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## Microarray analysis of gene expression following the formalin test in the infant rat<sup>☆</sup>

Gordon A. Barr<sup>a,b,c,\*</sup>, Puhong Gao<sup>a</sup>, Shaoning Wang<sup>a</sup>, Jianxin Cheng<sup>a</sup>, J. Qin<sup>d</sup>,  
Etienne L. Sibille<sup>b,e</sup>, Paul Pavlidis<sup>d</sup>

<sup>a</sup>Department of Developmental Psychobiology, New York State Psychiatric Institute, New York, NY, USA

<sup>b</sup>Department of Psychology, Hunter College, New York, NY, USA

<sup>c</sup>Department of Psychiatry, Columbia University Medical Center, New York, NY, USA

<sup>d</sup>Division of Bioinformatics, Columbia University Medical Center, New York, NY, USA

<sup>e</sup>Department of Neuroscience, New York State Psychiatric Institute, New York, NY, USA

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### Abstract

Injury and pain experienced by the infant results in immediate changes in pain sensitivity that last into adulthood. These long-term changes are likely initiated by altered gene expression. Here we measured how injury alters gene expression in the lumbar spinal cord early and late in the preweaning period of the rat. The expression of large numbers of genes was altered significantly at 3 days of age, when injury has long-term consequences. The functional classes of altered genes included transcription factors, cell death related and metal ion genes. The intensity of the stimulus in the 3-day-old pups induced changes in different classes of genes. Fewer changes were noted at 21 days of age. The increased expression of transcription factors and decreased expression of genes whose products are protective against cell death are hypothesized to underlie the long-term changes that are seen after injury in the neonate.

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### 1. Introduction

The infant of many species, including humans, reacts to noxious input with pain like responses. Neonates respond to noxious stimulation at or before birth, and nociceptive circuits continue to develop and change well into postnatal life [for example (Fitzgerald, 1991)]. Indeed, the young infant is more sensitive to noxious stimuli than is the older animal (Fitzgerald et al., 1988) and injury in the infant

results in long-term increases in subsequent reactivity to painful stimuli (Lidow et al., 2001; Ruda et al., 2000). Furthermore, qualitative changes occur in nociceptive processing at the end of the 2nd week of life in the rat, when adult like responses appear to noxious inflammatory stimuli and after which the increased plasticity to nociceptive stimuli declines. Many of these functional changes are due to changes in spinal cord physiology and anatomy.

There are functional postnatal changes in the afferent fibers to the spinal cord over the first 2 weeks of life (Fitzgerald et al., 1987). A-fibers mature early and are functional during the first week of life, terminate in the superficial cord, and withdraw at the end of the third week to their adult distribution (Fitzgerald and Jennings, 1999). C-fibers mature later, becoming functional during the second postnatal week (Fitzgerald et al., 1984; Jennings and Fitzgerald, 1998). Sensitization of the spinal cord can

<sup>☆</sup> The Cel files and details of the experimental design and analysis, in MIAME format, are available at <http://maxweber.hunter.cuny.edu/%7Egabbarr/research/form.htm>.

\* Corresponding author. Address: Department of Developmental Psychobiology, New York State Psychiatric Institute, Unit 40, NYSPI, 1051 Riverside Drive, 10021 New York, NY, USA. Tel.: +1 212 543 5694; fax: +1 212 543 5467.

E-mail address: gab5@columbia.edu (G.A. Barr).

113 occur before the maturation of C-fibers in complete and  
 114 differs from the classic C-fiber induced windup seen in the  
 115 adult (Jennings and Fitzgerald, 1998). This sensitization  
 116 peaks at postnatal day 6, decreasing until the sensitization is  
 117 no longer observed at postnatal day 21 (Jennings and  
 118 Fitzgerald, 1998). In rats 812-day-old, repeated low  
 119 frequency stimulation evokes a temporal summation of  
 120 synaptic activity that generates a progressive depolarizing  
 121 ventral root potential, a measure of windup (Hedo et al.,  
 122 1999; Herrero et al., 2000; Thompson et al., 1995, 1994,  
 123 1992). These studies demonstrate that infant rats respond  
 124 behaviorally to acute nociceptive stimuli, and can develop  
 125 hyperalgesia/allodynia after inflammation-induced injury.  
 126 The mechanisms underlying the short and long-term  
 127 plasticity in the spinal cord are almost completely unknown  
 128 in the infant.

129 Because there now exist a reasonable amount of data  
 130 describing the phenomenology of nociception in the infant,  
 131 we are poised to ask a number of questions related to the age  
 132 dependent neural responses to pain: what are the develop-  
 133 mental changes in the neural substrates that affect the  
 134 pattern of responses to noxious stimuli at different ages;  
 135 what changes in neural substrates are responsible for the  
 136 increased plasticity of the infant compared to the adult? Part  
 137 of the answer to these questions lies in the changing cascade  
 138 of genetic events that underlies the maturation of the  
 139 nervous system. Tissue injury results in a change in gene  
 140 expression that may account for both the acute and chronic  
 141 consequences of that injury. Gene expression induced by  
 142 injury can explain the qualitative differences in the response  
 143 to noxious insult that occur during the preweaning period.  
 144 Thus, the goal of this study is to identify changes in gene  
 145 expression shortly after injury in 3 and 21-day-old rats using  
 146 microarray technology.

## 149 2. Methods

### 151 2.1. Subjects

153 Pups were from litters bred in our colony at NYSPI. Breeding  
 154 was by methods previously described (e.g. Barr and Wang, 1992).  
 155 Dams were checked twice daily for births and any pups found on  
 156 that day were designated 0 days of age. Litters were culled to 1012  
 157 pups on the day after birth as needed. All work is done in  
 158 accordance with the provisions of the HHS 'Guide for the Care and  
 159 Use of Laboratory Animals' and the 'Principles for the Utilization  
 160 and Care of Vertebrate Animals' and were approved by IACUC at  
 161 NYSPI.

### 162 2.2. Treatment

164 At 3 or 21 days of age, the litter was removed from the dam and  
 165 kept warm in an incubator. In these experiments, we used the  
 166 formalin model. We chose formalin in part because it is a well  
 167 worked out nociceptive model in the infant (Guy and Abbott, 1992;  
 168 Teng and Abbott, 1998; Yi and Barr, 1995). Moreover, we wanted

169 a stimulus that was not acute but was reasonably limited in  
 170 duration. Classic thermal or mechanical stimuli are too brief.  
 171 Moreover, in our hands, pups do not respond to either CFA or  
 172 carrageenan with obvious pain responses; rather they show  
 173 increased sensitivity to subsequent noxious stimuli. We wanted a  
 174 stimulus that produced the clear nociceptive response that is  
 175 produced by the formalin injection. Three-day-old pups were  
 176 injected with 5 or 20  $\mu$ l of formalin (2%) and 21-day-old pups with  
 177 200  $\mu$ l, 2% in the plantar surface of the paw. The two volumes for  
 178 the three-day-old pups served two purposes. First, it was an  
 179 experimental manipulation to determine the effects of different  
 180 intensity stimuli at this young age; second, it was an attempt to  
 181 provide some match for the older pups. We state 'attempt' because  
 182 it is impossible to equate a priori levels of nociceptive stimuli at  
 183 different ages. Both the size and composition of the paw changes  
 184 with age and at different ages, different mechanisms are engaged.  
 185 For example, at 3 days of age, there is no evidence for neurogenic  
 186 edema (Fitzgerald and Gibson, 1984). Therefore, although we used  
 187 different volumes at 3 days of age and a higher volume at 21 days  
 188 of age, whether they produce equivalent levels of nociceptive input  
 189 is unknown. Moreover, other processes in response to injury differ  
 190 between the two ages. For example, neurogenic edema first  
 191 develops at about 14 days of age (Fitzgerald and Gibson, 1984)  
 192 although there is edema due to tissue damage at 3 days of age. In  
 193 published work (Gupta et al., 2001), we showed slight differences  
 194 in edema in 3- and 21-day-old pups given formalin (125 vs. 115%  
 195 of controls; 10  $\mu$ l, 2%). In unpublished data, we found that CFA  
 196 in different volumes also produced only slight differences in edema  
 197 (3-day-old 50  $\mu$ l, 142%; 21-day-old 200  $\mu$ l, 131%).

198 In these experiments, controls were removed but not injected.  
 199 Saline controls were not used because we did not want to risk  
 200 inducing gene changes even with a control injection. The omission  
 201 of that control does not allow us to distinguish between  
 202 inflammatory pain and acute pain from the injection. Because  
 203 formalin includes both an acute pain and sensitized phase, it is not  
 204 clear that the needle injection control would have allowed that  
 205 either. Four independent replicates from four different litters were  
 206 assayed. In our experimental design, we tested littermates,  
 207 extracted and labeled RNA and assayed the tissue at the same  
 208 time. We also assayed different ages at the same time

### 209 2.3. Dissection

210 All pups were killed 2 h after injection by decapitation and the  
 211 lumbar spinal cord (L4/5) rapidly removed. Two hours provided a  
 212 window into early changes in gene expression, although we do not  
 213 know which of the early changes results in later consequences. The  
 214 side ipsilateral or contralateral to the injury was dissected out by  
 215 midsagittal section. Controls were treated the same except that the  
 216 cord was not divided. Both dorsal and ventral horns were combined  
 217 in all tissue.

### 218 2.4. Isolation of total RNA

219 The frozen harvested tissue was ground into a powder by pestle  
 220 in eppendorf tubes. Total RNA was isolated from the frozen tissue  
 221 by the guanidinium isothiocyanate method (Ambion Research) and  
 222 treated with RNase free DNase (1 U/mg RNA) in the presence of  
 223 RNase inhibition (1 U/ml reaction volume) for 30 min at 37 °C.  
 224 Following DNase treatment, the total RNA samples were

extracted with phenochloroform and precipitated with two volumes of ice cold EtOH. Samples were resuspended in DEPC treated water and RNA concentrations assessed by UV spectrophotometry. RNA concentrations were normalized with DEPC treated water. Denaturing agarose gel electrophoresis measured the relative intensity of the 18S and 28S rRNA bands and assessed the integrity of the RNA sample. RNA samples were stored frozen in 0.2 ml eppendorf tubes in 96 well racks.

2.5. Reverse transcription

First strand cDNA synthesis was carried out using MMLV reverse transcriptase (GibcoBRL), DNasefree, total RNA (3 µg/reaction) and polyT primers. Samples were stored in 0.2 ml tubes in a 96 well format for organization and convenience at -80 °C.

2.6. Microarrays

For these studies, we used the rat neurobiology array from Affymetrix, which is a subset of neural specific gene probes. The chip contains oligonucleotide sequences representing about 1260 neurobiology related genes.

2.7. Microarray analysis

As a first step, each chip was visually inspected using MAS 5.0 software from Affymetrix, alignment checked and any contamination was masked (removed from analysis). These included dust, scratches, and water spots. About 50% of the chips required some minor masking. We then analyzed each chip with dChip (Li and Wong, 2001a,b) software to ascertain the percent of array and probe outliers (Note we did not use dChip to normalize data or to obtain expression values). The percent of array and probe outliers was consistently low and no arrays were discarded. Data were normalized and expression levels calculated using a variance stabilizing normalization method for all chips (Huber and Vignette, 2002; Huber et al., 2003). All data were then transformed to log 2 values.

It should be noted that we use only the perfect match data (PM). The mismatch data (MM), designed to provide controls for non-specific hybridization and background, shows hybridization that is systematic and of unknown source. Thus in our experience and as recommended by others (Bolstad et al., 2003; Irizarry et al., 2002; Naef et al., 2001), we ignore those data. We compared expression levels in normalized arrays, with both the same sample hybridized to different chips (e.g. technical replicates), or the same experimental conditions but different tissue samples hybridized to different chips (e.g. biological replicates).

2.8. Differential expression

To determine which genes were differentially expressed we compared the differences between treated pups and controls for each treatment condition separately using the permutation based method Statistical Analysis of Microarrays (SAM) as implemented in TIGR (Saeed et al., 2002; Tusher et al., 2001). SAM provided probability values that were then corrected using the False Detection Rate (FDR; Benjamini and Hochberg, 1995). The False Detection Rate controls false positive rates, while identifying

those genes that actually change. The FDR is the expected proportion of incorrect rejections of the null hypothesis among all rejections. FDR has increased power over Bonferroni and Holms correction schemes. Please note, however, that the FDR values and alpha levels are conceptually distinct. We used  $\alpha < 5\%$  cutoff for the FDR rate.

2.9. Data mining/clustering

We used a supervised method of clustering to identify clusters of genes whose expression was tightly grouped in an experimental group but not in the controls (Califano et al., 2000). The advantages of this method are that it identifies all statistically significant gene expression patterns in a phenotype group, evaluates the probability of a pattern occurring by chance in the control, provides levels of statistical confidence and discards genes that do not fit those patterns. The method has been applied to a variety of datasets with good results (Klein et al., 2001). The stringency of that phenotype was set by default and we required all treatment and control replicates to follow the phenotype. All described clusters were statistically significant.

2.10. Functional paths

Relating gene changes to functional pathways and biological processes is perhaps the most difficult of the analytic problems. We have approached this issue by use of the Class Score method to provide a level of statistical confidence (in this case  $P < .01$ ) to functional groupings (Pavlidis et al., 2002, 2003). This method determines whether or not there is overrepresentation of genes expressed in an experimental condition compared to chance, in a GeneOntology group. The algorithm combines probability values of genes that are members of single class and examines the likelihood that those combined probabilities occur by chance. Thus, significant classes are defined by the probability of the genes within them.

2.11. Validation of chip data

There is the need to validate the data independently using other methods. To confirm our results, we used quantitative real time PCR (SYBR green, qRT-PCR) and chose 20 genes at different levels of expression as determined by the microarray analyses. We used primers based on the sequence provided by Affymetrix and the literature. These sequences are found in Table 1. For each replicate in each experimental condition, we took RNA samples from the same tissue samples (ipsilateral vs. control only) as used for the microarray experiments. Samples were normalized to internal controls (e.g. actin). We then calculated expression levels of the treated animals relative to the controls for both the microarray data and the qRT-PCR data and averaged the data for the four replicates.

To determine if the message was converted to protein and the anatomical distribution of that protein, we assayed ERG-1 by immunocytochemistry. We chose this transcription factor because the mRNA was expressed differentially at both ages. We used standard ABC immunohistochemistry as described previously (Yi and Barr, 1995) on 30 µm sections from animals perfused 4 h after the formalin injection. The primary antibody was to Krox24 (identical to ERG-1; ●●●).

337 Table 1  
338 qRT-PCR primers name and their sequences

339 Primers	Gene name	Sense	AntiSense
340 AA957930	Tau Microtub. Asso. Prot.	5'CTCAGCCGCCATTAAGTC3'	5'TGCGGACAGGAGAGAAAG3'
341 AB017656	Cholinerg. Muscarin.	5'GCTTCCCATCCAGTTAGAGT3'	5'TGAGCGACATCCTCTTCC3'
342 AF023087	NGFI-Factor A	5'ACTGAGTAGGCGGTGACTTT3'	5'TTCTGAAGGATACACACCACA3'
343 AF030088	NT-I EG 3 (ania3)	5'TACACCTTCAAACAAACATTAGG3'	5'AAGAATCAACTTAACATCTCATGC3'
344 AI009806	Dynein	5'CTACTCAGGCGTTGGAGAA3'	5'GAAGTGTGGTCTCGTGTG3'
345 AI102562	Metallothionein	5'TGCCTTCTGTAGCTTACCC3'	5'CTTGCAGGAGGTGCATTT3'
346 AI176710	NOR-1	5'CCTTTGTTTGCAGTGACCT3'	5'GAAGCTACCGTGACATTGG3'
347 D25233 g	Retinoblastoma protein 1	5'GCCATCACACAGGTTAGTTG3'	5'TTCCTATGATTCTGTCTACTAATTCTAA3'
348 L08492	GABA-A	5'GTTTCATGGCCGTCTGTTAT3'	5'TGTTGAAGTGGTGTGCTT3'
349 M11596	NPY	5'TGAGGAATAACGCTCCAG3'	5'GATGCAAACATACACATCGTC3'
350 M18416	NGFI-A	5'ACTGCTCGACTGTAACCTCAC3'	5'TCACACAAAGGCACCAAG3'
351 M34643	Neurotrophin-3	5'CATGAATTGGCATCTGTCC3'	5'CTGTAAGGGTGTGTAAGTTT3'
352 S67770	Tr-GFBeta IIR	5'TTCACCTACCACGGCTTC3'	5'CCCGTCACTGGATAATGA3'
353 U17254 g	NGFI-B	5'CTC TTATCCCTCCCAGCTC3'	5'TTACAGCAGCGTCAGCTTAT3'
354 U75397	Krox-24	5'ATGCTGCGGTTACCTACTG3'	5'TTTAAGCAAACACAAGTACGAAG3'
355 U90610	CXCR4	5'TCTGTGACCGCCTTACC3'	5'TAGTCTTGAGGGCCTTGC3'
356 X06769	C-fos	5'CACCCTGCCTCTTCTCAA3'	5'CAAGAAGTCATCAAAGGGTTC3'
357 X95882	ATP Gated Ion Chann.	5'TGGATGTGGCTTGGTAGAG3'	5'TGTGGTGTGGTGTGTG3'
358 Y17607	Potassium Chann. Alpha	5'CGGCTTACGCATCATTTCT3'	5'CCGTGTCTGGAACATGAA5'3'

357 The primers are taken from Affymetrix sequences and the literature. The genes were chosen to include a range of expression values, not just overexpressed  
358 genes.

### 3. Results

#### 3.1. Reliability of the data

364 After normalization, we compared the expression values  
365 for different microarrays assayed with the same sample  
366 (technical replicates) or with different samples from the  
367 same experimental condition (biological replicates). In all  
368 cases, there were high correlations between arrays. Plots of  
369 intensity versus variation (M/A plots) show no intensity  
370 biased changes in variability (data not shown). Analysis of  
371 both genes and arrays by dChip, before normalization,  
372 showed small percentages of outliers for each (on average  
373 0.2 and 0.7%, respectively).

#### 3.2. Differential expression

378 To determine if there were age dependent changes in  
379 gene expression, we compared the control animals at 3 and  
380 21 days of age to each other. Approximately 600 genes were  
381 significantly different between the two ages using SAM with  
382 a 5% FDR rate (Benjamini and Yekutieli, 2001; Tusher et  
383 al., 2001). Therefore, separate tests were used for each age  
384 and volume. We compared each experimental group with its  
385 appropriate control using SAM with FDR correction  
386 (Benjamini and Yekutieli, 2001; Tusher et al., 2001). No  
387 genes were determined to be significant on the contralateral  
388 side at either age for either volume of injection; thus, only  
389 the data from the ipsilateral side, compared to the control,  
390 are presented.

391 Of the 1260 genes and EST's, 22 were determined to be  
392 significant for the 3-day-old high intensity group (FDR = <

5%). All were up regulated. For the 3-day-old low intensity  
416 group, 34 were down regulated and two were up regulated.  
417 The genes are listed in Table 2. At 21 days of age, only a  
418 single gene was up regulated (Early Growth Response 1;  
419 ERG-1). For the high volume group, the up regulated genes  
420 were of several classes. Most commonly seen were  
421 immediate early genes such as c-fos and ERG-1 and other  
422 transcription factors. A number of ion-gated channels were  
423 up regulated, most notably potassium channels. For the low  
424 volume group, there were not only more down regulated  
425 genes, but there was more diversity in the nature of those  
426 genes. These include many genes involved in cell growth or  
427 death including caspases, kinases, chemokines, and neuro-  
428 transmitter associated receptors and transporters. The up  
429 regulated genes for this group were both ERG-1 sequences  
430 that were also overexpressed in the high volume group and  
431 at 21 days of age.

#### 3.3. Coherent clusters

436 Supervised clustering identified patterns of expression  
437 that differed significantly between the experimental and  
438 control groups. In all cases, the pattern in the experimental  
439 phenotype was not seen in the controls. The results are  
440 shown in Fig. 1. For the low volume treated 3-day-old pups,  
441 there were also up regulated immediate early genes and  
442 other transcription factors, including ERG-1, JunB, c-fos,  
443 neurotrophin-3, and TNF. There were a large number of  
444 down regulated genes, including a number related to cell  
445 maintenance and cell death, including bcl-2 related genes.

447 For the 3-day-old high volume group, immediate early  
448 genes, transcription factors, and growth factors clustered

449 Table 2  
450 Significant genes as determined by SAM/FDR

451 Probe set ID	Title
452 <i>Three-day-old Ipsilateral side (5 µl)</i>	
453 <i>Up regulated</i>	
454 AF023087_s_at	early growth response 1
455 U75397_s_at	early growth response 1
456 <i>Down regulated</i>	
456 M91595_s_at	insulin-like growth factor binding protein-2
457 S61973_at	NMDA receptor glutamate-binding chain
458 M92076_at	glutamate receptor, metabotropic 3
459 rc_AI231354_at	stress activated protein kinase alpha II
460 D00688_s_at	rat monoamine oxidase A
461 U49930_g_at	caspace 3
462 D84450_at	ATPase, Na <sup>+</sup> /K <sup>+</sup> transporting, beta 3 polypeptide
463 L14323_at	phospholipase C-beta1
464 AF028784_s_at	glial fibrillary acidic protein alpha (GFAP)
465 U90610_g_at	chemokine receptor (LCR1)
466 D17521_at	protein kinase C-regulated chloride channel
467 U77933_at	caspace 2
468 D50093_s_at	prion protein
469 X99267_g_at	presenilin-2
470 M27925_at	synapsin 2
471 AB017912_g_at	MAD homolog 2 (Drosophila)
472 U90610_at	chemokine receptor (LCR1)
473 S73007_g_at	synuclein, alpha
474 M74494_g_at	ATPase, Na <sup>+</sup> /K <sup>+</sup> transporting, alpha 1
475 rc_AA892801_g_at	eukaryotic translation elongation factor 2
476 AF067727_s_at	MAD homolog 1 (Drosophila)
477 S53527_s_at	S100 calcium-binding protein, beta (neural)
478 X51992_at	gamma-aminobutyric acid A receptor, alpha 5
479 M58040_at	transferrin receptor
480 M15191_s_at	tachykinin
481 M28648_s_at	ATPase, Na <sup>+</sup> /K <sup>+</sup> transporting, alpha 3 subunit
482 E01789cds_s_at	protein kinase C, beta 1
483 D90258_s_at	proteasome (prosome, macropain) subunit, alpha type 3
484 AF090113_at	glutamate receptor interacting protein 2
485 AB016160_at	gamma-aminobutyric acid (GABA) B receptor, 1
486 E13541cds_s_at	chondroitin sulfate proteoglycan 5
487 AB004267_at	pregnancy up regulated non-ubiquitously expressed CaM kinase
488 M95762_at	GABA transporter GAT-2
489 X06554cds_s_at	Myelin-associated glycoprotein
490 <i>Three-day-old Ipsilateral side (20 µl)</i>	
491 <i>Up regulated</i>	
492 X06769cds_at	c-fos
493 M18416_at	early growth response 1
494 X06769cds_g_at	c-fos
495 M59980_s_at	K <sup>+</sup> voltage gated channel, Shal-related family, member 2
496 U75397UTR#1_s_at	early growth response 1
497 AF023087_s_at	early growth response 1
498 rc_AI102562_at	metallothionein
499 rc_AI1176662_s_at	early growth response 1
500 rc_AI1176456_at	strong similarity to rat metallothionein-II
501 rc_AI1176710_at	nuclear receptor subfamily 4, group A, member 3
502 M31837_at	insulin-like growth factor binding protein 3
503 X95882_at	purinergic receptor P2X, ligand-gated ion channel, 7
504 Z12152_at	neurofilament 3, medium
505 AF037071_at	C-terminal PDZ domain ligand of neuronal NOS
506 M26643_at	sodium channel, voltage-gated, type 4, alpha polypeptide

Table 2 (continued)

Probe set ID	Title
Z34264_at	K <sup>+</sup> voltage-gated channel, subfamily H, member 1
S49003_s_at	short isoform growth hormone receptor
L00981mRNA#1_at	lymphotoxin A
X60769mRNA_at	CCAAT/enhancer binding protein (C/EBP), beta
D00833_g_at	glycine receptor, alpha 1 subunit
X03347cds_g_at	FBR-murine osteosarcoma provirus genome
U14005exon#1_s_at	calcium channel alpha-1 subunit gene

Here, we present differentially expressed genes in 3-day-old pups following either low volume (5 µl; top) or high volume (20 µl; bottom). The analysis was by SAM (implemented in TIGR) with FDR rates under 5%. There were 36 and 21 genes that were significant for the low and high volume of formalin, respectively. They are presented from top to bottom in order of descending FDR significance. There was but one significant gene at 21 days. All data are from the side ipsilateral to the injection.

and were overexpressed. These include c-fos, several probes for ERG-1, and JunB. In addition, potassium voltage gated channels also clustered. A number of genes were clustered that were down regulated. These include a large number of chemokine related genes including interleukins, chemokine receptors, and mitogen activated protein kinase, ion channels and structural/synaptic genes. These genes did not appear as significantly down regulated in the tests of differential expression and thus require further validation. For 21-day-old pups, many fewer genes clustered into coherent patterns. A variety of genes was clustered, that were both up and down regulated.

3.4. Functional grouping

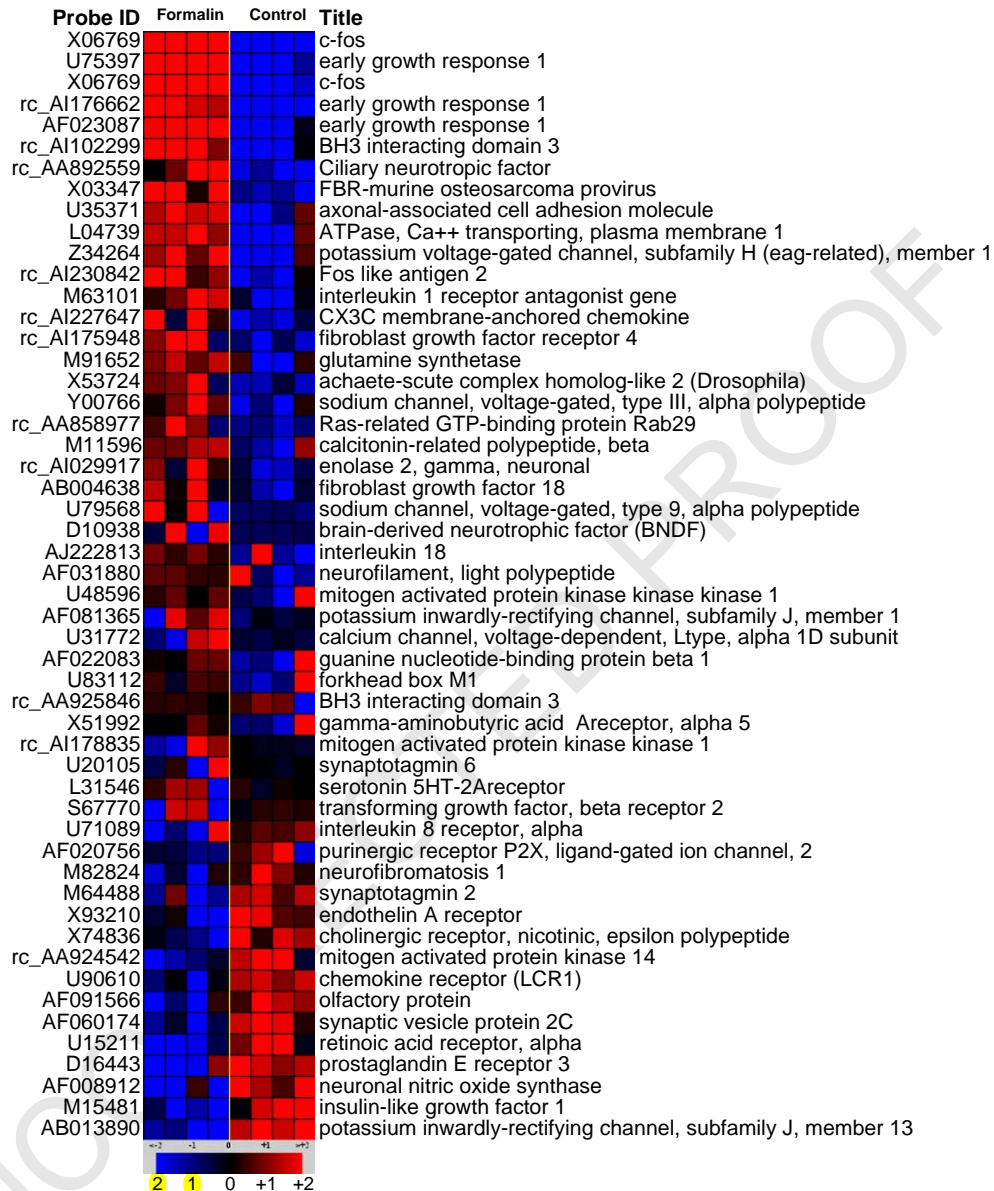
This approach relies on the hypothesis that common phenotypic endpoints may be attained through disruptions of closely related genes or biochemical pathways. In Table 3, we present the GeneOntology classes for both 3-day-old groups as constituted by alterations in related genes. Within each class, we present only members that were considered significant ( $P < .01$ ) in the uncorrected *t*-test. Although the *t*-test probabilities were uncorrected, for a class to be significant, multiple genes need to contribute to the GeneOntology class probability. Genes significant by chance are not likely to cluster in a single GeneOntology class and although some genes are likely to be significant by chance, significant classes are much less likely to be so. Moreover, many of these classes contain genes that were identified in the clustering method, and include transcription factors, metal, DNA binding genes and so forth. For the 3-day-olds, there were very different patterns, however, for the two intensities of stimuli. Significant GeneOntology classes and associated class probabilities are shown in Table 3. For the high volume group, as found by both differential expression and clustering analyses, the classes that were altered included strictly genes related to transcription regulation and zinc binding. In contrast, the

561 lower volume of formalin induced few classes of transcrip- 617  
 562 tion, but affected classes related to cell death, cell growth 618  
 563 and cell maintenance. Most of these genes are anti- 619  
 564 apoptotic. Table 4 lists significant genes in the identified 620  
 565 GeneOntology classes. 621

566 Although there was only a single gene that was 622  
 567 differentially expressed as determined by the FDR method 623  
 568 624  
 569 625  
 570 626

at 21 days of age, the aggregate probability of multiple 617  
 genes shows significant class structure. Formalin treatment 618  
 resulted in changes in amine biosynthesis related to 619  
 autonomic function, including genes coding for tyrosine 620  
 and tryptophan hydroxylase, neuropeptide Y, and vimentin. 621  
 Also altered were two classes of oxidoreductase involving 622  
 prostaglandin synthesis. 623  
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3 day old 20 µl



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Fig. 1. Gene expression clusters from supervised clusters. Red squares denote an increase, blue a decrease. Identical genes with different probe ID's represent different probe sequences for the same genes. In general, the different sequences show good concordance, with the exception of the apoptotic gene 'BH3 interacting domain 3' in the 3-day-old pups given 20 µl formalin. **IA:** ipsilateral side to injection, 20 µl, 3-day-old. Details as above. Note that the clusters overexpressed in the experimental animals (left) are largely growth and transcription factors and ion channels. Those under expressed are more varied but include a number of immune related genes. **IB:** ipsilateral side to injection, 5 µl, 3-day-old. The number of genes input into the program was filtered by raw p tests ( $P < .05$ ). Details as above. Although there are also transcription factors, growth factors and immune related genes overexpressed here, there are also more cell death related genes. **IC:** ipsilateral to the injection, 21 day old pups. Far fewer genes clustered here, consistent with data from all analyses and likely due to the relatively mild stimulus used. Those genes that do cluster are quite different from those of the 3-day-old pups and do not include transcription factors. Of note are the GABAA subunits that have been implicated in pain processing in infants.

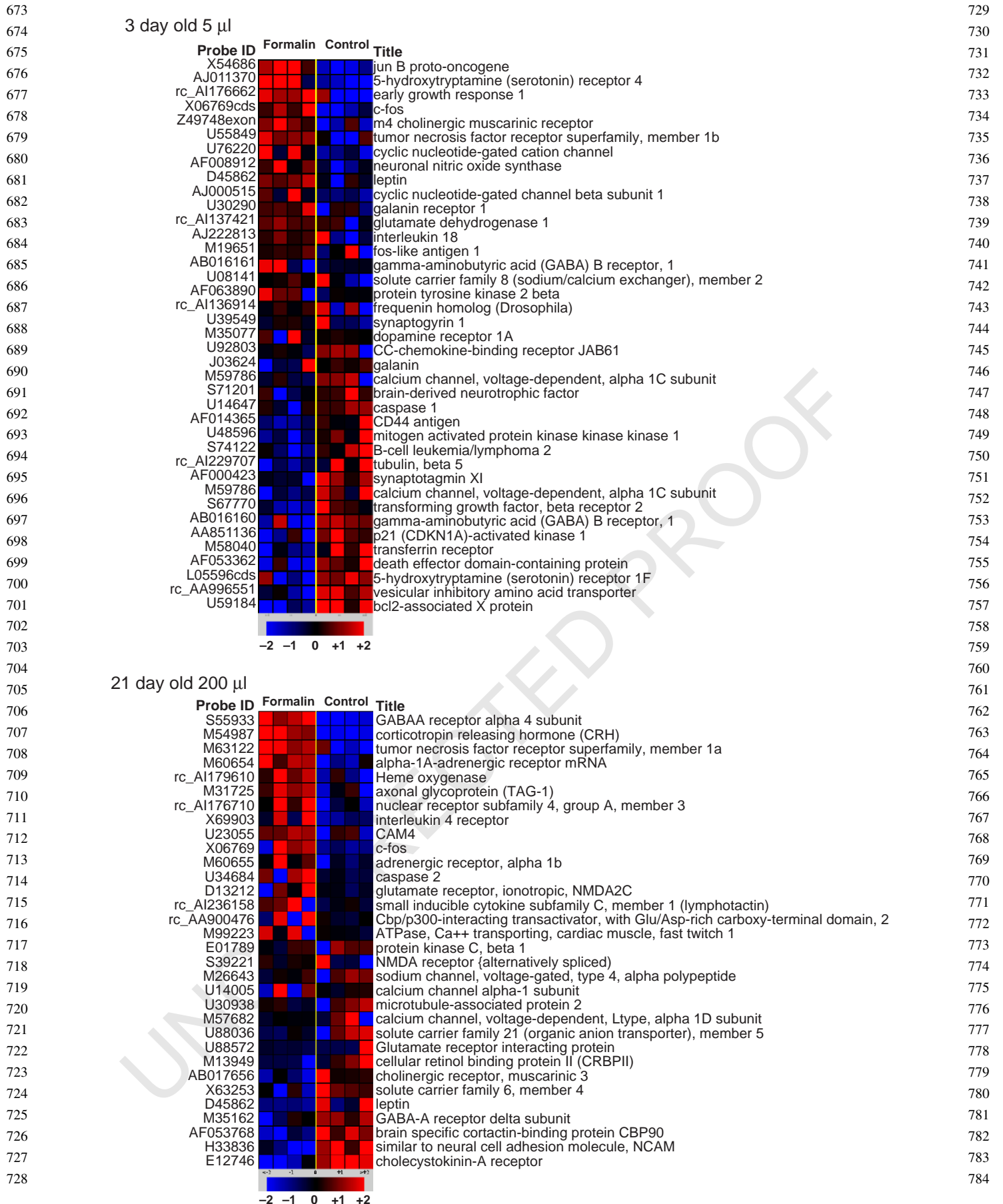


Fig. 1 (continued)

785 Table 3  
786 Class scores

787	GO class	GO number	Bioprocess	pval	843	
788	<i>Three-day-old (5 µl)</i>					844
789	<i>Biological function</i>					845
790	Cell death	GO:0008219	Cell death	0.00309	846	
791		GO:0006915	Apoptosis	0.00217	847	
792		GO:0006916	Anti-apoptosis	0.00315	848	
793	Cell growth	GO:0006818	Hydrogen transport	0.00983	849	
794	Embryogenesis	GO:0007345	Embryogenesis and morphogenesis	0.00900	850	
795	Death	GO:0016265	Death	0.00347	851	
796	<i>Cellular component</i>					852
797	Cytoplasm	GO:0005739	Mitochondrion	0.00172	853	
798	<i>Molecular function</i>					854
799	ATPase	GO:0015662	Ptype ATPase	0.00778	855	
800		GO:0005391	Sodium/potassium	0.00630	856	
801	<i>Three-day-old (20 µl)</i>					857
802	<i>Biological function</i>					858
803	Metabolism	GO:0006139	Nucleobase, nucleoside, nucleotide and nucleic acid metabolism	0.00616	859	
804		GO:0006350	Transcription	0.00202	860	
805		GO:0006355	Transcription regulation	0.00126	861	
806		GO:0006357	Transcription regulation from Pol II promoter	0.00481	862	
807	<i>Molecular function</i>					863
808	<i>Binding</i>					864
809	Metal ion	GO:0008270	Zinc binding	0.0027	865	
810	Nucleic acid	GO:0003676	Nucleic acid binding	0.00096	866	
811		GO:0003677	DNA binding	0.00106	867	
812		GO:0003700	Transcription factor	0.00107	868	
813	Transcription	GO:0030528	transcription regulator	0.00197	869	
814	Regulation	GO:0003704	specific RNA polymerase II transcription factor	0.00714	870	
815	<i>21-day-old FI (200 µl)</i>					871
816	Biosynthesis	GO:0016705	Oxidoreductase, acting on paired donors, with incorporation or reduction of molecular oxygen	0.00248	872	
817		GO:0009058	Biosynthesis	0.00594	873	
818		GO:0009309	Amine biosynthesis	0.00626	874	
819	Cardiovascular	GO:0008015	Circulation	0.00288	875	
820		GO:0008016	Control of heart	0.00331	876	

821 These are GeneOntology Classes that were significantly overrepresented in the set of all possible GO classes as determined by the Class Scoring method (Pavlidis et al., 2002, 2004). The method determines probability (pval) that genes in any specific Class are significantly overrepresented compared to chance. Only  $P < 0.01$  are shown here. Note the difference in class function for the two volumes. The high volume (top) includes almost exclusively transcription factors and regulators. The low volume group is far more diverse but is notable for cell growth and cell death.

822 **3.5. qRT-PCR validation**

823 We recognize that any internal standard is imperfect and subject to alteration by the experimental manipulation (Bustin, 2002). To obtain an approximation of the change in actin due to the experimental treatment, we compared the number of cycles to criterion at 3 days and 21 days of age, we measured At 21 days of age there was a significant increase in actin message in the experimental group (mean  $\pm$  one standard deviation:  $12.07 \pm 0.252$ ;  $12.409 \pm 0.359$ ). In contrast, there was no difference between the groups at 3 days of age ( $11.465 \pm 0.530$ ;  $11.370 \pm 0.564$ ).

834 Overall, there was good agreement between the Affymetrix analysis and the PCR data. The scatterplot in Fig. 2 shows the expression ratios (treated group/control group) for the microarray data and the qRT-PCR (summed over replicates). Although the magnitude of the expression is routinely higher for the PCR than for the microarray data,

877 the relative magnitudes and directions are similar. Correlating those averages for all treatment conditions showed that there was reasonable agreement between the two assay methods ( $r = 0.826$ ), accounting for about 68% of the variance. This provides independent confirmation of the validity of the microarray data.

885 **3.6. Protein**

886 mRNA for the transcription factor, ERG-1 was over-expressed at both 3 and 21 days of age ipsilateral to the injection site. We confirmed that the protein was also overexpressed immunohistochemically. At both 3 and 21 days of age, there was ipsilateral expression of ERG-1, but little seen on either the contralateral side or in untreated animals. Stained cells from six randomly chosen sections from three animals showed that staining was present only ipsilateral to the injury. Mean cell counts ( $\pm$  one SEM)

897 Table 4  
898 Gene members in identified geneontology groups

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899 Three Days of age (5 µl)  
900 *Biological function*

901 Cell death AF027954\_at Bcl-2 related ovarian killer protein  
902 AF044201\_at lifeguard  
903 L14680\_at Bcl-2  
904 M34643\_at Neurotrophin-3 (HDNF/NT3)  
905 rc\_AI229707\_s\_at class I betatubulin  
906 S61973\_at NMDA receptor glutamate-bind-  
907 ing subunit  
908 S73007\_g\_at synuclein alpha  
909 U49930\_g\_at caspase 3, apoptosis related  
910 U59184\_at Bcl-2 associated X protein  
911 U72350\_at Bcl-2 like  
912 U77933\_at caspase 2  
913 U90610\_g\_at chemokine receptor (LCR1)  
914 X99267\_g\_at presenilin2  
915 AF021923\_at solute carrier family 24 (Na+/  
916 K+/Ca++ exchanger), member  
917 2  
918 AF038571\_s\_at Glutamate transporter  
919 M74494\_g\_at ATPase, Na+/K+ transporting,  
920 alpha 1  
921 M90398\_at ATPase, H+/K+ transporting,  
922 non-gastric, alpha polypeptide  
923 M95762\_at solute carrier family 6 (neuro-  
924 transmitter transporter, GABA),  
925 member 13  
926 S68944\_r\_at Na+/Cl(-)-dependent neuro-  
927 transmitter transporter  
928 X63253cds\_s\_at mRNA for serotonin transporter  
929 AB017912\_g\_at MAD homolog 2 (Drosophila)  
930 AF067727\_s\_at MAD homolog 1 (Drosophila)  
931 AJ001029\_at SRYbox containing gene 10

932 *Cellular component*

933 Cytoplasm AF027954\_at Bcl-2 related ovarian killer protein  
934 AF030088\_at homer, neuronal immediate early  
935 gene, 1  
936 D00688\_s\_at monoamine oxidase A  
937 L14680\_at Bcl-2  
938 rc\_AA945583\_at hydroxysteroid (17beta) dehydro-  
939 genase 10  
940 U72350\_at Bcl-2 like

941 *Molecular function*

942 ATPase/ D84450\_at ATPase,Na+/K+ transporting,-  
943 transporter beta polypeptide 3  
944 M28648\_s\_at Na,KATPase alpha2 subunit  
945 M74494\_g\_at ATPase, Na+/K+ transporting,  
946 alpha 1  
947 M90398\_at ATPase, H+/K+ transporting,  
948 non-gastric, alpha polypeptide

949 *Three days of age (20 µl)*  
950 *Biological function*

951 Metab- AF053362\_g\_at death effector domain containing  
952 olism protein  
953 M15481\_at insulin like growth factor 1  
954 M17960\_at insulin like growth factor II  
955 (somatomedin A)  
956 M18416\_at early growth response 1  
957 M34253\_at interferon regulatory factor 1  
958 rc\_AI176710\_at nuclear receptor subfamily 4,  
959 group A, member 3  
960 rc\_AI230842\_at Fos like antigen 2

Table 4 (continued)

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953 U15211\_g\_at retinoic acid receptor, alpha  
954 U67777\_at neurogenic differentiation 3  
955 U75397\_s\_at early growth response 1  
956 X03347cds\_g\_at FBR murine osteosarcoma pro-  
957 virus genome  
958 X06769cds\_at c-fos  
959 X53724\_at Achaetescuta homolog 2  
960 X54686cds\_at pJunB  
961 X60769mRNA\_at CCAAT/enhancer binding protein  
962 (C/EBP), beta  
963 Z34264\_at K+ voltage-gated channel, sub-  
964 family H (eag-related), member 1

965 *Molecular function*

966 **Binding**

967 **Metal ion** AJ012603\_at disintegrin and metalloproteinase  
968 domain 17  
969 rc\_AI102562\_at metallothionein  
970 rc\_AI176456\_at highly similar to rat metallothio-  
971 nein II  
972 AF053362\_g\_at death effector domain containing  
973 protein  
974 M18416\_at early growth response 1  
975 M34253\_at interferon regulatory factor 1  
976 M34384\_at nestin  
977 rc\_AA892559\_at ciliary neurotropic factor  
978 rc\_AI176710\_at nuclear receptor subfamily 4,  
979 group A, member 3  
980 rc\_AI230842\_at Fos like antigen 2  
981 U15211\_g\_at retinoic acid receptor, alpha  
982 U67777\_at neurogenic differentiation 3  
983 U75397\_s\_at early growth response 1  
984 X03347cds\_g\_at FBR murine osteosarcoma pro-  
985 virus genome  
986 X06769cds\_at c-fos  
987 X53724\_at Achaetescuta homolog 2  
988 X54686cds\_at pJunB  
989 X60769mRNA\_at CCAAT/enhancer binding protein  
990 (C/EBP), beta

991 **Transcription regulation**

992 M18416\_at early growth response 1  
993 M34253\_at Interferon regulatory factor 1  
994 rc\_AI176710\_at nuclear receptor subfamily 4,  
995 group A, member 3  
996 rc\_AI230842\_at Fos like antigen 2  
997 U15211\_g\_at retinoic acid receptor, alpha  
998 U67777\_at neurogenic differentiation 3  
999 U75397\_s\_at early response gene1  
1000 X03347cds\_g\_at FBR murine osteosarcoma pro-  
1001 virus genome  
1002 X06769cds\_at c-fos  
1003 X53724\_at Achaetescuta homolog 2  
1004 X54686cds\_at pJunB  
1005 X60769mRNA\_at CCAAT/enhancer binding protein  
1006 (C/EBP), beta

This table summarizes the genes that are significant members of the GenoOntology Classes listed in Table 2. Red is up regulated; blue is down regulated determined from the expression values of the treated group compared to control. Note the GO classes and genes that mark up those classes appear in more than one function. Again there is a preponderance of genes in the low volume group that are related to cell growth and cell death and a number of transporter genes. Most of these are down regulated. The high volume groups show mostly up regulation of transcription and some regulation of metabolic function.

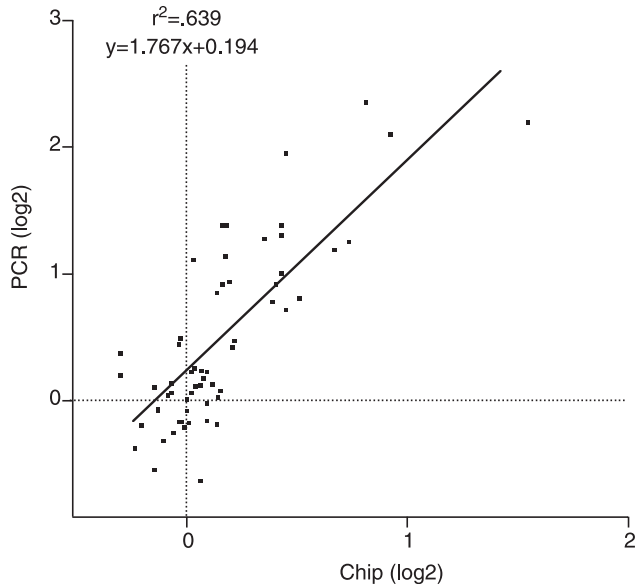


Fig. 2. Comparison of expression levels as determined by PCR and Microarray. Twenty samples were assayed by both methods for each condition. The data are the log (2) of the ratio of the expression values for the ipsilateral side to the control animals. The scatterplot is between the chip ratios and the PCR ratios for all three conditions, ipsilateral side. There is generally good agreement between the two methods, although the levels as determined by PCR are higher than those by array, as reported by others (note the different scales on the two axes). This is due largely to the normalization of the data, which reduces the magnitude of the ratios while reducing variability.

were  $69.7 \pm 16.5$  and  $134.8 \pm 10.7$  for the 3- and 21-day-old injected sides, respectively. Counts on the contralateral side were  $1 \pm 0.5$  and  $13.1 \pm 0.7$  for 3- and 21-day-old pups. One tailed *t*-tests between the ipsilateral and contralateral sides were significant at both ages. The photomicrograph demonstrates that the staining was almost exclusively limited to superficial dorsal horn (Fig. 3). Thus, changes in gene and protein expression for this transcription factor were consistent across methods. The microarray data showed an average 1.5–2.6 fold increase on the injured side compared to controls; qRT-PCR likewise showed about a 2.2–4.4 fold change. The immunohistochemistry showed protein expression largely limited to the side of injury.

4. Discussion

Injury to the infant induces alterations in a number of genes and these genes constitute coherent families that are putative candidates for mediating short and long-term changes in how the nervous system processes subsequent injury. These alterations were largely limited in these experiments to 3-day-old animals; far fewer changes were noted in the older animals when the ‘critical’ period of early injury has expired. Two major themes in the pattern of gene expression occurred. First, at both intensities of expression, large numbers of transcription factors were up regulated; second, for the lower intensity stimulus, neuroprotective

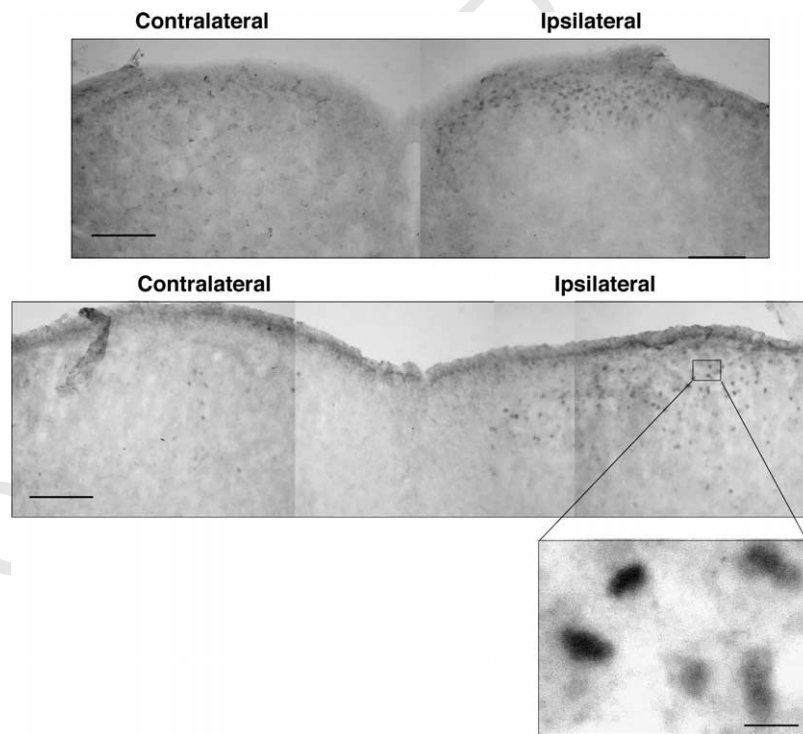


Fig. 3. Photomicrograph of ERG-1 staining in the dorsal horn of 3-day-old (top) and 20-day-old (bottom) pups injected with formalin two hours prior to sacrifice. Staining was superficial and concentrated on the side ipsilateral to the injection. Bar on lower power micrograph equals 50  $\mu$ m; on the higher power micrograph the bar is 10  $\mu$ m. Three-day-old, 20  $\mu$

and anti-apoptotic mechanisms were engaged. Below we discuss some of these changes in more detail.

#### 4.1. Transcription

The largest numbers of changes were in transcription factors. They include growth and tropic factors, immediate early genes, and transcription regulators. This is perhaps not surprising given the short time from the injury to measurement of gene changes. Other studies have identified immediate early gene protein products that are altered in the spinal cord of rat pups following injury including c-fos (Jennings and Fitzgerald, 1996; Yi and Barr, 1995) and ERG-1 (this study). Transcription factors have long-term consequences for later gene expression, gene function, and perhaps behavioral change. At this time, we do not know which expression differences are consequent to the changes in these transcription factors or whether or not they have functional consequences.

#### 4.2. Neuroprotection

##### 4.2.1. Cell death

A large number of classes and genes related to cell death, including anti-apoptotic Bcl-2 related genes and pro-apoptotic caspases that are downstream from Bcl-2 genes, were down regulated by formalin injection. Although there are a number of triggering events that initiate apoptosis, the biochemical cascade includes caspases, Par4, and others.

The substrates of caspases include cytoskeletal proteins, Bcl-2 family members, and presenilins (for review, see Mattson and Duan, 1999). Bcl-2 proteins are anti-apoptotic. That they were down regulated suggests increased vulnerability to cell loss. Further, disruptions of Na<sup>+</sup> ATPases, which maintain sodium and potassium gradients across the membrane, can facilitate apoptosis. Both the  $\alpha 1$  and 2 subunits of these pumps were down regulated; the  $\alpha 1$  subunit is ubiquitous, at least in adults, but loss of the  $\alpha 2$  subunit results in increased cell death (Ikeda et al., 2003). Thus, the net effect of down regulation of these genes in this treatment condition is to leave the cell more vulnerable to death. This is supportive of a hypothesized increase in apoptotic cell death following injury in the infant (Anand and Scalzo, 2000). In contrast, some caspases were also down regulated. Whether this is a primary effect resulting in less apoptosis or secondary to the down regulation of anti-apoptotic genes is an important question that cannot be answered by these data. Clearly, there are changes in cell survival that are occurring as a result of injury, and these are occurring only in the younger pups. More detailed studies, including time course analyses, are needed to determine the consequences of changes in cell death related genes.

##### 4.2.2. Metals

Metallothioneins I and II are involved in the processing of the metals such as zinc and copper. They are found in

the central nervous system in astrocytes in the adult brain (Nakajima and Suzuki, 1995) and are neuroprotective against oxidative stress (Ebadi et al., 1996; Hidalgo et al., 2002; Penkowa et al., 2003). Together the metallothioneins comprise a class of compounds whose function is well positioned to respond to injury. In the infant or adult brain, where expression is typically low, direct brain injury induces an increase in metallothionein protein starting 16–48 h after injury. This is likely due to reactive astrocytes both at the site of injury and at considerable distances from the original damage (Chung et al., 2004; Penkowa and Moos, 1995). Furthermore, metallothioneins are responsive both to cytokines and to corticosteroids (Ebadi et al., 1996; Hidalgo et al., 1997), making them candidates for a protective response to peripheral tissue injury. In the current study, metallothioneins were up regulated in the high volume 3-day-old treatment group. Thus, it is possible that in response to the formalin injection, metallothioneins up regulate rapidly in spinal cord to protect against neural damage. Whether or not regulation of these genes in response to peripheral injury is developmentally unique remains to be tested, but it should be noted that there were no comparable changes in these classes of genes in the 21-day-old animal

#### 4.3. Intensity differences

There were substantial differences not only quantitative, but also qualitative, in the effects of the two volumes of formalin in the infant. The high volume induced both transcription factors and metal ion genes. The low volume of formalin induced changes in cell growth and death, and in transcription factors. There are at least two explanations for these differences. First, gene expression may be intensity dependent. High and low intensity stimuli may recruit different classes of genes for regulation. This would imply different long-term and functional consequences of different intensity insults in the infant. Neither changed substantively at 21 days of age. Whether possible differences in tissue damage or intensity of ‘pain’ contribute to differences in gene expression cannot be determined with any certainty here.

Alternatively, the two volumes may induce the same change in gene expression but with different time courses. Thus the pattern of expression changes would be similar for the two intensities, but with different temporal properties. Specifically, here we hypothesize that the low volume also induced transcription factors, as did the higher volume, but with a shorter duration than in the higher intensity groups. At the time of analysis those transcription factors would be less intensely up regulated. There are data showing that Fos protein expression is intensity dependent in the infant (Yi and Barr, 1995), and the time course of the Fos protein expression is time dependent, peaking between 24 h and declining to 8 h after formalin injection (Barr, unpublished data). RNA message changes would occur earlier. If that

were the case, we would expect the changes in expression of genes related to cell maintenance and cell death, which occurred in the low volume group, to appear also in the higher intensity group but at a later time. More detailed analyses of the changes in gene expression over time are needed to understand more fully the detailed changes in gene expression in the infant.

In summary, we have identified a number of classes of genes whose short-term expression is altered by injury. Further, there appear to be age and intensity dependent differences, although the latter may be due to the timing of expression changes. At both ages and for each intensity group, growth factors, immediate early genes, and transcription related genes are up regulated, although much more intensely at the younger age and higher intensity stimulus. For the younger pup, with the lower intensity insult, a number of classes of genes that are protective to cell death were down regulated. The ultimate consequence of this is likely an increase in apoptotic and non-apoptotic cell death as has been hypothesized by others (Anand and Scalzo, 2000). These age specific changes in cell survival may account for the long-term effects of early injury (Anand and Scalzo, 2000; Lidow et al., 2001; Ruda et al., 2000).

## 5. Uncited reference

Yi and Barr (1995).

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