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CHRONIC NEUROPATHIC PAIN IS ACCOMPANIED BY GLOBAL CHANGES IN GENE EXPRESSION AND SHARES PATHOBIOLOGY WITH NEURODEGENERATIVE DISEASES

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Abstract—Neuropathic pain is induced by injury or disease of the nervous system. Studies aimed at understanding the molecular pathophysiology of neuropathic pain have so far focused on a few known molecules and signaling pathways in neurons. However, the pathophysiology of neuropathic pain appears to be very complex and remains poorly understood. A global understanding of the molecular mechanisms involved in neuropathic pain is needed for a better understanding of the pathophysiology and treatment of neuropathic pain. Towards this end, we examined global gene expression changes as well as the pathobiology at the cellular level in a spinal nerve ligation neuropathic pain model using DNA microarray, quantitative real-time PCR and immunohistochemistry. We found that the behavioral hypersensitivity that is manifested in the persistent pain state is accompanied by previously undescribed changes in gene expression. In the DRG, we found regulation of: (1) immediate early genes; (2) genes such as ion channels and signaling molecules that contribute to the excitability of neurons; and (3) genes that are indicative of secondary events such as neuroinflammation. In addition, we studied gene regulation in both injured and uninjured DRG by quantitative PCR, and observed differential gene regulation in these two populations of DRGs. Furthermore, we demonstrated unexpected co-regulation of many genes, especially the activation of neuroinflammation markers in both the PNS and CNS. The results of our study provide a new picture of the molecular mechanisms that underlie the complexity of neuropathic pain and suggest that chronic pain shares common pathobiology with progressive neurodegenerative disease. © 2002 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: persistent pain, gene regulation, dorsal root ganglion, spinal cord, DNA microarray, neuroinflammation.

Neuropathic pain is defined as pain initiated or caused by primary lesions or dysfunction in the nervous system. It is generally classified according to the etiology of the injury, which includes peripheral nerve injury such as polyneuropathy (e.g. diabetes, HIV), mononeuropathy or multiple mononeuropathy (e.g. diabetes, cancer, carcinomatous neuropathy, post-herpetic neuralgia), as well as CNS injury (e.g. post-stroke pain, spinal injury, multiple sclerosis). Neuropathic pain is clearly different from transient (physiological) pain in terms of etiology, the nature of the pain, as well as the mechanisms of pain generation. Physiological pain is transient in nature and elicits a coordinated, reflexive response that prevents further damage or promotes healing. Neuropathic pain usually is persistent, and nerve injury can produce sensory/motor deficits and other paradoxical sensations of a qualitative nature, such as hyperesthesias (hightened but non-painful appreciation of sensation), paresthesias (tingling and prickling sensation) and dysesthesias (unpleasant or painful sensations). The qualitative differences in sensation suggest that nerve injury leads to a reorganization of sensory transmission pathways that persists long after healing has occurred. Such a reorganization in the nervous system suggests that simple knowledge of pain pathways and neurotransmission is not enough to understand the complexity of chronic neuropathic pain (Suzuki and Dickenson, 2000; Woolf and Salter, 2000; Taylor, 2001; Zimmermann, 2001). So

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Abbreviations: APOB, apolipoprotein B; APOD, apolipoprotein D; ATF-3, activating transcription factor 3; DRG, dorsal root ganglion; GDNFR1, GDNF receptor alpha precursor; GFAP, glial fibrillary acidic protein; Go, guanine nucleotide-binding protein G(O), alpha subunit 1; IEG, immediate early gene; LIM, muscle LIM domain protein; MCP-1, monocyte chemotactic protein 1; MMP11, matrix metalloproteinase-11; NADH-DH, NADH dehydrogenase; NGFI-A, NGF induced gene; NPY, neuropeptide Y; PACAP, pituitary adenylate cyclase activating polypeptide 1; PAP, pancreatitis-associated protein; PBR, peripheral-type benzodiazepine receptor; PNS, peripheral nervous system; PTHrP, parathyroid hormone related peptide; P2Y6, pyrimidinergic receptor P2Y6; QRT-PCR, quantitative real-time PCR; RGP4, regulators of G-protein 4; SNL, spinal nerve ligation; SV2B, synaptic vesicle glycoprotein 2 b; VAMP-2, vesicle-associated membrane protein 2; VIP, vasoactive intestinal peptide.

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far, the pathophysiology of neuropathic pain remains poorly understood.

The pathophysiology of neuropathic pain has been investigated using rat peripheral nerve injury models such as chronic constriction injury of the sciatic nerve (Bennett and Xie, 1988), partial sciatic nerve ligation (Seltzer et al., 1990), and L5 and L6 spinal nerve ligation (SNL) (Kim and Chung, 1992). Both peripheral and central mechanisms contribute to the pathophysiology of these neuropathic pain models. There is compelling evidence demonstrating that hyperalgesia, allodynia, and ongoing pain associated with peripheral nerve injury reflect, at least in part, changes in the excitability of primary afferent neurons (Suzuki and Dickenson, 2000; Woolf and Salter, 2000; Taylor, 2001; Zimmermann, 2001). One particularly important change is the development of ongoing or ectopic activity of primary sensory neurons, that may induce changes in the CNS thought to be responsible for allodynia. Central mechanisms include central sensitization, disinhibition of dorsal horn inhibitory neurons, reorganization of neuronal circuits in the dorsal horn, and changes in descending pain facilitation and pain inhibition (Suzuki and Dickenson, 2000; Woolf and Salter, 2000; Taylor, 2001; Zimmermann, 2001). Both the peripheral and central mechanisms involve changes in gene expression. Changes in gene expression, protein expression and post-translational protein modification in dorsal root ganglion (DRG) and spinal cord have all been reported (Suzuki and Dickenson, 2000; Woolf and Salter, 2000; Befort et al., 2001; Taylor, 2001; Zimmermann, 2001). However, most of the studies revealed regulation of a few known genes at best, mostly focused on genes expressed in neurons. A global picture of gene regulation in the animal models of persistent pain is greatly needed in order to better understand the molecular mechanisms of neuropathic pain.

We, therefore, studied the global gene regulation in the SNL model in both the DRG and spinal cord. The SNL model involves the tight ligation and injury of spinal nerves L5 and L6, causing spontaneous pain, allodynia and hyperalgesia. Allodynia and hyperalgesia develop on the side of nerve injury (ipsilateral side) 3 days post-nerve ligation and persist for weeks to months (Kim and Chung, 1992). This chronic hypersensitivity may be the result of long-term changes in gene expression and subsequent pathobiology in the ipsilateral DRG and spinal cord. To reveal a global picture of the pathophysiology of neuropathic pain, we examined gene regulation in the ipsilateral L4–L6 DRGs of the SNL animals using DNA array technologies. In this gene profiling study, we identified regulation in injured L5 and L6 DRGs, as well as in intact L4 DRG. There is much evidence now that would argue in favor of the contribution of uninjured neurons to the overall pain profile. However, there are conflicting reports on the contribution of injured vs. uninjured afferents to neuropathic pain behavior (Gold, 2000). To understand the molecular mechanism of differential pathogenesis and to evaluate the contribution of each population of DRGs to the pathophysiological process, we examined gene regulation in injured L5–L6 DRGs as well as in the uninjured L4 DRGs separately by quantitative real-time PCR (QRT-PCR) and immunohistochemistry and found differential gene regulation in these two populations of DRGs. Furthermore, we studied global gene regulation in the spinal cord and identified common as well as differential gene regulation in the peripheral nervous system (PNS) and CNS following chronic SNL.

EXPERIMENTAL PROCEDURES

Animals and SNL

Male Sprague–Dawley rats (Taconic, Germantown, NY, USA) weighing 200–300 g at the time of testing were maintained in a climate-controlled room on a 12-h light/dark cycle (lights on at 06:00) with food and water available *ad libitum*. All of the handling of the animals and testing was performed in accordance with the policies and recommendations of the International Association for the Study of Pain (Zimmermann, 1983) and received approval from the Institutional Animal Care and Use Committee of MRL, West Point, PA, USA.

SNL injury was induced using the procedure of Kim and Chung (1992). Anesthesia was induced with 2% gaseous isofluorane (for induction 3–5% and O_2 500–700 μl, for maintenance 2–3% and O_2 400–500 μl). Following dorsal skin incision and muscle separation, the posterior interarticular transverse process of L/S1 was exposed and carefully removed with a micro Rongeur. The L5 and L6 spinal nerves were tightly ligated by a square knot with 6-0 silk thread. The muscles were closed with 4-0 absorbable sutures and the skin was closed with wound clips. Rats that exhibited motor deficiency (such as paw dragging) or failure to exhibit subsequent tactile allodynia were excluded from further testing (less than 5% of the animals were excluded). Sham control rats underwent the same operation and handling as the experimental animals but without SNL.

Behavioral testing

The assessment of tactile allodynia (i.e. decreased threshold to paw withdrawal following probing with non-noxious mechanical stimuli) consisted of measuring the withdrawal threshold of the paw ipsilateral to the site of nerve injury in response to probing with a series of calibrated von Frey filaments. Each filament was applied perpendicularly to the plantar surface of the ligated paw of rats kept in suspended wire-mesh cages. The withdrawal threshold was determined by sequentially increasing and decreasing the stimulus strength ('up-down' method), analyzed with a Dixon non-parametric test (Chaplan et al., 1994) and expressed as the mean withdrawal threshold. Animals were tested before surgery and only animals with a paw withdrawal threshold greater than 10 g were used for the subsequent study. Surgically treated animals were then tested on post-operative days 3 and 12. Only those animals that showed allodynia (paw withdrawal threshold smaller than 3 g) on both days were used for tissue collection on post-operative day 13 (less then 10% of the animals were excluded for tissue collection).

Tissue dissection and RNA preparation

Rat DRGs and spinal cord were dissected and rapidly frozen in liquid nitrogen. The spinal cord tissue was then partially thawed and further dissected on an ice-cold metal plate. Total RNA from each sample was prepared using Trizol™ (Life Technologies, Gaithersburg, MD, USA), followed by RNeasy™ (Qiagen, Hilden, Germany). RNA samples were analyzed by denatured gel electrophoresis. In addition, total RNA quality was assessed by capillary electrophoresis (Bioanalyzer 2100 Agilent, Palo Alto, CA, USA) to ensure that the 28S:18S rRNA ratio was >1.0 for each sample.

Affymetrix microarray hybridization and staining

Hybridization probes were prepared according to Affymetrix instruction (Lockhart et al., 1996). Five-micromolar primer encoding the T7 RNA polymerase promoter linked to oligo-dT_{24} was used to prime double-stranded cDNA synthesis from each total RNA sample (25 µg). cDNA synthesis reactions were carried out at 42°C using Superscript II RNaseH$^-$ reverse transcriptase (Life Technologies, Rockville, MD, USA). Second strand cDNA synthesis was finished using DNA polymerase I and T4 DNA ligase. Each double-stranded cDNA sample was purified by sequential phenol/chloroform extraction (Ambion, Austin, TX, USA) and adsorption to silica (Qiaquick™ kit, Qiagen, Hilden, Germany) according to the manufacturer's instructions. Half of each cDNA sample was transcribed *in vitro* into the copy RNA (cRNA) labeled with biotin-UTP and biotin-CTP using the BioArray High Yield RNA Transcript Labeling kit (Enzo Biochemicals, New York, USA). These cRNA transcripts were purified using RNeasy™ columns (Qiagen, Hilden, Germany) and quantitated by measuring absorption at 260 nm/280 nm. Fifteen-microgram aliquots of each cRNA sample were fragmented at 95°C for 35 min in 40 mM Tris-acetate, pH 8.0, 100 mM KOAc, and 30 mM MgOAc to a mean size of ~50–150 nucleotides. Hybridization buffer (0.1 M MES, pH 6.7, 1 M NaCl, 0.01% Triton, 0.5 mg/ml bovine serum albumin (BSA), 0.1 mg/ml H. sperm DNA, 50 pM control oligo B2, and 1× eukaryotic hybridization control) was added to each sample. Samples were then hybridized to RG-U34A microarrays (Affymetrix) at 45°C for 16 h. Microarrays were washed and sequentially incubated with streptavidin phycoerythrin (Molecular Probes), biotinylated anti-streptavidin antibody (Vector Laboratories, Burlingame, CA, USA), and streptavidin phycoerythrin on the Fluidic Station (Affymetrix). Finally, the microarrays were scanned with a dedicated Gene Array Scanner (Hewlett Packard Instruments, TX, USA) to capture a fluorescence image.

Affymetrix microarray data analysis

Samples were analyzed using Affymetrix microarray RG-U34A. For each probeset (an array of 16 pairs of oligonucleotides for a specific gene) an index of gene expression was calculated and analyzed using the SAFER algorithm (Holder et al., 2002) for all chip analysis. The SAFER gene expression index is a robust and resistant measure of gene expression which is an alternative to the 'average difference' calculated by the Affymetrix analysis software and the model-based expression index proposed by Li and Wong (2001). Like Li and Wong's procedure, the procedure for calculating the SAFER gene index involves both inter-array normalization and intra-array adjustment for probe-specific biases.

To analyze gene expression in DRG, differences in mean level of the gene expression index between ipsilateral and contralateral samples were assessed using a paired *t*-test for each probeset. This model facilitated estimation of ratios comparing the ipsilateral and contralateral samples and calculation of *P* values testing whether the ratios are different from one (i.e. a ratio of one implies no change between the means for the experimental conditions). By fitting separate models for each probeset, differences were assessed using an error term that included biological variability between samples and did not assume that this variability was the same for all genes. To analyze gene expression in the spinal cord, two models were used. As in the DRG analysis, gene expression between ipsilateral and contralateral spinal cord samples was assessed using a paired *t*-test for each probeset. Differences between ipsilateral or contralateral spinal cord samples with sham samples were assessed using analysis of variance (ANOVA) for each probeset.

Immunocytochemistry

Rat DRGs (L4, L5 and L6) were dissected from rats which had been perfused with 4% paraformaldehyde and post-fixed for 4 h. After cryoprotection in 30% sucrose and 30% sucrose:OCT = 1:1 mixture, the ipsilateral and contralateral L4–L6 DRGs from the same animal were placed in sequential order so that the identity of all DRGs was clear to the investigator and all DRGs were subsequently processed in parallel. The tissue was frozen and sections of 10 µm were cut with a cryostat. Tissue sections were washed several times with phosphate-buffered saline (PBS), then treated with 0.1% H_2O_2 for 10 min and then 0.3% H_2O_2 for 20 min followed by washing with PBS three times. The sections were then incubated with blocking buffer (3% BSA+3% donkey serum+0.1% Triton) for 1 h, followed by incubation with the primary antibodies for 2 h at room temperature. After washing with PBS 10 times, the sections were incubated with secondary antibodies, AB enzyme reagent (ABC kit, Vector) and developed using a Vector DAB staining kit according to the manufacturer's recommendations. The antibody to GFAP was purchased from Santa Cruz Biotechnology, and used at 1:20 dilution. Antibody to major histocompatibility complex (MHC) II was purchased from CEDARLANE® Laboratories and used at 1:20 dilution.

QRT-PCR

Total RNA was treated with DNase I, Amplification Grade (Invitrogen, Carlsbad, CA, USA), to remove DNA contamination before cDNA synthesis. cDNA was synthesized with oligo (dT)12–18 using Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). Real-time PCR analysis was performed on an Applied Biosystems ABI Prism 7700 Sequence Detection System. Matching primers and fluorescence probes were designed for each of the genes using the Primer Express program provided by Applied Biosystems. Both forward and reverse primers were used at 900 nM. In all cases, the final probe concentration was 250 nM. The PCR reaction was performed in a final volume of 50 µl using TaqMan Universal PCR Master Mix containing AmpliTaq Gold DNA Polymerase, AmpErase® UNG, dNTPs (with dUTP), Passive Reference 1, optimized buffer components (proprietary formulation) and 1 µl of cDNA template.

Primers and probes for GAPDH and interleukin (IL)-18 were obtained from Applied Biosystems. The sequence of the primers and probes used is listed in Table 1.

QRT-PCR data analysis

Average C_t values from triplicate PCR reactions were normalized to average C_t values for GAPDH RNA from the same cDNA preparations. The ratio of expression of each gene in ipsilateral vs. contralateral samples was calculated as: $2^{-(\text{mean}\Delta\Delta C_t)}$. C_t represents the threshold cycle and $\Delta\Delta C_t$ represents the difference C_t(test gene)$-C_t$(GAPDH RNA) for ipsilateral sample minus contralateral sample. Using the ANOVA method, 95% confidence intervals were determined for each ratio as:

$$2^{-(\text{mean}\Delta\Delta C_t)} \pm t_{0.975,N-m} s \sqrt{\frac{1}{n_i} + \frac{1}{n_j}}$$

where $t_{0.975}$ is the 97.5th percentile of the *t*-distribution with $N-m$ degrees of freedom, N is the total pooled sample size for a gene, m is the number of treatments including control, s is the pooled standard deviation, n_i and n_j are the number of ipsilateral and contralateral samples, respectively, being compared.

RESULTS

Time course of tactile allodynia development following SNL

Following L5–L6 SNL, tactile allodynia develops 3 days post-surgery and persists for weeks (Fig. 1) and

Table 1. Primers and probes used for real-time PCR

Gene (GenBank accession #)	Bases	Sequence (5′–3′)
NADH dehydrogenase (AA874803)	87–113	CTCATGAATCTCAGCACTTACATAGCA
	138–156	CACGCTGCCCCTCAGTAGA
	115–136	ATTCACAGCCGTCCAGTGCGGC
Calcium channel $\alpha_2\delta$ (M86621)	2869–2892	CTCAGCCTATGTGCCATCAATTAC
	2993–3017	CTGTGAAGTCATCCTCTTCCATTTC
	2964–2988	TGACATTTCCACGGCTCCTTGAGGC
PTHrP (M31603)	177–193	AAACGCGCGGTGTCTGA
	243–263	CAGATGGTGGAGGAAGAAACG
	195–220	CACCAGCTACTGCATGACAAGGGCAA
GDNFR1 (U97142)	813–831	TCTGCAACCGCCGTAAGTG
	872–891	AGCATCCCGTAGCTGTGCTT
	840–866	CCCTCAGGCAGTTCTTCGACAAGGTTC
PAP (M98049)	107–128	TACCCTCTGCACGCATTAGTTG
	154–176	TGAAACAGGGCATAGCAGTAGGA
	130–152	CCCAAAGGCTCCCAGGCATATGG
cAMP-dependent activator ATF3 (M63282)	147–168	CGAAGACTGGAGCAAAATGATG
	200–215	GCGGTCGCGCTGACTT
	172–196	CAACATCCAGGCCAGGTCTCTGCCT
Induced by rat carotid artery balloon angioplasty (U10894)	290–313	GGCCTAATGGAGGTGGATATAACA
	349–371	GCTCGCAACTGATTCTTCTCTTC
	322–344	CCGAGGACCTTCGTGTCAGCAGC
GluR5-2 (M83561)	163–185	CGGCATGAATTAAGAAGCTTGAA
	290–309	CCTCCGATCCTGAGCACTTG
	257–285	TTCCTGTGCTACATCCTCCCTCAGACCTC
Potassium channel $K_v1.4$ (M32867)	286–304	GGGCCTACTCCTCCCATGA
	364–382	CTGCTCTGCCTGTGGTGGA
	306–330	TCCGACTACCTCGGCTTCCTTGAGG
Potassium channel 9.3 (Y17607)	1660–1687	GCCACGAGCTACCTTACTTTAACATTAG
	1733–1751	GACCACGATGCCAATGGAA
	1690–1718	ACGTTTATGCACAGCAAGTACACGCCTTC
Sodium channel $Na_v1.9$ (AF059030)	5324–5345	CCAAGGTCAAGGTTCACAATGA
	5448–5468	GTCACCTCGTTCAGCCAAAAA
	5419–5445	CTCACTGAACACAGGCCGTTCGATCTG
Sodium Channel $Na_v1.1$ (M22253)	7040–7066	GCACCTACTAAGACTCAATGACCTGTC
	6985–7001	TTGCCGAGCTGGCTTTG
	7005–7036	ACTCTAGCCCTTAGTCCAAGGAGATTGATCGA
5-HT_3R (D49395)	850–872	ACGCTCCTTCTGGGATACTCAGT
	927–952	TGCACACTACAAAGTAGACACCAATG
	874–902	TTTCTCATCATCGTGTCAGACACACTGCC
Neural visinin-like protein (D10666)	69–91	CAATCCAAGAGGGATTTAAGCAA
	187–216	CGTCACTTAAGGAAAATGATAAAACTAAAC
	99–129	CTCCCAGAAAAGAAGCGAGAGAGAACCACTC
GADD45 (L32591)	244–265	ACCCTCATTCGTGCTTTCTGTT
	350–365	AGGCCCCCGCTCTCAG
	275–298	ACATCAACATCCTGCGGGTCAGCA
Striatum-enriched phosphatase (S49400)	1588–1604	GGCAGCCCAGCAAGAGG
	1644–1661	ACCCGGTTCTCCCAATCC
	1608–1632	CCCACTGTTCCCCGATCATTGTTCA
Peripheral-type benzodiazepine receptor (J05122)	131–153	TGGTATGCTAGCTTGCAGAAACC
	188–208	CGAATACAGTGTGCCCCAGAT
	161–182	CATCCGCCTCGCTGGACACTCG
B13 cytokine (AA892854)	236–261	CCCTCATGAAGATTCTCTCTAAAGGA
	289–310	TCCCTAAGAAGCAGACGCATTA
	263–288	ACCTGCTCTGCATCACCTTAGGCTGG
Monocyte chemotactic protein 1 (X17053)	2501–2525	TCTGAAGCTAATGCATCCACTCTCT
	2567–2593	TTCCAAATCACACTAGTTCTCTGTCAT
	2527–2556	TTCCACAACCACCTCAAGCACTTCTGTAGA
MRC-OX44 (M57276)	262–288	TTCTGGTCCAGAATACCTATGGAATAC
	349–370	CCCAAGAAGGCAACTACCATGA
	298–324	ATCTCCCCTTCCTGACACTTGGCAATG
Fibrinogen γ chain b (J00735)	796–819	CGGACTTTATCCAAGCAGAGAGAT
	860–882	TGCATCATGGATCCCATTAACTC
	825–853	TTTCCTATGAGACCATGGAACCCACTTCC
P2Y6 novel GPCR (D63665)	1184–1205	GTTATCAGCTTCCTGCCTTTCC
	1251–1270	CTCCAGTACCGGGCAAGAGA
	1219–1242	AGCCTACTTGGCTGTGCGCTCCAC
Mx1 protein (X52711)	2062–2084	TTCTCCAATTAATCAGGCTTGCT
	2120–2145	AAGGTAAACAGCTAAAGGGTCATTGT
	2090–2115	ACTTCCCTGGTCACAGCTAGCGTCCC

Table 1 (*Continued*).

Gene (GenBank accession #)	Bases	Sequence (5′–3′)
Interferon γ receptor (U68272)	345–366	CCCCTTTCTCCATGATGACAGA
	404–427	CCGGGACAACTATTGTAAGGAAGA
	374–400	CAATTTGGATGCTGCTCGTTGCTCCTC
C-C chemokine receptor 5 (Y12009)	918–937	CAGGCAATGCAGGTGACAGA
	971–992	CCCAACAAAGGCATAGATGACA
	939–965	ACTCTTGGGATGACACACTGCTGCCTC
Fc γ receptor (M32062)	813–835	CCCTGTCAGTCGGAAAGTACAAG
	865–883	GCTGCCGCTGTTTAGCCAT
	837–864	CTCCACAGGACAAATGACATCCCATCGT
NGF-induced gene (NGFI-A) (AF023087)	120–140	TATCCATGTTCGGGAGTTGGA
	171–200	AATGAACTTCATGTTCATAGCATACAAAGT
	142–170	CACCGCCTACTCAGTAGGTAACCACAGCA
complement protein C1q β (X71127)	712–733	CGGGAATCTGTGTGTGAACACT
	793–812	TGGTGACCTGGAAGGTGTTT
	764–792	AAAGTTCTCACCTTCTGCGACTATGCCCA
MHCII-like beta RT1.D (U31599)	157–178	AAATTGGCCGAATTCATTTCAA
	277–297	GACAGATGGCGTTCTCGTTCT
	227–253	TTCAGGACTGTTCCACACACACCCAGC
RGP4 (U27767)	723–742	GCCATGCAGGCTAAGAAAGG
	783–803	ACGTCAACGCTCCATCAAGAC
	745–779	CACTAACTTGTAAAAAGTGAGTTCCGTCAAGCCAC

The sequences of forward, reverse and specific probes are listed sequentially for each gene.

months (Kim and Chung, 1992). Throughout the time course, allodynia was mostly observed in the ipsilateral (same side as injury) hind paw of the animals but not in the contralateral hind paw (Kim and Chung, 1992). Correspondingly, electrophysiological, neurochemical, neuroanatomical, as well as gene expression changes thought to be important in neuropathic pain are mostly restricted to the ipsilateral side of the injury at the 2-week time point, consistent with the neuroanatomy of pain transmission pathways at the DRG and spinal cord levels (Suzuki and Dickenson, 2000; Woolf and Salter, 2000; Befort et al., 2001; Taylor, 2001; Zimmermann, 2001). We, therefore, compared the expression of genes in the ipsilateral DRGs with that in the contralateral DRGs from the same animals. Similarly, gene expression changes in the spinal cord were compared. We chose to study gene expression at around 2 weeks (13 days) post-surgery when allodynia is consistently demonstrated.

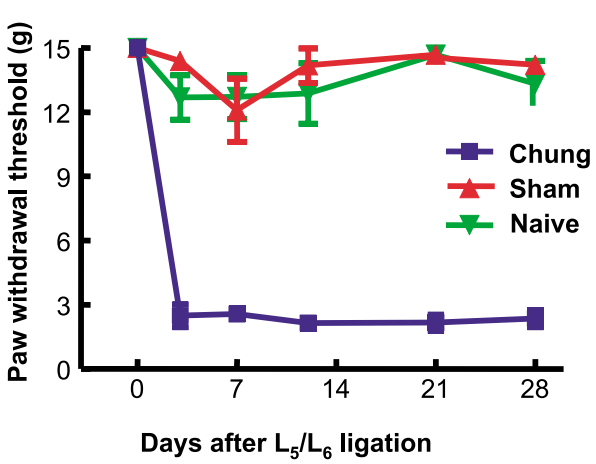

Fig. 1. Time course of allodynia development in rats with SNL. Data were expressed as mean ± S.E.M. $n = 10$ in each group.

Global gene expression changes between ipsilateral and contralateral DRGs as revealed by DNA microarray analysis

A total of 40 SNL-treated animals were used in this study. L4, L5 and L6 DRG samples on the ipsilateral side of nerve ligation from 10 rats with SNL were pooled (30 DRGs), and the L4–L6 DRG samples from the contralateral side of the same 10 animals were pooled (30 DRGs) to form one pair of samples. RNA was prepared from four such pairs of samples and expression of genes was analyzed by hybridization with Affymetrix chip RG-U34A, which contains 8799 probesets, each consisting of an array of 16 pairs of oligonucleotides for a specific gene. Using the SAFER analysis approach (see Experimental procedures), we compared gene expression between the ipsilateral DRG and contralateral DRG samples. We found 1164 probesets gave $P < 0.05$. Among these, 126 probesets (corresponding to 102 genes) show greater than two-fold up-regulation (Table 2), 52 probesets (corresponding to 46 genes) show greater than two-fold down-regulation (a total of 148 genes show greater than two-fold regulation). Twenty-three of these 148 genes were detected to be regulated by more than one probeset, demonstrating the consistency of these samples and analyses (Tables 2 and 3, genes with asterisks). In addition, we found evidence in the literature for regulation of 12 genes (corresponding to 14 probesets) among the 148 genes (Tables 2 and 3, genes in bold) observed. These include up-regulated genes such as galanin (Fukuoka et al., 1999; Ma and Bisby, 1999; Macdonald et al., 2001), vasoactive intestinal peptide (VIP) (Fukuoka et al., 1999; Macdonald et al., 2001), neuropeptide Y (NPY)(Abdulla and Smith, 1999; Fukuoka et al., 1999; Marchand et al., 1999; Macdonald et al., 2001), calcium channel $\alpha_2\delta$ (Luo et al., 2001; Newton et al., 2001), glial marker GFAP (Woodham et

Table 2. Genes that are up-regulated in ipsilateral DRGs based on microarray analysis

Description	Accession #	Fold increase
Neuropeptides		
NPY	M15880	45.6
Galanin	J03624	18.5
VIP	X02341	14.5
PACAP	AI228407	2.3
Cell cycle- and cell death-related		
NADH dehydrogenase**	AA874803	7.8–37.5
GADD45***	L32591	3.5–3.8
Cyclin D3**	D16309	2.0–2.2
Cyclin D1**	X75207	2.0–2.1
DNA synthesis–dihydrofolate reductase	AA900413	3.3
DNA replication protein	U17565	2.0
Cyclin D2	AA899106	2.0
Neuroinflammation and immune activation		
Complement protein C1q β**	X71127	6.5–9.0
MHCII RT1.D β**	X53054	5.7–6.5
MHCII(IA)-associated invariant chain****	X13044	5.5–7.4
MHCII RT1.u-D-α**	M15562	5.4–7.0
MHCII RT1.B β**	U65217	5.2–6.2
Fc γ receptor***	M32062	3.2–4.0
IL-18**	U77777	2.6–2.6
Complement protein C3**	M29866	2.3–2.7
MHCII A-α	K02815	5.7
MHCII β chain (RT1.DMb)	U31599	5.3
MHCII A-β RT1.B-b-β	M36151	4.6
MHCII RT1.B-α	X07551	4.5
MHCII M-β	AI171966	4.3
Complement protein C1S	D88250	3.7
Interferon-induced guanylate-binding protein 2	M80367	3.6
Complement protein C4	U42719	3.1
Allograft inflammatory factor-1	U17919	3.1
Complement protein C1r	AA799803	2.9
α-2-Macroglobulin	M23566	2.3
Complement C1 inhibitor	AA800318	2.3
TNF-converting	AJ012603	2.2
Interferon-induced gene	X61381	2.1
Ion channel		
Calcium channel $\alpha_2\delta$	M86621	2.9
Receptors and signaling molecules		
Striatum-enriched phosphatase	S49400	3.3
Stathmin-like protein RB3	AF026529	3.2
GTP cyclohydrolase I	M58364	2.8
ras-activating protein	U17035	2.7
GDNFR1	U97142	2.2
Tyr phosphatase D30	U28938	2.0
S-100	X06916	2.0
Dual-specificity protein tyrosine phosphatase (rVH6)	U42627	2.0
Transcription		
cAMP-dependent activator ATF3	M63282	8.3
Transcriptional repressor of myelin-specific genes	M72711	2.8
NGF-induced gene (NGFI-A)**	M18416	2.1–2.4
Tissue maintenance, remodeling and plasticity		
LIM**	X81193	4.2–5.0
Cornifin**	AA891911	3.3–7.0
Actin-related protein 2/3 complex subunit**	AF083269	2.4–2.6
Fibronectin***	L00191	2.3–2.4
PTHrP	M31603	10.1
GFAP	AF028784	6.1
Epididymal glycoprotein	M31173	6.0
Retinoid acid-binding protein	U23407	3.5
Retinoid acid-binding protein CRBP	M19257	3.1
Thrombospondin 4	X89963	2.9
VGF	M74223	2.6
Collagen-α2 chain	AI179399	2.4
Heparan sulfate proteoglycan core protein	S61865	2.4
GAS-7 protein	AJ131902	2.3
Corticotensin	U33935	2.3
Gal/GalNAc-specific lectin	J05495	2.3
Metalloproteinase inhibitor I	AI169327	2.3
GAP-43	L21192	2.2

Table 2 (*Continued*).

Description	Accession #	Fold increase
Matrix metalloproteinase MT3-MMP-del	D63886	2.2
Hepatic lectin	X07636	2.1
MMP-11	AB010960	2.1
Insulin-like growth factor-binding protein (rlGFBP-6)	M69055	2.0
Insulin-like growth factor-binding protein (lGF-BP3)	M31837	2.0
Others		
UDP-glucuronosyltransferase***	J05132	2.5–3.1
PAP	M98049	4.6
Induced by rat carotid artery balloon angioplasty	U10894	4.4
Arginine degradation	J02720	4.2
Cathepsin S	L03201	4.0
Transglutaminase K	M57263	3.8
Peripheral-type benzodiazepine receptor	J05122	3.2
Lysozyme	AA892775	3.0
Xenosine dehydrogenase/oxidase	AI172247	2.8
APOB	L07114	2.7
Hexokinase	D26393	2.4
APOD	X55572	2.4
Lumican	X84039	2.2
Anti-proliferative factor (BTG1	L26268	2.2
Estrogen responsive uterine RNA	U53184	2.2
Developmentally regulated protein	M22400	2.2
Proteasome subunit R-RING 12	D10757	2.2
Gelatinase	U65656	2.1
Tumor-associated glycoprotein E4	L12025	2.0
EST: 16 probesets corresponding to 15 genes		

Genes that show greater than two-fold up-regulation in the ipsilateral versus contralateral DRG with $P < 0.05$ are listed according to their broad functional categories. The gene expression fold increase for each probeset was calculated as the ratio of means of gene expression index between ipsilateral DRG and that of contralateral DRG. The genes that are shown to be up-regulated by more than one probeset are indicated with asterisks (the number of asterisks indicates the number of probesets that show up-regulation for that gene). The fold increase for these genes is given as a range of fold increase detected by all the probesets for that gene. Genes that previously have been demonstrated to be regulated are in bold. The genes that were tested by real-time PCR or immunohistochemistry are underlined.

al., 1989), GAP-43 (Cameron et al., 1991), muscle LIM protein, acidic epididymal glycoprotein (Newton et al., 2000) and activating transcription factor 3 (ATF3) (Tsujino et al., 2000), as well as down-regulated genes such as substance P, somatostatin, and sodium channels $Na_v1.8$ and $Na_v1.9$ (Marchand et al., 1994; Okuse et al., 1997; Dib-Hajj et al., 1999). The presence of these genes in our data set provides a level of validation of our experimental results.

The 102 up-regulated genes represent several functional categories including neuropeptides, cell cycle regulation and cell death, neuroinflammation, ion channels, receptor and signaling molecules, transcription factors, and genes involved in tissue maintenance and plasticity (Table 2). The 46 genes that are down-regulated encode neuropeptides, ion channels, ion transporters, proteins with synaptic functions, signaling molecules and others (Table 3). We observed immediate early genes (IEGs) such as ATF3 to be up-regulated about eight-fold, and NGF induced gene (NGFI-A, *krox-24/egr*) to be up-regulated two-fold. In addition, transcriptional repressor of myelin-specific genes is also up-regulated (Table 2).

Multiple ion channel genes that may directly contribute to altered excitabilities of ipsilateral sensory neurons were observed to be regulated in the ipsilateral DRG. Down-regulated ion channels include both voltage-gated and ligand-gated ion channels (Table 3). For example, three voltage-gated sodium channel genes are down-regulated. These include $Na_v1.8$ (PN3/SNS) and $Na_v1.9$ (NaN), which have previously been found to be down-regulated (Okuse et al., 1997; Dib-Hajj et al., 1999), and a novel observation that $Na_v1.1$ (Na channel I) is similarly decreased. Three voltage-gated delayed-rectifier potassium channel genes were found to be expressed two- to three-fold less in the ipsilateral DRGs. These include $K_v1.4$ (KCNA4), $K_v4.3$ (KCND3), and $K_v9.3$ (KCNS3). Ligand-gated ion channels that are down-regulated include kainate receptor GLUR5, serotonin receptor 5-HT_3R, and nicotinic acetylcholine receptor $\alpha3$ (Table 3). In contrast, the voltage-gated calcium channel $\alpha_2\delta$ shows up-regulation in our study (Table 2), consistent with the previous studies that demonstrated the up-regulation of this gene in the SNL model and the partial sciatic nerve ligation model (Luo et al., 2001; Newton et al., 2001).

A category of genes that may indirectly regulate the excitability of sensory neurons include ion transporters, neuropeptides, receptors, signaling molecules, and proteins with synaptic functions. We found ion transporters such as calmodulin-sensitive plasma membrane Ca^{2+}-transporting ATPase, potassium-dependent sodium–calcium exchanger, and Na^+,K^+-ATPase $\alpha1$ subunit are down-regulated (Table 3). Genes encoding neuropeptides appear to be most dynamically regulated in our studies. For example, galanin, VIP and NPY are up-regulated greater than 14-fold based on the estimation from microarray analysis (Table 2). Somatostatin and substance P, as well as amylin are down-regulated more than three-

Table 3. Genes that are down-regulated in ipsilateral DRGs based on DNA chip analysis

Description	Accession #	Fold decrease
Neuropeptide		
Somatostatin**	K02248	3.3–4.4
Amylin	X52820	4.4
Substance P	X56306	2.1
Ion channels		
5-HT 3R***	D49395	2.5–4.4
Sodium channel $Na_v1.9$	AF059030	3.6
Sodium channel $Na_v1.8$ (PN3)	X92184	2.8
Sodium channel $Na_v1.1$	M22253	2.2
Potassium channel $K_v4.3$	AI230211	3.5
Potassium channel $K_v9.3$	Y17607	2.4
Potassium channel $K_v1.4$	M32867	2.3
GLUR5	M83561	2.3
Nicotinic receptor α3	L31621	2.2
Synaptic protein and synaptic remodeling		
VAMP-1**	M24104	2.0–2.5
Neuronal pentraxin**	Al044716	2.0–2.5
ras-related mRNA rab3	X06889	2.8
SNAP-25B	AB003992	2.2
SNAP-25A	AB003991	2.1
SV2B	L10362	2.0
Carbonic anhydrase II	U60578	2.0
Neural plasticity and remodeling		
Neuritin	U88958	2.7
Collapsin response mediating protein 3	U52103	3.0
Ion pump		
Calcium-transporting ATPase	J05087	2.1
Na–Ca exchanger	AF021923	2.2
Na,K-ATPase	M28647	2.2
Receptors and signaling molecules		
Calmodulin homolog	AA893230	3.1
PLC δ_4	U16655	2.8
RGP4	U27767	2.7
Nel-like protein	U48246	2.4
Calcium-binding protein	AA892511	2.3
Go	M17526	2.1
Transferrin receptor	M58040	2.1
Neural visinin-like protein	D10666	2.1
Others		
Mitochondrial creatine kinase	X59737	2.0–2.0
Glycogenin**	L01793	2.0–2.0
Low molecular weight fatty acid-binding protein	J02773	2.4
Decay accelerating factor GPI-form precursor (DAF)	AF039583	2.3
lin-7	AF090133	2.3
Four-transmembrane protein	Y13275	2.2
Parvalbumin α	Al175539	2.1
Brain lipid-binding protein	U02096	2.0
EST		
Five ESTs		

Genes that show greater than two-fold down-regulation in the ipsilateral versus contralateral DRGs with $P < 0.05$ are listed according to their broad functional categories. The gene expression fold decrease for each probeset was calculated as the ratio of means of gene expression index between contralateral DRG and that of ipsilateral DRG. The genes that are shown to be down-regulated by more than one probeset are indicated with asterisks (the number of asterisks indicates the number of probesets that show down-regulation for that gene). The fold decrease for these genes is given as a range of fold decrease detected by all the probesets for that gene. Genes that previously have been demonstrated to be regulated are in bold. The genes that were tested by real-time PCR or immunohistochemistry are underlined

fold (Table 3). The genes for GDNFR1 and tyrosine phosphatases are up-regulated (Table 2), while GTP-binding protein, regulator of G protein RGP4, phospholipase C (PLC) δ_4, and genes that encode calcium sensor proteins such as calcium/calmodulin and protein kinase C (PKC)-binding proteins are down-regulated (Table 3). Consistent gene down-regulation events were also observed for synaptic proteins such as SNAP-25A, SNAP-25B, synaptic vesicle protein 2B (SV2B), and vesicle-associated membrane protein 2 (VAMP-2), as well as for proteins that potentially regulate synaptic functions such as neuronal pentraxin, ras-related mRNA Rab3, and carbonic anhydrase II (Table 3).

One of the striking outcomes of this microarray study was the identification of regulation of multiple genes within the same pathway. Genes involved in neuroinflammation, as well as genes important in cell cycle regulation and cell death are among these pathways. For example, complement protein genes C1q, C1r, and C1s are up-regulated three- to nine-fold; MHCII subunits are

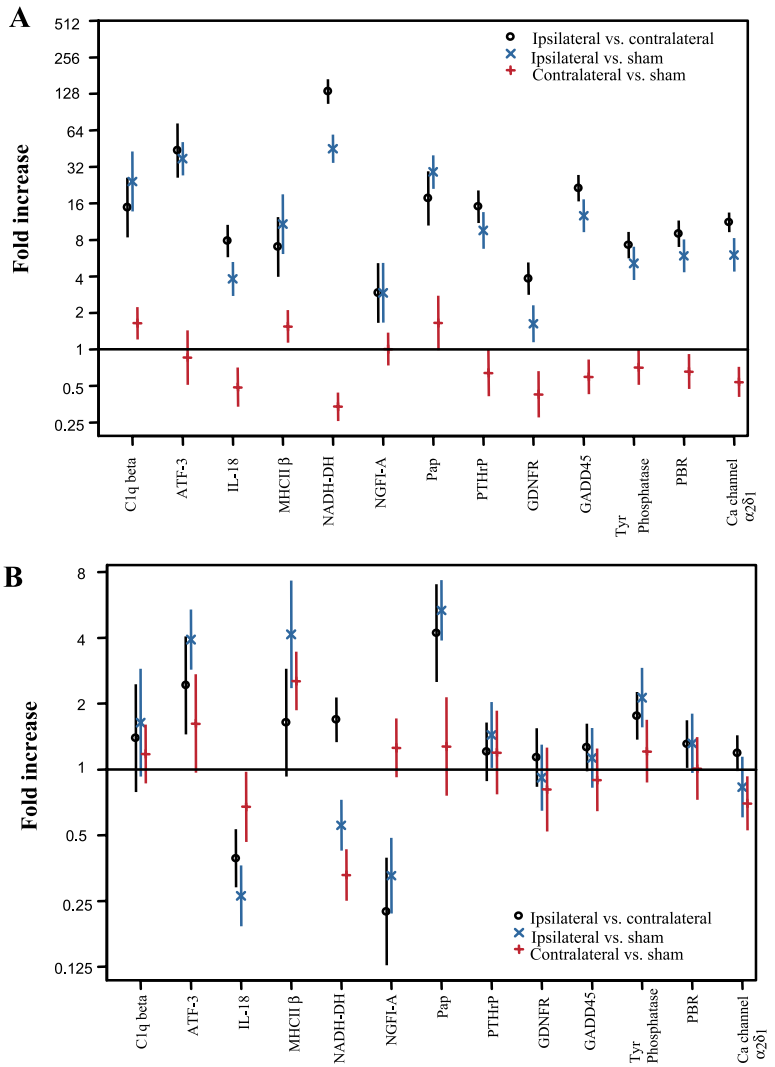

Fig. 2. Differential up-regulation of genes in the L5+L6 (A) and L4 DRGs (B) as revealed by QRT-PCR. For both L4 DRGs and L5+L6 DRGs, the level of gene expression was compared between ipsilateral and contralateral (I vs. C), between ipsilateral and sham (I vs. sham), and between contralateral and sham (C vs. sham) samples. The fold increase was calculated as the ratio of gene expression for each gene in ipsilateral versus contralateral, ipsilateral versus sham, and contralateral versus sham samples. Data were from QRT-PCR assays performed in triplicate for each RNA sample, each of which was prepared from DRGs pooled from four animals. The lengths of the bars represent 95% confidence intervals and the symbol on each bar is the mean of fold change estimated from the assay performed in triplicate. (A) All genes are up-regulated in the L5+L6 ipsilateral DRGs compared to contralateral or sham DRGs, whereas most of the genes are not up-regulated in the contralateral DRG compared to sham DRGs. (B) Only a subset of the genes show up-regulation in ipsilateral L4 DRGs. The up-regulation of these genes is smaller than that in L5+L6 DRGs. Note that the scale of (B) is much smaller than the scale of (A).

up-regulated 4.5–7.4-fold; Fc γ receptor is up-regulated 3.2–4.0-fold; allograft inflammatory factor-1, α-2-macroglobulin, IL-18, interferon-regulated genes and tumor necrosis factor (TNF)-converting enzyme are all expressed higher in injured DRG. All of these genes are indicators of neuroinflammation and immune activation. The regulation of these multiple genes in the same pathway provides clear evidence for the presence of dramatic neuroinflammation in the DRG 13 days following spinal nerve injury. In addition, genes important in cell cycle regulation including cyclin D1, D2, and D3, genes important for DNA synthesis and repair, and several genes that are indicative of cell death including GADD45 and NADH dehydrogenase are all up-regulated (Table 2). The up-regulation of these genes suggests the occurrence of cell death and regeneration in the DRG following peripheral nerve injury.

Other genes that are regulated can be broadly categorized as genes that are important for tissue maintenance, remodeling, and plasticity. These include the genes for LIM, epididymal glycoprotein and glial cell marker GFAP which were previously described to be up-regulated in a peripheral pain model (Woodham et al., 1989; Newton et al., 2000). In addition, our data show that PTHrP is up-regulated 10-fold. Extracellular matrix proteins such as fibronectin and collagen α2 are up-regulated two- to three-fold. Matrix metalloproteinase MMP-11, MT3-MMP-del, as well as metalloproteinase

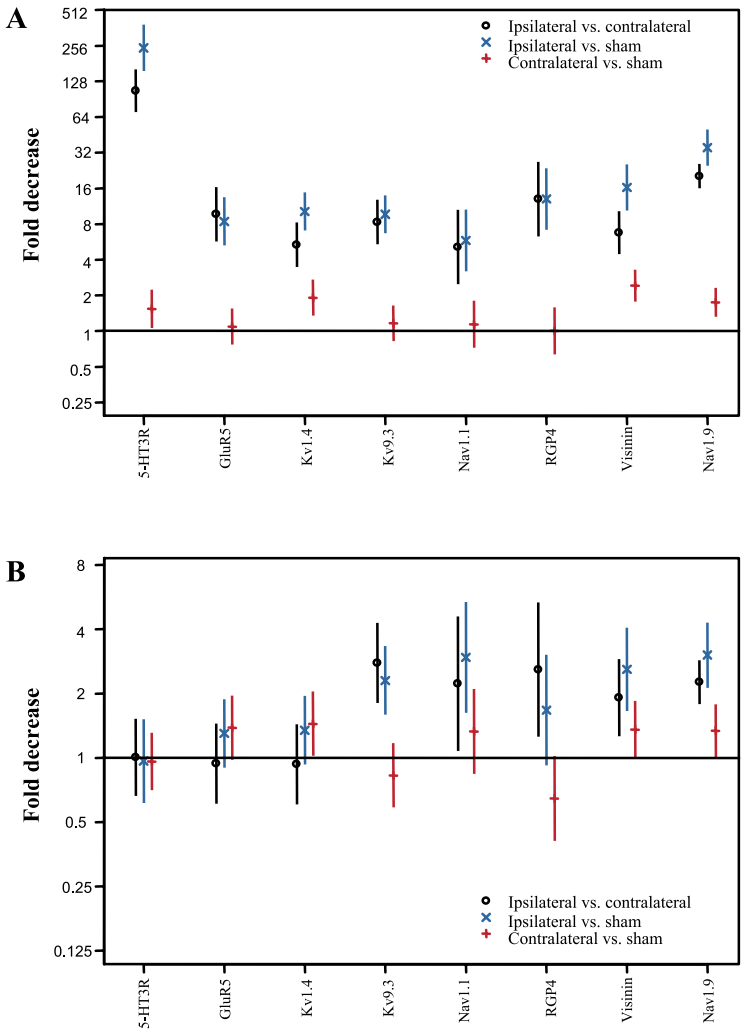

Fig. 3. Differential down-regulation of genes in the L5+L6 and L4 DRGs as revealed by QRT-PCR. For both L4 DRGs and L5+L6 DRGs, the level of gene expression was compared between ipsilateral and contralateral (I vs. C), between ipsilateral and sham (I vs. sham), and between contralateral and sham (C vs. sham) samples. The fold decrease was calculated as the ratio of gene expression for each gene in contralateral versus ipsilateral, sham versus ipsilateral, and sham versus contralateral samples. Data were from QRT-PCR assays performed in triplicate for each RNA sample, each of which was prepared from DRGs pooled from four animals. The lengths of the bars represent 95% confidence intervals and the symbol on each bar is the mean of fold change estimated from the assay performed in triplicate. (A) All genes are down-regulated in the L5+L6 ipsilateral DRGs compared to contralateral or sham DRGs, whereas most of the genes are not down-regulated in the contralateral DRGs compared to sham DRGs. (B) Only a subset of the genes show down-regulation in ipsilateral L4 DRG. The down-regulation of these genes is smaller than that in L5+L6. Note that the scale of (B) is much smaller than the scale of (A).

inhibitor 1 are also among the genes that are up-regulated in this category.

Gene expression changes as revealed by real-time PCR in injured and uninjured DRGs

We sought to confirm gene expression changes of a selected subset of genes using QRT-PCR. The genes selected show expression change between two- and 37-fold by microarray. Since both populations of injured L5–L6 DRGs and their uninjured ipsilateral L4 DRG sensory neurons develop some form of ongoing or ectopic activity following SNL, this model provides a tool for studying the relative contribution of the injured and uninjured neighboring DRGs to different aspects of pain behavior (Gold, 2000). We therefore compared gene expression changes between ipsilateral and contralateral samples, for each population of L5–L6 DRGs and L4 DRGs. Populations of L4 or L5–L6 DRGs in the ipsilateral and contralateral sides were also compared to the DRGs from sham-operated animals to reveal gene expression changes in relation to sham surgery. The results of these comparisons are depicted in Figs. 2 and 3. In general, (1) the regulation of all 21 genes selected were confirmed between ipsilateral and contralateral L5–L6 DRGs, and between ipsilateral and sham L5–L6 DRGs (Figs. 2A and 3A); (2) a subset of the genes were also shown to be regulated in ipsilateral L4 DRGs, however, the ratio of change was usually lower than that for L5–L6 DRGs (Figs. 2B and 3B).

We confirmed gene up-regulation in the injured L5–L6 DRGs of two IEGs (ATF3 and NGFI-A), calcium chan-

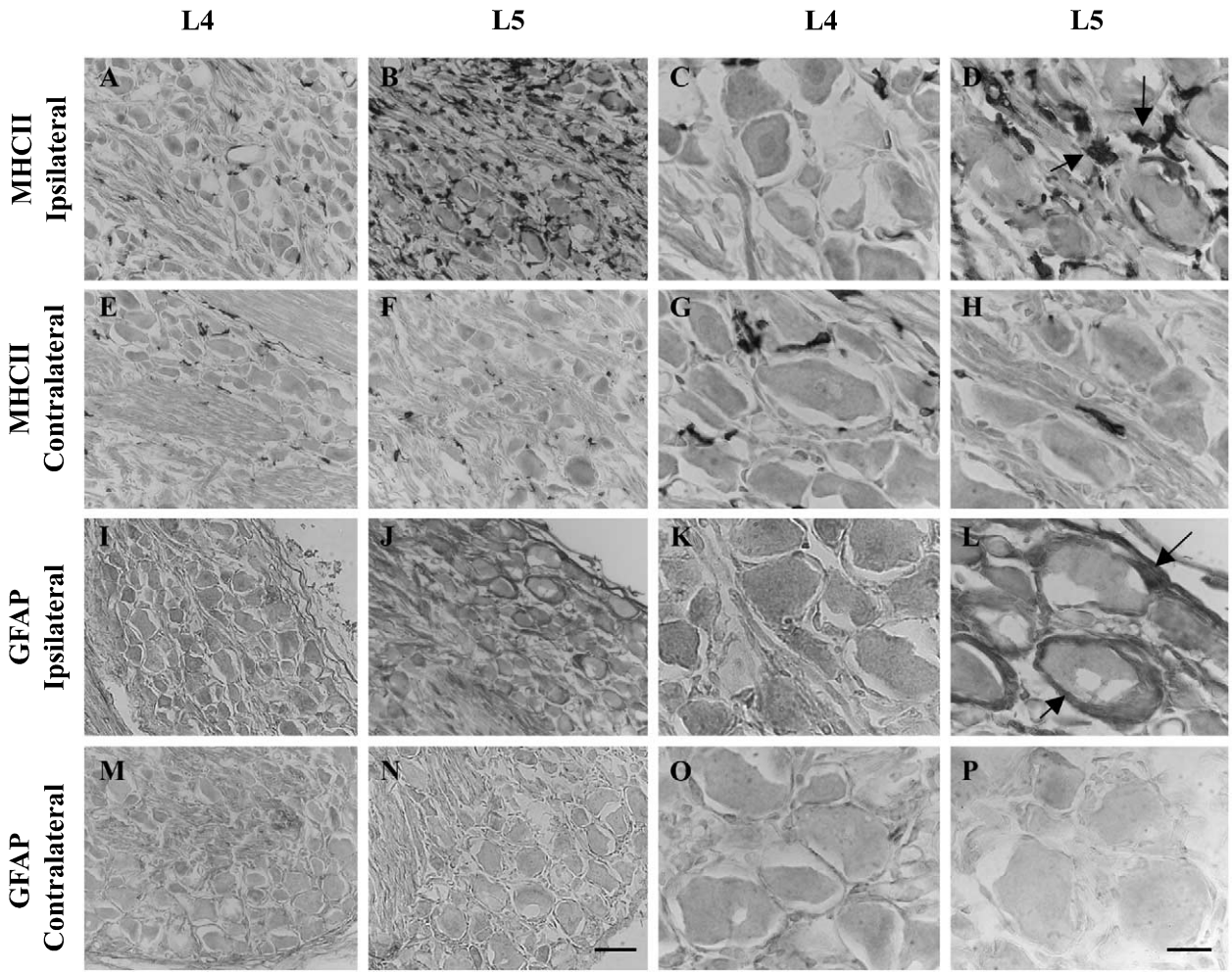

Fig. 4. Pathobiology at the cellular level in DRGs of SNL model as revealed by changes in MHCII β and GFAP immunohistochemistry. Pictures in the first two columns were taken at a lower magnification and the right two columns are taken at a higher magnification of the same sections. (A–H) The number of MHCII β positive cells increased in ipsilateral L5 DRG (B and D, arrows in D) as compared to contralateral L5 DRG (F and H). No change was detected between ipsilateral (A and C) and contralateral L4 DRG (E and G). (I–P) GFAP expression increased in the satellite cells (arrows in L) surrounding DRG neurons in the ipsilateral L5 DRG (J and L) as compared to contralateral L5 DRG (N and P). No change was detected between ipsilateral L4 DRG (I and R) and contralateral L4 DRG (M and P). Images were taken from sections of DRGs from the same animal, although similar regulations were found when two animals were examined. Scale bar for the left two columns = 30 μm; scale bar for the right two columns = 10 μm.

nel $\alpha_2\delta$, signaling molecule tyrosine phosphatase, neuroinflammation marker genes (MHCII β, IL-18 and C1q β), as well as genes that are indicators of cell death (GADD45 and NADH dehydrogenase) (Fig. 2). We also confirmed gene down-regulation in the ipsilateral L5–L6 DRGs of six ion channels (5-HT_3R, GLUR5, K_v1.4, K_v9.3, Na_v1.1, Na_v1.9), a regulator of G protein (RGP4), and a neuronal calcium sensor protein visinin (Fig. 3). Some of the genes that were confirmed to be regulated in L5–L6 DRG are not regulated in L4 DRG. However, several of these genes that are regulated in the L5–L6 DRG were also observed to be regulated in ipsilateral L4 DRG. These include up-regulated genes (ATF3, MHCII, pancreatitis-associated protein (PAP)) and down-regulated genes (K_v9.3, sodium channel Na_v1.1, RGP4 and visinin). The differential regulation of these genes in L4 DRG and injured L5–L6 DRG may account for the differential contributions of injured and intact sensory neurons to different aspects of pain behaviors.

Pathobiology at the cellular level in the DRG

We sought to investigate whether changes in gene expression as detected by QRT-PCR reflect changes at the cellular level using immunohistochemistry with antibodies to MHCII β and GFAP. Immunostaining with antibodies against MHCII β chain revealed that there was an increased number of positive cells present in the injured L5 and L6 ipsilateral DRGs but not in L4 ipsilateral DRGs (Fig. 4A–H and data not shown for L6 DRG), likely reflecting the recruitment of immune cells to the DRG. Moreover, consistent with the up-regulation of GFAP at the mRNA level, an increased immunostaining signal was detected in the satellite cells surrounding the DRG neurons in injured L5 and L6

Table 4. Genes that are up-regulated 1.4-fold or more, with $P < 0.05$ in the ipsilateral spinal cord in comparison to their regulation in DRGs

Description	Accession #	I vs. C ratio-SC	Ppair I vs. C-SC	I vs. S ratio-SC	Panova I vs. S-SC	Ppair I vs. C-DRG	I vs. C ratio-DRG
Chemokine							
Coronin 1A	AA892506	1.5	0.00	1.6	0.00	0.05	1.1
C-C chemokine receptor type 5	Y12009	1.4	0.01	1.3	0.00	0.42	1.3
Small inducible cytokine B13	AA892854	3.8	0.04	5.2	0.00	0.04	1.8
*Monocyte chemotactic protein 1***	X17053	1.5–3.5	0.00–0.01	1.5–3.5	0.00–0.00	0.00–0.04	1.5–1.7
Inflammation							
Complement protein C1q beta***	X71127	2.3–2.4	0.00–0.00	2.7–3.1	0.00–0.00	0.00–0.00	6.5–9.0
Complement protein C3**	M29866	2.1–2.2	0.01–0.01	2.8–2.9	0.00–0.00	0.01–0.01	2.3–2.7
Complement protein C4	U42719	2.3	0.00	2.4	0.00	0.00	3.1
Fc γ receptor	X73371	2.2	0.00	2.3	0.00	0.00	3.2
MHCII β chain (RT1.DMb)	U31599	1.5–1.5	0.00–0.01	1.5–1.8	0.00–0.00	0.00–0.00	4.3–5.3
Leukocyte antigen MRC-OX44	M57276	1.8	0.01	1.7	0.00	0.02	2.0
Allograft inflammatory factor-1	U17919	1.7	0.01	1.6	0.00	0.00	3.1
Membrane glycoprotein	D50558	1.4	0.02	1.6	0.04	0.01	1.5
Interferon-induced GTP-binding protein Mx1	X52711	1.5	0.00	1.6	0.00	0.05	2.0
MD-1	AA874924	1.6	0.00	1.6	0.00	0.07	1.2
Leukocyte antigen CD37	X53517	1.5	0.01	1.5	0.00	0.03	1.6
Fc γ receptor	M32062	1.4	0.02	1.5	0.00	0.00	3.9
Complement protain C1q c chain	AA891576	1.6	0.00	1.5	0.00	0.01	1.9
Interferon consensus sequence-binding protein	AA892259	1.4	0.00	1.5	0.00	0.00	1.6
Interferon γ receptor	U68272	1.4	0.01	1.4	0.01	0.80	1.0
Transcription							
cAMP-dependent activator ATF3	M63282	1.7	0.00	1.9	0.00	0.00	8.3
Stress resistance and actin polymerization							
Peripheral-type benzodiazepine receptor	J05122	1.4	0.00	1.4	0.00	0.00	3.2
Induced by rat carotid artery balloon angioplasty	U10894	2.1	0.00	2.0	0.00	0.00	4.4
PAP	M98049	3.3	0.02	3.6	0.00	0.01	4.6
Receptor							
P2Y6	D63665	1.7	0.00	1.7	0.00	0.00	1.7
Tissue growth and plasticity							
α-Tubulin-1	AA892333	1.6	0.02	1.5	0.04	0.96	1.0
Metalloproteinase inhibitor 1	AI169327	1.7	0.00	1.9	0.00	0.00	2.3
Serine protease inhibitor	D00753	1.5	0.01	1.4	0.00	0.06	1.2
Fibrinogen γ	J00735	1.7	0.00	1.2	0.32	0.60	0.9
EST							
EST	AA894029	1.6	0.02	1.6	0.00	0.00	3.4
EST	AI639255	1.5	0.02	1.6	0.00	0.09	1.6
EST	AA800908	1.4	0.04	2.5	0.10	0.86	0.9

Genes are broadly categorized by biological functions. Genes that are also regulated in the DRGs are in bold (> two-fold, $P < 0.05$ for DRG) or in italic (between 1.4- and two-fold, $P < 0.05$ for DRG). The ratios of the means of gene expression index for ipsilateral versus contralateral spinal cord (I vs. C ratio-SC), ipsilateral versus sham spinal cord (I vs. S ratio-SC), and ipsilateral vs. contralateral DRG (I vs. C-DRG) are listed. *P* values from the paired *t*-test for the ipsilateral versus contralateral samples (Ppair I vs. C-SC), *P* values from the ANOVA analysis for the ipsilateral versus sham spinal cord (Panova I vs. S-SC), and *P* values from the paired *t*-test for ipsilateral versus contralateral DRG (Ppair I vs. C-DRG) are listed for each gene. Genes that are detected by more than one probeset are indicated by asterisks following description (the number of asterisks indicates the number of probesets that show up-regulation for that gene). The values for these genes are given as a range. Genes that were tested by QRT-PCR are underlined.

ipsilateral DRGs, but not adjacent uninjured L4 DRGs or contralateral DRGs (Fig. 4I–P and data not shown for L6 DRG), indicating the activation of this type of glial cells following SNL.

Gene expression changes at the spinal level

Having validated our gene expression results in the DRGs, we sought to investigate whether gene expression changes that were initially detected in the peripheral DRG occur in other regions of the pain circuit such as in the spinal cord. In the spinal cord as well as DRG, we chose to study gene expression changes 13 days post-SNL, a time period when tactile allodynia is consistently demonstrated. We compared global gene expression in the ipsilateral lumbar spinal cord to that in the contralateral lumbar spinal cord and to that from the sham control by Affymetrix microarray analysis. For this study, a total of 24 animals were used. Ipsilateral lumbar spinal cord tissue from three animals was pooled and the contralateral lumbar spinal cord tissue from the same animals was pooled to make one pair of samples. Four such pairs of samples were prepared. In addition, we obtained four sham lumbar spinal cord samples, each sample was from three animals. Gene expression profiles were generated by hybridization to Affymetrix microar-

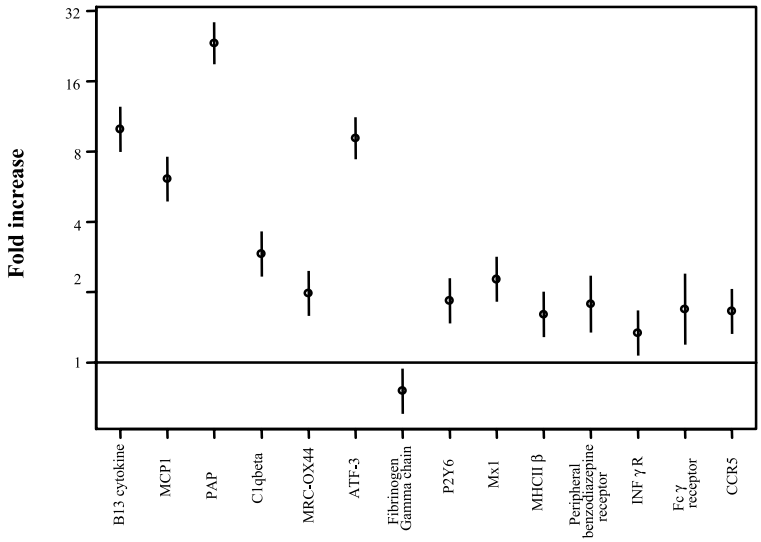

Fig. 5. Up-regulation of genes in the ipsilateral spinal cord as revealed by QRT-PCR. The fold increase was calculated as the ratio of gene expression for each gene in ipsilateral versus contralateral samples. Data were from QRT-PCR assays performed in triplicate for a pair of RNA samples, each of which was pooled from the ipsilateral or contralateral samples of the four pairs of RNA samples used for microarray analysis (from total of 12 animals). The lengths of the bars represent 95% confidence intervals and the symbol on each bar is the mean of fold change estimated from the assay performed in triplicate. All genes except fibrinogen γ were confirmed to be up-regulated in the ipsilateral spinal cord compared to contralateral spinal cord.

ray RG-U34A. Table 4 lists all the genes with greater than a 1.4-fold change and $P < 0.05$ in comparing ipsilateral and contralateral spinal cord gene expression. The *P* value and ratio for ipsilateral vs. sham comparison, as well as the *P* value and ratio for DRG ipsilateral vs. contralateral samples are listed for comparison purposes. As expected, ratios of gene expression between ipsilateral and contralateral spinal cord are generally highly correlated with those of the ipsilateral and sham comparison (Table 4). In addition, we found that 22 of the 31 genes that show greater than 1.4-fold change with $P < 0.05$ in the spinal cord also met this criterion in the DRGs (Table 4, genes in bold or italicized). Among the genes that are regulated in both the DRGs and the spinal cord are genes important for neuroinflammation and immune activation, transcription factor ATF3, peripheral benzodiazepine receptor, purine receptor P2Y6. Interestingly, a majority of the genes are regulated to a greater degree in the DRGs. However, several chemokines seem to be regulated to a greater extent in the spinal cord (Table 4).

The regulation of 14 genes in the spinal cord was tested by QRT-PCR, and 13 genes were confirmed (Fig. 5). Importantly, we confirmed the up-regulation of transcription factor ATF3 (eight-fold). We found that chemokines B13 cytokine and MCP1 are up-regulated by greater than six-fold, and other markers for neuroinflammation such as complement protein C1q β, MRC-OX44, interferon-induced GTP-binding protein, MHCII β, interferon γ receptor, Fc γ receptor and C-C chemokine receptor type 5 are all confirmed to be up-regulated, providing clear evidence for neuroinflammation in the spinal cord. PAP, peripheral benzodiazepine receptor, and purine receptor P2Y6, which were found to be up-regulated in the DRG, were also confirmed to be regulated in the spinal cord. The confirmation of the regulation of tested genes suggests that similar changes in gene expression may mediate the pathophysiology in the DRG and spinal cord.

DISCUSSION

We detected global changes of gene expression in DRGs and spinal cord in the SNL model of neuropathic pain. The categories of genes regulated include (1) IEGs, (2) those that contribute to the ectopic activity of primary sensory neurons, (3) those that indicate secondary pathogenesis events such as neuroinflammation and cell death, and (4) those that may indicate the common pathophysiology at the spinal cord level as well as in the DRG. This study reveals gene expression changes that are consistent with the literature. These results also provide new insight to the pathogenesis of neuropathic pain in animal models in the framework of both peripheral and central mechanisms, as well as secondary pathological events. These findings may provide a scientific basis for further studies seeking better diagnosis and treatment of neuropathic pain in patients.

Overall assessment of microarray analysis in our study

The reliability of these DNA microarray results is demonstrated by the following three observations: (1) a subset of the genes was observed to be consistently regulated by multiple probesets on the microarray; (2) we detected the regulation of 12 genes (by 14 probesets) which have previously been described to be regulated; (3) virtually all of the (34 out of 35) genes that we chose to study further were confirmed to be regulated

by QRT-PCR and/or immunohistochemistry. Based on the consistency and rate of independent confirmation, many of the genes listed in the tables are likely to be true positives.

Gene regulation reported here, however, is limited: the Affymetrix chips we used have 8000 genes and do not contain all the genes in the rat genome; genes that are expressed at levels too low to be detected by this method could also be missed. A few genes that have been reported to be regulated in the DRG or spinal cord in neuropathic pain models are not among those reported here because either the regulation is small ($<$ two-fold for DRG and <1.4-fold for the spinal cord) or gene expression changes were not detected. For example, ATP receptor P2X3 gene has been reported to be up-regulated in the intact sensory neurons and down-regulated in the injured sensory neurons (Tsuzuki et al., 2001). We found that the gene is down-regulated in L4–L6 DRGs and the expression ratio is 0.66 with $P<0.05$ in microarray analysis. In addition, α-III sodium channel was reported to be up-regulated (Dib-Hajj et al., 1999) but similar gene expression changes were not observed in this microarray analysis. In general, however, we confirmed most of the genes previously reported, in addition to many novel gene regulations.

Regulation of transcription factors

Dramatic regulation of transcription factor gene expression was found in the DRGs whose axons were injured. The regulation of these genes indicates that the induction of transcription may control the regulation of other genes that may be important for the maintenance of hypersensitive neuropathic pain state. At the time point we chose for our study, we found that ATF3 and NGFI-A are up-regulated. The transcription factor ATF3 has been found to be up-regulated in both sensory and motor neurons following axotomy of peripheral nerves (Tsujino et al., 2000). In our study, we examined the regulation of this gene and found that this transcription factor is up-regulated in both injured L5–L6 DRGs, as well as in non-injured neighboring L4 DRG. In addition, we found that this gene is up-regulated in the ipsilateral spinal cord. ATF3 has previously been demonstrated to co-express and co-localize with phosphorylated c-Jun (Takeda et al., 2000). Phosphorylated c-Jun is known to mediate regeneration and cell death (Zimmermann, 2001). It is possible that ATF3 works together with phosphorylated c-Jun in these pathways. One of the consequences of ATF3 up-regulation is neurodegeneration and cell death. Our finding that this gene is induced in injured DRG and their neighbors, as well as in the spinal cord suggests that the up-regulation of this gene could mediate the spread of pathobiology following nerve injury. In addition to the induction of ATF3, we also found the induction of another IEG, NGFI-A, in the injured ipsilateral DRG. This gene has been implicated in neuronal plasticity and may regulate the expression of genes important for neuronal sensitization and plasticity (Mataga et al., 2001).

Gene regulation in the uninjured neighboring DRG and spinal cord

There is increasing evidence that uninjured neighboring neurons contribute to the development and maintenance of the overall pain profile. However, there are conflicting reports on the relative contribution of injured versus uninjured afferents to neuropathic pain behavior (Ali et al., 1999; Gold, 2000; Li et al., 2000). Prior to our study, one might argue differential regulation in intact versus injured ganglia. We have been able to speak to this experimentally for 21 genes by comparing gene expression in the intact L4 DRG and the injured L5–L6 DRGs using QRT-PCR. We found that (1) all genes tested are regulated in the L5–L6 DRGs, (2) some genes such as MHCII β, PAP, $K_v9.3$, $Na_v1.1$, RGP4, visinin and $Na_v1.1$ are also regulated in the adjacent uninjured L4 DRGs. The genes tested are regulated in the ipsilateral L5–L6 DRG to a much greater extent than in L4 ipsilateral DRGs. The finding of the differential gene regulation in intact and injured neurons provides a molecular basis to evaluate the contribution of each population to the pathophysiological process.

It is not known exactly how the uninjured adjacent DRGs sense the injury and regulate gene expression. However, there might be pathologic interactions between the axons of L4 and L5–L6 DRGs. While the axons of injured L5–L6 DRG undergo Wallerian degeneration, electrical and chemical cross-excitations may occur between injured and uninjured axons. This in turn could send signals to the neuronal cell bodies to regulate gene expression, most likely through the regulation of IEGs such as ATF3, which regulates a host of other genes including those that affect neuronal excitability.

Some gene expression changes also occur in the spinal cord, these include genes involved in neuroinflammation and neuroimmune activation, transcription factor ATF3, peripheral benzodiazepine receptor, PAP, P2Y6, metalloproteinase-1 and others. To our knowledge, this is the first study that revealed the co-regulation of many genes in the PNS and the CNS in a peripheral neuropathic pain model and indicated that pathogenesis of neuropathic pain may be similar in the CNS and the PNS.

The mechanisms for gene regulation in the spinal cord after nerve injury are not entirely clear. However, ectopic and ongoing activity have been demonstrated in C-fibers and A-fibers following nerve injury, which induce changes in the excitability of dorsal horn neurons (Suzuki and Dickenson, 2000). It is possible that abnormal and persistent input to the dorsal horn neurons activates intracellular second messenger systems, which may induce IEGs which control expression changes of other genes. In addition, brain to spinal cord descending control pathways may also regulate gene expression in the spinal cord (Field and Basbaum, 1999; Urban and Gebhart, 1999; Ossipov et al., 2000).

Gene expression changes that may directly contribute to abnormal excitability of primary afferent neurons

We identified the regulation of a number of ion chan-

nels in the injured DRGs, some of which prior to this study were not known to be regulated. These include the down-regulation of voltage-gated sodium channels, voltage-gated potassium channels, as well as ligand-gated ion channels such as kainate receptor GLUR5, ionotropic serotonin receptor 5-HT_3R, and nicotinic receptor 3.

Electrophysiological recordings have previously demonstrated that C-fibers and A-fibers develop abnormal excitability following peripheral nerve injury, a phenomenon which implicates changes in gene and protein expression of ion channels. Sodium channels are among the best studied in terms of gene regulation among the ion channels in the neuropathic pain models. For example, sodium channels $Na_v1.8$ (SNS), and $Na_v1.9$ (NAN) have been found to be down-regulated but redistributed, and Na channel type III was shown to be up-regulated in the injured DRGs (Waxman et al., 1994). These changes in sodium channels, particularly the redistribution of the ion channels, have been described as the molecular mechanism of abnormal ectopic activity in afferent fibers. Our study using microarray analysis and QRT-PCR confirmation revealed an additional Na channel, $Na_v1.1$, is also down-regulated in injured DRGs. We do not know if $Na_v1.1$ is also redistributed in the peripheral nerve. However, it is important to point out that $Na_v1.1$ and $Na_v1.9$ are also down-regulated in the ipsilateral L4 DRG based on our real-time PCR analysis. Further study is required to understand whether the regulation of these genes might contribute to spontaneous activity in both injured and neighboring DRG neurons.

We discovered that multiple voltage-gated K-channels are down-regulated in the ipsilateral DRGs. This is consistent with electrophysiological studies which demonstrated that nerve injury induced by the Chung model of neuropathic pain leads to striking reductions in voltage-gated K^+ current in DRG neurons (Everill and Kocsis, 2000). Our study is the first that provides the molecular mechanisms for the down-regulation of K^+ current. We do not yet know the identity of the neurons which have reduced expression of each of the subtypes of potassium channel genes. However, $K_v1.4$ is the sole K_v1 subunit expressed in smaller diameter neurons, suggesting that homomeric $K_v1.4$ channels predominate in A- and C-fibers arising from these cells (Rasband et al., 2001). The reduced K^+ currents may contribute to higher excitability of the injured sensory neurons, which in turn can contribute to an abnormal pain state. In addition, we found that $K_v9.3$ is also down-regulated in the uninjured ipsilateral L4 DRG. The down-regulation of this channel could contribute also to the neuronal excitability of uninjured neighboring DRG.

We found that ligand-gated kainate receptor GLUR5 is down-regulated in the injured DRGs. GLUR5 has been found to be expressed specifically in the presynaptic terminals of C-fibers, especially in neurons that innervate the inner layer of lamina II in the dorsal spinal cord (Hwang et al., 2001). Electrophysiological studies have demonstrated that this channel has an inhibitory role in regulation of neurotransmission in presynaptic cells. Kainate receptor agonists, acting at a presynaptic locus, can reduce glutamate release from primary afferent sensory synapses (Kerchner et al., 2001). It is possible that down-regulation of this channel removes the inhibition of neurotransmission from primary sensory neurons, resulting in the hyperexcitability of these neurons.

Activation of peripheral 5-HT_3 subtype of serotonin receptor has been demonstrated to be pronociceptive. Injury-induced persistent pain is significantly reduced after functional elimination of this receptor subtype (Zeitz et al., 2002). We found that the 5-HT_3 receptor is dramatically reduced in the injured DRG. The decreased expression of this gene is likely to be a compensatory event.

Neuroinflammation in the SNL model of neuropathic pain

A remarkable concerted up-regulation of genes with roles in inflammation was found in our study, in both the DRGs and in the ipsilateral spinal cord. It is not surprising that such markers for inflammation are regulated in the DRGs, since DRG axons are the primary injury site where cells of monocyte origin can be recruited. Consistent with this finding, we detected the regulation of a textbook collection of genes that are markers for neuroinflammation. Using immunohistochemical methods, we found that the number of cells that are MHCII positive increased in injured DRGs, indicating the recruitment of immune active cells to the DRG. Interestingly, a majority of these markers for neuroinflammation are also up-regulated in the ipsilateral spinal cord. In addition, several chemokines and chemokine receptors are also up-regulated in the spinal cord. The up-regulation of these marker genes in spinal cord may indicate activation of glia such as astrocytes and microglia, which secrete chemokines (Wood, 2000; Boddeke, 2001). The induction of chemokines may in turn induce infiltration of immune cells into the CNS in the ipsilateral spinal cord. Indeed, microglia activation has been described in various inflammatory pain models (Watkins and Maier, 1999). Increased immunoreactivity to microglia marker OX44 and elevated levels of cytokines such as IL-1β, IL-6, TNF-α in the spinal cord have also been described in neuropathic pain models (DeLeo et al., 1996, 1997). We found, in a separate study by QRT-PCR, that the IL-1β, IL-1α, IL-6, and TNF-α genes are all up-regulated in the ipsilateral L5+L6 DRG and spinal cord of SNL animals compared to those from sham animals (Wang et al., unpublished results). Our finding of increased expression of many cytokines such as IL-18 and IL-1β, chemokines such as MIP-1, cell adhesion molecules such as MHCII, complement proteins, and the Fc γ receptor is consistent with the activation of microglia. In addition, it is suggested that following a focal peripheral nerve injury there is a selective alteration in the integrity of the blood–brain barrier at the level of the lumbar spinal cord (DeLeo and Yezierski, 2001). In fact, there is evidence that alterations in the tight junctions of the blood–brain barrier are induced by peripheral inflammation pain states (Huber et al., 2001). The alteration in the blood–brain

barrier may allow the infiltration of immune cells into the spinal cord.

The role of glial activation in neuropathic pain behaviors following peripheral nerve injury is not clearly understood. However, several possibilities may indicate that glial activation plays an important role in the pathophysiology of neuropathic pain: (1) glial activation may play an important role in the increase in spinal excitatory amino acids such as glutamate and aspartate, which in turn cause NMDA-mediated increase in intracellular calcium levels in nearby neurons and contribute to increased spinal neuron activity (Muller, 1992; Parpura et al., 1994; McKenna et al., 1996); (2) glia also release a variety of other substances thought to be important for pain including nitric oxide and prostaglandins (Zielasek and Hartung, 1996; Watkins and Maier, 1999; DeLeo and Yezierski, 2001); (3) we found that multiple chemokine genes are up-regulated. Secreted cytokines such as IL-1 and TNF-α may potentiate pain behavior (DeLeo et al., 1996; Wagner et al., 1998). Chemokines may play an important role in recruiting immune cells into areas of active inflammation. In addition to the chemoattraction of immune cells, chemokines may also contribute to the pathogenesis by directly affecting nociceptive signal transduction (Boddeke, 2001). All these possibilities suggest that glial activation may play a role in the pathophysiology of neuropathic pain. Once activated, astrocytes and microglia form a positive feedback circuit whereby substances released from microglia activate astrocytes to release substances that further stimulate microglia, and neuroexcitatory substances they release could drive exaggerated pain states. In the subcutaneous formalin-induced hyperalgesia model, disruption of glial function reduced pain and thus suggested an important role for spinal glia in the pathophysiology of pain (Watkins et al., 1997).

Our finding of complement protein up-regulation not only represents evidence of an ongoing and significant inflammatory attack, but could also suggest that areas of complement defense may co-localize with areas of degeneration and cell death. Consistent with this implication, we found that several genes such as NADH dehydrogenase, GADD45, and cyclin D1–D3, which are indicative of cell death are also up-regulated in the DRGs (Park et al., 2000).

An interesting phenomenon we observed is that the markers for neuroinflammation in our neuropathic pain model are similar to those described in Alzheimer's disease (AD) and stroke (Walker, 2000). Basic research and clinical studies have established that inflammation may play a role in the neurodegeneration that characterizes AD, stroke, Parkinson's disease, traumatic brain and spinal cord injury, and demyelinating diseases such as multiple sclerosis (Walker, 2000; Wood, 2000). Our study underscores that neuroinflammation may also play an important role in neuropathic pain, although inflammation is most likely to arise as a secondary response to primary events in the development of neuropathic pain. It is important to recognize that, secondary or not, CNS inflammation almost always carries with it the potential to do more damage than the etiology that gave rise to it. Once established, neuroinflammation may become a significant contributor to neurodegeneration and the behavioral consequence of neuropathic pain.

Apoptosis and cell death

We found that genes involved in cell cycle regulation and genes potentially involved in cell death are up-regulated in the DRG. These include cyclin D1–3, GADD45 and NADH dehydrogenase. In the case of cell cycle proteins, there is evidence that cell cycle regulators are up-regulated during some circumstances of neuronal death. Further evidence supporting the role of cyclin D in neuronal death comes from the fact that inhibition of cell cycle elements is protective in certain paradigms of neuronal death (Park et al., 2000). In addition to NADH dehydrogenase, GADD45 and cyclin Dl, D2 and D3, we also found the up-regulation of activation transcription actor ATF3. This transcription factor has been found to co-localize and co-express with phosphorylated c-Jun (Takeda et al., 2000). It is known that phosphorylation of c-Jun without c-fos is an initial event in a long-lasting cascade of transcriptional processes resulting in either axonal regeneration or in cell death (Zimmermann, 2001). Our results are consistent with other evidence that in the DRGs and the spinal cord, apoptosis and cell death are increased following peripheral nerve injury (Himes and Tessler, 1989; Lekan et al., 1997; Whiteside and Munglani, 2001). In addition, the genes that we discovered may be used as markers for potential cell death in neuropathic pain models.

Gene expression changes in the pathogenesis of neuropathic pain and therapeutic implications

Persistent abnormal pain develops following peripheral nerve injury in both animal models and patients. Our study of the genome-wide expression changes following peripheral nerve injury in animal models, when combined with early electrophysiological studies, as well as cellular and molecular characterizations, provides a clearer picture of gene expression changes that may contribute to the pathogenesis of neuropathic pain. Repeated and persistent abnormal input following nerve injury activates a number of intracellular second messenger systems, including the phosphorylation by protein kinases such as PKC and mitogen-activated protein kinase (Ji and Woolf, 2001). Intracellular signaling cascades result in IEG induction and maintenance which control widespread changes in gene and protein expression. These changes lead to changes in neuronal excitability and neuronal plasticity, as well as secondary events such as neuroinflammation and cell death. Careful study of the relationship of different gene regulation is important for further understanding of the pathogenesis of neuropathic pain.

In summary, we have identified global gene expression changes and novel molecular pathobiology following SNL in the DRG and spinal cord. While some of the changes in gene expression we discovered may reflect regeneration and repair, others may contribute to the

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pathogenesis of neuropathic pain. We found dramatic new changes in gene expression of ion channels and signaling molecules that directly affect the excitability of neurons in both DRGs and the spinal cord. In addition, we found gene expression differences that reflect secondary events which may cause a progression of the disease, but over time could become major contributors to the pathogenesis of neuropathic pain. Future clinical research for treatment of neuropathic pain should consider both the modulation of neuronal activity and the prevention of further disease progression.

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