; Woolf 1983).

Comparison of the effects of axotomy and CCI

Neither axotomy nor CCI has any major effect on excitability or current–voltage ($I$–$V$) characteristics of substantia gelatinosa neurons (Fig. 4 compared with Balasubramanyan et al. 2006). The similar effects of the two manipulations on synaptic transmission are illustrated in Fig. 8, which shows the percentage changes in four indices of synaptic transmission in various neuronal types. As seen with CCI (Balasubramanyan et al. 2006), the effects of axotomy on tonic neurons are qualitatively different from its action on other neuron types. IEIs of both sEPSC (Fig. 8A) and mEPSC (Fig. 8B) are increased by both axotomy and CCI in tonic neurons. By contrast, both manipulations lead to a decrease of IEIs in delay, phasic, and transient neurons.

There is less correspondence between the effects of axotomy and those of CCI on sEPSC amplitude (Fig. 8C). Axotomy-induced changes in sEPSC amplitude were small (Fig. 8C) and those for tonic, phasic, transient, and irregular neurons failed to reach significance on a t-test (Fig. 5B). We therefore suggest that axotomy exerted only minimal effects on sEPSC amplitude. This contrasts with the more obvious effects of CCI on sEPSC amplitude (Balasubramanyan et al. 2006; data are also illustrated in Fig. 8C). This difference may be explicable in terms of differential effects of axotomy and CCI on Ca$^{2+}$ channels in primary afferent terminals. Although CCI has been reported to increase expression of N-type Ca$^{2+}$ channels in spinal cord (Cizkova et al. 2002), the effect of axotomy on these channels remains to be investigated. Axotomy decreases Ca$^{2+}$ channel currents in DRG cell bodies and may exert a similar effect at presynaptic terminals (Abdulla and Smith 2001b; Baccei and Kocsis 2000). Alternatively, the relatively

![FIG. 7. Comparison of the effects of axotomy on the IEI of mEPSCs with its effect on the IEI of sEPSCs. Percentage changes calculated from the mean values shown in Figs. 5A and 6A.](image-url)
small effects on sEPSC amplitude may reflect the probability that axotomy is associated with less peripheral inflammation than that of CCI (see Introduction) or that it is less effective than CCI in increasing BDNF concentration in substantia gelatinosa (see following text).

For tonic neurons, the axotomy-induced decrease in mEPSC amplitude (Figs. 6, B and G and 8D) parallels the effects of CCI (Balasubramanyan et al. 2006). Axotomy does not increase mEPSC amplitude in delay neurons (Fig. 6, B and H), whereas small increases are seen after CCI (Fig. 8D; Balasubramanyan et al. 2006). This again may reflect a “weaker” effect of axotomy compared with that of CCI, perhaps as a consequence of the smaller amount of inflammation seen with axotomy compared with that seen with CCI.

Although, as mentioned in the Introduction, axotomy and CCI may send different types of information to the dorsal horn, both are capable of increasing aberrant spontaneous activity in primary afferent neurons (Amir et al. 2005; Govrin-Lippmann and Devor 1978; Ma et al. 2003; Wall and Devor 1983). Since their “electrophysiological signatures” in the substantia gelatinosa are so similar, it is likely that increased primary afferent activity is a major contributor to central sensitization for both types of injury. One small exception to this generalization is the difference between the effects of axotomy and CCI on mEPSC amplitude in transient cells (Fig. 8D). Sufficient data were not available to make comparisons for phasic and irregular neurons.

Dendritic location of synaptic inputs

One issue that deserves mention in connection with our analysis is that we have access to synaptic events only as they appear in the cell body. Since synaptic connections are often located on dendrites, altered transmission at these remote sites may not be accurately detected. It is thus possible that very small, slow synaptic events, which would not meet our criteria for bona fide sEPSCs or mEPSCs, may contain additional information about the effects of axotomy on synaptic transmission. It is also possible that alterations in dendritic cable properties following axotomy or CCI may alter the effectiveness of transfer of remote synaptic events to the cell body. Analysis of such phenomena is not feasible with the technology currently available. This issue may also have a bearing on the paucity of sIPSCs in the somata of lamina II neurons (see following text) as well as our inability to detect changes in excitability (see preceding text) because it is possible that changes in active Na\(^+\), K\(^+\), or Ca\(^{2+}\) conductances in dendrites may play a role in central sensitization.

Changes in inhibition?

It is well established that impediment of inhibition in the dorsal horn contributes to the onset of central sensitization (Coull et al. 2003; Laird and Bennett 1992; Moore et al. 2002; Sandkuhler 2009; Scholz et al. 2005). This is thought to involve downregulation of the Cl\(^-\) transporter KCC2 and collapse of the Cl\(^-\) concentration gradient such that GABA and glycine may produce smaller hyperpolarizing responses or even depolarizing responses (Coull et al. 2005; Keller et al. 2007; Miletic and Miletic 2008; Prescott et al. 2006). It should be noted, however, that these changes have been studied primarily in lamina I. By contrast, the role of postsynaptic inhibition in the physiology of lamina II is currently a matter of debate (Lu 2008). Although some authors (Labrakakis et al. 2009; Santos et al. 2007) argue that inhibition is of minor importance in this region, others have underlined the importance of specific inhibitory pathways (Lu and Perl 2003). Nevertheless, the apparent paucity of sIPSCs in substantia gelatinosa neurons seen under our experimental conditions led us to concentrate on the effects of axotomy on excitatory transmission. It is of course possible that some of the changes we observed result from nerve-injury-induced alterations in presynaptic inhibition of excitatory primary afferent transmission (Laird and Bennett 1992). Another possibility is that input to lamina II is altered following changes in inhibition in deeper laminae, where inhibition seems to play a greater role than that of excitation (Schneider 2008). These possibilities remain to be tested.

Another way in which peripheral nerve injury may impede inhibition involves apoptosis of GABAergic interneurons (Scholz et al. 2005). Since the percentage contribution of tonic neurons to the whole population was not reduced by axotomy (Fig. 3A), apoptosis may not have occurred under our experimental conditions. The difference between our observations and those of Scholz et al. (2005) may reflect our use of axotomy rather than various types of constriction injury. These authors also noted that apoptosis of GABAergic interneurons takes about 4 wk to fully develop, whereas in our experiments animals were subject to axotomy for <22 days.

BDNF, microglia activation, and peripheral nerve injuries

Several lines of evidence implicate microglial-derived BDNF in the onset of central sensitization (Coull et al. 2003; Lu et al. 2007, 2009; Millan et al. 2009; Trang et al. 2009; Tsuda et al. 2003, 2005). Thus we have also compared the effects of prolonged BDNF exposure on excitatory synaptic transmission with those of axotomy and CCI (Fig. 8). BDNF data were obtained from our previously published work, in which spinal cord organotypic cultures were exposed to BDNF for 5–8 days (Lu et al. 2007, 2009). In terms of IEI, the effects of BDNF nicely parallel the actions of both axotomy and CCI in both sEPSCs and mEPSCs (Fig. 8, A and B). There is, by contrast, much less correspondence between the actions of the three manipulations on event amplitude (Fig. 8, C and D). There appears to be more similarity between the actions of BDNF and CCI than that with BDNF and axotomy. In this regard, it is pertinent to mention that the differential patterns of microglial activation produced by axotomy compared with those produced by CCI are paralleled by their differential effects on the pattern of BDNF immunoreactivity (Cho et al. 1998). Thus CCI increases both Iba1 activation (Fig. 2A) and BDNF immunoreactivity in superficial laminae (Cho et al. 1998), whereas axotomy is more effective in increasing both neurotrophin content and microglial activation in deeper laminae (Fig. 2B and Cho et al. 1998). These differences may reflect the different patterns of primary afferent fiber damage seen with axotomy compared with those seen with CCI (Basbaum et al. 1991; Kajander and Bennett 1992) and the different sites of termination of these fibers throughout the dorsal horn (Mirnics and Koerber 1997; Todd and Koerber 2006).
Because there are now several reports describing actions of BDNF on voltage-gated Na\(^+\) and K\(^+\) channels, it is perhaps surprising that no marked changes in neuronal excitability were seen in either the present study, in that of Balasubramanyan et al. (2006), or in our previous studies of BDNF effects in the dorsal horn (Lu et al. 2007, 2009). Although this apparent discrepancy may be conveniently ascribed to the use of expression systems in some studies (Ahn et al. 2007; Colley et al. 2007), and to differential effects of acutely and chronically applied BDNF, a 2-h application of this neurotrophin has been reported to promote a marked increase in excitability of auditory brain stem neurons (Youssoufian and Walmsley 2007). Long-term application of BDNF, however, does not exert much effect on Na\(^+\) channels in primary afferent neurons (Oyelere et al. 1997). Thus the simplest explanation for the lack of effect of BDNF on excitability in our study is that different neuronal types are affected in different ways by this neurotrophin. Alternatively, BDNF, axotomy, and CCI may affect active conductances in dendrites (see earlier text)—a change difficult to detect using cell body recordings.

**Mechanism of axotomy-induced changes**

In delay neurons, axotomy was much more effective in increasing sEPSC frequency than in increasing mEPSC frequency (Fig. 7). This suggests that increases in the frequency of presynaptic APs, including those in primary afferent fibers, may account for axotomy-induced increased synaptic drive to these neurons. If this is so, how can excitatory drive to tonic neurons decrease? In these neurons, axotomy decreased both mEPSC and sEPSC (Fig. 7). This could reflect changes in the presynaptic AP activity and/or depression of the neurotransmitter release mechanism or perhaps disconnection of afferent axons from postsynaptic dendrites or cell bodies. Additional evidence to support the latter idea comes from ultrastructural studies that show that CCI promotes transient loss of the excitatory synaptic terminals of nonpeptidergic nociceptive fibers in substantia gelatinosa (Bailey and Ribeiro-da-Silva 2006). This is relevant because these fibers form the synaptic terminals of type 1 synaptic glomeruli (Ribeiro-da-Silva and Coimbra 1982) that associate with GABAergic neurons (Todd et al. 1996), many of which display a tonic firing pattern (Labrakakis et al. 2009; Lu and Perl 2003; Schoffnegger et al. 2006).

**Functional significance of axotomy-induced changes**

The axotomy-induced increase in sEPSC frequency in delay neurons may contribute to an overall increase in dorsal horn excitability. This is because two lines of evidence suggest that delay neurons are excitatory. First, their intracellular stimulation during paired recording experiments produces excitatory events in postsynaptic neurons (Lu and Perl 2005). Second, studies using a transgenic mouse that coexpresses enhanced green fluorescent protein under the control of the GAD-67 promoter have associated the GABA phenotype with initial burst (phasic), gap (irregular), or tonic neurons and not with delay neurons (Schoffnegger et al. 2006).

Since many tonic cells exhibit an islet cell morphology (Lu et al. 2009), islet cells are frequently GABAergic (Todd and Spike 1993) and 70% of GAD-expressing neurons (in mice) exhibit a tonic firing pattern (Labrakakis et al. 2009). The axotomy-induced decrease in sEPSC/mEPSC frequency in tonic neurons may be indicative of a decrease in synaptic drive to a population of inhibitory neurons. Although this too would be expected to contribute to an overall increase in dorsal horn excitability, the correlation between tonic firing neurons and an inhibitory phenotype is weaker than that between delayed firing and an excitatory phenotype. This is because some tonically firing neurons may be excitatory (Santos et al. 2007).

As already mentioned, axotomy-induced changes in mEPSC/sEPSC amplitude are modest compared with changes in frequency. It is thus unlikely that changes in event amplitude have a major bearing on dorsal horn excitability. The biological significance of changes in phasic, transient, and irregular neurons is difficult to assess because little is known about the neurotransmitter phenotype of the neuron types. In fact, whereas some phasic neurons have been reported to behave as excitatory interneurons (Lu and Perl 2005), others may be inhibitory (Heinke et al. 2004).

In general, the effects of axotomy and CCI are more similar than they are different. When all neuron types are considered they promote a similar “electrophysiological signature” in the substantia gelatinosa that may represent a “snapshot” of central sensitization following peripheral nerve injury. Unraveling the mechanism associated with the generation of this “electrophysiological signature” may therefore provide vital information relating to the etiology of the central sensitization that underlies neuropathic pain.

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