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Evaluation of phenoxybenzamine in the CFA model of pain following gene expression studies and connectivity mapping

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Abstract

Background: We have previously used the rat 4 day Complete Freund's Adjuvant (CFA) model to screen compounds with potential to reduce osteoarthritic pain. The aim of this study was to identify genes altered in this model of osteoarthritic pain and use this information to infer analgesic potential of compounds based on their own gene expression profiles using the Connectivity Map approach.

Results: Using microarrays, we identified differentially expressed genes in L4 and L5 dorsal root ganglia (DRG) from rats that had received intraplantar CFA for 4 days compared to matched, untreated control animals. Analysis of these data indicated that the two groups were distinguishable by differences in genes important in immune responses, nerve growth and regeneration. This list of differentially expressed genes defined a "CFA signature". We used the Connectivity Map approach to identify pharmacologic agents in the Broad Institute Build02 database that had gene expression signatures that were inversely related ('negatively connected') with our CFA signature. To test the predictive nature of the Connectivity Map methodology, we tested phenoxybenzamine (an alpha adrenergic receptor antagonist) - one of the most negatively connected compounds identified in this database - for analgesic activity in the CFA model. Our results indicate that at 10 mg/kg, phenoxybenzamine demonstrated analgesia comparable to that of Naproxen in this model.

Conclusion: Evaluation of phenoxybenzamine-induced analgesia in the current study lends support to the utility of the Connectivity Map approach for identifying compounds with analgesic properties in the CFA model.

Background

Several recent studies have characterized preclinical pain models with the goal of defining gene expression profiles related to different kinds of pain [1-9]. An understanding of gene alterations associated with pain is important as these may open up new pathways for targeting pain. While there are studies that describe gene expression profiles following nerve transection or nerve ligation models in animals [1-5,9], reports defining gene expression in studies that utilize inflammatory models such as the Complete Freund's Adjuvant (CFA) model are scarce. The rat CFA model yields acute inflammation and pain as measured by tactile allodynia [10-12], changes in weight bearing, and paw pressure withdrawal

thresholds [13]. We have used this model for screening potential compounds for osteoarthritic pain [11-13]. The non-steroidal anti-inflammatory drug (NSAID), naproxen [14], is active in the CFA model, reducing mechanical allodynia, weight bearing differentials and increasing the paw pressure withdrawal thresholds and is thus a clinically relevant positive control for this model.

The Connectivity Map was recently described by Lamb et al [15], as an approach to identify connections between diseases and drugs, based on their gene expression signatures. This resource consists of a large public database that includes hundreds of compound profiles termed the "reference gene signatures" obtained from cultured human cell lines treated with more than thirteen hundred pharmacologically active agents (Broad Build02 database) [15]. The database is attached to a pattern-matching tool. Gene signatures of interest from

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expression profiling studies can be compared to the Connectivity Map database, and subjected to a pattern matching algorithm that ranks similarities between the gene signature of interest and the reference signatures [15]. This can lead to identification of 'connections' between an investigator's gene expression signature (e.g. a disease tissue/model) and small molecule profiles in the database. Several recent studies have used this resource to identify novel agents for different indications, including hair growth and leukemia [16,17].

Dorsal Root Ganglia (DRG) are known to be important sites for pain processing. In this study, we first generated gene expression profiles of the ipsilateral L4 and L5 dorsal root ganglia (DRG) extracted from rats injected with CFA in their left paws for 4 days and compared them to profiles of L4 and L5 DRGs from naïve rats. The gene signature of CFA-treated versus naïve rats was created and queried in the Connectivity Map database. Phenoxybenzamine - an alpha adrenergic receptor antagonist and several other agents showed significant inverse connectivity to the CFA signature. We thus reasoned that these agents could potentially reduce pain processing and have antinociceptive properties in the CFA model. Here we describe the data leading to the generation of this hypothesis and our subsequent experimental confirmation of the activity of phenoxybenzamine in this model of inflammatory pain.

Methods and materials

Animals

Adult Male Sprague Dawley rats (200-250 g) were used in all experiments. Rats were purchased from Harlan, (Indianapolis, IN) and maintained on a 12/12 hr light/dark cycle with food and water ad libitum. Rats were acclimated for a week before use in experiments. The Pfizer Institutional Animal Care and Use Committee reviewed and approved the animal use in these studies. The animal care and use program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

CFA-induced inflammation

Rats were anesthetized briefly with isoflurane (5% induction, then 2% maintenance) and their left foot swabbed with ethanol. 0.15 ml Complete Freund's adjuvant (CFA, SIGMA, St. Louis, MO) was injected subcutaneously into the plantar surface of the left hind paw of the rat [10]. The CFA injection immediately induces local inflammation, paw swelling and pain, which persists for at least 2 weeks post-injection. For behavior studies, rats were placed on the equipment and left to acclimate for 30 minutes. On day 0, baseline measurements were read and rats were injected with CFA thereafter. On day 3, post-CFA reads were taken and only

rats that met criteria of hyperalgesia were placed on the study on day 4.

Mechanical allodynia and thermal hyperalgesia

To assess mechanical allodynia, rats were placed on an elevated wire mesh platform, and to confine their movement, a 15 × 22 × 25 cm plexiglass chamber was placed over each animal. Mechanical paw withdrawal thresholds (PWT) were measured by using a set of Semmes Weinstein monofilaments (Stoelting, Wood Dale, IL) using the Dixon up and down method [18]. Only rats that displayed a PWT of 8 g or less on day 3 (post-CFA) were placed on study. To assess thermal hyperalgesia rats were placed on glass plates with the source of heat applied from the bottom. On day 3 (post-CFA) rats that gave withdrawal latencies of 6 s or less were included in the experiments. Rats were then randomly assigned to either a vehicle group or drug group. On day 4, rats were treated with either the vehicle (saline), or drug and reads were taken 2 hrs after the treatment. All measurements were performed fully randomized and blinded and the reader did not know what treatment each rat received.

Drug administration

All drugs/vehicle were administered by oral gavage at 5 mg/kg/ml. Naproxen and phenoxybenzamine hydrochloride were purchased from SIGMA (St. Louis MO). Both were dissolved in saline, which was used as vehicle. All measurements were taken 2 hr post dosing. A group of naïve rats was also used in experiments, and these were not injected with CFA, and were not dosed.

Statistical analysis of behavioral data

This was done on the raw data using a one way ANOVA followed by the Student- Newman-Keul's Post-Hoc test.

RNA isolation and gene array profiling

A naïve (control) group of rats (n = 6) and a group of rats injected with CFA (n = 6) as described above, were used for gene expression profiling experiments. On day 4, rats were euthanized and ipsilateral L4 and L5 dorsal root ganglia (DRG) were extracted, pooled for each rat, and immediately frozen in liquid nitrogen. DRG were stored at -80°C until used. RNA isolation and processing for gene arrays was done according to standard protocols by Genelogic (Boston, MA). Briefly, dorsal root ganglia tissues were homogenized in guanidium isothiocyanate with β-mercaptoethanol (GITC+BME), using the Omni TH-115 homogenizer. Trizol was added and mixed by repeated inversions. RNA aqueous phase was separated using Heavy Phase Lock Gel tubes. Chloroform and sample homogenate were added to the tube

and shaken vigorously to remove protein contaminants, incubated on ice for 9-12 min, then centrifuged. The aqueous phase was cleaned using the Qiagen RNEasy Mini Kit (Cat # 74106). A bead cleanup was performed using Agencourt RNAClean magnetic beads (Cat # A29168) to further purify the RNA. RNA quality was evaluated by an Agilent Bioanalyzer. Only the RNA samples that passed the quality control measures proceeded for microarray. 12 high quality RNA samples each from an individual animal were prepared. 1 RNA sample from an animal in the control group had low yield and did not get processed further. The remaining 11 RNA samples were each run on a separate microarray. All 11 microarrays passed the quality control criteria and proceeded to downstream analysis. In total, 5 microarrays each from the 5 animals in the control group, and 6 microarrays each from the 6 animals in the CFA group were analyzed.

Rat whole genome 4X44K one color microarray processing

Sample preparation, hybridization, wash, scanning and feature extraction was performed according to the standard protocol, 'One-Color Microarray-Based Gene Expression Analysis v.5.7 (P/N G4140-90040)', by Agilent Technologies. One exception was that Triton was excluded from the wash buffer. Only the microarrays that passed the quality control criteria proceeded to downstream analysis.

Microarray data processing

The Agilent rat whole genome microarray signals were normalized with a program developed internally. Briefly, signal normalization against a common sample was performed using a smoothed piecewise linear curve, trained on the log intensities that differed by < 10% rank order between two samples. Local background subtraction was performed on each array prior to normalization. A computational naïve control pool was created from the averaged normalized signals of the individual naïve control arrays. Within the naïve control arrays, a median array was selected as the one least distant from the other naïve control arrays. All naïve control arrays were normalized against the median array prior to calculating the averaged normalized signals of the control pool. Finally each sample was normalized against the computational naïve control pool. Fold changes between samples were calculated, raising weak signals to a minimum value of 10 prior to comparison. Fold and p-values from student's t-test between groups was calculated.

Connectivity Map

The Broad's Connectivity Map algorithm was implemented in-house. All compound profiles were downloaded

from Broad Build02 database [15]. A nonparametric, rank-based pattern matching strategy based on Kolmogorov-Smirnov statistics was devised and used in the calculation of the Connectivity Score and p-value as described in [15] and [19].

Gene Expression Pathway Analysis

Oligo probes that met the fold and p-value cutoff (arbitrarily set at 1.5 fold increase or decrease and p-value < = 0.05) were used for Functional Analysis using Ingenuity Pathway Analysis (Ingenuity IPA 8.0 (content version 2602); Ingenuity® Systems, <http://www.ingenuity.com>). Functional Analysis identified the biological functions and/or diseases that were significant to the dataset. Fischer's exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that dataset is due to chance alone. Canonical Pathway Analysis identified the pathways from the Ingenuity Pathways Analysis library of canonical pathways that were significant to the dataset. The significance of the association between the data set and the canonical pathway was measured in 2 ways: 1) A ratio of the number of genes from the dataset that map to the pathway divided by the total number of genes that map to the canonical pathway is displayed. 2) Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

Hierarchical Clustering

The transcript expression signal intensity was first mean centered and normalized across samples and then a complete linkage clustering method was applied on the correlation similarity measure for hierarchical clustering and heat map in Spotfire® DecisionSite.

Results

CFA-behavior studies

Injection of CFA significantly reduced mechanical paw withdrawal thresholds (PWT) by 62% when compared to naïve rats ($P < 0.001$, Figure 1A). Naproxen (10 mg/kg) significantly reversed mechanical allodynia back to about 73% ($P < 0.01$, Figure 1A) of naïve mechanical thresholds. Injection of CFA significantly induced thermal hyperalgesia as measured by a decrease of latencies to withdraw by about 76% when compared to naïve rats ($P < 0.001$, Figure 1B). Naproxen significantly reversed the thermal hyperalgesia back to about 86% of naïve controls ($P < 0.001$, Figure 1B). These effects are in close agreement with previously reported data [11,12].

Gene expression microarray studies

Gene expression in DRG was compared between arrays obtained from 4 day CFA-injected rats and from naïve

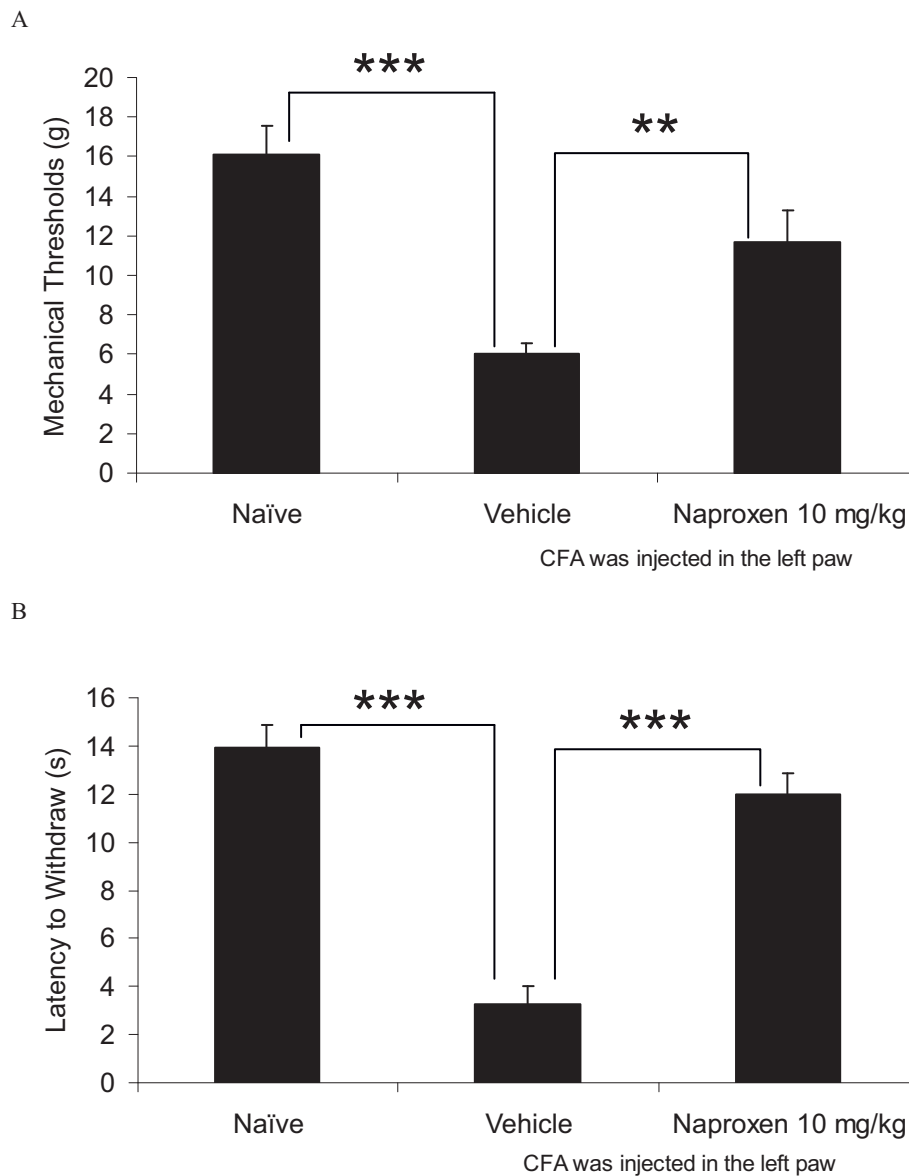
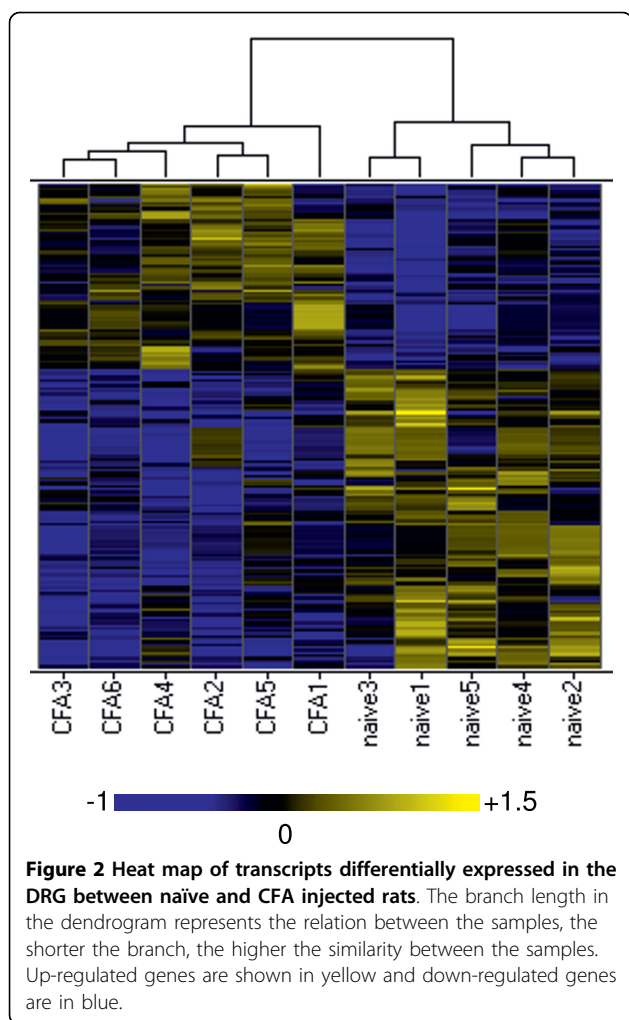


Figure 1 Oral naproxen is effective in reducing nociceptive behavior in 4 day CFA-injected rats. Naproxen reduced A) mechanical thresholds and B) paw withdrawal latencies taken 2 hr post dose. $n = 6-16$ rats per group. For this and other figures, data is presented as mean \pm s.e.m. ** $p < 0.01$ and *** $p < 0.001$, significant differences from vehicle (saline) - dosed rats.

rats. Genes that were up- or down- regulated by ≥ 1.5 fold ($p \leq 0.05$), were used to generate a differential expression list for CFA-treated versus naïve rat DRG. 235 oligo probes met these criteria. 178 oligo probes (representing 140 unique genes) have HUGO gene symbols and further analysis was focused on these genes. Hierarchical clustering indicated that these transcripts could separate the CFA samples from naïve samples, as shown in the heat map (Figure 2). We thus consider these transcripts as a molecular signature of the 4 day CFA model in the DRG tissue.

Overall, it appears that genes that mediate inflammation are suppressed, while genes that mediate neuron growth/survival are enhanced in this 4 day CFA gene signature. Ingenuity® Functional and Pathway Analysis (IPA) was conducted on the genes decreased by CFA treatment in the DRG to help understand the function and canonical pathways perturbed. IPA is a web-based application that enables the visualization, discovery and analysis of molecular functions and pathways within gene expression profiles. One function identified that was significantly affected (p -value < 0.01) is suppression



of a group of genes that have a role in immune response or inflammatory disorders (Table 1). Also noted is that leucine rich repeat interacting protein 1 (*Lrrfip1*), a gene that represses TNF alpha expression, is increased in the DRG from CFA-treated rats compared to that from naïve rats. This data suggests that the immune and inflammatory response may play a role in regulating pain processing in the CFA treated DRG. The eicosanoid signaling pathway was also significantly affected in this model (p -value = $4E-3$), with multiple pathway components down-regulated in the DRG tissue of the CFA-treated group 4 days after CFA treatment (Figure 3). These include the phospholipase A2 group IVA (*PLA2g4a*), prostaglandin-endoperoxide synthase 1 (*Ptgs1*), and rat arachidonate 12/15-lipoxygenase. In addition, several genes that might modulate the release of eicosanoids, such as *BCL6* [20], *BTK* [21] and *CNR2* [22], were also reduced 4 days post-CFA injection.

Another group of genes suppressed by CFA encode proteins that may contribute to T cell function. Among

these genes, *IL21R* [23], *IL2RB* [24], *RHOH* [25], *CNR2* [26], *IKZF1* [27], *BCL6* [28], *PTGS1* [29], *CASP8* [30], and *CD52* [31] were all down-regulated. In addition, several genes related to T-cell function were increased (Table 2) in the CFA treated rat DRGs. Examples include *IL22RA2* [32]; *CD7* [33]; *Lgals2* (also called galectin-2) [34], and *IL2RG* [35].

Genes that were up-regulated in the DRG of CFA treated rats, include some that have been previously shown to play a role in pain and neuronal growth, differentiation and prevention of neuronal death. These include neuromedin U, heptocyte growth factor, steroid-5-alpha-reductase (*SRD5A2*), *SLC30A3*, *PC4*, *SFRS1* interacting protein 1 (*PSIP1*), *PER2*, *ZHX2* and *ENC1*. The functions of these are discussed in the discussion section.

Connectivity Map Results

Using the list of differentially expressed genes between the CFA-treated and naïve rats as a DRG CFA signature, we evaluated an internal version of the Broad's Connectivity Map to identify compounds that display inverse connectivity with this CFA signature. This approach yielded five compounds that met the criteria with a p -val < 0.005 and a connectivity score < -0.5 (Figure 4). Based on their negative connectivity with the CFA signature, we hypothesized that these compounds could potentially reduce pain in the CFA model. To test this further, we selected one of the most strongly inferred compounds, phenoxybenzamine, for follow up experiments in the 4 day CFA model to examine its antinociceptive potential.

Phenoxybenzamine in the 4 day CFA assay

Phenoxybenzamine was evaluated in the 4 day CFA model using mechanical allodynia as an outcome measurement and with naproxen as a positive control. When compared to the vehicle treated rats, naproxen (10 mg/kg) reversed mechanical allodynia by 93% (P < 0.01, Figure 5). Phenoxybenzamine, at 10 mg/kg was almost as efficacious as naproxen and reversed mechanical allodynia by 82% (P < 0.05, Figure 5).

Discussion

In this study, we first conducted gene expression microarray experiments to examine transcriptional profiles in the ipsilateral dorsal root ganglia (DRG) of adult rats in a 4 day CFA model of inflammatory pain and generated a rat CFA model gene signature. Our second aim was to identify compounds that were negatively connected to this CFA gene signature using the Connectivity Map approach. Our third objective was to test the hypothesis that compounds identified in this way could reduce CFA-induced pain in this 4 day model. Reports on

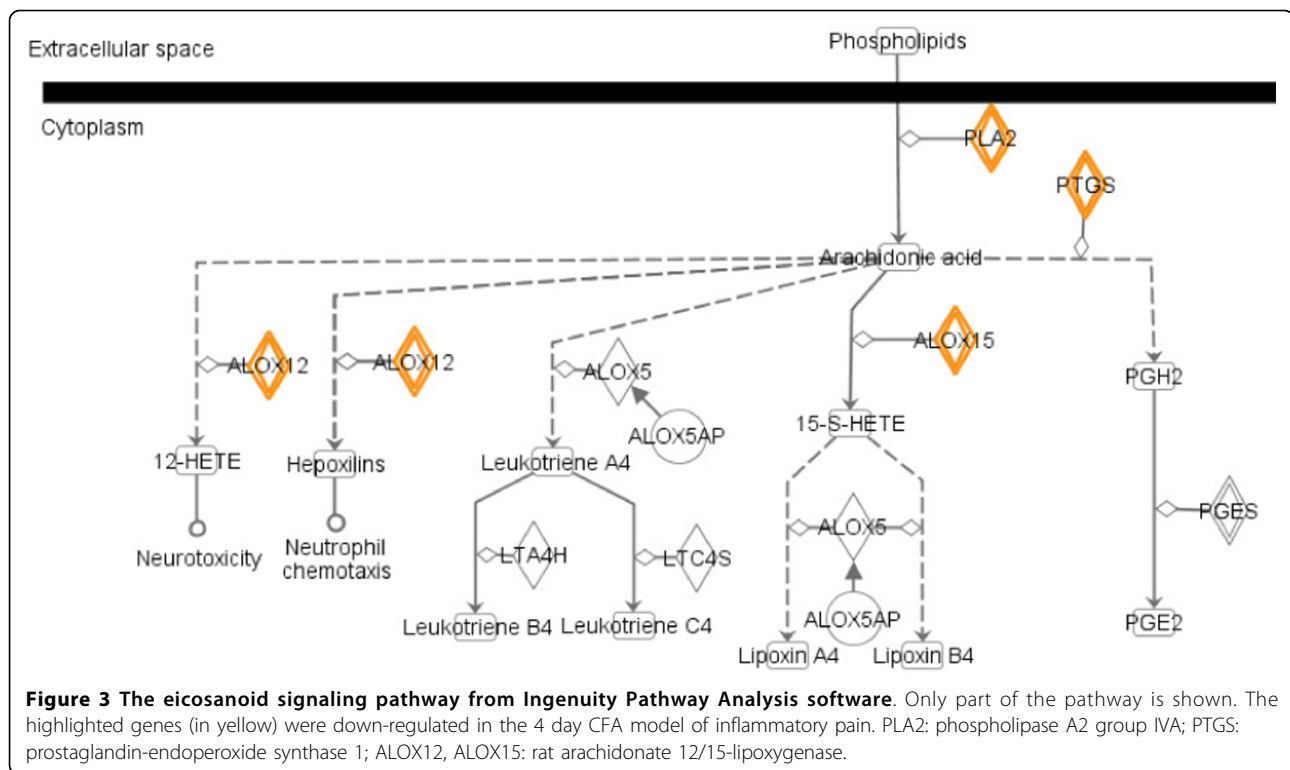
Table 1 Immune and inflammatory response genes down-regulated in the DRG by intraplantar CFA

Gene Symbol	Gene Name	Fold
Adamts12	ADAM metalloproteinase with thrombospondin type 1 motif, 12	-1.6
Alox15	arachidonate 15-lipoxygenase	-2.1
Bcl6	B-cell CLL/lymphoma 6	-1.6
Btk	Bruton agammaglobulinemia tyrosine kinase	-1.5
Ca6	carbonic anhydrase VI	-1.6
Casp8	caspase 8, apoptosis-related cysteine peptidase	-1.5
Cd22	CD22 molecule	-2.2
Cd52	CD52 molecule	-1.9
Cnr2	cannabinoid receptor 2 (macrophage)	-1.7
Colec12	collectin sub-family member 12	-1.9
Cpa3	carboxypeptidase A3 (mast cell)	-2.4
Ctgf	connective tissue growth factor	-1.6
Ctse	cathepsin E	-1.8
Cybb	cytochrome b-245, beta polypeptide	-1.6
Efna4	ephrin-A4	-1.5
F13a1	coagulation factor XIII, A1 polypeptide	-1.8
Fgr	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog	-1.5
Hcls1	hematopoietic cell-specific Lyn substrate 1	-1.8
Hecw2	HECT, C2 and WW domain containing E3 ubiquitin protein ligase 2	-1.6
Ikzf1	IKAROS family zinc finger 1 (Ikaros)	-1.8
Il1r2	interleukin 1 receptor, type II	-1.6
Il21r	interleukin 21 receptor	-1.5
Il2rb	interleukin 2 receptor, beta	-1.6
Klra7	killer cell lectin-like receptor, subfamily A, member 7	-1.6
Lox	lysyl oxidase	-1.6
Npas2	neuronal PAS domain protein 2	-1.7
Odz4	odz, odd Oz/ten-m homolog 4 (Drosophila)	-1.5
Pdgfrl	platelet-derived growth factor receptor-like	-1.7
Pla2g4a	phospholipase A2, group IVA (cytosolic, calcium-dependent)	-1.9
Ptgs1	prostaglandin-endoperoxide synthase 1	-1.9
Rara	retinoic acid receptor, alpha	-1.6
Si	sucrase-isomaltase (alpha-glucosidase)	-1.6
Sit1	signaling threshold regulating transmembrane adaptor 1	-1.5
Spag16	sperm associated antigen 16	-1.6
Tnfrsf14	tumor necrosis factor receptor superfamily, member 14	-1.7
Tpsb2	tryptase beta 2	-1.5
Xdh	xanthine dehydrogenase	-1.9

transcriptional profiling in inflammatory pain models are scarce. Yang et. al. examined gene expression changes in the inflamed tissues and the corresponding DRGs in a carrageenan model using a mini-array containing 100 cytokines, chemokines and related receptors [8]. Parkitna et. al. conducted transcriptional profiling of 4000 genes in the lumbar section of rat spinal cord of a 3-day and a 14-day CFA model [4]. The authors found a dramatic shift in the regulation of secretory vesicle trafficking in the spinal cord in the CFA model. The authors did not profile the L4-L5 DRG. However, they selected 4 transcripts that were changed in the spinal cord and tested them in the L4-5 DRG with qPCR.

Consistent with our results, none of the 4 transcripts were significantly changed in the CFA DRG comparing to the naïve rats. To the best of our knowledge, there are no published DRG whole genome microarray studies following 4 days CFA inflammation in rats. We used the CFA model since commonly used NSAIDs such as naproxen that are used to treat OA pain are effective in this model and it is thus frequently used pre-clinically to identify new therapies for OA pain [11,12].

By clustering differentially expressed genes by function or related pathways, we identified a number of genes that are implicated in the modulation of the immune system. Surprisingly, we found that the expression of



these genes is decreased in the CFA-treated rats, suggesting suppression of the immune system at day 4 post-CFA treatment in this model system. These include genes in the eicosanoid signaling pathway, including phospholipase A2 group IVA (PLA2g4a), prostaglandin-endoperoxide synthase 1 (Pgs1), and rat arachidonate 12/15-lipoxygenase. In addition, several genes such as BCL6, BTK and CNR2 that may modulate release of eicosanoids [20-22] are also reduced in the ipsilateral DRGs four days after CFA treatment. We speculate that these genes are early players that initiate inflammation and pain, and their signals are carried forth by other pathways that maintain chronic pain/inflammation with time.

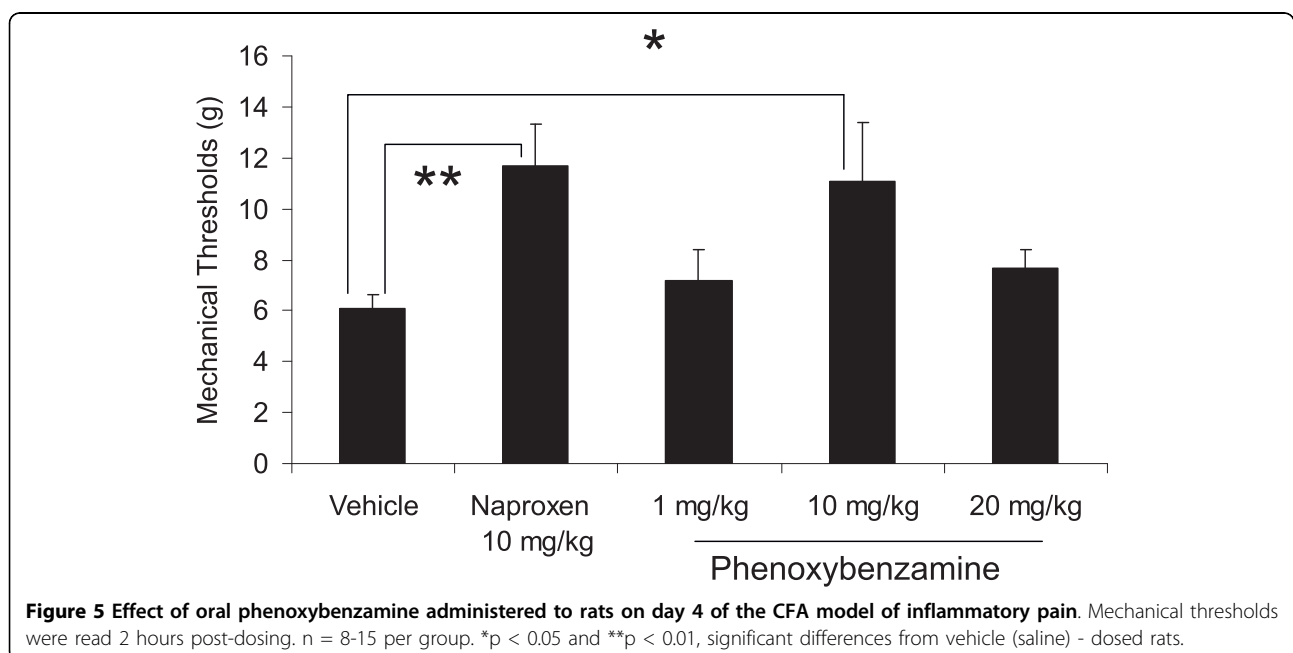
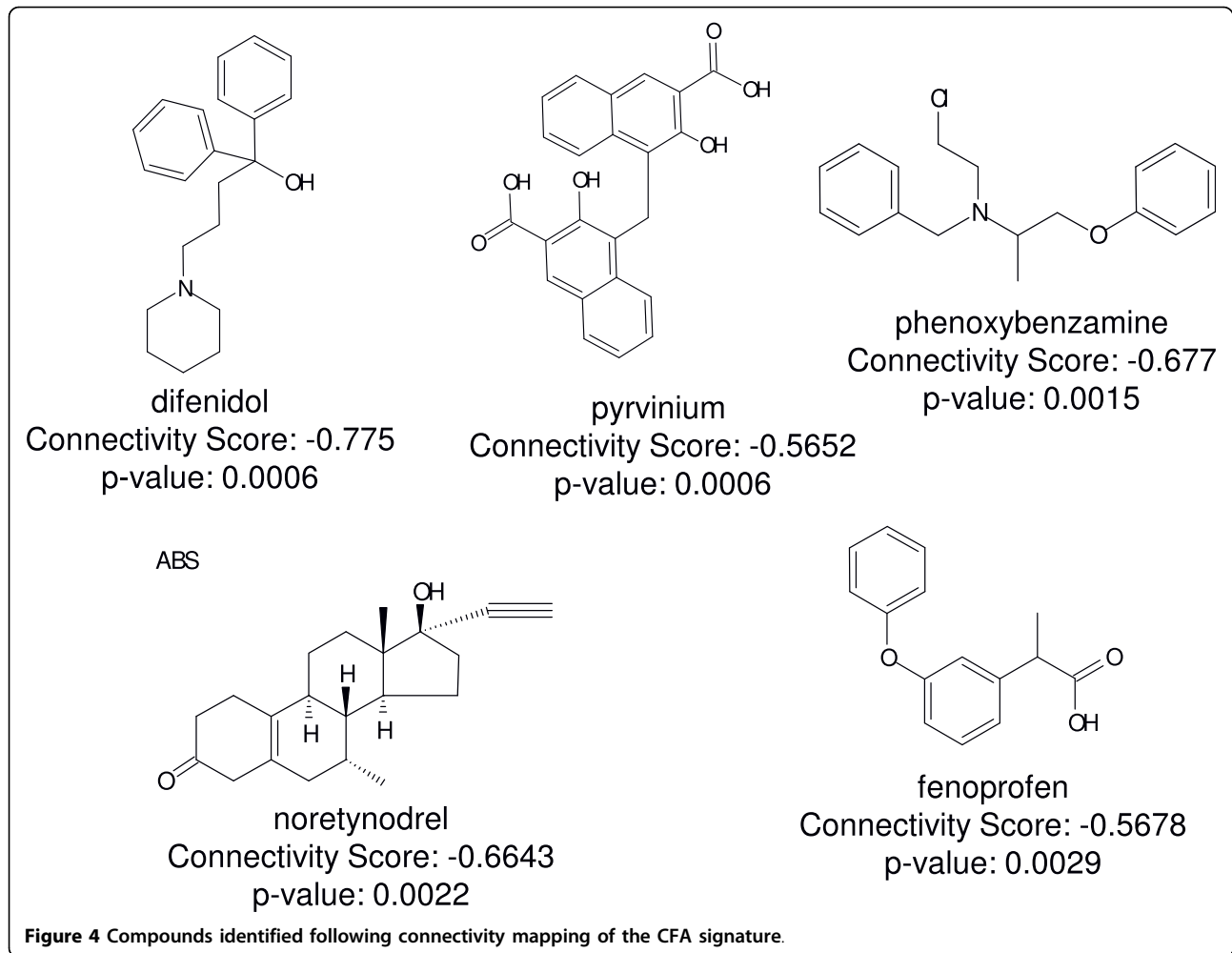
Another group of genes down-regulated by CFA treatment encode proteins that regulate T cell function. These include IL21 receptor subtype II, IL2 receptor beta, RHOH (Ras homolog gene family member H), cannabinoid receptor 2, IKZF1, CASP8, and CD52 [23-31]. It is also noteworthy that IL22RA2, a soluble receptor for IL22 that antagonizes IL22 which is primarily produced by activated T cells [32], CD7 which promotes T cell apoptosis [33], Lgals2 (galectin-2), a proapoptotic effector of activated T-cells [34], and IL2 receptor gamma [35] are all increased in the CFA group compared to the naïve group. This may represent a mechanism of communication by the DRG that modulates T cell activity during inflammation or pain

processing. In this regard, T cells have been recently suggested to play a role in neuropathic pain [36,37]. In a model where DRG inflammation was induced by administration of epidural zymosan in incomplete Freund's Adjuvant [7], an increase of CCL2 (MCP-1) among other cytokines was observed, while IL-2 and IL-12 were decreased on day 3 post inflammation. Similarly, in a peripheral inflammation model induced by hind paw injection of carrageenan, both the Scya2 (the CCL2 mRNA) and the gene product MCP-1 (CCL2) were up-regulated following nociceptive stimuli [8]. In that study, Scya2 mRNA was increased about 2-fold in the ipsilateral versus contralateral rat DRG and lasted up to 72 hour post carrageenan. Scya2 mRNA was localized to a subpopulation of vanilloid receptor 1 (TRPV1) containing neurons. Stimulation by a TRPV1 agonist, resiniferatoxin increased expression of Scya2 mRNA. These results are very similar to our findings where we found increased expression of the CCL2 gene in the DRG following CFA injection. CCL2 has also been reported to be up-regulated in the DRG in models of neuropathic pain ([38] and [39] for review). Thus, CCL2 seems to be a common signal elicited in different preclinical pain models.

Other genes that are induced in the DRG of CFA treated rats, include some that encode proteins either known to play a role in pain, promote neuronal growth/differentiation, or prevent neuronal death. One

Table 2 Genes up-regulated in the DRG by intraplantar CFA

Gene Symbol	Gene Description	Fold
Ccl2	chemokine (C-C motif) ligand 2	1.7
Enc1	ectodermal-neural cortex 1	1.8
Hgf	hepatocyte growth factor	1.5
Nmu	neuromedin U	1.5
Per2	period homolog 2	1.6
Psip1	PC4 and SFRS1 interacting protein 1	1.6
Slc30a3	solute carrier family 30	1.7
Srd5a2	steroid-5-alpha-reductase, alpha polypeptide 2	1.7
Zhx2	zinc fingers and homeoboxes 2	1.6
Cacna1d	calcium channel, voltage-dependent, L type, alpha 1D subunit	1.5
Cd7	Cd7 molecule	2.7
Defb50	defensin beta 50	1.6
Dennd4b	similar to brain specific protein 4	1.5
Dnah1	dynein, axonemal, heavy chain 1	1.5
Foxred2	similar to hypothetical protein FLJ23322	1.5
G1p2	interferon, alpha-inducible protein (clone IFI-15K)	1.5
Gmcl1	germ cell-less homolog 1	1.6
Hmgn2	high mobility group nucleosomal binding domain 2	1.6
Hnf4a	hepatocyte nuclear factor 4, alpha	1.8
Hpse2	heparanase 2	1.6
Htr2b	5-hydroxytryptamine (serotonin) receptor 2B	2.1
IgG-2a	gamma-2a immunoglobulin heavy chain	1.5
Il22ra2	interleukin 22 receptor, alpha 2	1.7
Il2rg	interleukin 2 receptor, gamma	1.5
Klhl34	similar to hypothetical protein FLJ34960	1.6
Klk9	kallikrein related-peptidase 9	1.7
Lgals2	lectin, galactoside-binding, soluble 2	1.5
Lrrfp1	leucine rich repeat (in FLII) interacting protein 1	1.6
Muc4	mucin 4, cell surface associated	1.6
Olr1092	olfactory receptor 1092	1.5
Olr1229	olfactory receptor 1229	1.6
Olr1366	olfactory receptor 1366	1.5
Olr233	olfactory receptor 233	1.8
Olr327	olfactory receptor 327	1.8
Olr651	olfactory receptor 651	1.6
Olr859	olfactory receptor 859	1.9
Podxl	podocalyxin-like	2.4
Pole4	polymerase (DNA-directed), epsilon 4 (p12 subunit)	1.6
Pot1b	similar to Protection of telomeres 1	1.9
Ppp1r8	protein phosphatase 1, regulatory (inhibitor) subunit 8	1.5
Rnf43	ring finger protein 43	1.5
Scn7a	sodium channel, voltage-gated, type VII, alpha	1.6
Sele	selectin, endothelial cell	2.1
Spetex-2F	Spetex-2F protein)	1.7
Stac2	SH3 and cysteine rich domain 2	1.6
Tm4sf20	transmembrane 4 L six family member 20	1.7
Tmbim4	transmembrane BAX inhibitor motif containing 4	1.6
Tmprss3	transmembrane protease, serine 3	1.5
Tmprss8	transmembrane protease, serine 8 (intestinal)	1.7
Ttl3	similar to tubulin tyrosine ligase-like family, member 3	1.6
Vom2r40	vomer nasal 2 receptor, 40	1.5



that stands out is neuromedin U (NMU) which has been shown to have an emerging physiological role in nociception upon binding to the NMU receptor 2 [40]. Mice deficient in NMUR2 displayed reduced thermal nociceptive responses in the hot plate test, decreased thermal hyperalgesia following capsaicin injection and reduced the late phase response in the formalin test [41]. In other studies, NMU inhibited inflammation-mediated memory impairment and neuronal cell-death in rodents [42]. Ketterer et. al. (2009) have shown recently that NMU may signal via the hepatocyte growth factor (HGF) c-Met pathway. In our study, HGF is also increased in the DRG following CFA treatment. HGF is a pleiotropic cytokine which partly functions to promote neuronal survival and growth [43]. HGF cooperates with nerve growth factor (NGF) to enhance axonal outgrowth from cultured dorsal root ganglion (DRG) neurons. HGF also enhances the neurotrophic activities of NGF in vivo where Met receptor signaling is required for the survival of a proportion of DRG neurons [44].

Another gene which is up-regulated in the DRG is the steroid-5-alpha-reductase (SRD5A2) which is a key enzyme in the conversion of several Δ^4 -3keto steroids, such as testosterone, progesterone, aldosterone and corticosterone, into their respective reductase derivatives. Morphine has been shown to increase SRD5A2 level and SRD5A2 inhibitor finasteride potentiates the antinociceptive effect of morphine, prevents the development of morphine tolerance in rats, suggesting that SRD5A2 plays a role in pain perception [45]. SLC30A3 which is also increased in the current study is responsible for transport of zinc into synaptic vesicles. It may have a role in neuropathic pain [46].

We also observed a number of genes whose expression in DRG is modulated by CFA, though their roles in nociception have not been established, they may have indirect linkages to pain plasticity. Two genes involved in regulating circadian rhythm are modulated in the DRG of CFA treated rats versus the naïve rats. Pain perception is influenced by the circadian rhythm in humans and in animals [47]. Expression of PER2 is increased, whereas expression of NPAS2 is decreased. Per/Cry form a heterodimer that interacts with the NPAS2/BMAL1 heterodimer to inhibit the transcription of Per and Cry [48]. SFRS1 interacting protein 1 (PSIP1, or Lens epithelium-derived growth factor (LEDGF)) is up-regulated in response to stress and enhances the survival of neurons in the retina and optic nerve [49]. We speculate that the increased expression of PSIP1 we observed may support neuronal growth in the DRG. ZHX2, a transcriptional regulator of neural progenitor cells, is also up-regulated in the DRG of CFA treated rats. Blocking ZHX2 function causes neuronal differentiation,

whereas overexpression of ZHX2 or its ephrin-B1 intracellular domain disrupts the normal differentiation of cortical neural progenitor cells [50]. ENC1 (or NRP/B), also a regulator of neuronal differentiation is increased in the DRG of CFA treated rats. Overexpression of NRP/B significantly induced neurite outgrowth in PC12 cells, whereas inhibition of NRP/B by antibodies or siRNA inhibited neurite outgrowth and suppressed the NGF-induced outgrowth of neurites [50,51]. In summary, we have found a number of genes which regulate neuronal growth in the CFA model, suggesting a neuronal growth component to this peripheral inflammation model.

Using the Connectivity Map approach, we identified 5 compounds in the Broad database that were negatively connected with the CFA signature. These compounds belong to different chemical classes and their structural properties are different. They also bind to different targets. Fenoprofen, an NSAID, was among them. Another compound we identified with this approach was pyrvinium, a non-competitive androgen receptor inhibitor [52]. Intrathecal administration of testosterone, an androgen derived from DHEA, has been shown to cause analgesia in neuropathic rats [53]. Difenidol, also identified by Connectivity Mapping is used for treatment of vertigo. The precise molecular target of this drug is not known, although recently it was found to be a ligand for muscarinic receptors: M1, M3 and M4 [54], and may thus play a role in processing of pain stimuli [55].

Phenoxybenzamine, a non-selective α -blocker [56] was also identified as having negative connectivity with the CFA pain signature. Previous studies have reported efficacy of phenoxybenzamine in patients with various pain conditions. In one study, 40 patients with causalgia were treated successfully for their pain with phenoxybenzamine [57]. In a case report, 3 out of 4 patients got relief from complex regional pain syndrome type I with phenoxybenzamine [58]. In the current study, we examined effects of phenoxybenzamine in the 4 Day CFA model to determine whether the Connectivity Map approach could identify compounds that had analgesic potential in this model. Our data confirm the antinociceptive properties of phenoxybenzamine; this effect may be due to a direct blockade of adrenergic receptors that may have become supersensitive to catecholamines or perhaps due to possible sympathetic sprouting, as a result of increased expression of nerve growth regulating genes. Sympathetic sprouting in the DRG has been shown to occur in a study that examined gene expression profiles at 3 days post zymosan in incomplete Freund's adjuvant [7]. Furthermore, antinociception has been reported for phenoxybenzamine in the rat hot plate test [59], the mouse tail flick test [60] and mouse writhing tests [61]. Although we do not yet understand

the basis for the diminished efficacy of phenoxybenzamine at the higher dose tested here (20 mg/kg), it is possibly due to excessive antagonism of the adrenergic receptors in addition to other mechanisms, such as its agonist-antagonistic properties [60]. Regardless, our data suggest that the CFA pain model may have broader utility for testing compounds that modulate pain involving the sympathetic nervous system.

Conclusion

We have established a 4 day CFA signature in the DRG that we subjected to a Connectivity Map approach and identified a compound already known to have a role in the treatment of pain, such as fenoprofen. Another of these compounds, phenoxybenzamine, has been reported in the literature to treat complex regional pain syndrome, and our studies confirm the analgesic properties of this compound in the 4 day CFA model. We conclude that use of the Connectivity Map approach to accompany gene expression microarray and behavior studies, represents a potentially fruitful way to identify novel pain therapies.

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Authors' contributions

MC conceived of the study, analyzed the gene expression microarray data, generated data from connectivity mapping and drafted the manuscript. SS performed part of the behavior studies. AT participated in design of the study. MB participated in design of the study and editing the manuscript. FK conceived of the study, designed and coordinated the study, performed part of the behavior studies, and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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