Pain 117 (2005) 6–18

PAIN

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Research papers

Microarray analysis of gene expression following the formalin test in the infant rat☆

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Received 1 December 2004; received in revised form 28 March 2005; accepted 25 April 2005

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.

Keywords: Postnatal; C-fibers; Microarray analysis

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

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

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doi:10.1016/j.pain.2005.04.024

occur before the maturation of C-fibers is complete and differs from the classic C-fiber induced windup seen in the adult (Jennings and Fitzgerald, 1998). This sensitization peaks at postnatal day 6, decreasing until the sensitization is no longer observed at postnatal day 21 (Jennings and Fitzgerald, 1998). In rats 8–12-day-old, repeated low frequency stimulation evokes a temporal summation of synaptic activity that generates a progressive depolarizing ventral root potential, a measure of windup (Hedo et al., 1999; Herrero et al., 2000; Thompson et al., 1995, 1994, 1992). These studies demonstrate that infant rats respond behaviorally to acute nociceptive stimuli, and can develop hyperalgesia/allodynia after inflammation-induced injury. The mechanisms underlying the short and long-term plasticity in the spinal cord are almost completely unknown in the infant.

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

2. Methods

2.1. Subjects

Pups were from litters bred in our colony at NYSPI. Breeding was by methods previously described (e.g. Barr and Wang, 1992). Dams were checked twice daily for births and any pups found on that day were designated 0 days of age. Litters were culled to 10–12 pups on the day after birth as needed. All work was done in accordance with the provisions of the HHS 'Guide for the Care and Use of Laboratory Animals' and the 'Principles for the Utilization and Care of Vertebrate Animals' and were approved by IACUC at NYSPI.

2.2. Treatment

At 3 or 21 days of age, the litter was removed from the dam and kept warm in an incubator. In these experiments, we used the formalin model. We chose formalin in part because it is a well worked out nociceptive model in the infant (Guy and Abbott, 1992; Teng and Abbott, 1998; Yi and Barr, 1995). Moreover, we wanted a stimulus that was not acute but was reasonably limited in duration. Classic thermal or mechanical stimuli are too brief. Moreover, in our hands, pups do not respond to either CFA or carrageenan with obvious pain responses; rather they show increased sensitivity to subsequent noxious stimuli. We wanted a stimulus that produced the clear nociceptive response such is produced by the formalin injection. Three-day-old pups were injected with 5 or 20 μl of formalin (2%) and 21-day-old pups with 200 μl, 2% in the plantar surface of the paw. The two volumes for the three-day-old pups served two purposes. First, it was an experimental manipulation to determine the effects of different intensity stimuli at this young age; second, it was an attempt to provide some match for the older pups. We state 'attempt' because it is impossible to equate a priori levels of nociceptive stimuli at different ages. Both the size and composition of the paw changes with age and at different ages different mechanisms are engaged. For example, at 3 days of age, there is no evidence for neurogenic edema (Fitzgerald and Gibson, 1984). Therefore, although we used different volumes at 3 days of age and a higher volume at 21 days of age, whether they produce equivalent levels of nociceptive input is unknown. Moreover, other processes in response to injury differ between the two ages. For example, neurogenic edema first develops at about 14 days of age (Fitzgerald and Gibson, 1984) although there is edema due to tissue damage at 3 days of age. In published work (Gupta et al., 2001), we showed slight differences in edema in 3- and 21-day-old pups given formalin (125 vs. 115% of controls; 10 μl, 2%). In unpublished data, we found that CFA in different volumes also produced only slight differences in edema (3-day-old 50 μl, 142%; 21-day-old 200 μl, 131%).

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

2.3. Dissection

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

2.4. Isolation of total RNA

The frozen harvested tissue was ground into a powder by pestle in eppendorf tubes. Total RNA was isolated from the frozen tissue by the guanidinium isothyocynate method (Ambion Research) and treated with RNAse free DNAse (1 U/mg RNA) in the presence of RNAse inhibition (1 U/ml reaction volume) for 30 min at 37 °C. 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), DNAfree, 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 Discovery Rate (FDR; Benjamini and Hochberg, 1995). The False Discovery 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 a 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, 2004). 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 19 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 Krox-24 (identical to EGR-1; Santa Cruz Biotech.).

Table 1
qRT-PCR primers name and their sequences

Primers	Gene name	Sense	AntiSense
AA957930	Tau Microtub.Asso. Prot.	5′CTCAGCCGCCATTAAGTC3′	5′TGCGGACAGGAGAGAAAG3′
AB017656	Cholinerg. Muscarin.	5′GCTTCCCATCCAGTTAGAGT3′	5′TGAGCGACATCCTCTTCC3′
AF023087	NGFI-Factor A	5′ACTGAGTAGGCGGTGACTTT3′	5′TTCTGAAGGATACACACCACA3′
AF030088	NT-I EG 3 (ania3)	5′TACACCTTCAAACAAACATTAGG3′	5′AAGAATCAACTTAACATCTCATGC3′
AI009806	Dynein	5′CTACTCAGGCGTTGGAGAA3′	5′GAAGTGTTTGGTCTCGTGTG3′
AI102562	Metallothionein	5′TGCCTTCTTGTAGCTTACCC3′	5′CTTGCAGGAGGTGCATTT3′
AI176710	NOR-1	5′CCTTTGTTTGCAGTGACCT3′	5′GAAGCTACCGTGACATTGG3′
D25233 g	Retinoblastoma protein 1	5′GCCATCACACAGGTTAGTTG3′	5′TTCCTATGATTCTGTCACTAATTCTAA3′
L08492	GABA-A	5′GTTCATGGCCGTCTGTTAT3′	5′TGTTGAAGGTGGTGCTTG3′
M11596	NPY	5′TGAGGAACTAACGCTCCAG3′	5′GATGCAAACATACACATCGTC3′
M18416	NGFI-A	5′ACTGCTCGACTGTAACTCTCAC3′	5′TCACACAAAGGCACCAAG3′
M34643	Neurotrophin-3	5′CATGAATTGGCATCTGTCC3′	5′CTGTAAGGGTTGCTGAAGTTT3′
S67770	Tr-GFBeta IIR	5′TTCACCTACCACGGCTTC3′	5′CCCGTCACTTGGATAATGA3′
U17254 g	NGFI-B	5′CTC TTATCCCTCCCAGCTC3′	5′TTACAGCAGCGTCAGCTTAT3′
U75397	Krox-24	5′ATGCTGCGGTTACCTACTG3′	5′TTTAAGCAAACACAAGTACGAAG3′
U90610	CXCR4	5′TCTGTGACCGCCTTTACC3′	5′TAGTCTTGAGGGCCTTGC3′
X06769	C-fos	5′CACCCTGCCTCTTCTCAA3′	5′CAAGAAGTCATCAAAGGGTTC3′
X95882	ATP Gated Ion Chann.	5′TGGATGTGGCTTGGTAGAG3′	5′TGTGGTGTGTGGTGTGTG3′
Y17607	Potassium Chann. Alpha	5′CGGCTTACGCATCATTCT3′	5′CCGTGTCTGGAACATGAA5′3′

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

3. Results

3.1. Reliability of the data

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

3.2. Differential expression

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

Of the 1260 genes and EST's, 22 were determined to be significant for the 3-day-old high intensity group (FDR = <5%). All were up regulated. For the 3-day-old low intensity group, 34 were down regulated and two were up regulated. The genes are listed in Table 2. At 21 days of age, only a single gene was up regulated (Early Growth Response-1; EGR-1). For the high volume group, the up regulated genes were of several classes. Most commonly seen were immediate early genes such as c-fos and EGR-1 and other transcription factors. A number of ion-gated channels were up regulated, most notably potassium channels. For the low volume group, there were not only more down regulated genes, but there was more diversity in the nature of those genes. These include many genes involved in cell growth or death including caspases, kinases, chemokines, and neurotransmitter associated receptors and transporters. The up regulated genes for this group were both EGR-1 sequences that were also overexpressed in the high volume group and at 21 days of age.

3.3. Coherent clusters

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

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

Table 2
Significant genes as determined by SAM/FDR

Probe set ID	Title
Three-day-old Ipsilateral side (5 μl)	
Up regulated	
AF023087_s_at	early growth response 1
U75397_s_at	early growth response 1
Down regulated	
M91595_s_at	insulin-like growth factor binding protein-2
S61973_at	NMDA receptor glutamate-binding chain
M92076_at	glutamate receptor, metabotropic 3
rc_AI231354_at	stress activated protein kinase alpha II
D00688_s_at	rat monoamine oxidase A
U49930_g_at	caspase 3
D84450_at	ATPase, Na+/K+ transporting, beta 3 polypeptide
L14323_at	phospholipase C-beta1
AF028784_s_at	glial fibrillary acidic protein alpha (GFAP)
U90610_g_at	chemokine receptor (LCR1)
D17521_at	protein kinase C-regulated chloride channel
U77933_at	caspase 2
D50093_s_at	prion protein
X99267_g_at	presenilin-2
M27925_at	synapsin 2
AB017912_g_at	MAD homolog 2 (Drosophila)
U90610_at	chemokine receptor (LCR1)
S73007_g_at	synuclein, alpha
M74494_g_at	ATPase, Na+/K+ transporting, alpha 1
rc_AA892801_g_at	eukaryotic translation elongation factor 2
AF067727_s_at	MAD homolog 1 (Drosophila)
S53527_s_at	S100 calcium-binding protein, beta (neural)
X51992_at	gamma-aminobutyric acid A receptor, alpha 5
M58040_at	transferrin receptor
M15191_s_at	tachykinin
M28648_s_at	ATPase, Na+/K+ transporting, alpha 3 subunit
E01789cds_s_at	protein kinase C, beta 1
D90258_s_at	proteasome (prosome, macropain) subunit, alpha type 3
AF090113_at	glutamate receptor interacting protein 2
AB016160_at	gamma-aminobutyric acid (GABA) B receptor, 1
E13541cds_s_at	chondroitin sulfate proteoglycan 5
AB004267_at	pregnancy up regulated non-ubiquitously expressed CaM kinase
M95762_at	GABA transporter GAT-2
X06554cds_s_at	Myelin-associated glycoprotein
Three-day-old Ipsilateral side (20 μl)	
Up regulated	
X06769cds_at	c-fos
M18416_at	early growth response 1
X06769cds_g_at	c-fos
M59980_s_at	K+ voltage gated channel, Shal-related family, member 2
U75397UTR#1_s_at	early growth response 1
AF023087_s_at	early growth response 1
rc_AI102562_at	metallothionein
rc_AI176662_s_at	early growth response 1
rc_AI176456_at	strong similarity to rat metallothionein-II
rc_AI176710_at	nuclear receptor subfamily 4, group A, member 3
M31837_at	insulin-like growth factor binding protein 3
X95882_at	purinergic receptor P2X, ligand-gated ion channel, 7
Z12152_at	neurofilament 3, medium
AF037071_at	C-terminal PDZ domain ligand of neuronal NOS
M26643_at	sodium channel, voltage-gated, type 4, alpha polypeptide
Z34264_at	K+ 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) or high volume (20 μl). 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 EGR-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 lower volume of formalin induced few classes of transcription, but affected classes related to cell death, cell growth and cell maintenance. Most of these genes are anti-apoptotic. Table 4 lists significant genes in the identified GeneOntology classes.

Although there was only a single gene that was differentially expressed as determined by the FDR method at 21 days of age, the aggregate probability of multiple genes shows significant class structure. Formalin treatment resulted in changes in amine biosynthesis related to autonomic function, including genes coding for tyrosine and tryptophan hydroxylase, neuropeptide Y, and vimentin. Also altered were two classes of oxidoreductase involving prostaglandin synthesis.

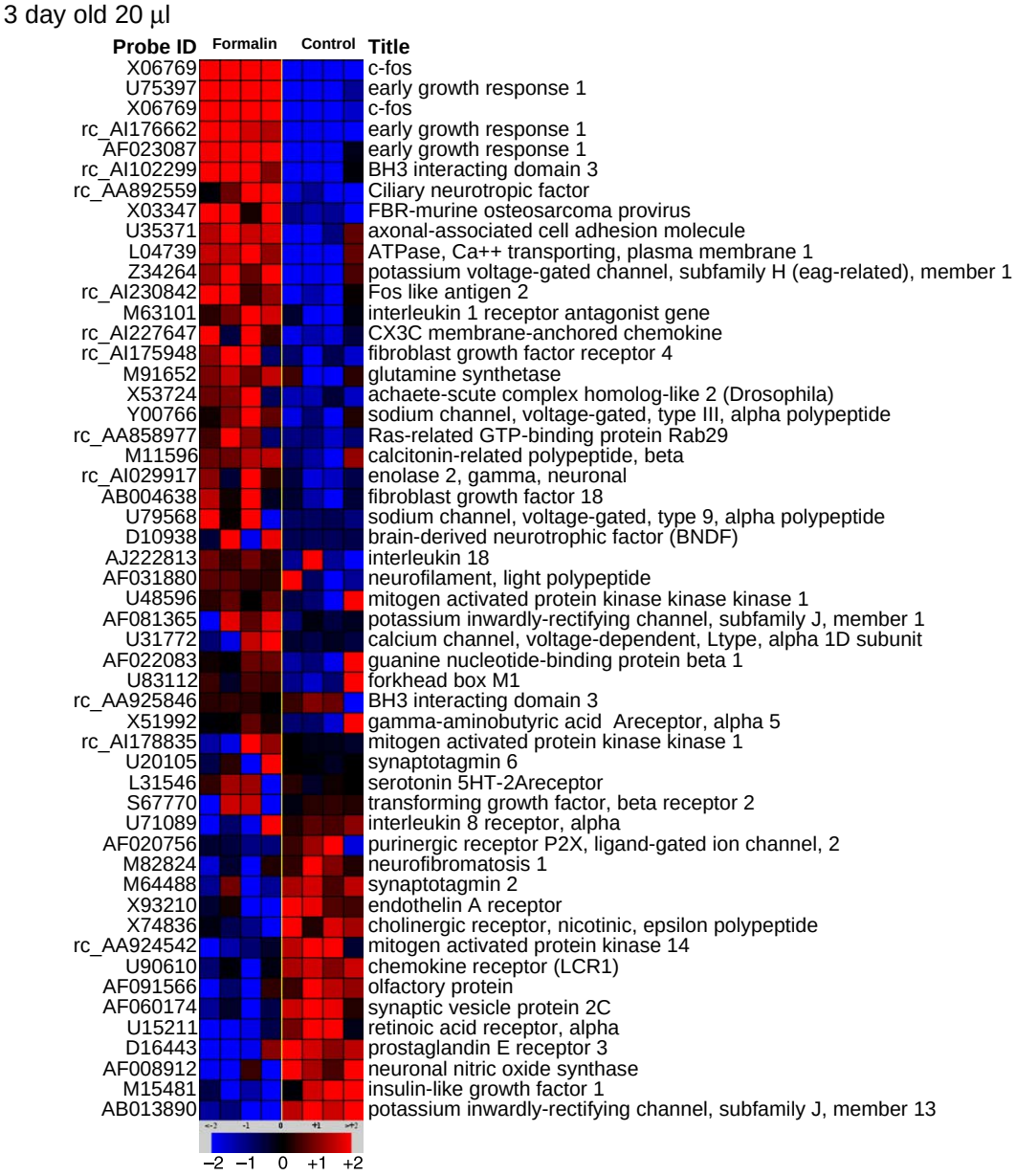
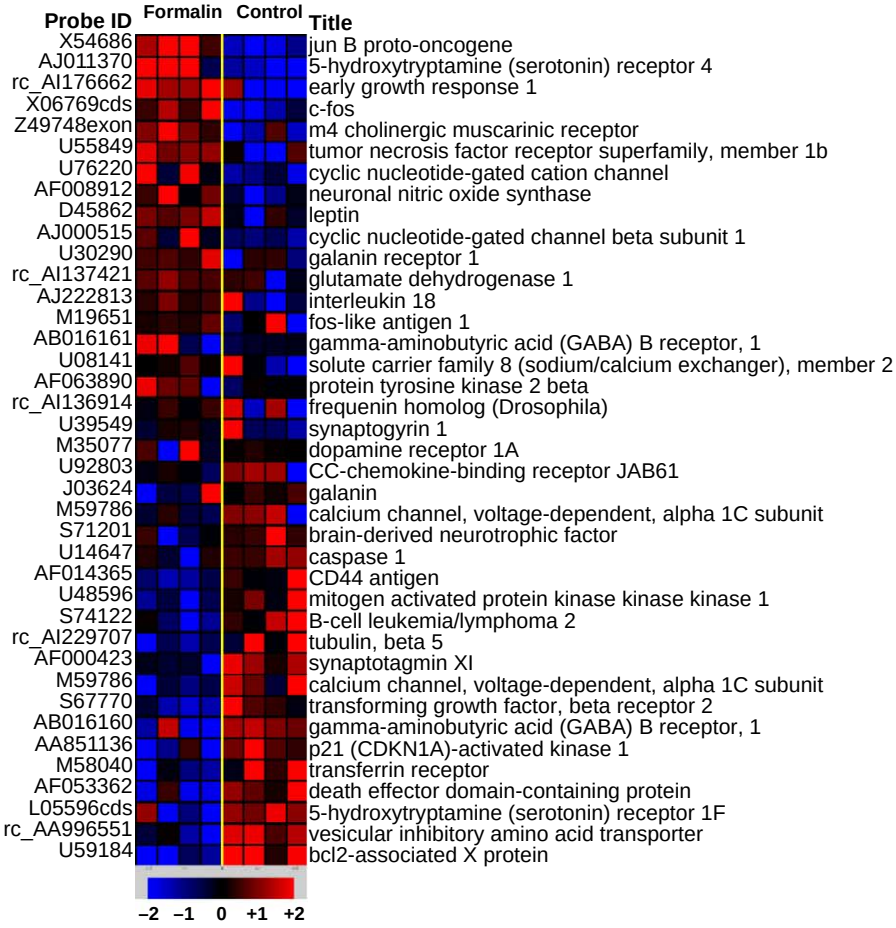


Fig. 1. *Gene expression clusters from supervised clusters*. Red squares denote an increase, blue a decrease. Identical genes with different probe ID's represent different probe sequences for the same genes. In general, the different sequences show good concordance, with the exception of the apoptotic gene 'BH3 interacting domain 3' in the 3-day-old pups given 20 µl formalin. *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. *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. *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.

3 day old 5 μl



21 day old 200 μl

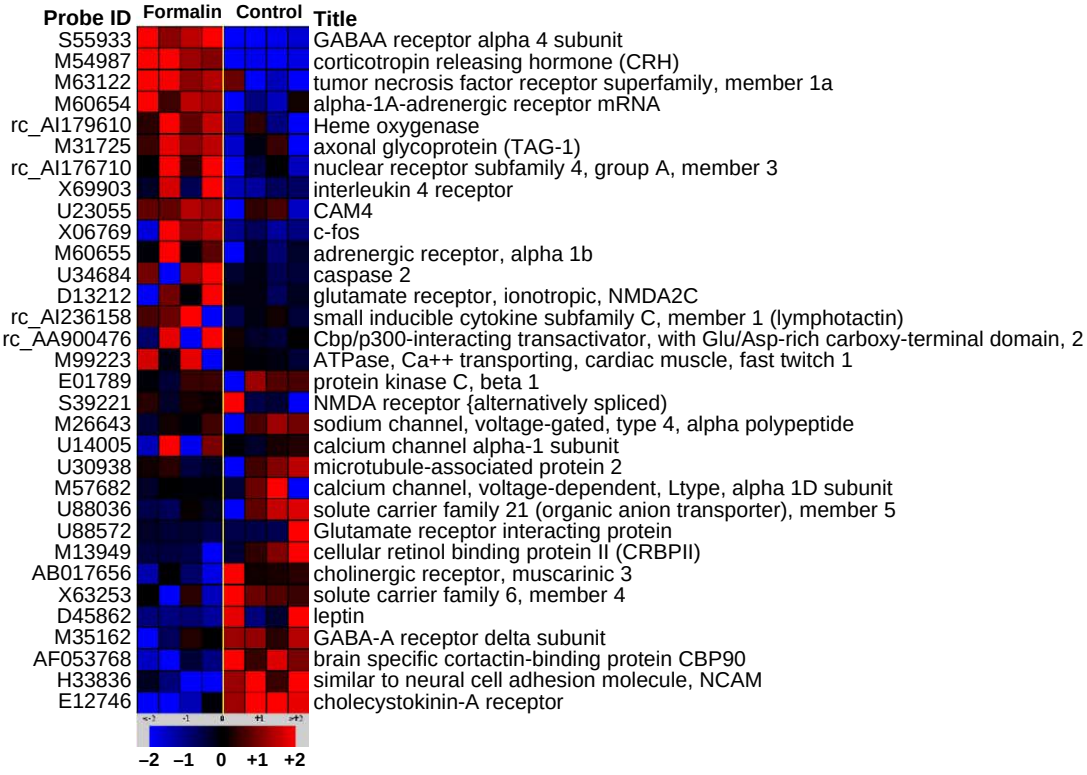


Fig. 1 (*continued*)

Table 3
Class scores

GO class	GO number	Bioprocess	pval
Three-day-old (5 μl)			
Biological function			
Cell death	GO:0008219	Cell death	0.00309
	GO:0006915	Apoptosis	0.00217
	GO:0006916	Anti-apoptosis	0.00315
Cell growth	GO:0006818	Hydrogen transport	0.00983
Embryogenesis	GO:0007345	Embryogenesis and morphogenesis	0.00900
Death	GO:0016265	Death	0.00347
Cellular component			
Cytoplasm	GO:0005739	Mitochondrion	0.00172
Molecular function			
ATPase	GO:0015662	Ptype ATPase	0.00778
	GO:0005391	Sodium/potassium	0.00630
Three-day-old (20 μl)			
Biological function			
Metabolism	GO:0006139	Nucleobase, nucleoside, nucleotide and nucleic acid metabolism	0.00616
	GO:0006350	Transcription	0.00202
	GO:0006355	Transcription regulation	0.00126
	GO:0006357	Transcription regulation from Pol II promoter	0.00481
Molecular function			
Binding			
Metal ion	GO:0008270	Zinc binding	0.0027
Nucleic acid	GO:0003676	Nucleic acid binding	0.00096
	GO:0003677	DNA binding	0.00106
	GO:0003700	Transcription factor	0.00107
Transcription	GO:0030528	transcription regulator	0.00197
Regulation	GO:0003704	specific RNA polymerase II transcription factor	0.00714
21-day-old FI (200 μl)			
Biosynthesis	GO:0016705	Oxidoreductase, acting on paired donors, with incorporation or reduction of molecular oxygen	0.00248
	GO:0009058	Biosynthesis	0.00594
	GO:0009309	Amine biosynthesis	0.00626
Cardiovascular	GO:0008015	Circulation	0.00288
	GO:0008016	Control of heart	0.00331

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.

3.5. qRT-PCR validation

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. At 21 days of age there was a significant increase in actin message in the experimental group (mean $\pm$ one standard deviation: 12.07 ± 0.252; 12.409 ± 0.359). In contrast, there was no difference between the groups at 3 days of age (11.465 ± 0.530; 11.370 ± 0.564).

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, 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 ($r=0.80$), accounting for about 64% of the variance. This provides independent confirmation of the validity of the microarray data.

3.6. Protein

mRNA for the transcription factor, EGR-1 was overexpressed 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 EGR-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)

Table 4
Gene members in identified geneontology groups

Three Days of age (5 μl)		
Biological function		
Cell death	AF027954_at	Bcl-2 related ovarian killer protein
	AF044201_at	lifeguard
	L14680_at	Bcl-2
	M34643_at	**Neurotrophin-3 (HDNF/NT3)**
	rc_AI229707_s_at	class I betatubulin
	S61973_at	NMDA receptor glutamate-binding subunit
	S73007_g_at	synuclein alpha
	U49930_g_at	caspase 3, apoptosis related cysteine protease
	U59184_at	Bcl-2 associated X protein
	U72350_at	Bcl-2 like
	U77933_at	caspase 2
	U90610_g_at	chemokine receptor (LCR1)
	X99267_g_at	presenilin2
Cell growth/ transport	AF021923_at	solute carrier family 24 (Na+/K+/Ca++ exchanger), member 2
	AF038571_s_at	Glutamate transporter
	M74494_g_at	ATPase, Na+/K+ transporting, alpha 1
	M90398_at	**ATPase, H+/K+ transporting, non-gastric, alpha polypeptide**
	M95762_at	solute carrier family 6 (neurotransmitter transporter, GABA), member 13
	S68944_r_at	**Na+/Cl(−)-dependent neurotransmitter transporter**
	X63253cds_s_at	**mRNA for serotonin transporter**
Embryogenesis	AB017912_g_at	MAD homolog 2 (Drosophila)
	AF067727_s_at	MAD homolog 1 (Drosophila)
	AJ001029_at	SRYbox containing gene 10
Cellular component		
Cytoplasm	AF027954_at	Bcl-2 related ovarian killer protein
	AF030088_at	**homer, neuronal immediate early gene, 1**
	D00688_s_at	monoamine oxidase A
	L14680_at	Bcl-2
	rc_AA945583_at	hydroxysteroid (17beta) dehydrogenase 10
	U72350_at	Bcl-2 like
Molecular function		
ATPase/ transporter	D84450_at	ATPase,Na+/K+transporting,-beta polypeptide 3
	M28648_s_at	Na,KATPase alpha2 subunit
	M74494_g_at	ATPase, Na+/K+ transporting, alpha 1
	M90398_at	**ATPase, H+/K+ transporting, non-gastric, alpha polypeptide**
Three days of age (20 μl)		
Biological function		
Metabolism	AF053362_g_at	death effector domain containing protein
	M15481_at	insulin like growth factor 1
	M17960_at	insulin like growth factor II (somatomedin A)
	M18416_at	**early growth response 1**
	M34253_at	**interferon regulatory factor 1**
	rc_AI176710_at	**nuclear receptor subfamily 4, group A, member 3**
	rc_AI230842_at	**Fos like antigen 2**
	U15211_g_at	retinoic acid receptor, alpha
	U67777_at	**neurogenic differentiation 3**
	U75397_s_at	**early growth response 1**
	X03347cds_g_at	**FBR murine osteosarcoma provirus genome**
	X06769cds_at	**c-fos**
	X53724_at	**Achaetescute homolog 2**
	X54686cds_at	**pJunB**
	X60769mRNA_at	**CCAAT/enhancer binding protein (C/EBP), beta**
	Z34264_at	**K+ voltage-gated channel, subfamily H (eag-related), member 1**
Molecular function		
Binding		
Metal ion	**AJ012603_at**	**disintegrin and metalloproteinase domain 17**
	rc_AI102562_at	**metallothionein**
	rc_AI176456_at	**highly similar to rat metallothionein II**
Nucleic acid	AF053362_g_at	death effector domain containing protein
	M18416_at	**early growth response 1**
	M34253_at	**interferon regulatory factor 1**
	M34384_at	nestin
	rc_AA892559_at	**ciliary neurotropic factor**
	rc_AI176710_at	**nuclear receptor subfamily 4, group A, member 3**
	rc_AI230842_at	**Fos like antigen 2**
	U15211_g_at	retinoic acid receptor, alpha
	U67777_at	**neurogenic differentiation 3**
	U75397_s_at	**early growth response 1**
	X03347cds_g_at	**FBR murine osteosarcoma provirus genome**
	X06769cds_at	**c-fos**
	X53724_at	**Achaetescute homolog 2**
	X54686cds_at	**pJunB**
	X60769mRNA_at	**CCAAT/enhancer binding protein (C/EBP), beta**
Transcription regulation	**M18416_at**	**early growth response 1**
	M34253_at	**Interferon regulatory factor 1**
	rc_AI176710_at	**nuclear receptor subfamily 4, group A, member 3**
	rc_AI230842_at	**Fos like antigen 2**
	U15211_g_at	retinoic acid receptor, alpha
	U67777_at	**neurogenic differentiation 3**
	U75397_s_at	**early response gene1**
	X03347cds_g_at	**FBR murine osteosarcoma provirus genome**
	X06769cds_at	**c-fos**
	X53724_at	**Achaetescute homolog 2**
	X54686cds_at	**pJunB**
	X60769mRNA_at	**CCAAT/enhancer binding protein (C/EBP), beta**

This table summarizes the genes that are significant members of the GenoOntology Classes listed in Table 2. Bold text is up regulated; normal text is down regulated etermined 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 then 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.

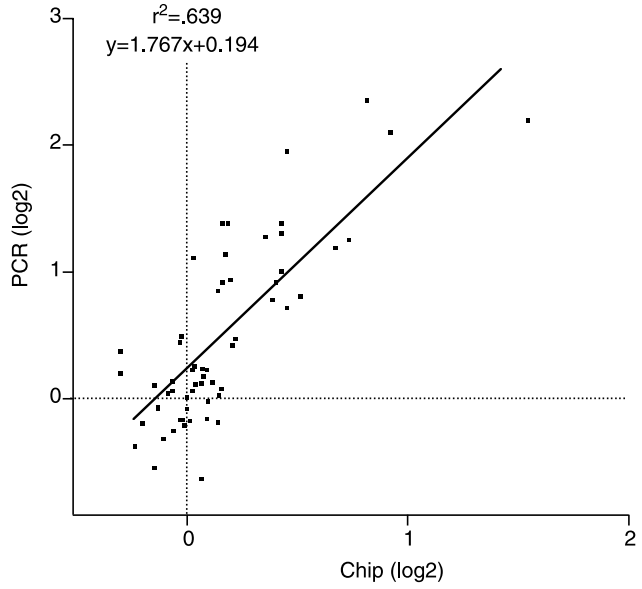


Fig. 2. *Comparison of expression levels as determined by PCR and Microarray*. Twenty samples were assayed by both methods for each condition. The data are the log (2) of the ratio of the expression values for the ipsilateral side to the control animals. The scatterplot is between the chip ratios and 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 ± 16.5 and 134.8 ± 10.7 for the 3- and 21-day-old injected sides, respectively. Counts on the contralateral side were 1 ± 0.5 and 13.1 ± 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

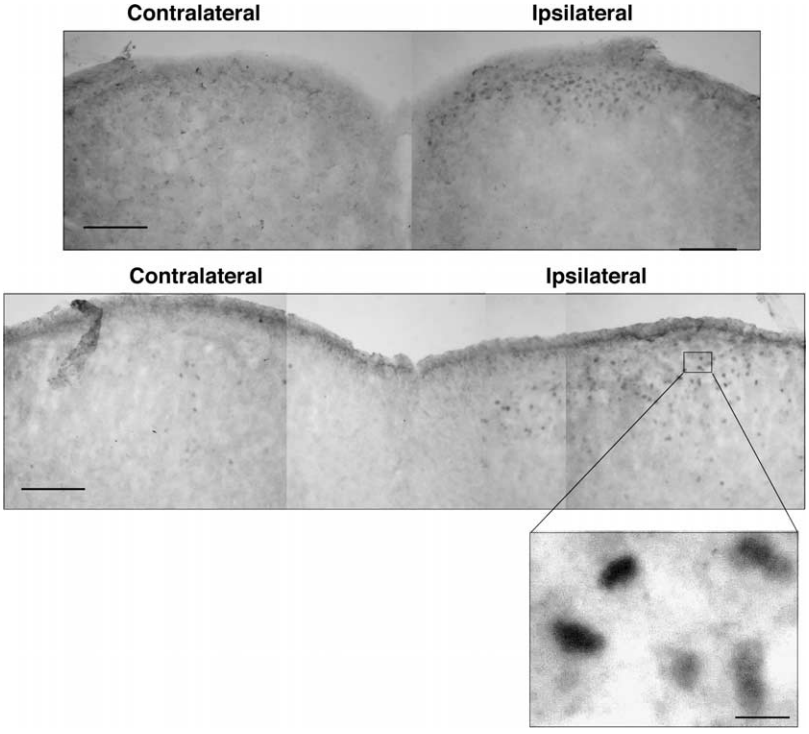


Fig. 3. Photomicrograph of EGR-1 staining in the dorsal horn of 3-day-old (top) and 20-day-old (bottom) pups injected with formalin four hours prior to sacrifice. Staining was superficial and concentrated on the side ipsilateral to the injection. Bar on lower power micrograph equals 50 μm; on the higher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

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 EGR-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 N + K + ATPases, which maintain sodium and potassium gradients across the membrane, can facilitate apoptosis. Both the α1 and 2 subunits of the these pumps were down regulated; the α1 subunit is ubiquitous, at least in adults, but loss of the α2 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 2–4 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).

Acknowledgements

Supported by NIDA K02-DA00325 to G.A. Barr and NIMH K01-MH067721 to E.L. Sibille.

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