Gene expression profiles of laser-captured adjacent neuronal subtypes

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Gene expression profiles of thousands of genes can now be examined *en masse* through cDNA and oligonucleotide microarrays[1–3]. Recently, studies have been reported that examined gene expression changes in yeast[4,5], as well as in mammalian cell lines[6], primary cells[7] and tissues[8]. However, present applications of microarray technology do not include the study of gene expression from individual cell types residing in a given tissue/organ (that is, *in situ*). Such studies would greatly facilitate our understanding of the complex interactions that exist *in vivo* between neighboring cell types in normal and disease states. We demonstrate here that gene expression profiles from adjacent cell types can be successfully obtained by integrating the technologies of laser capture microdissection[9] (LCM) and T7-based RNA amplification[10] with cDNA microarrays[11].

Neighboring small and large neurons are individually captured

To demonstrate this integration of technologies, we examined the differential gene expression between large- and small-sized neurons in the dorsal root ganglia (DRG). In general, large DRG neurons are myelinated, fast-conducting and transmit mechanosensory information, whereas small neurons are unmyelinated, slow-conducting and transmit nociceptive information[12]. We chose this system because numerous differentially expressed genes (small versus large) have been reported, thus the success of this experiment could be assessed; and because many small and large neurons are adjacent to each other, thus we could test whether individual neurons can be cleanly captured. Large (diameter of >40 μm) and small (diameter <25 μm and with identified nuclei) neurons were cleanly and individually captured by LCM from sections (10 μm in thickness) of Nissl-stained rat DRG (Fig. 1). For this study, two sets of 1,000 large neurons and three sets of 1,000 small neurons were captured for cDNA microarray analysis.

RNA amplification is reproducible between individual captures

RNA was extracted from each set of neurons and linearly amplified (independently) an estimated 10^6-fold using T7 RNA polymerase. After being amplified, one fluorescently labeled probe was synthesized from an individually amplified RNA (aRNA), divided equally into three parts and hybridized in triplicate to a microarray ('chip') containing 477 cDNAs (see Methods for chip design) plus 30 cDNAs encoding plant genes (for the determination of non-specific nucleic acid hybridization). Expression in each neuronal set (called S1, S2 and S3 for small and L1 and L2 for large neurons) was thus monitored in triplicate, requiring a total of 15 microarrays. The quality of the microarray data is demonstrated by pseudocolor arrays, one resulting from hybridization to probes derived from neuronal set S1 and the other from neuronal set L1 (Fig. 2*a*). In Fig. 2*a*, the enlarged part of the chip shows some differences in fluorescence intensity (that is, expression levels) for particular cDNAs and demonstrates that spots containing the different cDNAs are relatively uniform in size and that background between spots is relatively low. To determine whether a signal corresponding to a particular cDNA is reproducible between different chips, we calculated the coefficient of variation [c.v. or (standard deviation/mean) × 100%] for each neuronal set. From these values, the overall average c.v. for all 477 cDNAs per neuronal set was calculated to be: 15.81%, 16.93% and 17.75% for S1, S2 and S3, respectively, and 20.17% and 19.55% for L1 and L2, respectively.

Independent amplifications (about 10^6-fold) of different sets of the same neuronal subtype yielded quite similar expression patterns. For example, the correlation of signal intensities between S1 and S2 was $R^2 = 0.9688$, and between S1 and S3 was $R^2 = 0.9399$ (Fig. 2*b*). Similar results were obtained for the two sets of

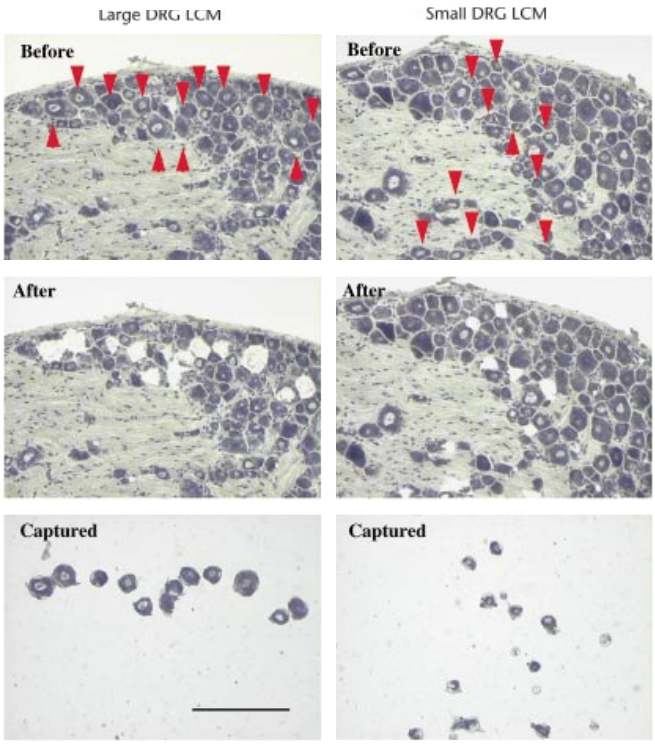

Fig. 1 Laser capture microdissection from Nissl-stained sections (10 μm in thickness) of adult rat large and small DRG neurons. Red arrows indicate DRG neurons to be captured (top panels). The middle and bottom panels show successful capture and film transfer, respectively. Scale bar represents 200 μm.

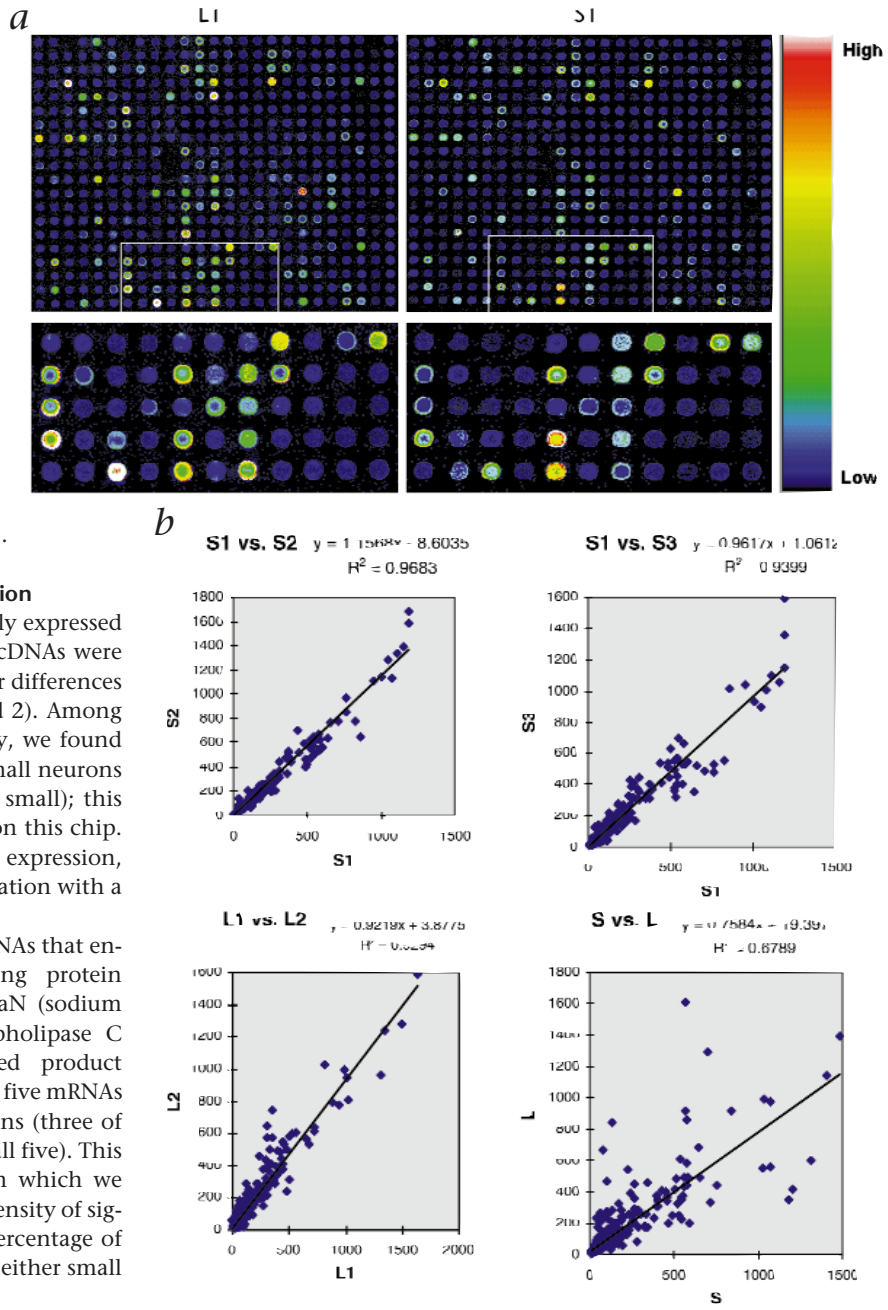

Fig. 2 cDNA microarray expression patterns of small (S) and large (L) neurons. ***a***, Example of the cDNA microarray data obtained (shown in pseudocolor representation). Boxed in white is an identical region of the microarray for L1 and S1 samples that are enlarged (shown directly below). ***b***, Scatter plots showing correlation between independent amplifications of S1 versus S2, S1 versus S3, L1 versus L2 and L (L1 and L2) versus S (S1, S2 and S3).

large neurons: $R^2 = 0.929$ for L1 compared with L2 (Fig. 2*b*). In contrast, a comparison between all three small neuronal sets (S1, S2 and S3) and the two large sets (L1 and L2) yielded a much lower correlation ($R^2 = 0.6789$), demonstrating, as expected, that a subset of genes is differentially expressed between the two neuronal subtypes (Fig. 2*b*).

Demonstration of differential gene expression

To identify the mRNAs that are differentially expressed between large and small neurons, all 477 cDNAs were examined and those with 1.5-fold or greater differences (at $P < 0.05$) were sequenced (Tables 1 and 2). Among the collection of cDNAs on the microarray, we found more mRNAs preferentially expressed in small neurons (14 mRNAs in large compared with 26 in small); this may simply reflect the set of cDNAs used on this chip. To confirm the observed differential gene expression, we subsequently performed *in situ* hybridization with a subset of these cDNAs.

For small neurons, we examined five mRNAs that encoded the following: Fatty acid binding protein (GenBank accession number M13501), NaN (sodium voltage-gated channel, AF059030), phospholipase C delta-4 (U16655), calcitonin gene-related product (CGRP, L00111) and annexin V (82462). All five mRNAs are preferentially expressed in small neurons (three of the five are shown in Fig. 3; see Table 3 for all five). This is based on quantitative measurements in which we measured for a given mRNA: the overall intensity of signal in small and large neurons, and the percentage of cells labeled within the total population of either small or large neurons (Table 3).

Our results confirm *in situ* hybridization studies for NaN mRNA (ref. 13, reported while this manuscript was in preparation) and CGRP mRNA (ref. 14) and are consistent with immunofluorescent studies with annexin V (ref. 15). Phospholipase C delta-4 is induced in S-phase of the cell cycle and resides in the nucleus[16]. Given that neurons are postmitotic, our observation indicates that this enzyme may play a different role in a subset of small neurons.

For large neurons, three cDNAs were examined: Neurofilaments NF-L (M25638) and NF-H (J04517), as well as the beta-1 subunit (M91808) of voltage-gated sodium channels. We found preferential expression of these mRNAs in large DRG neurons (Fig. 3 and Table 3). Recent *in situ* hybridization studies have also demonstrated preferential expression in large neurons for these three mRNAs (refs. 14–17). In addition, previous *in situ* hybridization studies are in agreement with our cDNA chip data for the differential expression in small and large neurons of P2X3 receptor mRNA (X90651)[18], NF-middle (J04517)[14], hsp 27 (ref. 19) and peripherin (M26232)[20]. One report, however, found no differences between small and large neurons for peripherin mRNA expression[14].

In general, for a given mRNA, the cDNA chip data and the results from the *in situ* hybridization studies are in agreement. However, in most cases, *in situ* hybridization studies indicate much greater differences in expression between small and large neurons than what is observed from cDNA chip data (Tables 1 and 2 compared with Table 3). For example, phospholipase C delta-4 expression is found almost exclusively in small neurons (Table 3 and Fig. 3), although the chip data indicate only about a threefold difference in expression (Table 2).

In part, this discrepancy can be explained by the fact that background signal due to non-specific nucleic acid hybridization (that is, the 75th percentile value obtained from the average hy-

Table 1 mRNA enriched in large DRG neurons

PRI ID	GB	Description	Mean ± s.e.m. (small)	Mean ± s.e.m. (large)	Ratio	*P*
192393	M25638	Rat smallest neurofilament protein (NF-L)	63.3 ± 6.12	551.56 ± 34.94	8.71	0.0005
191624	M14656	Rat osteopontin	53.4 ± 4.11*	218.52 ± 22.81	4.09	0.0005
192157	J04517	Rat high molecular weight neurofilament (NF-H)	475.86 ± 18.59	1319.77 ± 50.3	2.77	0.0005
192282	Z12152	*Rattus norvegicus* neurofilament protein middle	75.93 ± 3.75	206.55 ± 9.92	2.72	0.0005
192378	D87445	Human KIAA0256	30.26 ± 2.66*	77.42 ± 17.52	2.56	0.025
192283		Novel	50.9 ± 3.45*	128.56 ± 6.86	2.53	0.0005
192125	V00681	*R. norvegicus* mitochondrial genes for 16S rRNA, tRNA	186.5 ± 14.61	445.82 ± 23.95	2.39	0.0005
191851	X51396	Mouse MAP1B microtubule-associated protein	90.84 ± 5.91	215.55 ± 21.35	2.37	0.0025
192424	M91808	*R. norvegicus* sodium channel beta-1	83.99 ± 7.93	194.88 ± 20.61	2.32	0.0025
191862	S67755	hsp 27 = heat shock protein 27 (rats, Sprague-Dawley)	144.74 ± 10.14	265.94 ± 19.44	1.84	0.0005
192016	L10426	*Mus musculus* ets-related protein 81 (ER81)	43.85 ± 1.89*	80.04 ± 7.16	1.83	0.0025
192228		Novel	28.9 ± 1.11*	52 ± 3.41	1.80	0.0005
192411	M21551	Human neuromedin B	57.62 ± 5.56*	97.18 ± 6.61	1.69	0.0005
192422		Novel	110.06 ± 11.78	168.52 ± 12.14	1.53	0.0025

GB, gene bank accession number; mean, mean intensity of DNA chip microarrays; s.e.m., standard error of mean; ratio, mean intensity ratio of large DRG:small DRG neurons; *, mean intensity not significantly different ($P > 0.05$) from 75% of plant value.

bridization signals of all 30 plant cDNAs), has not been subtracted from each cDNA intensity. To show those mRNAs whose expression is not significantly different from background values, we noted them with an asterisk (*) in Tables 1 and 2. We chose not to subtract the 'non-specific hybridization' background because doing so may lead to very large and potentially spurious 'fold' differences.

For example, for phospholipase C delta-4 U16655 (Table 2), we report an expression ratio of 2.76. The 75th percentile value background signal for plant cDNAs is 48.68 for small and 40.94 for large neuronal sets. Therefore, the ratio of expression of this mRNA in small versus large neurons would go from a 2.76-fold difference (S = 146.33/L = 53.06) to an 8.1-fold difference (S = 97.65/L = 12.12) if the non-specific background were subtracted, respectively, from each intensity. Doing a similar analysis, for osteopontin M14656 (see Table 1), a 4.09-fold difference (L = 218.52/S = 53.4) becomes a 37.6-fold difference (L = 177.58/S = 4.72). Although these subtractions may give ratios that are closer to reality, without examining each mRNA via *in situ* hybridization, it would be difficult to ascertain whether all such subtractions would yield true results.

Overall, of the 40 mRNAs preferentially expressed in either large or small neurons, we or others (or both) have demonstrated similar results for 12 of these mRNAs via *in situ* hybridization studies. This level of success indicates that most of the other 28 mRNAs are also differentially expressed in small or large neurons.

Building expression databases with cell-type specificity

We demonstrate that by integrating LCM, aRNA and cDNA chips, one can successfully screen specific cell types obtained *in situ* from fixed tissue and subsequently identify differential gene expression. Although this study has identified mRNAs with differential expression within DRG neurons, there exists a great deal more heterogeneity within DRG neurons beyond simply small and large. For example, within small neurons (that is, nociceptive neurons) there is heterogeneity in gene expression, which presumably reflects, at least in part, the different sensory modalities transmitted. To approach this more complicated heterogeneity, the coupling of immunocytochemistry to LCM followed by aRNA and DNA chip analysis needs to be done. However, with this coupling, the number of neurons that can be possibly captured may be less. Here we used 1000 sectioned neurons 10 μm in thickness, but we have found similar results with 500 such neurons and so far have not attempted lower numbers of neurons. In addition, chips containing a larger number of cDNAs (that is, >10,000) need to be completed to identify more fully differential gene expression between large and small neurons.

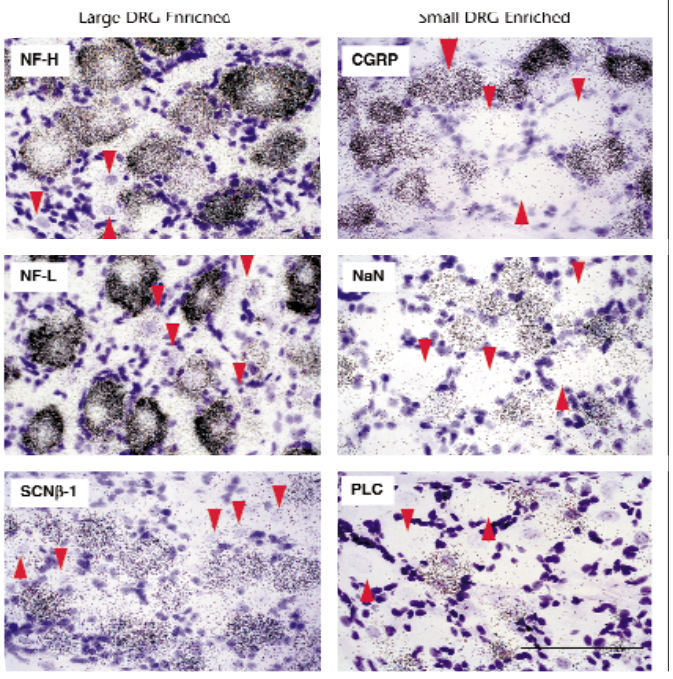

Fig. 3 Representative fields of radioisotopic *in situ* hybridization of rat DRG with selected cDNAs. The sections were Nissl counterstained. Left panels, Results with radiolabeled probes encoding neurofilament-high (NF-H), neurofilament-low (NF-L) and β-1 subunit of the voltage-gated sodium channel (SCN β-1). Red arrows (left panels) indicate identifiable small neurons. Right panels, Representative fields from radiolabeled probes encoding calcitonin gene-related product (CGRP), voltage-gated sodium channel (NaN) and phospholipase C delta-4 (PLC). Red arrows (right panels) indicate identifiable large neurons. Large red arrowhead indicates a large neuron, which is also labeled. Scale bar represents 100 μm.

Our positive results indicate that expression profiles generated with this integration can not only be useful for screening cDNAs, but also, more importantly, for producing databases that contain cell type-specific gene expression. Cell-type specificity within a database will give an investigator much greater leverage in understanding the contributions of individual cell types to a particular normal or disease state and thus allow for much finer hypotheses to be subsequently generated. Furthermore, genes that are coordinately expressed within a given cell type can be identified as the database grows to contain numerous gene expression profiles from a variety of cell types (or neuronal subtypes). Coordinate gene expression may also suggest functional coupling between the encoded proteins and therefore aid in one's attempt to determine function for most cDNAs now cloned.

Methods

Laser capture microdissection (LCM). Two adult female Sprague Dawley rats were used in this study. Animals were anesthetized with Metofane (Methoxyflurane; Mallinckrodt Veterinary, Mundelein, Illinois) and killed by decapitation. Using RNase-free conditions, cervical dorsal root ganglia (DRG) were quickly dissected out, placed in cryomolds, covered with Tissue-Tek frozen tissue embedding medium OCT (VWR, San Diego, California), and frozen in dry ice-cold 2-methylbutane (~ –60 °C). The DRG were then sectionedat 7–10 μm in a cryostat, mounted on plain (non-coated) clean microscope slides and immediately frozen on a block of dry ice. The sections were stored at –70 °C.

A quick Nissl (cresyl violet acetate) staining was used to identify the DRG neurons. Slides containing sections were quickly loaded on a slide holder, immediately fixed in 100% ethanol for 1 min followed by rehydration through subsequent steps of 95%, 70% and 50% ethanol diluted in RNase-free deionized H_2O (5 s each). Next, the slides were stained with 0.5% Nissl/0.1 M sodium acetate buffer for 1 min, dehydrated in graded ethanols (5 s each) and cleared in xylene (1 min). Once air-dried, the slides were ready for LCM.

The PixCell II LCM System from Acturus Engineering (Mountain View, California) was used for laser capture. Following the manufacturer's protocols, two sets of large and three sets of small DRG neurons (1,000 cells per set) were 'laser captured'. The criteria for large and small DRG neurons are as follows: A DRG neuron was classified as small if it had a diameter <25 μm plus an identifiable nucleus, whereas a DRG neuron with a diameter >40 μm was classified as large.

RNA extraction of LCM samples. Total RNA was extracted from the LCM samples with the Micro RNA Isolation Kit (Stratagene, San Diego, California) with some modifications. After incubating the LCM sample with 200 μl of denaturing buffer and 1.6 μl β-mercaptoethanol at room temperature for 5 min, the LCM sample was extracted with 20 μl of 2 M sodium acetate, 220 μl phenol and 60 μl chloroform: isoamyl alcohol. The aqueous layer was collected and then mixed with 1 μl of 10 mg/ml carrier glycogen, and precipitated with 200 μl of isopropanol. After a 70% ethanol wash and then air drying, the pellet was resuspended in 16 μl of RNase-free H_2O, 2 μl 10X DNase I reaction buffer, 1 μl RNasin and 1 μl of DNase I, and incubated at 37 °C for 30 min to remove any genomic DNA contamination. Next, the phenol-chloroform extraction was repeated as above. The pellet was resuspended in 11 μl of RNase-free H_2O, 1 μl of which was saved and used as a negative control for reverse transcription PCR (no RT control), and the remaining 10 μl was processed for RT–PCR and RNA amplification.

Reverse transcription (RT) of RNA. 10 μl of purified RNA from the preceding procedure was mixed with 1 μl of 0.5 mg/ml T7-oligodT primer (5'TCTAGTCGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGT$_{21}$-3') to initiate first-strand synthesis. The primer and RNA were incubated at 70 °C for 10 min, followed by incubation at 42 °C for 5 min. Next, 4 μl of 5× first-strand reaction buffer, 0.1M DTT (2 μl), 10 mM dNTPs (1 μl), 1 μl RNasin and 1 μl Superscript II (Life Technologies) were added, and incubation continued at 42 °C for 1 h. Next, 30 μl 5× second -strand synthesis buffer, 10 mM dNTPs (3 μl), 4 μl DNA polymerase I, 1 μl *E. coli* RNase H, 1 μl *E. coli* DNA ligase and 92 μl of RNase-free H_2O were added and the

Table 2 mRNA enriched in small DRG neurons

PRI ID	GB	Description	Mean ± s.e.m. (small)	Mean ± s.e.m. (large)	Ratio	*P*
191870	X03369	Rat beta-tubulin T beta15	996.81 ± 64.15	284.8 ± 38.79	3.50	0.0005
192273	M13501	Rat L-FABP (liver fatty acid binding protein)	232.87 ± 26.92	67.97 ± 14.68*	3.43	0.0005
192294	AF059030	*R. norvegicus* voltage-gated Na channel alpha subunit NaN	161.34 ± 20.07	51.3 ± 12.99*	3.15	0.0005
192195	D86642	Rat mRNA for FK506-binding protein	496.33 ± 40.11	158.8 ± 35.13	3.13	0.0005
192207	U16655	*R. norvegicus* phospholipase C delta-4	146.33 ± 10.03	53.06 ± 4.23	2.76	0.0005
192163	X90651	*R. norvegicus* P2X3 receptor	390.28 ± 10.4	164.81 ± 26.22	2.37	0.0005
191858	S69874	C-FABP = cutaneous fatty acid-binding protein (rat)	448.26 ± 30.01	196.97 ± 18.68	2.28	0.0005
192139	D45249	Rat proteasome activator rPA28 subunit alpha	104.46 ± 5.24	47.74 ± 6.97*	2.19	0.0005
192178	L12447	*M. musculus* insulin-like growth factor binding protein 5	288.97 ± 8.47	141.67 ± 5.61	2.04	0.0005
192306	X77953	*R.norvegicus* ribosomal protein S15a.	415.77 ± 54.08	204.19 ± 25.03	2.04	0.005
192129	M38188	Human unknown protein from clone pHGR74	114.72 ± 10.98	57.47 ± 11.64*	2.00	0.0025
192339		Novel	83.94 ± 6.26	42.42 ± 7.75*	1.98	0.001
191857	L00111	Rat CGRP	900.1 ± 45.83	459.99 ± 35.39	1.96	0.0005
192203	AF059486	*M. musculus* putative actin-binding protein DOC6	861.16 ± 32.58	448.32 ± 68.77	1.92	0.0005
192351	U25844	*M. musculus* serine proteinase inhibitor (SPI3)	271.95 ± 30.44	142.81 ± 6.93	1.90	0.0025
191837	M29472	*R. norvegicus* mevalonate kinase	94.44 ± 9.63	51.83 ± 5.95*	1.82	0.0025
191628		Novel	635.92 ± 73.01	363.86 ± 11.53	1.75	0.005
192175		Novel	181.28 ± 13.23	105.36 ± 10.39	1.72	0.0005
192284		Novel	188.28 ± 13	110.53 ± 7.27	1.70	0.0005
192330	Y10386	MMC1INH *M. musculus* C1 inhibitor	134.88 ± 11.01	79.3 ± 5.51	1.70	0.0005
192199	D42137	Rat annexin V gene	439.57 ± 13.62	265.21 ± 14.97	1.66	0.0005
192011	M98194	Rat extracellular signal-regulated kinase 1	319.35 ± 32.79	194.88 ± 6.83	1.64	0.005
192206	U59673	*R. norvegicus* 5HT3 receptor	139.96 ± 4.07	85.48 ± 6.17	1.64	0.0005
192167	U23146	*R. norvegicus* mitogenic regulation SSeCKS	456.44 ± 13.34	300.71 ± 23.25	1.52	0.0005
191848	M93056	Human monocyte/neutrophil elastase inhibitor	125.16 ± 14.76	82.56 ± 15.38	1.52	0.05
192309		Novel	463.17 ± 45.37	308.05 ± 25.45	1.50	0.01

GB, gene bank accession number; mean, mean intensity of DNA chip microarrays; s.e.m., standard error of mean; ratio, mean intensity ratio of small DRG:large DRG neurons; *, mean intensity not significantly different ($P > 0.05$) from 75% of plant value.

Table 3 *In situ* hybridization of selected cDNA clones

Clone ID	GB	Description	Small DRG		Large DRG	
			Intensity	% of labeled	Intensity	% of labeled
192393	M25638	Rat smallest neurofilament protein (NF-L)	±	100.0	+++	100.0
192157	J04517	Rat high molecular weight neurofilament (NF-H)	± / -	21.4	+++	98.6
192424	M91808	*R. norvegicus* sodium channel beta-1	± / -	10.0	++	96.3
192273	M13501	Rat liver fatty acid binding protein	+ / ++	62.2	+ / -	1.0
192294	AF059030	*R. norvegicus* voltage-gated Na channel NaN	++ / +	96.7	+ / -	4.2
192199	D42137	Rat annexin V gene	+ / ++	95.0	+ / ++	74.0
192207	U16655	*R. norvegicus* phospholipase C delta-4	++	42.2	-	0.0
191857	L00111	Rat CGRP	+++ / ++	83.7	++ / -	9.4

Intensity, estimated mRNA expression level per cell as follows: [-] no expression above background; [±] weak expression; [+] mild expression; [++] moderate expression; [+++] strong expression. %, percentage of DRG neurons expressing above background the mRNA of interest.

mixture was incubated at 16 °C for 2 h, followed by incubation of 10 min at 16 °C after the addition of 2 µl of T4 DNA polymerase. Next, the cDNA was extracted with phenol-chloroform and washed 3× with 500 µl of H_20 in a Microcon-100 column (Millipore). After collection from the column, the cDNA was dried down to 8 µl for *in vitro* transcription.

T7 RNA polymerase amplification (aRNA). The Ampliscribe T7 Transcription Kit (Epicentre Technologies, Madison, Wisconsin) was used: 8 µl double-stranded cDNA, 2 µl of 10× Ampliscribe T7 buffer, 1.5 µl each of 100 mM ATP, CTP, GTP and UTP, 0.1 M DTT (2 µl) and 2 µl of T7 RNA polymerase, incubated at 42 °C for 3 h. The aRNA was then washed three times in a Microcon-100 column, collected, and dried down to 10 µl.

Subsequent rounds of aRNA amplification. First, 10 µl of aRNA from first-round amplification was mixed together with 1 µl of 1mg/ml random hexamers (Pharmacia) and the mixture incubated at 70 °C for 10 min, chilled on ice, and equilibrated at room temperature for 10 min. Then 4 µl 5× first-strand buffer, 0.1 M DTT (2 µl), 10 mM dNTPs (1 µl), 1 µl Rnasin and 1 µl Superscript RT II were added and incubated at room temperature for 5 min followed by incubation at 37 °C for 1 h. Then, 1 µl of RNase H was added and incubated at 37 °C for 20 min, after which the reaction mix was heated to 95 °C for 2 min and chilled on ice. For second-strand cDNA synthesis, 1 µl of 0.5 mg/ml T7-oligo dT primer was added and the mixture incubated at 70 °C for 5 min and at 42 °C for 10 min. Next, 30 µl of 5X second-strand synthesis buffer, 10mM dNTPs (3 µl), 4 µl polymerase I, 1 µl *E. coli* RNase H and 90 µl of RNase-free H_2O were added and incubated at 16 °C for 2 h. Then, 2 µl of T4 DNA polymerase was added at 16 °C for 10 min. The double-stranded cDNA was extracted with 150 µl of phenol-chloroform to get rid of proteins and purified using a Microcon-100 column to separate out the unincorporated nucleotides and salts. The cDNA is then ready for second-round T7 *in vitro* transcription as above and then a subsequent third-round aRNA amplification.

Microarray design. The cDNAs present on the chip were obtained from two separate differential display[21] experiments. First, we did a screen to clone mRNAs preferentially expressed in DRG instead of brain, kidney and liver. Second, a screen was done to identify/clone mRNAs with decreased or increased concentration in ipsilateral ('treated') lumbar 5 and 6 DRG that were tightly ligated distal to the dorsal root ganglion ('Chung' model, see ref. 22) compared with contralateral ('control') lumbar 5 and 6 DRG.

Microarray printing. Here, 477 clones in vector PCR 2.1 from our previous differential display studies (as described above) were printed on silylated slides (CEL Associates, Houston, Texas). cDNAs were PCR-amplified with 5' amino-linked primers and purified with Qiagen 96 PCR Purification Kits. The print spots were about 125 µm in diameter and were spaced 300 µm apart from center to center. Thirty plant genes were also printed on the slides as a control for non-specific hybridization (gift from Mark Schena).

Microarray probe synthesis. Cy3-labeled cDNA probes were synthesized from aRNA of DRG captured using LCM with Superscript Choice System for cDNA synthesis (Gibco BRL). aRNA (5 µg) and random hexamers (3 µg) were mixed in a total volume of 26 µl (containing RNase-free H_2O), heated to 70 °C for 10 min and chilled on ice. Then, 10 µl 5× first-strand buffer, 0.1 M DTT (5 µl), 1.5 µl Rnasin, 25 mM d(GAT)TP (1 µl), 1 mM dCTP (2 µl), 2 µl Cy3-dCTP (Amersham) and 2.5 µl Superscript RT II were added and incubated at room temperature for 10 min and then at 37 °C for 2 h. To degrade the aRNA template, 3N NaOH (6 µl) was added and incubated at 65 °C for 30 min. Then, 1M Tris-HCl (20 µl, pH 7.4), 1N HCl (12 µl) and H_2O (12 µl) were added. The probes were purified with Microcon 30 columns (Millipore) and then with Qiagen's Nucleotide Removal Columns. The probes were vacuum-dried and resuspended in 20 µl of hybridization buffer (5× SSC, 0.2% SDS) containing mouse Cot1 DNA (Life Technologies).

Microarray hybridization and washes. Printed glass slides were treated with sodium borohydrate solution (0.066 M $NaBH_4$, 0.06 M Na AC) to ensure amino-linkage of cDNAs to the slides. Then, the slides were boiled in water for 2 min to denature the cDNA. Cy3-labeled probes were heated to 99 °C for 5 min, room temperature for 5 min and applied to the slides. The slides were covered with glass coverslips, sealed with DPX (Fluka, Milwaukee, Wisconsin) and hybridized at 60 °C for 4–6 h. The slides were washed in 1× SSC, 0.2% SDS at 55 °C for 5 min, 0.1× SSC, 0.2% SDS at 55 °C for 5 min. After a quick rinse in 0.1× SSC, the slides were air-blown dried to prepare them for scanning.

Microarray quantitation. cDNA microarrays (that is, microscope slides) were scanned for cy3 fluorescence using the ScanArray 3000 (General Scanning, Watertown, Massachusetts). ImaGene software (Biodiscovery, Los Angeles, California) was then subsequently used for quantitation. In total, 15 chips were processed, with 3 chips/neuronal set (see text). The intensity of each spot (that is, cDNA) was corrected by subtracting the immediate surrounding background. Next, the corrected intensities were normalized for each cDNA (that is, spot) using the following formula: Intensity (background corrected)/75th-percentile value of the intensity of the entire chip × 1000. To determine 'non-specific' nucleic acid hybridization, 75th-percentile values were calculated from the individual averages of each plant cDNA (for a total of 30 different cDNAs) from each neuronal set. The overall 75th-percentile value for S1, S2 and S3 = 48.68 and for L1 and L2 = 40.94.

Statistical analyses. To assess correlation of intensity value for each cDNA between individual sets of neurons (for example, S1 compared with S2 in Fig. 2*b*) or between two neuronal subtypes (that is, S1, S2 and S3 compared with L1 and L2 in Fig. 2*b*), scatter plots were used and linear relationships were measured. The correlation coefficient, R^2, that was calculated, indicates the variability of intensity values in one group vs. the other. To determine statistically whether or not intensity values measured from microarray quantitation are true signals, each intensity is compared, using a one-sample *t*-test, to the 75th-percentile value of 30 plant cDNAs that are present on each chip (representing non-specific nucleic acid hybridization). Values not significantly different from the 75th-percentile value are presented in Tables 1 and 2 and so denoted. To determine which cDNAs are statistically significant in their differential gene expression between large and small neurons, the intensity for each cDNA from neuronal sets for large neurons (L1 and L2) and small neurons (S1, S2, and S3) were grouped together respectively and intensity values were averaged for each corresponding cDNA. A two-sample

t-test for one-tailed hypotheses was used to detect a gene expression difference between small and large neurons.

***In situ* hybridization**. *In situ* hybridization was done as described[23]. DNAs were subcloned into pBluescript II SK (Stratagene). Each template was linearized and ^{35}S-UTP was incorporated through *in vitro* transcription with T7 or T3 RNA polymerase. The probes were then purified with Quick Spin columns (Boehringer). Probes (10^7 c.p.m./probe) were hybridized to 10 µm, 4% paraformaldehyde-fixed rat DRG sections, which were mounted on Superfrost Plus slides (VWR). After overnight hybridization at 58 °C and post-washes, the slides were exposed to film for primary data. Then, the slides were coated with Kodak liquid emulsion NTB2 and exposed in light-proof boxes for 1–2 weeks at 4 °C. The slides were developed in Kodak Developer D-19, fixed in Kodak Fixer and Nissl stained for expression analysis. Under light-field microscopy, mRNA expression levels of specific cDNAs were semi-quantitatively analyzed. This was done as follows: No expression (-, grains were less than 5-fold of the background); weak expression (±, grains were 5- to10-fold of the background); low expression (+, grains were 10- to 20-fold of the background); moderate expression (++, grains were 20- to 30-fold of the background); strong expression (+++, grains were >30-fold of the background). The percentage of small or large neurons expressing a specific mRNA was obtained by counting the number of labeled (above background) and unlabeled cells from either large or small neurons from four sections (at least 200 cells were counted).

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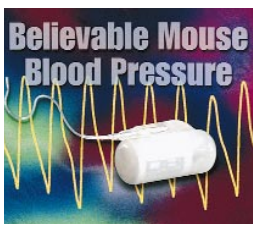

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