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Replicate high-density rat genome oligonucleotide microarrays reveal hundreds of regulated genes in the dorsal root ganglion after peripheral nerve injury.

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Published: 25 October 2002

Received: 29 August 2002

BMC Neuroscience 2002, 3:16

Accepted: 25 October 2002

This article is available from: <http://www.biomedcentral.com/1471-2202/3/16>

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Abstract

Background: Rat oligonucleotide microarrays were used to detect changes in gene expression in the dorsal root ganglion (DRG) 3 days following sciatic nerve transection (axotomy). Two comparisons were made using two sets of triplicate microarrays, naïve versus naïve and naïve versus axotomy.

Results: Microarray variability was assessed using the naïve versus naïve comparison. These results support use of a $P < 0.05$ significance threshold for detecting regulated genes, despite the large number of hypothesis tests required. For the naïve versus axotomy comparison, a 2-fold cut off alone led to an estimated error rate of 16%; combining a >1.5 -fold expression change and $P < 0.05$ significance reduced the estimated error to 5%. The 2-fold cut off identified 178 genes while the combined >1.5 -fold and $P < 0.05$ criteria generated 240 putatively regulated genes, which we have listed. Many of these have not been described as regulated in the DRG by axotomy. Northern blot, quantitative slot blots and *in situ* hybridization verified the expression of 24 transcripts. These data showed an 83% concordance rate with the arrays; most mismatches represent genes with low expression levels reflecting limits of array sensitivity. A significant correlation was found between actual mRNA differences and relative changes between microarrays ($r^2 = 0.8567$). Temporal patterns of individual genes regulation varied.

Conclusions: We identify parameters for microarray analysis which reduce error while identifying many putatively regulated genes. Functional classification of these genes suggest reorganization of cell structural components, activation of genes expressed by immune and inflammatory cells and down-regulation of genes involved in neurotransmission.

Background

Transection of the peripheral axons of primary sensory neurons results in profound alterations in their metabolism, regenerative capacity, survival, excitability, transmitter function and sensitivity to diverse extrinsic and intrinsic signals [1,2]. These changes are contributed to by transcriptional alterations triggered by a loss of trophic support from peripheral target organs, and by novel signals generated at the injury site. The transcriptional changes lead both to adaptive responses, such as the capacity to survive the injury and re-grow the injured axon, and maladaptive responses that can lead to a change in sensation, including the generation of neuropathic pain [3,4].

Measurements of mRNA and protein made in many laboratories using diverse methodologies have identified approximately 40 genes that are up-regulated and 25 down-regulated after peripheral nerve injury [3,5,6]. These regulated genes include members of several classes: G protein coupled receptors, ligand- and voltage-gated ion channels, receptor tyrosine kinases, growth factors, cytokines, neuropeptides, cell cytoskeletal genes, cell surface/extracellular matrix genes, and a miscellaneous group of enzymes.

We have now used high-density rat genome oligonucleotide microarrays to analyze changes in gene expression at an early time point (3 days) following a peripheral nerve injury in adult rats. Oligonucleotide microarrays provide the capacity to analyze parallel changes in many thousands of genes, and have been used successfully to examine expression profile changes in many neuronal and non-neuronal systems [7] including DRG neurons [8,9]. Studying the effect of peripheral nerve injury on gene expression profiles in the DRG offers several advantages. The DRG represents a dense collection of cell bodies of one general class of neuron, the primary sensory neuron. The lesion has a uniform impact on the cells, and the existence of a large pool of genes with known regulation allows for quality controls for changes identified by the microarrays [3,5,6].

Although microarray technology offers enormous potential advantages, there remain important concerns about representation, sensitivity, reproducibility, variability and the false positive and negative detection rates [10,11]. What, if any, is the threshold fold-difference between probe sets that reflects real regulation? Is fold-difference the most sensitive measure for detecting changes? Are genes with a low expression levels in all conditions detected? What is the concordance rate between genes identified as regulated by independent methods and genes identified by the microarray analysis? What criteria need to be

used to jointly minimize the false negative and the false positive rates? How many arrays need to be analyzed?

We now show that fold-difference alone results in a high degree of error whilst detecting regulated genes from microarrays, replicate arrays with statistical analysis reduces false positives and negatives. The oligonucleotide microarray screen indicates that hundreds of genes are regulated by neuronal injury, and that this technique is a powerful primary screen for such changes.

Results

Array sensitivity and variability

Affymetrix rat U34A oligonucleotide arrays were used to screen for changes in gene expression in DRG neurons three days following a peripheral nerve transection (axotomy, Ax) by comparing expression levels with non-injured DRGs (naïve, N). Nine biologically independent array hybridizations were performed (six naïve and three axotomy). DRG tissue (L4 and L5 from the left or ipsilateral side to the injury) from 5 male Sprague-Dawley rats were pooled for each RNA population. Each RNA sample was labeled separately and hybridized to a separate array. Genes were defined as detected if they received a present or marginal call in at least one of the arrays within each comparison. Of the 8799 annotated genes and expressed sequence tags (ESTs) represented on the rat U34A array, 52% (n = 6, naïve arrays) were detected in arrays hybridized with naïve rat DRG cRNA and 54% (n = 3, axotomy arrays) genes in the axotomy comparisons.

To assess the degree of variability inherent in the system, the mean probe set intensity values of the two independent sets of three naïve samples were compared on a scatter plot (Figure 1A). The majority of probe sets from the two control groups (N vs. N) lie on or very close to the identity line, with a linear regression of $r^2 = 0.9898$ (for all detected genes in the N vs. N comparison, Figure 1A). Figure 1A shows that most of the variation between the two naïve groups exists at the lower end of the gene expression levels. This suggests that this variability is in part due to array sensitivity. In consequence, differences in low intensity genes may be unreliable.

A plot of the mean intensity from triplicate naïves against triplicate 3 day axotomy (N vs. Ax) arrays (Figure 1B) show many data points lying both above and below the identity line, suggesting regulation. Since each individual sample was pooled from 5 male Sprague-Dawley animals of a similar age and from a single supplier (Charles River), biological variation is likely to be minimal.

Defining regulated genes

Many of the detected genes in both the N vs. N and the N vs. Ax array comparison groups show no difference, de-

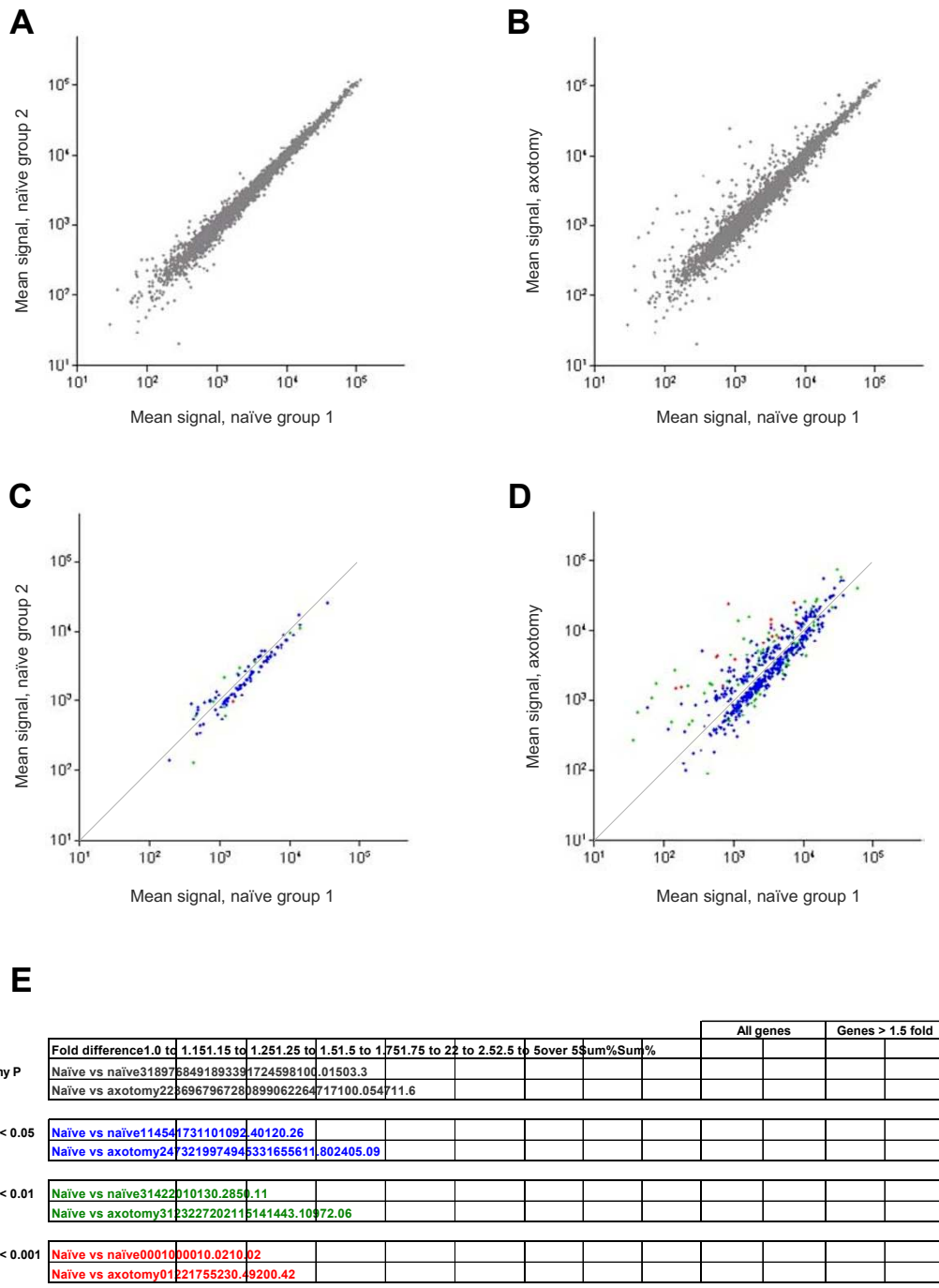


Figure 1
 Intensity plots of average values of all present genes for the naive group 1 versus naive group 2. (A) and the naive group 1 versus the 3 day axotomy group (B). Intensity plots of average values of all genes deemed to vary significantly using an unpaired two-tailed t-test, for the naive group 1 versus naive group 2 comparison (C) and the naive group 1 versus the 3 day axotomy group comparison (D). Plots are color coded so that P < 0.05 (blue points), P < 0.01 (green points), P < 0.001 (red points). Tabulated in (E) are the numbers of genes detected (grey) and those genes achieving P < 0.05 (blue), P < 0.01 (green), P < 0.001 (red) as a function of fold change.

defined as fold-difference up or down less than 1.15 (69%, N vs. N and 47%, N vs. Ax) (Figure 1E). Further, many show small fold-differences of 1.15 to 1.5 (27%, N vs. N and 41%, N vs. Ax) (Figure 1E). In total, genes displaying a fold-difference below 1.5-fold accounted for 97% of the N vs. N arrays and 88% of the N vs. Ax comparison.

When statistical significance is not taken into account, 122 genes display a difference of 1.5–2.0 fold and 28 a greater than 2-fold difference in the N vs. N group (2.6% and 0.6% of present genes respectively). In the N vs. Ax group 369 genes show a fold-difference of 1.5–2.0 and 178 greater than 2-fold (7.8% and 3.8% of present genes respectively). Although a greater than 2-fold change cut off eliminates most false positives (estimated error rate, 16%), this specificity is at the expense of many putatively regulated genes. In the 1.5–2.0 fold range in the N vs. Ax group, there are still three times as many genes as are in the N vs. N group. (369 genes [N vs. Ax] vs. 122 genes [N vs. N]; Figure 1E).

In order to distinguish systematic from random differences in the array intensity levels, a two-tailed t-test was performed for both comparison groups, which was possible since each group consisted of three independent experiments. One of the fundamental problems in analyzing microarray data is the risk of false positive results due to multiple hypothesis testing. The most conservative approach to this problem, a Bonferroni-corrected significance threshold, would not result in any significantly regulated genes, as the adjusted threshold would be 1.06×10^{-5} , while the lowest P value attained in our data set for the N vs. Ax. comparison was 1.51×10^{-5} . This would also rule out identification of genes via step-down methods based on the Bonferroni correction. Therefore, we attempted to assess the accumulation of false positives empirically, by testing multiple hypotheses in our system on a sample not expected to include any true positives, i.e. the N. vs. N. comparison. Those probe sets with a unadjusted significance of $P < 0.05$ (blue), $P < 0.01$ (green) or $P < 0.001$ (red) are indicated on the scatter plots in Figures 1C and 1D, while the distribution of significant differences at different fold-changes is illustrated in the table in Figure 1E. Of the 4599 detected genes in the naïve samples, 109 show a significant difference between the two sets (2.4%, $P < 0.05$). The vast majority of these genes, however, exhibit low fold changes (<1.5-fold, 89%, Figure 1E).

Of the 369 genes that show a fold-difference of 1.5–2.0 and the 178 that were above 2-fold in the N vs. Ax comparison, 223 (60%) and 84 (57%) respectively were not significant (Figure 1E, $P < 0.05$). These data indicate that using a fold-difference of greater than 2 from triplicate samples to select regulated genes will include many false positives. However, combining fold difference with signif-

icant difference in a two dimensional matrix can reduce the false positive error rate drastically. The combination of a >1.5-fold and significant difference ($P < 0.05$) identifies 12 genes in the N vs. N comparison compared with 240 genes for N vs. Ax, a 20-fold difference (Figure 1E). The estimated error of 5% using these two criteria is much less than that obtained using just above 2-fold (16%) or significance alone (20%) and prevents many genes regulated below 2-fold being excluded from consideration of regulation.

The inherent array error rate (determined by the significant difference rate in the N vs. N analysis) is clustered at low fold changes (Figure 1E). However, many more genes achieve statistical significance at these small fold changes in the N vs. Ax comparison than in the N vs. N group (1.15–1.5 fold: 6.2% [N vs. Ax] and 1.9% [N vs. N]; Figure 1E) indicating that some low fold differences may be real. The degree of significance ($P < 0.001$; 0.01, 0.05) helps distinguish erroneous changes at these low fold levels (Figure 1E).

Comparison of array data with the literature

Table 1 (see additional file 1) lists those genes previously shown to be regulated in various sciatic nerve injury models using a variety techniques, as well as the expression intensity, fold change and P value derived for these genes from the arrays. Of 69 genes reported in the literature to be expressed in the DRG (Table 1, see additional file 1) 16 are not detected by the arrays indicating that array sensitivity is an issue. Amongst these are particular functional classes, i.e. such as G protein coupled receptors, which are expressed at low levels [12].

25 of the 53 detected genes matched the criteria (>1.5-fold, $P < 0.05$) for defining regulated genes across the N vs. Ax comparison, and all of these were concordant with the changes reported in the literature (Table 1, see additional file 1). In addition 4 of the 53 detected genes whose levels had previously been described as unchanged by axotomy also did not vary on the arrays. Mismatches between changes expected from the literature and the arrays may be due to a failure of small differences to achieve statistical significance when using a triplicate analysis. Furthermore, differences in the timing, the nature of the injury models used and detection methods employed between this and earlier studies make detailed analysis of these data unfeasible. In order to detect true false positive and negative rates a direct comparison needs to be made between the array data set and transcript levels measured in samples equivalent to those used for array hybridization.

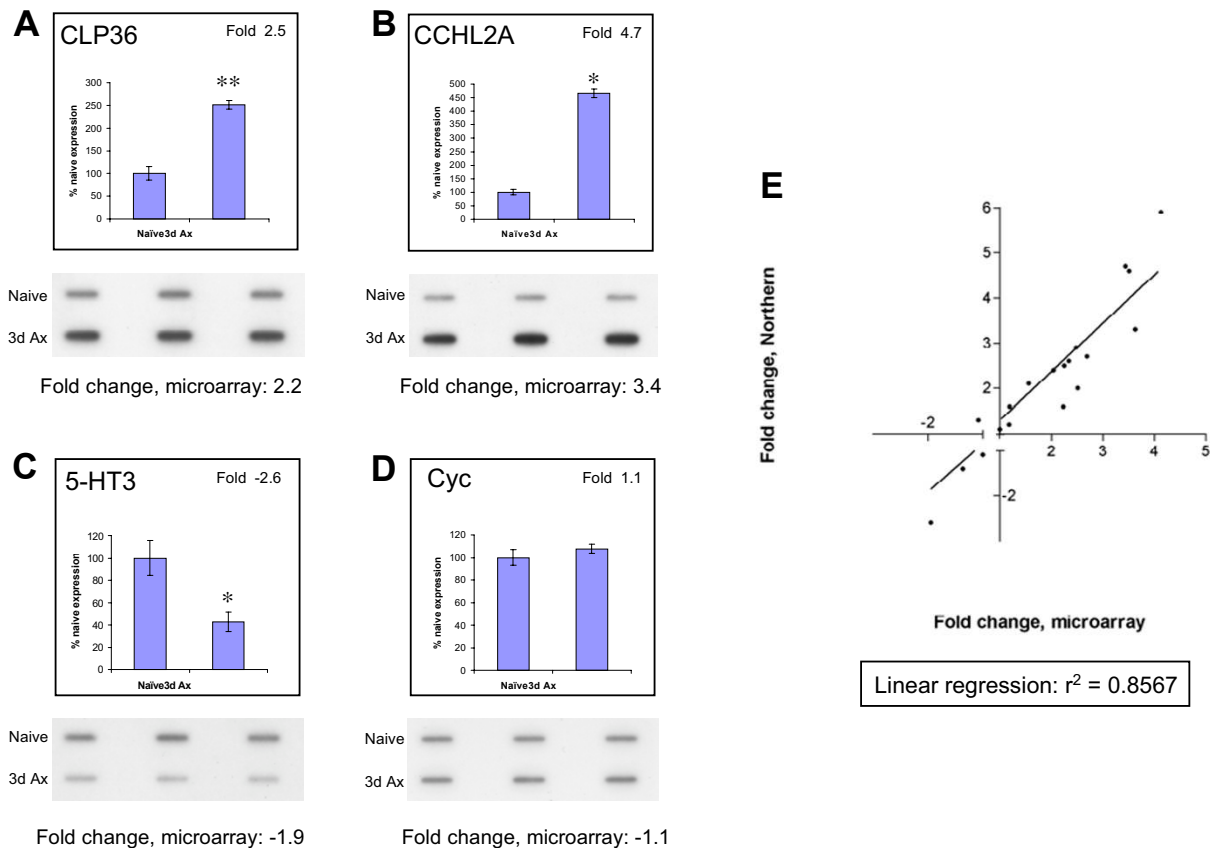


Figure 2

Triplicate Northern slot blots for three genes with altered expression, 2 showing an increase after axotomy (**A** and **B**), one a decrease (**C**). Cyclophilin a gene known not to alter following axotomy shows equal loading of the slot blots and acts as a normalization control (see methods) (**D**). The histograms indicate the Northern slot blot data expressed as percentage of naive expression levels or above the graphs as fold change. Below the blots is the gene expression fold change calculated from the triplicate microarray data. ** P < 0.01, * P < 0.05 (two-tailed unpaired t-test). **E**. Correlation between 19 separate mRNA fold-differences calculated by triplicate slot blot and those calculated from the triplicate arrays. Linear regression line of best fit is also plotted, scale does not represent values between -1 and 1 as fold change values within this range do not exist due to the method of calculation (see methods).

Northern blotting validation of transcriptional regulation

Twenty four genes detected by the arrays as being present in the DRG and displaying up-, down- or no regulation three days after sciatic nerve axotomy were analyzed by replicate quantitative slot blot (Figure 2) and Northern blot analysis (Figure 3). The genes were chosen in a non-random fashion to encompass the range of hybridization intensities seen on the arrays, with expression levels less than 1000 to intensity levels greater than 10,000. Genes were also chosen which displayed a wide range of fold-changes and varying p values and possible functions were taken into account (Table 2).

Figure 2 illustrates representative triplicate slot blots for three genes with altered expression, 2 showing an increase after axotomy (Figure 2A and 2B) and one a decrease (Figure 2C). Cyclophilin, a gene known not to alter following axotomy[13], was used to correct for loading (Figure 2D). Each blot was prepared from independent L4 and L5 DRG RNA samples extracted from different groups of animals than those used for the arrays.

Triplicate slot blots were produced for all 24 genes present in Table 2. The concordance rate between the microarray and slot blot fold changes for the 24 genes was 83% overall but concordance depended on gene expression level

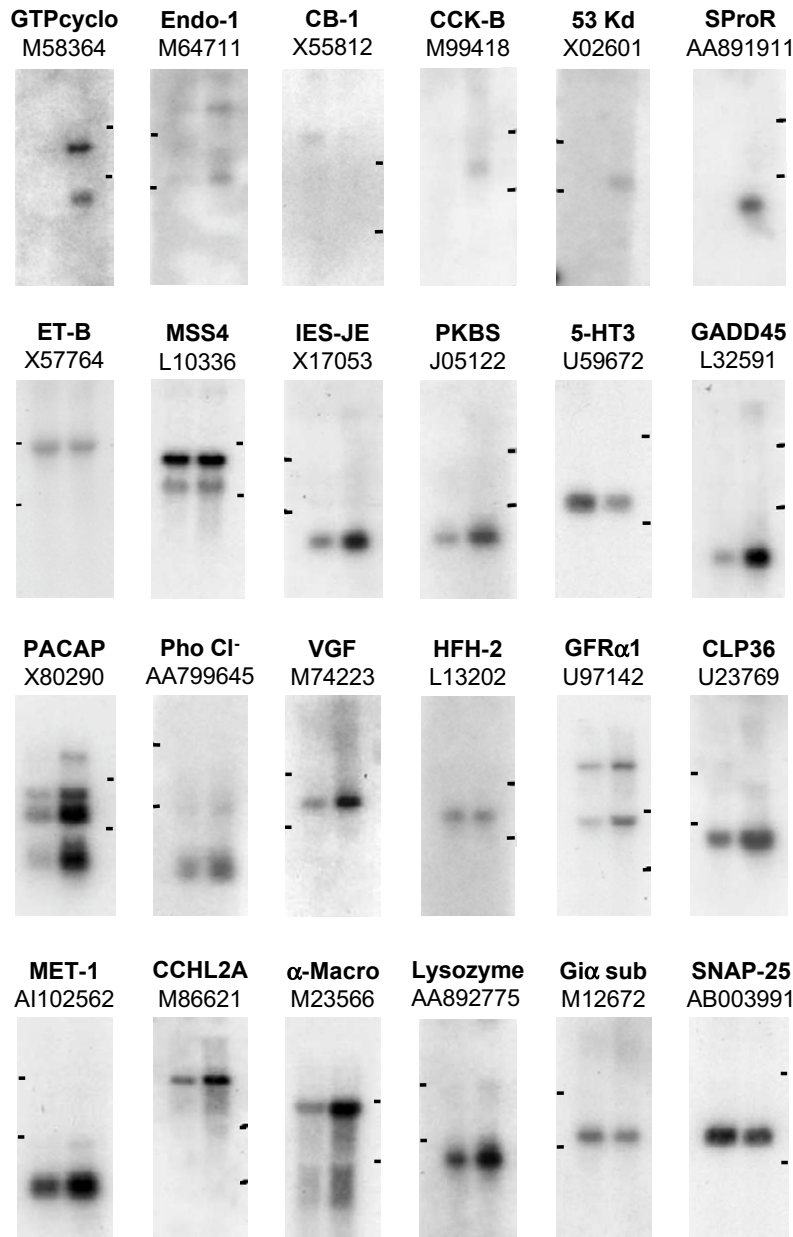


Figure 3

Northern blots showing differences in expression of 24 genes in naïve/control total DRG RNA (left lane) versus 3 day sciatic nerve axotomy total DRG RNA (right lane). Gene descriptions and accession numbers are shown above each blot. The relative positions of 28S and 18S ribosomal RNA are indicated which migrate at 4.7 kb and 1.9 kb respectively. Abbreviations: **GTPcyclo** GTP cyclohydrolase-1; **Endo-1** Endothelin-1; **CB-1** Cannabinoid receptor-1; **CCK-B** Cholecystokinin-B receptor; **53 Kd** Stromelysin-1 (matrix metalloproteinase-3); **SproR** Small proline rich protein-1A; **ET-B** Endothelin receptor-B; **MSS4** Guanine nucleotide releasing protein; **IES-JE** Monocyte chemoattractant protein-1; **PKBS** Peripheral benzodiazepine receptor; **5HT3** 5HT3A receptor; **GADD45** Growth arrest and DNA damage inducible gene alpha; **PACAP** Pituitary adenylate cyclase activating peptide; **Pho Cl-** Phospholemman chloride channel; **VGF** nerve growth factor inducible protein VGF ; **HFH-2** HNF-3/fork-head homolog-2; **GFRα-1** GDNF receptor alpha-1 subtype; **CLP36** PDZ and LIM domain protein-1 (ELFIN); **MET-1** Metallothionein-1L; **CCHL2A** Calcium channel α-2 subunit; **α-Macro** alpha(2)-macroglobulin; **Giα sub** Guanine nucleotide-binding protein G-1, α subunit; **SNAP-25** Synaptosomal associated protein 25A & B.

Table 2: Comparison of microarray, quantitative Northern slot blot and *in situ* hybridization data for genes validated in this study.

Descriptions	Accession Number	Array Data				Northern Data		<i>In situ</i> Regulation	Known Regulation	
		Naïve Mean intensity ± S.D.	Axotomy Mean intensity ± S.D.	Fold change	P value	Fold change	P value			
GTP cyclohydrolase I	M58364	222 ± 12	1605 ± 194	7.2	0.0064	**	3.8	0.0000	***	↑
Endothelin-1	M64711	538 ± 170	716 ± 34	1.3	0.2076		1.7	0.0004	***	
Cannabinoid CB1 receptor	X55812	576 ± 21	388 ± 49	-1.5	0.0118	*	-1.2	0.4221		
Cholecystokinin-B receptor	M99418	588 ± 145	534 ± 56	-1.1	0.6011		2.7	0.0008	***	↑(31,32)
53 kD polypeptide	X02601	645 ± 194	723 ± 113	1.1	0.5875		2.4	0.0433	*	
Small proline-rich protein 1a (EST195714)	AA891911	1063 ± 205	3856 ± 263	3.6	0.0002	***	3.3	0.0267	*	↑(8)
ET-B endothelin receptor	X57764	1228 ± 1015	1448 ± 1131	1.2	0.8145		1.2	0.1445		
Guanine nucleotide-releasing protein (MSS4)	L10336	1301 ± 335	1286 ± 174	1.0	0.9478		-1.1	0.0562		
Immediate-early serum-responsive JE (IES-JE)	X17053	1539 ± 473	3864 ± 818	2.5	0.0208	*	2.0	0.0089	**	↑
Peripheral-type benzodiazepine receptor	J05122	2664 ± 314	4148 ± 459	1.6	0.0132	*	2.1	0.0078	**	
5HT-3 receptor	U59672	2750 ± 254	1413 ± 276	-1.9	0.0036	**	-2.6	0.0140	*	↓
Gadd45	L32591	3394 ± 195	14,021 ± 644	4.1	0.0005	***	5.9	0.0047	**	
Pituitary adenylate cyclase activating peptide(PACAP)	X80290	3466 ± 434	12,182 ± 290	3.5	0.0000	***	4.6	0.0004	***	↑(6,55)
Phopholemmann chloride channel (EST189142)	AA799645	3629 ± 327	8094 ± 283	2.2	0.0001	***	1.6	0.0062	**	
VEGF (nerve growth factor-inducible protein)	M74223	3791 ± 619	10,200 ± 748	2.7	0.0004	***	2.7	0.0050	**	↑
HNF-3/fork-head homolog-2 (HFH-2)	L13202	3961 ± 396	3640 ± 202	-1.1	0.2994		1.3	0.0986		
GFRα1 (RET ligand 1)	U97142	4132 ± 755	8443 ± 581	2.0	0.0019	**	2.4	0.0029	**	↑(45)
CLP36 (Elfin)	U23769	5124 ± 532	11,527 ± 666	2.2	0.0003	***	2.5	0.0019	**	
Metallothionein-1 (EST211851)	A1102562	5628 ± 779	6715 ± 319	1.2	0.1230		1.6	0.0321	*	
Calcium channel α-2 subunit (CCHL2A)	M86621	7267 ± 1792	25,005 ± 1485	3.4	0.0002	***	4.7	0.0125	*	↑
α-2-macroglobulin	M23566	8995 ± 1046	21,057 ± 3221	2.3	0.0157	*	2.6	0.0023	**	
Lysozyme (EST196578)	AA892775	10,702 ± 3948	26,572 ± 2127	2.5	0.0082	**	2.9	0.0024	**	
Guanine nucleotide-binding protein G-i, α subunit	M12672	10,728 ± 1325	10,781 ± 1099	1.0	0.9608		1.1	0.0217	*	
SNAP-25A	AB003991	22,532 ± 3763	16,441 ± 1731	-1.4	0.0898		-1.4	0.0486	*	↓

Key to abbreviations, * P < 0.05, ** P < 0.01, *** P < 0.001 (two-tailed unpaired t-test). Down regulated genes are expressed as negative fold changes.

(Table 2). Genes expressed at low levels in the arrays tended to display fold changes that varied from that found by the slot blots. It is likely that this is a consequence of the unreliable average intensity estimation for low level genes due to limited array sensitivity (Figure 1A). If genes with a mean intensity expression level of less than 1000 are removed from the analysis (top five rows, Table 2), and the array fold changes of the remaining 19 genes are plotted against the slot blot fold changes, a very strong correlation is evident (linear regression $r^2 = 0.8567$) (Figure 2E). The concordance rate of this group is 95% (using >1.5-fold, P < 0.05 to predict regulation). This shows that within the bounds of array sensitivity, average fold changes for genes found to differ significantly between triplicate control and experimental arrays represent a reasonable estimate of the relative changes in gene expression.

Northern blot analysis, although requiring more tissue, both confirms that the correct transcript size is recognized by the probe and enables splice variants to be identified (Figure 3). All 24 genes in Table 2 were analyzed in this way and alternate splice forms were evident for 5 genes.

A list of all the putatively regulated genes within the DRG detected by the arrays (>1.5-fold and P < 0.05) 3 days following a sciatic nerve transection are given as additional data in table 3 (see additional file 2).

***In situ* Hybridization**

Dorsal root ganglia as well as containing the cell bodies of primary sensory neurons also contain satellite glial cells, Schwann cells and immune cells and it is important, therefore to determine which of these cell types express the regulated genes. Here we use isotopic- *in situ* hybridi-

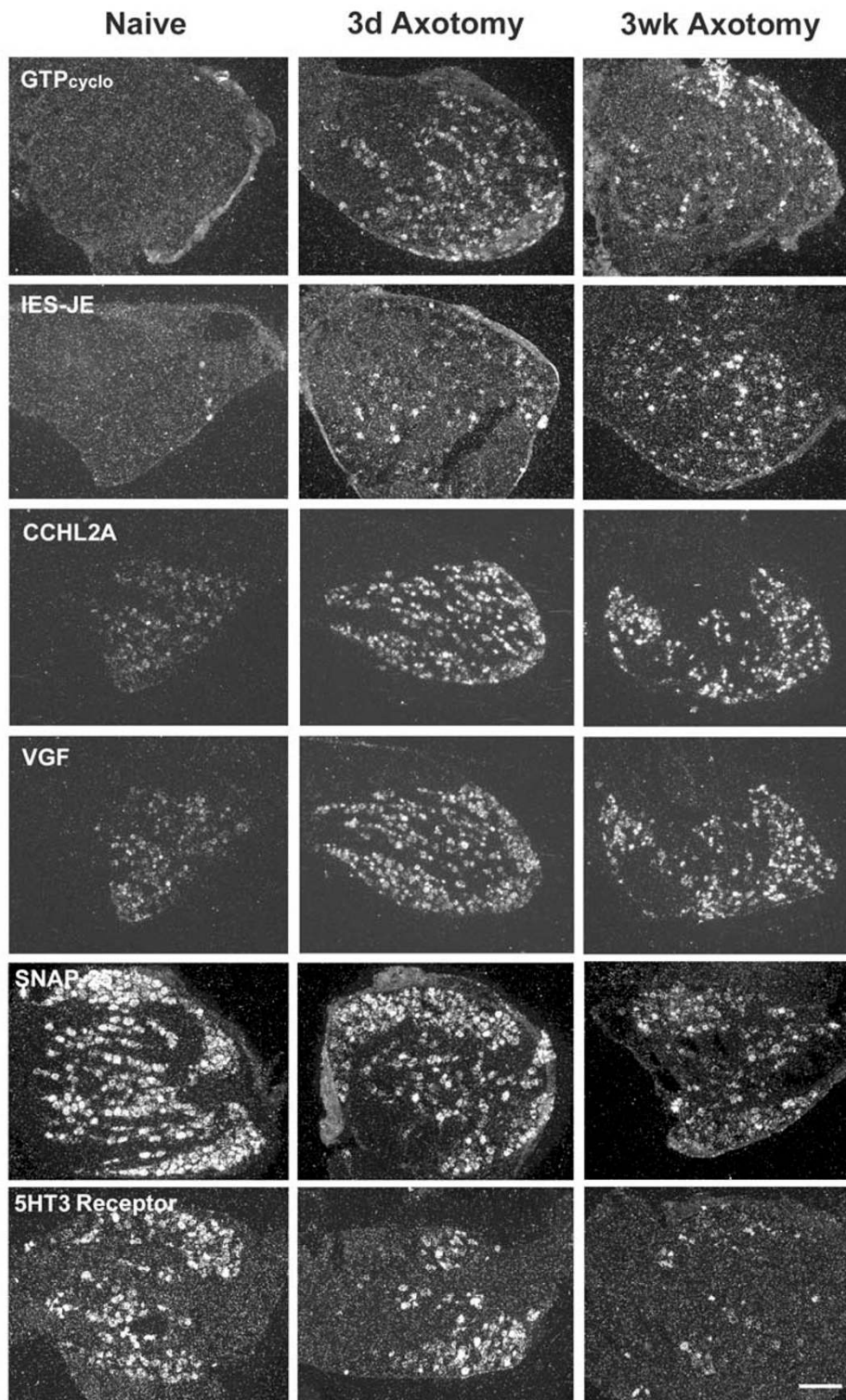


Figure 4
 Darkfield photomicrographs of 6 μm thick L4 DRG sections processed for *in situ* hybridization with probes indicated. GTPcyclo, IES-JE, CCHL2A and VGF transcripts show a strong upregulation following injury compared to the naïve. SNAP25, and 5HT3 receptor mRNA show a downregulation following injury to the peripheral neurons. Scale bar = 200 μm.

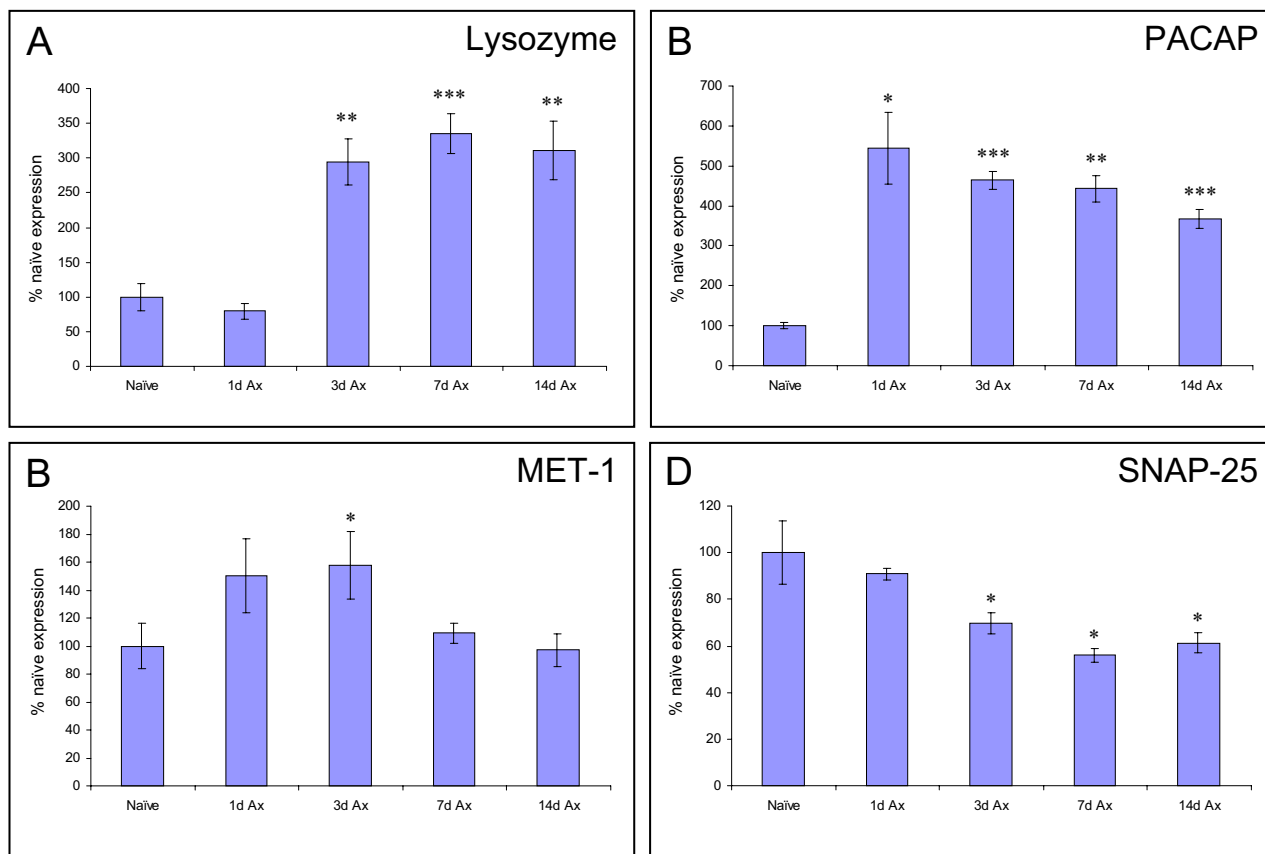


Figure 5
 Axotomy induced changes in expression of Lysozyme, PACAP, MET-1 and SNAP25 transcript levels over time. The histograms indicate the triplicate Northern slot blot data expressed as percentage of naïve expression levels 1, 3, 7 and 14 days post axotomy. ***P < 0.001 ** P < 0.01, * P < 0.05 (two-tailed unpaired t-test).

zation (ISH) to determine the cellular localization of 6 transcripts (Figure 4). All genes selected are neuronal. GT-Pcylo, IES-JE, CCHL2A and VGF all show a marked increase in the number of labeled neurons while SNAP25 and the 5HT3 receptor show a decrease in numbers and intensity of labeled DRG neurons following axotomy (Figure 4).

Time dependent changes in transcription

The pattern of expression of four genes within the DRG (Lysozyme, PACAP, MET-1 and SNAP25) were further quantified across a time course of 1, 3, 7 and 14 days post axotomy by triplicate northern slot blot (Figure 5). These genes show diverse patterns of regulation. While lysozyme, PACAP, MET-1 are all up-regulated, for MET1 this is only transient. Identical results were found by in situ hybridization (Fig. 4). Profiling changes in transcription over time is therefore, important.

Potential roles of injury-regulated genes

Table 4 (see additional file 3) indicates the pattern of expression, class, action, and functional role for 15 previously uncharacterized, and 9 novel injury-regulated genes, amongst the 24 validated genes. Five of these genes have been reported in DRG neurons previously and six of the genes are expressed in PC12 cells, a pheochromocytoma-derived cell line that shares neural crest origin with neurons of the DRG. Two genes are expressed in macrophages and 6 in glial cells. Three genes have been reported to show axotomy-induced regulation in non-DRG neurons (sympathetic and motor neurons). These genes belong to several different functional classes and from what is known about their functional role in other cells, may have a role in regeneration, cell survival, or alterations in sensory processing after nerve injury (Table 4, see additional file 3). Once a change in expression of the 240 genes in Table 3 have been validated, similar analyses of their putative function need to be made in order to begin to address

what role the genes play in the adaptive and maladaptive response to nerve injury.

Global changes in transcription

The 240 genes defined as putatively regulated (>1.5 -fold, $P < 0.05$) within the DRG 3 days after sciatic nerve section represent 5.1% of the detected genes, and of these 2.8% are up-regulated and 2.3% down-regulated (Figure 6A). These genes were assigned into functional classes and characterized as up- and down-regulated. Some gene classes are selectively regulated after axotomy (Figure 6B). Genes associated with the cell cytoskeleton, positive and negative regulators of apoptosis as well as immune markers tend to be up-regulated, whereas genes involved in membrane excitability (ion channels) or neurotransmission (neurotransmitters and vesicle trafficking genes) tend to be down-regulated.

Discussion

Although many studies have used microarray technology for expression profiling, the criteria for determining putatively regulated genes from such analyses remain poorly defined, the current standard being a >2 -fold difference in replicate arrays. Using Affymetrix high-density rat oligonucleotide-array data from control (naïve, non-injured) and experimental (axotomy, 3 day post peripheral nerve injury) DRGs, and comparing the differences detected in control vs. control and control vs. experimental arrays, we have explored the optimal criteria for minimizing the false positive error rate. A >2 -fold criterion alone was found to generate a substantial proportion of potential false positives, while also excluding many putatively regulated genes. Based on significant difference for each probe set ($P < 0.05$), 109 genes differed in the control vs. control group, and 556 in the control vs. experimental group, again a prohibitively high false positive rate (20%). However, a combination of fold and significant difference was found to maximize the difference between control vs. control and control vs. experimental array comparisons. Using >1.5 -fold and $P < 0.05$, 240 putatively regulated genes were detected in the experimental group compared with only 12 in the control group, an estimated error rate of 5%. If fold differences of 1.25 to 1.5 are included, many more significantly different genes are recruited (219 in the experimental vs. 41 in the control comparison) reducing the potential false negative rate. At the same time, the estimated false positive error rate rises to 11.5%, still lower than the 2-fold alone error. Producing a reduction in false positive without also producing false negative differences requires the use both of fold and significant difference. The established 2-fold criterion is much too conservative; when using replicate arrays with pooled samples from multiple animals to reduce biological variation, it loses over 50% of putatively regulated genes and fails to eliminate false positives. The very high concordance rate found

between genes predicted from the array analysis to be regulated and measurements of mRNA for 24 genes by quantitative Northern slot blots and *in situ* hybridization, validates utilization of a two dimensional matrix of fold and significant difference.

One problem with oligonucleotide arrays is sensitivity. Some groups of transcripts, particularly GPCRs, including the opiate receptors, which are expressed in the DRG, [14] fall below the microarray detection threshold. Lack of sensitivity may result from technical issues such as poor probe performance [11] or low copy number transcripts. Tissue heterogeneity and neuronal subpopulation-restricted gene expression may lower the concentration of a transcript in the total RNA sample to below detection threshold [10].

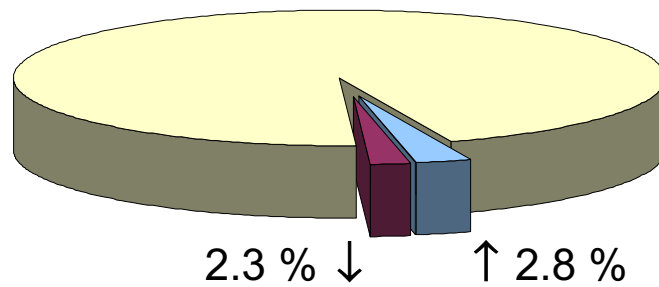
Recently Xiao et al [9] published a study using 7.5 K cDNA arrays to analyze genes regulated in the DRG 2, 7, 14 and 28 days following sciatic nerve axotomy. Of the genes listed in Table 4, seven were found in both studies (Peripheral Benzodiazepine receptor, 5HT3, Gadd45, CLP36, Lysozyme, SNAP25 and VGF). Of those genes whose expression was analyzed across time in this study (Figure 5) two, Lysozyme and SNAP25, were also detected by Xiao et al [9]. Both genes show very similar expression patterns over time in the two studies.

In a different cDNA array study, also recently published, 16 genes were identified to be regulated by at least 2-fold one week after sciatic axotomy [8]. Of these, seven were also identified by our study. Five of the non-concordant genes were not represented by known probe sets on the array used here. Others changed, but did not reach statistical significance, and likely represented differences in regulation levels relative to the time points used (1 week as opposed to 3 days). One gene verified in this study, the small proline-rich protein 1A (Table 2), was characterized in detail by Bonilla et al, who show that it colocalizes with filamentous actin in membrane ruffles and augments axonal outgrowth [8].

Comparison of the global lists of regulated genes between this study and other reports are complicated by the differing criteria used to identify regulated genes in the different studies. However, it is encouraging to note that a reasonable number of genes have been cross identified in three separate array studies looking at the effects of peripheral nerve injury on dorsal root ganglion gene expression, pointing to the reliability of array technologies across differing platforms.

Why is the number of genes regulated in sensory neurons after peripheral nerve injury so large? The injury constitutes an enormous stress to the cell and also deprives it

A



B

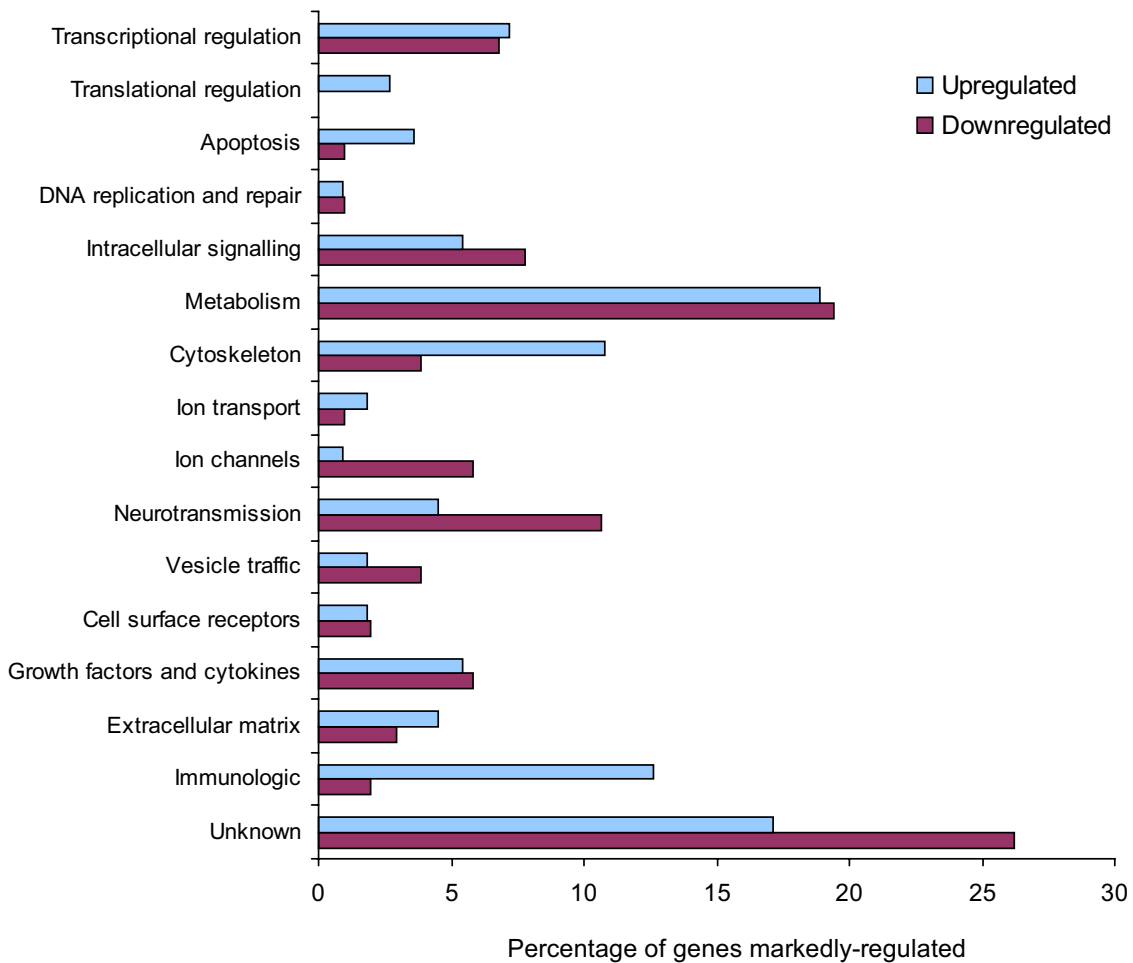


Figure 6
 Percentage of detected genes regulated above 1.5-fold ($P < 0.05$) with the proportion up and down regulated also shown **(A)**.
 Genes regulated above 1.5-fold ($P < 0.05$) were classified into functional classes, plotted are the percent of up and down regulated genes respectively in each functional class **(B)**.

from contact with its normal target-derived neurotrophic support. Both of these events represent perturbations from normal cellular functioning that require a variety of compensatory responses. A major issue is cell survival. Injured adult DRG neurons do not die after axonal injury [15] as a result of the upregulation of cell survival factors such as the small heat shock protein HSP27 [16]. We now find several genes that could have a survival-promoting role, including the peripheral benzodiazepine receptor, whose agonists are potent antiapoptotic compounds [17], GADD45A, which maintains genomic stability and appears to be anti-apoptotic in neurons after ischemic damage [18], metallothionein 1L, which is a free radical scavenger [19], GTP cyclohydrolase 1, which is obligatory for the actions of NGF on PC12 cells [20], and IES-JE (MCP-1) which attracts macrophages, which in turn have been reported to have a role in neuronal survival [21].

Neurons with axons in the peripheral nervous system can successfully regenerate when injured [22]. This is in part due to an environment permissive for axonal growth but also due to the upregulation of regeneration-associated genes (RAGs) which increase intrinsic growth capacity [23]. Three examples of RAGs are GAP-43 and CAP-23 [24] and sprp1A [8]. We now find several other potential candidates including: α 2 macroglobulin, CLP36, and VGF all of which have been described to promote neurite outgrowth or interact with the cytoskeleton in a way that may promote regeneration (see Table 4 [additional file 3] for references). MMP3 also known as 53 kD polypeptide, transin or stromelysin-1, a secreted protease, may facilitate neurite growth by dissolving the extracellular matrix of the basal lamina at the growing tip of the axon (see Table 4 for references).

Sensory neurons react to peripheral nerve injury by increasing their excitability, changing their constitutive synaptic transmitter profile and even their synaptic contacts in the spinal cord. Interestingly, an analysis of the functional classes of the genes differentially expressed (Figure 6) reveals the contribution that alterations in transcription make to these processes. Several of these changes are maladaptive in the sense that they contribute to the generation of the abnormal sensations that constitute neuropathic pain, by producing ectopic spontaneous activity into the CNS, altering synaptic drive, increasing excitability and diminishing inhibitory action in the dorsal horn of the spinal cord, and by making novel synaptic contacts with inappropriate neurons [1,4]. A number of the injury-regulated genes we describe here may contribute directly to this altered sensory processing; CB1 receptor, VGF, the phospholemman ion channel, SNAP25 A, endothelin-1 and the ligand-gated 5-HT₃ receptor (Table 4, see additional file 3). We have only validated a small fraction of the putatively regulated genes detected by the arrays. Our

analysis indicates that at a conservative estimate over two hundred genes (240 genes, >1.5-fold, $P < 0.05$) are regulated by the injury. These include genes of a number of distinct functional classes, some of which appear to change in a uniform way. Ion channels and neurotransmitter related genes tend to decrease their expression while those associated with the cytoskeleton increase. This raises the possibility that numbers of different genes may be regulated together by common changes in signal transduction and transcription induced by the injury, and contribute in this way to the coordinated changes that constitute the axotomy response.

Discovering and validating regulated genes by array analysis is clearly only the first step in elucidating their functional role. An evaluation of the cellular localization and distribution of the gene product as well as loss and gain of function manipulations *in vitro* and *in vivo* are required. Functional studies cannot yet be performed at a high throughput level and this represents one of the major limiting factors in analyzing the large numbers of candidate genes identified by microarray analysis. Elucidating the specific role of genes regulated in sensory neurons after peripheral nerve injury will provide insight though into many major biological issues including cell survival, growth, intercellular communication and the factors that contribute to sensory abnormalities. Microarray technology provides a powerful tool for beginning this analysis in a high throughput mode by revealing the extent of change in neuronal gene expression. Such analysis requires replicate measures to minimize variability, calculation of fold and significant differences to detect regulated genes with minimal false positive and negative confounders and validation of individual genes.

Conclusions

1: A comparison of two naïve (control) triplicate data sets allowed determination of the inherent variation present in the oligonucleotide array data. Analysis revealed a relatively high false positive error rate in the naïve versus axotomy (experimental) comparisons (16%) when using only a 2-fold criterion for regulated gene detection.

2: When statistical significance ($P < 0.05$) was combined with a fold difference of greater than 1.5 as the criteria for detecting putatively regulated genes, not only was the estimated false positive error substantially reduced (to 5%), but also many more genes were identified compared with the established 2-fold criterion, in the naïve versus axotomy comparison (240 as opposed to 178 genes).

3: All 240 genes displaying a fold change of greater than 1.5 and a P-value of less than 0.05 are listed.

4: To validate these findings we performed quantitative triplicate slot blots for 24 genes, enabling a direct comparison between the statistically analyzed array data and the mRNA measurements. These comparisons show strong concordance between putative and actual regulation as well as a direct relationship between array fold change and mRNA fold change.

5: Some genes were further quantified across a time course of 1, 3, 7 and 14 days post axotomy. These genes show diverse patterns of regulation in response to nerve injury and underlie the importance of including time as a dimension in any expression profile study.

6: Genes detected from the arrays, as being likely to be regulated, were assigned to functional groups. These data suggest the reorganization of cell structural components following peripheral nerve injury, activation of genes expressed by immune and inflammatory cells, and a down-regulation of genes involved in neurotransmission.

Methods

Surgical procedures

All procedures were performed in accordance with Massachusetts General Hospital animal care regulations. Adult male Sprague Dawley rats (200–300 g) were anesthetized with halothane. For the sciatic nerve transection (axotomy), the left sciatic nerve was exposed at the mid thigh level, ligated with 3/0 silk and sectioned distally. The wound was sutured in two layers, and the animals were allowed to recover.

Tissue and RNA preparation

Animals were terminally anesthetized with CO₂, the L4 and L5 DRGs rapidly removed, and stored at -80°C. Total RNA was extracted from homogenized DRG samples using acid phenol extraction (TRIzol reagent, Gibco-BRL). RNA concentration was evaluated by A₂₆₀ measurement and quality assessed by electrophoresis on a 1.5% agarose gel. Each RNA sample used for hybridization of each array was extracted from rat L4 and L5 DRGs (10 ganglia pooled from 5 animals, per sample).

Microarray Analysis

Affymetrix rat genome U34A oligonucleotide microarrays, representing 8799 known transcripts and expressed sequence tags (ESTs), were used (Santa Clara, CA [http://www.affymetrix.com]). Oligonucleotides are arranged in pairs corresponding to different regions of the target mRNA with multiple probe pairs. Each probe pair consists of a 25 nucleotide perfect match (PM) to the target region coupled with a 25-mer with a single mismatch (MM) at the 13th nucleotide. Transcript abundance is estimated by analysis of signal intensity of the PM/MM pairs. The arrays are hybridized with biotin-labeled cRNA, prepared as per

standard Affymetrix protocol. Briefly, total RNA (8 µg) from DRGs was reverse transcribed using an oligo-dT primer coupled to a T7 RNA polymerase binding site. Double-stranded cDNA was made and biotinylated-cRNA synthesized using T7 polymerase. The cRNA was hybridized for 16 hours to an array, followed by binding with a streptavidin-conjugated fluorescent marker, and then incubated with a polyclonal anti-streptavidin antibody coupled to phycoerythrin as an amplification step. Following washing, the chips were scanned with a Hewlett-Packard GeneArray laser scanner and data analyzed using GeneChip software. External standards were included to control for hybridization efficiency and sensitivity.

Hybridization levels for each species of mRNA detected on the arrays are expressed by intensity (signal) and as present (P), marginal (M) or absent (A) calls, calculated by Affymetrix software (MAS 5.0, $\alpha_1 = 0.04$ $\alpha_2 = 0.06$). To normalize the array data standard Affymetrix protocols were employed, each array was scaled to a target signal of 2500 across all probe sets (MAS 5.0).

The arrays were grouped for two comparisons: two triplicate sets of naïve data compared with one another, and one triplicate naïve set compared with one triplicate post-axotomy set. The individual naïve arrays included in each triplicate set were picked randomly. A probe set was determined undetected if it received an A call in all of the six arrays involved in the comparison. Detected were Present or Marginal by MAS5.0 in at least one array for each analysis. Mean signal and standard deviation were calculated for each detected probe set. The p-value for rejecting the null hypothesis that the mean signals were equal between the two triplicate sets was calculated using an unpaired, two-tailed t-test for independent samples with unequal variance (Satterthwaite's method). Fold-differences between the mean signals (A and B) in the two triplicate sets were calculated as $\max(A, B) / \min(A, B)$ with down regulation relative to naïve expressed as negative.

cDNA Probe production

To generate specific probes for Northern blot hybridization experiments, primers based on the rat accession number provided by Affymetrix were designed, primer pairs were chosen using the Primer3 software [http://www-genome.wi.mit.edu/] from the 1000 most 3' nucleotides within each accession sequence. PCR was performed on cDNA reverse transcribed from total RNA, extracted from lumbar DRGs, using poly-dT as a primer to obtain cDNA fragments (141 to 596 bp). These fragments were subsequently cloned into the PCR II vector (TA cloning Kit, Invitrogen) and the identity of each was confirmed by sequencing in both directions. These cDNAs were gel-purified and used to produce ³²P-labeled cDNA probes (Prime-It kit, Stratagene).

Northern blot analysis

Total RNA was size separated by electrophoresis on a 1.5% agarose/formaldehyde gel (10 µg of total RNA per lane) and transferred to a Hybond N+ nylon membrane. Membranes were hybridized with labeled-probes (see above) in ExpressHyb (Clontech) overnight at 65°C, washed and exposed to X-ray film with an intensifying screen at -80°C.

Slot Blots

Total RNA (1.25 µg) was directly transferred to Hybond N+ nylon membrane under vacuum using a Hoefer PR648 slot blot apparatus (Amersham Pharmacia Biotech) as described in [25]. The slot blots were produced in batches of ten from a relevant master mix and probed with the same cDNA under the same conditions as described for Northern blot analysis. Levels of hybridization were quantified using the 24450 phosphorimager system (Molecular Dynamics, Sunnyvale CA.). One of the blots in each batch was probed for cyclophilin to act as a loading control. The other nine blots were used to measure the expression levels of individual genes. Loading levels between samples on each blot were normalized using the cyclophilin levels from the control blot.

Isotopic in situ Hybridization

DRGs were rapidly removed, embedded in OCT (Tissue Tek) and frozen. Sections were cut serially at 6 µm. Isotopic-in situ hybridization was carried out using forty-eight base pair oligonucleotide probes, designed to have 50% G-C content and be complementary to the mRNAs whose accession numbers were provided by Affymetrix. Probes were 3'-end labeled with ³⁵S or ³³P-dATP using a terminal transferase reaction and hybridization carried out [26]. Autoradiograms were generated by dipping slides in NTB2 nuclear track emulsion and storing in the dark at 4°C. Sections were exposed for 1–8 weeks (depending on the abundance of transcript), developed, fixed and viewed under darkfield using a fiber-optic darkfield stage adapter (MVI). Controls to confirm specificity of oligonucleotide probes included hybridization of sections with labeled probe with a 1,000-fold excess of cold probe or labeled probe with a 1,000-fold excess of another, dissimilar cold probe of the same length and similar G-C content.

Authors' contributions

MC carried out array probe preparation, slot blot analysis and northern analysis, participated in the design of the study and drafted the manuscript. KB carried out northern analysis and data preparation, participated in the design of the study and drafted the manuscript. LK carried out in situ hybridization and participated in the design of the study. RSG carried out statistical data analysis and functional group analysis and participated in the design of the study. DD'U participated in data preparation and in the

design of the study. AA and JS carried out tissue preparation. JWM participated in data preparation. REP participated in the design of the study. CJW conceived the study and participated in its design and coordination.

Additional material

Additional File 1

Comparison of microarray and known regulation data for genes whose expression pattern within the DRG following nerve injury has been studied previously.

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[<http://www.biomedcentral.com/content/supplementary/1471-2202-3-16-S1.pdf>]

Additional File 2

Summary of genes identified as regulated >1.5-fold ($P < 0.05$) in the DRG 3 days after sciatic nerve axotomy.

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[<http://www.biomedcentral.com/content/supplementary/1471-2202-3-16-S2.pdf>]

Additional File 3

Summary of expression patterns and functional roles of uncharacterized nerve-injury regulated genes.

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[<http://www.biomedcentral.com/content/supplementary/1471-2202-3-16-S3.pdf>]

Acknowledgements

We thank Bayer AG and the NIH (NS38253, HD38533) for financial support.

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