Changes in Rat Spinal Cord Gene Expression After Inflammatory Hyperalgesia of the Joint and Manual Therapy

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Context: Mobilization of a joint affects local tissue directly but may also have other effects that are mediated through the central nervous system.

Objective: To identify differential gene expression in the spinal cords of rats with or without inflammatory joint injury after manual therapy or no treatment.

Methods: Rats were randomly assigned to 1 of 4 treatment groups: no injury and no touch (NI/NT), injury and no touch (I/NT), no injury and manual therapy (NI/MT), and injury and manual therapy (I/MT). We induced acute inflammatory joint injury in the rats by injecting carrageenan into an ankle. Rats in the no-injury groups did not receive carrageenan injection. One day after injury, rats received manual therapy to the knee of the injured limb. Rats in the no-touch groups were anesthetized without receiving manual therapy. Spinal cords were harvested 30 minutes after therapy or no touch, and spinal cord gene expression was analyzed by microarray for 3 comparisons: NI/NT vs I/NT, I/MT vs I/NT, and NI/NT vs NI/MT.

Results: Three rats were assigned to each group. Of 38,875 expressed sequence tags, 755 were differentially expressed in the NI/NT vs I/NT comparison. For the other comparisons, no expressed sequence tags were differentially expressed. Cluster analysis revealed that the differentially expressed sequence tags were over-represented in several categories, including ion homeostasis (enrichment score, 2.29), transmembrane (enrichment score, 1.55), and disulfide bond (enrichment score, 2.04).

Conclusions: An inflammatory injury to the ankle of rats caused differential expression of genes in the spinal cord. Consistent with other studies, genes involved in ion transport were among those affected. However, manual therapy to the knees of injured limbs or to rats without injury did not alter gene expression in the spinal cord. Thus, evidence for central nervous system mediation of manual therapy was not observed.

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E vidence[1-9] suggests that osteopathic manipulative treatment (OMT) is efficacious for the management of low back pain; however, the mechanisms of action are poorly understood. The end point associated with many OMT techniques—pain reduction—is a subjective experience difficult to measure in humans and animals. The effects of manual therapy on pain may be local or mediated through the central nervous system, neither of which is well understood. However, local effects may include improved lymphatic circulation[10] and fibroblast mechanosensation.[11,12]

In a study by Sluka et al,[13] mobilization decreased mechanical withdrawal latency after inflammatory insult in rats. Improvement in withdrawal latency of both the ipsilateral and contralateral limbs in a rat model of pain implied potential involvement of the central nervous system.[13] Further, improvement in withdrawal latency after mobilization of the joint proximal to the injured joint also implied potential involvement of the central nervous system.[14] Evidence for the role of the central nervous system in mediating the effects of manual therapy comes from the observations that inhibition of the serotonergic pathway prevented the antihyperalgesic effect of manipulation[15] and that OMT altered human serum levels of cannabinoids and opioids, which probably act centrally.[16]

Osteopathic manipulative treatment applied to the joint could act by means of the central nervous system through interconnected systems. Central processes of dorsal root ganglion cells that signal noxious stimulation enter the spinal cord and branch over several segments at the tip of the dorsal horn; there, the pain fibers synapse on the dendrites of cells in deeper layers of the spinal cord dorsal horn. These latter cells in laminae V and VI then send axons in the anterolateral tract to supraspinal levels for conscious perception and the arousal effects of pain. However, modulation of the relay of pain information within the spinal cord and to supraspinal levels takes place in the dorsal horn of the spinal cord. Enkephalinergic interneurons located in the substantia gelatinosa at the tip of the dorsal horn also branch over several spinal cord segments and make inhibitory synapse with the dendrites of the tract cells that signal pain to higher levels. These enkephalinergic interneurons are excited, in turn, by large A-alpha and A-beta fibers from nonnoxious cutaneous and proprioceptive afferents. As a consequence, stimulation of the large, rapidly conducting axons that convey nonnoxious and proprioceptive stimulation can inhibit the tract cells of the anterolateral system. This "gating" of pain information in the tip of the dorsal horn, first proposed by Melzack and Wall[17] and further confirmed and elaborated on by others,[18] is a mechanism by which manipulation could reduce joint pain.

Global gene expression in the central nervous system has been investigated as a means of understanding pain and its relief; of particular interest is the ability of alternative modalities to alter gene expression. In one study,[19] uninjured rats responsive to electroacupuncture (as measured by tail-flick latency) had differential expression of 63 genes in the hypothalamus compared with uninjured, unresponsive rats. In another study,[20] 68 genes that were differentially expressed in the spinal cords of rats with neuropathic pain returned to basal expression levels after electroacupuncture. More genes were differentially expressed at a lower electroacupuncture frequency than at a higher frequency in the arcuate nucleus of the hypothalamus compared with uninjured, untreated controls.[21] Yukhananov and Kissin[22] found differential expression of 67 pain-related genes in the spinal cord after hind paw inflammation.

From a previous study, Ruhlen et al[23] reported that injured rats treated with manual therapy did not recover voluntary running distance more quickly than untreated injured rats. Because behavioral end points in animal models can be subjective and prone to variability, we chose to assess a physiological end point, spinal cord gene expression, for the current study.

The purpose of the current study was to identify changes in spinal cord gene expression in rats with or without inflammatory joint injury of the ankle after manual therapy to the proximal knee or no treatment. We predicted that genes involved in pain-related pathways would be differentially expressed in the spinal cords of rats with inflammatory joint injury. Further, we hypothesized that manual therapy would return such genes to no-injury levels and that manual therapy in rats with or without a background of injury would induce differential gene expression in the spinal cord.

Methods

Animals

All experiments were approved by the Institutional Animal Care and Use Committee and were carried out in accordance with guidelines from the National Institutes of Health and from the International Association for the Study of Pain for pain research in animals. Male Sprague Dawley rats (250-350 g; Hilltop Lab Animals) were singly housed on a 12-hour light cycle in polypropylene cages with cedar bedding and were maintained on 5001 Laboratory Rodent Diet (Purina Mills). Rats were randomly assigned to 1 of 4 groups of 3 rats each: no injury and no touch (NI/NT), injury and no touch (I/NT), no injury and manual therapy (NI/MT), and injury and manual therapy (I/MT). Previously reported studies have shown that carrageenan injected into the ankles of rats induced swelling and hyperalgesia and reduced voluntary running wheel activity.[14,23]

Injury

Inflammatory joint injury was induced as described previously.[23] Briefly, 0.05 mL of 3% carrageenan in 0.9% saline (pH 7.4) was injected into the right ankle. Rats in the no-injury groups were not injected.

Manual Therapy

In the current study, we used a manual therapy protocol developed in our laboratory,[23] which was modified from that of Sluka et al[13,14] and Skyba et al.[15] We treated single knees in rats anesthetized with isoflurane (2%-5%) using 3 repetitions of 3 minutes with a 1- to 2-minute rest interval between repetitions. In injured or uninjured rats, the right knee was the knee treated. Knee mobilization/translation was applied by flexing and extending the ipsilateral knee joint to its end range of extension while the tibia was simultaneously translated in an anterior-to-posterior direction using grade III and IV mobilization forces, a technique previously shown to influence pain-related behavior in rats with inflamed ankles.[14] Flexion and extension were performed at a frequency of 20 times per minute. A board-certified osteopathic physician (E.J.S.) in neuromusculoskeletal and osteopathic manipulative medicine trained an osteopathic medical student in the manual techniques. Training consisted of a session of instruction and practice, followed by at least 1 supervised session. The student's technique was further observed at intervals during the study to minimize variability in performance. Manual therapy was performed 24 hours after carrageenan injection. Rats in the no-touch groups were anesthetized for the same 11- to 13-minute period, with no touch applied to the hind limb.

Euthanasia and Tissue Collection

After 30 minutes of anesthesia for the no touch group, rats were euthanized by carbon dioxide inhalation and cervical dislocation. This time frame was selected because gene expression changes were most prominent 30 to 60 minutes after an inducing stimulus in previous experiments.[24,25] The spine was severed just posterior to the ileal tuberosities and again approximately 2.5 cm anterior to the ileal tuberosities. The spinal cord was removed with hydraulic pressure by injecting saline from a 10-mL syringe and was placed in RNAlater solution (Qiagen) to prevent RNA degradation.

Microarray Hybridization and Data Analysis

RNA Extraction and Purification

The tissue was homogenized in QIAzol Lysis Reagent (Qiagen), and total RNA was isolated with the RNeasy Lipid Tissue Mini Kit (Qiagen), according to the manufacturer's instructions, within 2 hours of euthanasia. Messenger RNA (mRNA) was purified from total RNA using an Oligotex mRNA Mini Kit (Qiagen). To concentrate the 200 μL of purified mRNA sample, 20 μL of 3 mol/L sodium acetate and 600 μL of cold 80% ethanol was added to it, and the mixture was incubated for 2 hours at $-20°C$, centrifuged at 10,000 g for 15 minutes at 4°C, washed twice with cold 80% ethanol, and air dried for 30 minutes at 37°C.

*Complementary DNA Labeling
and Microarray Hybridization*

For the microarray hybridization experiments, the following comparisons were made: NI/NT vs I/NT (*Figure 1*), I/MT vs I/NT, and NI/NT vs NI/MT. The mRNA samples were labeled with fluorescent dye–labeled deoxynucleotide triphosphates for each comparative experiment: 1 sample was labeled with cyanine 3 (Cy3) deoxycytosine triphosphate, and the other with cyanine 5 (Cy5) deoxycytosine triphosphate (GE Healthcare). The complementary DNA (cDNA) labeling was carried out in the presence of a 16-mer deoxythymidine oligonucleotide with SuperScript III Reverse Transcriptase (Invitrogen), according to the manufacturer's instructions. The cDNA was purified using the SuperScript Indirect cDNA Labeling System (Invitrogen), according to the manufacturer's instructions.

The Cy3- and Cy5-labeled cDNA samples were combined, concentrated, and resuspended in 50% formamide, 0.1% sodium dodecyl sulfate (SDS), and 0.9% saline/0.44% sodium citrate (SSC). The combined cDNA samples were then denatured at 94°C for 3 minutes and placed under a coverslip on a Rat MI ReadyArray slide (Microarrays Inc) prehybridized with 0.1% bovine serum albumin solution in 5× SSC and 0.1% SDS for 30 minutes at 42°C. Rat MI ReadyArray slides have 38,875 probes representing 21,466 genes and 28,404 gene transcripts. The cDNA was hybridized for 16 to 20 hours at 42ºC; washed at room temperature sequentially in 2× SSC/0.1% SDS, 1× SSC, 0.2× SSC, and 0.05× SSC for 1 minute each; and dried by centrifugation. Slides were imaged using a GenePix 4000B scanner (Molecular Devices). The Cy3 and Cy5 intensities for each spot were quantified, followed by median background correction and normalization with a GenePix Pro 6.0 software scanner (Molecular Devices).

Statistical Analyses

Data were analyzed using Bioconductor (version 2.12), BiocInstaller (version 1.10.2),[26] and R (version 3.0.0)[27]
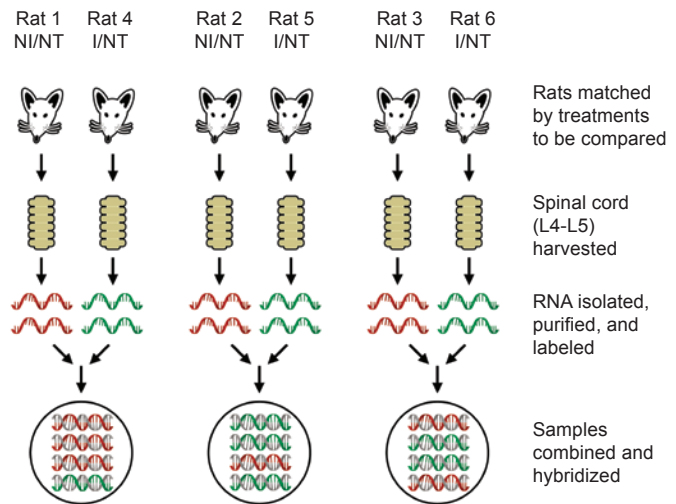

Figure 1.

Graphic illustration of the no injury and no touch (NI/NT) vs injury and no touch (I/NT) comparison in the current study. Each rat in the NI/NT group was paired with a rat in the I/NT group. After treatment, the L4-L5 sections of the rat spinal cords were harvested, and RNA was isolated, purified, and labeled with either cyanine 3 (Cy3; NI/NT samples, depicted as red) or cyanine 5 (Cy5; I/NT samples, depicted as green). The Cy3-labeled complementary DNA (cDNA) was combined with the Cy5-labeled cDNA and hybridized to probes. As indicated in this illustration, if a particular gene was downregulated in rat 4 vs in rat 1, there was a greater Cy3 than Cy5 intensity. Similarly, if the Cy5 intensity was higher for a gene than the Cy3 intensity (as shown for rat 5 vs rat 2), this indicates that the gene was upregulated in rat 5. Similar Cy5 and Cy3 intensities (as shown for rat 6 vs rat 3) suggest that these genes were equally expressed in spinal cord tissues of NI/NT vs I/NT rats. The comparisons of injury and manual therapy vs I/NT and NI/NT vs no injury and manual therapy were performed similarly.

software. As part of the preprocessing, within-array print-tip locally weighted scatterplot smoothing[28,29] and median normalization on the background-corrected red and green intensities (normexp method[30]) were performed for each spot, followed by between-array quantile normalization.[31] Differential expression analysis was performed by fitting linear models to the expression data for each gene and performing moderated *t* tests, which use an empirical Bayes modification of the *t* test to improve variance estimation for small sample sizes.[32] The Benjamini and Hochberg[33] correction method was used

to account for multiple testing and control the false discovery rate to less than 0.15. These methods were implemented using functions provided in the Limma package.[32]

Differentially expressed sequences were further analyzed for functional clustering using the Database for Annotation, Visualization, and Integrated Discovery (DAVID; National Institute of Allergy and Infectious Diseases, National Institutes of Health).[34] The DAVID software analyzes the representation of functional categories by performing a modified Fisher exact test. A group enrichment score is defined as the geometric mean (in $-\log_{10}$ scale) of these resulting P values in a corresponding annotation cluster to rank their biological significance. Thus, the top-ranked annotation groups most likely have consistent lower P values for their annotation members. Default DAVID settings are categories containing 2 or more genes with P values <.10.

Results

Of the 38,875 Rat MI ReadyArray probe sequences, 755 were differentially expressed in the NI/NT vs I/NT comparison (ie, rats without or with injury but not treated). More of these differentially expressed sequences appeared to be upregulated in the I/NT group (614 upregulated vs 141 downregulated) (*Figure 2*). For the NI/NT vs I/NT comparison, the largest significant difference came from gene ENSRNOG00000031399, which had 5.3 times higher expression in injured rats compared with uninjured rats ($\log_2$ fold change, −2.4).

No sequences were found to be differentially expressed in rats receiving manual therapy compared with controls in the no-touch groups, either with (I/MT vs I/NT) or without (NI/NT vs NI/MT) injury. A large average $\log_2$ fold change was observed for many genes in the NI/NT vs NI/MT comparison, where NI/MT rats had many upregulated genes but differences were not statistically significant, owing to large variability between the samples (*Figure 2*).

Clustering analysis of the differentially expressed sequences in injured rats, performed with DAVID, revealed overrepresentation in 75 categories. The *Table*

shows clusters with enrichment scores higher than 1 and categories within clusters with P values <.05.

Discussion

To our knowledge, the current study is the first to examine differential gene expression after manual therapy. We identified differential spinal cord gene expression between injured and uninjured rats, but we did not find evidence of differential gene expression after manual therapy.

There are several possible reasons for the outcomes of the current study. One possibility is that manual therapy does not influence gene expression in the spinal cord. Another is that the selected manual therapy techniques were applied to a joint (knee) adjacent to the injured joint (ankle), although based on OMT techniques used in humans, they may not be effective for treatment of a quadruped. Further, a single treatment may have been inadequate to demonstrate gene change in the spinal cord, and the time between treatment and harvesting of the spinal cord may have been inadequate to demonstrate an effect. As such, a longer course of manual therapy may influence gene expression.

A rat model of manual therapy would be helpful in future studies to investigate the molecular mechanism and the role of manual therapy in the central nervous system, but the current study failed to establish such a model. Although our protocol was based on a promising rat model that focused on the role of brain descending pathways in manual therapy,[13-15] our results suggest that the original studies showing central biochemical changes after manual therapy should be repeated to substantiate the animal model and to further delineate manual therapy's influence on the central nervous system.

The use of microarrays to analyze gene expression in nociception has blossomed in the past decade. In the current study, the functional clusters of spinal cord sequences that were differentially expressed in injured rats were consistent with those found in other studies of nociception.[19,20,22,35]

Yukhananov and Kissin[22] used methods similar to those of the current study, and there was overlap in the
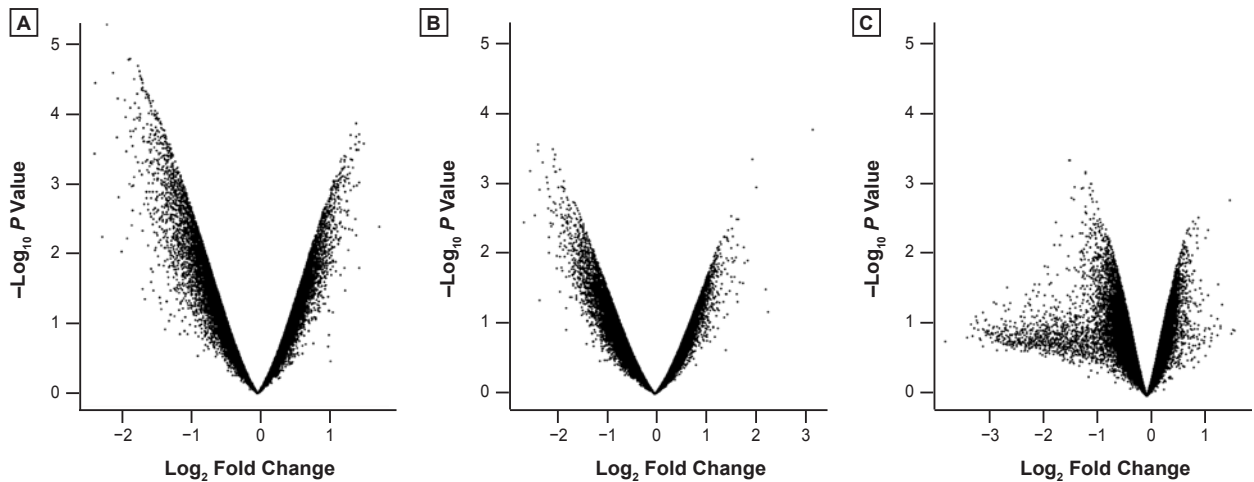
Figure 2.

Volcano plots representing the relationship between fold change in the gene sequence expression level for group 1 relative to group 2 and statistical significance. The horizontal axis represents the $\log_2$ fold change between the 2 groups compared for each analysis: (A) no injury and no touch (NI/NT) vs injury and no touch (I/NT), (B) injury and manual therapy (I/MT) vs I/NT, and (C) NI/NT vs no injury and manual therapy (NI/MT). The vertical axis represents the $-\log_{10}$ P value for testing whether the gene sequence is differentially expressed between the 2 groups. Each of 38,875 Rat MI ReadyArray probe gene sequences is represented by a point in each graph. In panel A, control of the false discovery rate to less than 0.15 resulted in a significant P value threshold of .003 or approximately 2.5 in $-\log_{10}$ on this graph (ie, there are 755 such gene sequences represented in panel A greater than 2.5. In panels B and C, control of the false discovery rate to less than 0.15 resulted in no significant P value threshold (ie, no gene sequences represented in panels B and C are considered significantly differentially expressed).

clustering analysis between their results and ours. Ion homeostasis sequences were enriched in both studies. Transmembrane sequences were enriched in the current study, and clusters that we feel are similar to transmembrane sequences (ie, membrane receptors and ligands) were enriched in the study by Yukhananov and Kissin.[22] However, there were no direct matches in the differentially expressed sequences between that study and the current one.

Also consistent with findings of other microarray studies,[19,20,22,35] we identified altered neural ion regulatory genes in the injured condition. These results support the known integral role of ion transport in nociception.[36] Further, the strong representation of differentially expressed genes in the disulfide bond cluster in the current study suggests membrane protein transport, such as ion channels. The categories within that cluster included signal peptide, glycoprotein, and the extracellular region—char-

acteristics associated with membrane proteins. Thus, not only do our findings support and expand on those of previous studies, but consistency with the findings of others also indicates that our methods were sound.

The differential gene expression we observed in injured vs uninjured rats supports the lack of differential gene expression we observed in treated vs untreated rats. Other studies have found differential gene expression in the central nervous system due to electroacupuncture,[19-21] rather than injury, as used in the current study. Our results also suggest that manual therapy does not induce differential gene expression in the central nervous system, either in injured rats or in uninjured rats. Finding differential gene expression in the central nervous system in other contexts but not with manual therapy suggests that manual therapy—at least the limb mobilization tested in the current study—does not affect gene expression in the spinal cord.

Table.
Classification of Differentially Expressed Sequences in Rats With Injury vs Rats Without Injury

Category	Sequences, No. (%)	P Value
Ion response (enrichment score, 2.29)[a]		
Response to metal ion	12 (1.9)	.003
Response to calcium ion	7 (1.1)	.004
Response to inorganic substance	14 (2.3)	.01
Disulfide bond (enrichment score, 2.04)		
Disulfide bond	56 (9.1)	<.001
Signal peptide	61 (9.9)	.002
Glycosylation site: N-linked	70 (11.3)	.004
Signal	61 (9.9)	.004
Glycoprotein	73 (11.8)	.009
Extracellular region	44 (7.1)	.02
Secreted	32 (5.2)	.03
Enzyme inhibitor (enrichment score, 1.55)		
Endopeptidase inhibitor activity	10 (1.6)	.007
Peptidase inhibitor activity	10 (1.6)	.01
Enzyme inhibitor activity	11 (1.8)	.05
Transmembrane (enrichment score, 1.55)		
Intrinsic to membrane	133 (21.5)	.003
Glycosylation site: N-linked	70 (11.3)	.004
Integral to membrane	128 (20.7)	.006
Glycoprotein	73 (11.8)	.009
Transmembrane	86 (13.9)	.03
Remodeling (enrichment score, 1.50)		
Macromolecular complex remodeling	3 (0.5)	.03
Plasma lipoprotein particle remodeling	3 (0.5)	.03
Protein-lipid complex remodeling	3 (0.5)	.03

(continued)

One limitation of the current study was that rats were anesthetized during manual treatment. Because anesthesia may alter gene expression,[37] we controlled for the effect of anesthesia by administering it to all rats, regardless of treatment. However, subtle changes in gene expression levels may have been present but beyond our ability to detect with microarray analysis, possibly masked by the effects of anesthesia. We investigated methods to administer manual therapy without anesthesia, such as by immobilizing the rats, but this would have stressed the animals. In addition to ethical concerns, stress could confound the study results as much as anesthesia, so we chose the less stressful option. The lack of a behavioral end point showing efficacy in this model[23] was another limitation. Our primary objective was mechanistic, but our ability to draw conclusions from the current study was hampered by the lack of a behavioral end point. In addition, the insult may have overwhelmed the rat's system, and we may have observed a treatment effect with a lesser injury. Finally, our small sample size was an additional limitation.

A better understanding of the role of the central nervous system in OMT will help elucidate the appropriate contexts of OMT. Mechanistic evidence is also needed to move OMT from the realm of alternative modalities into mainstream medicine. Using microarrays to determine differential gene expression will identify genes and pathways to target in future preclinical and clinical trials of OMT.

Conclusion

The results of the current study suggest that mobilization of the joint did not affect gene expression in the central nervous system. If any effect of joint mobilization as performed in the current study was mediated by the central nervous system, it was not by means of altered gene expression. Although this outcome may disappoint OMT practitioners, it was just one outcome of 1 type of manipulation in 1 type of tissue, and OMT includes many techniques in many tissues with many expected outcomes. Therefore, it is premature to rule out the effect or define the mechanism of OMT on the basis of our study

results. To truly elucidate the mechanisms of tissue manipulation and manual therapy in the full variety of OMT techniques, tissues, and outcomes, many similar studies will need to be performed. Only painstaking research with appropriate controls will yield the data needed to begin to understand the molecular mechanisms that are involved in the many situations in which OMT techniques have been shown to be effective in managing human dysfunction.

Acknowledgments

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Table (continued).
Classification of Differentially Expressed Sequences in Rats With Injury vs Rats Without Injury

Category	Sequences, No. (%)	P Value
Photodetection (enrichment score, 1.44)		
Phototransduction	5 (0.8)	.001
Detection of light stimulus	5 (0.8)	.003
Detection of external stimulus	6 (1.0)	.01
Detection of abiotic stimulus	5 (0.8)	.03
Cytoskeleton (enrichment score, 1.34)		
Cytoskeletal keratin	5 (0.8)	.01
Linker 12	5 (0.8)	.01
Coil 1A	5 (0.8)	.02
Rod	5 (0.8)	.02
Head	5 (0.8)	.02
Linker 1	5 (0.8)	.02
Coil 1B	5 (0.8)	.02
Intermediate filament protein, conserved site	6 (1.0)	.02
Tail	5 (0.8)	.03
Intermediate filament organization	3 (0.5)	.03
Filament	6 (1.0)	.03
Intermediate filament-based process	4 (0.6)	.03
Intermediate filament	7 (1.1)	.04
Intermediate filament cytoskeleton	7 (1.1)	.05
Immune (enrichment score, 1.18)		
Graft-vs-host disease	6 (1.0)	.01
Allograft rejection	6 (1.0)	.01
Type 1 diabetes mellitus	6 (1.0)	.01
Major histocompatibility complex	5 (0.8)	.04
Autoimmune thyroid disease	5 (0.8)	.05

[a] Enrichment scores calculated with DAVID (Database for Annotation, Visualization, and Integrated Discovery) software.[30]

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