Final Narrative Report
New Jersey Commission on Spinal Cord Research

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2. Name of Organization/Institution: **Rutgers University**

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Following Acute SCI

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Body of Report

1.   **Original Aims of the Project:**

Aim 1: What mRNAs are regulated following acute SCI in the period leading to secondary cell damage? We will perform a detailed time course of spinal cord injury in rats, prepare RNA and assess changes in mRNA levels using a custom rat cDNA microarray. Using clustering analysis, we will identify populations of mRNAs that are regulated during the period between primary and secondary injury.

Aim 2: Which of these regulated mRNAs are associated with inflammatory signaling? Using inhibitors or inducers of inflammation, we will associate specific mRNA changes with inflammatory signaling pathways. By comparing this pool with the population identified in Aim 1, we will restrict our candidate population of mRNAs to those most likely to be regulated by inflammation, and therefore to be good targets for drug development.

2.   **Project Successes**

Summary: Under NJCSCR support, we were the first to publish a comprehensive functional genomics study of acute spinal cord injury (SCI) in rats (1). During the course of our project, we built upon this success by working with other laboratories to investigate the role of glutamate receptors in the acute period (2), the role of MBP-specific T cells in exacerbating inflammatory response to injury (3), and ischemic preconditioning in spinal cord (Carmel et al., submitted). We extended these studies into specific inflammatory signaling mechanisms following spinal injury (4). Finally, additional results from each of the two aims are still being analyzed and prepared for publication, including the most detailed functional genomics analysis of acute spinal contusion ever attempted and a successful screen of anti-inflammatory compounds using microarray technologies (Pan et al., in preparation).

Our initial microarray study of spinal contusion began prior to this project, but the completion of the study was an important component of the project. We used Affymetrix technology to assess mRNA changes during the first 48 hrs following MASCIS contusion of rat spinal cords. Our publication (1) and the posted, downloadable dataset listed the changes in approximately 1,200 mRNAs in at least three replicate experiments for each time point. The most obvious finding demonstrated a spreading wave of inflammation/stress responses from the site of injury to the surrounding tissues, and a dramatic loss of neuronal cells and neuron-specific mRNAs immediately following contusion. Additional results identified the induction of several growth factor families as well as markers of astrocyte remodeling. Our results formed much of the initial basis for the creation of the SCIGenes web site at the University of Kentucky, cataloging all known mRNA changes after spinal cord injury. I now serve on the board of scientific advisors to SCIGenes.

We extended the use of Affymetrix technologies to assist the work of Dr. Regino Perez-Polo at the University of Texas Medical Branch at Galveston. Working with Dr. Perez-Polo's group, we showed that nearly 50% of all mRNAs regulated within 1 hr of injury were affected by the blockade of glutamate receptors by antagonists, demonstrating that excitatory amino acid neurotransmitters play a very large role in signaling cellular damage and death (2).

Another collaborator, Dr. Philip Popovich of Ohio State University, learned of our functional genomics work and asked for help identifying T-cell-specific effects in autoimmune models.

Previous work had suggested that autoimmune T cells may be beneficial in promoting spinal regeneration following injury (5-7). Dr. Popovich had obtained a transgenic mouse containing ~95% T cells specific for myelin basic protein (MBP), an autoantigen that is revealed following spinal injury. In our studies, the transgenic mice containing MBP-specific T cells exhibited far greater damage following spinal contusion (3). We also showed that the more inflammatory (T_H1) cytokine cascades, such as IL-2 and IFN-γ, were more highly induced following injury and more prolonged in their expression than in control animals. The more regulatory responses (T_H2), while slightly up-regulated, were much less prominent and delayed in transgenic animals. This study was important in demonstrating that autoimmune T cells may be quite damaging following spinal cord injury and their potential use as a therapy needs to be critically reassessed prior to human testing. Based on this work, we have been successful in obtaining NIH funding to continue the study of T cells in SCI (PI: Philip Popovich; Subcontractor: Ronald P. Hart).
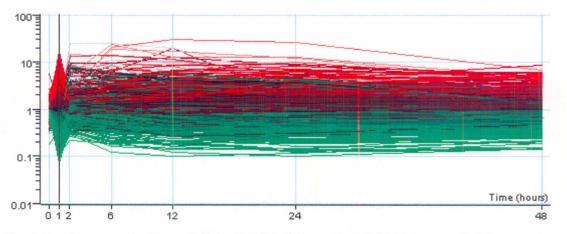
A complementary T cell study has been performed with Dr. Michal Schwartz of the Weizmann Institute. Dr. Schwarz's lab had found that T cells co-cultured with microglia enhanced their uptake of glutamate, potentially buffering its excitotoxic effect on neurons. We found that the patterns of mRNA in microglia co-cultured with T cells revealed by microarrays was consistent with a pattern induced by IFNγ stimulation. Dr. Schwarz's lab subsequently showed that microglia could be stimulated by IFNγ directly to enhance glutamate uptake. These studies have been submitted to the Journal of Neuroscience (Shaked et al., submitted; see Appendix). However, this work shows that inflammatory mechanisms may have selected, protective effects during the secondary phase of cellular damage, and that a pharmacologic approach to inducing glutamate buffering is possible.

We then examined mechanisms of cytokine activation following spinal injury (4). We described the time course of several inflammatory cytokine mRNAs following injury in vivo. Subsequent experiments with a novel culture system demonstrated an identical pattern of cytokine mRNA regulation, suggesting that all cells and mechanisms controlling cytokine mRNA response to injury exist within spinal cord tissue. It had been assumed that invading blood lymphocytes were required for inflammatory response, but our study showed that these cells were not necessary. We also found IL-1 precursor protein in uninjured spinal tissues. This is consistent with the presence of cells containing inflammatory cytokine precursors that are immediately activated and released upon injury. Indeed, our spinal culture system demonstrated the activation and release of mature IL-1 protein into the culture medium. Finally, since the appearance of inflammatory cytokine protein would be expected to act at its own receptor in the same tissues, and since cytokine receptors are know to signal cytokine mRNA accumulation, we asked whether IL-1 protein could contribute to IL-1 mRNA accumulation seen following SCI. We used either a pharmacologic blockade of cytokine receptors in culture, or transgenic "knockout" mice lacking IL-1 and TNFα receptors in vivo to show that the lack of receptor stimulation diminished the cytokine mRNA accumulation following SCI. This study showed the speed and complexity of the inflammatory response to SCI. It also demonstrated that anti-inflammatory treatments in patients probably are given too late to block the cytokine cascade itself. We argue that anti-inflammatory therapies probably diminish cellular mechanisms following primary inflammatory response, and this should be the focus for developing new acute therapies.

The microarray technology developed using NJCSCR support allowed us to contribute to a study on spinal ischemia and ischemic preconditioning. Working with Dr. Martin Marsala of UCSD, we examined gene expression following a preconditioning ischemic treatment of rat spinal cords

(Carmel et al., submitted, Cizkova et al., submitted; see Appendix). Our findings identify several classes of genes that correlate with the neuroprotection conferred by preconditioning, and lay the foundation for screening protective therapies.

Our unpublished work continues a more detailed functional genomics study of the acute phase of SCI (Aim 1). This was the goal of our first aim of the project, and in many ways, turned out to be the most difficult aim to perform well. As outlined below (Project Challenges) we worked to switch from our original plan of using cDNA microarrays to using spotted oligonucleotide microarrays before completing this study. Furthermore, we had the benefit of analyzing data from the NIH contract awarded to Dr. Alan Faden to perform microarray studies in spinal cord injuries (8). Our study included 0, 1, 2, 6, 12, 24, and 48 hrs following MASCIS contusion, with four independent animals used at each time point (2 males and 2 females). We dissected all five of the traditional MASCIS-defined 5 mm segments of spinal cord from each animal (D2, D1, I, P1, and P2). Below is shown the 1,683 mRNAs found by 1-way ANOVA to be affected significantly by spinal contusion in the I segment (the site of contusion) at any time point [$p < 0.05$ using the Benjamini and Hochberg False Discovery Rate to estimate multiple measurement error (9)]. Not shown is the pattern of mRNA changes in nearby D1 (distal to the contusion site) and P1 (proximal) regions, each of which represents the secondary effect of injury on either axotomized cells or those having lost neuronal input due to injury. Advanced analysis of these data will require at least another six months. We are working with our bioinformatics collaborator, Dr. Rebecka Jörnsten of the Rutgers Statistics Department, to identify patterns of gene responses and interpret biological function of these patterns. These data and their interpretation will be a valuable resource for many other scientists, so we will publicize the datasets and share them broadly.

Time course of gene expression changes following MASCIS spinal contusion in I (injury) segments. Each line represents a single gene of the 1,683 genes significantly changed from control (see text). The color of the line corresponds to the degree of change from control at 1 hr; with red representing increased levels, and green representing decreases. The y-axis depicts a log scale of the ratio of mean (n=4) expression compared with uninjured control (a pooled RNA preparation from six animals).

Our goal was to identify genes unregulated at 2 hrs, but increasingly regulated at 6-24 hrs, as candidates for markers of secondary injury—preliminary analysis identifies 243 genes that satisfy these criteria. Additionally, since a complementary dataset has been contracted by NIH and is publicly available, we plan to fold the NIH dataset and ours for one meta-analysis. We have already discussed this concept with our program officer at NINDS, who will consider our request for a supplement for a bioinformatics postdoc to perform these analyses. The

supplement would be added to my subcontract from Dr. Popovich's new NINDS grant based on our T cell collaboration.

Our second aim was to block inflammatory cascades with various inhibitors and then identify patterns of gene response associated with inflammatory mechanisms (Aim 2). We strategically redesigned the study to take advantage of our spinal cord slice culture system, since it had proven so valuable in studying inflammatory mechanisms (4). We prepared 1 mm slices of adult rat spinal cord (slicing the tissue was considered to be the injury) and cultured them in serum-free medium for 4 hrs (corresponding to 4 hrs following injury). Groups of cultures were treated with methylprednisolone (MP), acetaminophen (ACET), indomethacin (INDO), NS398, or a mixture of IL-1 receptor antagonist and soluble TNF receptor (CKIN; direct cytokine inhibition). RNA was prepared from each replicate (n=3) culture for each group and analyzed by microarray. We hypothesized that the major effect of all anti-inflammatory compounds would be similar, and so the patterns of gene response would be predominantly similar. This proved to be true. However, *our goal was to identify a pattern of gene response for one compound that was distinct from the others*. This way, we could quickly focus on one drug and determine whether the difference caused enhanced or diminished efficacy as compared with MP, the currently accepted therapy.

We first filtered the data by ANOVA to select genes with significant differences among the conditions tested (again using Benjamini Hochberg FDR to estimate multiple measurement error). This produced a list of 314 genes. This group was analyzed by Principal Components Analysis, K-medians clustering, and Relative Data Depth to identify and extract underlying patterns. *We found that NS398, the cyclooxygenase-2 selective antagonist, exhibited a distinct pattern of gene response, and, furthermore, we interpret the list of regulated genes to be protective in an injury environment.* This work is being prepared for submission (planned for September 2003).

We tested our hypothesis by repeating our NS398 treatments in vivo with MASCIS injured animals. Acute treatment with NS398 led to reduced lesion volumes 6 hrs after injury, increased number of activated macrophages at 24 hrs after injury, and, as reported by others, enhanced locomotor activity (10,11). These results will serve as preliminary data for an upcoming U01 grant to NINDS to perform pre-clinical animal testing of Vioxx and other cyclooxygenase-2 inhibits in spinal cord injured rats (planned for Oct 1 submission). The main thrust of the study will be to validate cyclooxygenase-2 as a viable therapeutic target, and to test whether antagonist therapies can be combined with MP to allow accelerated testing in human patients.

3.   Project Challenges

The major challenge of our study has been the development and refinement of our microarray technology. Our original proposal depended on rat cDNA microarrays. We constructed these arrays, but we found that they were difficult to produce in quantity with acceptable levels of quality. Our collaborators had successfully transitioned to using spotted "long" oligos (65-70 nt) instead of cDNA, and we chose to utilize this emerging technology. We also adapted a new labeling technology, which allows us to begin with small quantities of RNA without amplification (12). Finally, we combined these two technologies with a robotic hybridization workstation from Ventana Medical System, Inc. Our combination of technologies led to a strategic marketing alliance between our corporate technology sources, and our technology is

being acquired by microarray labs throughout the world. We delayed the completion of Aim 1 until the technology had been adequately validated to take the most advantage of our work.

4.   Implication for future research and/or clinical treatment.

We believe that our work with NS398 will lead to the development of a new therapy for cellular protection during the acute phase of SCI. The first goal arising from the project will be to perform pre-clinical animal testing to determine the feasibility and efficacy of these compounds. Following the philosophy of the NJCSCR, we wished to apply our findings to patients as soon as possible, and our NIH program officer agreed. A traditional R01 NIH grant focusing on the hypothesized mechanisms will be delayed until the U01 screening application is completed.

Our work with both Drs. Schwartz and Popovich will influence the proposed use of T cells as cell transplant therapy. Dr. Schwartz proposed the use of T cells as a regenerative therapy, but we have found that autoimmune T cells may exacerbate tissue damage following spinal contusion. However, one beneficial mechanism, T cell induced promotion of glutamate uptake by microglia, may be replaced by IFNγ treatment. We will continue examination of T cells in spinal contusion during our newly-funded NIH subcontract (from Dr. Philip Popovich).

Our anti-inflammatory studies continue beyond the cyclooxygenase-2 mechanisms. We have obtained a grant from the Philip Morris External Research Foundation to study nicotine as an anti-inflammatory therapy for SCI. Preliminary results demonstrate diminished accumulation of activated monocytes in spinal cord following contusion after a single injection of nicotine.

Finally, the technology we developed under this project has impacted many other projects. We have initiated a microarray study of descending neurons injured by SCI. We have completed microarray assays for the following spinal cord researchers: Drs. Martin Grumet, Wise Young, Renping Zhou, Michal Schwartz, Mary Bartlett-Bunge, Martin Masala, and Patrick Sullivan. We have initiated collaborative projects with others, including: Drs. Bonnie Firestein, Wilma Friedman, Monica Driscoll, and Arthur Brown. This genomics support work will continue.

5.   Plans to continue this research

As outlined above, we have obtained funding from NIH (subcontract with Dr. Philip Popovich) to continue our T cell work, the Philip Morris External Research Foundation to continue our anti-inflammatory screening, and the New Jersey Commission for Spinal Cord Research to provide microarray services and databases to spinal cord researchers, and to initiate a new project on cellular injury to descending neurons. An application is in progress to NIH for a U01 grant to screen cyclooxygenase-2 inhibitors in acute SCI, and a second application will follow for an R01 to study the mechanisms of protection by cyclooxygenase-2 antagonists.

6.   Publications included in report

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Carmel, J.B., O. Kakinohana, R. Mestril, W. Young, M. Marsala, R.P. Hart. Mediators of Ischemic Preconditioning Identified By Microarray Analysis of Rat Spinal Cord. Submitted to Experimental Neurology.

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Pan, J.Z., R. Jörnsten, and R.P. Hart. Screening anti-inflammatory compounds in spinal cord injury using microarrays: Identification of therapies and potential mechanisms using bioinformatic analysis. In preparation.

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