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DNA Microarray Analysis of the Contused Spinal Cord: Effect of NMDA Receptor Inhibition

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Spinal cord injury (SCI)-induced neurodegeneration leads to irreversible and devastating motor and sensory dysfunction. Post-traumatic outcomes are determined by events occurring during the first 24 hours after SCI. An increase in extracellular glutamate concentration to neurotoxic levels is one of the earliest events after SCI. We used Affymetrix DNA oligonucleotide microarrays (with 1,322 DNA probes) analysis to measure gene expression in order to test the hypothesis that SCI-induced N-methyl-D-aspartate (NMDA) receptor activation triggers significant postinjury transcriptional changes. Here we report that SCI, 1 hour after trauma, induced change in mRNA levels of 165 genes and expression sequence tags (ESTs). SCI affected mRNA levels of those genes that regulate predominantly transcription factors, inflammation, cell survival, and membrane excitability. We also report that NMDA receptor inhibition (with -(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine hydrogen maleate [MK-801]) reversed the effect of SCI on about 50% of the SCI-affected mRNAs. Especially interesting is the finding that NMDA receptor activation participates in the up-regulation of inflammatory factors. Therefore, SCI-induced NMDA receptor activation is one of the dominant, early signals after trauma that leads to changes in mRNA levels of a number of genes relevant to recovery processes. The majority of MK-801 effects on the SCI-induced mRNA changes reported here are novel. Additionally, we found that the MK-801 treatment also changed the mRNA levels of 168 genes and ESTs that had not been affected by SCI alone, and that some of their gene products could have harmful effects on SCI outcome.

Key words: spinal cord injury; NMDA receptor; MK-801; DNA microarray; Affymetrix; expression profile

The severity of sensory and motor loss after contusion spinal cord injury (SCI) depends on the size of the lesion surrounding the epicenter of the lesion. Initial tissue injury results from acute processes such as hemorrhage, edema, and excitotoxic cell death. For weeks after injury, the lesion cavity expands due to secondary damage resulting from processes that include inflammation, necrosis, apoptosis, and glial scar formation (Taoka and Okajima, 1998).

Considering the large number of components present in secondary damage after SCI, DNA microarray analyses are an appropriate methodological approach to determine the transcriptional changes that lead to secondary damage. While there have been effective DNA microarray analyses of gene expression profiles in some neuropathological conditions (Ginsberg et al., 2000; Mirnics et al., 2000), there are no published data on SCI-induced expression profiles.

Following the initial mechanical insult of SCI, there is accumulation of excessive glutamate at the injury site, lasting over 1 hour after SCI (Liu et al., 1991, 1999; McAdoo et al., 1999). The spatial and temporal distribution of the released glutamate correlates well with lesion

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size (10 mm) after SCI (Wrathall et al., 1994). Glutamate contributes to cell loss directly by inducing immediate excitotoxic cell death, when an excess of intracellular Ca^{2+} ions activates Ca^{2+}-dependent signaling cascades that eventually result in cell death (Sattler and Tymianski, 2000). Mobilization of free cytosolic calcium leads to the generation of oxygen radicals, energy failure, damage to the cytoskeleton, and protein missfolding (Sapolsky, 2001). However, early activation of glutamate receptors, and the resultant increase in intracellular calcium concentration, also trigger responses to injury that mediate recovery. For example, decreases in neuronal excitability, achieved by activating Ca^{2+} and ATP-dependent K^+ channels, or decrease in Ca^{2+} mobilization by inactivating N-methyl-D-aspartate (NMDA) channels via calpain and inducible nitric oxide (iNOS), are part of the recovery processes (see Sapolsky, 2001). However, there are also delayed postinjury processes that increase lesion cavity, instead of alleviating injury effects. Examples may be the unbalanced synthesis of pro-apoptotic and pro-inflammatory factors. How these delayed events are initiated and how they augment injury is not well understood. Glutamate receptor activation is likely to be one of the earliest major triggers for SCI-induced transcriptional changes that lead to secondary damage.

In order to characterize early SCI transcriptional changes that will be helpful to better understand the development of secondary damage, we used Affymetrix DNA microarrays (RN34A) to measure changes in mRNA levels 1 hour after SCI. We determined, via DNA microarray analyses, the effects of the NMDA receptor antagonist, -(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine hydrogen maleate (MK-801), in order to identify which SCI-induced changes in mRNA levels were affected or reversed by NMDA receptor inhibition. Furthermore, we showed that MK-801 treatment perturbed expression of genes irrelevant to SCI that could be a source of possible confounds.

MATERIALS AND METHODS

Animal Model for Spinal Cord Injury

Contusion injury to the rat spinal cord is closely related to the contusion/cyst type of injury (Basso et al., 1996), which is most often observed in a clinical setting (Bunge et al., 1993; van de Meent at al., 1996). Thus, we used a rodent spinal contusion model in these experiments (Gruner, 1992; Basso et al., 1995). Sprague-Dawley male rats (200–300 g) were anesthetized prior to surgery by administering pentobarbital (35 mg/kg) intraperitoneally. Anesthesia was deemed complete when there was no response to a foot pinch. Animals' backs were shaved, the area washed with the antiseptic betadine and a laminectomy performed over spinal segment T8. The spinal cord was injured by dropping an impactor probe (10 g, 2 mm diameter) from a distance of 12.5 mm onto the cord at spinal segment T8. Following injury, the animals were maintained in the anesthetized state and killed without revival from anesthesia if sampling was to occur during the next several hours. For longer-term experiments, the wound was closed by suturing the muscle and fascia and the skin closed with surgical staples. All procedures complied with the recommendations in the *NIH Guide for the Care and Use of Laboratory Animals* and were approved by the *UTMB Animal Care and Use Committee.*

Three groups of rats (sham, and two groups of injured rats) were given either artificial cerebrospinal fluid (ACSF; sham and one group of injured rats) or a treatment ((+)MK-801 hydrogen maleate; MK-801; RBI-Sigma, St. Louis, MO) was administered intrathecally for 1 hour before the injury (60 μl) and 1 hour after injury directly to the site of injury (30 μl).

Intrathecal administration. The initial incision of the skin exposed a superficial layer of neck muscles which was then separated by a midline incision beginning at the occipital crest and extending caudally about 2 cm. Separation of the superficial musculature exposed an underlying layer of muscles, which could be easily separated along the midline by blunt dissection. Next, a scraping tool was used to free the muscles from their point of origin on the occipital bone for about 0.5 cm on either side of the midline. The back of the skull could then be seen and gentle retraction of the neck musculature exposed the atlanto-occipital membrane. This fascial membrane connects the base of the skull with the first vertebra. A small hole (0.5 mm diameter) was drilled through the atlanto-occipital membrane, the stretched end of the catheter was inserted in the subarachnoid space, and clear cerebrospinal fluid filled the catheter. Catheter (Micor, Inc, Allison Park, PA) was cut to the appropriate length (5 cm) to allow positioning above T8 in a 250–260 g rat. ACSF or MK-801 was pumped through the catheter for 60 minutes at a flow of 1.0 μl/minute; therefore total injected volume was 60 μl. Laminectomy was performed, the T8 segment exposed, and ACSF or MK-801 pumped through a 30-gage needle directly into the site of injury (T8), during 60 minutes postinjury at a flow rate of 0.5 μl/minute. Therefore, total injected volume of ACSF or MK-801 (intrathecally + needle) was 90 μl. The dose of MK-801 used in this experiment (4.5 μg/hour) was chosen based on the results of Wong et al. (1998).

DNA Microarrays

Target preparation. The portion of the spinal cord containing segments T7–T9 was removed from anesthetized animals to provide tissue for the analyses. The animal was perfused transcardially with 120–150 ml of 0.9% NaCl containing heparin (1,000 units/l), to eliminate blood from the spinal cord tissue. Segments were then marked based on the location of dorsal roots and the desired section of the cord removed. The isolated length of cord was immediately placed in dry ice and allowed to freeze fully. Total RNA was prepared from frozen spinal cord segments (T7–T9) using TRI-Reagent (Molecular Research Center, Cincinnati, OH). Spinal segments were homogenized in 1,000 μl of TRI-Reagent, and total RNA extracted in chloroform, ethanol precipitated, and stored at −80°C. Total RNAs were assayed for integrity on 1% denaturing agarose gels. Approximately 10 μg of total RNA was used for each target. RNA was reverse-transcribed into double-stranded cDNA with a T_7 promoter-containing primer using Superscript II (Life Technologies, Bethesda, MD), as recommended by Affymetrix. Following extraction with phenol-chloroform and ethanol precipitation from ammonium acetate, the cDNA was used as a template in a biotin-labeled in vitro

TABLE I. Number of Genes Expressed in Spinal Cord Samples Analyzed Using Affymetrix (RN34A) DNA Oligonucleotide Microarrays

Experimental group	Number of expressed genes mean ± S.D.	Correlation coefficient (r) among sample pairs
Sham (n = 3)	604 ± 25	$0.98 > r > 0.99$
Injury (n = 3)	667 ± 30	$0.97 > r > 0.99$
Injury + MK-801 (n = 3)	518 ± 82	$0.95 > r > 0.98$

transcription reaction (Enzo BioArray, Affymetrix, Santa Clara, CA). Resulting target cRNA was collected on RNeasy columns (Qiagen, Chatsworth, CA) and then fragmented for hybridization to the microarrays.

Microarrays. The rat neurobiology U34 microarray from Affymetrix was used in all hybridizations. This array contains a representative 1,200 gene set chosen to represent important central nervous system (CNS) functions. Probes consist of 16 pairs of 25-mer oligonucleotides. One member of each pair contains a point mutation, and the signals of the pair are compared to assess specificity of hybridization. Biotinylated target cDNA was hybridized onto the array and then processed using the Affymetrix Genechip Fluidics Workstation 400, following the Mini Euk 2v2 protocol, except that only 3 μg of fragmented cRNA was added to the hybridization cocktail. Following binding with phycoerythrin-coupled avidin, microarrays were scanned on a Hewlett Packard GeneArray Scanner. Results were analyzed with Affymetrix GeneChip Analysis Suite 4.0 software. Individual microarrays were scaled to produce a mean signal intensity (average difference) of 2,500, excluding the top and bottom two percentile to remove outliers. The "average difference" describes the signal intensity difference between the match and mismatch probes for each gene averaged over the number of probe sets. Barring systematic error, the average difference reflects the amount of mRNA detected or expression level for each gene probed. In addition, the Affymetrix program calculates the absolute call for presence or absence of a transcript, where absence means, "not expressed." The absolute calls can be P (present), A (absent), and M (marginal). "Absent" means that nonspecific binding and noise are higher than specific binding of a probe; therefore, a zero in a table indicates that the specific transcript could not be detected.

To analyze the genomic data, we performed: (1) cluster analysis of overall expression profiles in order to establish similarities among microarrays; (2) cluster analysis of expression levels in order to classify similar groups of genes; (3) detection of genes with statistically significantly changed expression levels in the three experimental groups.

Cluster analysis

Clustering of arrays. Our data consisted of three different experimental groups (sham, injured, and MK-801-treated injured), with three samples (arrays) per group, i.e., nine arrays in all (Table I). We first compared the overall expression profiles of nine arrays by performing hierarchical cluster analysis. This is necessary in order to detect eventual gross differences among the arrays belonging to the same experimental group, as this could invalidate further statistical analysis of single gene expression changes. The clustering of arrays was performed by SPSS software, with the procedure that uses Average Linkage, and Manhattan or city-block distance measure between the expression vectors. This distance is defined between two points, $x=(x_1, x_2, \ldots x_n)$ and $y=(y_1, y_2, \ldots y_n)$, in n-dimensional space as $d(x,y) = \sum_{i=1}^{n} | x_i - y_i |$, here n = 1,322. The results of array clustering are shown in Figure 2. Other clustering procedures yielded similar results.

Clustering of genes. The second step involves clustering of different genes according to similarities in their expression levels. This global approach is usually used and is most useful to detect and compare expression profiles from among many different conditions, stimuli or phenotypes (Schultze and Downward, 2001). By using these cluster-analysis tools it is possible to identify potential co-regulated genes and genes with related functions. We performed cluster analysis by the use of the recently released CLUSFAVOR software developed by L.E. Peterson for the cluster and factor analysis of DNA microarray data (Tables IIC and VIIC). This software is freely available at: http://mbcr.bcm.tmc.edu/genepi/.

Statistical significance. Cluster analysis of expression levels of single genes can detect coherent patterns of gene expression (Eisen et al., 1998), but provides little information about the level of statistical significance present among different expression levels of the genes in the different experimental groups. Finding genes with significantly changed expression levels in one of the compared groups was done by: (a) identifying genes that are expressed ("present") only in one experimental group but not the other experimental group ("absent"), or (b) identifying genes that are expressed ("present") in both compared groups, with significantly changed expression levels in one group.

a. The decision process for absolute calls in one group (n = 3) was based on the significance level ($P<0.05$) obtained from the χ^2 test (df = 1). Only the combinations of three "absent" versus three "present calls" (and vice versa; $\chi^2 = 6$); and two "present" versus three "absent" calls (and vice versa; $\chi^2 = 4.5$) yielded a confidence level higher than 95%. All other present/absent combinations were discarded as not significant. Consistently different absolute call between two compared groups indicates the direction of change in mRNA levels: the change from consistently "absent" to consistently "present" suggests the increase in mRNA levels; vice versa change suggests a decrease in mRNA levels. The fold differences for the genes obtained from this "present/absent" analysis were not calculated, because the expression levels for genes with "absent" calls in at least two out of three samples, represents mainly noise and nonspecific binding-induced fluorescence, and not the expression level of the specific mRNA being measured.

b. If a gene was expressed in both groups (i.e., "present" in at least two samples of one group, and in all three samples in the other group), we calculated the fold change in expression levels and tested statistical differences by using modified *t*-tests, as well as statistical analysis of microarrays (SAM) corrections for multiple testing (see below). *t*-test scores for genes were determined on the basis of their change in expression levels, relative to the standard deviation of repeated measurement for that gene.

The genes with scores greater than some threshold could then be considered potentially significant. With this method, the list of potentially significant genes obtained in this way in microarray analysis usually contains hundreds of candidates. However, because of the large number of multiple tests, a certain number of genes on such a list are likely to be identified by chance. In the statistics of multiple testing, a familywise error rate (FWER) is defined as the probability of at least one false positive over a collection of tests. The basic method to reduce FWER in multiple tests is the Bonferroni correction, which imposes extremely conservative requirement for the threshold to be used for a single test but is not considered amenable to DNA microarray analyses (Tusher et al., 2001). Therefore, we used the recently developed (Tusher et al., 2001) SAM, a robust statistical method devised specifically for the analysis of microarray data. The complete version of SAM software is available for downloading from: http://www-stat.stanford.edu/~tibs/SAM/index.html.

This method computes the percentage of falsely identified genes for each dataset, or false discovery rate (FDR), by averaging the number of genes called significant over the appropriately chosen permutations of data among experimental groups. With the single tuning parameter (called Δ in the original work), the FDR for the dataset can be reduced, at the expense of a reduction in the number of significant genes. In our analysis, we selected the adjustable parameter Δ such that FDR was <5% for the list of significant genes. Note also that this method reliably identifies genes as statistically significant even in those instances where expression levels show relatively unremarkable fold changes. Because DNA microarrays are inherently unreliable (Butte et al., 2001), we introduced an additional cutoff to the list of significantly changed, according to the modified *t*-test, expression levels. We accepted only those mRNA values with a fold change that was higher than 1.5 for up-regulated genes and lower than 0.66 for down-regulated genes. These cutoff values are based on our analysis of identical samples (data not shown).

Therefore, the lists of identified significantly changed gene expressions presented in Tables III–VI are obtained from three types of analysis: (1) determination of significance based on χ^2 test ($P< 0.05$) for present/absent calls, (2) determination of significance based on modified *t*-test ($P< 0.05$) for changes in expression levels of genes present in both groups, with the use of appropriate multiple testing corrections (SAM), and (3) identification of genes with fold differences that are equal or higher than 1.5 (or equal or smaller then 0.66) for genes expressed ("present") in both compared groups.

Caveats of Affymetrix DNA microarrays

Negative values for expression levels for genes with "present" calls. These values were eliminated from the data set because they represent values for fluorescent intensities of mismatched pairs that mask perfect matched probes binding-induced fluorescent intensities.

Absolute call decision: to ignore or not to ignore?. Many researchers ignore absolute calls in their analysis of Affymetrix microarray data because of inherent problems with this component of the Affymetrix programs. In order to get more information about possible problems with absolute call decisions, we performed *t*-tests on two sets of data: (1) expression levels for all genes on microarrays (1,322 genes), ignoring the absent call decision, and (2) on preprocessed lists of genes, which were expressed ("present") in both groups (528 genes), according to the χ^2 test, as explained above. In both cases, we used a cutoff of 5% as an acceptable error. Significant *t*-values (regardless of fold of change) in this group of 528 genes were found for 36 up-regulated mRNAs, while in the group of 1,322 genes significant values were found for 39 up-regulated mRNAs. Among the 39 mRNAs, 22 were also in the "528 genes group," e.g., 22 genes were expressed ("present") in both groups, eight had negative expression values (therefore discarded), and nine genes that were among genes with "absent" and "present" calls. However, nine mRNAs seems unrealistically low for this experimental paradigm, considering that the number of genes with different absolute calls (736 genes). For example, we show (Fig. 3) that mRNAs for a number of pro-inflammatory factors increases significantly promptly after SCI. This finding is consistent with other published work (Bartholdi and Schwab, 1997; Kossmann et al., 1999; Hayashi et al., 2000; Nesic et al., 2001). The mRNAs for these inflammatory factors are robustly up-regulated and their activities are relevant to known SCI outcomes. All of these mRNAs have absent calls in sham samples, most likely due to the low sensitivity of Affymetrix microarrays to low abundant mRNAs. None of them are found among the significantly up-regulated nine genes, based on the SAM approach implemented on the "1,322 genes" data set. Alternatively, when we took into account the differences between "absent" and "present" calls as tested by the χ^2 test, we could identify about 20 inflammatory factors among the 87 genes detected, Table II showing increased mRNA levels after SCI (their expression levels changed from 0, not expressed; to +, expressed, indicating an increase in mRNA levels). For a majority of these, the SCI-induced increases are in agreement with observations in the literature (see Discussion).

Therefore, we believe that the *t*-test applied to the unprocessed data set (1,322 genes, e.g., when absent calls are ignored) results in a large number of false negatives, suggesting that when testing for statistical differences, only present calls should be taken into account and absence/presence differences analyzed and reported separately.

TABLE II A, B. Number of Genes Differentially Expressed After Injury:

A. Number of genes with consistently different absolute call after injury:

Sham (n = 3)	Injury (n = 3)
0	70 ($P < 0.05$; chi-square test)
17 ($P < 0.05$; chi-square test)	0

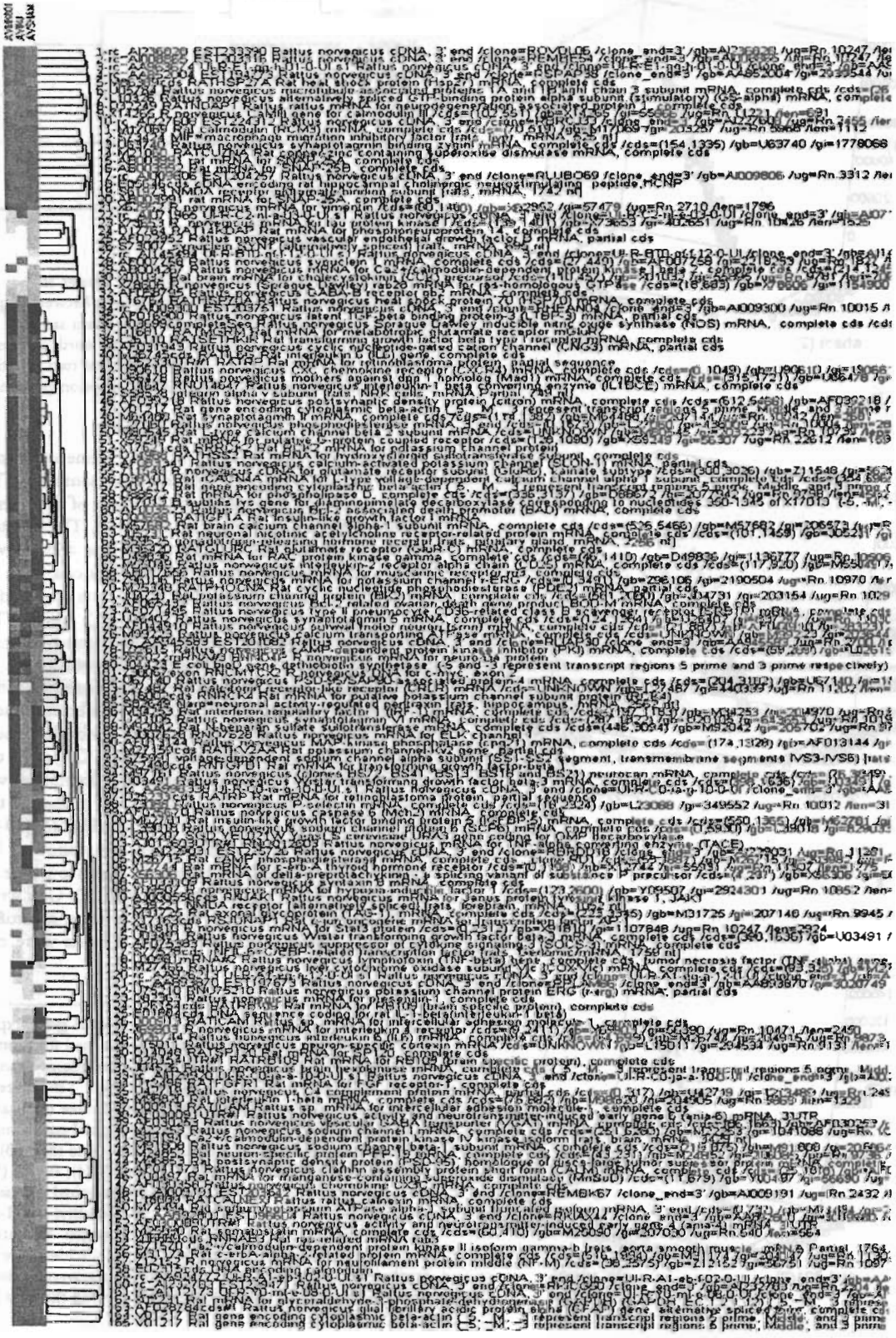
B. Number of genes expressed in injured and sham spinal cord samples and:

Up-regulated after injury:	36 ($P < 0.05$; *t*-test)
Down-regulated after injury:	42 ($P < 0.05$; *t*-test)

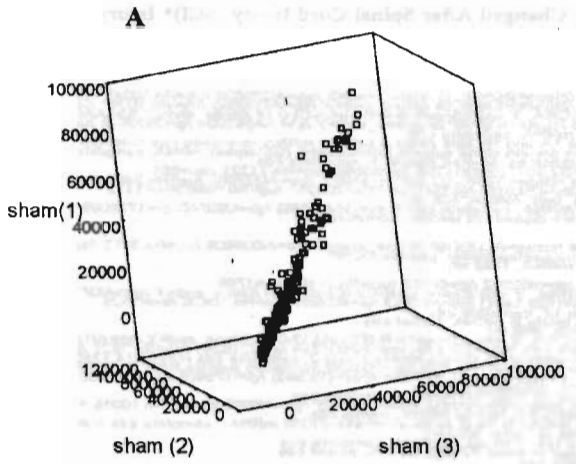
Ribonuclease Protection Assay for Cytokine Gene Expression

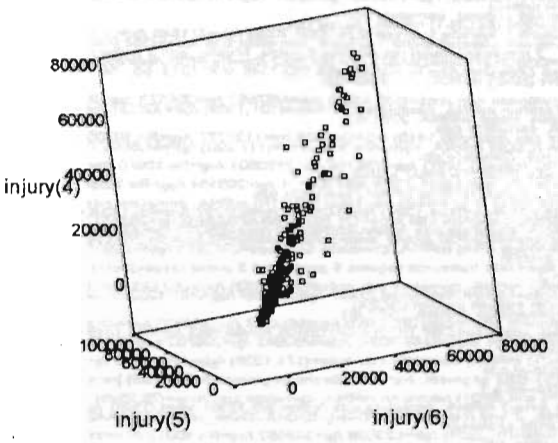
The portion of the spinal cord containing segments T5–T11 was removed from anesthetized animals after transcardial

TABLE II C. Cluster analysis of mRNA Expression Levels Significantly Changed After Spinal Cord Injury (SCI)* Injury:



*AVSHAM, Average expression levels in sham samples (n = 3); AVIN, average expression levels in injured samples (n = 3); AVMK801, average expression levels in MK801-treated injured samples (n = 3).





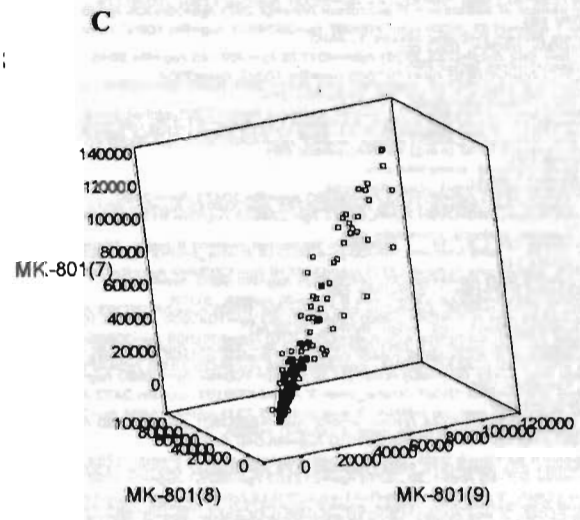


Fig. 1. Three-dimensional (3D) scatter plot of gene expression levels from nine microarrays (SPSS routines were used for plotting). Expression levels of 1,322 genes from a single microarray (one animal) are drawn along one axis in this "expression space"; the three axes (x, y, z) in the plot represent the expression levels of three microarrays from the same experimental group: (**A**) three sham spinal cord samples: sham (1) is drawn along y-axis, sham (2) along z-axis, and sham (3) along x-axis. **B:**Three injured spinal cord samples: injury (4); injury (5); injury (6), each along one of the axes. **C:** Three injured spinal cord samples treated with NMDA receptor antagonist, -(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine hydrogen maleate (MK-801): Mk-801 (7); MK-801 (8); MK-801 (9). In the ideal case, if expression levels from three samples in the same group were identical, the dots would fall along the main diagonal line passing through points (0,0,0) and (1,1,1).

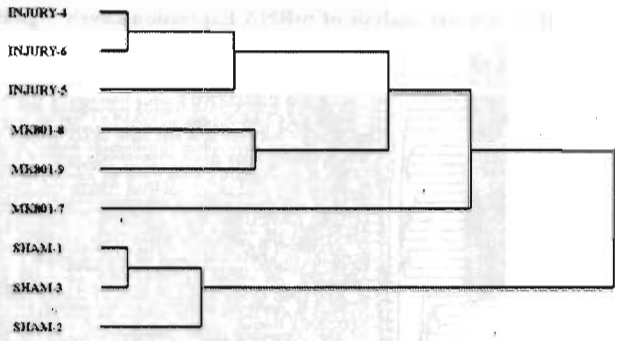


Fig. 2. Cluster analysis of samples/arrays: cluster analysis (City Block, or Manhattan, distance) using average linkage between expression levels for 1,322 probe pairs in nine chips: three sham samples, three injured spinal cord samples, and three injured spinal cord samples treated with MK-801. The more distant to the right the two junctions between samples are, the more dissimilar the transcription profiles.

perfusion with 100–150 ml of 0.9% saline containing heparin (1,000 units/l; to eliminate blood from samples). The segments were then labeled based on the location of the dorsal roots and the desired section of the cord removed. The isolated length of cord was immediately placed in dry ice and allowed to fully freeze, prior to being cut into seven sections, each constituting one spinal cord segment. Total RNA was prepared from seven frozen spinal cord segments (T5–T11) using TRI-Reagent (Molecular Research Center). Spinal segments were homogenized in 500 μl of TRI-Reagent, and total RNA extracted in chloroform, ethanol precipitated, and stored at −80°C. Cytokine expression was determined by a ribonuclease protection assay (RPA). The Riboquant Multi-Probe RNAse Protection Assay kit (PharmaMingen, San Diego, CA) was used for the measurement of rat cytokine mRNAs. Templates were used to generate a ^{32}P-labeled anti-sense RNA probe set that was hybridized in excess to target RNAs. Total RNA (10–20 μg) was hybridized with the labeled probe sets followed by RNAase treatment and analysis of protected bands on a denaturing 5% polyacrylamide gel electrophoresis (PAGE). Gels were subjected to PhosphorImage analysis and bands were quantified by densitometry. Relative cytokine mRNA levels were calculated by

TABLE III–VI. The Effect of MK-801 on SCI-Induced mRNA Level Changes: Lists of Genes Classifeid According to Their Putative Physiological Role†

Gene	Sham (1 hr)	Injury (1 hr)		Injury + MK-801 (1 hr)	
c-jun for AP-1	0[a]	+	*	+	
C/EBP[b] related	0	+	*	+	
HSP27	+	+3X	*	+3X	
Neuronal activity regulated pentraxin	0	+	*	0	*
HES-1	0	+	*	0	*
STAT 3	0	+	*	+	
Mad1	0	+	*	0	*
IRF-1	0	+	*	0	
Smad8	0	+	*	0	*
SOCS-3	0	+	*	+	
HIF-1	+	+2X	*	+0.6X	*
ANIA-6	0	+	*	+	
ANIA-4	+	+5X	*	+5X	
JAK 1	+	+7X	*	+7X	*
CaMI	+	+0.65X	*	+0.65X	
CaMKII (δ subunit)	0	+	*	0	*
CaMKII (γ subunit)	+	+0.6X	*	+1.63X	*
CaMIV	+	+0.65X	*	+0.65X	
RAC Prot.Kinase γ	0	+	*	0	*
MAPK-phosphatase (cpg21)	0	+	*	0	*
PDE2	0	+	*	0	*
Phospholipase D	0	+	*	0	*
PDE4	+	+4X		+4X	*

†Spinal cord injury (SCI)-induced changes in mRNA levels affected by MK-801 are in boldface, while designation of the lack of MK-801 effect is in medium type. Tables contain ONLY genes which mRNA level changed statistically significantly after injury (*$P < 0.05$).

[a]0 means mRNA levels for that gene were **not** detected; + means mRNa level for that gene was detected and (X) means the fold of increase/decrease in mRNA levels after injury. The change (expressed in fold of difference) was considered significant if the ratio of the mean expression levels in injured vs. sham samples was larger than 1.5 (or smaller than 0.66), and the confidence level for the difference more than 95% ($P < 0.05$). If the number in front of "X" is not changed in the MK-801 column that means that the change in mRNA level was not detected or was not statistically significant. If the number of X is changed, but the change is not labeled with * then the change was statistically significant (*t*-test, $P < 0.05$), but the fold of change was between 0.66 and 1.5, Also, the change in presence versus absence (+ versus 0, and vice versa) was significant ($P < 0.05$) if mRNA for a single gene was expressed in at least two samples in one group and not expressed in all three samples of compared group, or, if mRNA was not expressed in at least two samples of one group, and was expressed in all three samples of the compared group (Chi-square test, $P < 0.05$).

[b]C/EBP, CCAAT/enhancer-binding protein; NGFI, nerve growth factor induced factor; IESR, immediate early serum responsive gene; HSP, heat shock protein; HES, hairy/enhancer of split homologue; HIF, hypoxia inducible factor; STAT-3, signal transducers and activators of transcription; Mad, Mothers Against Decapentaplegic gene; IRF, interferon regulatory factor; SOCS, suppressors of cytokine signaling; ANIA, activity and neurotransmitter–induced early gene; JAK, Janus kinase; CaMK, Ca2+/calmodulin-dependent protein kinase; RAC Prot.Kinase, protein kinase B; PDE, phosphodiesterase.

TABLE IV. Inflammation

Gene	Sham (1 hr)	Injury (1 hr)		Injury + MK-801 (1 hr)	
Cytokines					
IL-1β[b]	0[a]	+	*	+	
TNF-α/β	0	+	*	+	
TNFα converting enzyme	+	+1.5X	*	+0.83X	
IL-6	**0**	**+**	*	**0**	*
IL-4 rec.	**0**	**+**	*	**0**	*
IL-2 rec. α chain	**0**	**+**	*	**0**	*
TGF-β 1	**0**	**+**	*	**0**	*
TGF-β binding protein 3	**0**	**+**	*	**0**	*
FGF receptor 1	**+**	**+1.8X**	*	**+0.5X**	*
IGF I	**0**	**+**	*	**0**	*
IGF-I rec	**0**	**+**	*	**0**	*
IGF-BP 5	**0**	**+**	*	**0**	*
VEGF B	+	+0.6X	*	+0.6X	
MIF	+	+0.6X	*	+0.6X	
Chemokines					
Receptor (CXCR4)	0	+	*	0	
Cell interaction					
C4 complement	0	+	*	+	
P-selectin	**0**	**+**	*	**0**	*
ICAM-1	0	+	*	+	
Integrin α V sub.	0	+	*	+	
Integrin α-M (Itgam)	**+**	**+2X**	*	**0**	*
Effectors					
iNOS	**0**	**+**	*	**0**	*
Neurocan	**0**	**+**	*	**0**	*

[a]Refer to footnote to Table III.

[b]IL-1, interleukin 1; TNF, tumor necrosis factor; TGF, transforming growth factor; FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor; IGF, insulin-like growth factor; IGFBP-5, insulin-like growth factor binding protein 5; MIF, macrophage migration inhibitory factor; ICAM, intercellular adhesion molecule; iNOS, inducible nitric oxide; rec., receptor; sub., subunit.

normalizing specific cytokine species measured to the ribosomal RNA L32 mRNA, which was included among probes provided as an internal control.

Double Immunoflourescence Staining

Animals were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer via the aorta and postfixed for 2–4 hours at 4°C. The injury segments and the segments immediately rostral and caudal to the injury site were removed and cryoprotected in 30% sucrose in phosphate buffer overnight and embedded in optimal cutting temperature (OCT). Tissue sections were cut transversely at 30 μm on a cryostat. Double immunofluorescence staining was performed to reveal simultaneously localization of Interleukin (IL)-6 protein and phosphorylated NMDA receptor subunit (NR1) in the spinal cord. Polyclonal antibodies raised in goat against IL-6 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA; sc-1266, 1:1,00). Polyclonal antibodies raised in rabbit against phosphorylated NR1 were obtained from Upstate Biotechnology (Lake Placid, NY; S897, 1:200).

Staining procedure. Floating tissue sections were incubated with 5% normal donkey serum in phosphate-buffered

TABLE V. Cell Survival

Gene	Sham (1 hr)	Injury (1 hr)		Injury + MK-801 (1 hr)	
Caspase-1	**0**[a]	+	*	**0**	*
Caspase-6	0	+	*	+	
BAD	**0**	+	*	**0**	*
BOD-M[b]	+	0	*	0	
Retinoblastoma p.	+	**+2X**	*	**+0.6X**	*
Synuclein 1	+	**+0.5X**	*	**+1.7X**	*
MnSOD	+	+1,5X	*	+1.5X	
Cu/Zn SOD	+	+0.6X	*	+1.25X	
Cytoskeleton					
Cytopl. β-actin	**0**	+	*	**0**	*
Axonal glycoprotein TAG1	0	+	*	+	
Vimentin	+	+2X	*	+2X	
GFAP	+	+1.5X	*	+1.5X	

[a]Refer to footnote to Table III.

[b]BOD, Bcl-2 related ovarian death gene; MnSOD, manganese-containing superoxide desmutase; GFAP, glial fibrillary acidic protein, and Vimentin are markers of injury-induced glial activation and glial scarring.

TABLE VI. Transmitters and Ion Channels

Gene	Sham (1 hr)	Injury (1 hr)		Injury + MK-801 (1 hr)	
Receptors					
NMDAR-2D1 rec.[b]	**0**[a]	+	*	**0**	*
GLUR-C rec.	**0**	+	*	**0**	*
GLUR 6 (KA) rec.	**0**	+	*	**0**	*
AMPA rec. binding protein	**0**	+	*	**0**	*
ACh, muscarinic M3 rec.	0	+	*	0	
GABA-B (gb2)	+	+0.6X	*	+1.23X	
Benzodiazepine rec. (PKBS) peripheral type	+	+4X	*	+4X	
Gonadotropin RH rec.	**0**	+	*	**0**	*
Substance P precursor	+	+0.5X	*	+0.5X	
Ion channels					
K^+-channel BK2	+	0	*	0	
RCK2 K^+ channel	**0**	+	*	**0**	*
K^+-channel r-ERG	+	+2.8X	*	+2.8X	
Ca^{2+} act. K^+ channel (SLON1)	**0**	+	*	**0**	*
L-type Ca^{2+} channel α1 subunit	**0**	+	*	**0**	*
Ca^{2+} channel α1 subunit	**0**	+	*	**0**	*
Na^+ channel protein 6 (SCP6)	+	+1.8X	*	+1.8X	
Na^+ channel (α subunit)	0	+	*	+	
Na^+ channel (β1 subunit)	+	+0.6X	*	+0.6X	
Cyclic nucleotide gated cation channel (CNG3)	**0**	+	*	**0**	*
Regulators					
Synaptotagmin II	**0**	+	*	**0**	*
Synaptotagmin VI	**0**	+	*	**0**	*
SNAP25	+	+0.5X	*	+	
PSD95/SAP90	**0**	+	*	**0**	*
Syntaxin 8	+	+0.65X	*	+0.65X	
Citron	**0**	+	*	**0**	*

[a]Refer to footnote to Table III.

[b]NMDA, N-methyl-D-aspartate; GLU, glutamate; AMPA, a-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid; Ach, acethylcholine; SNAP, synaptosomal associated protein; PSD, postsynaptic densities protein; GABA, gamma aminobutyric acid; rec., receptor.

saline (PBS) containing 0.3% Triton-X for 30 minutes. Sections were then incubated with a mixture of antibodies for IL-6 and phospho-NR1 diluted in PBS containing 1% normal donkey serum and 0.3% Triton overnight at room temperature; after rinsing three times (30 minutes) with PBS, sections were then incubated with mixed secondary antibodies of fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG 1:100 (Jackson ImmunoResearch Laboratories, West Grove, PA) and Alexa Fluor 594-labeled donkey anti-goat IgG 1:100 (Molecular Probes, Eugene, OR) for 4 hours. Tissue sections were then rinsed three times for 30 minutes and mounted onto gelatin-coated slides, and coverslipped with nonfade mounting medium. Omission of the primary antibodies or use of nonspecific secondary IgGs in the immunostaining process resulted in negative staining in the tissue. The sections were photographed on Olympus microscope equipped with a SPOT digital camera and all pictures were taken under the same exposure time.

RESULTS

Analysis of Variability Among DNA Microarray Results

To identify SCI-induced changes in gene expression, we used Affymetrix Rat Neurobiology U34 oligonucleotide microarrays containing 1,322 probes for known genes and ESTs. DNA microarrays were hybridized with cDNA probes synthesized from combined T7 + T8 + T9 spinal cord segments. The injury was inflicted to segment T8. Pooling of these three segments provided enough RNA to perform the DNA microarray and additional RPA analysis. We analyzed three samples in each experimental group: three sham spinal cords injected with ACSF, three injured spinal cords injected with ACSF, and three injured spinal cords treated with MK-801. The number of microarray probes for which hybridization with spinal cord RNA samples was detectable is shown in Table I. We found ~600 genes and ESTs expressed in uninjured spinal cords. In the absence of published data to provide a comparison, we relied on the high value of correlation coefficients among samples as a validation tool. The expression levels within uninjured experimental group showed high correlations ($0.99 > r > 0.98$; Table I), reflecting the low level of interindividual variability among samples. This finding is illustrated in Figure 1, which shows scatter plots of expression levels of three samples belonging to the same group (sham group is presented in Fig.1A). The expression levels of 1,322 genes detected on a given microarray are presented on one of three axes in the graph; three samples belonging to the same experimental group (three arrays) are presented on three (x, y, z) axes of each graph. Each data point in the three-dimensional (3D) space of the graph is defined by three values, e.g., expression values for one gene from three animals belonging to the same experimental group. For example, sham group consisted of samples: sham 1,

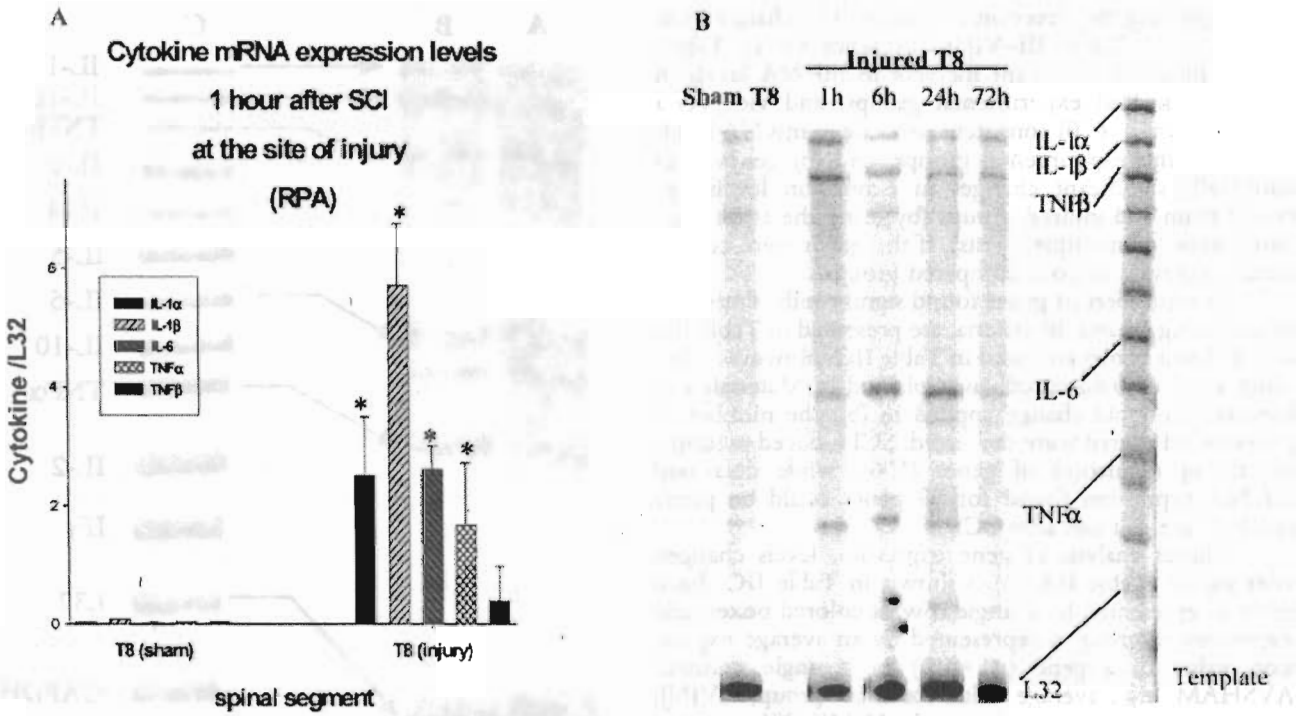


Fig. 3. Ribonuclease protection assay (RPA) analysis of pro-inflammatory cytokines in sham and injured samples: T8 spinal cord segments from sham and injured rats were assayed (n = 3). **A:** The pro-inflammatory cytokine mRNAs was measured one hour after spinal cord injury (SCI), at the site of injury (T8) and mRNAs for interleukin (IL)-1α/β, tumor necrosis factor (TNF)α/β, and IL-6 were detected in sham-treated samples. SCI induced a robust and significant increase in mRNAs of these pro-inflammatory cytokines; for example, IL-6 mRNA increased 200-fold, ($P = 0.0004$) and IL-1β mRNA by 70-fold ($P = 0.0008$). **B:** Representative gel displaying SCI-induced increases in cytokine mRNA levels at different time points. Pro-inflammatory cytokine mRNA increase peaked at one hour after SCI, except for IL-6 mRNA, which reached maximum at 6 hours after injury, and returned to basal levels by 72 hours.

sham 2, and sham 3 (Fig. 1A). Ideally, expression levels of all genes in three samples belonging to the same group would be identical, and all expression levels would fall on the line passing through (0,0,0) and (1,1,1) if there were no interanimal or interarray differences. The extent to which experimental values deviate from that line indicates the extent of interindividual variations among experiments. Figure 1A shows that there is a tight correlation among sham samples. SCI induced a significant increase in the number of hybridized probes (666; $P < 0.05$) and as expected, injury also slightly increased variability among samples ($0.97 < r < 0.99$). This is displayed as a broader scatter of data points in Figure 1B. MK-801 treatment significantly decreased the number of detected probes (518; $P = 0.04$), indicating that treatment with the NMDA receptor antagonist significantly changed the expression profile of injured spinal cords.

This result was confirmed by cluster analysis (Fig. 2) based on similarities among different microarrays. Hierarchical cluster analysis of the expression levels showed (Fig. 2) that the expression profiles of spinal cord tissues from rats belonging to any given experimental group were the most similar. The MK-801 treatment-induced gene expression changes clearly distinguished these samples from their untreated but injured counterparts. Cluster analysis of arrays also shows that injury and, more interestingly, MK-801 induces dramatic mRNA changes for a large number of genes (otherwise the changes would not be detectable by using clustering based on similarities among different microarrays). This finding supports the use of microarrays in analyzing the effect of MK-801 on SCI-induced changes. Sham-treated animals were expectedly dissimilar from the two other groups.

Based on the results of clustering, we assumed that the average expression, calculated as the mean of expression values in the three samples, appropriately represented the mRNA level for any given gene in any given experimental group, and that *t*-tests could be reliably used to detect genes with significantly changed expression levels among different experimental groups.

The Effect of SCI on mRNA Levels One Hour After Trauma

We first identified all transcription changes taking place 1 hour after injury by comparing three sham and three injured rats. As explained in Materials and Methods, we identified: (a) genes that have consistently different absolute calls in compared groups (by using chi-square

test), suggesting the direction of change. The change from absence (0 in Tables III–VI) to presence (+ in Tables III–VI) indicates consistent increase in mRNA levels in one of compared experimental groups, and vice versa changes (from + to 0) consistent decrease in mRNA levels in one of the experimental groups; and (b) genes with statistically significant changes in expression levels between sham and injured groups (by using the *t*-test with corrections for multiple *t*-tests), if the genes were consistently expressed in both compared groups.

The numbers of genes found significantly altered by injury, using (a) and (b) criteria, are presented in Table IIA and B. Their names are listed in Table IIC. However, after using a 1.5 or 0.66 cutoff (as explained in Materials and Methods) for fold change applied in (b), the number of genes found altered were decreased. SCI induced predominantly up-regulation of genes (106), while decreased mRNA expression found for 59 genes could be partly result of the cell loss after SCI.

Cluster analysis of gene expression levels changed after injury (Table IIA, B) is shown in Table IIC. Each gene is represented by a single row of colored boxes, and experimental group is represented by an average expression value of a gene (n = 3) in a single column: AVSHAM, e.g., average value for sham group; AVINJ, average value for injured group; AVMK-801, average expression values in MK-801-treated injured group. The color coding for gene expression levels is presented as a horizontal bar on the top of the cluster. It is obvious that injured (middle) column shows consistently different color than sham group. However, colors in the cluster analysis do not precisely define the extent and statistical difference of the change in mRNA levels. Thus, we listed these genes in Tables III–VI with stated direction, fold, and statistical significance of the change in mRNA levels. Moreover, cluster analysis identified only small groups of closely related expression level changes (blue-green segment). These genes regulate excitability, cell survival, and inflammation. Therefore, we used a priori determined categories to group genes based on their known biological function, in order to better address the specific hypotheses being tested with respect to the role of glutamate in SCI-induced cell loss and recovery. The majority of identified transcripts are related to transcription factors and signaling molecules (Table III), inflammation (Table IV), cell survival (Table V), and cell excitability (Table VI).

We confirmed some of the results presented in Table IV using an RPA method for the quantitative analysis of cytokine mRNA expression (Fig. 3). Figure 3 shows the change in pro-inflammatory cytokine mRNAs changes after SCI, at the site of injury (T8; Fig. 3A) at different time points (Fig. 3B). Furthermore, we performed RPA cytokine analysis on seven separate thoracic segments (T5 to T11) in order to assess spatial changes in the expression of mRNAs induced by injury. We found that the most robust change happened at the site of injury, T8. Changes were smaller at T7 and T9 segments and returned to basal levels rostrally and caudally from the site of injury at

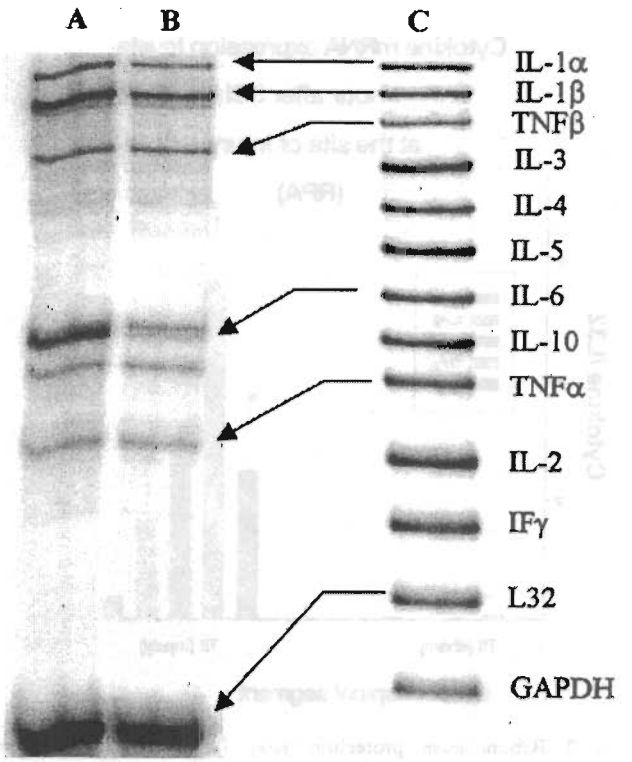


Fig. 4. RPA analysis of MK-801-treated samples. **A:** mRNA cytokine profile of injured spinal cord segments (T7, T8, and T9 combined), pooled from three rats (15 μg of total RNA). **B:** Cytokine mRNA expression in injured spinal cord treated with MK-801. **C:** Template set used in RPA experiments. Considering small differences (7%) in L-32 mRNA expression levels between A and B, it is obvious that MK-801 treatment caused decrease in IL-6 mRNA levels (39%), and to a lesser extent, in IL-1β mRNA levels. Other cytokine mRNA levels remained unchanged. IFγ, interferon gamma; GAPDH, glyceraldehyde-3-phosphate dehydrogenase, housekeeping gene.

segments T5, T6, T10, and T11 (Nesic et al., 2001). We expected to detect significant but smaller injury-induced mRNA changes in pooled T7, T8, and T9 segments, than in T8 alone. As shown in Figure 4A and B, RPA analysis of pooled T7 + T8 + T9 samples showed very similar changes in cytokine expression when compared to examples of the injured T8 segment. Thus, pooling of T7, T8, and T9 did not affect detection of SCI-induced changes in cytokine mRNAs.

The only discrepancy detected between DNA microarray and RPA analysis of pro-inflammatory cytokines were: (a) that RPA could detect low expression levels of cytokines in sham samples and microarrays could not. As shown in Table IV, almost all sham cytokine mRNAs are labeled with zero, indicative of the low sensitivity of Affymetix microarrays for low-abundant mRNAs, and (b) that RPA could detect IL-1α mRNA after SCI (Fig. 3) and microarrays could not.

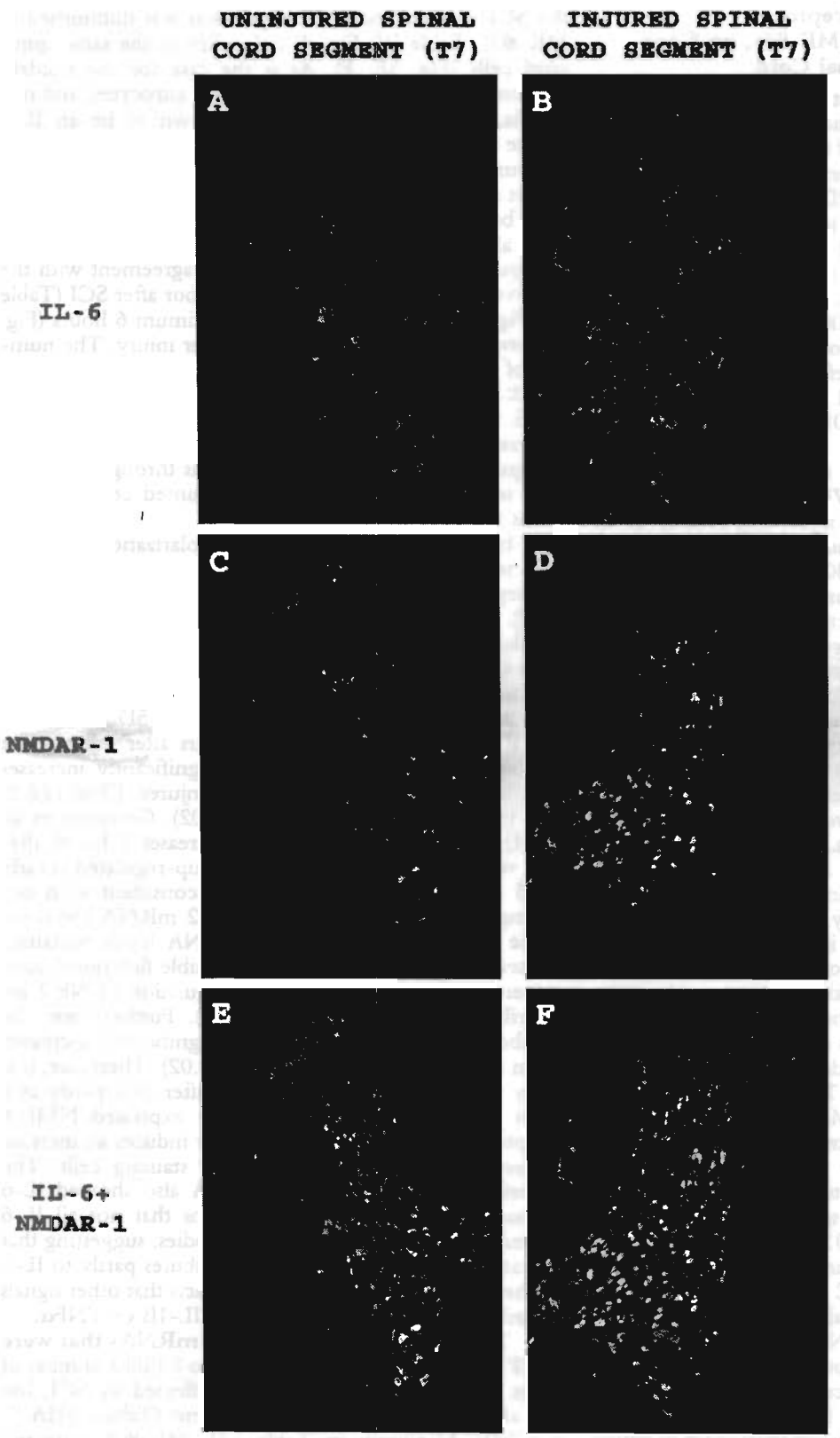


Fig. 5. Double immunoflourescence staining IL-6 and N-methyl-D-aspartate (NMDA) receptor. **A:** IL-6 immunoreacivity was detected in uninjured spinal cord segment (T7). **B:** More cells stained for IL-6 in injured spinal cord (T7) 12 hours after SCI. **C:** NMDA receptor subunit 1 (NR1) immunostaining in the uninjured and (**D**) injured spinal cord segments. NMDAR-1 was targeted with antibodies because NMDAR-1 coexpression is necessary for functional expression of all other cloned NMDA receptor subunits. **E:** Double-labeled cells indicate colocalization of IL-6 and NR1 (e.g., functional NMDA receptors) in uninjured and (**F**) injured spinal cord segments. Magnification: 10×.

The Effect of the NMDA Receptor Noncompetitive Antagonist, MK-801, on Gene Transcription in Injured Spinal Cord

The extracellular concentration of glutamate rises to neurotoxic levels after trauma and returns to basal levels within 2 hours. We hypothesized that post-traumatic high concentrations of glutamate overactivate NMDA receptors, and consequently, that NMDA receptor overactivation induces considerable change in gene transcription. To test this hypothesis, we measured mRNA levels in injured spinal cords treated with 9μg of MK-801, a noncompetitive NMDA receptor antagonist.

As shown in Figure 2A, MK-801 treatment significantly changed the expression profile of the injured spinal cord segments. We assigned the effects of MK-801 to two categories: (1) effects of MK-801 on mRNAs affected by injury, and (2) effects of MK-801 on mRNA levels not affected by injury.

The effects of MK-801 on mRNA levels affected by injury (Tables III–VI). As shown in Table IIC, gene expression affected by injury and then reversed in the presence of MK-801 is observable in all cases where the color of the sham and MK-801 column is similar and differs from the color in the injured column.

Out of 23 transcription factors and signaling molecules' mRNAs for which levels were significantly changed after SCI (Table III), 11 changes were reversed by the presence of MK-801 (indicated in boldface), suggesting that NMDA receptor activation after injury participates in transcriptional stimulation of 47% of these genes. Also, 56% of inflammatory (Table IV), 50% of survival (Table V), and 56% of excitability-related (Table VI) SCI-induced mRNA level changes were reversed by MK-801 treatment of injured spinal cords.

RPA analysis, as shown in Figure 4, confirmed the DNA microarray results describing the effect of MK-801 on some of the pro-inflammatory cytokines. For example, IL-6 mRNA decreased by 39% in MK-801-treated samples, while tumor necrosis factor (TNF)α, TNFβ, and IL-1α were unaffected. Interleukin-1β decreased by 19%, which is in agreement with the decrease in the same pro-inflammatory interleukin, as detected with DNA microarrays (30%). However, this decrease is not statistically significant, and is omitted from Table III.

It is very likely that NMDA receptor activation affects gene transcription (or mRNA maturation processes) directly via intracellular mechanisms in a given spinal cord cell. NMDA receptor subunit expression is widely distributed throughout spinal cord neurons and glial cells (Grossman et al., 2001). We also showed the conspicuous presence of NR1 immunostaining in spinal white and gray matter (Fig. 5C and D). For most gene products, presented in red in Tables III, V, and VZI, we predicted colocalization with NMDA receptors. However, colocalization of inflammatory factors and NMDA receptors has not been well documented. Therefore, we tested the hypothesis that both, NMDA receptors (NR1 subunit) and IL-6 (one of the inflammatory genes in which the SCI-induced increased expression was diminished in MK-801; Table IV; Fig. 4) colocalize to the same spinal cord cells (Fig. 5E, F). As is the case for the resident immunocompetent cells of the CNS, astrocytes, and microglia, neurons have also been shown to be an IL-6 source in vivo and in vitro. Consistent with reports of IL-6 in neurons under normal physiological conditions (Schöbitz et al., 1993; Gadient and Otten, 1994), we also found IL-6 being expressed in the uninjured spinal cord (Fig. 5A), albeit more robustly in injured spinal cord after 12 hours (Fig. 5B). This finding is in agreement with the observed increase in IL-6 mRNA 1 hour after SCI (Table IV, Figs. 3 and 4), that reaches a maximum 6 hours (Fig. 3B) and stays elevated at 24 hours after injury. The number of cells stained for IL-6 significantly increases from 120 ± 10.2 per section in uninjured T7 (n = 3) to 171 ± 15.15 in injured T7 ($P < 0.02$). Therefore, significant increase in IL-6 mRNAs (Fig. 3, Table IV) seems to lead to significant increase in IL-6 synthesis throughout spinal cord section T7. The identity of counted cell types remains to be investigated.

Interleukin-1, TNFs, and depolarization have all been reported to stimulate IL-6 expression in cortical and sensory neurons (Ringheim et al., 1995; Sallmann et al., 2000). Sallmann et al. (2000) also found that depolarization-induced IL-6 transcription depends on calcium and calcium calmodulin-dependent kinases, suggesting that NMDA receptor activation may contribute to IL-6 mRNA increases after SCI. Figure 5D also shows that (NR1 staining increases 12 hours after injury. The number of cells stained for NR1 significantly increases from 50.1 ± 13.12 per section in uninjured T7 to 122 ± 20.7 (n = 3) in injured T7 ($P < 0.02$). Grossman et al. (2001) found that NR1 mRNA increases 4 hours after SCI, while NR2 mRNAs are already up-regulated as early as 15 minutes after trauma. This is consistent with our finding that 1 hour after injury, NR2 mRNA levels increase (Table VI), while NR1 mRNA levels remained unaltered at 1 hour after trauma. Possible functional consequences of early and robust up-regulation of NR2 are described in Grossman et al. (2001). Furthermore, the number of double-stained cells also significantly increased (from 37 ± 8.8 to 77.5 ± 9.7; $P < 0.02$). Therefore, it is likely that IL-6 increases 12 hours after SCI partly as a result of activation of constitutively expressed NMDA receptors, but also because SCI injury induces an increase in the number of NMDA receptor staining cells. The majority of cells stained for NMDA also showed IL-6 immunoreactivity. However, it seems that not all IL-6 stained cells reacted with NR1 antibodies, suggesting that activation of NMDA receptors contributes partly to IL-6 synthesis, in agreement with known facts that other signals contribute to IL-6 synthesis, such as IL-1β or TNFα.

The effect of MK-801 on the mRNAs that were NOT affected by trauma. We also found a number of genes whose transcription was not affected by SCI, but was affected by the MK-801 treatment (Tables VIIA–C and VIII).As shown in Table VII, MK-801 primarily

TABLE VII A, B. Number of Genes Differentially Expressed After MK-801 Treatment and not Affected by Injury

A. Number of genes with consistently different Absolute call after MK-801 treatment:

Sham	Injury	Injury + MK-801	Number of genes
0	0	+	0 ($P < 0.05$; chi-square test)
+	+	0	48 ($P < 0.05$; chi-square test)

B. Number of genes expressed in sham, injured and MK-801 treated spinal cord samples and:

Up-regulated only in MK-801:	47 ($P < 0.05$; *t*-test)
Down-regulated only in MK-801:	73 ($P < 0.05$; *t*-test)

decreases mRNA levels: 121 down-regulated versus 47 up-regulated genes. It is observable in the Table VIIC that genes in MK-801-treated samples have different shades of color, indicating different expression levels compared to sham and injured samples. For example, a series of "blue" genes (Table VIIC) has increased expression levels in MK-801treated samples compared to the sham and injured column. However, cluster analysis (e.g., color coding) does not give precise information about the extent and statistical significance of changes that take place under different conditions. Similarly to the analysis shown in Section II.a, we presented genes (Table VIII) that have statistically significant fold of change higher than 1.5 and lower than 0.66 and genes with consistently different absolute call in MK-801-treated samples compared to sham and injured samples. We categorized them according to their known function. Furthermore, the analysis of the biological role of genes listed in Table VIII may explain some of the consequences of MK-801 used to diminish the excitotoxic outcome of SCI-induced activation of NMDA receptors. As shown in Figure 6, some of the MK-801 effects on mRNA levels could be deleterious.

DISCUSSION

Expression Profile of the Contused Spinal Cord One Hour After Injury

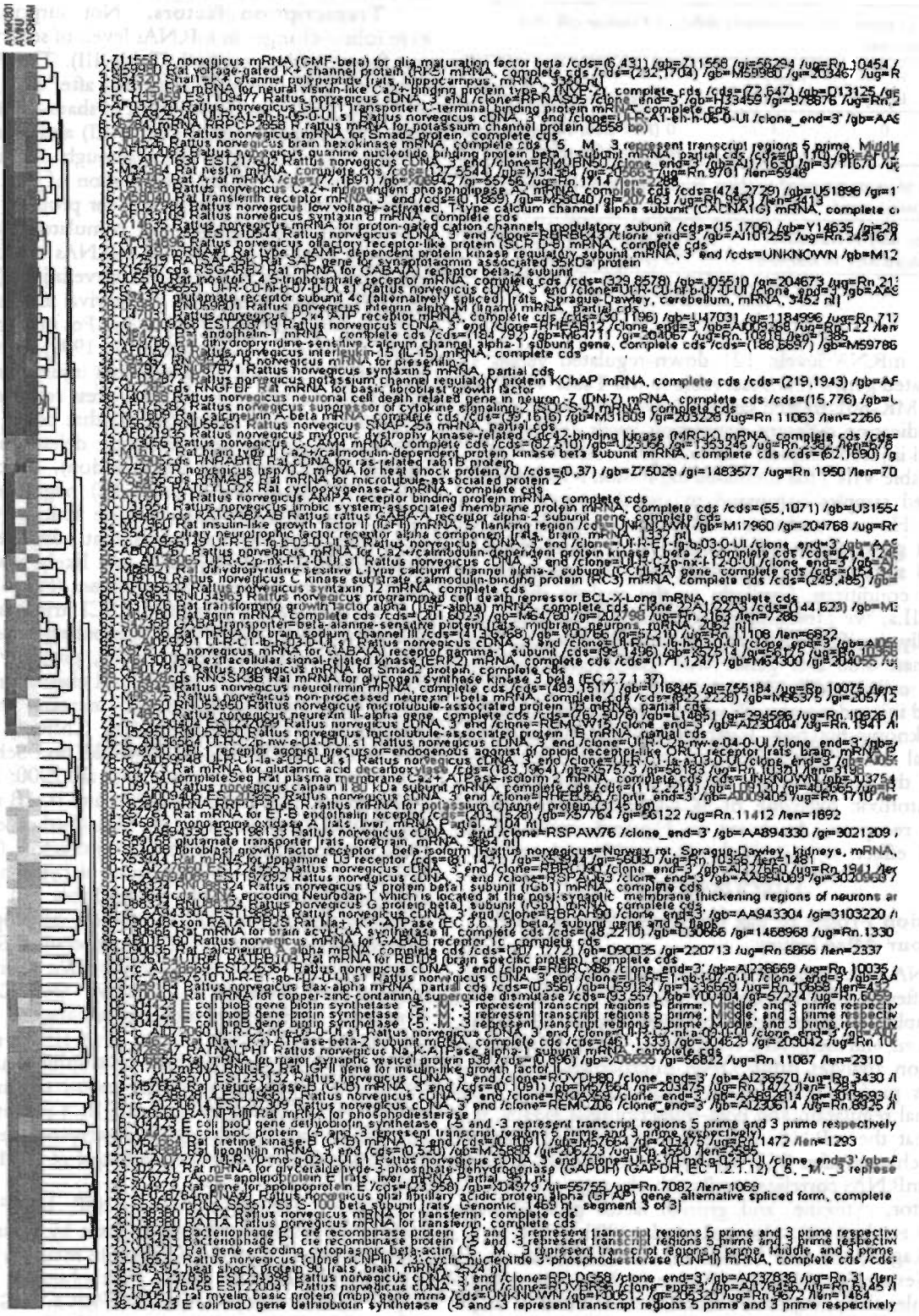
DNA microarray analysis of transcriptional changes 1 hour after SCI showed altered expression for a number of genes implicated in transcriptional regulation, inflammation, cell survival, and membrane excitability. Although large-scale expression analyses using DNA microarrays only yield mRNAs profiles without distinguishing between transcriptional regulation and post-transcriptional events, it is likely that the mRNA changes described here result in protein changes after SCI. CNS trauma-induced stimulation of mRNAs correlates well with changes in transcription factor, cytokine, and growth factor protein levels (Taupin et al., 1993; Humpel et al., 1993; Yang et al., 1994; Raghupathi and McIntosh, 1996; Holmin et al., 1997; Nesic et al., 2001). In Figure 6, there is an outline of the roles that the gene products, here identified, might have in neurodegeneration and recovery after SCI. The diagram emphasizes our current lack of knowledge about the post-SCI molecular sequel.

Transcription factors. Not surprisingly, there were robust changes in mRNAs levels of several transcription factors early after SCI (Table III). The AP-1 genes, c-jun and c-fos, increase promptly after SCI (Hayashi et al., 2000). Here *c-jun* not present in sham-treated rats, was expressed in injured cords (Table III) and *c-fos* expression increased 3.5-fold after SCI, although not significantly. SCI also induced robust upregulation of JAK and STAT mRNAs (Table III), which code for proteins that constitute a main signaling pathway stimulated by cytokines. Increased levels of JAT/STAT mRNAs are found in developing CNS and in peripheral nerve injury (Cattaneo et al., 1999), suggesting a role in survival and nerve regeneration in the injured CNS. Jun and Fos promoters are also targets for STAT 3 (Cattaneo et al., 1999), consistent with the positive feedback loop between injury-induced cytokines (STAT3 target genes) and stress response regulated by immediate early genes (AP-1) that could lead to the cytokine overproduction and their deleterious effects in injured spinal cord (see next section). In contrast, increased SOCS mRNA (Table III) negatively regulates JAK/STAT signaling and cytokine actions (Cattaneo et al., 1999). However, it seems that adaptive regulatory mechanisms after severe SCI are likely insufficient to counterbalance degenerative processes (Fig. 6).

Inflammatory factors. SCI triggers inflammation early on (Table IV), contributing substantially to secondary damage (Bethea, 2000), probably via unbalanced production of cytokines and chemokines. For example, SCI stimulates robust increase in the pro-inflammatory cytokines, IL-1β, TNFα/β and IL-6 mRNAs (Table IV; Fig. 3; Bartholdi and Schwab, 1997; Kossmann et al., 1999; Hayashi et al., 2000; Nesic et al., 2001). While SCI-induced TNFα and IL-1β increases are reported to be deleterious in SCI (Bethea et al., 1999; Nesic et al., 2001), IL-6 may be protective (Tuna et al., 2001). SCI induces up-regulation of cytokines and chemokines (McTigue et al., 1998; Table IV) which then stimulate endothelial cells to mediate neutrophil, monocyte and T-cell attachment, excarberating SCI-induced cell losses (McTigue et al., 2000). We found that SCI induced an increase in mRNA for ICAM-1 and P-selectin, which also mediate binding of neutrophils, monocytes, and T-cells. Taoka et al. (1997) showed that antibodies to P-selectin reduce neutrophil accumulation in the injured cord and attenuate motor dysfunction. The observation of increased IL-2 and IL-4 receptor mRNAs after SCI is novel, although IL-2 receptors are reported to increase in SCI patient plasma (Segal et al., 1997). Their role in SCI is not known.

After CNS injury, NGF, IGF, bFGF, and TGF increase in what may be an attempt by injured CNS to limit secondary injury and promote recovery (Hughes et al., 1999; Kent et al., 1999). We observed increases in IGF-I, IGF-I receptor and IGFBP-5, FGF receptor, TGFα/β, and TGFβ receptor mRNA levels (Table IV).

TABLE VII C. Cluster Analysis of mRNA Expression Levels Significantly Changed in MK-801-Treated Samples; but not Affected by Injury



AVSHAM, average expression levels in sham samples (n = 3); AVINJ, average expression levels in injured samples (n = 3); AVMK801, average expression levels in

TABLE VIII. The Effect of MK-801 on mRNAs not Affected by SCI

Gene	Sham (1 hr)	Injury (1 hr)	Injury + MK-801 (1 hr)	
Transcription factors and signaling regulators				
HSP70	+[a]	+	+2.3X	*
CaCAMK β subunit	+	+	+1.6X	*
PDE I	+	+	+0.6X	*
Inflammation				
IGF II	+	+	0	*
bFGF	+	+	0	*
IL-15	+	+	0	*
IL-3 rec. β subunit	+	+	0	*
SOCS 2	+	+	0	*
Endothelin	+	+	0	*
Cox-2[b]	+	+	0	*
Cell survival				
Bcl-2 BOK	+	+	0	*
Bcl-w	+	+	0	*
Microtubule-associated protein 1B	+	+	+0.3X	*
Neuronal cell death related gene DN-7	+	+	0	*
Neurexin III-α	+	+	+0.4X	*
Myelin basic protein	+	+	+2X	*
Transmitters and ion channels				
GABA B 1d rec.	+	+	0	*
GABA(A) rec. γ sub.	+	+	+0.6X	*
GLYCINE R. α 1	+	+	0	*
MGLUR6	+	+	0	*
Glutamate transporter	+	+	+0.6X	*
5HT 1c Rec.	+	+	0	*
Syntaxin 5	+	+	0	*
Syntaxin 8	+	+	0	*
K^+-channel (RK5)	+	+	0	*
K^+-chann. regul. prot. KChAP	+	+	0	*
T-Ca^{2+} chann. α sub.	+	+	0	*
ClC-5 Cl^- channel	+	+	0	*
Ca^{2+} chann. α sub.	+	+	0	*
G-protein β1 subunit (rGb1)	+	+	+0.5X	*
Na, K-ATPase β2 subunit	+	+	+0.6X	*

[a]Refer to footnote to Table III.
[b]COX-2, cyclooxygenase 2; bFGF, basic fibroblast growth factor; TACE, TNF-alpha converting enzyme; SCI, spinal cord injury.

Although it is shown that exogenously applied IGF-I improves hindlimb reflex after SCI (Pulford et al., 1999), the role of the IGF family of proteins in SCI is not known. Similarly, the role of the TGF family in SCI is unknown except for a dual effect of TGFβ, which improves motor recovery but also enhances scarring after SCI (Hamada et al., 1996).

Cell death regulators. Functional outcomes after SCI depend on cell loss occurring during the first 24 hours postinjury interval (Grossman et al., 2001). Although apoptosis after neuronal injury can have both beneficial and harmful effects (Jackson and Perez-Polo, 1996), apoptosis after SCI is most often associated with neurodegeneration. Apoptotic inducers appear as early as 1 hour after injury (Zurita et al., 2001) and by 6 hours, Caspases -8 and -9 are present in the gray matter within the lesion (Keane et al., 2001), while Caspase-3 becomes evident by 24 hours (Citron et al., 2000; Keane et al., 2001). We found that mRNA levels for Caspase-6, a significant inducer of Caspase-3 (Allsopp et al., 2001), increased 1 hour after SCI (Table V), a novel finding. Other pro-apoptotic factors present at one hour after SCI were Caspase-1, Calpain, BAD, and iNOS; novel anti-apoptotic agents present after SCI were Synuclein and Retinoblastoma protein, which could be potentially interesting therapeutic candidates.

Neurotransmitter receptors and ion channels. While a detailed discussion of gene expression changes associated with cell excitability (Table VI) is beyond the scope of this discussion, the overall impression is that SCI caused changes likely to lead to a deleterious enhancement of membrane excitability via increases in mRNAs for ionotropic glutamate receptors, Ca^{2+} and Na^+ channels and decreases in mRNAs for gamma aminobutyric acid (GABA) receptors and K^+ -channels. This interpretation is consistent with the observation that chlometiazol, which activates GABA and glycine inhibitory receptors, improves motor recovery after SCI (Farooque et al., 1999) and that hyperexcitability promotes post-SCI devastating chronic central pain development (Woolf and Salter, 2000).

The Effect of NMDA Receptor Inhibition on SCI-Induced Change in mRNA Levels

We propose that early SCI-induced NMDA receptor overactivation triggers gene expression changes that significantly affect degeneration/regeneration processes after SCI. We asked whether treatment with MK-801, an NMDA receptor antagonist known to affect glutamatergic transmission in spinal cord (Li and Tator, 2000), would alter SCI-induced gene expression patterns. It is known that pretreatment with the MK801 prevents transcription factor, activin β or growth-associated protein-43 (GAP-43) expression after brain trauma (Lai et al., 1997; Hughes et al., 1999; Luque et al., 2001).

As shown in Figure 2 and Table IIC, the expression profile of MK-801-treated spinal cords differed distinctly from ACSF-treated injured spinal cords, suggesting that NMDA receptor inhibition profoundly changed SCI-induced transcription. Specifically, about half of all SCI-induced changes were reversed by MK-801 treatment (Tables III–VI), indicating that NMDA receptor hyperactivity after SCI considerably contributed to SCI-induced transcriptional changes. Especially interesting and novel is the finding that NMDA receptor activation participates in the up-regulation of a number of inflammatory molecules (Table IV). Although Jander et al. (2000) suggested that NMDA receptor activation stimulates inflammatory gene expression after brain ischemia, this has not been shown in SCI. For example, NMDA receptor activation reversed the stimulatory effect of SCI on IL-6 expression (Table IV; Fig. 4). We also showed that NMDA receptors and IL-6

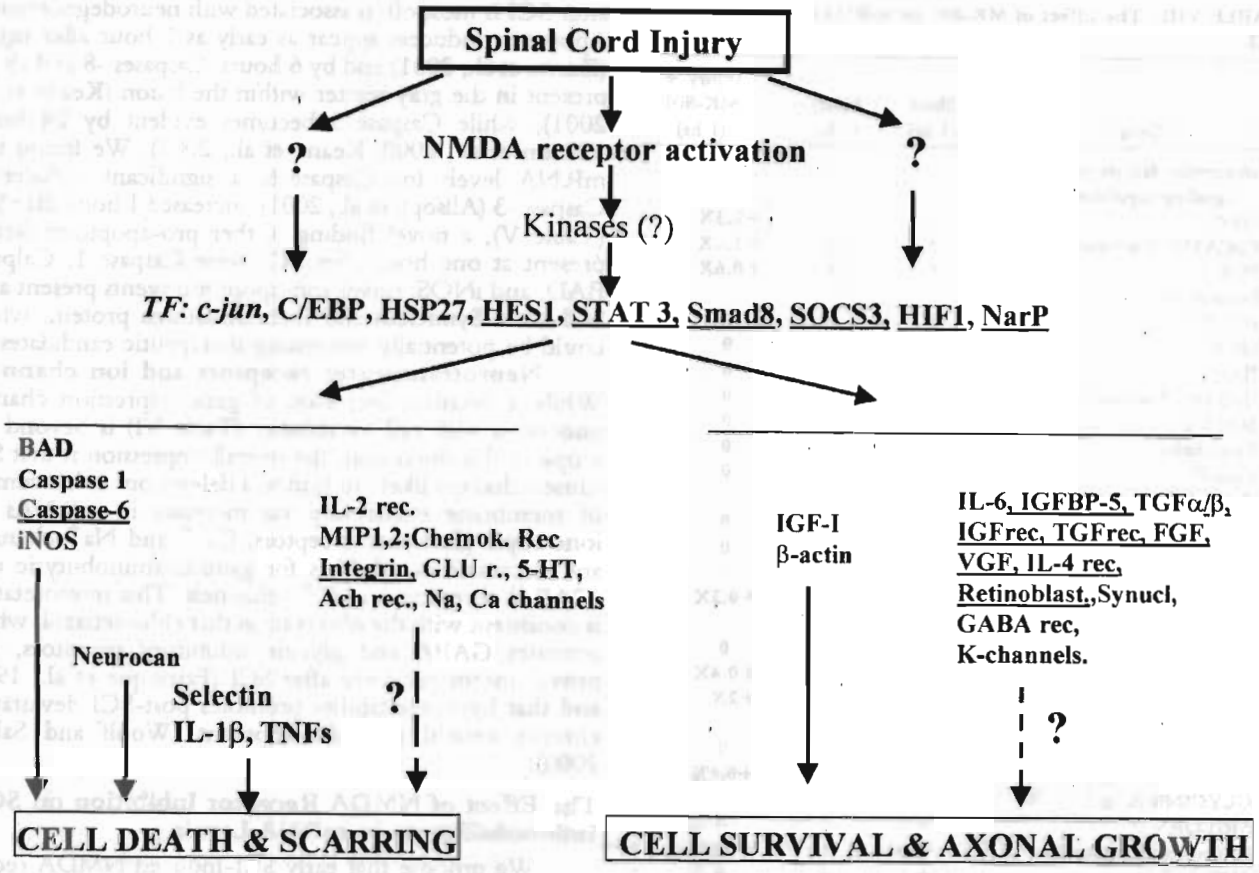


Fig. 6. Gene expression changes detected 1 hour after SCI, and their possible effect on cell death and recovery. A majority of the transcriptional changes observed at 1 hour after SCI have not been reported before (underlined gene names). Question marks next to dashed arrows label genes for which a role in SCI is not known. Based on published results describing their role in other tissues or models, we predict that while some of these transcriptional changes are neuroprotective, the others will be deleterious. Outcomes will depend on balance between the two. NMDA receptor activation affects mRNA levels of genes written in boldface.

colocalize to the same spinal cord cells (Fig. 5), suggesting that NMDA receptor activation can directly affect IL-6 transcription. One of the transcription factor candidates for mediating NMDA receptor activation is NF-κB, known to regulate IL-6 in the CNS (van Wagoner and Benveniste, 1999) and at least 27 other cytokines and inflammatory factors (Pahl, 1999). NMDA receptor activation elicits NF-κB activation in striatal neurons (Qin et al., 2000). It has also been shown that SCI induces increased p50/p65 NF-κB subunit nuclear binding (Bethea et al., 1998; Xu et al., 1998). We found that p50/p65 binding activity to a cognate consensus sequence decreased by 43% after MK-801 treatment of injured spinal cords, although the decrease was not statistically significant ($n = 3$, data not shown). Consistent with this result is the finding that only 21% of analyzed genes affected by MK-801 contain NF-κB binding sites in their promoter region. Other transcriptional factors and promoter activating mechanisms that mediate the effect of NMDA receptor activation on SCI-induced gene transcription remain to be characterized.

Noninjury-Related Effects of MK-801 on Spinal Cord Expression Profile

Although MK-801 prevents some SCI-induced cell loss (Liu et al., 1997; Wada et al., 1999), it does not improve blood flow, edema, or locomotor function (Li and Tator, 1999; Haghighi et al., 2000; Li and Tator, 2000). Our results (Tables III–VI and VIII) offer one explanation for the lack of long-term beneficial effects for MK-801 treatment on SCI outcomes. Some of the SCI-induced gene expression changes that can cause cell death were not reversed by MK-801 (Tables III–VI, letters not in boldface). For example, MK-801 induced decreases in growth factors, Retinoblastoma protein or hypoxia inducible factor (HIF), which are likely to increase cell death

and hinder recovery processes. There were also genes relevant to cell loss events that were affected by the MK-801 treatment, but not by SCI (Table VIII). For example, the anti-apoptotic Bcl-w (Yan et al., 2000) was not expressed in MK-801-treated injured spinal cords, although it was expressed in sham and injured spinal cords (Table VII). Similarly, bFGF, important to recovery (Rabchevsky et al., 2000), were down-regulated by MK-801. Therefore, DNA microarrays are useful, not only for assessing the potentially beneficial effects of therapeutics, but also for elucidating possible adverse effects.

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