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Dorsal horn-enriched genes identified by DNA microarray, in situ hybridization and immunohistochemistry

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Abstract

Background: Neurons in the dorsal spinal cord play important roles in nociception and pain. These neurons receive input from peripheral sensory neurons and then transmit the signals to the brain, as well as receive and integrate descending control signals from the brain. Many molecules important for pain transmission have been demonstrated to be localized to the dorsal horn of the spinal cord. Further understanding of the molecular interactions and signaling pathways in the dorsal horn neurons will require a better knowledge of the molecular neuroanatomy in the dorsal spinal cord.

Results: A large scale screening was conducted for genes with enriched expression in the dorsal spinal cord using DNA microarray and quantitative real-time PCR. In addition to genes known to be specifically expressed in the dorsal spinal cord, other neuropeptides, receptors, ion channels, and signaling molecules were also found enriched in the dorsal spinal cord. In situ hybridization and immunohistochemistry revealed the cellular expression of a subset of these genes. The regulation of a subset of the genes was also studied in the spinal nerve ligation (SNL) neuropathic pain model. In general, we found that the genes that are enriched in the dorsal spinal cord were not among those found to be up-regulated in the spinal nerve ligation model of neuropathic pain. This study also provides a level of validation of the use of DNA microarrays in conjunction with our novel analysis algorithm (SAFER) for the identification of differences in gene expression.

Conclusion: This study identified molecules that are enriched in the dorsal horn of the spinal cord and provided a molecular neuroanatomy in the spinal cord, which will aid in the understanding of the molecular mechanisms important in nociception and pain.

Background

The dorsal horn of the spinal cord plays important roles in sensory information processing. The dorsal horn contains the neural circuitry conveying nociceptive information, including pain and temperature, from the periphery by the primary afferents [1–3]. Nociceptive afferent fibers

terminate predominately in the dorsal horn of the spinal cord. Activation of the nociceptors transmits afferent messages to the spinal cord dorsal horn through neurotransmitters such as glutamate. Initial processing of nociceptive information occurs in the spinal cord dorsal horn by excitatory and inhibitory interneurons. The projecting neurons, spinalthalamic tract cells, then convey primary nociceptive information to higher centers, signaling localization and encoding the character of the nociceptive input. Other inputs related to the subjective components of pain and related to motor and autonomic control are also relayed to higher centers. The projection neurons of the dorsal horn also activate the descending control system, which in turn controls the gain of dorsal horn neurons either through excitatory or inhibitory mechanisms. In this manner, the initial nociceptive information may be further modulated by signals descending from higher centers [1–3].

The dorsal horn can be subdivided into six distinct laminae on the basis of the cytological features of its resident neurons [2,4]. Classes of primary afferent neurons that convey distinct modalities terminate in distinct laminae of the dorsal horn. Thus, there is a close correspondence between the functional and anatomical organization of the neurons in the dorsal horn of the spinal cord. Nociceptive neurons are mostly located in the superficial dorsal horn, in the marginal layer (lamina I) and in the substantia gelatinosa (lamina II), and receive direct synaptic input from Aδ and C fibers. Laminae III and IV are located ventral to the substantia gelatinosa and contain neurons that receive monosynaptic input from Aβ fibers. Lamina V primarily contains wide-dynamic-range neurons that project to the brain stem and to regions of the thalamus. These neurons receive monosynaptic input from Aδ and Aβ fibers. They also receive input from C-fibers, either directly on their dendrites, or indirectly via interneurons. Many neurons in lamina V also receive nociceptive input from visceral structures. Neurons in lamina VI receive input from large-diameter afferents from muscles and joints and respond to nonnoxious manipulations of joints. These neurons are thought not to contribute to the transmission of nociceptive messages.

Consistent with the important role of the dorsal horn of the spinal cord in pain transmission and modulation, neurochemical studies have implicated an enriched expression of neurotransmitters, neuropeptides, ion channels, and receptors in these neurons, including substance P, enkephalin, CGRP, somatostatin, and GABA. The morphology of primary afferent central terminals, dorsal horn neurons, and descending systems, together with their chemical neuroanatomy, synaptic arrangements, transmitter systems, and functional properties have been extensively documented [1–5]. However, the molecular/chemical neuroanatomy of the dorsal horn is a subject that is constantly being revised and updated as improved techniques reveal new insights into classical pathways or substances that are localized in the spinal cord. In this study, we performed a large-scale screening for genes that are enriched in the dorsal spinal cord. In addition to molecules that are known to be highly expressed in the dorsal spinal cord, we identified other neuropeptides, ion channels, and signaling molecules enriched in the dorsal spinal cord. We then further characterized the cellular localization of a subset of these genes in the spinal cord, as well as the regulation of a subset of the genes in a neuropathic pain model.

Results

Global identification of genes that are enriched in the dorsal spinal cord using DNA microarray analysis

In order to identify genes that are enriched in the dorsal spinal cord, we took advantage of DNA microarray technology which is powerful in identifying regional-specific gene expression. We screened globally for genes that are expressed at higher levels in the dorsal spinal cord as compared to the ventral spinal cord. A total of 9 rats were used in this study. Dorsal spinal cord and ventral spinal cord are separated by cutting through the central cannel. Dorsal spinal cord samples from 3 rats were pooled and ventral spinal cord samples from the same 3 rats were pooled to form each pair of samples. RNA was prepared from 3 such pairs of samples, each was analyzed by hybridization with a Affymetrix chip RG-U34A, which contains 8799 probesets. Using the SAFER analysis approach (see methods), we compared gene expression between the dorsal and ventral spinal cord samples using a paired t-test and analysis of variance (ANOVA). Using a paired t-test we found 31 probesets with p-value smaller than 0.05 and greater than 2-fold higher expression in the dorsal spinal cord (Table 1). An ANOVA analysis detected 30 of these 31 probesets along with 21 additional probesets that met these criteria. We found evidence in the literature for enriched expression in the dorsal spinal cord of 23 genes among the 51 genes detected by ANOVA (Table 1). In addition, we independently tested the expression of 7 genes by QRT-PCR, 2 genes by in situ hybridization, and 2 genes by immunohistochemistry. All but one of these genes were confirmed to be enriched in the dorsal spinal cord. Both paired t-test and ANOVA analysis detected genes that are enriched in the dorsal spinal cord as revealed by independent confirmation. As ANOVA analysis provides more candidate genes, we chose to subsequently present the results based on ANOVA analysis.

The 51 genes that are expressed higher in the dorsal spinal cord represent several functional categories including neuropeptides, ion channels, receptor and signaling molecules, calcium/calmodulin binding proteins, transcrip-

Table 1: Confirmation and comparison of ANOVA and paired t-test for microarray analysis.

Accession#	Description	D vs V Ratio	Confirmation	P_{ANOVA}	P_{pair}	Reference
K02248	Somatostatin	11.1	literature	<0.05	<0.05	[21–24]
S49491	Proenkephalin	4.9	literature	<0.05	<0.05	[25–28]
L09119	Neurogranin	4.1	literature	<0.05	<0.05	[29]
AF058795	GABA b2	2.8	literature	<0.05	<0.05	[30]
AB004267	CAMK I b2	2.7	literature	<0.05	<0.05	[31]
M15880	NPY	2.4	literature	<0.05	<0.05	[24,32]
X04139	PKC	2.3	literature	<0.05	<0.05	[33][34]
M25890	Somatostatin	12.4	literature	<0.05	<0.05	[21–24]
X56306	Protachykinin	6.5	literature	<0.05	<0.05	[24,27,35]
M15191	Beta-tachykinin	5.0	literature	<0.05	<0.05	[24,27,35]
X55812	Cannabinoid receptor	2.9	literature	<0.05	<0.05	[36]
M16410	Neurokinin B	2.3	literature	<0.05	<0.05	[35,37]
X62840	K channel Kv 3.1	2.2	literature	<0.05	<0.05	[38]
S79730	Nociceptin	2.1	literature	<0.05	<0.05	[39]
S39221	NMDA receptor NR1	2.5	literature	<0.05	<0.05	[40]
X57573	GAD	2.9	literature	<0.05	<0.05	[24,41,42][43]
AI102205	Vesicle associated calmodulin protein	4.6	Test confirmed	<0.05	<0.05	
D17764	Synuclein beta	2.2	Test confirmed	<0.05	<0.05	
AF023087	NGFI-A	2.2	Test confirmed	<0.05	<0.05	
D12573	Hippocalcin	2.2	Test confirmed	<0.05	<0.05	
AI639118	EST	2.5	Test confirmed	<0.05	<0.05	
X54249	Zinc finger protein	2.4	Test not confirmed	<0.05	<0.05	
X67241	Guanine nucleotide releasing factor	2.2	Not tested	<0.05	<0.05	
L25633	Neuroendocrine-specific protein	2.0	Not tested	<0.05	<0.05	
D10666	Neural visinin-like protein	2.4	Not tested	<0.05	<0.05	
D90219	CNP	2.2	Not tested	<0.05	<0.05	
AF007758	Synuclein-1	2.0	Not tested	<0.05	<0.05	
U88958	Neuritin	2.0	Not tested	<0.05	<0.05	
E13644	Neurodap-1	2.0	Not tested	<0.05	<0.05	
AI639213	EST	3.1	Not tested	<0.05	<0.05	
AF041107	Tulip2	2.1	Not tested	**>=0.05**	<0.05	
AF019974	Chromogranin B	2.5	literature	<0.05	**>=0.05**	[44][45]
D10392	Syntaxin 1A	2.4	literature	<0.05	**>=0.05**	[46]
M93669	Secretogranin	2.4	literature	<0.05	**>=0.05**	[44,47]
X62839	K-channel 3.1	2.3	literature	<0.05	**>=0.05**	[38]
AA894330	CAMKII beta	2.2	literature	<0.50	**>=0.05**	[31]
L09119	Neurogranin	2.1	literature	<0.05	**>=0.05**	[29]
AA925248	Sodium channel SCN6A	10.0	literature	<0.05	**>=0.05**	[48]
AF078779	4-repeat ion channel	6.9	Test confirmed	<0.05	**>=0.05**	
S80376	G alpha(olf)	2.6	Test confirmed	<0.05	**>=0.05**	
AI014091	MRG1	2.1	Not tested	<0.05	**>=0.05**	
AI639036	EST	11.3	Not tested	<0.05	**>=0.05**	
AI639062	EST	2.9	Not tested	<0.05	**>=0.05**	
AI639470	EST	2.8	Not tested	<0.05	**>=0.05**	
M20722	Proline rich protein	2.7	Not tested	<0.05	**>=0.05**	
AF034899	Olfactory receptor (SCR D-9)	2.3	Not tested	<0.05	**>=0.05**	
AF019043	Dynamin-like protein	2.2	Not tested	<0.05	**>=0.05**	
AF091834	NSF	2.2	Not tested	<0.05	**>=0.05**	
X67877	Resiniferatoxin-binding protein	2.1	Not tested	<0.05	**>=0.05**	
AF089839	N-ethylmaleimide sensitive factor	2.1	Not tested	<0.50	**>=0.05**	
AI072943	EST	2.1	Not tested	<0.05	**>=0.05**	
AA866291	EST	2.0	Not tested	<0.05	**>=0.05**	

Genes that show greater than 2-fold expression in the dorsal spinal cord versus ventral spinal cord with $P < 0.05$ are listed for each analysis method. The gene expression fold increase for each probeset was calculated as the ratio of means of gene expression index between dorsal and ventral spinal cord.

Table 2: Genes that are enriched in the dorsal spinal cord based on microarray analysis.

Description	Accession#	D vs V ratio	Description	Accession#	D vs V ratio
Neuropeptide			**Calcium calmodulin binding protein**		
Somatostatin**	M25890	11.1–12.4	Vesicle associated calmodulin protein	AI102205	4.60
Protachykinin	X56306	6.45	Neurogranin**	L09119	2.1–4.1
Beta-tachykinin	M15191	5.02	Chromogranin B	AF019974	2.49
Proenkephalin	S49491	4.89	Neural visinin-like protein	D10666	2.42
NPY	M15880	2.44	Secretogranin	M93669	2.35
Neurokinin B	M16410	2.27	Hippocalcin	D12573	2.16
Nociceptin	S79730	2.10			
CNP	D90219	2.17	**Others**		
			Syntaxin 1A	D10392	2.43
Channel			Synuclein-1	AF007758	2.03
Sodium channel SCN6A	AA925248	10.02	Synuclein beta	D17764	2.21
Four repeat ion channel	AF078779	6.92	Neuritin	U88958	2.03
NMDA receptor NR1	S39221	2.48	Neurodap-1	E13644	2.03
K channel Kv 3.1**	X62840	2.2–2.3	GAD	X57573	2.86
			Proline-rich protein	M20722	2.73
Signaling			NSF	AF091834	2.16
Cannabinoid receptor	X55812	2.93	Resiniferatoxin-binding protein	X67877	2.13
GABA-B R2	AF058795	2.79	N-ethylmaleimide sensitive factor	AF089839	2.08
Olfactory receptor (SCR D-9)	AF034899	2.35	Tulip2	AF041107	2.08
G alpha (olf)	S80376	2.63	Neuroendocrine-specific protein	L25633	2.01
Guanine nucleotide releasing factor	X67241	2.21	Dynamin-like protein	AF019043	2.22
CAMK I beta 2	AB004267	2.73			
CAMKII beta	AA894330	2.15	**EST**		
PKC beta	X04139	2.29	EST	AI639036	11.34
			EST	AI639213	3.13
Transcription factor			EST	AI639062	2.88
Zinc finger protein	X54249	2.40	EST	AI639470	2.77
NGFI-A	AF023087	2.21	EST	AI639118	2.50
MRG1	AI014091	2.12	EST	AI072943	2.06
			EST	AA866291	2.01

Genes that show greater than 2-fold expression in the dorsal spinal cord versus ventral spinal cord 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 dorsal and ventral spinal cord. The genes shown to be up-regulated by more than one probeset are indicated with asterisks. The fold increase for these genes is given as a range of fold increase detected by all the probesets for that gene. The genes that were tested by real-time PCR or immunohistochemistry are underlined.

tion factors, synaptic proteins, and others (Table 2). Neuropeptide genes are among the category that have the highest number of genes expressed specifically in the dorsal spinal cord. These include somatostatin, protachykinin, beta-tachykinin, neuropeptide Y (NPY), neurokinin B, and nociceptin. In addition, we also detected another peptide, C-type natriuretic peptide (CNP), which is expressed at 2-fold higher levels in the dorsal spinal cord. A number of ion channels were found to be expressed higher in the dorsal spinal cord, including, voltage-gated sodium channel type VI (SCAN6A), a putative four repeat ion channel, NMDA receptor NR1, and potassium channel Kv3.1. A few G-protein coupled receptors such as cannabinoid receptor, GABA-B R2 receptor, and olfactory receptor-like protein (SCRD-9), as well as molecules important in signaling such as Galpha(olf), Guanine nucleotide releasing factor, and CAM kinase I beta 2, CAM kinase II beta, and PKC beta were also identified to be expressed more in the dorsal spinal cord. We found that the dorsally enriched genes also include several members of the calcium sensor protein family, such as vesicle associated calcium calmodulin binding protein (CAMK IV homolog), PKCγ substrate neurogranin, chromogranin, neurovisinin-like protein, secretogranin, and hippocalcin. In addition, several transcription factors and synaptic proteins were also expressed higher in the dorsal spinal cord (Table 2).

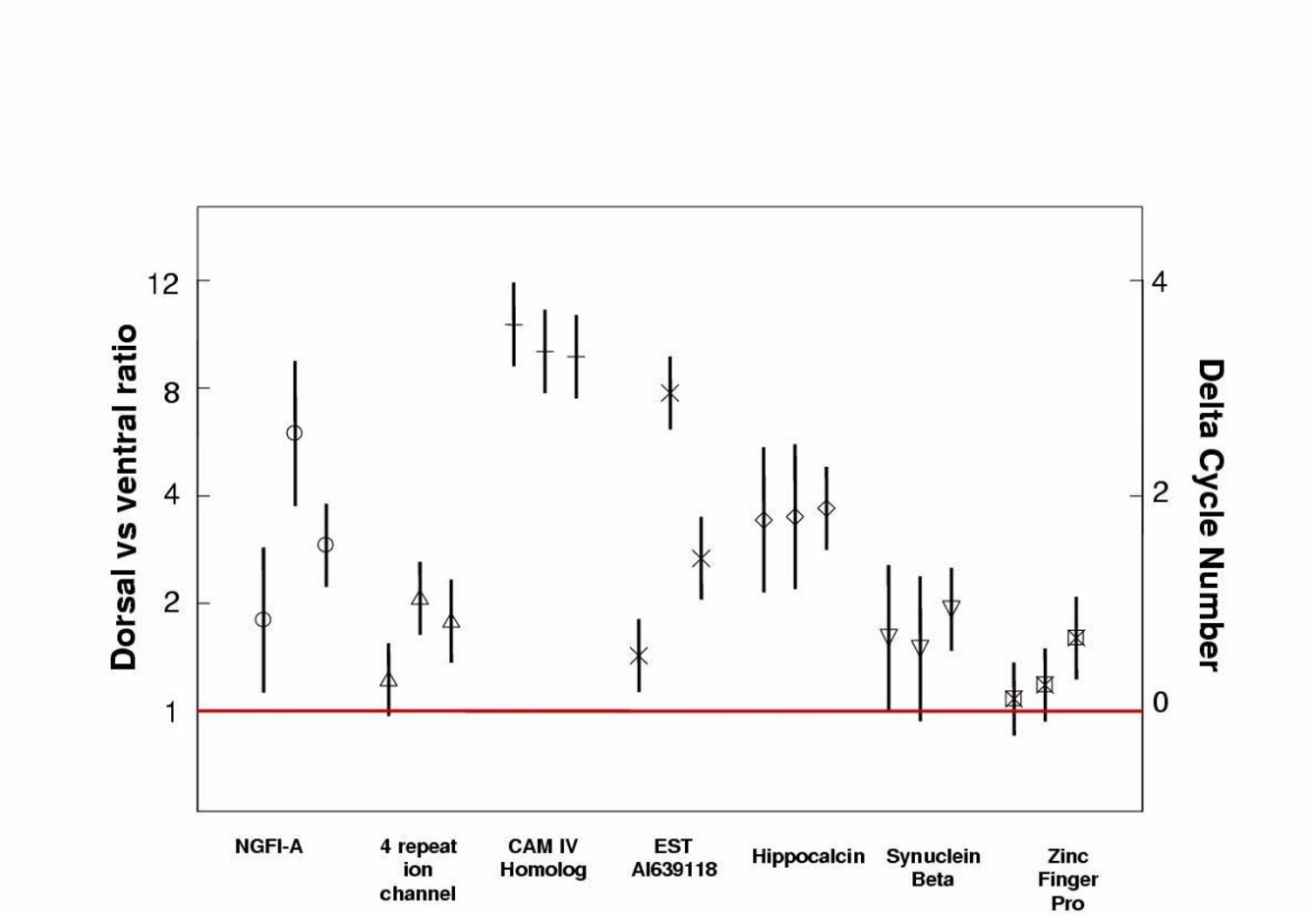

Figure 1
Differential expression of genes in the dorsal versus ventral spinal cord as revealed by QRT-PCR. The ratio of expression between dorsal and ventral spinal cord for each gene in each dorsal and ventral spinal cord sample pair is shown. Data were from QRT-PCR assays performed in triplicate for a pair of RNA samples, each of which was pooled from the dorsal or ventral samples of the 3 pairs of RNA samples used for microarray analysis (from a total of 9 animals). The length of bars represent 95% confidence intervals and the symbol on each bar is the mean of ratios estimated from the assay performed in triplicate.

QRT-PCR confirmation of genes enriched in the dorsal spinal cord

We sought to confirm the differential gene expression of a selected subset of genes in the spinal cord along the dorsal-ventral axis using QRT-PCR. The genes selected showed expression differences between 2 to 7-fold by DNA microarray. We compared gene expression between dorsal and ventral spinal cord samples. And the results of these comparisons are depicted in Fig. 1. In general, higher expression was confirmed for 6 out of the 7 genes tested in the dorsal spinal cord, despite some degree of variation among the 3 pairs of samples from different animals (Fig. 1).

We confirmed the enriched expression of the following genes: a transcription factor NGFI-A, a putative four repeat ion channel, a vesicle associated calcium/calmodulin binding protein that is homologous to CAMK IV, a neuronal calcium sensor protein hippocalcin, synuclein beta, and an EST gene. The Zinc finger protein mRNA was found to be only marginally enriched in the dorsal spinal cord (Fig. 1).

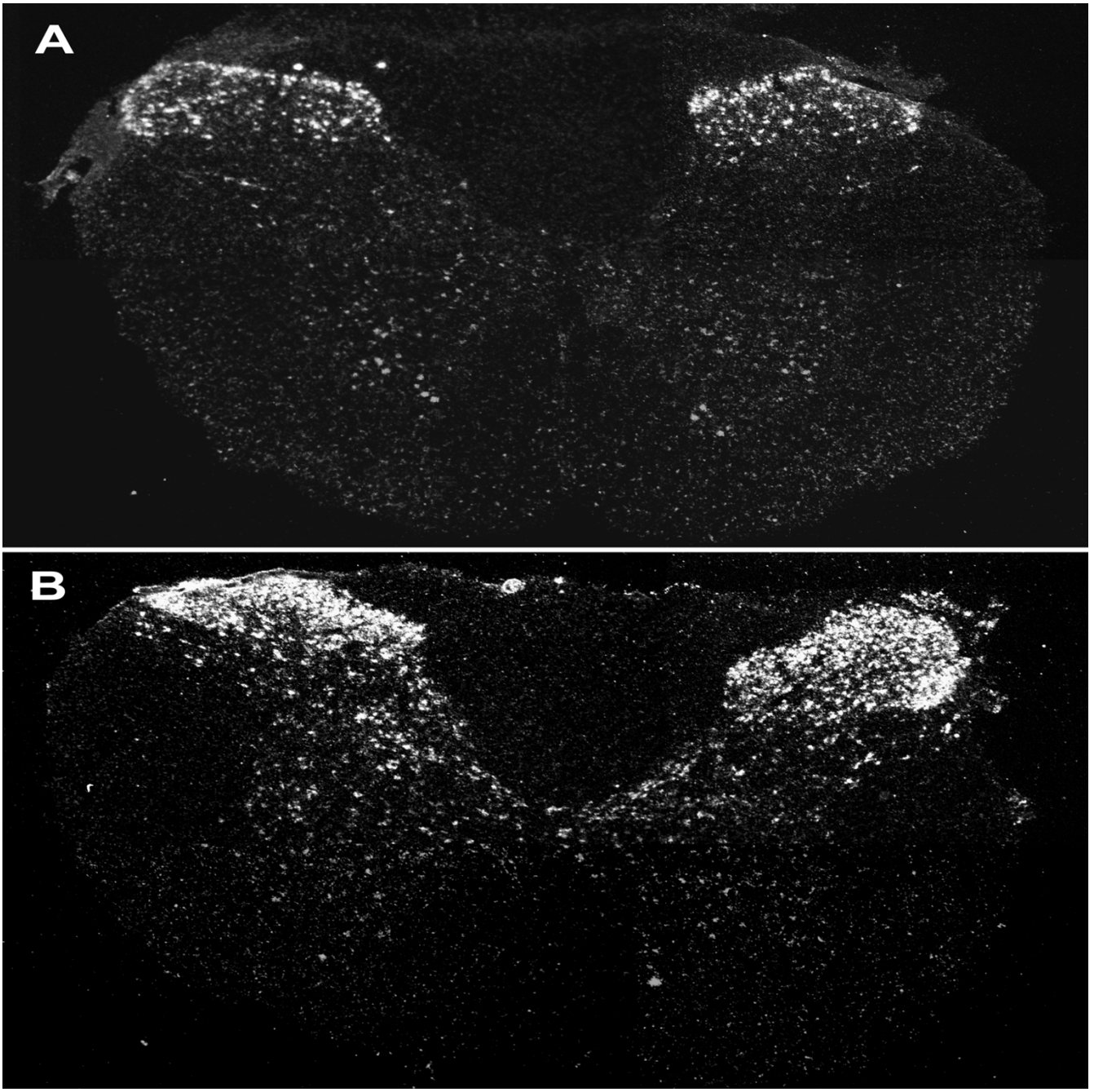

Figure 2
mRNA localization of neurogranin and CAMK IV homolog. Neurogranin (A) and CAMK IV homolog (B) are located in the superficial dorsal horn and exhibit different patterns with the antisense riboprobes. Pictures were pieced together from 4 images which were taken using 4X objectives.

Cellular expression of dorsal spinal cord-enriched genes

Using in situ hybridization and immunohistochemistry, we sought to investigate whether differences in gene expression as detected by DNA microarray reflect differences at the cellular level. In situ hybridization revealed that PKCγ substrate neurogranin and vesicle-associated calcium/calmodulin binding protein (CAMK IV homolog) mRNA are both highly enriched in the superficial layers of the dorsal horn of the spinal cord (Fig. 2). Neurogranin expression seems to be enriched in subsets of the cells of lamina II and III (Fig. 2A), similar to PKCγ localization (Fig 3C). CAMK IV homolog seems to be broadly ex-

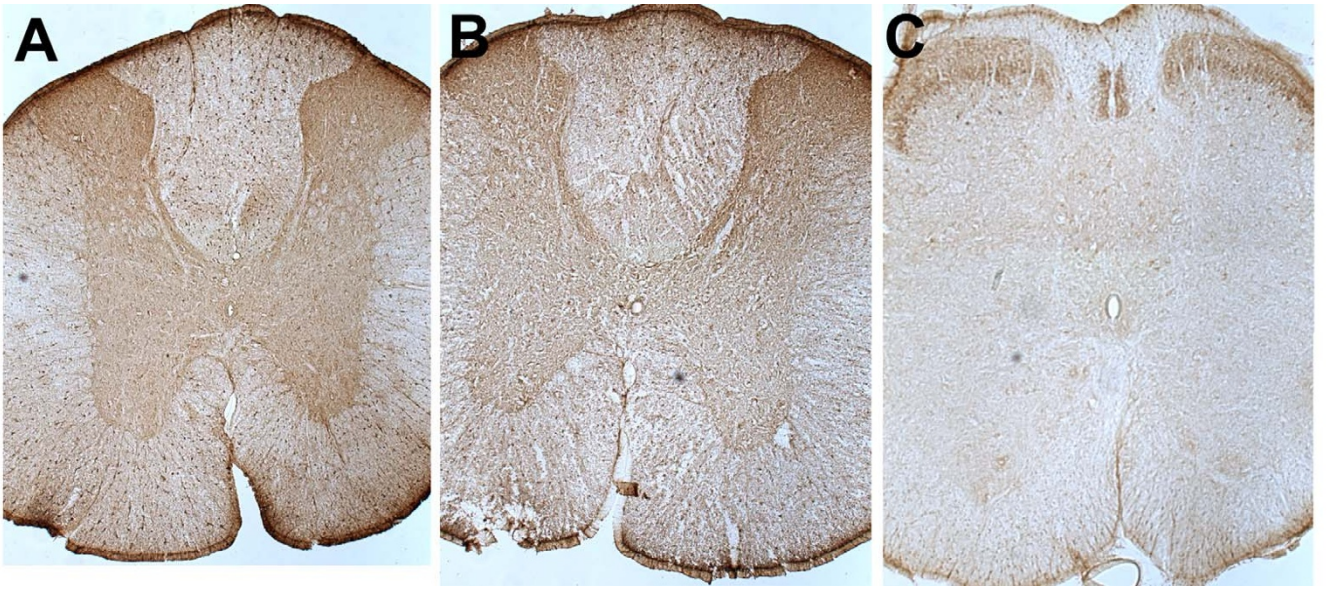

Figure 3
Cellular localization in the spinal cord as revealed by immunohistochemistry. Galpha(olf) (A), Syntaxin 1A (B), and PKCγ (C) are localized primarily in the dorsal spinal cord. Images were taken from sections of spinal cord from the same animal although similar patterns of staining were found when two animals were examined.

pressed in layers I-III of the dorsal horn (Fig. 2B). Immunohistochemistry with antibodies against Galpha(olf), syntaxin 1A, and PKCγ revealed that these proteins are also enriched in the dorsal spinal cord (Fig. 3). Galpha(olf) and Syntaxin 1A appear to be localized in the neuropil of the dorsal horn (Fig. 3A and 3B) whereas, PKCγ appears to be expressed in layer IIi of the dorsal horn (Fig. 3C) as previously described [6].

Regulation of dorsally enriched genes in a chronic neuropathic pain model

We sought to study whether the genes that are enriched in the dorsal spinal cord are regulated during a persistent pain state such as the spinal nerve ligation (SNL) model of neuropathic pain. For a subset of genes enriched in the dorsal spinal cord, we used QRT-PCR to compare the gene expression in the lumbar spinal cord between spinal nerve ligation rats and rats subjected to sham surgery, and to compare expression between ipsilateral and contralateral sides (separated by cutting through the midline in the segment of lumbar spinal cord) in spinal nerve ligation rats. In general, we found that genes that are enriched in the dorsal spinal cord are not among those found to be upregulated in the spinal nerve ligation model of neuropathic pain (Table 3).

Discussion

The dorsal spinal cord is a region implicated in sensory perception, receiving, transmitting, and modulation of signals from peripheral sensory system. It is important in the integration of computational and neuromodulatory functions. In a pathological state, the changes in the dorsal horn of the spinal cord may contribute to prolonged abnormal pain. The aim of this study was to find genes that are enriched in the dorsal spinal cord that can potentially play important roles in pain transmission, pain modulation, and pathophysiological conditions. Since microarray technology represents a potentially powerful method for identifying cell type- and regionally restricted genes expressed in the nervous system [7,8], we conducted a large-scale screening using this technique for genes that are expressed higher in the dorsal spinal cord. Genes found in this screen can then be further studied for their cellular localization in the spinal cord.

Validation of microarray data

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 21 genes (by 23 probesets) which have previously been described to be enriched in the dorsal spinal cord; (3) Genes that we chose to study further were confirmed to be expressed higher in the dorsal spinal cord by QRT-PCR (6 out of 7), in situ hybridization (2 out of 2), and immunohistochemistry (2 out of 2). Based on the consistency and the rate of independent confirmation, many of the genes listed in the tables are likely to be true positives.

Table 3: Relative gene expression in the spinal cord of SNL (Chung) neuropathic pain model as revealed by QRT-PCR.

	Chung Ipsilateral vs Contralateral Relative Expression Scale			Chung Ipsilateral vs Sham ipsilateral Relative Expression Scale		
	Estimate	Lower 95% CI	Upper 95% CI	Estimate	Lower 95% CI	Upper 95% CI
NGFI-A	1.211	0.887	1.654	1.217	0.959	1.544
4 Repeat Ion Channel	0.837	0.613	1.143	1.197	0.944	1.519
CAMK IV Homolog	0.968	0.709	1.322	1.206	0.950	1.530
EST (AI639118)	0.831	0.609	1.135	0.859	0.677	1.089
Hippocalcin	1.089	0.798	1.487	1.237	0.975	1.569
Synuclein beta	1.079	0.791	1.473	1.278	1.007	1.621
Neurogranin	0.897	0.501	1.606	0.977	0.562	1.698

The expression between Chung ipsilateral and contralateral spinal cord, as well as between Chung ipsilateral spinal cord and sham ipsilateral spinal cord were compared and fold changes estimated with 95% confidence interval given.

We note that our list of dorsal spinal cord-enriched genes is likely to be incomplete since the Affymetrx DNA microarrays do not represent the entire rat genome and our study may not have been sensitive enough to discover all the genes, especially those that are expressed at low levels.

Toward a molecular anatomy of the spinal cord, particularly in the pain sensory pathways

We found that some genes are expressed in a lamina-specific manner, while others may have a gradient of expression. Neurogranin is highly enriched in the superficial laminae. PKCγ has been demonstrated to be expressed in lamina IIi. The colocalization of neurogranin and PKCγ in similar regions in the spinal cord suggests that neurogranin may be an endogenous substrate of PKCγ in the spinal cord. Neurogranin has been shown to be phosphorylated by PKCγ, and this phosphorylation is greatly decreased in PKCγ knockout mice, suggesting neurogranin is a PKCγ-specific substrate [9]. Evidence that strongly suggests the phosphorylation of neurogranin plays an important role in neural plasticity comes from the studies that demonstrated neurogranin knockout mice show essentially the same deficits in behaviors related to learning and memory as that of the PKCγ knockout mice [10]. Neurogranin is not only a substrate of PKCγ, but also plays a important role in regulating PKC signaling [11]. Interstingly, PKCγ knockout mice demonstrate reduced neuropathic pain [12]. Similar to neurogranin, we found that a calmodulin-binding, vesicle-associated, CAM kinase IV-like protein is highly expressed and enriched in the superficial layer of the dorsal spinal cord. The function of this gene is not yet known. However, the protein was also found to be enriched in forebrain neurites [13].

Using immunostaining, we found that Syntaxin 1A and Galpha(olf) proteins were enriched in the dorsal spinal cord. Syntaxin 1A has previously been shown to be preferentially expressed in the dorsal spinal cord neuropil. Galpha(olf) is a G protein that was initially found to be expressed in the olfactory epithelium. The Galpha(olf) has been shown to be specifically expressed in the striatum in the brain and was found to colocalize with and activated by adenosine A_{2A} receptors [14]. It is possible that the specific expression of Galpha(olf) in the dorsal spinal cord implies its mediation of functions for specific G-protein-coupled receptors in these neurons.

Conclusions

1. We conducted a large-scale screening using DNA microarray for genes that are specifically expressed in the dorsal spinal cord. We found additional neuropeptides, receptors, ion channels, and signaling molecules to be enriched in the dorsal spinal cord.

2. The regulation of a subset of the genes was confirmed by QRT-PCR. Six out of the seven genes were confirmed to be enriched in the dorsal spinal cord.

3. In situ hybridization and immunohistochemistry revealed that neurogranin, CAMK IV homolog, Galpha(olf), and Syntaxin 1A, are indeed enriched in the dorsal spinal cord.

4. Through the detection of a large number of genes which were previously determined to have enriched expression in the dorsal spinal cord and our QRT-PCR, in situ hybridization and immunohistochemical confirmations, this study provides a level of validation for the case of Affyme-

trix DNA microarrays in conjunction with SAFER algorithm to detect differences in gene regulation.

Materials and Methods

Animals

Male Sprague-Dawley rats (Taconic, Germantown, NY.) 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 [15] and received approval from the Institutional Animal Care and Use Committee of MRL, West Point, PA.

Spinal nerve ligation (SNL) injury was induced using the procedure of Kim and Chung [16]. 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 spinal nerve ligation.

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 [17] and expressed as the mean withdrawal threshold. Animals were tested before surgery and only animals with a paw withdrawal threshold greater than 10 grams were used for the subsequent study. Surgically treated animals were then tested on postoperative 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 postoperative day 13 (less then 10% of the animals were excluded for tissue collection).

Tissue dissection and RNA preparation

Total RNA from each sample was prepared using Trizol™ (Life Technologies, Gaithersburg, MD), 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) 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 [18]. 5 µM primer encoding the T7 RNA polymerase promoter linked to oligo-dT_{24} primer 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). 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) and adsorption to silica (Qiaquick™ kit, Qiagen, Hilden, Germany) according to manufacturers' 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, NY). These cRNA transcripts were purified using RNeasy™ columns (Qiagen, Hilden Germany) and quantitated by measuring absorption at 260 nm/280 nm. 15 µg aliquots of each cRNA sample were fragmented at 95°C for 35 min in 40 mM Tris-acetate, pH8.0, 100 mM KOAc, and 30 mM MgOAc to a mean size of ~50–150 nucleotides. Hybridization buffer (0.1 M MES, pH6.7, 1 M NaCl, 0.01% Triton, 0.5 mg/ml BSA, 0.1 mg/ml H. Sperm DNA, 50 pM Control Oligo B2, and 1X 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, Eugene, OR), biotinylated anti-streptavidin antibody (Vector Laboratories, Inc., Burlingame, CA), and streptavidin phycoerythrin on the Fluidic Station (Affymetrix, Santa Clara, CA). Finally, the microarrays were scanned with a dedicated Gene Array Scanner (Hewlett Packard Instruments, TX) to capture a fluorescence image.

Affymetrix microarray data analysis

A total of 9 animals was divided into 3 groups, and the dorsal and ventral spinal cord were pooled for each group to give rise to 6 samples. Each sample was analyzed on one 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 [19] for all chip analysis. The SAFER gene expression index is a robust and

Table 4: Primers and probes used for QRT-PCR. The sequences of forward, reverse and specific probes are listed sequentially for each gene (accession #).

Gene (GenBank accession #)	Bases	Sequence (5'-3')	Product size (bp)
NGFI-A (AF023087)	120–140	TATCCATGTTCGGGAGTTGGA	81
	171–200	AATGAACTTCATGTTCATAGCATACAAAGT	
	142–170	CACCGCCTACTCAGTAGGTAACCACAGCA	
Four Repeat Ion Channel (AF078779)	5127–5150	GGAGGAAGGACAACAATGAAGTCT	75
	5183–5201	TTCGGAGCCACAGGAAGCT	
	5155–5177	TGTGCAAGATGAACCCCATGCCA	
CAMK IV homolog (AI102205)	263–283	TGTGAAAAGCAAGCTCCCAAA	81
	323–343	CAGGCTCAGGCCATAAATCCT	
	299–319	CTGCCATGCCTCCCTGGGAGG	
EST (AI639118)	56–80	GAAGTTGCTCTGACTGAATGGATTT	123
	153–178	TCACAGACTTACATCCTGTTTCTGAA	
	93–121	AGCTGTCACACTTGTTGTCGGAAGCACAC	
Hippocalcin (D12573)	607–628	CCGGAAAAGAGGACTGAGAAAA	134
	723–740	CTGCTGGGATCGCATTGC	
	630–656	CTTCCGCCAAATGGACACAAACAATGA	
Synuclein beta (D17764)	227–247	AACAAAGGAGCAGGCATCTCA	127
	336–353	TTGGGCCACTTCCTCTGG	
	274–296	CTGGGAACATTGCAGCAGCCACC	
Zinc Finger Protein (X54249)	1870–1893	GAGAGTGCACACATCAGCATTAGA	98
	1944–1967	TCCTTTTCACCATCGTGTAAGTCA	
	1918–1941	CAGCTCTGTACCTCAGCCGCCCAC	

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 [20]. Like Li and Wong's procedure, the procedure for calculating the SAFER gene index involves both between-array normalization and an adjustment for probe-specific biases.

To analyze gene expression in the spinal cord, differences in mean level of the gene expression index between dorsal and ventral samples were assessed for each probeset using a paired t-test and ANOVA. These models facilitated estimation of ratios comparing the dorsal and ventral 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.

Quantitative Real-Time PCR (QRT-PCR)

Total RNA was treated with DNase I, Amplification Grade (Invitrogen, Carlsbad, CA) 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). Real-time PCR analysis was performed on a Applied Biosystems ABI Prism7700 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^R DNA Polymerase, AmpErase^R UNG, dNTPs (with dUTP), Passive Reference 1, optimized buffer components (proprietary formulation) and 1 µl of cDNA template.

Primers and probes for GAPDH and IL-18 were obtained from Applied Biosystems. The sequence of the primers and probes used is listed in Table 4.

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 between dorsal and ventral samples was calculated as: $2^{-(\text{mean}\Delta\Delta\text{Ct})}$. C_t represents the threshold cycle and $\Delta\Delta C_t$ represents the difference $C_{t(\text{test gene})} - C_{t(\text{GAPDH RNA})}$ for dorsal sample minus ventral sample. Using the ANOVA method, 95% confidence intervals were determined for each ratio as:

$$2^{-}\left(mean\Delta\Delta Ct\right) \pm t_{0.975,N-m^{S}}\sqrt{\frac{1}{n_i}+\frac{1}{n_j}}$$

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 dorsal and ventral samples, respectively, being compared. Similarly, expression between ipsilateral and contralateral samples were analyzed.

Immunocytochemistry

Rat spinal cords were dissected from rats which had been perfused with 4% paraformaldehyde and post fixed for 4 hours. After cryoprotection in 30% sucrose overnight and 30% sucrose/OCT (1:1) mixture for 8 hours, the tissue was frozen and sections of 30 um were cut with cryostat. Tissue sections were floated, washed several times with PBS, then treated with 0.5% H_2O_2 for 30 minutes followed by washing with PBS 3 times. The sections were then incubated with blocking buffer (3% BSA + 3% donkey serum + 0.1% Triton) for 1 hour, followed by incubation with primary antibodies for 2 hours 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 manufacturer's recommendations. The antibody to syntaxin-1 and Galpha(olf) were purchased from Santa Cruz Biotechnology, Inc. and used at 1: 20 dilution.

In situ hybridization

Twenty micron sections were collected on gelatin-coated slides, dried and then stored at -80°C in desiccated boxes. At the time of processing, the slides were warmed to room temperature, postfixed in paraformaldehyde, treated with acetic anhydride and then delipidated and dehydrated. Processed section-mounted slides were hybridized with antisense or sense (control) riboprobes (8–12 × 10^6 DPM/slide) in 50% formamide hybridization mix and incubated overnight at 55°C in an open-air humidified slide chamber. In the morning, the slides were immersed in 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate; pH 7.0)/10 mM DTT, treated with RNase A (20 mg/ml) and washed 2 × 30 min at 65°C in 0.1× SSC to remove nonspecific label. After dehydration, the slides were apposed to BioMax (BMR-1; Kodak) X-ray film for 1–2 days and then dipped in NTB2 nuclear emulsion (Eastman Kodak; diluted 1:1 with 600 mM ammonium acetate). The slides were exposed for 21 days in light-tight black desiccated boxes, photographically processed, stained in Cresyl violet and coversliped.

Authors' contributions

H.S. carried out the tissue dissection, molecular biology study, in situ hybridization studies. J. Xu carried out Affymetrix hybridization, K. D. P. conducted QRT-PCR analysis. R.J.B participated in the Affymetrix microarray hybridization. F. K conducted immunohistochemistry. D. J. H conducted data analysis for Affymetrix microarray and performed the statistical analysis. D. L. G and K. S. K. participated in the design of the study. H. W. conceived the study, conducted in its design and coordinations, and wrote the manuscript.

All authors read and approved the final manuscript.

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