

Author



The Construction of *GFP* Fusion Genes For Transgenically Labeled Glial Cells in Zebrafish

Christine Vo

Biological Sciences

Christine has taken advantage of many on-campus resources that support undergraduate research at UCI. She first got involved in research in 2000, as part of the Bridge to Biomedical Research Program. In the following academic year, Christine began her own project, for which she received a UROP grant. She presented her findings at the 2002 UCI Undergraduate Research Symposium and for the School of Biological Sciences' Excellence in Research Program. She hopes to continue her research in an M.D./Ph.D. program and one day practice medicine. Christine is active on campus as a member of the American Medical Student Association, and off campus as a volunteer in the emergency room of Garden Grove Hospital.

Abstract

The olfactory bulb is the brain region responsible for the sense of smell. Previous studies suggest that glial cells play an important role in the formation and patterning of the olfactory bulb. This project was conducted to further investigate the roles and behaviors of glial cells in olfactory bulb formation. Zebrafish were used due to their transparent anatomy and rapid rate of development. The primary experimental approach was to construct fusion genes to transgenically label glial cells with green fluorescent protein (GFP). To accomplish this, the promoter region of the glial fibrillary acidic protein (*GFAP*) gene, which encodes a protein specifically expressed in glial cells, was isolated by screening a zebrafish genomic DNA library via polymerase chain reaction (PCR). Subsequent restriction mapping and Southern blot hybridization were used to localize the promoter region of *GFAP* within the PCR-positive clones. A *GFP* fusion gene was constructed from a vector containing the *GFP* gene and another vector containing a polylinker and polyadenylation site. In subsequent experiments, the fusion gene construct will be injected into zebrafish embryos and screened for GFP expression. The movement of the labeled glial cells during olfactory formation will then be visualized in living embryos under the confocal microscope. These future studies will serve to further elucidate the involvement of glial cells in olfactory bulb development.

Faculty Mentor



Two types of cells populate the nervous system: neurons and glial cells. In the traditional view, glial cells are primarily supporting cells that function in simple ways to support the neuronal signaling underlying nervous system function. Recent studies have revealed unexpectedly direct and complex interactions between glial cells and neurons, however, and indicate that glial cells may undergo profound changes in structure in the course of their normal activity.

Christine Vo's work enables this class of cells to be imaged and studied at the single cell level in living organisms. Her work sets the stage for experiments now beginning to study glial patterning in the central nervous system of the zebrafish. Christine's example shows how intellectual curiosity, hard work, and a commitment by UCI undergraduates can help establish new research directions for the laboratory in which they work.

Key Terms

- ◆ Fusion Genes
- ◆ Glial Fibrillary Acidic Protein (*GFAP*) Gene
- ◆ Glomerulus/Glomeruli
- ◆ Green Fluorescent Protein (*GFP*)
- ◆ Olfactory Bulb

Oswald Steward

College of Medicine

Introduction

The sense of smell is mediated by a region of the brain called the olfactory bulb. This structure possesses hundreds of processing units, called glomeruli, arrayed on its surface. These spherical structures contain the sites of synaptic contact between sensory neurons from the nose and projection neurons connecting to higher levels of the brain. In addition, there is a layer of glial cells that separates glomeruli from each other. During olfactory function, each glomerulus responds to a specific set of odorants. The position of glomeruli that respond to the same set of odorants is conserved between different individuals (Mombaerts et al., 1996). Ultimately, the brain relies on this stable pattern of glomeruli in order to generate olfactory perceptions. In order to study the formation of the olfactory bulb, it is important to understand how glomeruli are formed and arranged in the correct spatial pattern.

Previous studies indicate that glial cells participate in the formation of the olfactory bulb, and more specifically are required for glomerular development (Tolbert et al., 1989). In insects, removal of glial cells by irradiation causes immature glomeruli to stop development and rapidly disassemble. Circumstantial evidence suggests that glial cells may also be required for very early stages of insect glomerular development. In rodents, the possible initiating step in glomerular formation may occur when glial cells accompany sensory axons as they project to their nascent targets. Moreover, some populations of glial cells change shape and orientation near the future sites of glomerular formation (Valverde et al., 1992). Other experiments by Bulfone *et al.* (1998) suggest that glial cells may actively participate in the patterning of glomeruli across the surface of the bulb. The mutational removal of two of the principal cell types of the olfactory bulb, projection neurons (also known as mitral cells) and inhibitory neurons, clearly showed that these olfactory bulb cell types are not involved in glomerular patterning. As these cells do not contribute to glomerular patterning, the remaining cell types must instead contribute spatial information for the patterning of the bulb. Taken together, these studies strongly support a model of olfactory development in which glial cells are the most likely candidates for contributing essential spatial information used to construct the stereotyped glomerular map.

The hypothesis of this project is that glial cells not only contribute to the formation of the olfactory bulb in general, but also actively participate in the patterning of the intricate sensory map contained within the bulb. The zebrafish embryo is a suitable system in which to study these pro-

cesses due to its transparent anatomy and fast rate of development. In addition, the stereotyped olfactory sensory map can be imaged in living zebrafish during embryonic development. Using zebrafish, this hypothesis will be addressed by visualizing the movement of glial cells during the patterning of the olfactory bulb, and ultimately by specifically manipulating glial cells in living embryos. The experimental approach was to transgenically label glial cells with green fluorescent protein (GFP). The first step in this approach was to isolate the promoter from glial fibrillary acidic protein (*GFAP*) gene that encodes a protein specifically expressed in glial cells. *GFAP* is typically expressed in astrocytes, a glial cell subtype, and has been previously cloned from zebrafish (Dynes, unpublished data). This promoter region was fused to the *GFP* gene's open reading frame and the resulting construct will be used for generating transgenic zebrafish.

Methods and Materials

P1 Artificial Chromosome Library Organization and PCR Screening

The P1 Artificial Chromosome (PAC) library (obtained from C. Amemiya) was constructed with *Sau* 3A partially-digested zebrafish genomic DNA (average insert fragment size of 115 kb) that was cloned into the PAC vector pCYPAC 6. The library contains 271 pools of clones with 384 clones in each pool. The pools were arrayed in a matrix, and those pools lying along each row and column were mixed to form individual superpools (17 horizontal and 16 vertical superpools).

Basic molecular biology techniques were performed according to Maniatis *et al.* (1982). Four different primers, PCR 1-PCR 4, were designed to bind to the *GFAP* 5' untranslated-region (UTR). *GFAP* sequences were amplified via Polymerase Chain Reaction (PCR) using a Hybrid Omnigene PCR machine with the designed primers and FastStartTaq polymerase (Roche). Library fractions were amplified using a 55 °C annealing temperature and either 35 or 39 cycles. Fragments amplified from the PAC library were size-separated using agarose gel electrophoresis and compared to fragments amplified from genomic DNA (positive control) and 1 kb ladder (Life Technologies). PAC library pool DNA for PCR screening was isolated using an Eppendorf DNA preparation kit. After positive clones were identified, filters for hybridization were custom-spotted using a programmable spotter. The filters were hybridized, washed at high stringency, and exposed as detailed below.

Restriction Mapping and Southern Blot Hybridization

Restriction digests of positive PAC DNA were size-separated on an agarose gel and blotted onto a nylon membrane. The GFAP 5' UTR hybridization probe was isolated by PCR and radioactively labeled with ^{32}P -dCTP using the random hexamer method (TaKaRa). Next, the labeled probe and hybridization solutions (formamide and 3x SSC) were added to the membranes for hybridization at 37 °C. After overnight hybridization, filters were washed at 37 °C at high stringency and the hybridized fragments were detected using a phosphorimager (Molecular Dynamics).

Construction of Promoter-testing Vector CV-1 and GFAP-GFP Fusion Gene

Vector JLD 111 (Dynes, unpublished) contains the EF-1a promoter, IRES region, unc-76, *EGFP* gene, and Ampicillin resistance marker. Vector pEGFP-NI (CloneTech) contains the *EGFP* gene and a multiple cloning site. These vectors were cut with Xho I and Not I restriction enzymes, and the generated fragments were purified and ligated together using TaKaRa ligation kit. The ligation mixture was transformed into the *Escherichia coli* strain DH5a, and the construct was termed CV-1. To clone a promoter fragment into this vector, CV-1 was cut with Nco I and Sac I. A 10 kb fragment of the PAC positive clone 239-9P was generated by cutting with Nco I. The digested fragment was gel-purified and further digested with Sac I. The *GFP* gene was ligated to the *GFAP* promoter sequence from the PAC and transformed as above, resulting in the clone 2.0 GFAP-CV1.

In situ Hybridization

Solutions and reagents for *in situ* hybridization were prepared using Schilling's laboratory protocol (personal communication). Zebrafish embryos at 36 hr post-fertilization were fixed in 4% paraformaldehyde overnight at 4 °C. The embryos were dehydrated in methanol, re-hydrated, and hybridized with an RNA probe. The RNA probe was made by *in vitro* transcription using digoxigenin-labeled nucleotides. The detection system contained digoxigenin antibody coupled to alkaline phosphatase, 4-nitroblue tetrazolium chloride (NBT), and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). The stained embryos were then visualized for GFAP expression using bright field and Nomarski microscopy.

Results

Screening the PAC Library Using PCR and Southern Blot Hybridization

Polymerase chain reaction (PCR) was used to screen the library for positive pools containing the *GFAP* promoter

gene sequence. Initially, the superpools were screened in order to identify potential positive pools. Once positive pools were identified, they were screened by hybridization to identify the individual positive clones. For these experiments, PCR amplification of zebrafish genomic DNA was used for the positive control, and PCR amplification of a mixture without DNA was used for the negative control.

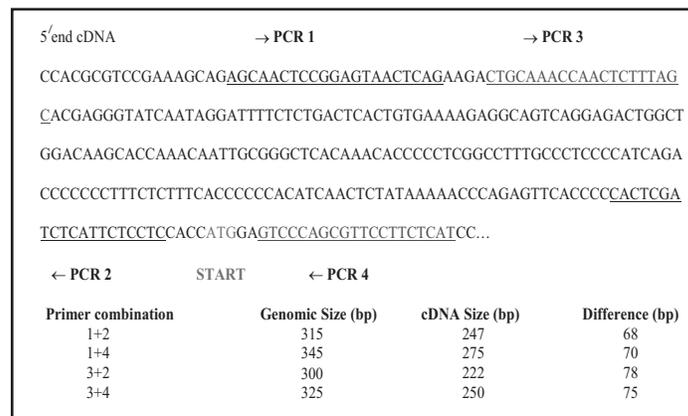


Figure 1
The GFAP 5' UTR primer binding sites and the expected sizes of the amplified fragments. The genomic sizes are derived from the agarose gels in Figures 2 and 3.

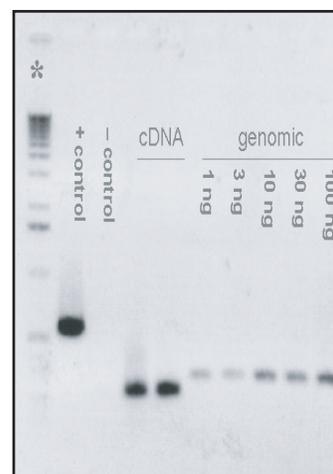


Figure 2
(The following agarose gel pictures are in the inverted color format to make them more legible.) Comparison of PCR-amplified fragments from cDNA and genomic DNA templates, and titration of genomic template DNA. Positive control is from the promoter region of a zebrafish odorant receptor. Asterisk (*) indicates a 1 kb ladder (Life Technologies).



Figure 3
PCR-amplification of genomic DNA with four different primer combinations (PCR 1-PCR 4). Asterisk (*) indicates a 1 kb ladder (Life Technologies).

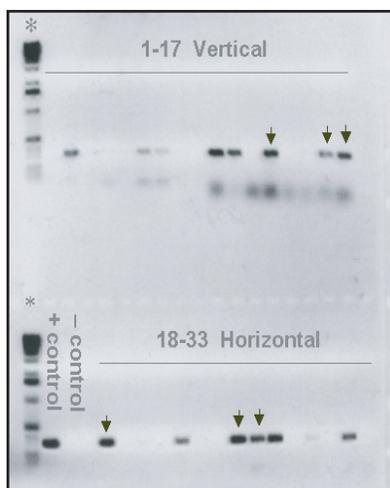


Figure 4
PCR screening of the zebrafish genomic PAC superpools: 1-17 vertical superpools and 18-33 horizontal superpools. Arrows indicate those positive superpools that were ultimately confirmed by hybridization. Asterisk (*) indicates a 1 kb ladder (Life Technologies).

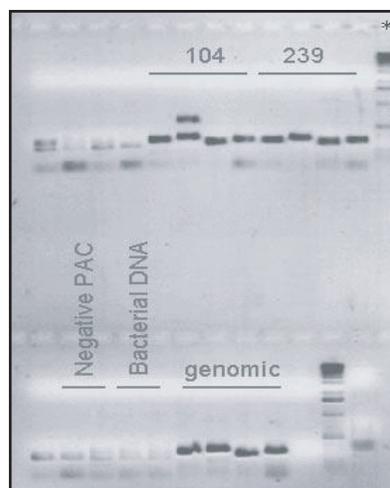


Figure 6
PCR screening of positive pools 104 and 239 with different primer combinations. Genomic DNA, bacterial DNA and negative PAC DNA are used as controls. Asterisk (*) indicates a 1 kb ladder (Life Technologies).

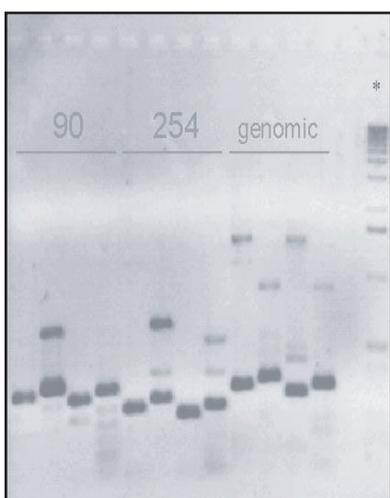


Figure 5
PCR screening of positive pools with different primer combinations. Positive pools are numbered (90, 254). Asterisk (*) indicates a 1 kb ladder (Life Technologies).

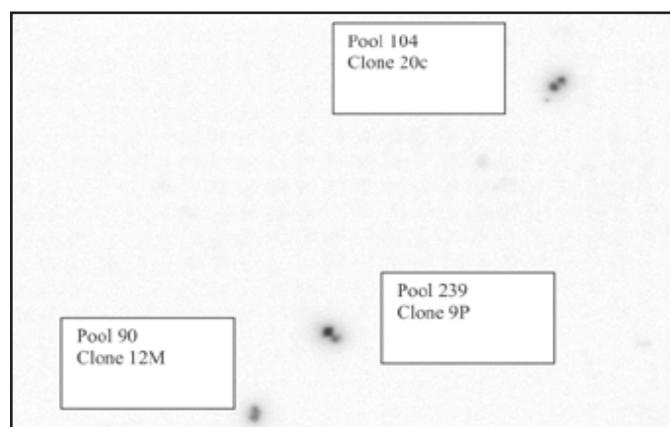


Figure 7
Radioactive hybridization of arrayed clones from positive pools 90, 104 and 239.

All four pair-wise combinations of primers (PCR 1-PCR 4) were then used to amplify the 5' UTR region of GFAP. These primer combinations amplified fragments that were about 70 bp larger than predicted from the cDNA sequence. This finding was consistent with the presence of a 70 bp intron in the 5' UTR (Figures 2 and 3). Using primers PCR 1 and PCR 2, 8 positive superpools (2, 5, 7, 8, 16, 23, 24, 32) were identified (Figure 4).

The four potential positive pools (90, 104, 239, and 254) were identified in subsequent PCR screenings using different primer combinations (Figures 5 and 6). Individual clones from these positive pools, 384 clones per pool, were spotted in pairs onto a filter and screened using radioactive hybridization.

The film exposure showed pairs of spots that were used to identify positive clones (Figure 7). The three identified positive clones were 90-12M, 104-20C, and 239-9P. Cloned 239-9P was randomly selected for further study.

Using restriction mapping and Southern blot hybridization, the proximal portion of the promoter region in the positive PAC clone 239-9P was identified (Figure 8). A GFAP 5' UTR probe hybridized to a 10 kb Nco I and a 2 kb Nco I - Sac I fragment. These fragments were chosen for sub-cloning and testing because they were small enough to be cloned easily, yet large enough to potentially contain the entire set of *cis*-acting regulatory sequences. In addition, by summing the sizes of fragments generated by restriction enzyme digestion, the genomic DNA insert size was approximated as 75 kb. Analysis of Nco I - Not I double digest identified the fragments at the ends of the genomic DNA insert. These fragments are termed junction fragments and are 6 and 3.5 kb in size.

Two plasmids were made for testing promoter activity in the GFAP-hybridizing restriction fragments. The first vector construct was CV-1, which is composed of Xho I - Not I fragment of vectors JLD 111 (containing the polylinker and polyadenylation sites) and pEGFP-NI (containing the GFP

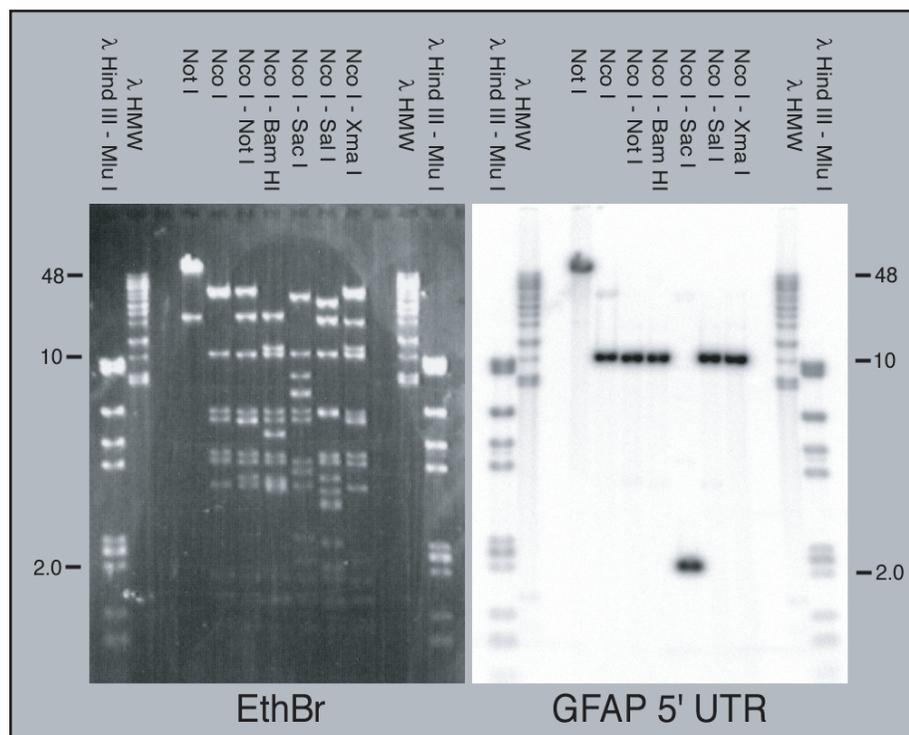


Figure 8
Restriction Mapping and Southern blot hybridization of the PAC positive clone 239-9P

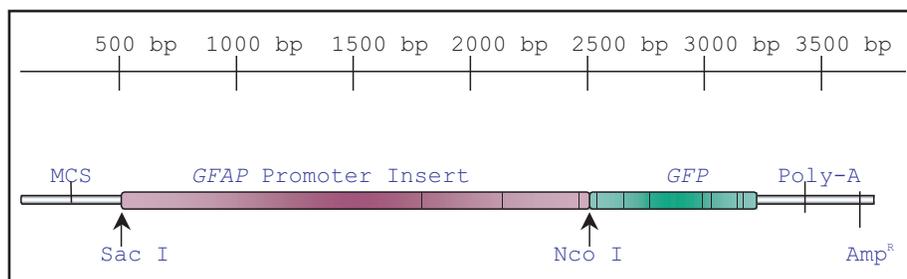


Figure 9
The GFP-GFAP fusion genes construct.

gene region). The 2.0 kb Nco I - Sac I fragment was fused to EGFP in CV-1 at the start codon, which is overlapped by an Nco I site. This clone, 2.0 GFAP-CV1, will be injected into zebrafish blastomere in future experiments to test for promoter activity.

Parallel to the GFP fusion gene construction, *in situ* hybridization was performed to visualize the protein expression of GFAP mRNA by using an RNA probe. The stained embryos were visualized for GFAP expression using bright field and Nomarski microscopy. GFAP expression was robust in the developing forebrain, midbrain and hindbrain regions, as well as in the spinal cord when compared to the negative control embryos.

Discussion and Conclusions

The initial approach of this project was to localize and isolate the promoter region of the *GFAP* gene from a PAC library. In these experiments, we saw a high level of background amplification, potentially due to contamination, but ultimately positive pools and superpools could be identified. In retrospect, it appears that there was a contamination of one of the PCR reagents. The size of the contaminating fragment was consistent with the amplification using the cDNA as a template. In order to avoid this problem, four different combinations of primers were used in the subsequent screening processes. Based on the size difference between the fragments amplified for cDNA and genomic DNA, we concluded that there was an intron with an approximate size of 70 bp in the genomic 5' UTR of *GFAP*. Ultimately, four potential positive pools were identified, but pool 254 was excluded from subsequent processing due to an error in identifying the pool number. The identified positive pools and clones potentially contain the promoter of the *GFAP* gene.

Restriction mappings and Southern blot hybridization indicated that the *GFAP* promoter is at least partially contained within the 10 kb Nco I fragment. Because additional junction fragments of

6 and 3.5 kb lie between the 10 kb Nco I fragment and the PAC vector the size of the promoter fragment contained in clone 239-9P is 13.5 kb at minimum. Fragments in the 2-13 kb size range are expected to be large enough to contain all necessary *cis*-acting DNA sequences to recapitulate *GFAP* gene expression. This expectation is based on the finding that a 2.2 kb 5'-flanking sequence from the human *GFAP* gene was sufficient to direct astrocyte-specific expression in transgenic mice (Brenner et al., 1994). In addition, the construction of GFP fusion genes (Figure 9) was simplified by the finding of an overlapping Nco I site at the start codon of both GFP and *GFAP*. Currently, zebrafish embryos are being injected with 2.0 GFAP-CV1 vector in order to determine whether the 2.0 kb Nco I - Sac I fragment is able to recapitulate the *GFAP* expression pattern.



Figure 10
In situ hybridization of embryos probed with antisense GFAP probe

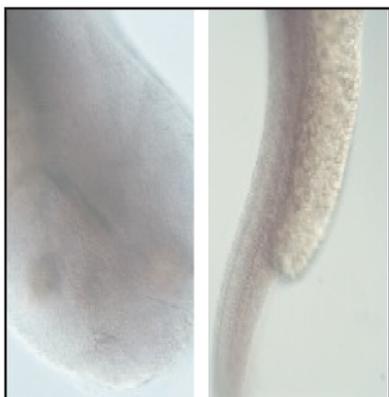


Figure 11
In situ hybridization of control embryos probed with sense GFAP probe

In parallel experiments using *in situ* hybridization, GFAP expression was shown to be robust in the embryos hybridized with GFAP antisense probe (Figure 10) in contrast to the control embryos hybridized with GFAP sense probe (Figure 11). As the isolation of the *GFAP* gene from zebrafish has not been published, the GFAP expression that was visualized in these experiments via *in situ* hybridization could not be compared to other published data. However, similar results were found by using antibodies against the GFAP protein (Marcus and Easter, 1995). In this study, the first immunopositive cells in the central nervous system were identified in the embryo hindbrain at 24 hr post-fertilization. Furthermore, initial patterning events occur in zebrafish between 36 and 48 hr (Dynes and Ngai, 1998). Therefore, it was expected that 36-hr embryos would strongly express GFAP throughout the central nervous system. The results from using GFAP antibodies suggest that glial cells contribute to the development and organization of the central nervous system by supporting early axon outgrowth and by forming the boundaries around the tracts and between neuromeres. It should be noted that these general findings mirror the hypothesis of glial cell function in olfactory development. The expression pattern of our cloned GFAP promoter is expected to be the same as the expression pattern detected using *in situ* hybridization. The main advantage of the fusion gene approach is that the

movement of glial cells can be visualized in living embryos, which is impossible when using *in situ* hybridization.

Finally, the construction of fusion genes that express GFP in glial cells will be useful for studying glial cell function throughout the nervous system. This project has focused on the olfactory system, as it has been particularly useful in imaging approaches to studying neural development and function. However, this project indirectly benefits the study of spinal cord injury as well. Because GFAP expression is also found along the spinal cord, glial cells might be important for the study of cell regeneration after injury.

Many potential experiments can be performed in the future with the functional GFAP-GFP fusion gene construct. Individual GFAP and glial cells can be studied using transgenic manipulation, ablation, or molecular genetic approaches. With a better understanding of glial cells and the mechanism of their regeneration, it may be possible to find improved treatments for conditions such as spinal cord injury, which involve massive cellular damage.

Acknowledgements

I would like to thank Professor Oswald Steward for allowing me to pursue this project. I deeply appreciate all the guidance and support of postdoctoral scientist Joseph Dynes throughout my research experience. Also, thanks to Professor Thomas Schilling, Chris Amemiya, Edward Modafieri, and Kelli Sharp for all their help in the laboratory. This project was supported by the Summer Undergraduate Research Program at UCI.

Works Cited

- Brenner, M.K., W.C. Kisseberth, Y. Su, F. Besnard, and A. Messing. "GFAP Promoter Directs Astrocytes-Specific Expression in Transgenic Mice." Neuroscience 14 (1994): 1030-1037.
- Bulfone, A., F. Wang, R. Hevner, S. Anderson, T. Cutforth, S. Chen, J. Meneses, R. Pedersen, R. Axel, and J.L.R. Rubenstein. "An Olfactory Sensory Map Develops in the Absence of Normal Projection Neurons or GABAergic Interneurons." Neuron 21 (1998): 1273-1282.
- Dynes, J., and J. Ngai. "Pathfinding of Olfactory Neuron Axons to Stereotyped Glomerular Targets Revealed by Dynamic Imaging in Living Zebrafish Embryos." Neuron 20 (1998): 1081-1091.
- Marcus, R.C., and S.S. Easter Jr. "Expression of Glial Fibrillary Acidic Protein and its Relation to Tract Formation in Embryonic Zebrafish (*Danio rerio*)." Journal of Comparative Neurology 359 (1995): 365-381.
- Maniatis, T., E.F. Fritsch, and J. Sambrook. Molecular Cloning—A Laboratory Manual. New York: Cold Spring Harbor Laboratory, 1982. 107-217.
- Mombaerts, P., F. Wang, C. Dulac, S.K. Chao, A. Nemes, J.E. Mendelsohn, and R. Axel. "Visualizing an Olfactory Sensory Map." Cell 87 (1996): 675-686.
- Tolbert, L.P., and L.A. Oland. "A Role for Glia in Development of Organized Neuropilar Structures." Trends in Neurosciences 12 (1989): 70-75.
- Valverde, F., M. Santacana, and M. Heredia. "Formation of an Olfactory Glomerulus: Morphological Aspects of Development and Organization." Neuroscience 49 (1992): 255-275.

