Gene expression profiling of experimental traumatic spinal cord injury as a function of distance from impact site and injury severity

Andrea De Biase,1,*, Susan M. Knoblach,1,§ Simone Di Giovanni,2,§ Chenguang Fan,1 Annamaria Molon,1 Eric P. Hoffman,1 and Alan I. Faden2

1Children’s National Medical Center, Center for Genetic Medicine; and 2Department of Neuroscience, Georgetown University School of Medicine, Washington, District of Columbia

Submitted 7 April 2005; accepted in final form 28 May 2005

Traumatic spinal cord injury (SCI) induces widespread, coordinated changes in gene expression as a function of distance from impact site and injury severity. Physiol Genomics 22: 368–381, 2005. First published June 7, 2005; 10.1152/physiolgenomics.00081.2005—Changes in gene expression contribute to pathophysiological alterations following spinal cord injury (SCI). We examined gene expression over time (4 h, 24 h, 7 days) at the impact site, as well as rostral and caudal regions, following mild, moderate, or severe contusion SCI in rats. High-density oligonucleotide microarrays were used that included ~27,000 genes/ESTs (Affymetrix RG-U34; A, B and C arrays), together with multiple analyses (MAS 5.0, dChip). Alterations after mild injury were relatively rapid (4 and 24 h), whereas they were delayed and prolonged after severe injury (24 h and 7 days). The number and magnitude of gene expression changes were greatest at the injury site after moderate injury and increased in rostral and caudal regions as a function of injury severity. Sham surgery resulted in expression changes that were similar to mild injury, suggesting the importance of using time-linked surgical controls as well as naïve animals for these kinds of studies. Expression of many genes and ESTs was altered; these were classified functionally based on ontology. Overall representation of these functional classes varied with distance from the site of injury and injury severity, as did the individual genes that contributed to each functional class. Different clustering approaches were used to identify changes in neuronal-specific genes and several transcription factors that have not previously been associated with SCI. This study represents the most comprehensive evaluation of gene expression changes after SCI to date. The results underscore the power of microarray approaches to reveal global genomic responses as well as changes in particular gene clusters and/or families that may be important in the secondary injury cascade.

Traumatic spinal cord injury (SCI) induces widespread, coordinated changes in gene expression (3, 11, 15–17, 19, 23, 64). These may be associated with pathogenic auto-destructive events such as hemorrhage, metabolic failure, inflammatory/immune activation, loss of ionic homeostasis, lipid degradation, production of free radicals, and neurotransmitter/neuro-modulator imbalances (for review see Refs. 8, 26). Such alterations may contribute to death of neurons and oligodendroglial cells, glial proliferation (scarring), demyelination, and axonal loss. Alternatively, reactive biochemical and expression changes that are neuroprotective and/or promote recovery (re-generation/plasticity) may also be induced (17, 18, 26, 51).

Recently, many investigators have utilized high-throughput methods such as differential-display PCR, subtractive PCR, or microarrays to delineate global changes in gene expression patterns that may underlie the complex response to SCI (3, 11, 22, 58, 62, 64, 67). Indeed, several groups have used spotted cDNA or high-density oligonucleotide microarray platforms to evaluate expression of from ~1,200 to 15,000 genes. Such studies have revealed global changes in expression of multiple genes involved in inflammatory/immune (3, 11) and wound-healing responses to injury (64). Others have used microarrays to identify expression clusters that led to studies of specific novel pathways that have not previously been associated with SCI, including cell cycle genes involved in neuronal cell death (17) as well as genes involved in axonal plasticity and remodeling (15, 16). The majority of such studies have focused on mild and/or moderate levels of SCI. Of these, only a few have examined regions distant from the site of impact (3, 11). No study has evaluated the potential effects of injury severity.

We describe an extensive gene expression profiling study of rat experimental SCI that assessed changes in gene expression after three levels of injury (mild, moderate, and severe) and across several spinal cord regions (injury site and adjacent rostral and caudal regions). The analysis utilized the Affymetrix complete rat genome GeneChip set (~27,000 genes; GeneChips RG-U34A, RG-U34B, RG-U34C), which includes not only full-length or annotated genes (A array), but also thousands of expressed sequence tags (ESTs) (B and C arrays). In addition, the data were analyzed with two different probe set algorithms.

Materials and Methods

Experimental SCI and expression profiling. SCI was performed in rats as previously described (17). Briefly, male Sprague-Dawley rats (275–325 g) were anesthetized with intraperitoneal pentobarbital sodium (65 mg/kg). Injury was induced using a weight drop method (10-g weight dropped from 17.5 mm, 30 mm, and 50 mm, respectively, for the three levels of injury) that included stabilization of the vertebral column before impact. The site of impact was vertebral T9, which corresponds to spinal T10–T11. At the appropriate time after injury, animals were deeply anesthetized with pentobarbital sodium (100 mg/kg) and decapitated. Complete laminectomy of the entire vertebral canal was rapidly performed, and the cord was dissected into injury, rostral, or caudal sections (each 1 cm long) (see Fig. 1). These were immediately removed and frozen on dry ice. The injury site and both rostral and caudal regions were collected from four injured and two sham-operated rats (that received only laminectomy surgery) for mild and severe injury levels at 4, 24, and 168 h (7 days) after injury.

27,000 genes;
Only the injury site was collected from rats subjected to moderate SCI, and samples were taken at 0.5, 4, 12, 24, 48, 72, and 168 h after injury. Two naive controls (rats that did not undergo any surgical procedure or any anesthesia at the time of death) were also included. In parallel, additional animals were prepared (mild, n = 10; moderate, n = 12; severe, n = 11) to examine the effects of the injury parameters used on locomotor recovery and histopathology. Performance on the Basso, Beattie, and Bresnahan (BBB) locomotor test was assessed from 1–28 days after injury of each severity, as previously detailed (7). At 28 days after injury animals were anesthetized and perfused, and their cords were removed and processed using standard paraffin embedding and hematoxylin-eosine staining, to assess pathology. All animal procedures and experiments complied fully with the principles set forth in the “Guide for the Care and Use of Laboratory Animals” prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [DHEW Pub. No. (NIH) 85–23, 1985].

Microarray (GeneChip) quality control. Expression profiling was performed as described previously (17). Briefly, RNA was extracted from each cord sample individually using TRIzol reagent (Invitrogen). Seven micrograms of total RNA was used for cDNA and biotinylated cRNA synthesis and fragmentation as detailed in the manufacturer’s protocol (Affymetrix). The same amount of total RNA (7 μg) was used to generate gene expression profiles regardless of injury level or spinal cord location. Affymetrix rat RG-U34A, RG-U34B, and RG-U34C arrays were used. Each spinal cord region from each individual animal was hybridized to all three RG-U34 arrays (A, B, or C).

Stringent quality control methods were employed as previously published (17) and detailed on our Public Expression Profiling Resource web site: http://pepr.cnmcresearch.org/home.do. Each array fulfilled the following quality control measures: cRNA fold changes from 5 to 10, scaling factor from 0.3 to 1.5, percentage of “present” (P) calls from 40 to 55%, and average signal intensity levels between 900 and 1,100. Housekeeping genes and internal probe set controls showed >80% present calls, consistent values, and 5/3 ratios <3.

Experimental normalization, data filtering, and statistical analysis of gene expression profiles generated with MAS 5.0 and dChip. Prior to any type of experimental-based normalization, array normalization was applied to generate expression values using two different algorithms: dChip and MAS 5.0. When we used MAS 5.0, an array-to-array normalization using a scaling factor of 800 was employed, generating MAS 5.0-type CHP files that were later loaded into GeneSpring for experimental normalization and analysis. In parallel, the same arrays were analyzed with the dChip probe set algorithm (34, 35) to establish a project-based normalization, rather than single-array normalization, generating new “dChip-type” expression values “homologous” to the CHP files generated with MAS 5.0. These new dChip-type CHP files were also loaded into GeneSpring for experimental normalization and analysis. Finally, data from both algorithms were analyzed using GeneSpring software (Silicon Genetics) and normalized to the average of the naive signal intensities for each gene (experimental normalization). Experimental normalization to naive was entirely arbitrary, and additional types of normalization can be performed online on our publicly available SAS server database (http://sas.cnmcresearch.org/). Genes that were significantly altered...
under both algorithms were retained for functional analysis, whereas genes that were altered in one or the other algorithm were not included in this study.

Experiment normalization was performed, prior to any type of statistical or fold change filtering, by normalizing arrays from injured and sham controls to the mean of the two arrays from naive animals considered as the baseline gene expression level. Normalized data were then compared for differential gene expression analysis across time points between sham and injured groups. We have recently shown that use of signal intensity values, together with a “present call” noise filter achieves an excellent signal/noise balance relative to other probe set analysis methods (56). Thus, data analyses were limited to probe sets that showed one or more “present” (P “calls”) across the complete data set. Initial data analysis also included a fold change filter of ≥1.5 (increase or decrease) relative to sham-operated animals (Affymetrix MAS 5.0).

Statistical analysis was performed on GeneSpring software, using a Welch ANOVA t-test P value of <0.01 between sham and injured time-matched groups. Although a P value of <0.01 alone would give many false positives, the combination of present call filters, fold change thresholds, and P value thresholds eliminates most false positives that are obtained with only P < 0.01.

Genes having at least one “present” call across the entire data set, a fold change ratio >1.5 and a significant expression (P < 0.01) were subsequently sorted into functional groups, using annotations provided in the Database for Annotation, Visualization and Integrated Discovery (DAVID) (National Institute for Allergy and Infectious Diseases; http://david.niaid.nih.gov/david) and SwissProt/TrEMBL (http://www.expasy.org). In cases of genes with multiple functions/activities, assignment was based on the most frequently attributed activity or to any central nervous system (CNS)- or SCI-specific activity, if stated.

Real-time RT-PCR. In previous reports, real-time PCR data was described that validated selected transcripts from different clusters derived from this data set (15–17). For the present study, additional real-time RT-PCR was performed to validate additional genes from several major functional classes altered by injury. Three injured and two sham rats (biological replicates) were used in triplicate (experiments) for RT-PCR. Animals were different from the ones used in the array analysis. Genes included in the analysis were selected from a wide range of expression changes (small to large) to assess the accuracy of such validation across this variable. RT-PCR was performed for the following functional classifications/genes: inflammatory genes (Tgfβ1), neurotransmission (KCh, Scn1a, P2rx4), transcription factors (c-fos), extracellular matrix (Col1a1), and growth factors/differentiation (Syngr1). The following primers were used: Tgfβ1 754F 5′-CACCAAACCGGTGCTATATGG(FAM)G-3′, Tgfβ1 856R 5′-GCTCTGACGGGACAGCAATG-3′; Kch 2469 5′-CTACTGAGTGGTGACGCTG-3′; Scn1a 739F 5′-GACAGCGGAAACAAGTAGTGCAAGC(TGT)-3′, Scn1a 7493 5′-TgAGATTCTGAGTTGTTGGAAAGAGAA-3′, e-fos 1393F 5′-CAAGCCTGGGAGCGGCTCCT-3′; e-fos 1446F 5′-CGGGAAGTGGAACTGAC-3′, Col1a1 395FL 5′-CACATGGGCTGATATGTGCAAAGCTG-3′, Col1a1 479R 5′-GGGACGCTGCTCATCCATGT-3′, P2rx4 114FL 5′-CACTTTATCCCGGAGAAGGG-3′, KCh 2469R 5′-GCCGTAAAGG-3′, P2rx4 114FR 5′-GAGAAACGTAGAGTGCAAGCTG[FAM]C-3′, Tgfb1 2201 5′-GGTCAGATATGGGGTCACTG5G-3′, e-fos 1393R 5′-TGTGGAGTGGTTTCTGGCAAGTAC-3′, KCh 2546R 5′-CTGAGGAGGAGCGGTCCTT-3′; c-fos 1446R 5′-GAGACGGTGGAACTGAC-3′, c-fos 1446R 5′-GCCGAAGTGGAACTGAC-3′.

Fluorophore-labeled LUX primers (forward) and their unlabeled counterparts (reverse) were purchased from Invitrogen. LUX primers were designed within Affymetrix probe set sequences for each gene, and all primers were designed using LUX Designer software (http://www.invitrogen.com/lux). A multiplex PCR method that combined each experimental gene with the housekeeping gene GAPDH in each PCR mix was used. For each sample, 20 µl PCR mix contained 2 µl cDNA (first diluted 1:10 after reverse transcription), 200 nM of each gene-specific primer (two pairs for multiplex PCR), 1× Platinum Quantitative PCR SuperMix-UDG (Invitrogen), and 1× ROX reference dye (500 nM in SuperMix). PCR conditions were standard (Invitrogen, http://www.invitrogen.com/lux), and reactions were conducted in a 96-well spectrophotometric thermal cycler (ABI 7700 Sequence detector system, Applied Biosystems). Fluorescence was monitored during every PCR cycle at the annealing or extension step and during the post-PCR temperature ramp. Fold changes were measured according to manufacturer’s instructions. GAPDH was used as a reference and subtracted for net changes. Student’s t-test was used to calculate P values.

RESULTS

Motor scores and histopathology. In the parallel dose-response study, lesion size at the site of impact and surrounding white matter damage was increased with injury severity (Fig. 1A). In addition, severity-dependent locomotor differences on the BBB rating scale were observed over 28 days after injury (Fig. 1B). The dissection scheme for rostral, caudal, and injury sites is shown (Fig. 1C).

Database, Public Expression Profiling Resource (PEPR), and SAS visualization tool. All microarray data are available on the Public Expression Profiling Resource web site (http://pepr.cnmrsearch.org/home.do). This website contains .DAT, .CEL, and .TXT files for every microarray as well as online time query and visualization tools to assist with analysis of the database. In addition to Supplemental Tables and Supplemental Figures for this report, the web site contains data from additional acute time points (0.5, 12, 48, and 72 h) (RG-U34; A, B, and C arrays). (The Supplemental Material for this article is also available at the Physiological Genomics web site.)

In addition to PEPR, all biological transcripts on the RG-U34 A, B, and C arrays can be visualized across time, injury levels, and spinal cord locations in three-dimensional graphical format in our SAS server and visualization tool (http://sas.cnmrsearch.org/). Moreover, normalization of the entire data set to either naive or sham samples is made optional, and graphical outputs can be zoomed and rotated for better visualization. In addition to Supplemental Tables and Figures for this report (PEPR), both web sites contain data from all time points (RG-U34; A, B, and C arrays) and regions as detailed in MATERIALS AND METHODS. We have previously reported gene changes induced by mild injury at selected time points (17) and specific clusters involved in cell cycle, plasticity, and regeneration (15, 16). However, here we focus on a global analysis of selected time points after injury (4, 24, 168 h) that are widely studied with respect to the pathophysiology of secondary injury, and we focus on a few potentially related clusters.

Global changes in expression after SCI: effect of injury severity, spinal cord region, and laminectomy. Hierarchical clustering and intensity maps revealed broad expression patterns related to injury severity, spinal cord region, and laminectomy alone (sham). Sham surgery, mild, moderate, and severe injury resulted in marked overall changes in expression profiles compared with naive animals (Fig. 2, A–C). After mild injury, expression of many genes was increased, with the
highest fold changes observed relatively early (4 and 24 h), followed by gradual reductions (although still significantly elevated compared with sham surgery) over time (Fig. 2A, boxed genes). After severe injury, the highest fold changes were comparatively delayed (24 h and 7 days) (Fig. 2B, solid boxes). Severe and moderate injuries generally showed the greatest number and magnitude of alterations; indeed, expression of some genes was directly proportional to injury severity (Fig. 2C). Expression of many genes was reduced at the injury site, but increased in rostral and caudal regions (Fig. 2B, dashed boxes).

Spinal cords from animals that received laminectomy only (sham injured controls) also showed overall changes in expression compared with naive animals. These alterations in expression induced by laminectomy alone extended not only from 4 h to 7 days after surgery, but also to rostral and caudal regions.
away from the site of impact (shown as example in mild injury Fig. 2A, ovals). Thus, laminectomy alone induced widespread relatively prolonged effects on gene expression that resembled a mild injury effect.

Table 1 shows data presented in Fig. 2 in terms of gene numbers. Since the intensity maps indicated that laminectomy alone altered gene expression, separate analyses compared injury-induced changes to either naive or to sham controls for each respective time point and region (Table 1, A). As expected from the intensity maps, comparison of injuries to sham-operated animals rather than naive animals reduced the number of genes that met criteria for significant changes in expression, particularly at the injury site (Table 1, A). Generally, more genes were altered after moderate or severe injury than after mild injury (Table 1, A), although such changes differed between regions. At the injury site, the number of changes increased, and then decreased, with progressive injury severity (from mild to moderate to severe). In rostral and caudal regions, the greatest number of changes was observed after severe injury. The direction of changes (up or down) depended on injury severity, with increased expression of most genes observed after mild or moderate injury and with decreased expression of genes predominating after severe injury (Table 1B).

Functional classifications. Approximately 50% of all gene changes were significant by both the MAS 5.0 and dChip algorithms. Injury-induced changes in gene expression that met all significance criteria (significant by MAS 5.0 and dChip, significant vs. sham, ≥1.5-fold change) were classified into functional categories as detailed in MATERIALS AND METHODS. These were then grouped into gene lists with functional groups ranked by overall expression level and actual fold change values (Supplemental Tables S1–S7). Functional classes with the largest number of genes altered by injury included those associated with metabolism, inflammation/immune responses, signal transduction, transcription/protein synthesis, and the cytoskeleton (Fig. 3).

There were no marked alterations in global representation of the functional classes (% individual functional group/all changes) that could be consistently associated with location relative to the injury site or to injury severity. However, these factors did alter representation of the classes such that they were not precisely identical. They varied slightly between different regions and injury severities, with the actual number and identity of genes within each class fluctuating widely with these variables (Fig. 3 and Supplemental Tables S1–S7). Further sorting of the initial functional classes into subgroups of two or more functionally similar genes also failed to reveal any marked trends or patterns related to distance from the impact or injury severity (Supplemental Tables S1–S7).

ESTs and RG-U34B/RG-U34C arrays. Enough information was available on public databases to allow functional classification of ~65% of the ESTs that were altered by SCI (see Supplemental Materials). After the functional classification was completed, we calculated how many classified genes/ESTs were represented on RG-U34B or RG-U34C arrays to gauge what level of information was obtained by including them in the analysis. We found that ~50% of functionally classified ESTs were derived from RG-U34B or RG-U34C arrays (Table 2).

Genes common to SCI profiling studies. Table 3 summarizes published studies of expression profiling of traumatic SCI. Many gene expression changes that were initially described in these studies were also observed in the present experiment. Examples include specific genes associated with inflammation (i.e., cathepsins, interleukin-1β, intercellular adhesion molecule-1) (3, 23), secretory/synaptic processes (SNAP proteins, synaptogyrin) (3, 11), and oxidative and/or stress-responses (Hsp70, Fos, metallothioniens) (11) (see Supplemental Material). Additional genes in each of these groups were identified.
with the RG-U34B and RG-U34C arrays (for example of genes that clustered with IL-1β, see Supplemental Fig. S1). Several other functionally related gene subclasses were also identified that have not previously been a focus of reported SCI array data. These included genes involved in the ubiquitin cycle, G protein-coupled signal transduction, translation initiation/elongation, and RNA splicing (Supplemental Material).

Identification of neuronal-specific genes and a transcription factor binding cluster. Highlighted below are some gene clusters that were significantly altered after injury but have not previously been associated with SCI. They serve as examples of genes that may represent potential candidates for further study.

An association was derived by initially comparing expression profiles of two potassium channel genes [Kcncl2 (M59980), Kcnk1 (AF022819)] that were previously associated with CNS injuries in multiple array studies (25) with that of two potassium channel genes that showed marked down-regulation in our data set [Kcnc2 (X62829), Kcnc1 (X62840); Kcnc1 was also selected for RT-PCR analysis, described below] (Fig. 4). Next, we used the average signal of these related channel genes as an anchor for a new gene cluster (Fig. 5). The resultant cluster (correlation coefficient \(r = 0.993\)) contained a large group of genes whose expression was inversely correlated with injury severity (Fig. 5). Many of these genes have important neuronal-specific functions (synuclein, brain glyco-
Di Giovanni et al. Aimone et al. Velardo et al. analysis, since it was the most responsive to injury and is one seemed that Cebp\textsubscript{H9254} (AA900875), and Copeb (AI044674) (Fig. 6). Of these, it after mild injury. The cluster included Cebp\textsubscript{H9254} and Copeb (AI044674) (Fig. 6). Overexpression of Cebp\textsubscript{H9254} in neuroblastoma cells increases expression of a specific group of target genes [ornithine decarboxylase (ODC), GRO, lipocalcin 2, decorin, and xanthine dehydrogenase] that may be involved in CNS injury (14). Cebp\textsubscript{H9254} is not present on the RG-U34 arrays, but it shows pleiotropic activity with Cebp\textsubscript{H9252}, so we looked at the potential relationship between Cebp\textsubscript{H9254} and these genes in our data set.

Table 3. Microarray studies of experimental traumatic spinal cord injury

<table>
<thead>
<tr>
<th>Study</th>
<th>Model</th>
<th>Anesthesia</th>
<th>Subject</th>
<th>Injury Severity</th>
<th>Study Groups</th>
<th>Design</th>
<th>Platform</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Song et al. 2001 (Ref. 58)</td>
<td>MASCIS T9</td>
<td>Halothane nitrous oxide</td>
<td>Rat strain unspecified, female (275–325 g)</td>
<td>10 g × 25 mm (moderate)</td>
<td>Sham, 3 h, 24 h</td>
<td>pooled 3 rats per n; n = 3/group; -0.8 cm epicenter</td>
<td>Affymetrix RN-U34</td>
<td>MAS &lt; 4.0; P calls 7 of 9 crosswise comparisons; hierarchical clustering in Cluster, TreeView; confirmation: RT-PCR</td>
</tr>
<tr>
<td>Carmel et al. 2001 (Ref. 11)</td>
<td>MASCIS T9–10</td>
<td>Pentobarbital Sodium</td>
<td>Long-Evans Rat, male/female</td>
<td>10 g × 25 mm</td>
<td>6h, 12h, 24h, 48h; time-yoked shams</td>
<td>pooled 1–3 rats/sample; M/F rats; n = 3/group; 0.5 cm epicenter; 0.5 cm caudal</td>
<td>Affymetrix RG-U34A</td>
<td>MAS 4.0, difference call in 8 of 9 replicate comparisons; hierarchical clustering in Cluster, TreeView; GeneCluster normalized to mean of 0, variance 1; Confirmation: RT-PCR</td>
</tr>
<tr>
<td>Di Giovanni et al. 2003 (Ref. 17)</td>
<td>OSU T8</td>
<td>Ketamine/ xylazine</td>
<td>Fischer rat, female (165–200 g)</td>
<td>1.0 mm displacement (moderate)</td>
<td>0.5 h, 4 h, 24 days; time-yoked shams</td>
<td>unpoled; n = 4/group; 1.0 cm epicenter</td>
<td>Affymetrix RG-U34A</td>
<td>MAS 4.0. P calls in 40% or more profiles; 2.0-fold change restriction; clustering in GeneSpring; confirmation: QMF-PCR, Immunoblot, immunocytochemistry</td>
</tr>
<tr>
<td>Resnick et al. 2004 (Ref. 51)</td>
<td>MASCIS T8–9</td>
<td>Halothane</td>
<td>Long-Evans Rat, male (300–340 g)</td>
<td>12.5 g × 10 mm</td>
<td>12 h, 42 days; 12 h sham controls</td>
<td>unpoled; n = 3/group; 1.0 cm rostral and caudal; samples 1.5–2.5 cm; away from epicenter</td>
<td>Affymetrix RG-U34A</td>
<td>MAS 4.0, dChip, drop method, Naef algorithm; 1.2-fold change restriction; confirmation: RT-PCR</td>
</tr>
<tr>
<td>Velardo et al. 2004 (Ref. 64)</td>
<td>MASCIS C4–5</td>
<td>Ketamine/ xylazine</td>
<td>Sprague-Dawley Rat, athymic nude Rat, female (220–250 g)</td>
<td>10 g × 12.5 mm</td>
<td>1, 3, 10, 30, 90 days; &quot;normal&quot; vs. athymic nude naive, sham</td>
<td>pooled 4 rats/sample; n = 3 per group</td>
<td>Affymetrix RG-U34A</td>
<td>MAS9;0; SAM; hierarchical clustering in Cluster, TreeView; confirmation: immunocytochemistry</td>
</tr>
</tbody>
</table>

A second cluster focused on transcription factors, since alterations in these might affect expression of multiple downstream target genes. The transcription factor Egr-2/Krox-20/SCIP/Oct-6 (U78102) was significantly altered after mild and severe injury and is involved in myelination, plasticity, and ischemic CNS injury (46, 57, 59). Therefore, we selected this gene to nucleate ($R^2 = 0.99$) a cluster of transcription factors after mild injury. The cluster included Cebp\textsubscript{H9256} (M65149), Cit-ed4 (AA900875), and Copeb (AI044674) (Fig. 6). Of these, it seemed that Cebp\textsubscript{H9256} might be the best candidate for further analysis, since it was the most responsive to injury and is one of a group of CCAAT enhancer binding proteins that are involved in inflammation, growth arrest, and stress response (Fig. 6). Overexpression of Cebp\textsubscript{H9256} in neuroblastoma cells increases expression of a specific group of target genes [ornithine decarboxylase (ODC), GRO, lipocalcin 2, decorin, and xanthine dehydrogenase] that may be involved in CNS injury (14). Cebp\textsubscript{H9256} is not present on the RG-U34 arrays, but it shows pleiotropic activity with Cebp\textsubscript{H9252}, so we looked at the potential relationship between Cebp\textsubscript{H9256} and these genes in our data set. Elevations in Cebp\textsubscript{H9256} correlated with Gro and preceded elevations of ODC and lipocalcin 2 after mild and moderate injury (Fig. 7, A and B), but neither decorin nor xanthine dehydrogenase were increased after injury (Fig. 7, A and B).

Technical confirmation of microarray data with multiplex RT-PCR. Using quantitative and multiplex real-time PCR, we have extensively confirmed our mild injury site data set in
In the present study, we performed additional confirmation with multiplex PCR of seven genes from different functional groups (cell growth/differentiation, neurotransmission, inflammation, extracellular matrix, and transport) that were expressed over a range of levels (low to high). Fold changes were statistically significant ($P < 0.01$). We then compared the fold changes declared after analysis of the array data with each probe set algorithm to those detected by RT-PCR, and we found that fold changes in array data derived with either algorithm were confirmed by RT-PCR, although actual values were scaled differently (Table 4). Fold changes of dChip values correlated linearly with RT-PCR values ($P = 0.0478$), whereas MAS 5.0 values did not ($P = 0.521$) (Table 4), which suggests a closer alignment between dChip values and those from RT-PCR.

DISCUSSION

Comprehensive evaluation of changes in gene expression that contribute to secondary injury, tissue loss, and neurological dysfunction may lead to the development of novel interventions that promote recovery. To that end, we have undertaken an extensive study to assess temporal and spatial changes in expression after injuries of different severities. Our results indicate that both laminectomy and impact trauma result in prolonged changes in gene expression in the rostral and caudal regions, as well as the impact site. Laminectomy showed a profile consistent with mild injury. This unexpected observation depended upon the use both sham and naive controls, the combination of which has not been previously published in SCI microarray studies. The greatest number of gene changes was observed at the injury site after moderate injury. Perhaps surprisingly, both severe and mild injury led to relatively fewer changes in gene expression in this region. However, the number and magnitude of alterations in the rostral and caudal regions generally increased with injury severity. Moreover, decreases in gene expression correlated with severity in all regions. Major contributors to the global expression response included genes involved in metabolism, inflammation/immune response, signal transduction, transcription/protein synthesis, and cytoskeleton. Nevertheless, there was considerable heterogeneity in the proportional representation of these classes, as well as for other functional groups and individual genes, when responses to injury severity were compared spatially. Last, several specific clusters of genes were identified that may correlate with cell death and/or neuronal injury. In previous reports, we have focused on subsets of these data that are involved in cell cycle, synaptic plasticity, and regeneration (15–17).
It should be underscored that sham injury (lamineotomy surgery alone) produced temporally extended (7 days) responses in the rostral and caudal regions that resembled mild injury. The fact that sham surgery alters gene expression emphasizes the importance of study designs that include sham controls that are temporally yoked to experimental injury, as well as the inclusion of naive controls. Not only do the naive controls help determine the magnitude of change in shams, but they can protect against false negatives arising from comparisons of experimental groups to sham groups, in cases where the sham response is robust.

Functional classifications and novel clusters. Consistent with findings from other groups (3, 11, 64), transcripts from genes with roles in inflammation, transcription and protein synthesis, metabolism, and the cytoskeleton were well represented after injury; however, changes in genes involved in oxidative stress, apoptosis, cell growth/differentiation, proteolysis, cell adhesion/extracellular matrix, and transport were less common. Representation of most of the functional classifications was broadly similar but also showed considerable severity and spatially dependent heterogeneity, rather than a specific injury or spatially dependent pattern. Our analysis rejected all but the most robust differences (see later DISCUSSION) and therefore is less sensitive to smaller yet potentially physiologically meaningful alterations. It is unlikely that the functional classifications were too broad to reveal important changes, as further sorting into additional subgroups did not suggest any distinct trends. However, the classifications themselves are subjective; therefore, a different sorting scheme may reveal different expression patterns. In addition, pathophysiological mechanisms typically involve groups of genes that cross several different functional groups and thus may not be as readily apparent from functional gene lists. Pathway driven or temporal clustering approaches may assess such changes more effectively (see Supplemental Fig. S2 for pathway analysis).

We applied temporal clustering approaches in search of novel clusters that might be related to mechanisms or markers of injury and/or cell death. The first cluster was anchored with potassium channel expression levels; therefore, it was not unexpected that many of the genes in the cluster were neuronal-specific. However, it is interesting that several genes in this cluster are particularly associated with neurites/synapses and plasticity. For example, HuD is a developmentally regulated neuronal-specific Hu/ELAV-like protein that stabilizes
GAP-43 and promotes neurite outgrowth and/or differentiation of PC12 cells (6, 13, 42). Ntab is a noncoding transcript that is only expressed during later stages of development and in adult mammalian brain (21). Although its presence has not previously been demonstrated in spinal cord, in brain it is apparently transported to neuronal processes; however, its function is unknown (21). Synucleins (α and β) are also synaptic proteins; their decreased expression contrasts to findings in chronic neurodegeneration or traumatic brain injury, where synucleins accumulate and/or increase (12, 44, 63). Visinin-like 1 is a neuronal calcium sensor/binding protein that is reduced in Alzheimer’s disease and translocated to the extracellular space, where it is associated with plaques, tangles, and dystrophic neurites (9, 53). Because many of these genes are “neuronal-specific” and are both decreased over time and with increasing injury severity, it is possible that these changes may reflect cell death within the epicenter, rather than downregulated expression. These issues require additional study for clarification.

A second cluster included a group of transcription factors (Cited4, Coped, Cebp, Cebp) that may be related to injury. Cited4/ Cebp-interacting transactivator participates in the regulation of hypoxia-inducible factor 1α (HIF-1α) as well as downstream HIF-1α-controlled hypoxia-mediated gene activation (36, 40); thus, it may be involved in hypoxia-induced signaling in the CNS after injury. Coped/Zif9/CPBP/Kruppel-like factor 6 (Klf6) is one of several Kruppel-like transcription factors that have roles in angiogenesis and tumor suppression (glioblastoma) (28, 33). Increases in Klf6 have previously been associated with tissue injury or systemic stress, including hemorrhagic shock, radiation-induced microvessel damage, and balloon-injury to endothelial cells, where it may be involved in fibrosis and deposition of extracellular matrix (27, 29, 32).

Within the latter cluster, we focused primarily on Cebp, which has been associated with neuronal differentiation, plasticity, and acute-phase and inflammatory responses (10, 41, 54, 60, 66).

In our data set, expression of Cebp was similar to that of ODC, Gro, and lipocalin 2, which are potential downstream Cebp target genes (14). ODC is a rate-limiting enzyme in the synthesis of polyamines (putrescine, spermidine, spermine) (61). Polyamines and ODC are altered after various types of brain injury, and they may be associated with antioxidant activity, altered ionic and/or excitatory amino acid signaling, blood-brain barrier breakdown/vasogenic edema, and astrogliosis (1, 24, 50, 68). Gro is an inflammatory chemokine, and lipocalin 2 is associated with the acute phase response, two features that have been associated with Cebp in non-CNS tissue (49, 55). Interestingly, TNF and IL-1β were significantly increased early after SCI, and both induce Cebp synthesis (see Supplemental Material)(10). Therefore, both upstream and downstream gene changes are consistent with the concept that Cebp may be important in the secondary injury response.

Additional studies will be necessary to clarify the role of the CCAAT/enhancer-binding proteins in SCI and to determine which specific cell-types may upregulate expression of this transcription factor family in response to injury.

Comparison to previous studies. Many studies have recently utilized microarray analysis to profile changes in gene expression in a variety of rodent models of SCI (3, 16, 17, 20, 22, 23, 43, 64). The present study differs from most previous approaches (summarized in Table 2) not only in focus (injury severity, rostral/caudal examination, inclusion of sham and naive animals) but also in other differences in sampling, platform, and analysis. Our sampling/analysis utilized individ-
ual (unpooled) animals, time-yoked sham animals, and groups with relatively high numbers \((n = 4/\text{group})\). In addition, although we presently report data from selected time points within the acute postinjury period, the entire study includes temporally repetitive sampling \([0.5, 4, 12, 24, 48, 72, 168 \text{ h (7 day)}]\) over this time frame (data are available at http://pepr.cnmcresearch.org/home.do), which is convenient for temporal validation of results, as well as alternative statistical approaches to analysis \((31, 45)\). In addition, we have now implemented a publicly available SAS server database and visualization tool for better visualization across injuries, locations, and time, offering three different project normalization options (naïve animals, time-matched sham animals, and absolute expression values). The present experiments are also unique in that we used the complete set of rat RG-U34 Affymetrix arrays, which covers \(\sim 27,000\) genes/ESTs, whereas previous studies utilized either spotted arrays, RG-U34A, or Affymetrix rat neurobiology arrays. RG-U34A or neurobiology arrays cover \(\sim 7,000\) genes, the majority of which are full-length and/or annotated \((\sim 1,000\) are ESTs) \((\text{http://Affymetrix.com})\). However, the entire set of RG-U34 arrays \((A, B, C)\) contains over 16,000 ESTs; therefore, it was important to show that the majority of genes whose expression was altered by injury have been studied and annotated well enough to be classified and thus represented in the global assessment of the functional genomic response. Nearly half of all genes that were altered by injury were identified through the use of RG-U34B and RG-U34C arrays, which have not previously been used in SCI studies. In many cases, ESTs from the RG-U34B and RG-U34C arrays also increased the number of genes associated with clusters that have already been verified and/or are under currently under investigation \((3, 11, 15–17, 23)\).

Table 4. Fold change comparison across different algorithms

<table>
<thead>
<tr>
<th>Probe Set No.</th>
<th>Common Name</th>
<th>Accession No.</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z78279_at</td>
<td>Colla1</td>
<td>Z78279</td>
<td>2.19</td>
</tr>
<tr>
<td>X62840mRNA_s_at</td>
<td>Knc1</td>
<td>X62840</td>
<td>0.21</td>
</tr>
<tr>
<td>X52498cds_at</td>
<td>Tgfb1</td>
<td>X52498</td>
<td>6.43</td>
</tr>
<tr>
<td>X06769cds_at</td>
<td>c-fos</td>
<td>X06769</td>
<td>6.93</td>
</tr>
<tr>
<td>U47031_at</td>
<td>P2rx4</td>
<td>U47031</td>
<td>5.32</td>
</tr>
<tr>
<td>U39549_g_at</td>
<td>Syngr1</td>
<td>U39549</td>
<td>10.27</td>
</tr>
<tr>
<td>M22253_at</td>
<td>Scn1a</td>
<td>M22253</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Fold changes are statistically significant \((P < 0.01)\).
REFERENCES


ACKNOWLEDGMENTS

We thank Andres Lerner, Jorge Garay, and Cinzia Brandoli for expert technical assistance.

GRANTS

This work was supported by National Institutes of Health contract NIH-NINDS-01 (NS-1-2339).


