Molecular mechanisms of targeted-activity dependent spinal stimulation mediated recovery in rats with cervical spinal cord injuries with RNA-Seq

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***Abstract*— Spinal cord injury (SCI) is a debilitating neurological condition without effective treatments. Previously, we have shown that targeted, activity-dependent spinal stimulation (TADSS) is effective in improving long-term functional recovery after cervical spinal cord injury. Here, we apply RNA Sequencing (RNA-Seq) technology to characterize changes in gene expression over 6 weeks of treatment. Additionally, we identified potential pathways that are affected by the change in gene expression associated with TADSS. These pathways include cell growth/cell death, immune, and endocrine systems, which have previously been connected with functional recovery after SCI. These results provide a valuable start for a better understanding of how TADSS improves functional recovery in a chronic model of spinal cord injury.**

I. INTRODUCTION

There are about 230,000 people in the United States living with spinal cord injury (SCI), with approximately 12,000 new cases each year [1]. More than half of all traumatic SCI in humans occur at the cervical level of the spinal cord, which causes deficits in movements, particularly in the control of the forelimb [2]. Restoration of motor function, particularly of arm and hand movements, is an area of great concern for individuals with cervical SCI, though current treatments have significant limitations [1].

Electrical stimulation has been used as a therapeutic option for individuals with SCI. It is well known that electrical stimulation can cause muscle contraction, and functional electrical stimulation (FES) of muscle tissue has been shown to activate innervating nerves to strengthen muscles and restore hand grasp and release [1]. FES has also been applied to neural tissues to restore control over bodily functions [3]. However, the effectiveness of FES functional recovery is limited, particularly in the long term.

A novel strategy — targeted, activity-dependent spinal stimulation (TADSS) — when paired with physical retraining improves fine motor function after spinal cord injury during and after treatment better than open-loop stimulation with retraining or retraining alone [4]. Targeted, open-loop intraspinal microstimulation (TOLSS) stimulates the spinal cord at regular intervals, without feedback to control the interstimulus intervals. TADSS was designed to strengthen connections that may have been damaged by SCI. TADSS delivers spinal stimulation at a current just below the threshold to elicit muscle movement to a site in the spinal cord below the lesion following sufficient electromyographic (EMG) activity in the triceps or wrist. Compared to TOLSS or only physical retraining (RT), the TADSS group exhibited twice the performance enhancement by the end of therapy. Furthermore, these benefits persisted 3 weeks after the treatment ended, distinguishing TADSS from other treatments that show improvement only during stimulation [4].

While TADSS improves reaching performance post-SCI, the mechanisms of this recovery are unknown. We hypothesize that the improved recovery observed with TADSS induces plasticity, possibly through modifications of synapses at or below the level of injury. Hebbian plasticity suggests that synapses that are used will be made more effective, and synapses that are ineffective will weaken over time. Presynaptic activity that precedes postsynaptic action potentials or depolarization is known to be able to induce long term potentiation (LTP). So, synaptic plasticity is sensitive to the timing of postsynaptic action potentials, providing a mechanism of inducing plasticity [5]. By coupling the spinal stimulation to occur immediately after EMG activity in forelimb muscles, TADSS may strengthen connections in descending motor pathways in accordance with spike-timing dependent plasticity (STDP).

Uncovering the molecular changes, especially genes and pathways, that are modified in TADSS treated animals would inform our understanding of the mechanisms underpinning recovery and possibly identify novel therapeutic targets. RNA-Seq is a next generation sequencing technology which profiles the transcriptome, the complete set of mRNA transcripts in a cell and their quantity [6]. RNA-Seq has advantages over existing technologies that we hope to use to map and quantitatively analyze RNA sequences. For instance, unlike hybridization-based approaches, RNA-Seq can detect transcripts that do not correspond to existing genomic sequences. In addition, unlike DNA microarrays, RNA-Seq has a low background signal and has a greater dynamic range [6]. Analyzing differential gene expression with RNA-Seq will help us find quantitative differences in expression between TADSS and unstimulated control groups. These molecular changes could be future targets to enhance TADSS and recovery after SCI.

Previous work used RNA-Seq to understand how SCI affects the spinal cord. For example, a 2017 study found that differentially expressed genes were enriched in several pathways such as immune response, MHC protein complex, antigen processing and presentation, translation-related genes, and Toll-like receptor [7]. Additionally, a study into injury responses following SCI found an upregulation of genes associated with cell death after injury and found new recovery associated genes[8]. Few, if any, studies focus on

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how treatment affects gene expression at or near the site of the injury and how it improves recovery.

In this study, we find that there are distinct expression patterns between the treatment and control groups, suggesting TADSS is associated with axonal growth or other neuroremodeling processes, and other systemic neuroprotective benefits.

II. METHODS

All procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Reaching Task

We trained animals under a standard forelimb reaching task, which has been previously described [4]. After we identified the dominant forelimb of a rat, we trained it to reach through a slot in their Plexiglass arena for a chocolate pellet placed on a nearby dimpled block. There was a gap between the arena and block so that the pellet would fall outside the arena if it was not grasped tightly enough, and the slot was only wide enough for one forelimb to reach the pellet. In each session, we gave a rat 5 warm-up trials. Then, we counted the number of pellets (out of 20) that the rat grasped and brought to its mouth. [4].

Spinal Contusion

We have previously described our rat model of incomplete cervical SCI. [4]. In total, 6 female Long-Evans rats received a moderate to severe unilateral dorsal spinal contusion at the C4/C5 border, ipsilateral to the dominant forelimb. All surgical procedures have been described previously [4].The animals were anesthetized with an intraperitoneal injection of ketamine (70 mg/kg) and xylazine (10 mg/kg). A hemilaminectomy was performed on the C4 vertebrae ipsilateral to the dominant forelimb. Each rat then received a moderate-severe contusion with the Ohio State impact device [4]. After injury, musculature and skin were closed, and all animals received post-operative care, including baytril, buprenorphine, lidocaine, and manual bladder expression.

Intraspinal Microwire and EMG Implants

Four to six weeks post-SCI, we implanted the rats with 5 intraspinal platinum-iridium microwires and 5 stainless steel EMG wires in the triceps and extensors. We used an approach originally developed for the lumbar spinal cord by Mushahwar et al [9]. Following a hemilaminectomy at C7 and T1 ipsilateral to the lesion, we inserted each of the microwires at the C6 to C8 spinal segments near the dorsal roots. Stainless steel wires were also placed in the ipsilateral tricep and extensor muscles.

RNA Isolation, Quantification, and Qualification

3 rats with 6 weeks of EMG-triggered spinal stimulation and 3 rats with no stimulation were sacrificed 12 weeks post-SCI and perfused with PBS. Their spinal cords were flash frozen in liquid nitrogen and separated into two segments: one at the level of the lesion (around C4-C5) and one just caudal to the lesion but rostral to the intraspinal microwire implants (about C5-C6), as seen in Figure 1. The RNA from each segment was isolated and purified using the QIAGEN RNeasy Mini kit. The RNA sequencing was done at the Weill Cornell Genomics and Epigenomics Core Facility using the Illumina sequencing platform.

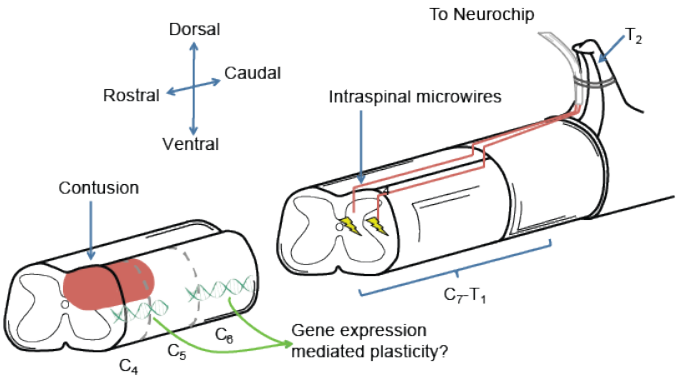


Fig. 1: Experimental setup. A contusion damages half of the spinal cord at C4-C5, causing deficits in the dominant forelimb. Intraspinal microwires are placed in C6-C8, caudal to the injury site and delivers TADSS. After perfusion, the spinal cord is dissected and is divided into two sections, one with the lesion, and one caudal to the lesion. Adapted from McPherson 2015 [4].

RNA-Seq Analysis

To analyze the RNA sequences, we downloaded the Rattus norvegicus genome (Rnor_6.0) from the Ensembl website. We performed the data analysis with the Galaxy Project, an open-source software that contains common data analysis tools. HISAT2 v2.1.0 was used to align the paired-end reads to the reference genome with paired end reads and report alignments tailored for transcript assemblers including StringTie. HISAT2 was selected for its speed and low memory requirements. The transcriptome assembly reporting was set to report alignments tailored for transcript assemblers for better integration with Stringtie. Stringtie v1.3.4 was used to assemble the alignments from HISAT2 into possible transcripts. Stringtie uses a novel network flow algorithm to assemble and quantify full-length transcripts representing multiple splice variants for each gene locus [10]. We only used reference transcripts to skip novel transcripts. Additionally, the output files were tailored for differential expression by DESeq2, average read length was set to 52. DESeq2 v2.11.40.2 was used to perform differential expression analyses using transcript counts output by Stringtie. The adjusted p-value < 0.05 was adopted as the standard for judging statistically significant differences in gene expression between control and treatment groups. Adjustment was done with the Benjamini-Hochberg procedure which controls the false discovery rate [11].

DESeq2 outputs a tabular file containing gene identifiers (Ensembl transcript IDs), mean normalized counts, the logarithm (base 2) of the fold change (log2FC), standard error estimate for log2FC, Wald statistic, p-value of the change, and the adjusted p-value. Transcripts were categorized as upregulated or downregulated based on the log2FC. Negative values indicate that relative to the control, there was a decrease in expression of the transcripts in TADSS subjects. A positive value indicates upregulation of those genes. Ensembl transcript IDs were translated to gene

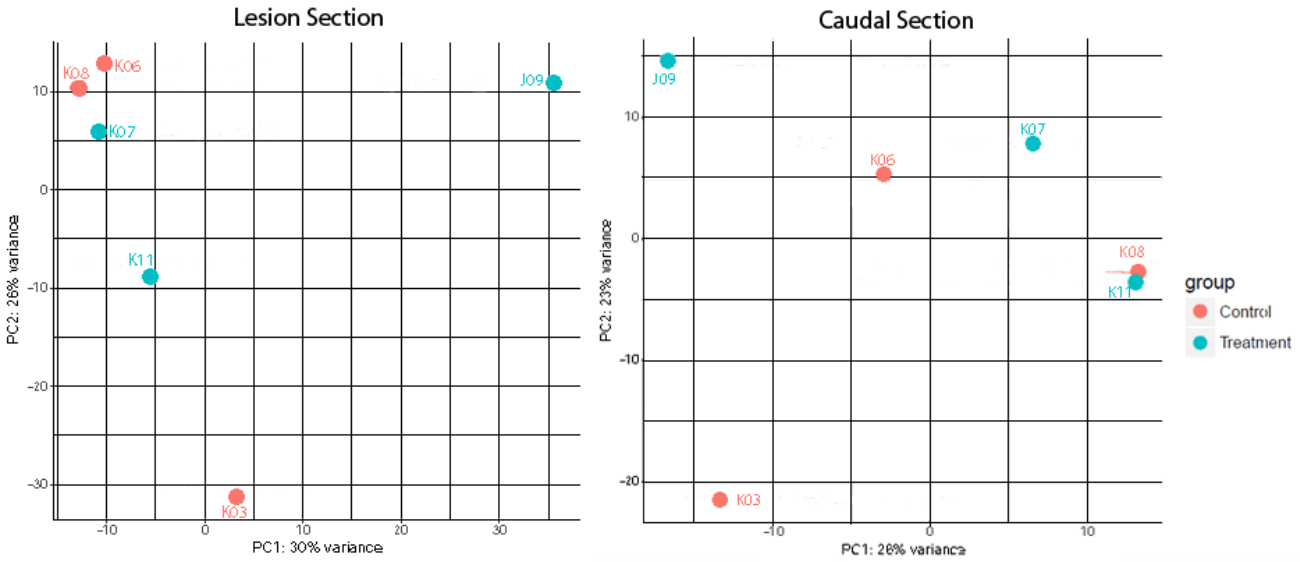


Fig. 2: PCA analysis with two principal components of expressed transcripts in A) the lesion section and B) the caudal section (n=3)

symbols using BioMart.

Principal Components Analysis

Principal components analysis (PCA) was performed with the DESeq2 R package within Galaxy on each section of the spinal cord. These analyses included all genes that were differentially expressed in control and treatment groups, not just one that were significantly differentially expressed.

Clustering Analysis

Clustering analysis was performed using the heatmap2 R package through Galaxy. Some genes had multiple transcripts that were differentially expressed. Genes with transcripts that were both upregulated and downregulated were excluded from this analysis. Across each gene that was included, the transcript counts were scaled so that we could compare the relative expression of each gene across subjects.

Enrichment Analysis

Gene set enrichment analysis (GSEA) was performed to understand the biological pathways involved in TADSS. We used the PANTHER Pathway database to analyze gene expression and up- and downregulated pathways in all sections of the cord.

III. RESULTS

Transcriptome dynamics

Transcriptomes aligned using HISAT2 had 93% of all reads mapped to the rat reference genome. 49 differentially expressed transcripts were identified in the lesion section, and 48 were identified in the caudal section. Of these, 4 transcripts in the lesion section and 12 from the caudal section were excluded from analysis because the gene had transcripts that were both up- and downregulated. Of these transcripts, 22 in the lesion section and 19 in the caudal section were significantly upregulated in rats that received TADSS, and 23 in the lesion section and 17 in the caudal section were downregulated.

Principal Components Analysis

We performed PCA to examine the source of variance in our data across 2 principal components in each section of the spinal cord. As shown in Figure 2, the contribution of PC1 was 30% and 26% and the contribution of PC2 was 26% and 23% in the lesion and caudal section, respectively. The 3 samples from each group do not appear to cluster together. However, this pattern may be due to batch effects: sub-groups of measurements that have qualitatively different behaviour across conditions and are unrelated to the biological or scientific variables in a study. [12] It is true that the animals were sacrificed on different days which could have introduced variation to the dataset. As PCA evaluates both biological and technical variability, the lack of clustering within treatment groups may be due to batch effects [12].

Heatmaps

The selected differentially expressed genes were classified into cluster groups based on their expression across samples. From the heatmaps in Figure 3, it appears that the control group cluster together and the TADSS group also cluster together. Additionally, we can see numerous distinct clusters of genes that are upregulated and downregulated in the TADSS group.

Pathway Analysis

We analyzed the pathways that were enriched in the significantly up- and downregulated genes in the both the lesion and caudal sections (Figure 4). From this pathway analysis, we suggest that many pathways could be involved in the TADSS recovery mechanism. Pathways of note that represented upregulated genes in the lesion section include: angiogenesis, cholecystokinin receptor (CCKR) signaling map, gonadotropin-releasing hormone (GnRH) receptor pathway, heterotrimeric G-protein signaling pathway—G_i alpha and G_s alpha mediated pathway, inflammation mediated by chemokine and cytokine signaling pathway, platelet-derived growth factor (PDGF) signaling pathway, and the Ras pathway. Pathways upregulated in the caudal

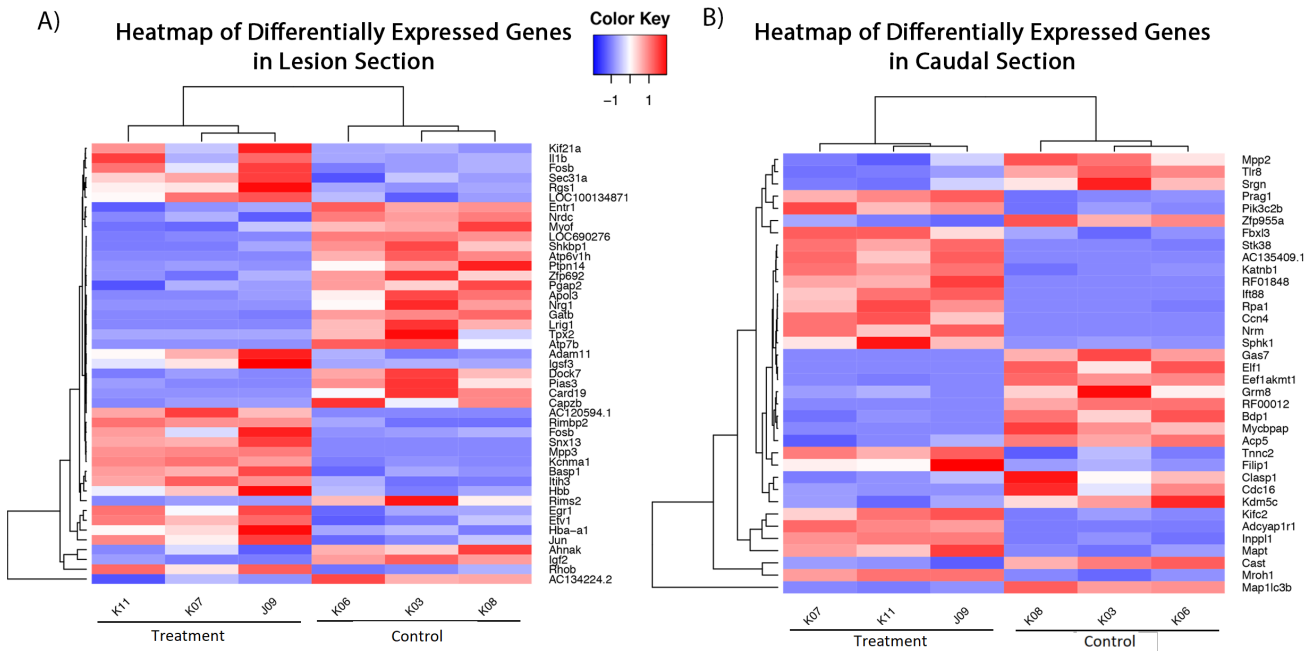


Fig. 3: A) Heatmap of differentially expressed genes in the 6-week lesion section, with clustering. B) Heatmap of differentially expressed genes in the 6-week caudal section, with clustering. Each column represents one subject. K11, K07, and J09 received TADSS. K08, K03, and K06 are controls. Each row in the heatmap represents a gene that is differentially expressed. As each gene is scaled, a higher relative transcript count is represented in red while a lower relative transcript count is represented in blue. White represents unchanged gene expression.

section include: angiogenesis, insulin/insulin-like growth factor (IGF) pathway-protein kinase B signaling cascade, and vascular endothelial growth factor (VEGF) signaling pathway. No pathways of note were downregulated in either individual section. When the differential expression of genes were analyzed across sections, upregulated genes were also found to be enriched in the integrin signalling pathway. The toll receptor (TR) signaling pathway was indicated to be enriched in downregulated genes when the two sections were analyzed together.

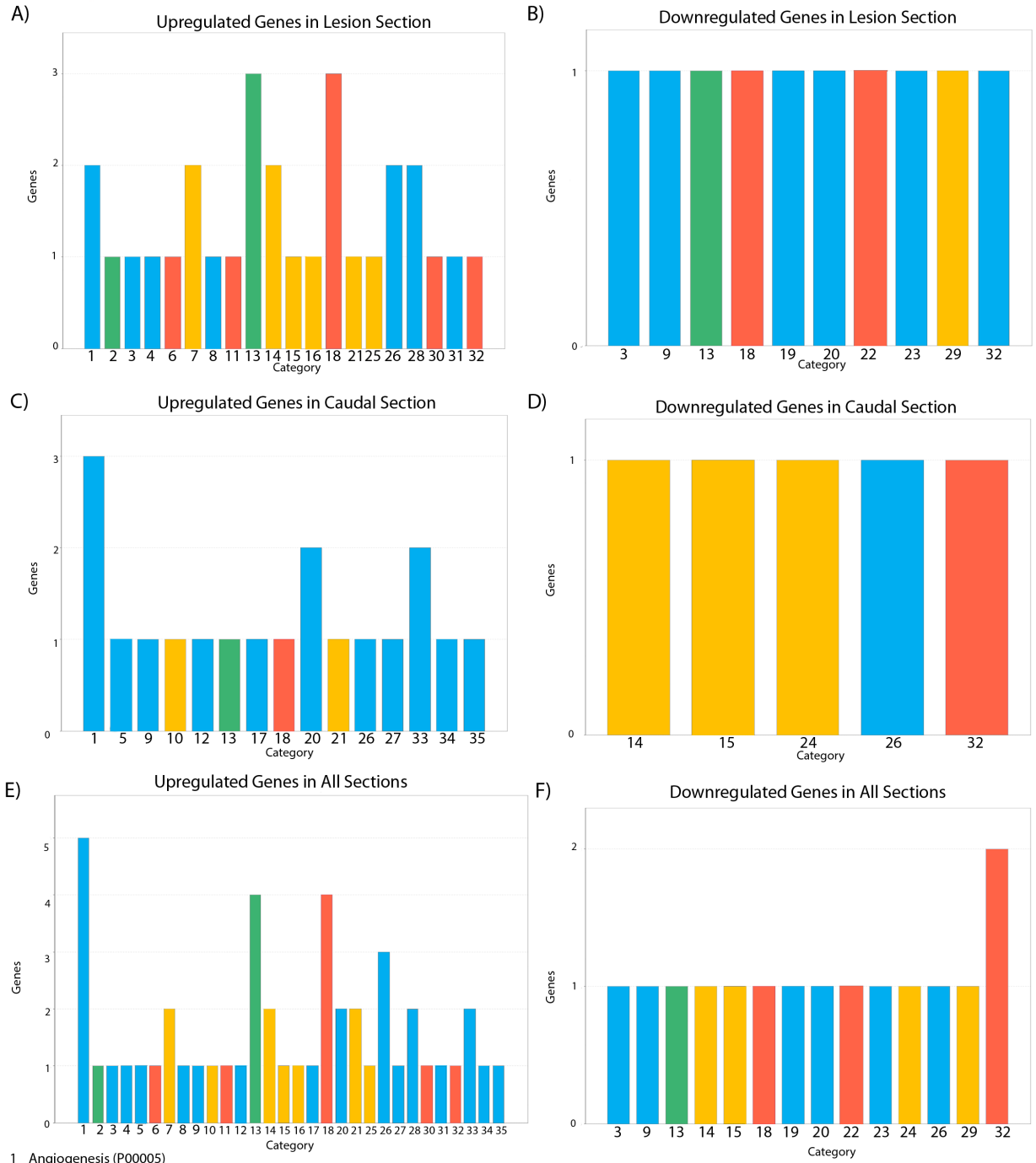
IV. Discussion

In this study, we have systematically analyzed patterns of gene expression change using RNA-Seq to characterize changes in the spinal cord with TADSS and without TADSS. From previously published and unpublished data, we have shown that TADSS produced improved functional recovery after SCI. Our analysis of gene expression changes suggests that TADSS is associated with axonal growth or other neuronal remodeling processes, and improved recovery through the immune and endocrine systems, all of which seem to contribute to enhanced recovery after SCI.

Our pathway analysis revealed numerous pathways that are involved with cell growth, a possible indication of increased axonal growth post-SCI with spinal stimulation. In the lesion section of the spinal cord, we saw that the Ras pathway, a known regulator of cell growth in eukaryotic cells, was enriched [13]. It has also been implicated in apoptosis in neural cells and spinal cord to improve recovery post-SCI [13], [14]. Also enriched in the lesion section, the insulin/IGF pathway and the protein kinase B signaling cascade, which play roles in the functional recovery following TADSS, are also involved in cell growth. The insulin/IGF pathway is best known for extending the lifespan of organisms in a wide range of species [15]. In the context of SCI, the insulin/IGF pathway may help preserve existing neurons or even aid in the growth of new neurons. Protein kinase B has a role in the regulation of metabolism, cell survival, motility, transcription and cell-cycle progression.

In the caudal section of the spinal cord, three pathways of note were enriched. The angiogenesis, the PDGF pathway, and VEGF pathway are involved in the growth of new blood vessels and the regulation of blood vessel formation [16],[17]. These blood vessels are essential for normal cell growth and development, and are implicated in different pathologies [17]. Furthermore, other studies have suggested that the combination of VEGF and PDGF pathways can reduce cell death after SCI and reduce the size of the resulting lesion [18]. The functions of these pathways all suggest that there is cell growth and possible axonal growth following TADSS in an injured spinal cord. Confirmation of this thinking can be done through functional techniques in future experiments such as immunohistochemistry or viral vectors.

Cellular processes were also implicated in pathways enriched in the lesion section of the spinal cord. The CCKR signaling map is a part of the gastrointestinal system, though numerous studies have shown that CCK is involved in neuropathic pain pathways and is upregulated with nerve injury [19],[20],[21]. In one of these studies, the authors



1 Angiogenesis (P00005)
2 Angiotensin II-stimulated signaling through G proteins and beta-arrestin (P05911)
3 Apoptosis signaling pathway (P00006)
4 Axon guidance mediated by Slit/Robo (P00008)
5 Axon guidance mediated by netrin (P00009)
6 B cell activation (P00010)
7 CCKR signaling map (P06959)
8 Cytoskeletal regulation by Rho GTPase (P00016)
9 EGF receptor signaling pathway (P00018)
10 Endothelin signaling pathway (P00019)
11 FAS signaling pathway (P00020)
12 FGF signaling pathway (P00021)
13 Gonadotropin-releasing hormone receptor pathway (P06664)
14 Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway (P00026)
15 Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway (P00027)
16 Huntington disease (P00029)
17 Hypoxia response via HIF activation (P00030)
18 Inflammation mediated by chemokine and cytokine signaling pathway (P00031)
19 Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade (P00032)
20 Insulin/IGF pathway-protein kinase B signaling cascade (P00033)
21 Integrin signalling pathway (P00034)
22 Interferon-gamma signaling pathway
23 JAK/STAT signaling pathway (P00038)
24 Metabotropic glutamate receptor group III pathway (P00039)
25 Oxidative stress response (P00046)
26 PDGF signaling pathway (P00047)
27 PI3 kinase pathway (P00048)
28 Ras Pathway (P04393)
29 Synaptic vesicle trafficking (P05734)
30 T cell activation (P00053)
31 TGF-beta signaling pathway (P00052)
32 Toll receptor signaling pathway (P00054)
33 VEGF signaling pathway (P00056)
34 p53 pathway feedback loops 2 (P04398)
35 p53 pathway (P00059)

Fig. 4: Results of PANTHER Pathway analysis. Each bar represents a different pathway in which genes that were upregulated (left) downregulated (right) in the lesion (A,B), caudal (C,D), or all (E,F) are involved. The height of the bar shows the number of genes in the gene list that are involved in that pathway. Blue bars are pathways involved in cell growth or death. Red bars are involved in the immune system. Yellow bars represent cellular processes, and green bars are pathways that involve the endocrine system.

suggest that the increase in CCK can facilitate an increase in prostaglandin and serotonin release at the site and modulate nociceptive transmission [21]. These results may suggest that TADSS could have some effects on neuropathic pain. Alternatively, it could be that the increased transmission with CCK upregulation is also applicable to motor pathways and TADSS brings on changes in the spinal cord on the cellular level which facilitate signal transmission. Similarly, a heterotrimeric G-protein signaling pathway was enriched by upregulated genes in the lesion section. Generally, these G-proteins act as signal transducers in the cell [22]. The G_i alpha protein subtype is the inhibitory conformation of the stimulatory G-protein, G_s alpha [23]. These proteins are a part of G-protein coupled receptor (GPCR) pathways and activate and inhibit the cAMP cascade [22]. In the context of TADSS and SCI, perhaps there is a cell-level change in the expression of these G-proteins, which changes how action potentials or other cellular signals reach motor neurons.

When we analyzed the upregulated genes across both sections, the analysis indicated that the integrin signaling pathway was enriched. Integrins are a family of receptors that are involved in cellular responses such as migration, survival, differentiation and motility, and provide a context for responding to other inputs, including those transmitted by growth-factor- or G-protein-coupled receptors [24]. Given the other pathways that were enriched in the lesion and caudal sections and the breadth of pathways that the integrins are involved in, it is likely that TADSS has a similar effect on the integrins.

We also observed that pathways involved with the immune system were enriched, specifically with inflammation and the toll-like receptor. These changes may have a mediatory effect which improves recovery after SCI [25].

Inflammation is known to occur in both short- and long-term studies of SCI and is generally thought to be damaging for neural cells, so it was not entirely unexpected that the inflammation mediated by chemokine and cytokine signaling pathway was enriched [26],[27]. However, recent data suggest that inflammation may have neuroprotective effects during the acute phase of SCI and that complete depletion of neutrophils hinders functional recovery [25]. These findings suggest that the increased expression of genes in the inflammation pathway in the TADSS group could be due to some neuroprotective effect or other recovery mechanism which is brought on by the targeted spinal stimulation.

The toll-like receptor (TLR) pathway, which was enriched by downregulated genes in both the lesion and caudal spinal cord section, is also a part of the immune system. More specifically, they activate macrophages in defense against pathogens, though it has been suggested that trauma (such as that from a SCI) could also activate those pathways, and the TLR could mediate recovery [28]. With two somewhat competing functions, it is not immediately clear how the TLR contributes to the changes seen with TADSS. However, the finding that the TLR pathway regulates inflammation and gliosis is promising as there could be protective effects that enhance recovery after SCI.

The enrichment of gonadotropin-releasing hormone receptor pathway with TADSS is a bit more puzzling, though previous studies have shown that GnRH could have neurotrophic effects and that GnRH treatment improved recovery of rats after SCI [29]. Furthermore, the endocrine system and immune system are regulated through the hypothalamic-pituitary-adrenal (HPA) axis, which can be disrupted by SCI [30]. This connection suggests that these pathways that are changed in association with TADSS are a part of a more complex, inter-related mechanisms of spinal cord injury recovery.

Moving forward, there are still more analyses that should be done to improve our understanding of the molecular mechanism of TADSS. From these animal groups, there are still RNA sequences from spinal cord sections that have not yet been analyzed. Additionally, we have samples from rats that received 2 weeks of treatment rather than 6 weeks of treatment. With those data, we could examine the transcriptome at different time points of treatment and better understand how gene expression changes in a chronic model of SCI. Furthermore, we will implement corrections to reduce the impact of batch effects on the variability of our data through study design and analysis techniques.

Final Thoughts

We have provided a first look at molecular mechanisms underlying a neuroprosthetic strategy of improving motor recovery post-SCI. Our results suggest that the growth, immune, and endocrine pathways are activated in rats treated with TADSS than injured control rats, which may indicate axonal growth or other neuronal remodeling processes and other protective benefits as a result of TADSS. Our study reveals genes and pathways consistent with other approaches to SCI treatment, and suggest that TADSS brings on a broader multi-system change that enhances functional recovery.

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