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Patterns of pain: Meta-analysis of microarray studies of pain

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ABSTRACT

Existing microarray gene expression profiling studies of tonic/chronic pain were subjected to meta-analysis to identify genes found to be regulated by these pain states in multiple, independent experiments. Twenty studies published from 2002 to 2008 were identified, describing the statistically significant regulation of 2254 genes. Of those, a total of 79 genes were found to be statistically significant "hits" in 4 or more independent microarray experiments, corresponding to a conservative $P < 0.01$ overall. Gene ontology-based functional annotation clustering analyses revealed strong evidence for regulation of immune-related genes in pain states. A multi-gene quantitative real-time polymerase chain reaction experiment was run on dorsal root ganglion (DRG) and spinal cord tissue from rats and mice given nerve (sciatic chronic constriction; CCI) or inflammatory (complete Freund's adjuvant) injury. We independently confirmed the regulation of 43 of these genes in the rat-CCI-DRG condition; the genetic correlates in all other conditions were largely and, in some cases, strikingly, independent. However, a handful of genes were identified whose regulation bridged etiology, anatomical locus, and/or species. Most notable among these were *Reg3b* (regenerating islet-derived 3 beta; pancreatitis-associated protein) and *Ccl2* (chemokine [C–C motif] ligand 2), which were significantly upregulated in every condition in the rat.

1. Introduction

The development of high-density oligonucleotide microarray technologies has allowed for the simultaneous assessment of the expression levels of several thousand mRNA transcripts in a single high-throughput procedure [46] (see [29,55] for review). From 2002 to 2008, 20 studies were published describing the use of microarray-based technologies—commonly (albeit not always accurately) referred to as "gene chips"—to profile global gene expression patterns in the central and peripheral nervous system following a variety of neuropathic and inflammatory pain states in rodents. These studies each identified up to hundreds of candidate genes, many of which have yet to be confirmed by follow-up genetic and/or functional assays. The confirmation and analysis of these genes would greatly increase our understanding of the complex molecular cascades causal to chronic pain, affected by chronic pain, and/or useful as correlative biomarkers of chronic pain.

How to interpret the vast amounts of data generated by microarray experiments into meaningful patterns and clusters that can guide the development of novel hypotheses has remained a consistent challenge. In addition, the problems of false-positive findings on a potentially massive scale, and the difficulty in identifying what constitutes a biologically relevant change in gene expression amongst the tens of thousands of mRNA transcripts represented on a modern microarray chip, has led many to question the value these types of studies provide. Simply put, of the hundreds of candidate genes identified by multiple microarray studies as "pain relevant," how should one prioritize single gene-focused follow-up studies? These decisions are usually made with respect to a priori hypotheses, but this strategy obviously limits the potential heuristic value of microarray gene expression profiling as a systematic vehicle for gene discovery. In some cases, regulated genes are chosen for further study based on their ontologies or via bioinformatics analyses (eg, [11,17]). An alternate and completely agnostic approach is to use meta-analytic techniques for the reanalysis of primary data obtained in different published investigations.

Herein, we collected lists of significantly regulated genes from relevant microarray studies in order to identify genes appearing several times in independent experiments. We were thus able to

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find genes that are consistently regulated—that is, over-expressed or under-expressed in pain-relevant tissues taken from animals in chronic pain compared to controls—in different pain states, microarray platforms, species, strains, and laboratories. The results of this analysis identified several genes that are known to have strong links to pain processing, as well as many genes that have never been implicated in pain but now emerged as especially strong candidates for further study. A multi-gene quantitative real-time polymerase chain reaction (qPCR) study was performed to independently confirm these genes; this was broadly successful only for genes expressed in the dorsal root ganglia (DRG) and spinal cord of nerve-injured rats. Altering the pain-causing injury (inflammation) or species (mouse) yielded completely different regulation patterns. However, a few genes were more consistently regulated—especially *Reg3b* (pancreatitis-associated protein) in rats and mice, and *Ccl2* (chemokine [C–C motif] ligand 2) in rats—these regulations were largely confirmed in a new, single-gene qPCR experiment.

2. Materials and methods

2.1. Literature search and criteria

A search of the PubMed database (http://www.ncbi.nlm.nih.gov/entrez/) was performed in March 2009 to identify published manuscripts describing microarray experiments utilizing tissues obtained from rodents following the induction of a tonic or chronic neuropathic or inflammatory pain state, and performing gene-expression profiling in these tissues compared to control tissues taken from pain-free rodents. We read each individual manuscript closely in order to ensure relevance. All published manuscripts meeting the following criteria were included for analysis: (1) the manuscript must describe the use of a microarray-based assay containing at least 100 distinct genes analyzed in parallel; (2) the study must have used an established rodent model of either neuropathic or inflammatory pain [34] as the source of tissue for microarray analysis; and (3) the microarray analysis must have been performed on one or more pain-relevant tissues (ie, stimulated peripheral tissue, peripheral nerve carrying afferent information from the stimulated tissue, dermatome-appropriate dorsal root ganglion, dermatome-appropriate dorsal spinal cord). Note that studies involving pain-relevant tissues higher in the neuraxis than the spinal cord would have been considered, but none was identified.

2.2. Meta-analysis and bioinformatics

The lists of significantly regulated genes from each identified paper were compiled onto a single Excel spreadsheet. As the original gene lists from individual papers were encoded using several different coding systems (eg, GenBank Accession, Affymetrix ID, gene name), it was necessary to convert these into a single identification code to standardize the information. This was accomplished using the online Gene IDConverter tool (http://idconverter.bioinfo.cnio.es/) [2]. After standardization, we searched for the number of times that an individual gene was found to be significantly regulated (by the authors' definitions) in the studies examined. Statistical significance was determined by the binomial test. The calculation of binomial probabilities was performed using an online tool (http://faculty.vassar.edu/lowry/binomialX.html), and used the variables n (number of independent microarray experiments; ie, 20), k (the number of times the gene appears as a "hit" in independent microarray experiments; the definitions of a "hit" in each case are provided in Table 1), and p (the empirical probability of a specific gene being regulated in any one experiment; calculated from the mean of data in the far-right column of Table 1 as 3.87%, or $p = 0.0387$) in order to calculate the probability (P) of observing any particular regulated gene k or more times in independent microarray experiments.

Each gene significantly regulated in 4 or more studies was coded on an arbitrary 4-point scale as to its level of validation as a "pain gene" based on extensive PubMed searches and a consultation of the PainGenes Database [25] (Table 2). The scale was applied as follows: 0—no apparent evidence; 1—weak correlational evidence (protein or mRNA expressed in pain-relevant cells and/or anatomical regions); 2—strong correlational evidence (previously demonstrated increase/decrease in protein/mRNA expression in a pain state); and 3—causational evidence (selective pharmacological and/or genetic manipulation alters pain behavior).

Finally, lists of genes were analyzed using the Gene Functional Classification tool from the DAVID Bioinformatics Resources website (http://david.abcc.ncifcrf.gov/) in order to find groups of genes with similar functions based on the controlled Gene Ontology vocabulary [12]. Lists of genes were entered using a classification stringency of "low" due to the relatively small number of total genes in each list. The output was a series of functionally related clusters of genes (called gene groups) ranked by enrichment score, a statistical measure of the overall biological significance of each gene group to the total gene list.

2.3. Chronic constriction injury

All behavioral studies were approved by the local animal care and use committee and were conducted in accordance with the guidelines for animal research by the International Association for the Study of Pain [62].

Adult (175–200 g) male Sprague–Dawley rats (Harlan Inc, Indianapolis, IN, USA; Frederick, MD, USA breeding colony) were housed in groups of 3 on sawdust bedding in plastic cages. Adult (25–35 g) male CD-1 (ICR: Crl) mice (Charles River Laboratories, Boucherville, QC) were similarly housed in groups of 4. These strains (and sex) were chosen based on their common use in the original microarray studies being meta-analyzed here. Artificial lighting was provided on a fixed 12-hour light–dark cycle (lights on at 7:00 am) with food and water available ad libitum. Rodents were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal) and received either a unilateral chronic constriction injury (CCI) or sham procedure (exposure of the sciatic nerve without manipulating it) as described elsewhere [8]. The CCI was chosen over other nerve injuries as the most generalizable, because it has the smallest number of uniquely regulated genes (compared to 2 other surgical procedures leading to neuropathic allodynia: spinal nerve ligation and spared nerve injury) according to the analysis of Griffin and colleagues [17] (also see [11]). Rats (but not mice) receiving the CCI injury showed the expected changes in the posture of the ipsilateral hind paw (ventroflexed toes and paw inversion) [8]. Although rodents in this particular experiment were not tested behaviorally, all rat and mouse surgeries were performed by highly experienced surgeons in the Bennett and Mogil laboratories, respectively. At 14 days postsurgery, corresponding to the time of peak mechanical allodynia based on our extensive previous experience with these protocols, animals were euthanized for tissue harvesting.

2.4. Complete Freund's adjuvant

Rats and mice (see Section 2.3 above) were injected with 50% complete Freund's adjuvant (CFA) in the plantar surface of the right hind paw (20 μL volume in mice; 150 μl volume in rats), or physiological saline as a control. Of the 3 inflammatory mediators used

Table 1
Methodological summaries of the pain-relevant microarray experiments analyzed.

References	Year	Species strain/ sex	Pain assay	Tissue	Microarray platform	Definition of "regulated"	Number of regulated genes reported (% of total)[a]
Ko et al. [23]	2002	Rat/SD/Male	S1/S2 transection	Spinal cord	Incyte Gene Discovery Array Mouse 1.1	>2-fold	42 (0.5%)
Costigan et al. [10]	2002	Rat/SD/male	Sciatic nerve transection	DRG	Affymetrix Rat RGU34A	>1.5-fold, P < 0.05	197 (2.2%)
Xiao et al. [56]	2002	Rat/SD/not stated	Sciatic nerve transection	DRG	Atlas Rat 6.5 k	>2-fold	117 (1.8%)
Wang et al. [55]	2002	Rat/SD/male	SNL	Spinal cord, DRG	Affymetrix Rat RGU34A	P < 0.05	166 (2.4%)
Sun et al. [48]	2002	Rat/SD/male	SNL	Spinal cord	Affymetrix Rat RGU34A	>2-fold, P < 0.05	44 (0.6%)
Bonilla et al. [9]	2002	Mouse/B6/male	Sciatic nerve transection	DRG	Incyte Mouse LifeArray GEM1	>2-fold	13 (0.2%)
Kubo et al. [24]	2002	Mouse/ICR/male	Sciatic nerve transection	Sciatic nerve	Incyte Mouse LifeArray GEM2	>2-fold	53 (0.6%)
Valder et al. [53]	2003	Rat/SD/not stated	SNL	DRG	Affymetrix Rat RGU34A	>2-fold, P < 0.05	139 (2.0%)
Yang et al. [58]	2004	Rat/SD/male	Sciatic nerve transection	Spinal cord	Atlas Rat 1.2	>2-fold, P < 0.05	169 (2.6%)
Ren et al. [43]	2005	Rat/SD/male	0.25% carrageenan[b]	Spinal cord	Custom spotted array (rat)	P < 0.05, FDR[c]	31 (15.1%)
Barr et al. [6]	2005	Rat[d]/not stated/ not stated	2% formalin	Spinal cord	Affymetrix Rat Neurobiology	P < 0.05, FDR[c]	47 (3.7%)
Nesic et al. [35]	2005	Rat/SD/male	SCI	Spinal cord	Affymetrix Rat RGU34A	>1.5-fold up, > 0.66-fold down, P < 0.05	36 (0.1%)
Rodriguez Parkitna et al. [45]	2006	Rat/Wistar/male	150 μL CFA, CCI	Spinal cord, DRG	Atlas Rat 4 k	Z-score > |3.5|	40 (1.0%)
LaCroix-Fralish et al. [27]	2006	Rat/SD/male	SNL, L5 nerve root ligation	Spinal cord	Affymetrix Rat RAE230A	P < 0.01	805 (17.1%)
Yang et al. [57]	2007	Rat/SD/male	4% carrageenan	DRG, hind paw	SuperArray GEA (rat)	>2-fold	35 (16.1%)
Geranton et al. [16]	2007	Rat/SD/male	10 μL CFA	Spinal cord	Affymetrix Rat RAE230	>1.3-fold, P < 0.05	74 (0.2%)
Griffin et al. [17]	2007	Rat/SD/male	CCI, SNI, SNL	Spinal cord	Affymetrix Rat RGU34A	>1.25-fold, P < 0.01	96 (1.1%)
Yukhananov et al. [59]	2008	Rat/SD/male	2% carrageenan	Spinal cord	Affymetrix Rat RAE230	P < 0.01	798 (2.6%)
Nishida et al. [36]	2008	Rat/SD/male	Paclitaxel-induced neuropathy	DRG	Affymetrix Rat RG230 2.0	>2-fold	51 (0.3%)
Levin et al. [28]	2008	Rat/not stated/ not stated	SNL	Spinal cord, sciatic nerve, DRG	Affymetrix Rat RGU34A, B, C	>3-fold	195 (1.3%)

SD, Sprague Dawley rat strain; DRG, dorsal root ganglion; SNL, spinal nerve ligation; B6, C57BL/6 mouse strain; ICR, Institute for Cancer Research stock; SCI, spinal cord injury; CFA, complete Freund's adjuvant; CCI, chronic constriction injury; SNI, spared nerve injury.

[a] Genes significantly up- or downregulated at any postinjury time point are included. Numbers represent the total number of comparable annotated genes (ie, those with unique gene IDs) in each study; they do not necessarily correspond exactly to the number of regulated genes reported in the published papers. Note also that due to improvements in annotation with time, the total number of annotated genes on the same array can be very different in experiments performed at different times.

[b] Injections given at 3 days of age and in adulthood, 1 day before tissue extraction.

[c] False discovery rate (FDR) correction for multiple comparisons [7].

[d] Rats tested were 3 or 21 days of age.

Table 2
List of genes found to be significantly regulated by 4 or more independent microarray experiments.

Entrez gene ID	No. of studies (out of 20)	Direction	Neuropathic/ inflammatory (out of 14/6)	Gene name	Protein name	Validation level [a]
50654	9	Up	8/1	*Ctss*	Cathepsin S	3
29687	8	Up	7/1	*C1qb*	Complement component 1, q subcomponent, beta	2
25211	8	Up	7/1	*Lyz1*	Lysozyme	1
362634	7	Up	7/0	*C1qc*	Complement component 1, q subcomponent, gamma	2
24604	7	Up	7/0	*Npy*	Neuropeptide Y	3
116510	7	Up	5/2	*Timp1*	Tissue inhibitor of metalloproteinase 1	1
29461	7	Up	4/3	*Vgf*	VGF nerve growth factor, inducible	3*
25399	6	Up	6/0	*Cacna2d1*	Calcium channel, voltage-dependent, alpha2/delta1	3
25599	6	Up	6/0	*Cd74*	Cd74 antigen	0
81657	6	Down	3/3	*Gabbr1*	Gamma-aminobutyric acid (GABA) b receptor 1	3
29141	6	Up	6/0	*Gal*	Galanin	3
24387	6	Up	6/0	*Gfap*	Glial fibrillary acidic protein	2
24588	6	Down	5/1	*Nefm*	Neurofilament 3, medium	2
294273	6	Up	5/1	*RT1-DMb1*[b]	Major histocompatibility complex, class II, dm beta	0
25012	6	Down	6/0	*Snap25*	Synaptosomal-associated protein 25	1
24797	6	Up	6/0	*Sst*	Somatostatin	3
117556	6	Down	5/1	*Sv2b*	Synaptic vesicle glycoprotein 2b	1

Table 2 (*continued*)

Entrez gene ID	No. of studies (out of 20)	Direction	Neuropathic/ inflammatory (out of 14/6)	Gene name	Protein name	Validation level [a]
24806	6	Up	5/1	*Tac1*	Tachykinin 1	3
24877	6	Down	6/0	*Vsnl1*	Visinin-like 1	0
29427	5	Up	4/1	*Aif1*	Allograft inflammatory factor 1 (Iba1)	2
25239	5	Up	5/0	*Apod*	Apolipoprotein d	1
24211	5	Down	4/1	*Atp1a1*	ATPase, Na+/K + transporting, alpha 1 polypeptide	1
24233	5	Up	4/1	*C4a*	Complement component 4a	2
24241	5	Up	4/1	*Calca*	Calcitonin/calcitonin-related polypeptide, alpha	3
361673	5	Up	4/1	*Cxcl10*	Interferon-inducible protein variant 10	2
25112	5	Up	5/0	*Gadd45a*	Growth arrest and DNA-damage-inducible 45 alpha	2
29423	5	Up	5/0	*Gap43*	Growth associated protein 43	1
81682	5	Up	5/0	*Lum*	Lumican	1
83613	5	Down	5/0	*Nefl*	Neurofilament, light polypeptide	1
29480	5	Down	4/1	*Rgs4*	Regulator of G-protein signaling 4	2
294274	5	Up	3/2	*RT1-DMa*[c]	Major histocompatibility complex, class II, dm alpha	0
29571	5	Down	5/0	*Scn10a*	Sodium channel, voltage-gated, type 10, alpha	3
24230	5	Up	5/0	*Tspo*	Benzodiazepine receptor, peripheral	3
25624	5	Down	5/0	*Vamp1*	Vesicle-associated membrane protein 1	1
117064	5	Up	5/0	*Vip*	Vasoactive intestinal polypeptide	3
25291	4	Up	3/1	*Anxa3*	Annexin A3	2
100363366	4	Up	3/1	*Aplp2*	Amyloid beta (a4) precursor-like protein 2	0
25728	4	Up	3/1	*Apoe*	Apolipoprotein E	3
29221	4	Up	4/0	*Arg1*	Arginase 1	2
54227	4	Up	4/0	*Arpc1b*	Actin related protein 2/3 complex, subunit 1b	0
25389	4	Up	4/0	*Atf3*	Activating transcription factor 3	2
25390	4	Up	2/2	*Atp1b3*	ATPase, Na+/K + transporting, beta 3	3*
192262	4	Up	4/0	*C1s*	Complement component 1, s subcomponent	2
24232	4	Up	4/0	*C3*	Complement component 3	2
24770	4	Up	4/0	*Ccl2*	Monocyte chemoattractant protein-1	3
58919	4	Up	3/1	*Ccnd1*	Cyclin D1	0
25101	4	Down	4/0	*Chrna3*	Cholinergic receptor, nicotinic, alpha polypeptide 3	3
64036	4	Down	3/1	*Cd55*	Decay accelerating factor 1 (CD55, complement)	0
29593	4	Down	4/0	*Ckmt1*	Creatine kinase, mitochondrial 1, ubiquitous	1
155151	4	Up	4/0	*Coro1a*	Coronin, actin binding protein 1a	1
29563	4	Up	4/0	*Crabp2*	Cellular retinoic acid binding protein 2	0
117505	4	Up	4/0	*Csrp3*	Cysteine and glycine-rich protein 3	1
171293	4	Up	3/1	*Ctsd*	Cathepsin D	0
25425	4	Up	4/0	*Ctsh*	Cathepsin H	0
25417	4	Down	4/0	*Dpysl4*	Dihydropyrimidinase-like 4	0
24330	4	Up	2/2	*Egr1*	Early growth response 1	3
289211	4	Up	4/0	*Fcgr2b*	Fc receptor, IgG, low affinity IIb	0
29707	4	Up	2/2	*Gabra5*	Gamma-aminobutyric acid a receptor, alpha 5	1
25454	4	Up	4/0	*Gfra1*	Glial cell line derived neurotrophic factor family receptor alpha 1	3
29559	4	Down	4/0	*Grik1*	Glutamate receptor, ionotropic, kainate 1	3
79246	4	Down	3/1	*Htr3a*	5-Hydroxytryptamine (serotonin) receptor 3a	3
24484	4	Up	3/1	*Igfbp3*	Insulin-like growth factor binding protein 3	1
25641	4	Up	3/1	*Igfbp6*	Insulin-like growth factor binding protein 6	1
246153	4	Down	3/1	*Kcnc2*	Potassium voltage gated channel, shaw-related subfamily, 2	1
498335	4	Up	4/0	*LOC498335*	Similar to small inducible cytokine b13 precursor (Cxcl13)	0
24567	4	Up	1/3	*Mt1a*	Metallothionein 1a	0
24587	4	Down	4/0	*Nefh*	Neurofilament, heavy polypeptide	2
60355	4	Down	4/0	*Nsf*	N-ethylmaleimide sensitive fusion protein	2
64636	4	Down	3/1	*Ntsr2*	Neurotensin receptor 2	3
25531	4	Down	4/0	*Rab3a*	Rab3a, member ras oncogene family	1
24618	4	Up	4/0	*Reg3b*	Pancreatitis-associated protein	2*
309622	4	Up	4/0	*RT1-Bb*[d]	Rt1 class II, locus bb	0
294269	4	Up	4/0	*RT1-Da*[e]	Rt1 class II, locus da	0
294270	4	Up	4/0	RT1-Db1 [f]	Rt1 class II, locus db1	0
24615	4	Up	3/1	*S100a4*	S100 calcium-binding protein a4	2
29701	4	Down	4/0	*Scn11a*	Sodium channel, voltage-gated, type XI, alpha (NaV1.9)	3
499660	4	Up	4/0	*Sprr1al*[g]	Similar to cornifin a (small proline-rich protein 1a)	0
79423	4	Up	4/0	*Stmn4*	Stathmin-like 4	1
56010	4	Down	3/1	*Ywhag*	14–3–3gamma	0

[a] Evidence for involvement in pain: 0—no evidence; 1—weak, correlational evidence; 2—strong, correlational evidence; 3—causational evidence (see text for details).
[b] The equivalent mouse ortholog is H2-Dmb1.
[c] The equivalent mouse ortholog is H2-Dma.
[d] The equivalent mouse ortholog is H2-Ab1.
[e] The equivalent mouse ortholog is H2-Ea-ps.
[f] The equivalent mouse ortholog is H2-Eb1.
[g] The equivalent mouse ortholog is Sprr1a.
* Evidence published in the literature after our validation effort.

in the 6 relevant microarray studies—formalin, carrageenan, and CFA—we chose CFA because in our hands it produces the most robust and long-lasting evidence of pain-related behavior. At 24 h postinjection, corresponding to the time of peak mechanical

allodynia based on our previous experience, animals were euthanized for tissue harvesting. Hind paws were weighed immediately post mortem to confirm inflammation. Data from one mouse with insufficient inflammation was discarded from further analysis.

2.5. Quantitative real-time RT-PCR (qPCR)

Rat and mouse quadrisected (ipsilateral dorsal) lumbar spinal cord and L4–L5 DRG were harvested and total RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA, USA) and the RNeasy kit (Qiagen, Mississauga, ON, Canada) followed by a DNase treatment. To obtain enough total RNA for qPCR, DRG and spinal cord tissue was pooled (2 rats/spinal cord, 3 rats/DRG, 3 mice/spinal cord, 4 mice/DRG) from similarly treated animals. RNA quality was assessed on a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA) and the concentration measured on a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

For the multi-gene qPCR experiment, custom 384-well qPCR plates were prepared by SA Biosciences (Custom RT^2 Profiler PCR Arrays; Frederick, MD, USA) containing species-specific primers corresponding to transcripts of the 79 genes listed in Table 2, as well as 5 housekeeping genes (*Actb*, *Gapdh*, *B2m*, *Gusb* and *Hprt1*). Quality control was achieved via the observation of single peaks by RT-PCR, Ct < 30, and >80% efficiency by DART-PCR (www.sabiosciences.com/pcrarrayperformance.php). Total RNA was reverse transcribed (RT) using the RT^2 First Strand Kit, and after quality control for RT efficiency transcripts were amplified on the custom qPCR plates with RT^2 Master Mixes containing SYBR Green on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA). Data were analyzed by the manufacturer's supplied software, using the $2^{-\Delta Ct}$ method as a ratio compared to the average of the housekeeping genes. In the multi-gene study, each reported fold-regulation ratio (CCI vs sham, CFA vs saline) comprises pooled biological (between-plate replicates) with n = 2–3 mice or rats represented. Statistical significance was assessed by Student's *t*-test among the biological replicates.

For the single-gene (*Reg3b* and *Ccl2*) qPCR assays, total RNA from newly obtained tissue was reverse transcribed using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems) and transcripts were amplified with TaqMan probe and primers sets specific to *Reg3b* and *Ccl2* on an ABI Prism 7700 Sequence Detection System (Mm00440616_g1 and Mm00441242_m1 for mouse *Reg3b* and *Ccl2*, respectively; Rn00583920_m1 and Rn00580555_m1 for rat *Reg3b* and *Ccl2*, respectively). Data were analyzed using the $2^{-\Delta Ct}$ method as a ratio compared to the housekeeping gene, *Gapdh*, using the TaqMan Rodent GAPDH Control Reagents kit (Applied Biosystems). In the single-gene study, each reported fold-regulation ratio (CCI vs sham, CFA vs saline) comprises 3 technical (within-plate) replicates and 2–3 (pooled) biological replicates.

We note that these data are characterized by great heterogeneity in the ratios of regulated genes across tissues compared to housekeeping genes. The multi-gene study, like the meta-analysis before it, can be considered primarily hypothesis-generating in nature. For the single-gene qPCR study, by contrast, we were interested in an unbiased, apples-to-apples comparison of fold-regulations by the chronic pain state in each case, using statistically conservative methods. To accomplish this, we employed confidence limits analysis as follows. For each condition and in each biological replicate, the technical error variance was used to generate minimum and maximum point-estimates (95% confidence intervals [CI]) of fold-regulations (pain vs control). Reported are the means of these point estimates along with their 95% CIs of the biological variance. Statistical significance was defined as the nonoverlap of the 95% CIs with 1.

3. Results

3.1. Analysis of significantly regulated genes from 20 independent microarray experiments

We identified 20 papers from the literature from the years 2002–2008 that describe a microarray analysis of tissue obtained from rodents experiencing a tonic/chronic pain state. The general characteristics of these studies can be found in Table 1. Of these 20 studies, 14 used a neuropathic assay and 6 used an inflammatory assay. Eighteen studies were performed on tissue obtained from rat, 2 studies on tissue obtained from mouse. The tissue from which total RNA was isolated was (or included) spinal cord in 13 studies, DRG in 9 studies, sciatic nerve in 2 studies, and hind paw skin in one study. A compilation of all of the lists of significantly regulated genes from the individual papers revealed a total of 2254 unique genes (not shown; interested parties can obtain the full list from the corresponding author). Of these 2254 genes, 355 genes were observed to be regulated in 2 independent studies, 98 genes were observed to be regulated in 3 studies, 44 genes were observed to be regulated in 4 studies, 16 genes were observed to be regulated in 5 studies, 12 genes were observed to be regulated in 6 studies, 4 genes were observed to be regulated in 7 studies, 2 genes were observed to be regulated in 8 studies, and 1 gene was observed to be regulated in 9 independent studies (Fig. 1a). The binomial probability (*P*) of observing the same gene in 2, 3, and 4 independent microarray studies was calculated as $P = 0.14$, 0.03, and 0.006, respectively. Thus, a unique gene found to be significantly regulated in 3 or more independent microarray studies (out of 20 total) was considered to be statistically significant ($P < 0.05$). The more conservative list of genes that were significantly regulated in 4 or more microarray studies ($P < 0.01$) is reported in Table 2; of the 79 genes on this list, 57 were consistently upregulated by the pain state and 22 were consistently downregulated. The list of genes that were significantly regulated in 3 microarray studies is provided as Supplementary Table 1 online (Appendix).

The average validation score (see Materials and methods) as a function of the number of studies showing regulation of that gene is shown in Fig. 1b. Of interest is the fact that in the intervening period after these validation scores were assigned, new data supporting at least 3 genes' involvement in pain were published: *Atp1b3* [26], *Reg3b* [19], and *Vgf* [44].

3.2. Multi-gene qPCR confirmation of genes with 4 or more hits

In order to validate the ability of this meta-analysis of pain microarray studies to identify true-positive regulated genes with generalizable effects, we performed an independent study using a custom multi-gene qPCR plate. The expression of 79 genes showing 4 or more hits was assessed, de novo, in DRG and spinal cord of rats and mice given a neuropathic (CCI) or inflammatory (CFA) injury, at a single time point associated with maximal levels of mechanical allodynia in each case (14 days postoperative for CCI; 24 hours postinjection for CFA) based on our extensive experience with these algesiometric tests. Note, however, that allodynia was not measured in the subjects providing tissue for the qPCR experiments, and thus it remains possible that the tissue was not obtained at time points corresponding to maximum allodynia. Although qPCR assays were run in biological (between-plate) triplicate; of the 8 conditions (2 species $\times$ 2 tissues $\times$ 2 injuries) examined, one of the replications failed in 2 cases (rat spinal cord CCI and mouse spinal cord CCI) such that statistical significance could not be evaluated. It should be noted, however, that as for the meta-analysis itself, the point of this endeavor was heuristic rather than hypothesis-testing, and thus we considered >2-fold

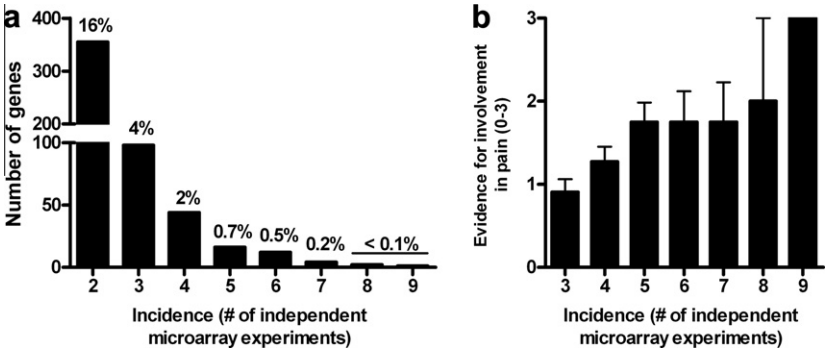

Fig. 1. Summary data from the meta-analysis of published pain microarray studies. (a) The total number of genes reported as being significantly regulated in a pain state in 2 or more independent microarray experiments. Corresponding percentages are provided above the respective bars. (b) The mean prior validation score (see Table 2 and main text) for genes reported as being significantly regulated in a pain state in 3 or more independent microarray experiments (mean ± SD).

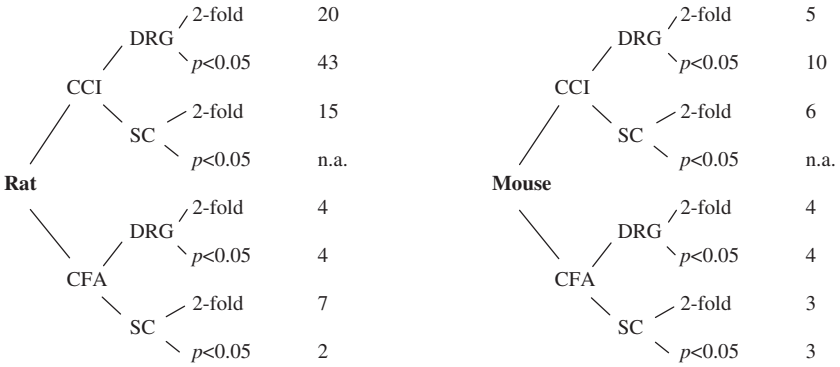

Fig. 2. Gene regulations (out of a total of 79) "confirmed" by the multi-gene quantitative real-time polymerase chain reaction experiment. DRG, dorsal root ganglion; CCI, chronic constriction injury; SC, spinal cord; CFA, complete Freund's adjuvant; n.a., not applicable, as statistical analyses could not be performed due to low sample size.

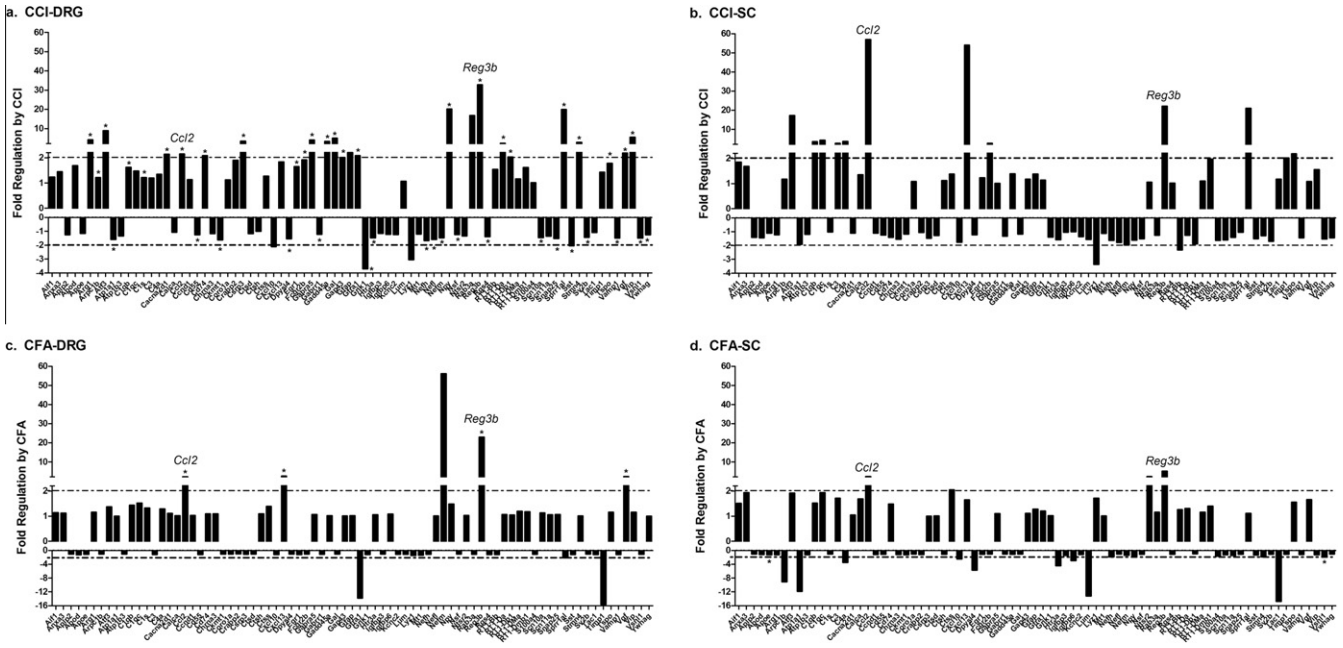

Fig. 3. Rat multi-gene quantitative real-time polymerase chain reaction experiment results. Bars represent fold regulation compared to control group (sham surgery in graphs a and b; vehicle injection in graphs c and d) in dorsal root ganglion (DRG) (a and c) and ipsilateral dorsal spinal cord (SC; b and d) tissue from rats given chronic constriction injury (CCI) surgery (a and b; 7 days postoperative) or complete Freund's adjuvant (CFA) injection (c and d; 24 h postinjection). Horizontal dashed lines indicate 2-fold regulation compared to control in each direction. Note the different *y*-axis scale below zero in graphs c and d. $^{*}P < 0.05$ (note that statistics could not be performed due to low sample size in graph b).

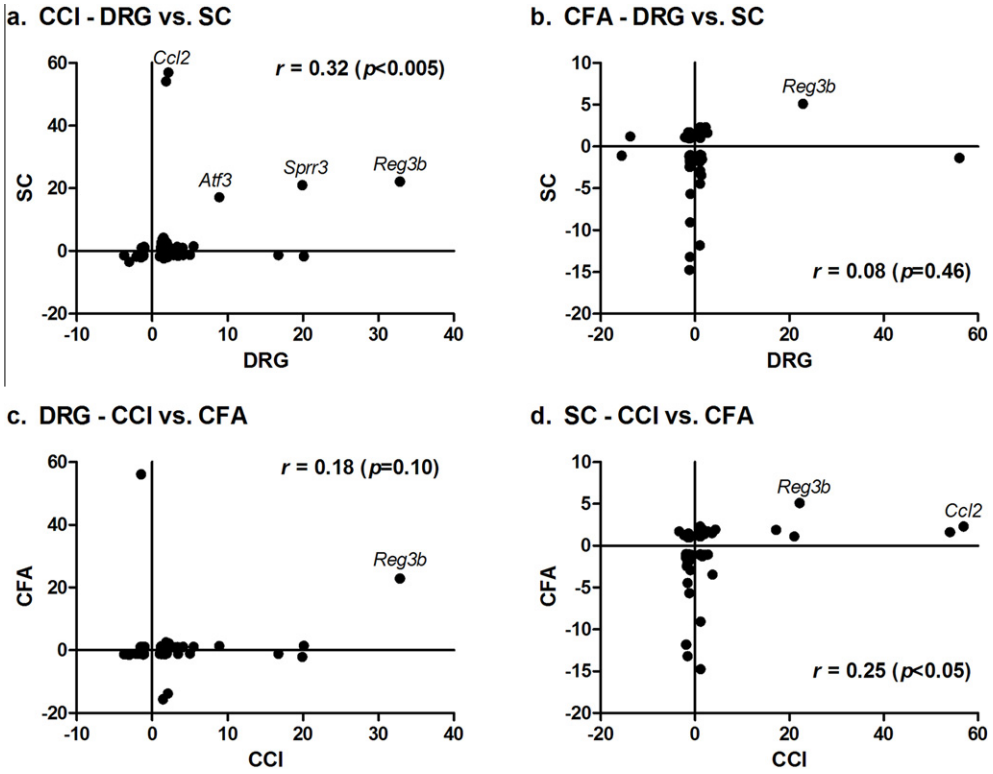

Fig. 4. Correlations (Pearson's *r*; with uncorrected *P*-values) among fold-regulations shown in the different conditions in Fig. 3. Genes whose fold-regulations exceed >2 on both axes are denoted by name. CCI, chronic constriction injury; DRG, dorsal root ganglion; SC, spinal cord; CFA, complete Freund's adjuvant.

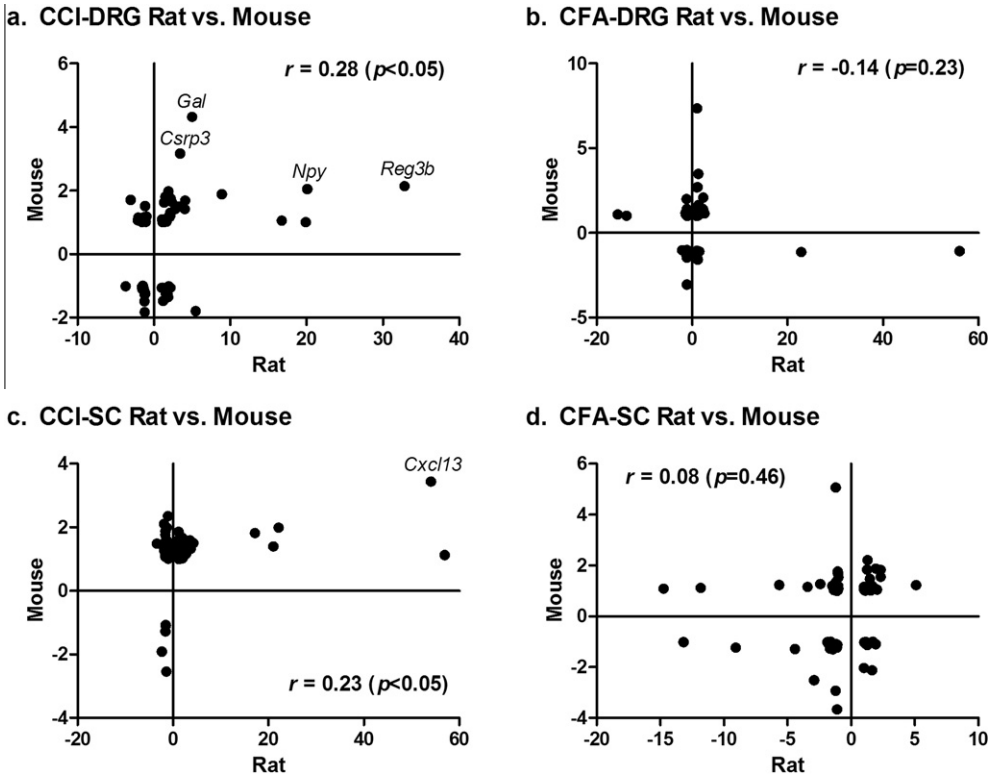

Fig. 5. Correlations (Pearson's *r*; with uncorrected *P*-values) among fold-regulations of all analogous conditions between mouse and rat. Genes whose fold-regulations exceed >2 on both axes are denoted by name. Note the different *y*-axis (mouse) and *x*-axis (rat) scales. CCI, chronic constriction injury; DRG, dorsal root ganglion; CFA, complete Freund's adjuvant; SC, spinal cord.

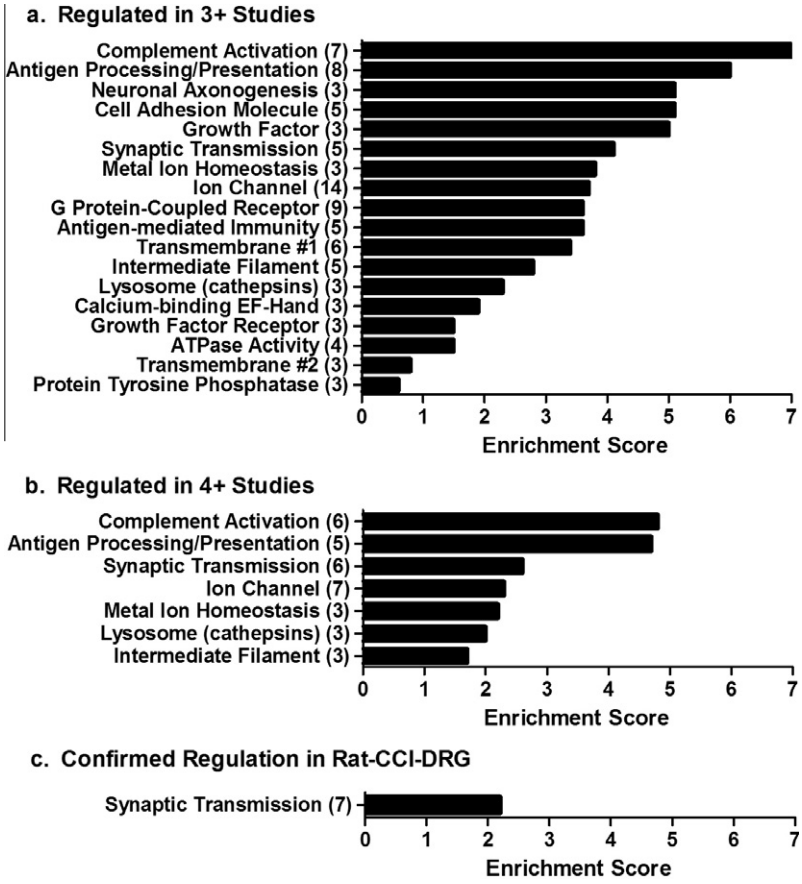

Fig. 6. Functional annotation clustering analyses of (a) genes regulated in 3 or more microarray studies ($P < 0.05$; see Supplementary Table 4); (b) genes regulated in 4 or more microarray studies ($P < 0.01$; see Supplementary Table 5); and (c) genes confirmed to be regulated in rat dorsal root ganglion (DRG) after chronic constriction injury (CCI) by quantitative real-time polymerase chain reaction (see Supplementary Table 6). Cluster names are from the Gene Ontology "biological process" domain. Numbers in parentheses represent the number of individual genes in the cluster. The enrichment score is a measure of the significance of the gene group to the total gene list.

regulations in the spinal cord-CCI conditions as suggestive of true regulation.

Results of the rat and mouse multi-gene qPCR experiment are shown in Figs. 2 and 3 and Supplementary Tables 2 and 3. As can be seen, a large number of these genes were "confirmed" to be regulated in the direction seen in the original microarray studies in the rat-CCI-DRG condition, with 20 genes displaying >2-fold regulation and 43 genes showing statistically significant changes at $P < 0.05$ (and 20 genes displaying both >2-fold regulation and significance at $P < 0.05$). Although statistical significance could not be evaluated in the rat-CCI-spinal cord condition, a similar number of genes (15 vs 20) displayed >2-fold regulation. In all other conditions, however, far fewer genes were found with *either* fold-change or statistical evidence of regulation. Pearson correlations of fold-regulation levels in the rat are shown in Fig. 4. The only correlation surviving Bonferroni correction ($P = 0.05/4 = 0.0125$) was between DRG and spinal cord expression levels following CCI.

An unexpected finding was the greatly diminished number of statistically significant or >2-fold regulated genes after CCI in the mouse compared to the rat. Fig. 5 shows Pearson correlations of fold-regulation levels between rat and mouse. In fact, the raw correlations in the CCI conditions were significant, although not after Bonferroni correction.

3.3. Functional annotation clustering analyses

Functional clustering of genes based on Gene Ontology annotation was performed on lists of genes found to be regulated in 3 or more independent microarray experiments (177 genes), 4 or more independent microarray experiments (79 genes), and confirmed by subsequent qPCR analysis (rat DRG-CCI; 43 genes) using the Gene Functional Classification tool. The overall pattern of gene clusters by function demonstrated a particular enrichment of genes involved in immune function, cell structure and growth, and cell signaling, among others (Fig. 6). The lists of specific genes identified in these clusters can be found in Supplementary Tables 4–6.

3.4. Single-gene qPCR of Reg3b and Ccl2

The *Reg3b* and *Ccl2* genes were the only genes of the 79 showing reliable regulation across the 4 injury/tissue combinations in the rat (Fig. 3), and *Reg3b* additionally was upregulated >2-fold in both DRG and spinal cord after CCI in the mouse. To confirm these observations with proper hypothesis-testing procedures, we collected tissue in separate cohorts of rats and mice and performed qPCR on these 2 genes individually, using standard qPCR methods. As shown in Fig. 7, we largely confirmed the results of the multi-gene qPCR study with respect to these 2 genes. *Reg3b* was significantly upregulated (ie, fold-regulation significantly >1) in every condition except mouse-CFA, and *Ccl2* was significantly upregulated in every condition in the rat except CCI-DRG, but none in the mouse. Of interest is the extremely high correlation ($r = 0.91$, $P < 0.001$) between the fold-regulations in this experiment and those obtained in analogous conditions in the multi-gene qPCR study (Fig. 7c), strongly supporting the accuracy of the multi-gene study.

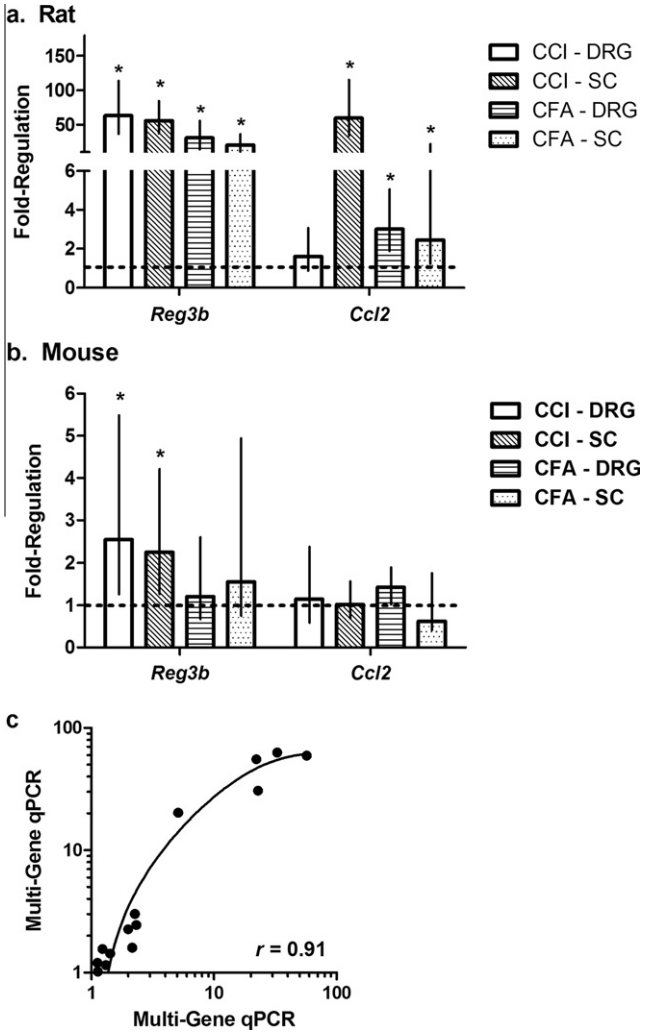

Fig. 7. Single-gene quantitative real-time polymerase chain reaction (qPCR) of *Reg3b* and *Ccl2* in a new cohort of rats (a) and mice (b). Bars represent average fold-regulation compared to control (sham surgery for chronic constriction injury [CCI] groups and saline injection for complete Freund's adjuvant [CFA] groups); note the difference in scale in the 2 graphs. Stems represent the 95% confidence interval over 3 biological replicates. The dashed horizontal line indicates no regulation by pain; statistical significance (*$P < 0.05$; conservatively defined) is achieved for stems not touching this line. Graph c shows the correlation of fold-regulations in the 18 conditions shown in graphs a and b with the analogous conditions in the multi-gene quantitative real-time polymerase chain reaction experiment. SC, spinal cord; DRG, dorsal root ganglion.

4. Discussion

Experimental use of high-density oligonucleotide microarrays has provided novel insights into the molecular mechanisms involved in the pathogenesis of chronic pain, in at least 2 cases leading to a successful prediction of genetic association in humans [11,50]. However, these insights do not come easily, as highly parallel analyses of gene expression produce large, cumbersome lists of regulated genes, and it remains difficult to tell true positives from false positives and establish both biological significance and generalizability of the implicated proteins.

We performed an analysis of unique microarray experiments from 20 published papers in order find genes that are repeatedly found to be regulated in a persistent pain state. We found a positive relationship between the incidence of significant regulation of a gene in independent microarray experiments and the level of existing "validation" of that gene/protein in the published literature. Of the 79 regulated genes that appeared in 4 or more independent microarray experiments, our meta-analysis found 41 genes with a prior evidence score of 2 or 3, establishing validity of both this analysis and the microarray profiling approach as a whole. Of particular interest to us was the appearance of *Atp1b3* (Na^+, K^+-ATPase, β_3 subunit) on the list, which we recently demonstrated via genetic haplotype mapping to play a role in mediating variability in pain behavior among mouse strains [26], despite the existence of no prior evidence whatsoever linking this protein to pain. With *Atp1b3* as an example, the 38 genes that have little to no prior validation are of potentially considerable import.

4.1. "Confirmation" of regulated genes via multi-gene qPCR

We found that when using DRG tissue from nerve-injured rats, over 50% of the regulations identified by the meta-analysis could be statistically confirmed in a de novo qPCR experiment. Although many of microarray studies used spinal cord tissue, we unfortunately lost one biological replicate due to technical error and could not evaluate our replication "success" in the spinal cord. However, the comparable number of genes displaying >2-fold regulation in the spinal cord compared to the DRG and the highly significant correlation between DRG and spinal cord regulations both suggest that many of these genes were likely "confirmed" in this tissue as well.

The inability to confirm gene regulations in all other conditions (rat CFA and *all* mouse conditions) is likely explained by the paucity of their representation in the original microarray studies. Only 2 of 20 studies (accounting for <3% of the unique genes considered) were performed on the mouse, and only 6 of 20 studies (accounting for <25% of the unique genes considered) used inflammatory stimuli. It is also likely that nerve damage causes true regulation of a larger number of genes than does inflammation.

It is important to note that our inability to "confirm" many of these gene regulations does not necessarily mean they are false positives. Obviously, none of the study designs from the original papers exactly match each other, nor do they match exactly the design of the multi-gene qPCR experiment. The data of Griffin and colleagues [17] are instructive in this respect: of 612 genes found to be significantly regulated by 3 surgical neuropathic injury models (CCI, spared nerve injury and spinal nerve ligation), only 54 genes (<9%) were commonly regulated by all 3.

Of course, many of the currently identified 79 regulations might indeed be false positives. Much has been said about the reliability and false-positive rates of these techniques from a statistical perspective [3,18,20], but far less is known about the error associated with actual biological differences across multiple experiments over time. Of interest perhaps is the comparison of genes identified by the current meta-analysis with the results of microarray studies published after the current study was conducted. Two studies of DRG expression in rats with surgical nerve injuries revealed a great deal of overlap: 15 genes on the current list were found to be significantly regulated by Vega-Avelaira et al. [54] (including *Reg3b* and *Ccl2*), and 51 genes by Maratou et al. [32]. A study of trigeminal ganglion mRNA expression after CFA revealed only 5 common genes [39]. Other more recent microarray studies couldn't be analyzed in this way because not all regulated genes were reported [41,60].

4.2. Specificity of gene regulation

Although very few individual genes were identified that were commonly regulated across the varying species, injury states, and tissues studied herein, the patterns of regulation observed overall were supportive of certain commonalities. Most notably, the overall correlation between gene regulation in the DRG vs spinal cord

(comparing all analogous rat and mouse conditions) was $r = 0.56$ ($P < 0.001$) (see also [55]). Not surprisingly, much evidence was uncovered for specificity of gene regulation as well. For example, some genes appeared to be neuropathic-specific (at least in the rat), including *Atf3*, *C1qb*, *Fcgr2b*, *Nefm*, *Tspo*, and *Sprr1al*. The clearest example was *Sprr1al* (small proline-rich protein 1a-like), with 20-fold upregulation after CCI but no evidence for upregulation after CFA. This gene, like the immediate-early gene, *Atf3*, is known to be involved in peripheral nerve regeneration [9,47]. Neuropathic-specific upregulation of cytoskeleton-related genes, such as *Nefm* (neurofilament, medium chain), has also been reported [45].

Perhaps the most striking dissociation is that the range of fold-regulations observed in the rat is considerably greater than that observed in the mouse (Fig. 5). Whether this points to species differences in homeostasis or simply to the fact that the list of genes examined was enriched in rat-relevant genes—and that a *different* list of mouse-relevant genes might be discovered—is not clear. Either possibility would represent a great challenge in comparative pain research.

4.3. Clusters of pain-regulated genes

Functional cluster analyses revealed the importance of gene clusters involved in cell signaling (eg, synaptic transmission, ion channels, G protein-coupled receptors), cell structure and growth (eg, intermediate filaments), and especially, immune system activity. Of particular note, a large number of genes involved in the complement cascade were regulated. Their involvement in pain has been previously demonstrated [17,22,52]; our analysis confirms the importance of these findings. Furthermore, the analysis identified a number of additional immune-related gene clusters involved in antigen presentation, cell adhesion, and antigen-mediated immunity. These findings serve to emphasize the importance of the immune system in peripheral neuropathic and inflammatory pain. Finally, a number of relatively novel gene clusters were identified by our functional analyses, including ATPase activity (see [26]), metal ion homeostasis, and protein tyrosine phosphatases.

4.4. Reg3b and Ccl2 as putative biomarkers of pain

In light of the profound dissociations by injury and species revealed by the multi-gene qPCR experiment, it is particularly noteworthy that 2 genes—*Reg3b* and *Ccl2*—showed regulation by pain generalizing across injury, tissue, and (for *Reg3b*) species. Genes showing somewhat less robust generalization include *Atf3*, *C1qc, C4a, RT1-Dmb1*, and *Vgf*.

Reg3b, part of a family of "regenerating genes" originally identified in regenerating pancreatic islet cells [51], encodes a secretory protein most commonly called pancreatitis-associated protein (PAP; also known as PAP1, HIP/PAP, Reg-2, peptide 23 and Reg-IIIβ), as it is expressed in this inflammatory condition [21]. PAP is massively upregulated in motor neurons and some sensory neurons (including IB4-positive cells in the DRG) after nerve injury [4,19,30]. Suggesting a role beyond mitogenesis, and increasing the relevance to pain, are the recent demonstrations that PAP is upregulated in the DRG after CFA [5,19] and cyclophosphamide [49] administration in rats. To date, there is no direct causal evidence linking *Reg3b*/PAP to pain.

On the other hand, *Ccl2*, encoding the chemokine (C-C motif) ligand 2 (CCL2)—commonly known as monocyte chemoattractant protein-1 (MCP-1)—has with its receptor CCR2 a well-studied role in pain processing, especially in neuropathic pain (see [1,14]). Upregulation in the DRG by nerve injury and inflammation at the mRNA and protein level, in both neurons and glial cells, has been demonstrated multiple times, but evidence at the spinal cord level is more controversial [14]. Although CCL2 involvement in pain in the mouse has been amply demonstrated via transgenics [33], to our knowledge, only 2 studies have demonstrated upregulation of *Ccl2* after common neuropathic injuries [13,15] in this species, and none after inflammatory injury. The rat vs mouse difference seen here might relate to differences in the timing of *Ccl2* regulation: in a study of DRG expression after CCI in rats, the peak upregulation was seen at postoperative day 7–14 (the latter being the time point employed here) [61], whereas in a recent study of DRG expression after CCI in mice, the peak upregulation was seen on postoperative day 1 [13].

Intriguingly, for both genes, evidence exists—in humans, no less—that protein levels in accessible tissues and body fluids are increased in inflammatory disorders that often feature pain [21,31,37,38,40,42]. In one case, a significant correlation between urinary PAP levels and bladder pain in patients with interstitial cystitis was demonstrated [31], but of course it remains unclear whether this was directly related to pain intensity or disease severity. Overall, in light of the present findings, CCL2 and especially REG3B/PAP ought to be further considered as potential biomarkers of chronic pain.

Conflict of interest statement

None of the authors have financial or other relationships that might lead to a conflict of interest in the study.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pain.2011.04.014.

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