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Final Narrative Report

Principal Investigator Name, Address, Telephone Number
Melitta Schachner, Ph.D.
W.M. Keck Center for Collaborative Neuroscience
604 Allison Road, D-251
Piscataway, NJ 08854
Ph: 732-445-1780
Fx: 732-445-2063

Name of Organization/Institution
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Genes involved in spinal cord regeneration in zebrafish

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1. Original aims of the project:

(1) Identify changes in gene expression associated with the early regenerative response to axotomy after SCI. To identify genes that are involved in the initiation of an axon growth response, gene expression profiles will be compared between isolated uninjured NMLF neurons and those that were axotomized 4 and 12 hours prior to analysis.

(2) Identify changes in gene expression associated with the growth phase of descending axons after SCI. To analyze growth-associated genes, expression profiles of isolated NMLF neurons during the phase of active axon growth (11 days post-lesion) will be compared to that of uninjured NMLF neurons.

(3) Identify changes in gene expression associated with axonal injury in non-regenerating neurons. To dissociate gene regulation in axotomized neurons that is part of a regenerative response from potentially injury-related changes in gene expression that are not part of a program for axon regrowth we will employ expression profiling of the individually identifiable, axotomized Mauthner neuron, which shows a particularly weak regenerative response after SCI. We will analyze expression profiles of Mauthner neurons for the same time points as for the NMLF (unlesioned and 4 hours, 12 hours, 11 days post-lesion).

(4) Identify changes in gene expression downstream of L1.1 upregulation after SCI. To identify genes that may be regulated via the signal transducing functions of L1.1, expression profiles of axotomized NMLF neurons that received anti-sense morpholino oligonucleotides to "knock down" expression of L1.1 will be compared to those that received control morpholino at 11 days post-lesion.

(5) Verify the relevance of genes identified by expression profiling for axon regrowth in vivo after SCI. We will analyze regulatory genes, such as transcription factors, found to be upregulated also in other systems, to identify a general program of axon regrowth and analyze those genes specifically changed in expression after L1.1 knock down to further evaluate the regulatory role of L1.1 in axon regrowth. Expression of identified genes in regenerating and non-regenerating populations of neurons in the CNS after SCI will be assessed by in situ hybridization to find correlations with the capacity to regrow an axon. In addition, we will reduce expression of newly identified genes after SCI by applying gene-specific anti-sense morpholino oligonucleotides to brainstem neurons and assess locomotor recovery and axon regrowth under these conditions.

2. Project successes:

Our project successfully identified candidate genes involved in spinal cord regeneration. While the technology turned out to be more difficult than we

expected, the candidate genes are now proving to be integrally involved in the regeneration process that we sought to investigate. However, the gene-by-gene validation studies are time-consuming and painstaking, so these studies will continue for a time until we have sufficient impact for publication of this valuable work.

We have successfully extracted RNA with high quality from laser microdissected NMLF of zebrafish brain, amplified RNA from nano-gram to micro-gram range of quantities, completed the Affymetrix microarray analysis, confirmed the microarray results with quantitative real-time PCR, investigated the mRNA expression of some genes of interest by NMLF neurons and investigated the effects of some of these genes on the regeneration of NMLF neurons after spinal cord injury with specific anti-sense morpholino molecules. Each of these accomplishments is detailed below.

It is well known that RNA gets very easily degraded and that the quality of RNA is the key point for the success of microarray analysis. We had proposed to get RNA from laser microdissected tissue, but it is much harder to get RNA with good quality than that from fresh tissue, because we need to make sections of the brain, stain the sections to help recognize the NMLF and microdissect single cells as proposed with a laser. All of these procedures increase the possibility of RNA degradation. We then tried to get RNA with good quality as well as we possibly could. Although liquid nitrogen is colder than dry ice, which might help to keep RNA intact, it turned out that it was very hard to get good sections with brains frozen on liquid nitrogen. With brains frozen on dry ice and sections stained with cresyl violet, RNA with good quality was extracted from microdissected single cells from zebrafish brain. However, microarray analysis needs large amounts of RNA and it turned out that it was impossible to get enough RNA from microdissected NMLF neurons (approximately 50 neurons from 4 fish), even after RNA amplification. We then decided, finally, to dissect the NMLF area, and not single NMLF neurons for the RNA preparation. We collected RNA samples from zebrafish NMLF at 4hrs, 12 hrs and 11d after spinal cord transection at the brain stem-spinal cord junction and used unlesioned zebrafish as control (n=3/group). Each sample contained sections from 3 zebrafish brains. After RNA amplification, the amount of RNA increased dramatically from nano-gram to micro-gram quantities. Real-time PCR for several common neuron-specific genes was performed to check the quality of the amplified RNA. The RNA sample would be used for Affymetrix microarray analysis only if all of the selected genes could be detected with real-time PCR.

When compared with an unrelated study, but using similar methods as we did, regarding the array data (Veldman et al., 2007), we found that the fold changes in our data were not as impressive as those reported in the published study. One possible reason for this might be that the tissue for the laser microdissection was

the total NMLF area with mixed cell types, not single NMLF neurons. RNA from other types of cells in NMLF area, not only the regenerating neurons, might dilute the gene expression changes in the NMLF neurons. Although the fold changes for the genes we found up- or down-regulated were not high, our results of the Affymetrix analysis showed a reliable data quality. On the list of up-regulated genes of 11 days after spinal cord injury (SCI), we found Gap43, L1.1 and L1.2, and their fold changes (11d) to be 2.2, 1.6 and 1.2 respectively (Fig. 1, left). This result is consistent with the previously published data (Becker et al., 1998). Gap43 is a well-known regeneration-associated protein and its fold change is relatively higher. The importance of L1.1 for the regeneration of NMLF neurons has also been demonstrated very clearly (Becker et al., 2004). That the fold change of L1.1 (1.6) is a little higher than that of L1.2 (1.2) is also in agreement with the observation that, of the two homologs, L1.1 is most prominently up-regulated after spinal cord lesion. On the list of up-regulated genes, except those known for axonal regeneration, there are also many new genes whose role in axonal regeneration has not been reported before, for example, C/EBP (Fig. 1, right). Quantitative real-time PCR was performed to confirm the microarray result. From Fig. 1, we can see that the real-time PCR data is consistent with that of the microarray data. The fold changes seen by real-time PCR were even higher (Fig. 1, right).

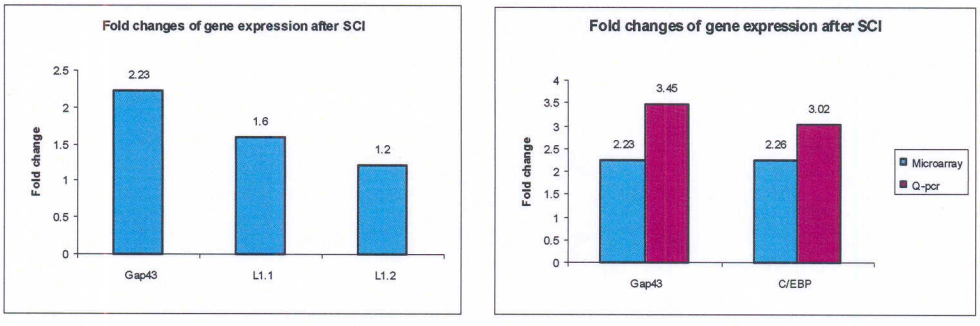

Fig. 1. Fold changes of gene expression after SCI. The fold changes of Gap43, L1.1 and L1.2 are 2.23, 1.6 and 1.2 respectively (left). Quantitative RT-PCR confirmed the results of the array (right).

After evaluating the microarray results, we selected several interesting genes and performed in situ hybridization on the NMLF of lesioned and unlesioned zebrafish brain. The sections incubated with sense RNA probe did not give any signal demonstrating that our antisense probes were specific. The in situ hybridization results showed that most of the genes studied were expressed by NMLF neurons, e.g. C/EBP (Fig. 2). And the expression of C/EBP was significantly up-regulated at 11 days after SCI. The injured fish showed almost 4.5 more C/EBP positive

neurons when compared with the unlesioned fish which did not receive spinal cord transection (Fig. 2). This data confirmed the result from microarray and quantitative real-time PCR.

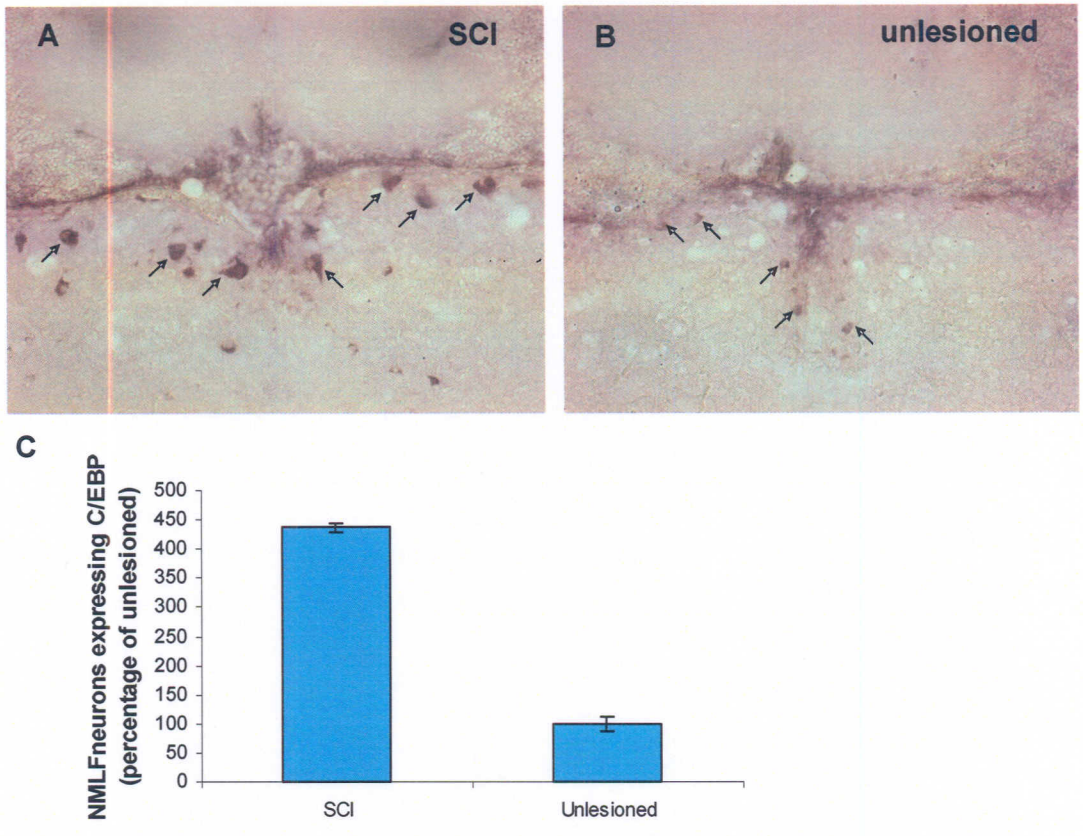

Fig. 2. The expression of C/EBP was significantly upregulated at 11 days after spinal cord injury. More NMLF neurons express C/EBP (arrows in A) at 11 days after spinal cord injury (A) when compared with the unlesioned control (B). The number of positive neurons for C/EBP of injured fish was almost 4.5 times higher than that from unlesioned fish (C).

Anti-sense morpholino molecules can block the translation of the targeted genes, in this way the functions of the targeted gene are explored. In our experiments, the gel-foam containing morpholino solution was placed between the two vertebral stumps after SCI. The transected axons of neurons take up the morpholino molecules and retrogradely transport them to the cell body which locates in brain. If the targeted gene is important for axonal regeneration, after its translation is blocked by morpholino, the axon will not regenerate well. The swimming distance of the moropholino-treated fish 6 weeks after SCI then indicates how axons regenerate. The regenerated neurons can be retrogradely labeled with biocytin which is injected at 4 mm caudal to the first SCI, 24 hours before sacrificing the zebrafish. Our preliminary results of the morpholino experiment showed that the anti-sense morpholino for C/EBP significantly

inhibited spinal cord regeneration. Six weeks after the SCI, fish treated with C/EBP morpholino showed significantly reduced total swimming distance values to maximally only 29% of the values of fish treated with control morpholino which is set to 100% (Fig. 3, left). In animals that had received C/EPB morpholino, the number of retrogradely labeled neurons was also significantly reduced, to only about 23% of the control group set to 100% (Fig. 3, right). Thus, our data indicate that C/EBP plays an important role in the spinal cord regeneration of zebrafish.

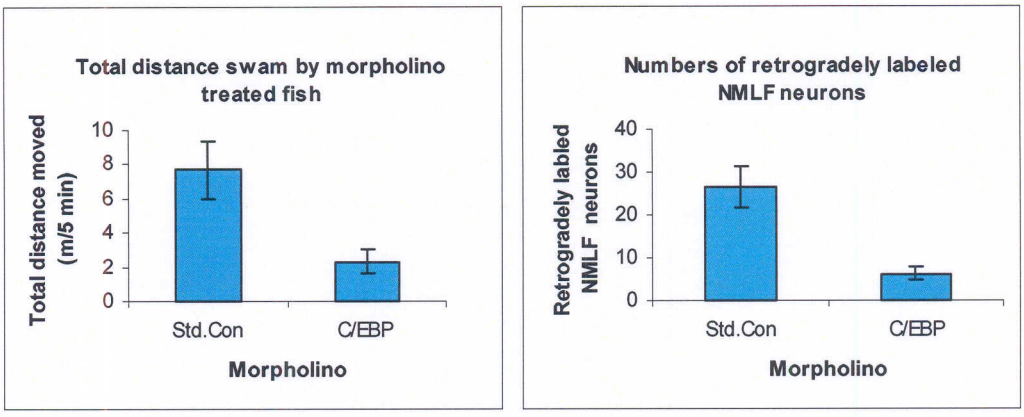

Fig. 3. The anti-sense morpholino for C/EBP significantly inhibits spinal cord regeneration. When compared with fish treated with standard control morpholino, fish that had received C/EBP morpholino showed much less swimming distance (29% of Std. Con, left) and retrogradely labeled neurons (23% of Std. Con, right).

Now, we are still working on seven other genes, the roles of which in spinal cord regeneration are largely unknown. The morpholino experiments for these genes are in progress and solid data about their roles in spinal cord regeneration will be obtained soon. Results from these studies are likely to expand our knowledge of spinal cord regeneration to include many new genes, pointing to novel approaches for future therapy.

3. Project challenges:

After the painstaking work to optimize the snap-freezing method, we tried to isolate RNA and amplify them from microdissected NMLF neurons. About 4 fish are required to get only 50 neurons. But it turned out that it was also impossible to get enough RNA even from about 100 neurons of 8 fish. One reason for this might be that the RNA amount in post-mitotic neurons in adult brains is very low when compared with other kinds of cells and tissues. Also, the RNA amount was too low to be amplified. The extremely low amount of the RNA increased the possibility for the RNA to get lost during the complicated amplification procedures. When unfortunately failed after very hard efforts, then it was decided that the NMLF area, not single neurons, would be microdissected. Even from the NMLF area, three fish were needed to get enough amplified RNA for the microarray analysis.

The second problem was to collect perfect sections. In our experiments, membrane-coated slides were used to get RNA with good quality. To reduce the possibility of RNA degradation, those membrane-coated slides had been kept at –20°, and they needed to be heated by putting hands on the other side of the slide to collect the sections. This increased the difficulties of collecting good sections. Since the best sections for the laser microdissection should include all of the sections containing NMLF, it was not so easy to get a complete set of serial sections and collect all NMLF containing sections with a hand-heated slide. If only one of the sections was missed, the tissue source for the RNA preparation would not be reliable. In order to get reproducible microarray results, we decided only to use the tissues containing all well preserved NMLF sections, in this way the variation between samples were reduced as much as possible. But it was not easy to get perfect sections every time, generally, at least 6 fish were needed to get good sections from 3 fish. Than means, if three samples for the SCI and unlesioned control (each from 3 individual fish) at each time point (4 hrs, 12 hrs and 11 d) were needed, we used at least 100 fish for the RNA preparation.

The last problem, also the biggest one, was that the fish we got from the vendors were not always reliable. Sometimes, the fish were too young, sometimes too old. Over longer stretches of sometimes months, the fish did not survive the spinal cord injury for unknown reasons. We also tried to find other fish vendors, but it turned out others were even less reliable than the original one. We also tried to get fish from Dr. Stephen Moorman at UMDNJ, but the numbers of fish they could provide was very limited. Till now, we still suffer from this problem, and we plan to breed our own fish by buying expensive breeding racks. This problem with the vendors was not at all anticipated by the PI from her experience with vendors in Germany which were highly reliable. The PI had experience with two vendors whom she used when one vendor did not have sufficient numbers of fish, the other was used.

4. Implications for future research:

With this zebrafish spinal cord injury platform, many candidates which are promising for spinal cord regeneration were discovered. Thus, our work provides good candidates which might improve recovery when expressed in the mouse, and possibly even human, injured spinal cord and other parts of an injured central nervous system.

Among the genes we chose from the microarray results, many of them are novel genes for spinal cord regeneration. The discovery of the roles of these genes in spinal cord regeneration found in zebrafish and then in mouse will improve research in the field of spinal cord injury. Some identified genes are unknown in their functions. We expect that our work will greatly increase our knowledge of

genes positively involved in spinal cord regeneration.

5. Plans to continue this research:

After finishing the studies in zebrafish, the next step of this work will focus on the mouse. The first question would be, whether the mouse analog of a gene of interest can promote neurite outgrowth and/or neuronal survival. This will be investigated first with mouse cell lines and primary cultured neurons. Secondly, the functions of these identified genes will be explored in vitro by using assays which cannot be specified at present because they depend on the specific functions of these genes. Thirdly, the effect of these genes on mouse spinal cord injury will be examined by addition of the recombinant molecules to the animal via Alzet pumps.

6. List of publications emerging from this research:
None.

References Cited:

Veldman MB, Bemben MA, Thompson RC, Goldman D. (2007) Gene expression analysis of zebrafish retinal ganglion cells during optic nerve regeneration identifies KLF6a and KLF7a as important regulators of axon regeneration. Dev Biol. 312:596-612

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 24:7837-42.

Becker T, Bernhardt RR, Reinhard E, Wullimann MF, Tongiorgi E, Schachner M. (1998) Readiness of zebrafish brain neurons to regenerate a spinal axon correlates with differential expression of specific cell recognition molecules. J Neurosci 18:5789-803.