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Harnessing pain heterogeneity and RNA transcriptome to identify blood–based pain biomarkers: a novel correlational study design and bioinformatics approach in a graded chronic constriction injury model

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Abstract

A quantitative, peripherally accessible biomarker for neuropathic pain has great potential to improve clinical outcomes. Based on the premise that peripheral and central immunity contribute to neuropathic pain mechanisms, we hypothesized that biomarkers could be identified from the whole blood of adult male rats, by integrating graded chronic constriction injury (CCI), ipsilateral lumbar dorsal quadrant (iLDQ) and whole blood transcriptomes, and pathway analysis with pain behavior. Correlational bioinformatics identified a range of putative biomarker genes for allodynia intensity, many encoding for proteins with a recognized role in immune/nociceptive mechanisms. A selection of these genes was validated in a separate replication study. Pathway analysis of the iLDQ transcriptome identified Fcγ and Fcε signaling pathways, among others. This study is the first to employ the whole blood transcriptome to identify pain biomarker panels. The novel correlational bioinformatics, developed here, selected such putative biomarkers based on a correlation with pain behavior and formation of signaling pathways with iLDQ genes. Future studies may demonstrate the predictive ability of these biomarker genes across other models and additional variables.

Keywords: allodynia, dorsal horn, Fc receptor, microarray, neuropathic pain, pathway analysis.

J. Neurochem. (2012) 10.1111/j.1471-4159.2012.07833.x

Neuropathic pain may arise as a consequence of a lesion or disease affecting the somatosensory system (Jensen *et al.* 2011). Such pain is a major health problem that afflicts up to 8% of the general European population (Torrance *et al.* 2006; Bouhassira *et al.* 2008), substantially reduces quality of life for the afflicted individual and poses a significant economic burden to the health system and society. Symptoms may include hypersensitivity to noxious (hyperalgesia) and non-

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noxious (allodynia) stimuli, and spontaneous pain. Neuropathic pain presents challenges for diagnosis and treatment selection given its heterogeneity with respect to pain intensity, disease mechanisms, and treatment responses (Mogil *et al.* 1999a,b; Woolf and Decosterd 1999; Woolf and Mannion 1999; Finnerup and Jensen 2006; Costigan *et al.* 2009b; von Hehn *et al.* 2012), which are all incompletely understood.

The problems posed by disease heterogeneity are not unique to neuropathic pain. However, other fields facing such difficulties, like oncology, have addressed these clinical challenges through the development of biomarkers, defined by the NIH Biomarkers Working Group (2001) as 'a characteristic that can be measured and evaluated as an indicator of normal biologic processes, pathologic processes or pharmacologic responses to therapeutic intervention'. Biomarkers should be distinguished from surrogate endpoints, for which stringent qualification is required to enable the surrogate to substitute for a clinical endpoint (NIH Biomarkers Working Group 2001). Biomarker development for neuropathic pain has had some potential leads in genetics (Foulkes and Wood 2008), quantification of primary afferent activity (Chizh and Hobson 2007), sampling the cerebrospinal fluid (Uceyler *et al.* 2007; Zin *et al.* 2010), and imaging of the pain matrix (Chizh and Hobson 2007; Tracey and Mantyh 2007), and more recently via pain phenotyping (von Hehn *et al.* 2012), but none are well accepted as objective biomarkers at present. An objective measure of pain intensity may prove a useful adjunct to the visual analogue scale and other subjective measures of pain reporting in a clinical scenario, to overcome the difficulties associated with inter-individual differences in reporting experiences or using rating scales, and when a subjective report cannot be solely relied upon (e.g. compensation claims, inability to communicate). An objective measure of pain intensity may be useful to assess analgesic efficacy during drug development, where a notoriously high placebo response and Type II error in clinical trials in pain is recognized as the single biggest hurdle in assessing drug efficacy (Dworkin 2011).

In fields such as oncology, the most recent advances in biomarker discovery and development have used transcriptome profiling of the blood as a window into disease processes that are not always easily accessible (Hanash *et al.* 2011). However, with regards to neuropathic pain, the RNA transcriptome has not been applied to biomarker screening in the blood or at any other site, but rather to mechanistic studies in rodent neuropathic pain models at the peripheral nerve injury site, dorsal root ganglia, and spinal cord (Lacroix-Fralish *et al.* 2011). Transcriptome analyses of the blood are likely to show promise for neuropathic pain, given the recognition that peripheral immune cells contribute to pain hypersensitivity at both peripheral and central sites in rodent models of nerve injury (Austin and Moalem-Taylor 2010; Grace *et al.* 2011a,b).

Therefore, we aimed to test whether the whole blood transcriptome could identify genes that would correlate with heterogeneity in mechanical allodynia intensity in the peripheral nerve-injured adult male rat, at a single timepoint. Specifically, regarding the NIH biomarker definition (NIH, Biomarkers Working Group 2001), such an approach aimed to identify a potential measurement of the pathological pain process, namely mechanical allodynia intensity. If so, this approach may provide a useful strategy for future explorations of the broad applicability of such putative biomarkers for pre-clinical neuropathic pain assessment, neuropathic pain in general, and whether the whole blood transcriptome may prove useful for clinical evaluations. A key element in this novel approach is pain heterogeneity, as the shortcoming in virtually all the studies reviewed by Lacroix-Fralish *et al.* (2011), and in the field at large, is that means are compared between a largely homogeneous neuropathic pain group and a sham control. This simple pain versus no pain comparison of group means is not sufficient to discover biomarkers of pain intensity, as it is unable to differentiate whether degree of gene expression is related to the nature of the nerve injury and surrounding graded sequelae or the intensity of consequent pain behavior. Alternatively, by individually pairing the degree of allodynia with the respective gene expression from each rat, the variability in behavior can be powerfully harnessed to elucidate the gene expression strongly correlated with allodynia. Persson *et al.* (2009) correlated gene expression with the contrasting pain response phenotypes of different mouse strains, but this design is limited by other potential genetic differences between the strains. In contrast, our graded chronic constriction injury (CCI) model (Grace *et al.* 2010) presents an opportunity to intentionally generate a graded pain population that is not confounded by such genetic differences, allowing correlational investigation of transcriptional changes. The graded approach also has superior face validity, as it is consistent with the variable spectrum of clinical pain.

Therefore, we hypothesized that biomarkers of pain intensity, related to graded CCI, could be identified in peripheral blood, by correlating the whole blood transcriptome with pain behavior. Given the absence of prior correlational studies, new analyses were developed using Pathway Analysis to refine the gene list to a size suitable for testing in a validation group, so to enable future studies examining parameters beyond those tested here. To focus such analyses, the whole blood transcriptome was analyzed together with the transcriptome of the dorsal quadrant of the lumbar spinal cord, ipsilateral lumbar dorsal quadrant to sciatic injury (iLDQ). The inclusion of these conjoint whole blood and iLDQ analyses was based on the premise that signaling pathways of both tissues contributed to allodynia induced by sciatic nerve injury. Although it could be argued that the injured peripheral nerve or the dorsal root ganglia

should also be included, given the prominent role of peripheral immune cells at these sites (Austin and Moalem-Taylor 2010), the iLDQ alone was selected at this time owing to the success of a recent transcriptome study in identifying peripheral immune contributions at this region (Costigan *et al.* 2009a). By analyzing the whole blood and iLDQ transcriptomes together, it was hypothesized that putative biomarkers identified in the whole blood would have a closer relationship with the mechanisms contributing to allodynia, than a simple correlation between the whole blood transcriptome and pain behavior. Thus, this study provides a new analytical approach that, based on the results to be reported here, bears future testing and investigation.

Materials and methods

Given the absence of prior correlational transcriptome studies, a new data pipeline was developed (summarized in Fig. 1). Development of the correlational data pipeline, detailed here, required the generation of data that were used to justify progression to the next step. Therefore, a significant proportion of the manuscript is dedicated to methods development to arrive at a list of putative blood-based biomarkers.

Subjects

Pathogen-free adult male Sprague-Dawley rats were used for the transcriptome study (300–350 g; Animal Resource Centre, Perth, WA, Australia; $n = 10$) and the prospective validation study (300–350 g; Harlan Laboratories, Madison, WI, USA; $n = 12$). Rats were housed in temperature- (18–21°C) and light-controlled (12 h light: dark cycle; lights on at 07:00 hours) rooms with standard rodent food and water available *ad libitum* and allowed to habituate to the holding facility for 1 week prior to experimentation. The transcriptome study was performed at the University of Adelaide, Australia, and all relevant procedures were approved by the University of Adelaide Animal Ethics Committee. The prospective validation study was performed at the University of Colorado at Boulder, USA, giving strength to translation of results across disparate research sites, and all relevant procedures were approved by the Institutional Animal Care and Use Committee of the University of Colorado at Boulder. Both studies were conducted in accordance with the ARRIVE guidelines and the NHMRC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Surgery

The CCI model of graded sciatic nerve injury was performed aseptically at the mid-thigh level of the left hindleg, as previously described, so to create a graded intensity of pain behavior (Grace *et al.* 2010). Briefly, animals were anesthetized with isoflurane, the skin of the hindquarters was shaved and the sciatic nerve gently elevated. Zero, 1, or 4 sterile chromic gut sutures (cuticular 4-0 chromic gut; Ethicon, Somerville, NJ, USA) were loosely tied around the isolated sciatic nerve (N; approximately 3–4 mm in length). Once the superficial muscle overlying the nerve was sutured with silk, and prior to surgical stapling of the skin incision, additional equal lengths of chromic gut were placed subcutaneously (S; approximately 3–4 mm in length), such that each animal was

exposed to four equal lengths of chromic gut in total. Thus, the treatment groups (n = transcriptome, prospective validation studies), differing in pain behavior, were: sham (N0S0; $n = 2, 3$), low pain (N0S4; $n = 2, 3$); medium pain (N1S3; $n = 3$); and high pain (N4S0; $n = 3$).

Mechanical allodynia

Testing was conducted blind with respect to group assignment. Rats received at least three 60 min habituations to the test environment prior to behavioral testing. The von Frey test (Chaplan *et al.* 1994) was performed within the sciatic innervation region of the hindpaws as previously described in detail (Chacur *et al.* 2001; Milligan *et al.* 2001). Assessments were made prior to (baseline) and at post-operative days 3, 10, and 21. A logarithmic series of 10 calibrated Semmes-Weinstein monofilaments (von Frey hairs; Stoelting, Wood Dale, IL, USA) were applied randomly to the left and right hindpaws to define the threshold stimulus intensity required to elicit a paw withdrawal response. Log stiffness of the hairs was determined by $\log_{10}$ (milligrams $\times$ 10) and ranged from manufacturer designated 3.61 (0.407 g) to 5.18 (15.136 g) filaments. The behavioral responses were used to calculate absolute threshold (the 50% paw withdrawal threshold) by fitting a Gaussian integral psychometric function using a maximum-likelihood fitting method (Harvey 1986; Treutwein and Strasburger 1999), as described previously (Milligan *et al.* 2000, 2001). This fitting method allows parametric analyses that otherwise would not be statistically appropriate (Milligan *et al.* 2000, 2001). A reduction in von Frey threshold was interpreted as an increase in allodynia.

Tissue preparation, RNA extraction, chip hybridization, and PCR

Transcriptome study

Following final behavioral testing on day 21, the rats were anesthetized with sodium pentobarbital and circulating blood collected via cardiac puncture into RNAprotect Animal Blood Tubes (QIAGEN, Doncaster, VIC, Australia), and stored at −80°C. Following transcardial perfusion with isotonic saline, the L4-L6 segment of spinal cord (innervating the sciatic nerve) was carefully excised and the iLDQ was dissected, rapidly frozen in liquid nitrogen, and stored at −80°C.

Total RNA (including microRNA) was purified from blood using a RNeasy Protect Animal Blood Kit (QIAGEN), as directed by the manufacturer. Spinal cord tissue was disrupted over dry ice in an RNase-free (DEPC treated) loose fitting glass homogenizer. Total RNA (including microRNA) was purified using a miRNeasy kit (QIAGEN), according to the manufacturer's instructions. The RNA quality was assessed using the Agilent 2100 Bioanalyzer (Santa Clara, CA, USA) and RNA integrity numbers were > 7 for all samples.

cDNA was reverse transcribed from the total RNA and hybridized to the Affymetrix Rat Gene 1.0 ST Array (Santa Clara), according to the manufacturer's instructions. Array intensity was quantified using the Affymetrix GeneChip 3000 7G scanner (Santa Clara) by the Adelaide Microarray Centre (SA, Australia).

Prospective validation study

Following final behavioral testing on day 21, the rats were anesthetized with sodium pentobarbital and 100 μL of circulating blood collected via cardiac puncture into tubes containing 1 mL of

TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) for immediate processing. Total RNA was purified from whole blood using the standard phenol:chloroform extraction with TRIzol Reagent (Invitrogen) according to the manufacturer's instructions, and stored at −80°C.

Primer sequences were obtained from Genbank at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov; Table 1). cDNA was reverse transcribed from the total RNA and amplification performed using Quantitect SYBR Green PCR kit (QIAGEN, Valenica, CA, USA) in iCycler iQ 96-well PCR plates (Bio-Rad, Hercules, CA, USA) on a MyiQ single Color Real-Time PCR Detection System (Bio-Rad). The reaction mixture (26 μL) was composed of QuantiTect SYBR Green (containing fluorescent dye SYBR Green I, 2.5 mM $MgCl_2$, dNTP mix, and Hotstart Taq Polymerase), 10 nM fluorescein, 500 nM of each forward and reverse primer (Invitrogen), nuclease-free water, and 1 μL of cDNA from each sample. Each sample was measured in duplicate. The reactions were initiated with a hotstart at 95°C for 25 min, followed by 40 cycles of 15 s at 94°C (denaturation), 30 s at 55–65°C (annealing), and 30 s at 72°C (extension). Melt curve analyses were conducted to assess uniformity of product formation, primer-dimer formation, and amplification of non-specific products. The PCR product was monitored in real-time, using the SYBR Green I fluorescence, using the MyiQ single Color Real-Time PCR Detection System (Bio-Rad). Threshold for detection of PCR product was set in the log-linear phase of amplification and the threshold cycle (C_T) was determined for each reaction. The level of the target mRNA was quantified relative to the housekeeping gene (β-actin). The expression of β-actin was not significantly different between samples, suggesting that differences were likely caused by true changes in mRNA expression, rather than reflecting changes in cell number per sample.

Data pipeline and analyses

Data pre-processing: correlational pathway analysis of whole blood and iLDQ genes using 'Adelaide plots'
Array CEL files (Affymetrix) for the whole blood and iLDQ transcriptomes were normalized to a set of manufacturer designated control genes, that were unchanged between treatment groups, to ensure that differences were caused by true changes in mRNA expression, rather than reflecting changes in cell number per sample.

Probes without signal significantly above background were eliminated. Following this, we departed from traditional pre-processing of microarray data that aims to identify significant differential gene expression between treatment group means (Ko *et al.* 2002; Yang *et al.* 2004; LaCroix-Fralish *et al.* 2006a; Lacroix-Fralish *et al.* 2006b; Griffin *et al.* 2007; Costigan *et al.* 2009a; Persson *et al.* 2009; Vega-Avelaira *et al.* 2009). Instead, independent Pearson correlations were performed between von Frey threshold and gene expression for the whole blood and iLDQ transcriptomes, which therefore accounted for the variability in behavior and were independent of treatment group (see Fig. 2c as example). The resulting gene list, consisting of 14 825 whole blood and 14 099 iLDQ genes, had correlation coefficients continuously ranging from $|0.01|$ to $|0.91|$ for the whole blood, and $|0.01|$ to $|0.94|$ for the iLDQ. Pathway Analysis programs are unable to deal with datasets of this size, and open source software are unable to weight genes, using the correlation coefficient, for example. Traditional data pre-processing addresses the problem of data reduction by selecting genes that are significantly differentially regulated between the treatment group means. In contrast, the continuous range of 14 825 or 14 099 correlations essentially posed a sorites paradox (Hyde 2011); that is, at what point along this continuous range of correlations does one define the breakpoint between meaningful and non-meaningful associations? The solution to this problem required that a threshold be determined to split the dataset according to a true association with allodynia. Such a threshold was identified by developing an analysis that was based on two premises: that the significance of a particular gene cannot be understood in isolation but only in the context of gene networks and signaling pathways (Kitano 2002), and that the genes most strongly correlated with allodynia would contain the signaling pathways relevant to allodynia.

Therefore, genes from the whole blood and iLDQ transcriptomes from each rat were independently ranked according to the absolute correlation coefficient (RAACC) between gene expression and von Frey threshold (hereafter referred to as the RAACC Gene list; Fig. 1). Beginning at the top (i.e. the highest correlation coefficient) of the RAACC Gene lists, bins were defined across the entire 14 825 whole blood gene and 14 099 iLDQ gene transcriptomes by selecting the top 1000 genes and then progressively frame-shifting downward by units of 100 genes (such that bin 1 contained genes

Table 1 PCR primer specifications

Gene	Primer sequence (5′-3′)	GenBank Accession No.
Tnfrsf11b	F: GGAATAGATGTCACCCTGTG R: CGAGCTGTGTCTCCGTTTTA	NM_012870.2
Thra	F: TTCCTGCCGGATGACATTGG R: AAGGCAGCTCGGAGAACATG	NM_001017960.1
Avpr1a	F: TTGTGTCAGCAGCGTGAAGA R: GGTTTTCTGAATCGGTCCAG	NC_005106
Csf3	F: CAGCTGTGTGCCACCTACAA R: TCAGGCACTTTGTCTGCTGC	NC_005109.2
Xylb	F: TAACATGGAGGTCTCGGCAT R: CTTAGGCATGATTCGGTAGC	NC_005107
Olr952	F: TCTACTGTCCACGTTGGCAT R: TCATGTGAGAGGAACAGGTG	NC_005106
Hook1	F: AGCAAAGGTTGAGCAAAGAG R: GCTGGAACTTTCACCTTCAG	NC_005104
Trim30	F: TGTCCTGAGAAGGAGTCAGA R: TCCATGAGCCCTTCAAATGC	NC_005100
Cx3cl1	F: TGTACTCTGCTGGCGGGTCA R: TCGTCTCCAGGATGATGGCG	NC_005118
Irf2bp1	F: CTGGTGACATCAAGGTCAAG R: GGGTGGGGTAGGGAATAATT	NC_005100
LOC686781	F: TACAGAGGGATCTGAGGCCA R: AGCGCATCTCCATGCTCTGA	NC_005100
β-actin	F: TTCCTTCCTGGGTATGGAAT R: GAGGAGCAATGATCTTGATC	NM_031144.2

1–1000, bin 2 contained genes 101–1100, and so forth), so that each subsequent bin progressively excluded the prior 100 top ranked genes. This was done, as noted above, because pathway analysis programs are unable to handle such large datasets; hence, binning in this manner created a means for systematic analysis without losing data. Each 1000-gene bin was analyzed in DAVID 6.7 Pathway Analysis software (http://david.abcc.ncifcrf.gov) (Huang da *et al.* 2009a,b), which classified the binned genes into signaling pathways, based on the Kyoto Encyclopedia of Genes and Genomes database. To identify where the threshold between meaningful and non-meaningful correlations lay, it was necessary to determine the number of pathways that would be identified by chance using this approach. To do this, 1000 genes were randomly re-sampled from the iLDQ transcriptome and analyzed in DAVID 6.7 ten times. Subjecting these 10 bins of 1000 random genes to the DAVID 6.7 pathway analysis yielded an average of 1.8 pathways. Thus, as the bins created from the entire transcriptomes were progressively analyzed from bin 1 (highest correlations), the threshold was inferred at point at which the pathway number per bin fell below that expected by chance (≤ 1.8), that is the excluded genes (i.e. the preceding RAACC genes that were excluded from the bin) were the major contributors to signaling pathways related to allodynia.

The number of pathways classified from each bin by DAVID 6.7 was quantified for the binned iLDQ- and whole blood-RAACC Gene lists (Fig. 1). Within the iLDQ-RAACC Gene list (Fig. 1), bins 1 through 3 yielded between 12 and 21 pathways, but beyond these bins (i.e. beyond the top 300 genes), ≤ 2 pathways were identified per bin, suggesting that the top 300 genes (corresponding to $r > |0.7|$) were the major contributors to signaling pathways related to allodynia. Binned analysis of the whole blood-RAACC Gene list (Fig. 1) did not identify such an unequivocal threshold. Bins 1 through 5 yielded between 0 and 3 pathways, which inconsistently exceeded the number of pathways expected by chance. Beyond bin 5 (i.e. beyond the top 500 genes), ≤ 1 pathway per bin was identified, suggesting that the top 500 genes (corresponding to $r > |0.7|$) made only a meager contribution to the signaling pathways related to allodynia. Such a result is to be expected, given that the factors in the blood contributing to pain pathophysiology probably represent only a very small proportion of the total compartment.

It was hypothesized that the meager organization of the top 500 genes from the whole blood-RAACC Gene list (Fig. 1) into signaling pathways could be improved, if analyzed with genes from the iLDQ, given that peripheral immunity has a recognized contribution to neuropathic pain pathophysiology within the CNS (Grace *et al.* 2011a,b). Therefore, the top 500 whole blood and 300 iLDQ genes from the respective RAACC Gene lists were merged to form an 800 gene combination list. To determine how many genes in the combination list might be false positives (Type I errors), the following analysis was undertaken: the whole blood- (14 825 genes) and iLDQ- (14 099 genes) RAACC Gene lists were combined and the binned analysis performed. As hypothesized, merging the whole blood- and iLDQ-RAACC gene lists resulted in an increased number of bins that yielded signaling pathways (exceeding the number expected by chance), beyond either the binned analysis of the whole blood or iLDQ in isolation. Bins 1 through 7 yielded between 2 and 23 pathways, but beyond these bins (i.e. beyond the top 700 genes), ≤ 1 pathways were identified per bin, suggesting

that the top 700 genes were the major contributors to signaling pathways related to allodynia. This result indicated that when the top 500 whole blood and 300 iLDQ gene lists were merged to form an 800-gene combination list, ~ 100 genes were unlikely to be related to the severity of allodynia. Thus, the whole blood genes contributing to signaling pathways may be better defined when interpreted together with the iLDQ genes in this form of binned analysis. However, it was posited that the potential Type I errors present in the 800-gene list would be adequately eliminated through the identification of common pairs (Putative biomarker panel 1) or pathway derived gene list (Putative biomarker panel 2), thus the full combined 800-gene list was taken forward for further data refinement. It should be noted that 7 genes derived from both the iLDQ and whole blood transcriptomes are duplicated in the combination list (800 genes), and these are used to generate Putative biomarker panel 1: direct whole blood-iLDQ correlates (see results). As the combination list is eventually analyzed in DAVID 6.7, where duplicated genes are only counted once, any overlap is inconsequential.

The graphical representation of these binned analyses, depicted in Fig. 3, demonstrate that the most highly correlated RAACC genes are highly organized in functional signaling pathways, before abruptly diminishing. We are naming these new analyses 'Adelaide plots', akin to a 'Manhattan plot', because of the related function and etymological basis. Thus, the whole blood (500 genes), iLDQ (300 genes), and combination (800 genes) gene lists generated here are referred to as 'Adelaide Gene lists' (Fig. 1).

Data pre-processing: traditional differential expression of iLDQ genes between treatment groups

We next determined whether the new Adelaide plot analysis was better matched to our study design than a traditional analysis. As the iLDQ transcriptome would contain genes that were more closely related to allodynia than the whole blood, the iLDQ was selected to afford the differential analysis the best possible chance of success.

Microarray data processing was performed in the freely available statistical programming and graphics environment R (http://cran.r-project.org), with open source software available through Bioconductor project (http://www.bioconductor.org). Background subtraction was performed with RMA (Irizarry *et al.* 2003) and followed with quantile normalization, both implemented in affy library. Differential expression was determined with limma (Smyth 2005), using a linear model to compare all treatment groups with each other and isolate changes specific to iLDQ samples. Statistical significance of the results was low, as could be expected in an experiment with very few replicates in each treatment group.

In view of the large number of Type I errors that would be inevitably present in any selection of potentially regulated genes, functional analysis was undertaken to identify those with high likelihood of being associated with nerve injury. Forty-six named genes with $p < 0.05$, unadjusted for multiple testing, were identified. Gene networks were constructed in MetaCore (http://www.genego.com) and 22 of the initial 46 genes were found to have interconnecting relationships. The low number of significant iLDQ genes (i.e. significantly differentially regulated genes with interconnecting relationships) is unsurprising given the low number of replicates in each group, in contrast to the larger groups sizes that are typically required to overcome within-group variability. However, our

approach suggests that when the variability is used as an endpoint, additional information can be gleaned with smaller group sizes.

Putative biomarker panel 1: direct whole blood-iLDQ correlates
The iLDQ- (300 genes) and whole blood- (500 genes) Adelaide Gene lists (Fig. 1) were compared. Genes common to both lists were identified (Common pairs identified, Fig. 1) and the magnitude of gene expression correlated between each whole blood-iLDQ pair, to quantify the reliability of gene expression in the blood to mirror that in the iLDQ.

Putative biomarker panel 2: contribution of whole blood genes to iLDQ signaling pathways
The iLDQ- (300 genes) and combination- (800 genes) Adelaide Gene lists were analyzed in DAVID 6.7. When the genes contributing to these pathways were identified, it was found that only 77 of the original 300 iLDQ genes and 106 of the original 800 combination genes contributed to the signaling pathways defined using DAVID 6.7. This resulted from the fact that 1 gene may contribute to multiple pathways. For example, in the iLDQ-Adelaide Gene list 77 of 300 genes generated 19 pathways, and the other 223 genes did not contribute. Therefore, the 77 and 106 genes noted above were selected for further analysis and referred to as 'Pathway derived Gene lists' (Fig. 1). The iLDQ genes (77 genes) that contributed to the combination Pathway derived gene list (106 genes) were then removed, leaving 29 whole blood genes that were both correlated with allodynia and also formed signaling pathways with iLDQ genes.

Prospective validation study
To validate the putative biomarkers identified via our novel data pipeline, a prospective validation study was performed in which a separate group of graded CCI rats were generated in different laboratory and whole blood collected (see 'Prospective validation study' above). Real-time PCR was performed on a selection of the top correlation coefficient ranked whole blood genes from each potential biomarker panel in Fig. 1: the whole blood-RAACC Gene list (top 2 genes); the 7 gene candidate biomarkers panel (top 2 genes); the 29 candidate biomarkers panel (top 4 genes); and 2 genes not correlated with von Frey threshold (r < 0.01) from the whole blood-RAACC Gene list. The derivation method of the 29 candidate biomarkers panel directly tested the study hypothesis, which if proved true, would increase the potential of the panel to yield suitable biomarkers. Therefore, a greater number of genes were selected from the 29 candidate biomarkers panel to subject the panel to more rigorous validation testing. In addition, *Cx3cl1* [Entrez ID:89808] was also analyzed using real-time PCR because of the unique correlation with allodynia and its cognate receptor expression in the iLDQ (see Results). A Pearson correlation was performed between the magnitude of gene expression (relative to housekeeping gene) and von Frey threshold, recorded immediately prior to tissue collection at day 21 post surgery. The coefficient of variance (% CV) was determined between the pipeline and prospective correlation coefficients.

Distribution of published 'pain genes' within the iLDQ transcriptome dataset
The 39 genes that were identified as having 'strong correlational' or 'causational' evidential links with rodent neuropathic pain models in the recent meta-analysis by Lacroix-Fralish *et al.* (2011) were identified within the iLDQ transcriptome dataset. The relevant correlation coefficient was paired with each gene, and the 'pain genes' dataset was then split according to the concordance (23 genes; Table S2) or discordance (16 genes; Table S3) between the direction of correlation in this study and the published direction of gene regulation (Lacroix-Fralish *et al.* 2011). The data were binned into correlation coefficient tertiles and the distribution frequency plotted.

Results

The data pipeline used to identify the putative pain biomarkers is summarized in Fig. 1.

Mechanical allodynia
Behavior was quantified at 21 days post graded CCI surgery for both the transcriptome and prospective validation studies (Fig. 2). The degree of allodynia correlated with the graded CCI treatment group (transcriptome study: Pearson $r = -0.99$ [95% CI: -0.99, -0.50]; prospective validation study: Pearson $r = -0.99$ [95% CI: -1.00, -0.98]), similar to that found previously (Grace *et al.* 2010, 2011a). Whereas the experimental design employed different treatment groups, once graded allodynia had been achieved at day 21, all animals were treated as one heterogeneous group (transcriptome study: $n = 10$; prospective validation study: $n = 12$) for correlational analyses (e.g. Fig. 2c).

Putative biomarker panel 1: direct whole blood–iLDQ correlates
After derivation of Adelaide plots (Fig. 3), Adelaide Gene lists (Fig. 1) were determined for whole blood (500 genes) and iLDQ (300 genes). Following comparison of these lists, 7 genes were identified as common to both. These genes are ranked according to the correlation coefficient in Table 2, indicating the ability of gene expression in the blood to mirror that in the iLDQ, and are presented together with references to link the protein encoded by each gene with nociceptive hypersensitivity and immune signaling.

Putative biomarker panel 2: contribution of whole blood genes to iLDQ signaling pathways
Pathway derived Gene lists (Fig. 1) were generated by analyzing the iLDQ- (300 genes) and combination- (800 genes) Adelaide Gene lists (Fig. 1) in DAVID 6.7. The genes of the iLDQ-Pathway derived Gene list (77 genes) were removed from the combination-Pathway derived Gene list (106 genes). Thus, the 29 remaining whole blood genes were those that formed signaling pathways with iLDQ genes (Fig. 4), and were correlated with the severity of allodynia. These genes are ranked in Table 3 according to the correlation coefficient, and are presented together with references to link the protein encoded by each gene with nociceptive hypersensitivity and immune signaling.
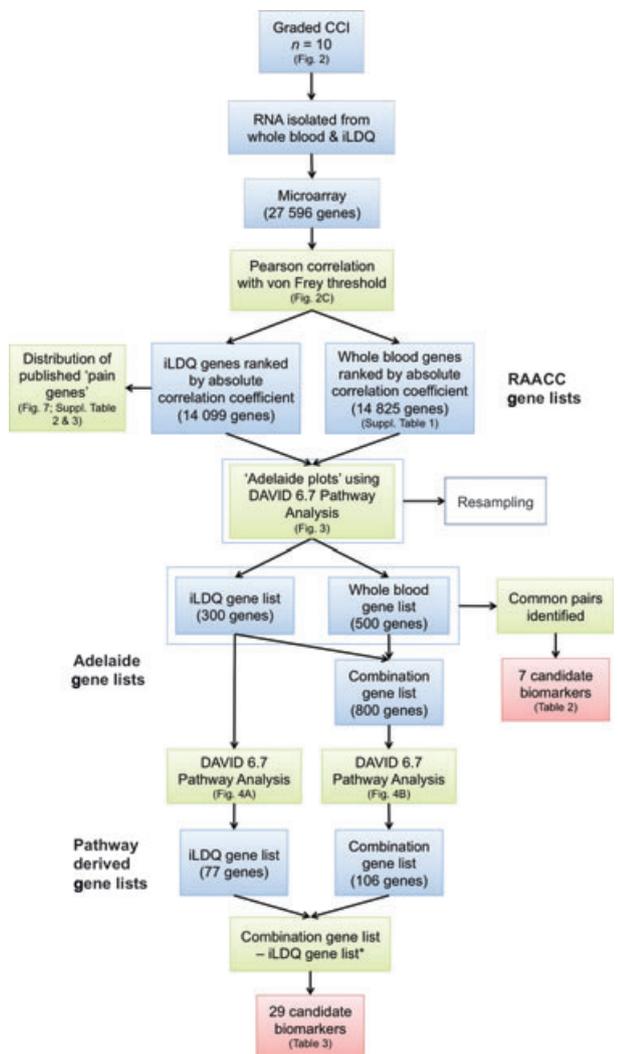
Fig. 1 Transcriptome data pipeline developed and employed to identify putative biomarker panels. Red boxes represent putative biomarker panels; blue boxes represent experimental steps and results; green boxes represent bioinformatic steps; white boxes represent supplementary analyses. CCI, chronic constriction injury; iLDQ, ipsilateral lumbar dorsal quadrant; suppl, supplementary. *Genes common to both the iLDQ and whole blood gene lists were retained.

Fig. 2 Graded chronic constriction injury of the sciatic nerve produces graded allodynia at day 21 post surgery. Graded neuropathy was induced by varying the distribution of four equivalent chromic gut pieces across the nerve (N) or subcutaneous (S) compartments. As such, treatment design was N0S0 (transcriptome study, $n = 2$ prospective validation study, $n = 3$), N0S4 ($n = 2, 3$), N1S3 ($n = 3$), and N4S0 ($n = 3$) to generate a single, heterogeneous group with respect to allodynia for correlational analysis in (a) the transcriptome study and; (b) the prospective validation study. (c) As an example, the correlation between ipsilateral lumbar dorsal quadrant *P2ry13* gene expression and von Frey threshold is presented. Each point represents an individual rat.

Many of the 29 putative whole blood biomarker genes encode for proteins that form ligand-receptor pairings in the CNS. Of each potential pairing, the only ligand or receptor genes from the 29 putative whole blood biomarker genes to form cognate pairs with genes in the iLDQ-Pathway derived Gene list (77 genes; Fig. 1) were *Csf3r* [Entrez ID:298518] and *Cx3cr1* [Entrez ID:171056]. When the cognate pairs were correlated, an insignificant inverse relationship was found between whole blood *Csf3* [Entrez ID:25610] and iLDQ *Csf3r* ($r = -0.47$, $p = 0.17$; Fig. 5a), but a significant correlation was found between whole blood *Cx3cl1* and iLDQ *Cx3cr1* ($r = 0.84$, $p = 0.003$; Fig. 5b).
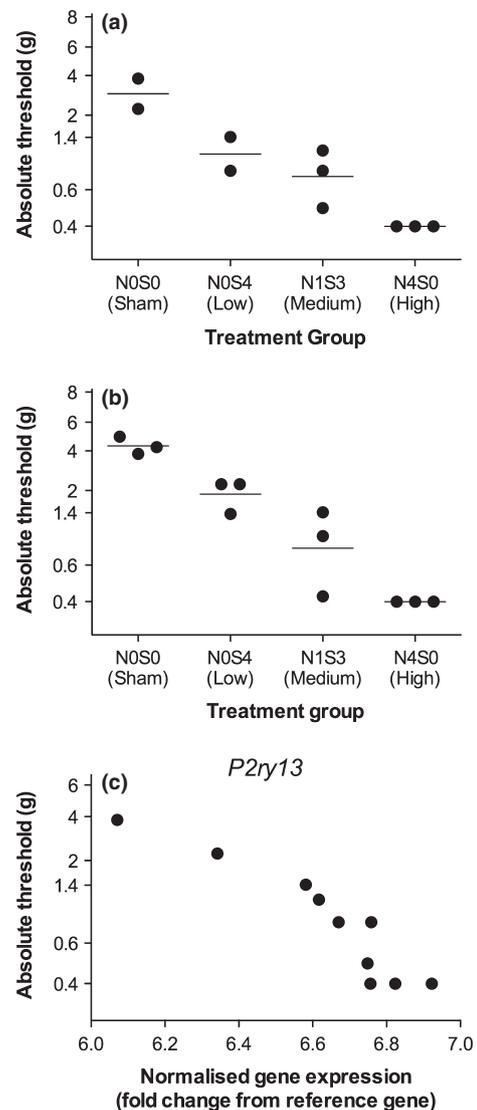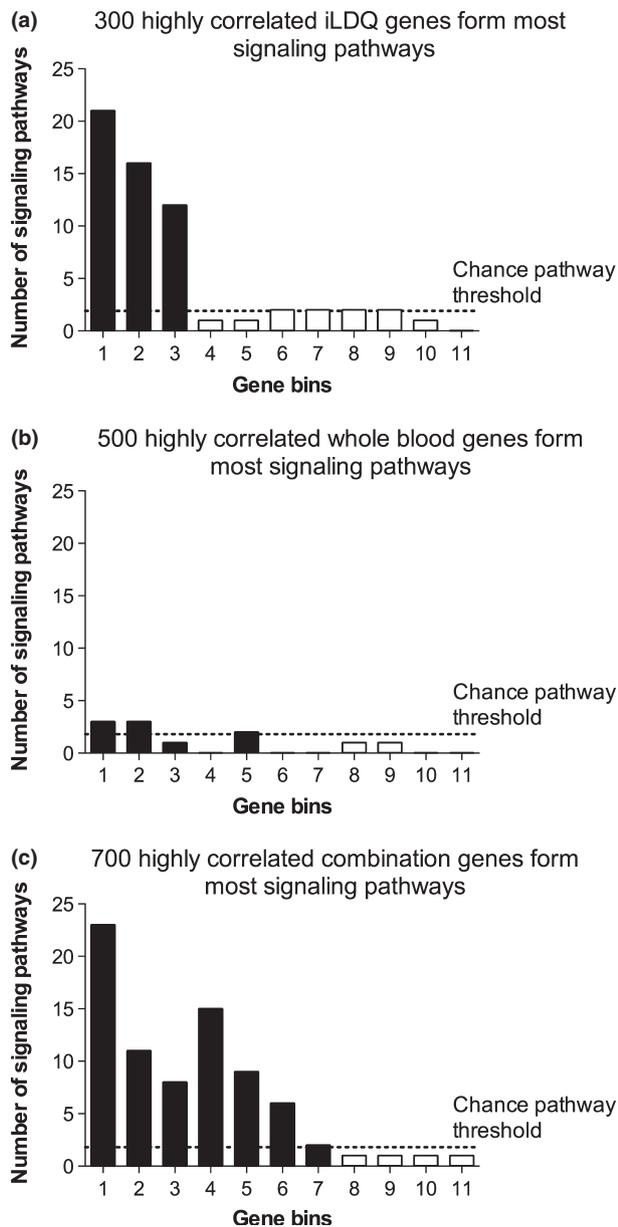
Comparison between putative whole blood biomarkers from RAACC gene list and those derived from the Adelaide and pathway derived gene lists

Although it could be argued that whole blood biomarkers could be identified on the strength of the correlation between whole blood gene expression and von Frey threshold, we

(a)

300 highly correlated iLDQ genes form most signaling pathways

(b)

500 highly correlated whole blood genes form most signaling pathways

(c)

700 highly correlated combination genes form most signaling pathways

Fig. 3 'Adelaide plots' define the gene list thresholds. Genes from the entire transcriptome were independently ranked according to absolute correlation coefficient (RAACC), for (a) the ipsilateral lumbar dorsal quadrant (iLDQ) transcriptome; (b) the whole blood transcriptome; (c) the combined iLDQ and whole blood transcriptomes. Beginning at the top of the RAACC Gene lists, bins were defined across the RAACC gene lists by selecting 1000 genes and then progressively frame-shifting by 100 genes (such that bin 1 contained genes 1–1000, bin 2 contained genes 101–1100 and so forth), so that each subsequent bin progressively excluded the prior 100 top ranked genes, and each bin analyzed in DAVID 6.7. The number of pathways classified from each bin was quantified and are displayed here as 'Adelaide plots', identifying the degree of organization into signaling pathways among the most highly correlated genes. As each bin was progressively analyzed in DAVID 6.7, the RAACC genes being major contributors to signaling pathways related to allodynia were identified as (a) the top 300 iLDQ genes, (b) the top 500 whole blood genes, (c) the top 700 combined iLDQ- and whole blood-RAACC Gene lists. These thresholds were inferred, as exclusion of these genes in Pathway Analyses (i.e. analyses beyond bins 4, 6, and 8, respectively) resulted in the pathway number per bin falling below that expected by chance (≤ 1.8, dotted line defined by random re-sampling of 1000 genes). Thus, Adelaide plots make it possible to identify the division between genes that contribute to signaling pathways and those that do not.

Prospective validation study

To validate the putative biomarkers identified via our novel data pipeline, a prospective validation study was performed in which a separate group of graded CCI rats were generated in a different laboratory and whole blood collected. Real-time PCR was performed on the top correlation coefficient ranked whole blood genes from the RAACC Gene list (top 2 genes; See Table S1), the 7 gene candidate biomarkers panel (top 2 genes; Table 2); the 29 candidate biomarkers panel (top 4 genes; Table 3); *Cx3cl1*, owing to its unique correlation with its cognate receptor in the iLDQ; and 2 genes not correlated with von Frey threshold (r < 0.01) from the whole blood-RAACC Gene list. Hence, a total of 9 putative biomarker genes and 2 negative control genes were analyzed. A Pearson correlation between gene expression (relative to the housekeeping gene, β-actin) and von Frey threshold revealed that 8 of 9 putative biomarker genes positively replicated, in addition to the replication of 2 negative controls. The results of the prospective validation study are presented in Table 4, and the genes correlated with von Frey threshold presented in Fig. 6, together with the regression line from the original transcriptome study. As the expression of β-actin was not significantly different between samples, this suggests that mRNA changes were likely caused by true changes in expression, rather than reflecting changes in cell number per sample.

Distribution of published 'pain genes' within the iLDQ transcriptome dataset

The recent meta-analysis by Lacroix-Fralish *et al.* (2011) identified a list of spinal cord and dorsal root ganglion genes

posited that genes more closely related to the mechanisms contributing to allodynia could be identified by taking the iLDQ signaling pathways into account. Therefore, the top 35 whole blood RAACC genes (equivalent to the number of non-repeated genes across both biomarker panels [Table 2, Table 3]) are presented in Table S1, together with references to link the protein encoded by each gene with nociceptive hypersensitivity, and immune signaling. Nineteen genes were found to contribute to nociceptive hypersensitivity and/or immune signaling in the Adelaide and Pathway derived biomarker panels (Table 2, Table 3), whereas only 7 such genes were identified in the whole blood-RAACC panel (Table S1).

Table 2 Putative biomarker panel 1: Seven direct whole blood-iLDQ correlates

Gene name	Symbol	Entrez ID	Nociceptive hypersensitivity	Immune signaling	Paired lumbar DH gene correlation		von Frey theshold correlation	
					r	p	r	p
Hook homolog 1 (Drosophila)	Hook1	[Entrez ID:313370]	-	-	0.755	0.011	−0.784	0.007
Similar to Tripartite motif protein 30-like	Trim30	[Entrez ID:499223]	-	(Shi et al. 2008)	−0.711	0.021	0.791	0.006
Similar to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	LOC295452	[Entrez ID:295452]	-	-	0.658	0.039	−0.718	0.019
Structural maintenance of chromosomes 2	Smc2	[Entrez ID:362519]	-	-	−0.633	0.050	0.743	0.014
Complement component 1, q subcomponent, A chain	C1qa	[Entrez ID:298566]	(Twining et al. 2005; Griffin et al. 2007)	(Baruah et al. 2009; Schmid et al. 2009)	0.560	0.092	−0.702	0.024
Transmembrane protein 176B	Tmem176b	[Entrez ID:171411]	-	(Louvet et al. 2005; Schmid et al. 2009; Condamine et al. 2010; Melchior et al. 2010)	0.525	0.119	−0.778	0.008
SP100 nuclear antigen	Sp100	[Entrez ID:363269]	-	(Negorev et al. 2009)	−0.518	0.125	0.706	0.022

Following a Pearson correlation between the whole blood and ipsilateral lumbar dorsal quadrant transcriptomes with von Frey threshold, gene segments identified as making a major contribution to signaling pathways in Adelaide plots were selected. The resulting whole blood- and ipsilateral lumbar dorsal quadrant-Adelaide Gene lists were compared and genes common to both were selected as putative biomarkers. The table is ranked according to the correlation coefficient between the gene expression and von Frey threshold. A non-exhaustive list of references is cited for the link of the protein encoded by each gene to nociceptive hypersensitivity and immune signaling. Omitted references indicate that, to the best of our knowledge, the protein encoded by that gene currently has no known role.

with 'strong correlational' or 'causational' evidential links with rodent neuropathic pain models. The distribution of these genes was identified within our iLDQ transcriptome, and the relationship between the direction of correlation and the published direction of gene regulation was classified as either concordant (23 genes; Table S2) or discordant (16 genes; Table S3). The distribution of concordant genes is more heavily weighted toward those that are negatively correlated with von Frey threshold (positively correlated with allodynia) than those that were discordant (Fig. 7). The discordant genes showed a normal distribution, clustered around $r = 0$, as did the distribution of the iLDQ transcriptome r frequency.

Discussion

We used our graded CCI model (Grace *et al.* 2010) to generate graded mechanical allodynia, based on the premise

that elements of such behavior could be explained by graded pathology in the iLDQ, such as microglial activation. Tissues were collected from male rats at a single timepoint after full development of allodynia. Given that there is a recognized peripheral immune component to the CNS mechanisms of rodent neuropathic pain models (Grace *et al.* 2011a,b), we proposed the novel hypothesis that biomarkers of graded CCI could be identified in the blood. It was also hypothesized that whole blood genes correlated with von Frey threshold (RAACC-Gene list; Fig. 1) with a closer relationship to the mechanisms contributing to allodynia could be identified as putative biomarkers, based on their contribution to signaling pathways with iLDQ genes, leading to the development of new bioinformatic analyses. We successfully tested the first hypothesis by correlating the whole blood transcriptome with von Frey threshold and, after developing a new correlational bioinformatic pipeline, identified a range of putative biomarkers (Tables 2 and 3, Table S1). The majority of select
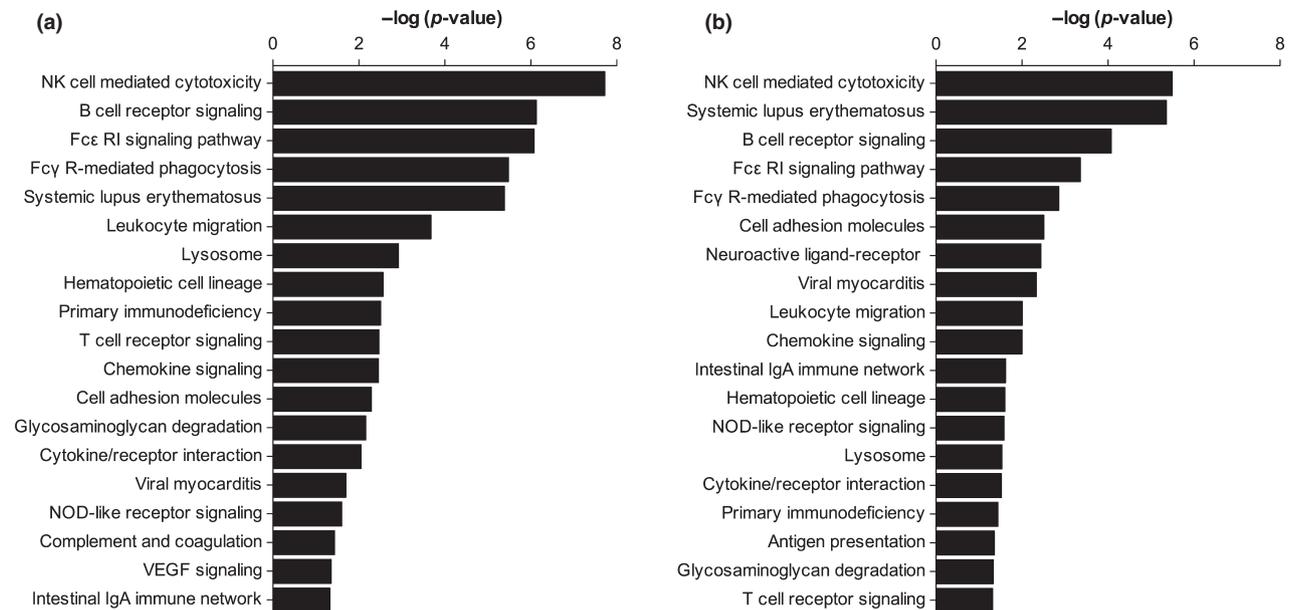
(a)

−log (*p*-value)

(b)

−log (*p*-value)

Fig. 4 Signaling pathways derived from (a) the ipsilateral lumbar dorsal quadrant (iLDQ), and (b) the combined whole blood and iLDQ gene lists. (a) The iLDQ-Adelaide gene list (300 genes; Figure 1) was classified into signaling pathways using DAVID 6.7. (b) The combined iLDQ and whole blood gene lists B (800 genes; Figure 1) were classified into signaling pathways using DAVID 6.7. Bars represent the -log(*p* value) for that pathway within the gene list (two-tailed Fisher's exact test; threshold of 1.3 (*p* = 0.05) used).

putative biomarkers were successfully tested in an independent prospective validation study (Table 4). By comparing the number of genes that are linked with nociceptive hypersensitivity and/or immune signaling, according to current literature, across the RAACC and the bioinformatically derived biomarker panels, the second hypothesis was also confirmed.

Each approach yielded a panel of putative biomarkers accessible via the blood, which is in keeping with the call from Rolan *et al.* (2003) to use biomarker panels for complex diseases, such as neuropathic pain. This is especially relevant for the present study, where inflammatory processes, independent of neuropathic pain, may regulate and hence confound the interpretation of individual gene expression. Nevertheless, a few notable features and examples will be highlighted, regarding individual genes, to provide support for the potential of the biomarker panels, and the methods used to derive them. Firstly, many of the genes identified encode for proteins that have a known link to immune or nociceptive signaling (Table 2 and 3), thus providing significant face validity to our panels and validating previous results from disparate laboratories. For example, of the 9 genes that were tested in the prospective validation study, 4 have a recognized role in nociceptive signaling, 4 have a recognized role in immune signaling, and 4 have no recognized role in either process (Tables 2 and 3; *Hook1* [Entrez ID:313370]; *ThrA* [Entrez ID:81812]; *Xylb* [Entrez ID:316067]; *Olr952* [Entrez ID:288880]). *Tnfrsf11b* [Entrez ID:25341] encodes for a soluble cytokine receptor, also known as osteoprotegerin, which has been linked with many

diseases, including painful diabetic neuropathy (Doupis *et al.* 2009). *Avpr1a* [Entrez ID:25107] encodes for an arginine vasopressin receptor, polymorphisms of which influence pain sensitivity to capsaicin across different mouse strains and in humans (Mogil *et al.* 2011). *Csf3* encodes for an anti-inflammatory chemokine that is associated with painful diabetic neuropathy and has been proposed as a potential therapeutic for neuropathic pain (Doupis *et al.* 2009; Ro *et al.* 2009). *Cx3cl1* encodes for the pro-inflammatory chemokine fractalkine, that is posited as a neuron-to-glial signal in pre-clinical neuropathic pain models (Milligan *et al.* 2008). *Trim30* [Entrez ID:499223] encodes for a protein in the toll like receptor signaling cascade that functions as a negative regulator of NFκB activation (Shi *et al.* 2008). Secondly, some cross-validity is lent to the methods used to derive the putative biomarker panels, as *C1qa* [Entrez ID:298566] was identified in both panels. *C1qa* encodes for a subunit of the C1q complement protein, which may be causally linked to nociceptive hypersensitivity, if peripheral nerve injury induces activation of the classical complement pathway (Twining *et al.* 2005; Griffin *et al.* 2007). Finally, of the ligand/receptor cognates across the blood and iLDQ, a significant correlation was only found between *Cx3cl1* in the blood and *Cx3cr1* in the iLDQ (Fig. 5b). These genes encode for the chemokine fractalkine, and its cognate receptor, CX$_3$CR1, which have a recognized mechanistic role in pre-clinical models of neuropathic pain. Fractalkine is posited to be a neuron-to-glia signal within the CNS (Milligan *et al.* 2008), whereas its cognate receptor is considered to be exclusively expressed by activated microglia in the dorsal

Table 3 Putative biomarker panel 2: Twenty-nine contributors to iLDQ signaling pathways

Gene name	Symbol	Entrez ID	Nociceptive hypersensitivity	Immune signaling	r	p
Tumor necrosis factor receptor superfamily, member 11b	Tnfrsf11b	[Entrez ID:25341]	(Doupis et al. 2009)	(Caidahl et al. 2010)	−0.900	0.001
Thyroid hormone receptor alpha	ThrA	[Entrez ID:81812]	-	-	0.898	0.001
Arginine vasopressin receptor 1A	Avpr1a	[Entrez ID:25107]	(Mogil et al. 2011)	-	−0.805	0.005
Colony stimulating factor 3 (granulocyte)	Csf3	[Entrez ID:25610]	(Doupis et al. 2009; Ro et al. 2009)	(Ro et al. 2009)	−0.802	0.005
Potassium large conductance calcium-activated channel, subfamily M, beta member 2	Kcnmb2	[Entrez ID:294961]	-	-	−0.794	0.006
Galanin receptor 1	Galr1	[Entrez ID:50577]	(Alier et al. 2008)		−0.784	0.007
Calcitonin receptor-like (precursor to CGRP receptor)	Calcrl	[Entrez ID:25029]	(Ma et al. 2003; Lee and Kim 2007)	(De Corato et al. 2011)	0.777	0.008
Contactin 1	Cntn1	[Entrez ID:117258]	-	-	−0.776	0.008
Protein phosphatase 3, regulatory subunit B, alpha isoform	Ppp3r2	[Entrez ID:29749]	(Jung and Miller 2008; Miletic et al. 2011)	(Jung and Miller 2008)	−0.774	0.009
Adenylate cyclase 8	Adcy8	[Entrez ID:29241]	-	-	−0.774	0.009
Adenosine A2B receptor	Adora2b	[Entrez ID:29316]	(Abo-Salem et al. 2004)	(Ham and Rees 2008)	−0.768	0.009
Protein kinase, cAMP dependent, catalytic, beta	Prkacb	[Entrez ID:293508]	(Gold and Gebhart 2010)	(Riether et al. 2011; Sugimoto et al. 2011)	0.767	0.010
Heat shock protein 1	Hspb1	[Entrez ID:24471]	(Kim et al. 2001)	(Kim et al. 2009)	−0.767	0.010
Myosin, heavy polypeptide 2, skeletal muscle, adult	Myh2	[Entrez ID:691644]	-	-	−0.761	0.011
Deoxyribonuclease II beta	Dnase2b	[Entrez ID:59296]	-	(Okabe et al. 2008)	−0.759	0.011
Purinergic receptor P2X, ligand-gated ion channel, 3	P2rx3	[Entrez ID:81739]	(Wirkner et al. 2007)	-	0.757	0.011
Chemokine (C-X3-C motif) ligand 1	Cx3cl1	[Entrez ID:89808]	(Milligan et al. 2008)	(Milligan et al. 2008)	−0.754	0.012
Angiotensin II receptor, type 1b	Agtr1b	[Entrez ID:81638]	-	(Downie et al. 2009; Chon et al. 2011; Fuchtbauer et al. 2011)	−0.751	0.012
Caspase recruitment domain family, member 6	Card6	[Entrez ID:294770]	-	(Dufner et al. 2006)	0.747	0.013
RT1 class II, locus Ba	Rt1-ba	[Entrez D:309621]	(Grace et al. 2011b)	(Grace et al. 2011b)	0.739	0.015
Coxsackie virus and adenovirus receptor	Cxadr	[Entrez ID:89843]	-	-	−0.738	0.015
Adrenergic, beta-1-, receptor	Adrb1	[Entrez ID:24925]	-	(Walker-Brown and Roberts 2009)	−0.730	0.016

Table 3 (continued)

Gene name	Symbol	Entrez ID	Nociceptive hypersensitivity	Immune signaling	r	p
Inducible T-cell co-stimulator ligand	*Icoslg*	[Entrez ID:499415]		(Simpson *et al.* 2010)	0.728	0.017
Tumor necrosis factor receptor superfamily, member 10b	*Tnfrsf10b*	[Entrez ID:364420]	-	(Hoffmann *et al.* 2009)	−0.728	0.017
Purinergic receptor P2X, ligand-gated ion channel, 7	*P2rx7*	[Entrez ID:29665]	(Sperlagh *et al.* 2006; He *et al.* 2012)	(Sperlagh *et al.* 2006; Tsukimoto *et al.* 2009; He *et al.* 2012)	−0.725	0.018
Histone cluster 3, H2ba	*Hist3h2ba*	[Entrez ID:303175]	-	-	−0.725	0.018
Purinergic receptor P2Y, G-protein coupled 2	*P2ry2*	[Entrez ID:29597]	(Malin *et al.* 2008)	(Kronlage *et al.* 2010)	−0.723	0.018
Prostaglandin E receptor 2, subtype EP2	*Ptger2*	[Entrez ID:81752]	(Kunori *et al.* 2011)	(Kunori *et al.* 2011)	−0.712	0.021
Glutamate receptor, metabotropic 1	*Grm1*	[Entrez ID:24414]	(Kohara *et al.* 2007)	(Hermans and Challiss 2001; Chiocchetti *et al.* 2006)	−0.703	0.023
Complement component 1, q subcomponent, alpha polypeptide	*C1qa*	[Entrez ID:298566]	(Twining *et al.* 2005; Griffin *et al.* 2007)	(Baruah *et al.* 2009; Schmid *et al.* 2009)	−0.702	0.024

Following a Pearson correlation between the whole blood and ipsilateral lumbar dorsal quadrant (iLDQ) transcriptomes with von Frey threshold, gene segments identified as making a major contribution to signaling pathways in Adelaide plots were selected. The whole blood- and iLDQ-Adelaide Gene lists were combined and classified into signaling pathways using DAVID 6.7 Pathway Analysis software. The iLDQ genes that contributed to signaling pathways were removed, leaving a list of whole blood genes that were correlated with the severity of allodynia, and were linked with iLDQ genes in signaling pathways. The table is ranked according to the correlation coefficient between the gene and von Frey threshold. A non-exhaustive list of references is cited for the link of the protein encoded by each gene to nociceptive hypersensitivity and immune signaling. Omitted references indicate that, to the best of our knowledge, the protein encoded by that gene currently has no known role.

horn of the spinal cord (Verge *et al.* 2004). Therefore, fractalkine gene expression in the blood may also serve as a biomarker for other diseases where microglial activation plays a role.

While the preceding discussion highlights the face validity of our biomarker panels, we have successfully demonstrated the strength of our approach by beginning the process of qualification (construct validation), in that 8 of 9 putative biomarker genes were positively replicated, in addition to the replication of 2 negative controls (Table 4, Fig. 6). The unsuccessful validation of 1 gene (*Hook1*) may be as a result of a Type I error in the transcriptome, which
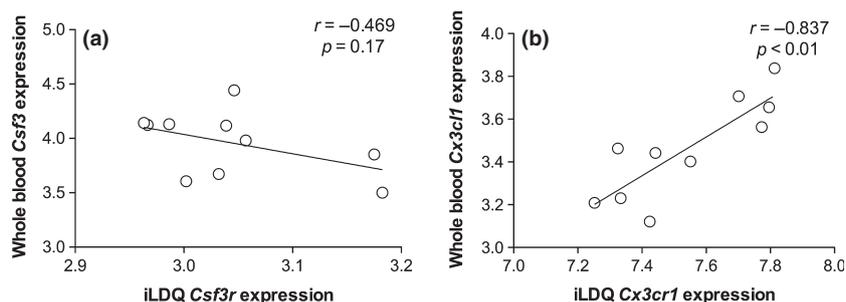

Fig. 5 Relationship between blood ligand and ipsilateral lumbar dorsal quadrant (iLDQ) receptor gene expression in the graded chronic constriction injury model. Of the potential ligand/receptor pairing between the 29 whole blood genes contributing to iLDQ signaling pathways biomarker panel (Table 3) and the iLDQ-Pathway derived Gene list (Fig. 1), the only pairs to form with their cognate receptors in the iLDQ were *Csf3* and *Cx3cl1*. A correlation between the ligands and their cognate receptors found (a) an insignificant inverse relationship between *Csf3* and *Csf3r*; but (b) a significant relationship between *Cx3cl1* and *Cx3cr1*.

Table 4 Prospective validation of select putative biomarker genes

Gene name	Symbol	Entrez ID	Gene list	r	p	% CV
Tumor necrosis factor receptor superfamily, member 11b	Tnfrsf11b	[Entrez ID:25341]	29 candidate biomarkers	−0.881	0.001	1.53
Thyroid hormone receptor alpha	Thra	[Entrez ID:81812]	29 candidate biomarkers	0.639	0.01	23.88
Arginine vasopressin receptor 1A	Avpr1a	[Entrez ID:25107]	29 candidate biomarkers	−0.735	0.01	6.42
Colony stimulating factor 3 (granulocyte)	Csf3	[Entrez ID:25610]	29 candidate biomarkers	0.753	0.01	4.49
Chemokine (C-X3-C motif) ligand 1	Cx3cl1	[Entrez ID:89808]	29 candidate biomarkers	−0.713	0.01	3.96
Xylulokinase homolog (H. influenzae)	Xylb	[Entrez ID:316067]	Whole blood RAACC Gene list	−0.664	0.05	21.75
Olfactory receptor 952	Olr952	[Entrez ID:288880]	Whole blood RAACC Gene list	−0.635	0.05	24.58
Hook homolog 1 (Drosophila)	Hook1	[Entrez ID:313370]	7 candidate biomarkers	−0.479	0.11	34.11
Similar to Tripartite motif protein 30-like	Trim30	[Entrez ID:499223]	7 candidate biomarkers	0.587	0.05	20.90
Interferon regulatory factor 2 binding protein 1	Irf2bp1	[Entrez ID:308404]	Negative control	0.148	0.67	40.83
Similar to NFkB interacting protein 1	LOC686781	[Entrez ID:686781]	Negative control	0.093	0.77	9.35

A prospective validation study was performed using a separate group of graded chronic constriction injury rats from different laboratory. Whole blood was collected and real-time PCR performed on select putative biomarker genes. The table presents the genes analyzed, the list from which they were derived, the Pearson correlation between gene expression and von Frey threshold, and the coefficient of variance (% CV) between the pipeline and prospective correlation coefficients.

is potentially supported by Table 2, showing that the gene has no known function in either nociceptive or immune signaling. The replication study was also performed in a different laboratory, demonstrating the robustness of our findings. To build on our positive replication, it is necessary that other variables are tested, such as additional neuropathic pain models, additional pain modalities, sex, allodynia timecourse, and sensitivity to drug effect. Although beyond the scope of this study, the genes identified via our novel data pipeline may be useful to investigate additional variables, as they have a closer relationship with the mechanisms contributing to allodynia (Table 2, Table 3), compared with the whole blood-RAACC Gene list (Table S1). The targeted investigation of the putative biomarker genes (Tables 2 and 3) is more economical and has greater feasibility than conducting further transcriptome studies, and a potential causal relationship with allodynia suggests that the putative biomarker genes may be a more robust and reliable measure under different circumstances, compared with those simply correlated with allodynia (Table S1). Importantly, the pre-clinical development of the genes identified here, or their respective proteins, may lead to their translation as clinical neuropathic pain biomarkers that serve as tools for mechanism-based diagnoses; to target therapy by patient stratification; and/or to expedite drug development by enriching patient populations in clinical trials, quantify pain intensity and to detect treatment effect (Rolan 1997; Chizh et al. 2008; Lathia et al. 2009; Woodcock 2009).

The iLDQ transcriptome raises some additional points, regarding the mechanisms underlying nerve injury-induced nociceptive hypersensitivity. Firstly, despite the fact that the iLDQ is considered a neural tissue, many of the signaling pathways classified from the transcriptome of this tissue are in fact immune (Fig. 4a). These data further support the important role of central immunity in models of neuropathic pain (Austin and Moalem-Taylor 2010; Ren and Dubner 2010; Grace et al. 2011a,b), which may explain why neuronally targeted therapies for neuropathic pain are not disease modifying. Of these immune pathways, both the Fcγ and Fcε signaling pathways have potential, but as yet unrecognized, significance for neuropathic pain (see representative correlations in Figure S1). The antibody receptors, Fcγ and Fcε, are expressed by the innate immune cells of the CNS, microglia (Ulvestad et al. 1994; Inoue et al. 1999; Suemitsu et al. 2010), but also by neurons, leading to direct activation by IgG and IgE, respectively (Andoh and Kuraishi 2004a,b; Suemitsu et al. 2010; Qu et al. 2011), the former having been shown to induce mechanical allodynia when intrathecally administered to naïve mice (Cao et al. 2009). The consequences of Fc receptor activation on antigen presenting cells, such as microglia include cytokine secretion, and stimulation and modulation of T cells (Joller et al. 2011), which may have profound implications for pain pathophysiology (Grace et al. 2011b). Significant expression of spinal dorsal horn Fcγ receptor mRNAs have been identified by other transcriptome studies in pain models (Griffin et al. 2007; Costigan et al. 2009a). Therefore, the role of Fc receptors in neuropathic pain pathophysiology requires further investigation. As an aside, caution should be exercised when drawing conclusions from the signaling pathway classifications, as the Kyoto Encyclopedia of Genes and Genomes database contains only previously defined signaling pathways, and at present, the database is further limited by the absence of pathways that specifically encompass neuro-immune signaling.
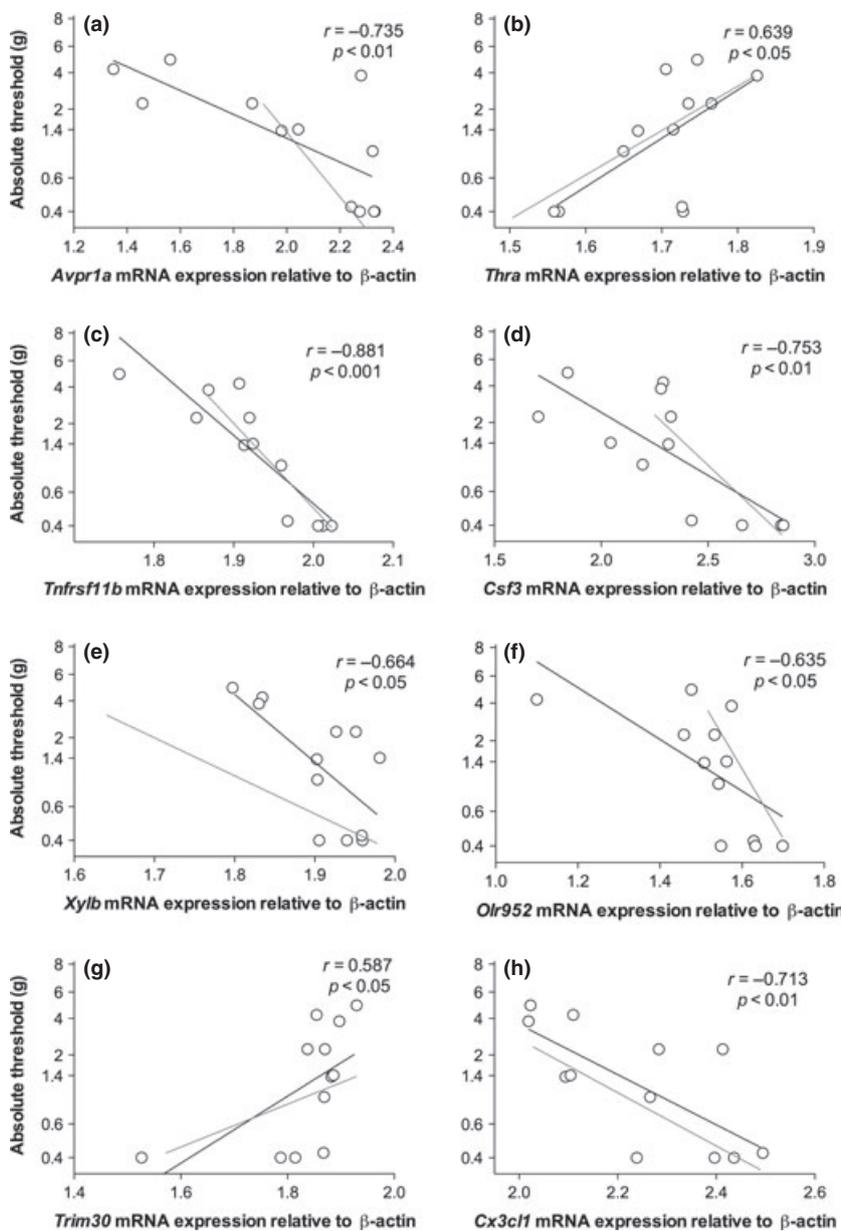
Fig. 6 Prospective validation of select putative biomarker genes. To validate the putative biomarkers identified via our novel data pipeline, a prospective validation study was performed using real-time PCR. A Pearson correlation was performed between gene expression (relative to housekeeping gene) and von Frey threshold. (a–d) The top 4 genes, from the 29 candidate biomarkers panel (Table 3) (*Tnfrsf11b*; *Thra*; *Avpr1a*; *Csf3*). (e, f) The top 2 genes, ranked by correlation coefficient, from the whole blood RAACC-Gene list were significantly correlated with von Frey threshold (*Xylb*; *Olr952*). (g) Of the top 2 genes from the 7 gene candidate biomarkers panel, only *Trim30* was significantly correlated with von Frey threshold. (h) *Cx3cl1* was also significantly correlated with von Frey threshold. The replication study regression line (black) and statistics, and the transcriptome study regression line (gray) are indicated for reference.

Secondly, examination of the distribution of published 'pain genes' (Lacroix-Fralish *et al.* 2011) within our iLDQ transcriptome identified genes that were either concordant or discordant with the direction of correlation and the published direction of gene regulation (Fig. 7; Table S2, Table S3). Provided that these data are not confounded by artifacts of the transcriptome, they lead to a number of potential conclusions. The concordant genes are skewed toward up-regulation as compared to discordant genes in our model ($r < -0.16$), with ~60% of concordant 'pain genes' falling within the lower tertile and therefore negatively correlated with von Frey threshold (positively correlated with allodynia). These data suggest that severity of allodynia is generally associated with activation and recruitment of new

genes and processes, rather than down-regulation of existing ones. Of the genes in the lower tertile ($r < -0.16$), the Adelaide plot (Fig. 3a) suggests that the top 300 genes (corresponding to $r < -0.7$) are probably the most important predictors of pain severity (*Gfap* [Entrez ID:24387]; *Ctss* [Entrez ID:50654]; *C1qc* [Entrez ID:362634]; *C1qb* [Entrez ID:29687]; *Aif1* [Entrez ID:29427]; *C3* [Entrez ID:24232]). Of the discordant genes, those that fall in the outer tertiles (*Rgs4* [Entrez ID:29480]; *Scn11a* [Entrez ID:29701]; *Chrna3* [Entrez ID:25101]; *C1s* [Entrez ID:192262]; *Gfra1* [Entrez ID:25454]; *Calca* [Entrez ID:24241]; *S100a4* [Entrez ID:24615]; *Gadd45a* [Entrez ID:25112]) are especially noteworthy, as they have an opposite relationship with pain severity to that previously reported (Lacroix-Fralish *et al.*
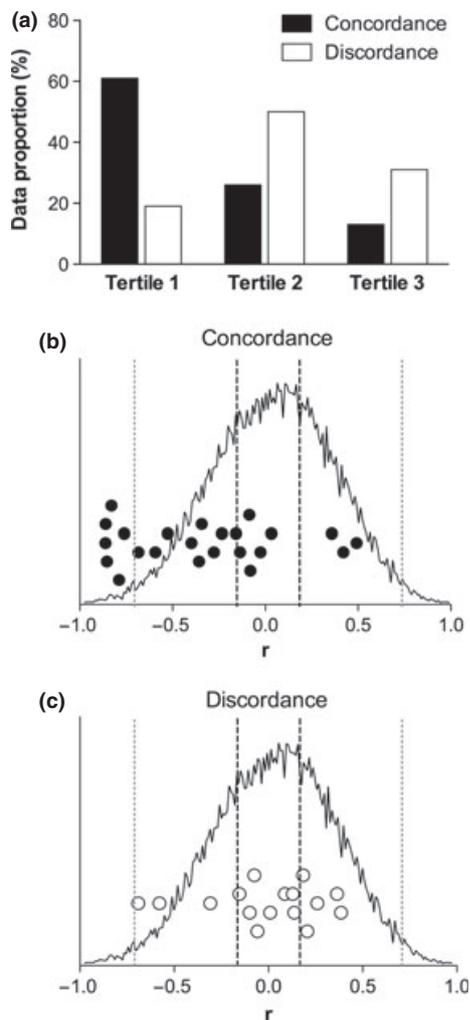
Fig. 7 Distribution and concordance of published spinal cord and dorsal root ganglia 'pain genes' within the ipsilateral lumbar dorsal quadrant (iLDQ) transcriptome dataset. Genes that were identified as having 'strong correlational' or 'causational' evidential links with rodent neuropathic pain models in the recent meta-analysis by Lacroix-Fralish *et al.* (2011) were identified within the iLDQ transcriptome dataset (RAACC-Gene list; Figure 1). The 'pain genes' dataset was then split according to the concordance or discordance between the direction of correlation between gene expression and von Frey threshold, and the published direction of gene regulation (Lacroix-Fralish *et al.* 2011). (a) The data was binned in correlation coefficient tertiles and the distribution frequency plotted. (b) Individual points are plotted for concordant genes and overlaid with the correlation coefficient frequency of the entire iLDQ transcriptome. (c) Individual points are plotted for discordant genes and overlaid with the correlation coefficient frequency of the entire iLDQ transcriptome. Dashed lines indicate correlation coefficient tertiles; dotted lines indicate correlation coefficient thresholds defined here using Adelaide plots.

2011). However, the meaning and behavioral significance of this is as yet unknown, and requires further study and validation.

A final note concerns the conclusions drawn from studies of single factors in models of neuropathic pain, for which a number of recent studies serve as useful, but not isolated, examples (Ren and Neugebauer 2010; Gao *et al.* 2011; He *et al.* 2012; Wilson *et al.* 2011). These studies use exclusive terms, such as 'mediate', to describe the effect of a single factor on pain behavior. The Adelaide plots generated from this study suggest that at least 300 genes contribute to allodynia at the spinal level (Fig. 3a). Therefore, it is inaccurate to use such exclusive terms to describe single factor pain studies, and we suggest that such conclusions are avoided in the future. Moreover, these data suggest that pharmacologically targeting a single site well down the mechanistic pathway to treat pain may be a futile approach, owing to the apparent widespread adaptations in multiple systems and possible redundancy of nociception amplification systems.

Our novel, proof-of-concept, graded CCI transcriptome study design departed from previous pain transcriptome studies in two main ways. Firstly, heterogeneity was intentionally created in a single population for analysis, thus avoiding the difficulties associated with few replicates in each treatment group. Gene selection was based on relationship with the endpoint of interest, allodynia, rather than on the treatment group. Thus, we were able to differentiate, at least in part, between gene expression specific to the model (such as changes in whole blood related to the chromic gut sutures placement in each surgery group) and that specific to allodynia. Furthermore, we did not attempt to limit variability within treatment groups by pooling RNA prior to chip hybridization, for example, as has been done previously (Griffin *et al.* 2007; Costigan *et al.* 2009a). Secondly, we used a novel data pipeline to analyze the graded transcriptome. Data pre-processing for a binary study design is relatively straightforward, in that only the genes significantly up- or down-regulated compared to controls are carried through to the next stage of analysis (Ko *et al.* 2002; Yang *et al.* 2004; LaCroix-Fralish *et al.* 2006a; Lacroix-Fralish *et al.* 2006b; Griffin *et al.* 2007; Costigan *et al.* 2009a; Vega-Avelaira *et al.* 2009). Even some correlational studies have employed a similar pre-processing step by excluding non-significant expression of normalized fold changes compared to a baseline (Cameron *et al.* 2007; Persson *et al.* 2009). However, it is possible that selecting genes for further analysis based on overabundance may overlook subtle changes in gene expression. This is especially relevant for highly regulated and controlled genes, or those whose product is highly potent. Therefore, our approach differed, in that we developed the Adelaide plot to identify genes of interest. The use of pathway analysis in the data pre-processing is likely to minimize and eliminate Type I errors, which is supported by the fact that we identified genes encoding for proteins that have established or potential links with the mechanisms underlying neuropathic pain (Table 2,

Table 3). Adelaide plots are a powerful and versatile tool to identify the level of organization in signaling pathways of large datasets and would be valuable to employ in future studies. A potential weakness in using Pathway Analysis software, such as DAVID 6.7, is that genes are selected on the basis of a known function and contribution to known signaling pathways. Hence, potential biomarker candidates may have been overlooked among the genes that have not yet been characterized. Tools using next-generation sequence data that identify novel expressed sequence tags or gene products may be a valuable alternative to partially circumvent this limitation. Our study design is based on the premise that the rats with graded CCI differ only in respect to the mechanisms underlying graded allodynia, as discussed previously (Grace *et al.* 2010). However, there may be other mechanisms that could confound pre-clinical validation and clinical translation, such as graded Wallerian degeneration (Lin *et al.* 2001), which may lead to a graded immune response (Griffin *et al.* 1992). Therefore, future studies should consider examining additional neuropathic pain models that have the capacity to produce a heterogeneous response.

We are the first to use the transcriptome to identify putative blood-based biomarker panels in a rodent model of nerve injury. The criteria for such biomarkers were that they were correlated with von Frey threshold and that they contributed to signaling pathways with iLDQ genes. To achieve this aim, we developed a novel data pipeline to integrate graded CCI, pain behavior, whole blood and iLDQ transcriptomes, using the power of Pathway Analysis. The panels identified from this approach include a direct correlation between gene expression common to both the whole blood and iLDQ, and identification of whole blood genes that directly contribute to the iLDQ signaling pathways. Future qualification studies may demonstrate the predictive ability of these biomarker genes across further models and additional variables.

Acknowledgments

Supporting information

Additional supporting information may be found in the online version of this article.

Figure S1. Representative correlations from Fcγ and Fcε signaling pathways.

Table S1. The top 35 whole blood genes, ranked according to absolute correlation coefficient between gene expression and von Frey threshold.

Table S2. Concordant gene list.

Table S3. Discordant gene list.

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