Final Narrative Report
New Jersey Commission on Spinal Cord Research

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Body of Report

1. Original Aims of the Project:

Regeneration of axons from descending nuclei of the brain is the ultimate goal of spinal cord injury research. In the past few years, much has been learned about inhibitory mechanisms of regeneration, and because of this, many new regenerative therapies have shown great promise. Our research group has developed methods for identifying gene expression changes in large numbers of genes using the advanced techniques of microarrays. This approach has proved useful in identifying previously unknown mechanisms for protection from acute spinal cord injury and in comparing different regeneration paradigms for similar mechanisms. We now propose to extend our studies to two promising regenerative therapies. Our goal is to identify fundamental gene expression mechanisms that mediate the neuronal effects of regenerative therapies. Knowledge of such mechanisms will drive the refinement of therapies, and, potentially, the search for small molecule drugs that may be more practical for curing spinal cord injury.

1. What is the pattern of descending motor cortex gene expression associated with *spinal contusion* or *axotomy* of the corticospinal tract? We will perform functional genomic analysis of gene expression in motor cortex following contusion or dorsal hemisection (*in the absence of regeneration*). We expect to see regulation of neurotrophin signaling intermediates, reduced expression of GAP-43, tubulins, and other regeneration-associated genes by 2-4 weeks after injury due to inhibition of regenerative mechanisms.
2. What is the pattern of descending motor cortex gene expression associated with *regeneration* of the corticospinal tract? We will compare regeneration methods either using chondroitinase ABC to degrade extracellular inhibitory networks, or using silencer RNA to prevent inhibition of regeneration through common intracellular pathways.

2. Project Successes

The project was awarded a reduced budget and only one year of support instead of the two requested. Nonetheless, this project provided an important milestone in our research effort—the development of laser capture microdissection (LCM) of retrograde-labeled brain neurons as an effective tool for studying cell-specific gene expression in spinal cord injury and regeneration. It will take several years of work to reap the benefits of this important pilot project.

Since the scope of the project was reduced, we chose to focus on two models: (1) Hemisection of corticospinal tract and the effect on axotomized descending neurons; and (2) Spinal contusion followed by radial glial transplant and the effect on growing raphe neurons.

Each of these experiments depended on our ability to (1) identify injured and/or regrowing neurons using retrograde tracers compatible with LCM techniques, (2) develop and validate LCM techniques compatible with microarray assays, (3) accurately amplify vanishingly small quantities of mRNA collected by LCM, (4) assess differential gene expression on microarrays and perform the appropriate statistical analyses, and (5) validate selected microarray data with quantitative real-time PCR (qPCR) and/or immunohistochemistry. As the reviewers feared, each of these steps required significant time and ingenuity.

First, we hired a talented postdoctoral scientist, Dr. Sophie Parmentier-Batteur, with experience in rodent CNS surgery, immunohistochemistry and ;microarray techniques. Dr. Batteur was the

appropriate complement to our molecular biology expertise for developing these methods. Dr. Batteur tested several retrograde tracers and found two that were effective and compatible with our LCM techniques, Fluorogold (FG) and RDX/RITC. This gave us two fluorescent colors required for our differential cell collection in LCM.

Dr. Batteur then attempted to use the standard Arcturus microscope for LCM but was unable to select small, independent cells by this method and she could not visualize fluorescent retrograde tracers. We turned to the newly-developed PALM/Zeiss LCM system, which allowed fluorescent detection, identification of multiple populations of neurons depending on multiple fluorescent tags, and efficient dissection of large numbers of individual cells. The use of the PALM/Zeiss system was essential for the success of our experiments.

Next, we worked with Genisphere, Inc., to develop an amplification technique compatible with our oligonucleotide-based microarrays. We assayed 192 genes by qPCR using several variations of the amplification technique and helped Genisphere to select their eventual commercial product, SenseAmp. We validated that this technique was capable of amplifying RNA from as few as 5 cells with good fidelity (Goff et al., 2004). Again, selection and validation of this technique was essential for the project.

We first applied our techniques to a simple model of spinal axotomy. Rats were injured by dorsal hemisection to lesion the corticospinal tract (CST). A Gelfoam pledget soaked in Fluorogold was inserted into the injury site to retrograde label injured neurons. At 1 or 7 days after injury, rats were sacrificed and the cortex was dissected, frozen-sectioned and used for LCM collection of pools of individual labeled cells. Retrograde-labeled CST neurons from uninjured animals were used as controls. FG-labeled injured CST nuclei and RDX/RITC-labeled CST nuclei with intact fibers were captured by LCM (~100 cells per rat, n=3 rats). After RNA extraction and double amplification, microarray analysis compared injured CST nuclei with a pool of intact CST nuclei. Statistical analysis (one sided Student's t-test) of the ratios (injured CST/intact CST) yielded 272 genes. A subset based on gene ontology (GO) classification for signal transduction included genes involved in cellular remodeling (including Nudt6, 3.8-fold; VEGFc, 2.6-fold; TGFb3, 2.6-fold) and axonal growth (including axon-associating molecule, 3.6-fold; Wdr7 WD repeat domain 7, 1.9-fold). Selected results were validated by qPCR and immunohistochemistry. Interestingly, the genes that were not validated fell into two categories: (1) those that were regulated in neighboring cells such as astrocytes, and (2) those that were at or near the limits of detection on our microarrays. A manuscript summarizing these results is nearly ready for submission.

A second experiment built on the successes of Dr. Martin Grumet with the transplantation of a radial glial cell clone, RG3.6 (Hasegawa et al., 2005). After spinal contusion, Dr. Grumet's lab showed enhanced spinal tissue architecture and functional recovery following RG3.6 transplant as compared with fibroblast-transplanted animals. We replicated this experiment, labeling injured fibers immediately after contusion with Fluorogold. At 5.5 weeks following injury, we injected RITC/RDX tracer 1 cm caudal from the injury site. At 6 weeks, we collected brain and inspected raphe for double-labeled neurons (injured, re-grown to 1 cm caudal from the injury). Quantification showed that the number of growing raphé neurons is significantly increased in RG3.6-transplanted rats compared to medium-treated rats (Fig. 1, 15.3% ± 1.1 vs. 8.2% ± 3.0, n=4, $p<0.05$, Student's t-test). To select against labeling sprouted neurons, we collected single-labeled neurons from the same sections, and we tested multiple time points.

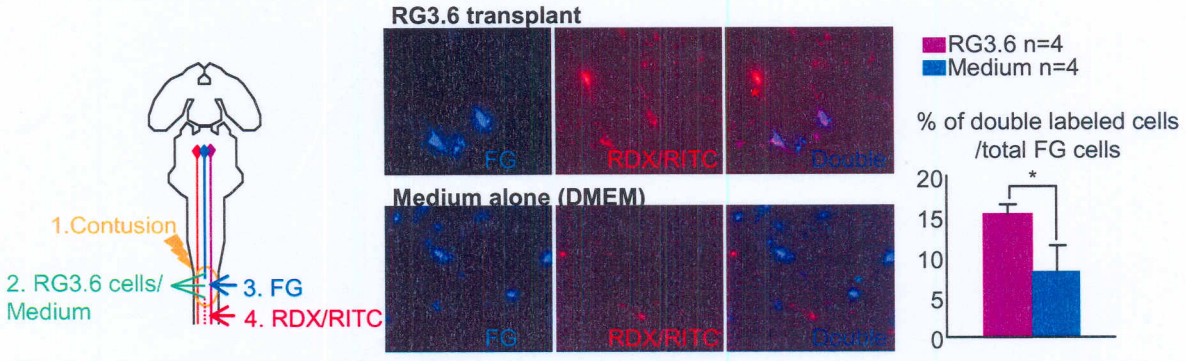

Figure 1. Retrograde tracing of the Raphé-spinal tracts to differentiate raphé neurons with injured 5-HT fibers from raphé neurons with growing 5-HT fibers. The number of growing raphé neurons is significantly increased in RG3.6-transplanted rats compared to medium-treated rats ($p<0.05$ Student's t-test).

Microarray results compared double-labeled growing raphé cells from RG3.6-transplanted animals to injured FG-labeled cells from medium-treated animals. Statistical analysis (Welch's t-test) found 75 genes differentially expressed in growing neurons compared with injured neurons; of these genes, 18 (24%) are consistent with a program of repair, including neural growth, differentiation and survival. The results provide evidence that double labeled growing neurons of the RST are involved in regenerative (Nr2f1, Slit3, Gsk3B) and sprouting (LAR-interacting protein 1, Attractin, Collapsin response mediator protein 1) processes. Again, selected results have been validated by qPCR. Immunohistochemistry is in progress.

A more comprehensive experiment is currently underway. Contused rats were treated with Chondroitinase ABC in an attempt to reduce inhibitory extracellular components. RG3.6 cells were transplanted 30 days after injury. FG was applied at injury, and RDX/RITC was added 5.5 weeks following RG3.6 cell transplant. At 6 weeks after transplant, regrowing cortical neurons will be collected by LCM and analyzed on microarrays. In this version of the experiment, contusion completely ablates the CST, and delayed cell transplant has been shown to promote growth of CST neurons, so we should be able to model true regeneration in a more acceptable model. Our plan is to complete these experiments as pilot data for a future grant submission.

Two novel projects arose from these studies. First, we have begun a collaboration with Dr. Melitta Schachner (formerly of the University of Hamburg, currently Rutgers University) to apply LCM studies to spinal cord injury in zebrafish. In this model, zebrafish spinal cord injury has been shown to produce functional regeneration (Becker et al., 1997). Furthermore, Dr. Schachner's laboratory has shown that the neural adhesion molecule L1.1 is essential for regeneration (Becker et al., 2004) and that different tracts regenerate with different efficiencies (Becker et al., 2005). We propose to use LCM and microarrays to compare regenerating and non-regenerating neural tracts following spinal cord injury. This project was recently funded by the NJCSCR, and preliminary results are encouraging.

Finally, we adapted our LCM/microarray technique for use with microRNAs. We fractionated RNA collected from small numbers of cells (~50 cells) using the Ambion mirVana kit to produce high molecular weight fraction (containing mRNA) and low molecular weight fraction (containing microRNAs). The newly-discovered microRNAs have been shown to regulate translation of mRNAs by binding the 3' untranslated region in a RISC complex (for reviews, see: Bartel, 2004; Rogelj and Giese, 2004). By adapting the SenseAmp method we had previously

validated (Goff et al., 2004) with a microRNA labeling technique we described recently (Goff et al., in press), we were able to amplify the microRNA population and display it on a custom microarray. Adapting these techniques to an ongoing study of microRNA regulation of stem cell differentiation, we proposed to microdissect neural stem cells from embryonic rat brain and identify microRNA expression patterns that may mediate differentiation. Our goal is to manipulate the differentiation of neural stem cells for use as therapeutic transplants for spinal cord injury. This proposal was submitted to NIH and received a score of 137 and a 5.7%ile, which we are hoping will be funded (R21 NS054028-01, PI: M. Grumet, Co-PI: R.P. Hart).

3. Project Challenges

The project was extremely challenging technically and it was hard to be productive with the reduced budget and scope. We have attempted to extend or renew the project with NJCSCR and NIH but have thus far been unsuccessful. Our conclusion is that our models of regeneration in these proposals are not yet considered acceptable by the scientific community. We are committed to continuing to apply our novel technology to other projects (zebrafish and microRNAs, see above) as well as to continue with regeneration projects as they develop and are validated.

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

I believe this project is the most important one I have begun for the eventual treatment of spinal cord injury. First, our description of gene expression patterns in regrowing or regenerating neurons may serve as proxies for more time-consuming and expensive behavioral studies of therapies. Second, we plan to build on the zebrafish regeneration system to assemble a biological assay for regenerating genes. Our plan is to select genes from our LCM/microarray studies in rat as future knock-down experiments in zebrafish, similar to what was done with L1.1 (Becker et al., 2004).

5. Plans to continue this research

Two projects have already been funded arising from this project (Schachner, NJCSCR and Hart, NJCSCR). A third project has received a good score at NIH (Grumet and Hart, NIH). We also plan to incorporate our techniques derived from this project into future NIH proposals from Grumet studying radial glial transplants.

6. Publications included in report

Goff, L.A., J. Bowers , J. Schwalm , K. Howerton , R.C. Getts and R.P. Hart (2004) Evaluation of sense-strand mRNA amplification by comparative quantitative PCR. BMC Genomics 5: 76.

Parmentier-Batteur, S., Y.-W. Chang, M. Grumet and R.P. Hart (2004) Profiling neuronal degeneration and regeneration after spinal cord injury: Comparing mRNA patterns after hemisection or radial glial cell transplant into contused spinal cord. Society for Neuroscience Annual Meeting, Poster.

Parmentier-Batteur, S., Y.W. Chang, M. Grumet and R.P. Hart (2005) Host effects on gene expression profiles of radial glial cells transplanted into rat spinal cord. Society for Neuroscience Annual Meeting, Poster.

Parmentier-Batteur, S., Y.-W. Chang, M. Grumet and R.P. Hart. Gene expression in cortical neurons following spinal cord hemisection. Manuscript in preparation.

References

Bartel DP (2004) MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116:281.

Becker CG, Lieberoth BC, Morellini F, Feldner J, Becker T, Schachner M (2004) L1.1 is involved in spinal cord regeneration in adult zebrafish. J Neurosci 24:7837.

Becker T, Lieberoth BC, Becker CG, Schachner M (2005) Differences in the regenerative response of neuronal cell populations and indications for plasticity in intraspinal neurons after spinal cord transection in adult zebrafish. Mol Cell Neurosci.

Becker T, Wullimann MF, Becker CG, Bernhardt RR, Schachner M (1997) Axonal regrowth after spinal cord transection in adult zebrafish. J Comp Neurol 377:577-595.

Goff LA, Bowers J, Schwalm J, Howerton K, Getts RC, Hart RP (2004) Evaluation of sense-strand mRNA amplification by comparative quantitative PCR. BMCGenomics 5:76.

Hasegawa K, Chang YW, Li H, Berlin Y, Ikeda O, Kane-Goldsmith N, Grumet M (2005) Embryonic radial glia bridge spinal cord lesions and promote functional recovery following spinal cord injury. Exp Neurol 193:394-410.

Rogelj B, Giese KP (2004) Expression and function of brain specific small RNAs. RevNeurosci 15:185.

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W.M. Keck Center for Collaborative Neuroscience
The Spinal Cord Injury Project

Profiling neuronal degeneration and regeneration after spinal cord injury: Comparing mRNA patterns after hemisection or radial glial cell transplant into contused spinal cord

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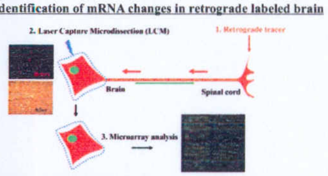
Abstract

We used a combination of SCI models, cell transplants, laser capture microscopy (LCM) and microarrays to identify mRNA patterns associated with neuronal degeneration and regeneration. Axotomy of the corticospinal tract leads to degeneration of cortical neurons. Axotomized cells were retrograde-labeled with fluorogold (FG) and intact neurons were labeled distal to the injury with rhodamine dextran (RDX). Individually-labeled neurons were isolated using LCM. RNA was prepared, amplified, and used to probe spotted oligo microarrays. The predominant effects of axotomy were on genes associated with cell growth, signaling, and transcription factors. The profile of neuronal response to axotomy is consistent with a program of cellular remodeling. Transplanting RG3.6, an immortalized radial glial cell line, elicits axonal growth following spinal contusion. Six weeks after injury, serotonergic fibers originating in the raphe can be detected distal to the contusion. We retrograde labeled injured neurons upon contusion with FG and growing fibers 5.5 wks later using RDX injected distal to the injury. At 6 wks, 15% of FG neurons were RDX -positive (growing) in the raphe after RG3.6 transplant, as compared with 8% double-labeling after transplant of culture medium (DMEM) alone. Microarray array results compared growing raphe cells from RG3.6-transplanted animals to injured but not growing cells from DMEM-treated animals. Axonal growth was associated with enhanced markers of growth, differentiation, neurogenesis and axon guidance. This profile is distinct from that of axotomy and predicts specific cellular mechanisms mediating regeneration.

Methods

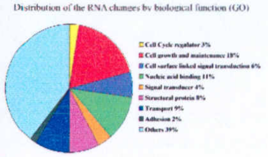
Specific identification of mRNA changes in retrograde labeled brain



- Retrograde tracer introduced into the spinal cord.
- Laser Capture microdissection performed using the Palm Microlaser Systems (Carl Zeiss Inc., NY, USA) on brain coronal sections (10 µm-thickness).
- Microarray analysis:
  - RNA was extracted using a microRNeasy kit (Qiagen, CA).
  - RNA was amplified twice using a SenseAMP kit (Genisphere, Inc.; Goff et al., BMC Genomics 2004). ~25 µg aRNA was obtained from 150 cells isolated by laser capture microdissection.
  - 1 µg of aRNA was reversed transcribed using a sequence-tagged primer (Genisphere, PA). The resulting cDNA was hybridized to microarrays containing 7,793 unique oligonucleotides, specific for 11,749 unique mRNAs.

Degeneration-associated genes are principally involved in cell growth/maintenance, cell structure, signal transduction and nucleic acid binding.

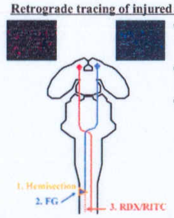
Distribution of the RNA changes by biological function (GO)



Identification of degeneration -associated genes

What genes are regulated in cortical neurons following hemisection of descending CST?

Retrograde tracing of injured fibers of the corticospinal tract to label neurons

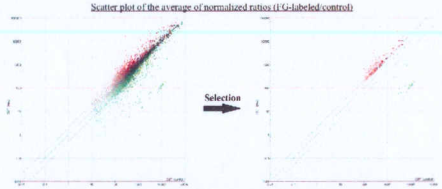


- **Hemisection:** Right hemisection of the spinal cord (T9-T10) was performed on anesthetized (pentobarbital 45-55 mg/kg) female rat Sprague-Dawley (77 ± 2 days old; n=3). Control rats received only the injection of RDX/RITC to label the cortical neurons (n=3).
- **Fluorofold injection:** FG (3%) was introduced into the incision via gelfoam pledgets. This 1st tracer labeled **blue cortical neurons with injured fibers** (see diagram).
- **RDX/RITC injection:** a 1:1 mixture of rhodamine dextran 10,000 (RDX; 20% in 0.2% DMSO) and rhodamine-b-isothiocynate (RITC; 10% in 0.2% DMSO) was injected 0.5 cm caudal to the incision (0.5 µl). This 2nd tracer labeled **in red cortical neurons with intact fibers** (see diagram).

Microarray analysis:

- Microarrays were run with 2 samples per array: one sample of aRNA from FG-labeled neurons (labeled with Cy5) vs. a portion of a control pool of uninjured cortical neurons (labeled with Cy3). Microarrays were analyzed using GeneSpring (Silicon Genetics, Inc.). Ratios indicated fold change between injured and uninjured samples.
- Statistical analysis of the ratios between FG-labeled neurons from injured rats and RDX/RITC-labeled neurons from control rats was performed on 8,449 gene probes using a one-sided Student t test, selecting 272 genes.

Scatter plot of the average of normalized ratios (FG-labeled/control)



Signal transducers activated following axotomy of the corticospinal tracts reflect both an inhibition of axonal growth and a trend for regeneration.

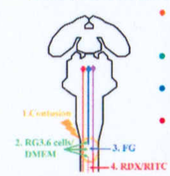
Signal transducers			
Ratios	Description		Biological process
3.8	Nudt6	antisense basic fibroblast growth factor	cell growth
3.6		Axin-associating molecule	axonal growth
2.6	Vegfc	vascular endothelial growth factor C	cell growth and/or maintenance
2.4	Ide	insulin degrading enzyme	proteolysis and peptidolysis
2.3	Tgfb3	transforming growth factor, β3	cell growth; cell proliferation
2.0	Fat2	MEGF1	homophilic cell adhesion
1.9	Wdr7	WD repeat domain 7. alias TGFβ resistance associated gene	cell growth
1.9	Gdf15	growth differentiation factor 15	TGFβ receptor signaling pathway
1.8		transforming growth factor-β binding protein 4S	cell growth
1.7		semaphorin 3D	cell growth; cell proliferation
0.1	Esdn	endothelial and smooth muscle cell-derived neuropilin-like protein	cell guidance

Conclusion: Axotomy is associated with a program of cellular remodelling.

Identification of genes associated with axonal growth

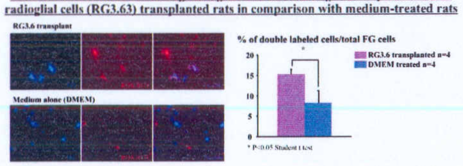
What genes are regulated in raphé neurons during renewed axonal growth after spinal contusion?

Retrograde tracing of the Raphé-spinal tracts to differentiate raphe neurons with injured 5-HT fibers from raphe neurons with growing 5-HT fibers.



- **Contusion:** Spinal cord contusion was performed using the MASCIS Impactor (at T9/T10) on female rat Sprague-Dawley anesthetized with pentobarbital (45-55 mg/kg; n=4 per group). Control rats received only the injections of the retrograde tracers.
- **Radial glial cell (RG3.6) transplant:** 3-point injections of $1x10^5$ cells were performed in the contusion area. Vehicle-treated rats received the same volume of medium (DMEM).
- **Fluorogold injection:** 3% FG was injected in the center of the lesion. This first tracer primarily labeled raphe neurons with injured fibers in blue (see figure below).
- **5 1/2 weeks later, RDX/RITC injection:** a 1:1 mixture of rhodamine dextran 10,000 (RDX; 20% in 0.2% DMSO) and rhodamine-b-isothiocynate (RITC; 10% in 0.2% DMSO) was injected 1 cm caudal to the lesion center. This second tracer labeled raphe neurons with intact fibers in red and double-labeled (purple) FG-labeled raphe neurons with fibers that have grown across the lesion (see figure below).

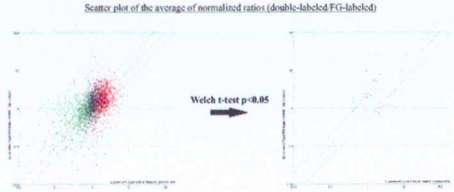
The number of double-labeled growing 5-HT neurons is significantly increased in radioglial cells (RG3.63) transplanted rats in comparison with medium-treated rats



Microarray analysis:

- Microarrays were run with 2 samples per array: one sample of aRNA from either FG-labeled neurons or double-labeled neurons (labeled with Cy5) vs. a portion of a control pool of uninjured raphe neurons (labeled with Cy3). Microarrays were analyzed using GeneSpring (Silicon Genetics, Inc.). Ratios indicated fold change between experimental and control samples.
- Statistical analysis of the ratios between FG-labeled neurons from DMEM-treated rats and double-labeled neurons from RG3.6 treated rats was performed on 3185 genes with at least three replicates in one group using the Welch t-test at $p<0.05$, producing 56 significantly different genes.

Scatter plot of the average of normalized ratios (double-labeled/FG-labeled)



Do retrograde tracers label the same 5HT neuronal phenotype?

- Arrows depict expression ratio relative to uninjured control raphe neurons.
- Both FG-labeled and double labeled neurons express high levels of 5-HT markers.
- The expression pattern of the 5-HT markers is nearly identical in the two cell populations.

5-HT markers	Normalized ratio vs. control levels	
	FG-labeled	Double-labeled
5-HT receptor 3b	↓	↓
5-HT receptor 2B	↓	↓
5-HT receptor 4	↓	↓
5-HT receptor 6	↓	↓
5-HT1B receptor.	↓	↓
5-HT receptor 1A	↓	↓
5-HT receptor 7	↔	↔
5-HT receptor 1D	↔	↔
5-HT receptor 1F	↔	↔
5-HT receptor 2 A	↑	↑
5-HT receptor 7	↑	↑
5-HT receptor 5A	↓	↑
5-HT receptor 2C	↑	↓

Gene expression changes in double-labeled growing neurons involve genes implicated in cell growth, cell defense, axonal growth and axonal guidance.

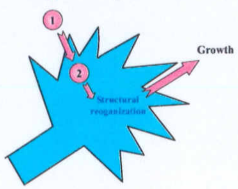
Double-labeled	FG-labeled	Common name	Description	Major Biological function
9.07	2.51	Anxa4	ZAP 36/annexin IV	Anti-inflammatory process-Synaptic vesicules exocytosis
7.79	2.24		nucleotide binding protein long form	Development
7.02	0.35	Slc24a2	solute carrier family 24, member 2	Calcium clearance in axon
5.47	1.63	Chrna2	cholinergic receptor, nicotinic, alpha polypeptide 2	Cell growth - Response to BDNF and FGF2
5.10	1.93		cysteine and histidine-rich cytoplasmic protein	Cell differentiation
4.99	0.78		Nonhistone chromosomal protein HMG-14	Synaptic plasticity in aged animal
3.31	1.47	Ugt2a1	UDP glycosyltransferase 2 family, polypeptide A1	Protection- Cell defense
3.29	0.57	G3bp	Ras-GTPase-activating SH3-domain binding protein	Cell growth and differentiation
3.25	1.66	Shank2	proline rich synapse associated protein 1	Post-synaptic density - Associated with regeneration
2.51	0.90	Igf1	insulin like growth factor 1	Neurogenesis - Regeneration
2.36	0.73	Nr2f1	nuclear receptor subfamily 2, group F, member 1	Neurogenesis
2.31	0.90	Znf291	KIAA1154-like protein	Zinc finger protein - neural development
2.21	0.66	Hes3	transcription factor HES-3	Axonal growth (notch-1 ligand)
1.93	0.61	Siat7e	sialyltransferase 7e	Polysialation of N-CAM for neurite outgrowth
1.90	0.81	Crmp1	collapsin response mediator protein 1	Neurite extension - semaphorin pathway
1.89	1.00	PSMD1	26S proteasome, subunit p112	Neuroprotection - Excitotoxicity
1.78	0.44		alpha 2 type I collagen	Cell adhesion
1.59	0.68		Similar to Fc fragment of IgG binding protein	Cell adhesion
1.07	0.53	Cfdp1	craniofacial development protein 1	Neural developpment
0.74	2.11	Cyp4f14	leukotriene B4 omega hydroxylase	Inflammation
1.32	2.78	Eaf2	ELL associated factor 2	Induction of apoptosis
1.15	3.50	Cct5	chaperonin containing TCP1, subunit 5 (epsilon)	Present at the edge of growth cone
1.91	3.84	Cited4	Cbp/p300-interacting transactivator 4	Inhibits VEGF expression

Values are mean ratio (experimental/uninjured control; n=4).

Conclusion: Putative mediators and signaling mechanisms for renewed raphe-spinal tract growth after SCI

(1) **External cues/receptors:**
insulin-like growth factor 1
craniofacial development protein 1
cell adhesion: alpha-2 type I collagen
Fc fragment of IgG binding protein

(2) **Intracellular pathways:**
Ras-GTPase-activating protein
collapsin response mediator protein 1
cholinergic receptor, nicotinic, alpha polypeptide 2
nuclear receptor subfamily 2, group F, member 1
transcription factor HES-3



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