

Rho-Kinase Inhibition Enhances Axonal Plasticity and Attenuates Cold Hyperalgesia after Dorsal Rhizotomy

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Dorsal rhizotomy results in primary deafferentation of the dorsal horn with concomitant sprouting of spared intraspinal monoaminergic axons. Because descending monoaminergic systems are thought to mitigate nociceptive transmission from the periphery and because dorsal rhizotomy can result in neuropathic pain, we sought to determine whether the rhizotomy-induced sprouting response could be further augmented. Because myelin-derived molecules mask endogenous plasticity of CNS axons and because myelin-inhibitory signaling occurs through the Rho-GTPase pathway, we inhibited Rho-pathway signaling after cervical dorsal rhizotomy in rats. An increase in the density of serotonergic- and tyrosine hydroxylase-positive fibers was seen in the dorsal horn 1 week after septuple rhizotomy, and axon density continued to increase for at least 1 month. One week after septuple rhizotomy, administration of intrathecal Y-27632, an antagonist of Rho-kinase (ROCK), increased the density of both fiber types over vehicle-treated controls. To examine behavioral effects of both cervical rhizotomy and ROCK inhibition, we examined responses to evoked pain: mechanical and thermal allodynia and cold hyperalgesia in the forepaw were examined after single, double, and quadruple rhizotomies of dorsal roots of the brachial plexus. The most notable behavioral outcome was the development of cold hyperalgesia in the affected forepaw after rhizotomies of the C7 and C8 dorsal roots. Application of Y-27632 both attenuated cold hyperalgesia and induced monoaminergic plasticity after C7/8 rhizotomy. Thus, inhibition of Rho-pathway signaling both promoted the sprouting of intact supraspinal monoaminergic fibers and alleviated pain after dorsal rhizotomy.

Key words: Rho-kinase; pain; dorsal root; monoaminergic axons; serotonin; tyrosine hydroxylase

Introduction

Deafferentation of the spinal cord can occur via spinal cord injury (SCI), which severs supraspinal connections, or dorsal rhizotomy, which disconnects primary sensory neurons from their spinal targets. Both injuries result in persistent loss of input to the dorsal horn, because supraspinal axons in the CNS do not regenerate their axons, and although primary afferents regenerate axons in the PNS, they do not cross the spinal PNS/CNS interface at the dorsal root entry zone (DREZ). Clinically, a lack of primary afferent regeneration across the DREZ manifests as both loss of sensation in the affected limb and, paradoxically, the development of severe pain, with sensitivity to cold being a prevalent component (Bruxelle et al., 1988; Bonica, 1990).

Dorsal rhizotomy is well suited to examining plasticity of spinal axons, because this injury occurs peripherally without disruption of spinal tissue or the glia limitans. Dorsal root injury in-

duces both terminal sprouting of spared afferents (Sengelaub et al., 1997; Belyantseva and Lewin, 1999; Darian-Smith, 2004) and of supraspinal axons, particularly those expressing serotonin (5-HT) (Wang et al., 1991a,b; Zhang et al., 1993; Kinkead et al., 1998). Injury-induced sprouting may contribute to both spontaneous locomotor recovery (Weidner et al., 2001) and neuropathic pain (Bruce et al., 2002). Because recovery of useful motor and sensory function may depend on different axonal subpopulations, it is important to determine manipulations that optimize the plasticity of each.

Rhizotomy-stimulated growth of intraspinal axons is most likely attributable to spinal expression of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) (Johnson et al., 2000). The effects of these growth factors are diminished by an inhibitory myelin-rich environment (Mukhopadhyay et al., 1994). Known myelin-derived inhibitors (NogoA, myelin-associated glycoprotein and oligodendrocyte myelin glycoprotein) hinder the growth of axons through their interaction with the Nogo receptor (Fournier et al., 2001; Liu et al., 2002; Wang et al., 2002a), which in turn cooperates with the p75 neurotrophin receptor (Wang et al., 2002b) to activate the small GTPase RhoA (Yamashita et al., 1999, 2002; Yamashita and Tohyama, 2003). RhoA activation leads to actin depolymerization and growth cone collapse (Jalink et al., 1994; Gallo and Letourneau, 2004). After SCI, RhoA is highly activated at the lesion site (Dubreuil et al., 2003; Madura et

Received July 6, 2004; revised Oct. 3, 2004; accepted Oct. 18, 2004.

This work was funded by the International Spinal Research Trust and the National Science and Engineering Research Council of Canada. M.S.R. is a Michael Smith Foundation for Health Research Scholar. We thank Maggie Hampong and Stuart Marshall for excellent technical assistance and Andrew Gaudet, Angela Scott, and Dr. Lowell McPhail for critical review of this manuscript.

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DOI:10.1523/JNEUROSCI.3337-04.2004

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al., 2004), and antagonism of RhoA or its downstream effector Rho-kinase (ROCK) enhances both regeneration and recovery after SCI (Dergham et al., 2002; Fournier et al., 2003). Recent data indicate that Rho or ROCK inhibitors also prevent pain that normally develops as a consequence of sciatic nerve injury, possibly by preventing injury-induced upregulation of the protein kinase C γ -isoform (PKC γ) in the dorsal horn (Inoue et al., 2004).

Here we applied intrathecal Y-27632 after dorsal rhizotomy to investigate the effects of ROCK inhibition on intact descending monoaminergic fibers. Because these axons are involved in pain modulation in the spinal dorsal horn (Millan, 2002), we also examined the effects of dorsal rhizotomy and ROCK inhibition on nociception in the forepaw.

Materials and Methods

Surgery. Animal procedures were performed in accordance with the guidelines of the Canadian Council for Animal Care and approved by the University of British Columbia Animal Care Committee. Male Wistar rats ($n = 59$; 150–200 gm) were anesthetized with ketamine hydrochloride and medetomidine hydrochloride (75 and 0.5 mg/kg, i.p., respectively). A septuple cervical rhizotomy (complete deafferentation of the ipsilateral forelimb) was performed to examine the effects of Rho-kinase inhibition on plasticity of spinal axons ($n = 17$). The left cervical and upper thoracic dorsal roots (C4–T2) were exposed after a lateral hemilaminectomy, and the dura was opened with microscissors. All dorsal roots (C4–T2) were cut with microscissors. Osmotic minipumps (Alzet, Cupertino, CA) delivered sterile saline ($n = 5$) or Y-27632 ($n = 7$; Calbiochem, San Diego, CA) intrathecally (4 mg/ml in sterile saline, 48 μ g/d) throughout the survival time (7 d). An additional 5 vehicle-treated rats with septuple rhizotomies were killed after 28 d. In three uninjured rats, Fluorogold (FG) was injected into the digits of the left forepaw (1 μ l per digit, 5% in distilled H₂O). After all surgeries, the effects of anesthesia were reversed with a 50 μ l intramuscular injection of 5 mg/ml atipamezole hydrochloride.

Single (C8; $n = 5$), double (C7 and C8; $n = 5$), or quadruple (C5, C6, T1, and T2; $n = 5$) rhizotomies were performed to assess the effects of partial forelimb denervation on sensation–nociception in the forepaw. Dorsal roots (C5–T2) were exposed as described above and cut with microscissors; sham-operated rats ($n = 5$) received the same hemilaminectomy (including cutting of the dura) without root injury. Because we found that cold hyperalgesia was reproducibly induced by C7/8 rhizotomy (see Results), we then sought to study the effects thereupon of Y-27632 treatment. Osmotic minipumps (Alzet) delivered sterile saline ($n = 9$) or Y-27632 ($n = 5$; Calbiochem) intrathecally (4 mg/ml in sterile saline, 48 μ g/d) in rats with C7/8 rhizotomies for a period of 10 d.

Behavior. Behavioral testing was done blind and was conducted twice preoperatively in all animals. In developing a model of rhizotomy-induced pain, animals were tested on postoperative days 5, 10, and 25; animals that received quadruple or double rhizotomies were also tested on postoperative day 15. In examining the effects of Y-27632 treatment on rhizotomy-induced pain, animals were tested on postoperative days 2, 5, and 10. Daily test scores are averages of three trials per forepaw. Animals were placed alone in each behavioral apparatus for 15 min before testing, and trials of a particular test were separated by at least 1 hr.

Mechanical allodynia was examined using the Dynamic Plantar Aesthesiometer (Ugo Basile, Comerio, Italy). Rats were placed in a raised cage with a wire mesh floor over the stimulator unit. The metal filament was applied to the center of the palmar surface of the forepaw, and upward force was increased from 1 to 50 gm over 7 sec. Force at withdrawal was recorded for both forepaws.

Cold pain was assessed by squirting acetone (10 μ l) onto the palmar surface of each forepaw (through the wire mesh floor) and measuring the duration of withdrawal, biting, or licking behavior that ensued. A brief withdrawal with no further attention to the forepaw was assigned a duration of 1 sec.

The plantar tester (Ugo Basile) was used to test for thermal allodynia. Rats were placed in a cage with a glass floor, over a moveable infrared

light source. The light source was positioned under the center of the palmar forepaw; the time from stimulus onset to withdrawal was recorded for both forepaws.

Immunohistochemistry. Animals were killed with an overdose of chloral hydrate (1 gm/kg, i.p.) and transcardially perfused with sterile saline, followed by 4% paraformaldehyde (PF). In addition to the animals described above, five unoperated (intact) rats were killed for immunohistochemistry. Spinal cords were removed, postfixed for 12 hr in 4% PF, cryoprotected in 24% sucrose in 0.1 M phosphate buffer for 24 hr, frozen, and cut into 16- μ m-thick transverse sections. Sections were incubated with 10% donkey serum (in 0.1% Triton X-100 for 20 min) to prevent nonspecific binding. Rabbit anti-5-HT (1:16,000; Immunostar, Hudson, WI), sheep anti-tyrosine hydroxylase (TH) (1:200; Chemicon, Temecula, CA), and rabbit anti-PKC γ (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) were applied overnight at room temperature. Secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were raised in donkey, conjugated to Alexa 488 or Cy3, and applied at 1:200 for 2 hr at room temperature.

Image analysis. Digital images were captured using Northern Eclipse software (Empix Imaging, Mississauga, Ontario, Canada) via a digital camera (QImaging, Burnaby, British Columbia, Canada) mounted on an Axioplan 2 microscope (Zeiss, Jena, Germany). SigmaScan Pro (SPSS, Chicago, IL) was used for subsequent quantitative measurements of axon density. Immunopositive axon density measurements were made on threshold overlays of dorsal horn images (in which all pixels overlying immunopositive axons had a grayscale value of 68, and all other pixels had a value of 0), such that average intensity could be converted to axonal density measurements by dividing the output by 68. Averaged axonal density measurements from each treatment group were plotted as line graphs using graphing software (Sigma Plot 2001; SPSS). A two-way ANOVA was used to detect differences between groups in depth profile measurements, and a one-way ANOVA was used to detect differences between averaged density measurements. In all cases, significance was taken as $p < 0.05$. All measurements were taken from five transverse sections of cervical spinal cord per animal.

Results

Y-27632 accelerated plasticity of serotonergic and tyrosine hydroxylase-positive axons in the deafferented dorsal horn

We and others have shown that the ROCK antagonist Y-27632 promotes neurite outgrowth over inhibitory myelin and chondroitin sulfate proteoglycan substrates *in vitro* (Borisoff et al., 2003; Fournier et al., 2003). *In vivo*, Y-27632 treatment has enhanced plasticity of injured corticospinal axons (Dergham et al., 2002; Fournier et al., 2003); however, the effects of ROCK inhibition on intact spinal axons have not been reported. Here, we examined the effects of intrathecal Y-27632 on growth of monoaminergic supraspinal axons within the deafferented region of the cervical spinal cord. Because serotonergic, dopaminergic, and noradrenergic axons descend to the dorsal horn and modulate nociception (Millan, 2002), we were particularly interested in the response of 5-HT- and TH-positive axons to both rhizotomy and Y-27632. We therefore examined axon density in the dorsal horn in rats that received rhizotomy plus intrathecal sterile saline (vehicle) (Figs. 1*a,b,d,e*, 2*a,b,d,e*) and in rats that received rhizotomy plus intrathecal Y-27632 (Figs. 1*g,h*, 2*g,h*). For reference, measurements of monoaminergic axon density from the dorsal horn of intact (unoperated) rats are shown in Figures 1*c* and 2*c*; representative images from these animals appear in Figure 6, *a* and *b*.

In all groups, the density of 5-HT-positive axons/axon terminals was highest in the superficial laminae of the dorsal horn (Fig. 1). Also apparent in all groups was the effect of rhizotomy, because the density of 5-HT-positive axons in the dorsal gray matter was higher ipsilateral to rhizotomy than on the contralateral, uninjured side (Fig. 1*c,f,i*). In 7 d vehicle-treated animals, the rhizotomy-induced increase in the density of 5-HT-positive ax-

ons was restricted to laminae I and II (i.e., within the first 200 μm of the dorsal horn) (Fig. 1c). Serotonergic axon density continued to increase over 28 d (Fig. 1d,e,j,k) and extended into deeper laminae. In 7 d Y-27632-treated animals, serotonergic axon density also increased in the superficial laminae ipsilateral to rhizotomy; however, the increase in axon density penetrated deeper into the dorsal horn than in 7 d vehicle-treated controls (Fig. 1g). One week after rhizotomy, serotonergic axon density was greater in animals treated with Y-27632, and augmented serotonergic axon density was observed throughout the dorsal horn both ipsilaterally (Fig. 1j) and contralaterally (Fig. 1k).

To summarize changes in serotonergic innervation of the dorsal horn and to examine contralateral effects of rhizotomy, axon density measurements were averaged and compared within superficial [0–100 μm (Fig. 1m)] and deeper [120–400 μm (Fig. 1n)] laminae. In superficial laminae, axon density was increased bilaterally in 28 d vehicle-treated animals and 7 d Y-27632-treated animals but only ipsilateral to rhizotomy in 7 d vehicle-treated animals (Fig. 1m, asterisks). In addition, 28 d vehicle-treated animals and 7 d Y-27632-treated animals had greater serotonergic axon density than 7 d vehicle-treated animals, both ipsilateral and contralateral to rhizotomy (Fig. 1m, daggers). In deeper laminae, the density of serotonergic axons was not different between 7 d vehicle-treated animals and intact animals (Fig. 1n). However, 28 d vehicle-treated animals and 7 d Y-27632-treated animals had increased serotonergic axon density compared with both intact animals (Fig. 1n, asterisks) and 7 d vehicle-treated animals (Fig. 1n, daggers). These results indicate that dorsal rhizotomy induced serotonergic sprouting throughout the dorsal horn, that injury-induced increases in serotonergic axon density were most dramatic ipsilateral to rhizotomy and in superficial laminae, that rhizotomy-induced sprouting was persistent and continued to increase ipsilateral and contralateral to injury over 28 d, and that Y-27632 treatment accelerated serotonergic sprouting induced by rhizotomy.

Tyrosine hydroxylase-positive axons in the dorsal horn were also stimulated by septuple dorsal rhizotomy. In 7 d vehicle-treated animals (Fig. 2a,b), TH-positive axon density increased in the superficial laminae of the dorsal horn ipsilateral to rhizotomy (Fig. 2c). Twenty-eight days after rhizotomy, TH-positive axon density had increased both ipsilateral and contralateral to injury at all levels of the dorsal horn (Fig. 2d–f,j,k). In Y-27632-treated animals, TH axon density was increased above intact levels

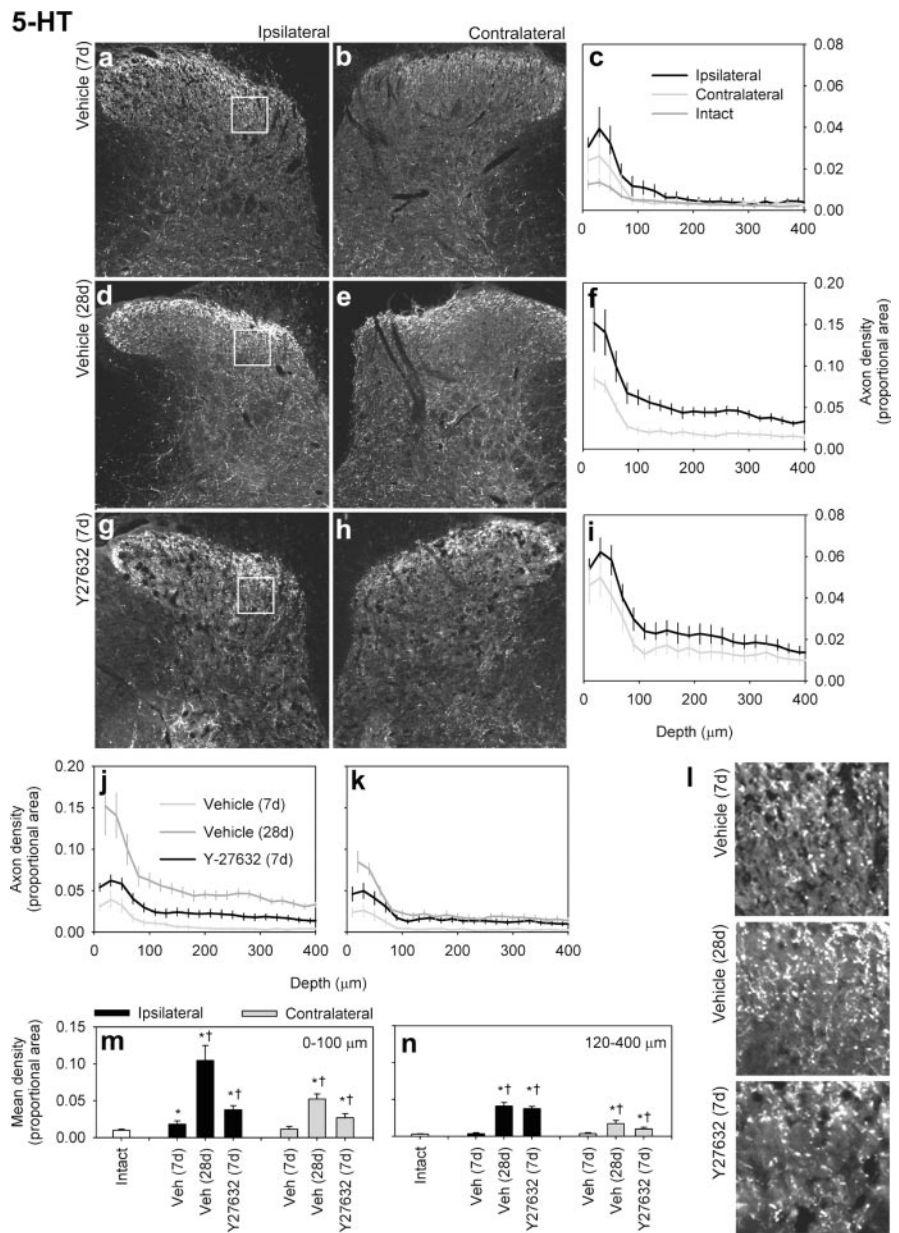


Figure 1. Y-27632 accelerated rhizotomy-induced sprouting of serotonergic axons in the dorsal horn. *a, b*, Serotonin immunoreactivity in the dorsal horn of vehicle-treated rats 7 d after septuple dorsal rhizotomy; densitometric measurements revealed a significant increase in serotonin-positive axon density ipsilateral to rhizotomy (c ; $n = 5$). *d, e*, Serotonin immunoreactivity in the dorsal horn of vehicle-treated rats 28 d after septuple dorsal rhizotomy; serotonergic axon density was greater ipsilateral to rhizotomy (f ; $n = 5$). *g, h*, Serotonin-positive axon density in the dorsal horn of Y-27632-treated rats 7 d after septuple dorsal rhizotomy also increased on the side of injury (i ; $n = 7$). Y-27632-treated animals had increased serotonergic axon density both ipsilateral (j) and contralateral (k) to rhizotomy. Higher-power images (l) illustrate the increased density of axons in Y-27632-treated animals. *m, n*, Average serotonergic axon density in the superficial (m) and deeper (n) laminae of the dorsal horn. Asterisks indicate significant differences from intact animals, and daggers indicate significant differences from 7 d vehicle (Veh)-treated animals. Boxes in *a, d*, and *g* are $150 \times 150 \mu\text{m}$. Error bars indicate SEM.

throughout the dorsal horn both ipsilateral (Fig. 2g,i,j) and contralateral (Fig. 2h,i,k) to rhizotomy.

Density measurements were averaged and compared within superficial [0–100 μm (Fig. 2m)] and deeper [120–400 μm (Fig. 2n)] laminae to summarize changes in TH axon density after rhizotomy with and without Y-27632. In superficial laminae, TH axon density was increased bilaterally in 28 d vehicle-treated animals and 7 d Y-27632-treated animals but only ipsilateral to rhizotomy in 7 d vehicle-treated animals (Fig. 2m, asterisks). Ad-

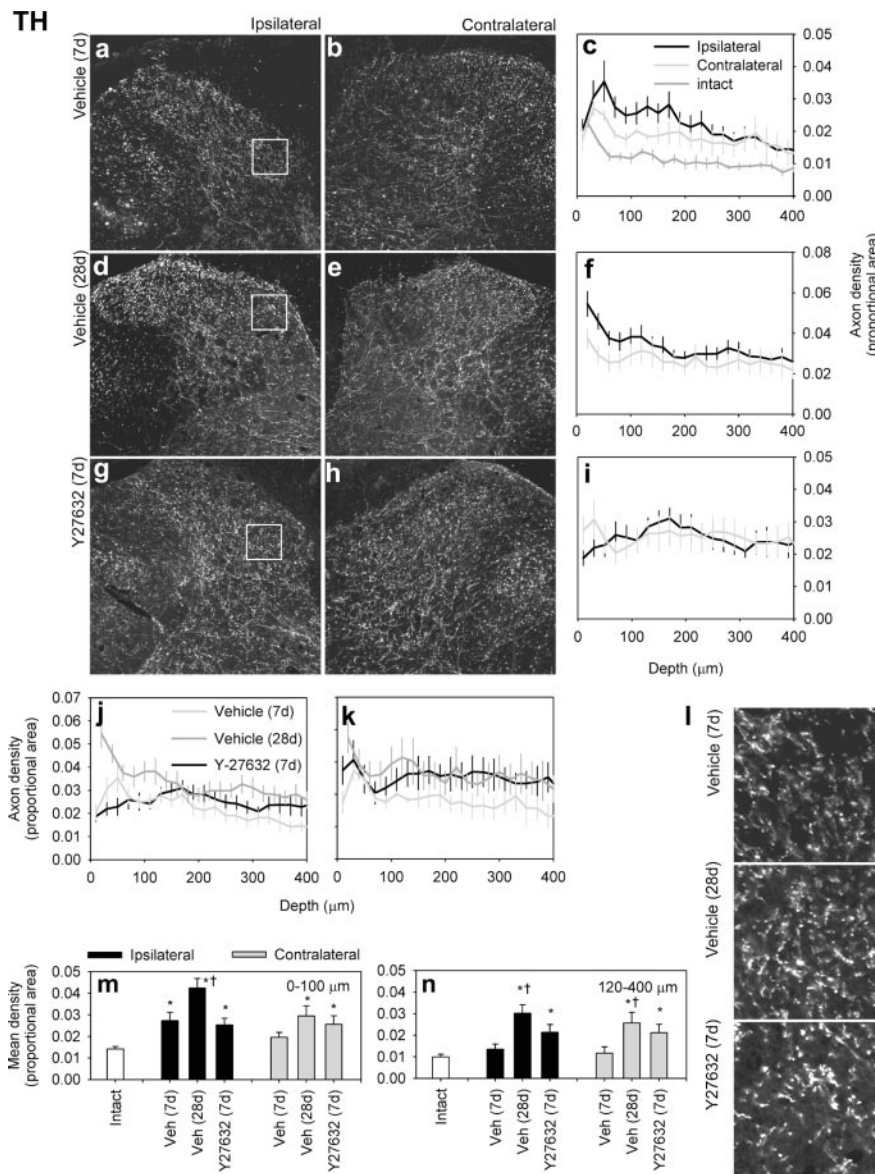


Figure 2. Y-27632 accelerated rhizotomy-induced sprouting of TH-expressing axons in the dorsal horn. *a, b, d, e*, TH immunoreactivity in the dorsal horn of vehicle-treated rats 7 d (*a, b*) and 28 d (*d, e*) after septuple dorsal rhizotomy. Densitometric measurements indicated that vehicle-treated animals had increased density of TH-positive axons at 7 d after injury (*c*; $n = 5$) and that axon density was further augmented by 28 d after rhizotomy (*f, j, k*; $n = 5$). In Y-27632-treated rats, TH axon density was not different between injured and uninjured sides 7 d after rhizotomy (*g–i*; $n = 7$). Akin to its effects on serotonergic axons, Y-27632 increased TH axon density both ipsilateral (*j*) and contralateral (*k*) to rhizotomy; higher-power images (*l*) illustrate the increased density of axons in Y-27632-treated animals. *m, n*, Average TH-positive axon density in the superficial (*m*) and deeper (*n*) laminae of the dorsal horn. Asterisks indicate significant differences from intact animals, and daggers indicate significant differences from 7 d vehicle (Veh)-treated animals. Boxes in *a, d*, and *g* are $150 \times 150 \mu\text{m}$. Error bars indicate SEM.

ditionally, 28 d vehicle-treated animals, but not 7 d Y-27632-treated animals, had greater TH-positive axon density than 7 d vehicle-treated animals ipsilateral to rhizotomy (Fig. 2*m*, dagger). In deeper laminae, the density of TH-expressing axons was not different between 7 d vehicle and intact animals (Fig. 2*n*). However, 28 d vehicle-treated animals and 7 d Y-27632-treated animals had increased TH-positive axon density both ipsilateral and contralateral to rhizotomy (Fig. 2*n*, asterisks). Interestingly, 28 d vehicle-treated animals, but not 7 d Y-27632-treated animals, had increased TH-positive axon density compared with 7 d vehicle-treated animals (Fig. 2*n*, daggers). These results indicate that dorsal rhizotomy induced persistent sprouting of TH-positive axons in the dorsal horn and that Y-27632 treatment accelerated this sprouting response.

Double (C7 and C8) cervical rhizotomy induced cold hyperalgesia

Rhizotomy-induced sprouting of monoaminergic axons in spinal gray matter prompted us to investigate the effects of partial denervation on the development of stimulus-evoked hyperalgesia and/or allodynia in the forepaw. Although several models of peripheral nerve injury produce long-lasting pain that has been well characterized (Bennett and Xie, 1988; Seltzer et al., 1990; Kim and Chung, 1992), little is known about the painful sequelae of injury to the central projections of primary afferents. Because we wanted to investigate rhizotomy-induced pain in the rat forepaw, it was first necessary to characterize its afferent innervation. Previous work has suggested that the forepaw is innervated solely by the C7 and C8 dorsal root ganglia (DRGs) (Takahashi and Nakajima, 1996). Here, we investigated afferent contribution to forepaw innervation by injecting FG into the digits of the forepaw (Fig. 3*a*) and examining ipsilateral DRGs from C5 to T2 (Fig. 3*b–g*). Two days after FG injection, the majority of FG-positive neurons were found in the C7 and C8 DRGs (Fig. 3*d, e*). However, FG-labeled neurons were also encountered in C6 and T1 (Fig. 3*c, f*). We then assessed evoked pain behavior in three models of injury: single rhizotomy (at C8), rhizotomy at C5, C6, T1, and T2 (i.e., sparing C7 and C8), and double rhizotomy at C7 and C8 (Fig. 4). In each of these injury models, some innervation of the forepaw was preserved. A sham-operated group of animals was included, and all behavioral experiments were conducted by an experimenter who was blind to the injury model being tested.

Mechanical allodynia was examined using the Dynamic Plantar Aesthesiometer (Ugo Basile). When increasing force was applied to the center of the palmar forepaw, no differences in force at withdrawal were detected between forepaws after sham surgery (Fig. 4*a*), single rhizotomy (Fig. 4*b*), quadruple rhizotomy (Fig. 4*c*), or double rhizotomy (Fig. 4*d*). Using the plantar (in this case palmar) test (after Hargreaves et al., 1988), the same animals were tested for thermal allodynia. No decreases in latency to withdrawal from infrared heat were observed in the partially denervated forepaws of any experimental group (Fig. 4*b–d*). After C7/8 rhizotomy, some rats had increased latency to withdrawal in the forepaw ipsilateral to rhizotomy (Fig. 4*d*, N.B.). It should be noted, however, that this effect was transient, variable among animals, and was not observed in vehicle-treated animals (Fig. 5*a*) or in untreated animals tested subsequently (our unpublished observations).

Cold pain was assessed by measuring the duration of withdrawal, biting, or licking after a squirt ($10 \mu\text{l}$) of acetone onto the

palmar surface of the forepaw (Choi et al., 1994). Rats in sham surgery, single rhizotomy, or quadruple rhizotomy groups responded to acetone application with a brief withdrawal, accompanied by little licking and no biting of the forepaw, and no difference in duration of response was observed between left and right forepaws in any of these groups (Fig. 4*a–c*). A similar, brief response was evoked by acetone application to the normally innervated (contralateral) forepaw of the double-rhizotomized group (Fig. 4*d*). However, when acetone was applied to the denervated (ipsilateral) forepaw 5 or 10 d after double rhizotomy, it typically provoked prolonged elevation of the forepaw accompanied by vigorous licking and biting. The duration of response was significantly greater in the partially denervated forepaw only in animals that received rhizotomies at both C7 and C8 (Fig. 4*d*, asterisks). This cold-induced pain developed by 5 d after rhizotomy and resolved in all animals by 25 d after injury. It should be noted that none of the rats, in any group, showed signs of autotomy or self-induced mutilation of the affected forepaw during the experiment.

Cold pain was attenuated by intrathecal Y-27632

Because Y-27632 treatment induced sprouting of monoaminergic axons, which are known to play both facilitatory and inhibitory roles in processing painful stimuli in the dorsal horn, we hypothesized that intrathecal administration of Y-27632 might modulate the cold pain observed after rhizotomy at C7 and C8. Rhizotomized rats with vehicle- or Y-27632-filled pumps were tested preoperatively and over a period of 10 d after surgery. The identity of the rats was coded to prevent bias. Rats that received rhizotomies at C7 and C8 plus vehicle pumps developed cold pain in the forepaw ipsilateral to injury that was similar in onset and extent to rats with the same injury and no pump (Fig. 5*a*). In contrast, no significant difference in the duration of response to cold was observed between forepaws in Y-27632-treated rats (Fig. 5*b*). ROCK inhibition had no apparent effect on mechanical or thermal thresholds, which were not different between the affected and unaffected forepaws. Qualitatively, Y-27632-treated rats typically had a non-nocifensive response to acetone application, with little or no biting of the forepaw. Thus, Y-27632 treatment ameliorated cold pain induced by C7/8 dorsal rhizotomy.

Y-27632 induced plasticity of serotonergic and tyrosine hydroxylase-positive axons after C7/8 rhizotomy

After septuple rhizotomy, sprouting of 5-HT- and TH-expressing axons increased over 28 d and was accelerated by intrathecal Y-27632 (Figs. 1, 2). To determine whether monoam-

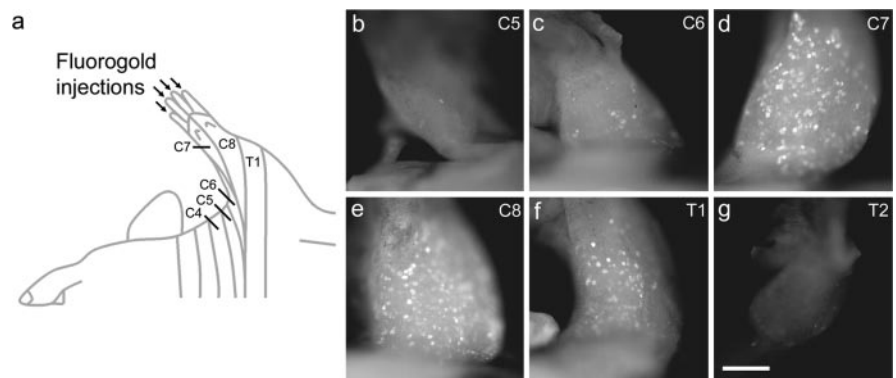


Figure 3. The rat forepaw is innervated by neurons in C6–T1 dorsal root ganglia. *a*, Diagram of the rat (ventral view), showing the positions of the C6–T1 dermatomes (Takahashi and Nakajima, 1996) and sites of FG injections (arrows). *b–g*, FG-positive neurons in whole-mounted DRGs. Although C7 and C8 DRGs contained the majority of FG-positive neurons (*d, e*), labeled neurons were also detected in C6 and T1 (*c, f*). Occasional FG-labeled neurons were encountered in C5 or T2 (*b, g*) DRGs. Scale bar, 500 μ m.

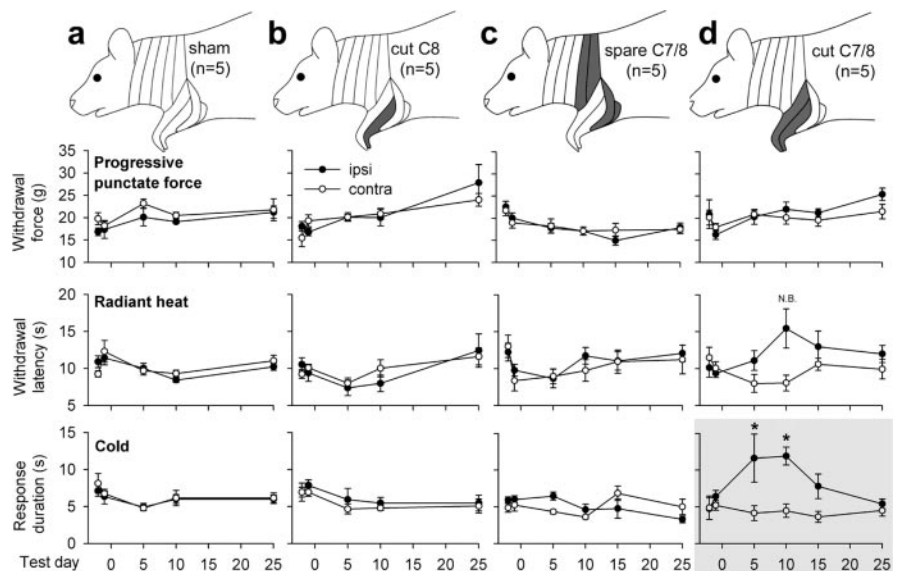


Figure 4. Section of the C7 and C8 dorsal roots induced cold pain in the rat forepaw. *a–d*, Rats were tested twice preoperatively and then over 25 d after sham surgery (*a*), single rhizotomy at C8 (*b*), quadruple rhizotomy at C5, C6, T1, and T2 (i.e., sparing C7 and C8; *c*), or double rhizotomy at C7 and C8 (*d*). No differences in pain responses were detected between forepaws after sham surgery (*a*), single rhizotomy at C8 (*b*), or quadruple rhizotomy at C5, C6, T1, and T2 (*c*). However, when the C7 and C8 dorsal roots were cut, cold pain developed in the forepaw ipsilateral to rhizotomy (ipsi; black) by 5 d after injury (indicated by shading). There was no evidence of accompanying mechanical or thermal allodynia. N.B., At 10 d after C7/8 rhizotomy, some animals had an increase in thermal withdrawal latency in the ipsilateral forepaw (*d*). Asterisks indicate significant differences between ipsilateral and contralateral (contra; white) forepaws. Error bars indicate SEM.

nergic projections to the dorsal horn respond similarly after double rhizotomy, we examined serotonergic (Fig. 6) and TH-positive (Fig. 7) axon density in intact (unoperated) animals and in animals that received rhizotomies at C7/8 plus vehicle or Y-27632. Tissue was examined 10 d after injury, because this is when cold hyperalgesia peaked in untreated and vehicle-treated animals.

As expected, serotonergic axon density increased in response to C7/8 rhizotomy. In 10 d vehicle-treated animals (Fig. 6*b*), serotonergic axon density was increased above uninjured levels in the superficial laminae of the dorsal horn (Fig. 6*d,e*, asterisks). However, serotonergic axon density in 10 d, double-rhizotomized animals was lower than serotonergic axon density in 7 d, septuple-rhizotomized animals (compare the scales in Figs. 1*c*, 6*d*). Intrathecal Y-27632 enhanced serotonergic plastic-

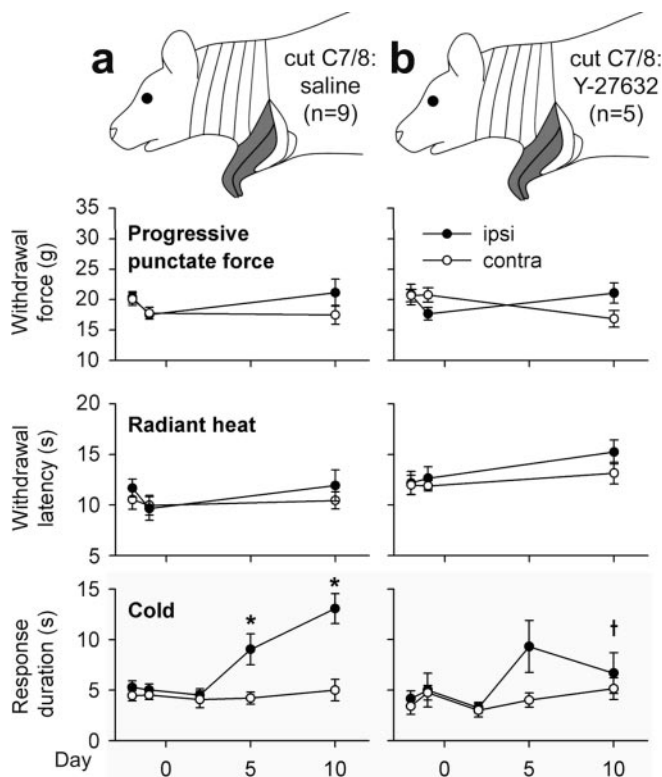


Figure 5. Cold pain was attenuated by intrathecal administration of Y-27632. Rats were tested twice preoperatively and then over 10 d after double rhizotomy at C7 and C8. *a*, Rats that received intrathecal saline developed cold pain in the forepaw ipsilateral to rhizotomy (ipsi; black). *b*, Cold pain was reduced or absent in Y-27632-treated animals. Asterisks indicate significant differences between ipsilateral and contralateral (contra; white) forepaws, and the dagger indicates a significant difference between ipsilateral forepaws in vehicle-treated and Y-27632-treated rats. Error bars indicate SEM.

ity after C7/8 rhizotomy because, in 10 d Y-27632-treated animals (Fig. 6*c*), serotonergic axon density was elevated above intact levels in both superficial and deeper laminae (Fig. 6*d,e*, asterisks) and was increased compared with vehicle-treated animals throughout the dorsal horn (Fig. 6*d,e*, daggers). It is of interest, however, that serotonergic axon density in Y-27632-treated animals was lower at 10 d after double rhizotomy than at 7 d after septuple rhizotomy (compare the scales in Figs. 1*i*, 6*d*).

In vehicle-treated animals, density of TH-expressing axons was not increased at 10 d after C7/8 rhizotomy (Fig. 7*b,e,f*). However, Y-27632 treatment increased density of TH-positive axons relative to both intact animals (Fig. 7*c,e,f*, asterisks) and vehicle-treated animals (Fig. 7*c,e,f*, daggers) in superficial laminae of the dorsal horn. Given that septuple rhizotomy induced sprouting of TH-positive axons (Fig. 2) and TH-positive axon density increased in Y-27632-treated, double-rhizotomized animals, we hypothesized that TH axon density simply increased slowly after C7/8 rhizotomy relative to serotonergic axon density. We therefore examined axon density in vehicle-treated animals 25 d after C7/8 rhizotomy (Fig. 7*d*). Surprisingly, density of TH-positive axons was not increased in 25 d, vehicle-treated animals (Fig. 7*e,f*). Thus, it appears that, in contrast to serotonin-expressing axons, TH-positive axons were not stimulated by C7/8 rhizotomy.

Spinal PKC γ expression was not affected by C7/8 dorsal rhizotomy or intrathecal Y-27632

PKC γ is upregulated in the dorsal horn subsequent to several types of peripheral nerve injuries that cause pain (Mao et al.,

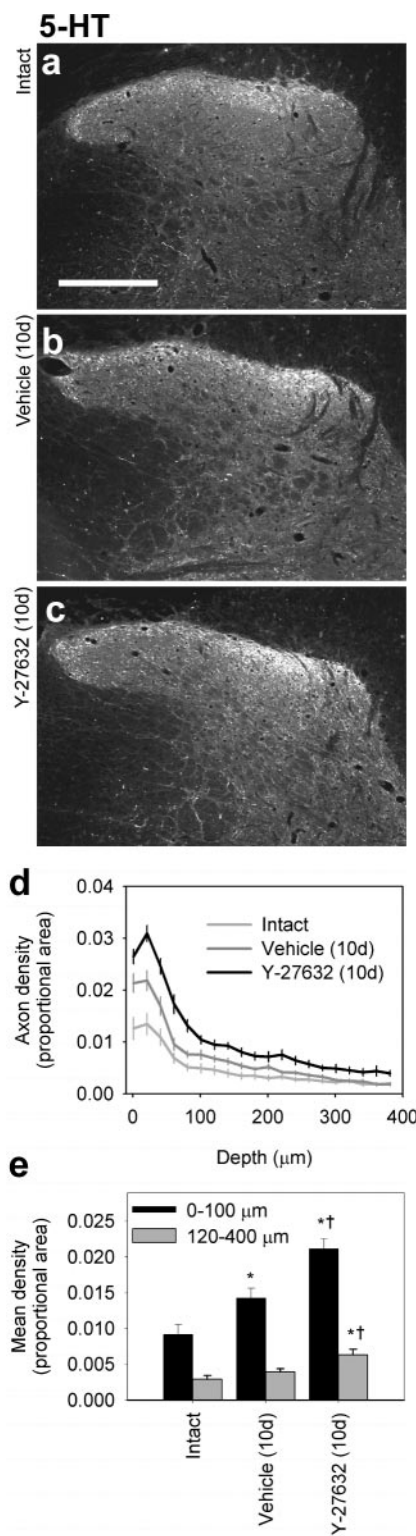


Figure 6. Y-27632 induced plasticity of serotonergic axons after C7/8 rhizotomy. *a–c*, Serotonin immunoreactivity in intact animals (*a*), 10 d vehicle-treated animals (*b*), and 10 d Y-27632-treated animals (*c*). In injured animals, all data shown are ipsilateral to C7/8 rhizotomy. *d*, Densitometric measurements revealed injury-induced increases in serotonergic axon density in superficial laminae and Y-27632-induced increases throughout the dorsal horn. *e*, Average density of serotonergic axons in the superficial (black) and deeper (gray) laminae of the dorsal horn. Asterisks indicate significant differences from intact animals, and daggers indicate significant differences from 10 d vehicle-treated animals. Scale bar, 500 μ m. Error bars indicate SEM.

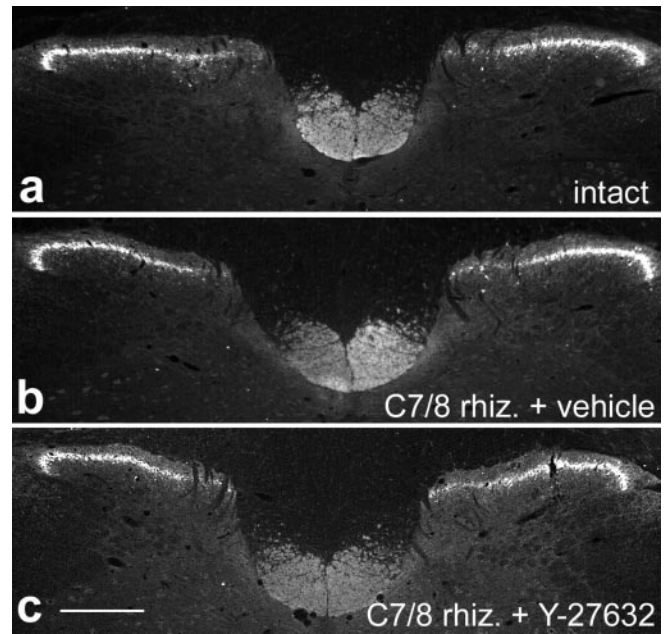
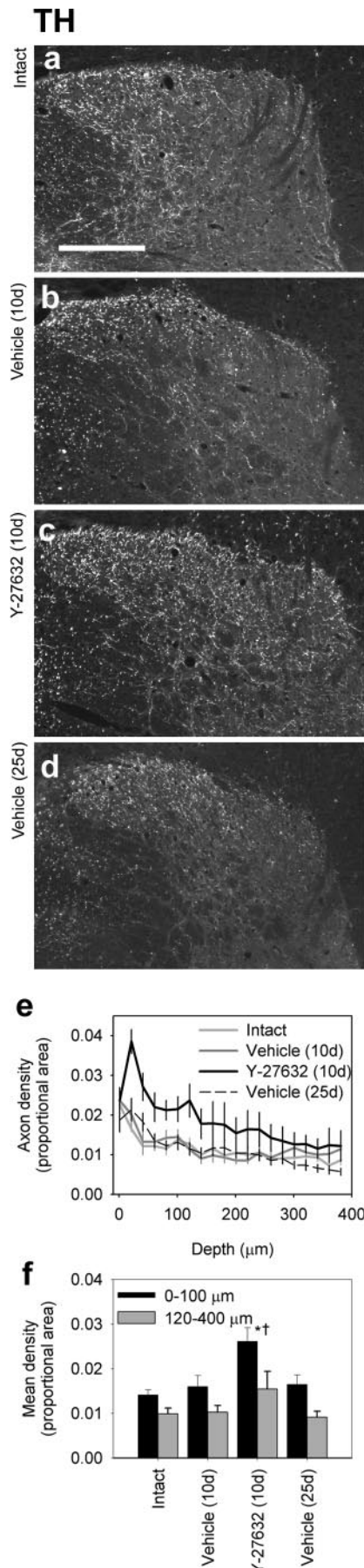


Figure 8. PKC γ expression in the dorsal horn was not affected by C7/8 rhizotomy (rhizo) or intrathecal Y-27632. *a–c*, PKC γ immunoreactivity in the dorsal horn of intact animals (*a*), 10 d vehicle-treated animals (*b*), and 10 d Y-27632-treated animals (*c*). There was no change in PKC γ expression after C7/8 rhizotomy. Scale bar, 500 μm .

1995; Malmberg et al., 1997; Miletic et al., 2000; Inoue et al., 2004). This second messenger is also thought to participate in sensitizing dorsal horn neurons to glutamate to induce persistent neuropathic pain (Lin et al., 1996; Ji et al., 2003). In a recent experiment, an intrathecal injection of the *Clostridium botulinum* C3 exoenzyme before partial sciatic nerve injury prevented both allodynia and upregulation of PKC γ in the dorsal horn (Inoue et al., 2004). To determine whether PKC γ was involved in cold pain after C7/8 rhizotomy, we examined PKC γ immunoreactivity in intact (unoperated) animals and in animals that received rhizotomies at C7/8 plus vehicle or Y-27632 (Fig. 8). In all animals examined, PKC γ was expressed in lamina II of the dorsal horn and in the corticospinal tract (Fig. 8). Expression of PKC γ in 10 d vehicle-treated animals (Fig. 8*b*) and 10 d Y-27632-treated animals (Fig. 8*c*) was comparable with that in intact animals (Fig. 8*a*). Thus, changes in PKC γ expression did not accompany cold pain, or its Y-27632-induced resolution, after C7/8 rhizotomy.

Discussion

Intrathecal administration of the ROCK inhibitor Y-27632 enhanced plasticity of intact serotonergic and noradrenergic axons in the spinal cord. After septuple rhizotomy, monoaminergic axon density in Y-27632-treated animals was intermediate between 7 d and 28 d vehicle-treated animals, indicating that ROCK inhibition accelerated rhizotomy-induced sprouting. Partial denervation of the forepaw via dorsal rhizotomies at C7/8 induced

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Figure 7. Y-27632 induced plasticity of TH-expressing axons after C7/8 rhizotomy. *a–d*, TH immunoreactivity in intact animals (*a*), 10 d vehicle-treated animals (*b*), 10 d Y-27632-treated animals (*c*), and 25 d vehicle-treated animals (*d*). In injured animals, all data shown are ipsilateral to C7/8 rhizotomy. *e*, Densitometric measurements revealed Y-27632-induced increases in density of TH-positive axons in superficial laminae. *f*, Average density of serotonergic axons in the superficial (black) and deeper (gray) laminae of the dorsal horn. The asterisk indicates a significant difference from intact animals, and the dagger indicates a significant difference from 10 d vehicle-treated animals. Scale bar, 500 μm . Error bars indicate SEM.

cold hyperalgesia, and ROCK inhibition assuaged the development of this pain. In this injury model, serotonergic but not TH-expressing axons were stimulated by rhizotomy, whereas plasticity of both populations was induced by Y-27632.

Plasticity of monoaminergic axons

Septuple rhizotomy increased the density of serotonergic and TH-expressing axons in the dorsal horn, in agreement with previous reports of rhizotomy-induced sprouting (Polistina et al., 1990; Wang et al., 1991a,b; Zhang et al., 1993; Kinkead et al., 1998). Dopamine- β -hydroxylase-positive noradrenergic and adrenergic axons are not stimulated by rhizotomy (Wang et al., 1991a,b), whereas dopaminergic axons do undergo rhizotomy-induced plasticity (Mitchell et al., 2000). Thus, the increase in TH-positive axons observed in the present work is probably attributable to sprouting of dopaminergic axons, but we cannot exclude a contribution of noradrenergic sprouting to the increase in TH-positive fiber density.

The differential effects of septuple and double rhizotomy on monoaminergic spinal axons are of great interest. Whereas serotonergic axon density increased in both injury models, both injury- and Y-27632-induced increases were largest after septuple rhizotomy. These results are suggestive of a dose-dependent effect of rhizotomy on intraspinal sprouting, i.e., intraspinal sprouting increases with the number of roots injured. The most likely candidates for mediating rhizotomy-evoked plasticity of monoaminergic axons are BDNF and NT-3 because the expression of both increases in the spinal cord within 1 week of cervical rhizotomy (Johnson et al., 2000) and because bulbospinal monoaminergic neurons express TrkB and TrkC, conferring responsiveness to BDNF and NT-3, respectively (King et al., 1999; Madhav et al., 2001). If spinally produced neurotrophins do underlie rhizotomy-induced sprouting, one might surmise that neurotrophin levels are proportional to the number of dorsal roots involved.

We also found that septuple rhizotomy provoked an increase in density of both serotonergic and TH-expressing spinal axons, whereas double rhizotomy increased density of serotonergic but not TH-positive axons. If rhizotomy does stimulate spinal plasticity in a dose-dependent manner, these results suggest that serotonergic axons are more responsive to dorsal root injury and that TH-positive axons require a threshold number of roots to be injured to mount a sprouting response. Serotonergic axons have an increased propensity for growth relative to other bulbospinal systems (Borisoff et al., 2000), which may underlie our present finding that double rhizotomy had more pronounced effects on serotonergic axons than on TH-expressing axons.

Monoaminergic sprouting in the dorsal horn was observed both ipsilateral and contralateral to septuple rhizotomy, and the contralateral effect deserves some mention. Contralateral effects are widely recognized as consequences of unilateral nervous system damage, such as peripheral nerve injury or dorsal root section (for review, see Koltzenburg et al., 1999). Although both humoral and neural mechanisms have been proposed to underlie such contralateral changes, the latter are more likely, because contralateral effects are typically restricted to the same level of the neuraxis as the injury. The loss of afferent input to spinal neurons results in their atrophy and process retraction (Krassioukov and Weaver, 1996) (M. S. Ramer, unpublished observations). Because many spinal neurons have contralateral projections that would also be expected to undergo deafferentation-induced atrophy, unilateral dorsal rhizotomy may have contralateral effects mediated by spinal interneurons with contralateral projections.

After dorsal rhizotomy, both ipsilateral and contralateral dorsal horn neurons lose their afferent input (primary afferent in the case of ipsilateral and midline-crossing in the case of contralateral); thus, it is expected that neurotrophins would be upregulated bilaterally after unilateral dorsal rhizotomy. Y-27632 would therefore be expected to enhance rhizotomy-induced sprouting bilaterally in the dorsal horn, as observed in the present work.

It is perhaps surprising that contralateral sprouting occurs in the absence of a behavioral correlate. It might be expected that increased density of monoaminergic axons would be associated with greater descending tonic inhibition, resulting in increased pain thresholds. Because this was not the case, we conclude that loss of primary afferent input and the pain that ensues are prerequisites for analgesic effects of monoaminergic sprouting.

Cold pain induced by cervical rhizotomy

The importance of developing animal models of pain after cervical root injuries is underscored by the clinical frequency of painful and debilitating avulsion injuries to the brachial plexus (Bruxelle et al., 1988) versus the relatively rare phenomenon of lumbosacral nerve root avulsion (Chin and Chew, 1997). Autotomy after cervical dorsal root injuries in rodents has been described and is suggestive of chronic pain (Lombard et al., 1979; Abad et al., 1989); however, most available data on rhizotomy-induced pain in animals describes mechanical allodynia that develops ipsilateral to single lumbar rhizotomy at L5 (Colburn et al., 1999; Eschenfelder et al., 2000; Li et al., 2000). Interestingly, we found no evidence of mechanical allodynia subsequent to cervical rhizotomy. Although it remains plausible that mechanical allodynia might develop after injury to a different combination of cervical roots (C6 and C8, for example), the absence of mechanical hypersensitivity may be attributable to intrinsic differences between nociceptive circuitry at cervical and lumbar levels: for example, we showed previously that lumbar DRGs contain fewer P2X₃-positive neurons than their cervical counterparts (Ramer et al., 2001).

The physiological basis of cold hyperalgesia remains to be defined. Ion channels specific to cold sensation have been identified [CMR1/TRPM8 (McKemy et al., 2002; Peier et al., 2002); ANKTM1/TRPA1 (Story et al., 2003; Bandell et al., 2004)], but it remains unknown how these might be regulated by dorsal rhizotomy. If changes in temperature-activated TRP (transient receptor potential) channels are involved in the development of cold pain, these would occur in adjacent ganglia that maintain a connection between the periphery and the spinal cord. Similar changes in gene expression in uninjured ganglia have been described after L5 spinal nerve injury (Fukuoka et al., 1998, 2001, 2002) and chronic constriction of the sciatic nerve (Obata et al., 2003); although these have been primarily attributed to the peripheral intermingling of degenerating and intact axons, degenerating centrally projecting axons may likewise influence their intact neighbors within spinal gray matter. Alternate mechanisms for the development of cold hyperalgesia involve increases in the excitability in the peripheral terminals of cold-sensitive neurons and/or increases in the sensitivity of higher-order neurons to glutamate (Woolf and Salter, 2000). If central sensitization does contribute to cold pain after C7/8 rhizotomy, it appears to occur in the absence of PKC γ upregulation.

Spinal monoaminergic axons and neuropathic pain

Although a wealth of information is available on the modulatory roles of spinal monoaminergic systems on pain induced by peripheral nerve injury or inflammation (for review, see Millan,

2002), data surrounding the monoaminergic modification of nociception after dorsal rhizotomy are comparatively scant. Some of the available data suggest that monoaminergic projections to the dorsal horn facilitate nociception and may contribute to pain that develops after dorsal rhizotomy. Stimulation of Raphe nuclei and locus ceruleus inhibits the responses of neurons in the intact dorsal horn, whereas stimulation of the same nuclei excites dorsal horn neurons ipsilateral to dorsal rhizotomy (Hodge et al., 1983). Serotonin also activates silent glutamatergic synapses in the dorsal horn (Li and Zhuo, 1998), potentially amplifying the response to noxious or non-noxious stimuli (Kerchner et al., 1999). Other data indicate that monoaminergic projections to the dorsal horn are antinociceptive after dorsal rhizotomy: systemic administration of amitriptyline, which inhibits reuptake of both 5-HT and noradrenaline, attenuates autotomy after quintuple cervicothoracic rhizotomy (Abad et al., 1989).

Seemingly paradoxical data on inhibitory and facilitatory influences of spinal monoamines on pain might be reconciled by receptor-specific effects: each transmitter can exert both facilitatory and inhibitory effects on pain by signaling through different receptors, although the pronociceptive and antinociceptive character of specific receptors remains contentious (Millan, 2002). Rhizotomy and/or ROCK inhibition may induce changes in the spinal complement of neurotransmitter receptors, which may underlie both the development and the attenuation of cold hyperalgesia that we observed. Alternatively (or in combination), Y-27632-induced sprouting of monoaminergic axons in the dorsal horn may result in increased transmitter release and subsequent dose-dependent receptor interactions. It is reasonable to speculate that low and high concentrations of serotonin, norepinephrine, and dopamine may have opposing effects on descending inhibition–facilitation (Jones, 1991; Eide and Hole, 1993; Millan, 2002).

After C7/8 rhizotomy, serotonergic axon density increased with time in vehicle-treated rats, and, although pain behavior developed within the first postoperative week, it resolved by the end of the second. In Y-27632-treated animals, both the sprouting response and the pain resolution were accelerated. These results suggest that there is a threshold density of serotonergic innervation of the dorsal horn above which the pain-exacerbating effects of dorsal rhizotomy are counteracted. Pharmacological experiments will be required to establish a causal relationship between spinal plasticity resulting from ROCK antagonism and attenuation of pain. However, these data suggest that enhancing spinal plasticity by alleviating myelin-derived inhibitors, already shown to improve locomotor recovery (Merkler et al., 2001; Dergham et al., 2002; Fournier et al., 2003), may also reduce the development of neuropathic pain.

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