

EXPRESSION OF *C-JUN* FOLLOWING OPTIC NERVE INJURY
IN *DANIO RERIO*

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**EXPRESSION OF *C-JUN* FOLLOWING OPTIC
NERVE INJURY IN *DANIO RERIO***

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This thesis is dedicated to my mother for her sacrifice and dedication in providing me with the opportunities she never had.

ABSTRACT

Unlike mammals, fish and amphibians exhibit remarkable regenerative properties of the central nervous system. This study focused on the changes in *c-jun* expression consequent to optic nerve injury in *Danio rerio* (zebrafish). Known for its function in regulating transcription, *c-jun* binds with other transcriptional regulators to affect nerve regeneration and cell death in ways that seem paradoxical. I sought to determine if *c-jun* was differentially expressed in the retina following optic nerve injury as compared to fish which had undergone a similar operation but whose optic nerve had not been injured (sham-operated). Based on previous studies, I hypothesized that expression of *c-jun* in the retina would significantly increase in fish that suffered injury to the optic nerve compared to those that only underwent collateral tissue damage. Following RNA extraction and cDNA synthesis with reverse transcription polymerase chain reaction (RT-PCR), quantification of *c-jun* expression was determined by quantitative real time-PCR (qRT-PCR). Expression levels were normalized to expression of a reference gene (*β -actin*), yielding relative expression levels. A two way analysis of variance was carried out in order to determine whether there were significant differences in gene expression 3 hours, 24 hours and 168 hours after injury between sham-operated and optic nerve injured fish. Significant differences ($p < 0.05$) between sham-operated and optic nerve injured retinas were observed at 24 hours and at 168 hours. The significant differences in expression of *c-jun* corroborated and extended past studies (Veldman et al., 2007, Herdegen et al., 1993). Furthermore, my findings raised the possibility that *c-jun* expression may be important for optic nerve regeneration. If the ability of fish to regenerate optic nerve has a genetic basis, then one may be able conduct gene therapy treatment in glaucoma patients by identifying the genes that are differentially expressed following optic nerve injury.

INTRODUCTION

Amphibians and fish regenerate nerves after injury to the central nervous system (CNS) whereas mammals lack the ability to do so. The ability of fish and amphibians to restore the nerves of the CNS after injury has led to numerous studies of the regeneration of the optic nerve, which is an accessible part of the CNS (Broude et al., 1997; Sperry, 1944; Veldman et al., 2007). The events associated with optic nerve regeneration are controlled by specific gene products encoded in the fish and amphibian genome. The regeneration process involves changes in the complement of genes that are transcribed, which in turn reflects changes in the expression of the protein factors that regulate transcription (Petrausch et al., 2000).

The CNS includes the brain and the spinal cord whereas the peripheral nervous system (PNS) involves the rest of the nerves that connect the CNS to organs and muscles. Unlike the CNS, the PNS is not protected by bone or the blood brain barrier and thus, is more prone to injury (Maton et al., 1993); therefore, it not surprising that the PNS has regenerative properties. However, injuries to the CNS have distinct and typically irreversible effects such as paralysis from spinal cord injury and blindness from optic nerve injury. The cells unique to the CNS are responsible for its inability to regenerate. Oligodendrocytes are glial cells which myelinate axons in the vertebrate central nervous system. In mammals they are thought to inhibit axonal growth. However, in fish, they are known to contribute to the elongation of retinal axons (Stuermer et al., 1992). Astrocytes are another type of macroglial cell in the CNS thought to be involved in the inhibition of optic nerve regeneration. These cells undergo chemical and morphological changes responsible for a cascade of events that lead to the formation of the glial scar in mammals (Stichel & Muller, 1998). Fish however,

do not form a glial scar, implying that their astrocytes do not undergo astrogliosis, the process that occurs when astrocytes become activated in response to injury.

The mammalian CNS is inhibited from regenerating by the cells that myelinate the central nervous system, by glial scarring which inhibits the use of a portion of the nerve, and by the cascade of events induced by gene expression, which is the aspect of particular interest to me (Broude et al., 2007; Bandtlow & Schwab, 2000). In contrast, the peripheral nervous system shows morphological and metabolic changes when injured, changes which are absent prior to injury (Broude et al., 2007). Furthermore, a study by Bandtlow & Schwab (2000) seems to indicate that CNS neurons regenerate when grown in PNS tissue, but PNS neurons do not regenerate when grown in CNS tissue. This finding hints that the tissue type and environment of the CNS is responsible for lack of neuronal regeneration.

The decision to injure and monitor the regeneration of the optic nerve was made because, like nerves in the peripheral nervous system, it is easily accessible, but it is part of the central nervous system. The eye is a model organ for studying the effects of central nervous system regeneration due to the properties of the retina. The decision to take RNA samples from the retinal portion of the eye was made because it is where retinal ganglion cells (RGC) are found. These cells are the source of axons that form the neuronal portion of the optic nerve and represent the site where transcription related to the re-growth of axons occurs. Furthermore, Bernhardt (1999) demonstrated that if RGCs' regeneration enables the optic pathway to be restored. Also, fish demonstrate growth associated cell surface molecules stemming from the retinal ganglion cell portion of the eye; these cell surface molecules are thought to have a direct connection to axonal re-growth and guidance of axons back to the

optic tectum (Stuermer et al., 2004). More specifically, fish and amphibians can regenerate the axons connected to the retina and replace damaged retinal cells (Cameron et al., 2005). Other researchers have demonstrated that axonal regeneration is involved in the restoration of the retina (Cameron et al., 2005) and that a specific type of intermediate filament protein (plasticin) is present in both newly generated retinal ganglion cells and post optic nerve injury (Asch et al., 1998; Glasgow et al. 1992) provides further evidence for relating the axons' re-growth to the selection of retina as my tissue of interest

It has been suggested that the factors involved in the inhibition of regeneration in mammals are related to the collection of genes expressed in the neuronal cell body or proteins that act as physical barriers to the regeneration process or by the interaction of both (Plunet et al., 2002). In mammals, regeneration associated genes (RAGs) exhibit significant up regulation when peripheral axons are severed. However, if there is damage to central axons, these genes are not up regulated (Broude et al., 1993). Fernandes and Tetzlaff (2000) demonstrated that these genes code for proteins that are transcription factors, ion channels and regulatory proteins (proteins that bind to specific regulatory sequences of DNA in order to switch genes on and off and regulate the transcription of genes) involved in peripheral nerve regeneration, and the absence of these proteins is responsible for the lack of regenerative properties in the central nervous system.

A transcription factor is a protein that binds to specific regions of DNA in order to regulate gene expression. This experiment focused specifically on the AP-1 transcription

factor, which is composed of proteins that belong to the c-Jun¹, c-Fos, ATF, and CREB families (Raivich et al., 2004); *c-jun* was selected for this particular experiment because it is found to be upregulated following nerve injury in both mammals and fish (Cameron et al., 2005; Raivich et al., 2004; Veldman et al., 2007). According to Raivich et al. (2004), *c-jun* is notably expressed after injury to the central nervous system as part of the response to damage. Furthermore, Raivich et al. (2004) demonstrated paradoxically that *c-jun* is expressed both as axons regenerate and as neurons undergo apoptosis. *c-jun* is a transcription factor involved in the regulation of a large set of genes (Hai and Hartman, 2001), and it showed significant increases in expression based on microarray analysis in retinal ganglion cells taken from zebrafish three days after optic nerve injury (Veldman et al., 2007). In mammals, *c-Jun* is a gene that is part of the subfamily Jun, which is part of the Fos/Jun family; this family is known to have certain similarities with the ATF/CREB family (Hai & Curran, 1991). Both the Fos/Jun family and the ATF/CREB family are known to be part of the response system during optic nerve injury. This observation strikes another area of interest with the genes contained in the two families. Furthermore, *c-jun* is known to be expressed as a response to neurodegenerative diseases in both the CNS and the PNS. The paradoxical nature of *c-jun* attracted my attention for experimentation in hopes of learning more about its expression and localization.

This study focused specifically on quantifying the expression of *c-jun* during different time intervals following optic nerve injury of zebrafish (*Danio rerio*) using qRT-PCR and comparing it to quantification of *c-jun* caused by damage to the muscle tissue around the eye

¹ Zebrafish protein names are written in Roman text with a capital letter, whereas gene names are italicized and all letters are lower case. Human protein and gene names are capitalized and in Roman text. (http://zfin.org/zf_info/nomen.html)

(sham-operated). In addition, this study focused on the effect on gene expression created by laceration of the muscle tissue surrounding the eye. In order to isolate the gene expression specific to injury as opposed to tissue damage I compared retinal RNA from sham operated (SO) fish to optic nerve injured (ONI) fish.

MATERIALS AND METHODS

Zebrafish Maintenance

Wild-type zebrafish (ZDR) were purchased from Aquatica Tropicals (Plant City, FL) and maintained at a 12 hour light/ 12 hour dark cycle for at least two weeks prior to use for experimentation. Use of animals was approved according to the Texas State University IACUC (protocol approvals # 0703-0122-07)

Experimental Design

This experiment dealt specifically with the comparison between zebrafish that were sham-operated and those whose optic nerve had been injured. The purpose was to compare gene expression of *c-jun* when only the outside muscles of the eye are severed to when the optic nerve has been severed as well.

Injury

Zebrafish injury and dissection were performed by Katherine Saul as follows: Zebrafish were anaesthetized in 0.2% tricaine methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, WA) dissolved in aquarium water. After fish became disoriented, they were placed under a dissecting scope. The muscles around the left eye were cut, the left eye's optic nerve was severed (90%) and the eye was placed back into the socket (see Figure 1). The right eye was treated similarly but did not receive damage to the optic nerve (sham-operated). The fish were placed back in their tanks (three fish for 3 hours, three fish for 24 hours and three fish for 168 hours) after which they were sacrificed. After the allotted time period, zebrafish were euthanized in 1% MS222 solution, the eyes were removed, immediately placed in RNAlater® (Ambion; Austin, TX) and the lens, retina, RPE and choroid were dissected for use in RNA extraction.

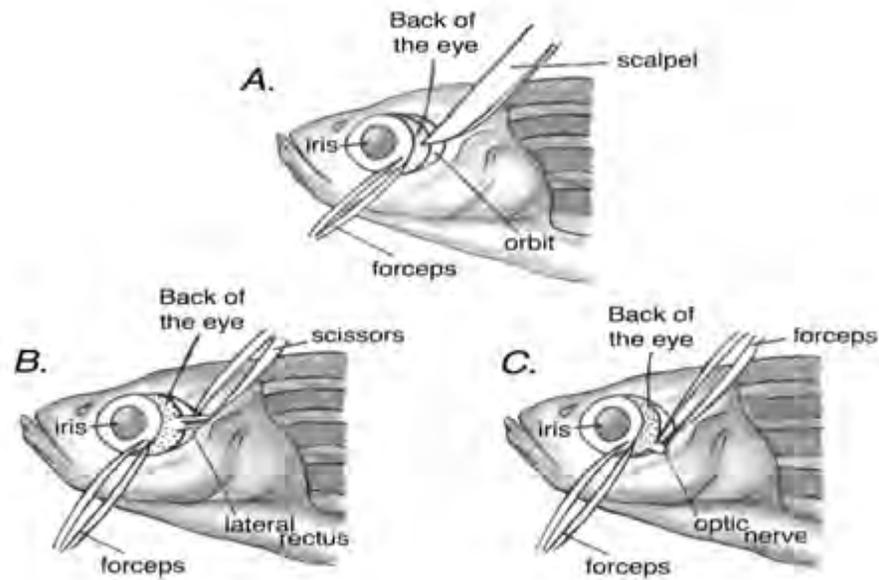


Figure 1. Method of Optic Nerve Injury. (A) Separation of dorsal connective tissue and cutting of the lateral rectus muscle enables to angle the eye rostrally in order to expose the optic nerve. (B) Severing of approximately 90% of the optic nerve with microscissors, care was taken to avoid damaging the ophthalmic artery. (Figure modified from Liu and Londraville, 2003; illustrated by Lauren Bradshaw).

RNA Extraction

RNA extraction was performed by Katherine Saul as follows: Zebrafish retina, RPE and choroid were collected for each time period as biological triplicates and were each placed in 1ml of TRI-Reagent (Ambion; Austin, TX). A 27 gauge needle and syringe were used to triturate tissue. Chloroform (200 μ l) was added and tubes were vigorously shaken by hand to induce precipitation of RNA. The aqueous layer was removed after incubating at room temperature for 15 minutes and placed in a new tube; the organic layer was discarded. Isopropanol (500 μ l) was added to the fresh tube and the contents were incubated for 20 minutes at -20°C . Supernatant was extracted and a small white pellet was left undisturbed at the bottom of the tube. Ethanol (75%) was added, spun at 14,000 rpm in an Eppendorf

tabletop centrifuge for 10 minutes, and supernatant was removed and discarded. In order to eliminate pigments arising from the inclusion of retinal pigment epithelium, RNA was placed in RNeasy spin columns (QIAGEN, Valencia, CA) and a clean-up was conducted by adding water containing diethylpyrocarbonate (DEPC) and buffer RLT (lysis buffer) (QIAGEN, Valencia, CA). Filtrate was discarded and buffer RPE (binding buffer) (QIAGEN, Valencia, CA) was added and spun at 10,000 RPM for 2 minutes, addition of buffer RPE was repeated and the spin was repeated. DEPC water (30 μ l) was added and spun at 10,000 RPM for 1 minute. Filtrate in the tube contained the RNA and the filter was discarded. Quality of RNA was evaluated with a Nanodrop spectrophotometer (ThermoFisher Scientific, Waltham, MA)

Reverse Transcription

Samples of 24 hour and 168 hour ONI and SO RNA were obtained from Katherine Saul and diluted to a concentration of 350 ng to match the concentration of 3 hour cDNA samples also supplied by Saul. Reverse transcription was conducted using MMLV Reverse Transcriptase solution (Promega), random primers, oligo dT primers (Promega), 5X reaction buffer, premixed dNTPs and RNase inhibitor (Promega) at 37 $^{\circ}$ C for 60 minutes – conditions identical to those used by Saul.

Design and Selection of Primers

Primers for *c-jun* were designed based on characteristics that made them suitable for qRT-PCR conditions. Homodimerization, primer length, hair pin loop formation, self dimerization, melting temperature and Δ G values were considered and analyzed using an online oligo-nucleotide calculator (<http://www.idtdna.com/SCITOOLS/scitools.aspx>).

The resulting primers are shown in Table 1.

Table 1. Forward and reverse primers used for PCR.

Gene	Forward	Reverse
<i>c-jun</i>	GGCTCATCATCCAGTCTAGCAACG	TCCTGCTCATCCGTCACGTTTC
β -actin	ATCAGCATGGCTTCTGCTCT	GTGAGGAGGGCAAAGTGGTA

Quantitative Real Time PCR

Using a SYBR® Green ER Two-Step qRT-PCR Universal Kit (Invitrogen, Carlsbad, CA) on an Eppendorf Realplex² Mastercycler (Hamburg, Germany), qRT-PCR was conducted. The cycling program followed was: 50 °C for 2 min, 95 °C for 10 minutes, 40 cycles of 3 steps (95 °C for 15 seconds, 60 °C for 15 seconds, 68 °C for 15 seconds), terminated with a melt curve to determine product homogeneity. As mentioned before, samples were collected in triplicate for each time point, with each observation representing RNA collected from a single eye. Additionally, technical triplicates were also performed. Triplicate samples for analysis of *c-jun* expression and duplicate samples for analysis of β -actin expression. The analysis of qRT-PCR results followed the Livak and Schmitt (2001) method of $2^{-\Delta\Delta C_t}$ β -actin was used as reference gene and *c-jun* was our gene of interest; this procedure allowed us to calculate a fold change in expression. A two-way independent ANOVA was conducted followed by post-hoc tests using StatPlus (Analysis Soft. <http://www.analysisoft.com/en/>) in order to determine significance of differences among categories.

RESULTS

Expression of *c-jun*

Retinas that were extracted at the 3-hour time period showed no significant difference in the levels of *c-jun* expressed between sham operated and optic nerve injured. In the 24-hour samples, there was statistically significant ($p < 0.05$) higher levels of expression in the optic nerve injured retina compared to the sham operated. The 24-hour ONI samples demonstrated a mean fold difference that was nearly double the SO 24-hour samples. The 168-hour ONI samples also demonstrated significant ($p < 0.05$) differences from 168-hour SO samples.

Comparison within the sham operated samples demonstrated significant differences between 168 hours v 24 hours and 24 hours v 3 hours. In contrast, when comparing the optic nerve injured samples, all tests rejected any difference between 24 hour vs. 168 hour comparisons. Furthermore, a Tukey-Kramer post hoc test demonstrated a significant difference ($p < 0.05$) between 168 hours vs. 3 hours and 24 hours vs. 3 hours. Gene expression levels are expressed in terms of relative Ct method shown in Figure 2. The lower the relative Ct, the more difference between housekeeping gene and gene of interest mRNA expression. Therefore, the lower the relative Ct, the more mRNA present and consequently, elevated gene expression.

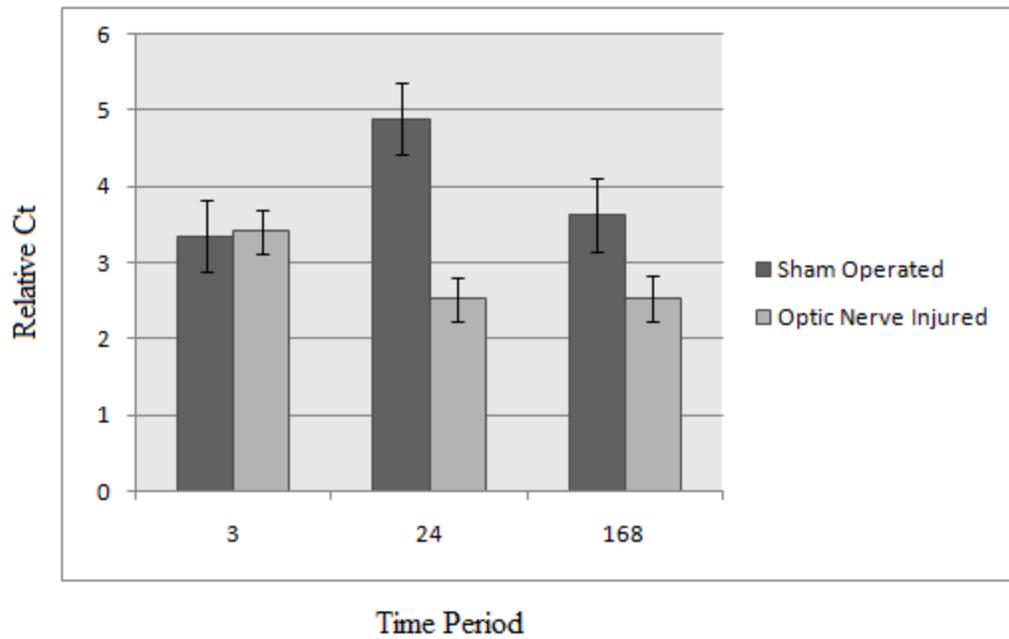


Figure 2. Relative Ct is represented on y axis and time points represented on x axis.

A significant increase between 3 hour and 24 hour time periods is demonstrated in fold changes. A decrease in fold change was observed between 24 hour and 168 hour time periods.

DISCUSSION

Overall, this project focused on isolating the gene quantification specific to optic nerve injury as opposed to tissue damage suffered when performing optic nerve injury. This study demonstrated significant increases in expression of *c-jun* following optic nerve injury compared to sham operated for the later time periods, 24 hours and 168 hours. This experiment gave insights on the effects *c-jun* has on CNS injury by demonstrating that in addition to changes in gene expression at different time points, it also shows significant changes in gene expression within the time point between sham operated and optic nerve injury.

This research may have an application in contrasting the effects of nerve injury in central and peripheral nerves. My findings corroborated a study by Broude et al. (1997) which states that *c-jun* is differentially expressed after both peripheral and central nervous system injury, only they did it through the use of dorsal root ganglion neurons. However, Bernhardt (1999) finds that *c-jun* expression is much less pronounced following CNS injury of dorsal root ganglion cells compared to PNS injury. Herdegen et al. (1997) found that the level of *c-jun* expression is correlated with the intensity of the cell body reaction. This contradiction can lead to further studies of differences in gene expression within the central nervous system. Furthermore, it was demonstrated that the manner in which the optic nerve is injured will determine the level of *c-jun* expression post-injury (Herdegen et al., 1997). Taking into account that the type of injury influences gene expression, the effect of distinct injuries on gene expression merits further scrutiny.

Furthermore, knowing that *c-jun* is considered part of the AP-1 transcription factor, and therefore involved in early response (Herdegen et al., 1997; Vaudano et al., 1996), one

would think that our results would yield immediate up-regulation of *c-jun* at the 3 hour time point. However, this inconsistency can be explained by several factors. One reason can be because a 3-hour time point is too soon to have any difference in gene expression to compare between CNS and PNS injury response. According to Bernhardt (1999), the early period of RGC re-growth lasts about 30 days in an adult goldfish. Another reason can be that *c-jun* is known to elicit only a delayed response following optic nerve injury and has a prolonged role in regeneration (Herdegen et al., 1997). These findings can lead one to hypothesize that monitoring expression at later time periods may lead to the discovery of patterns in *c-jun* expression post-injury.

The effects of c-Jun post-injury are paradoxical and warrant further investigation. It is known that there is an over expression of *c-jun* following injury (Veldman et al., 2007; Herdegen et al., 1993; Herdegen et al., 1997). However, it is unknown whether these effects promote the transcription of factors that aid regeneration or if it promotes the transcription of inhibitory elements that lead to apoptosis (Crocker et al., 2001). c-Jun's promiscuous binding nature with members of the ATF/CREB family and c-Fos is known to be the reason for the uncertainty of effects caused by the gene (Herdegen et al., 1997). In other words, c-Jun's ability to stimulate regeneration or promote apoptosis depend on whether it binds to itself or another transcription factor. Hai and Hartman (2001) speculate that the formation of homodimers act as transcription repressors, whereas the formation of heterodimers lead to the activation of gene expression. A recent study by Saul et al., demonstrated that *atf-3* and *c-jun* have common patterns of gene expression following optic nerve regeneration. Seijffers et al. (2007) help explain this finding by stating that c-Jun and Atf-3 form a heterodimer which enhances peripheral nerve regeneration. Thus, interactions with some transcription

factors may lead to the promotion of regeneration; whereas interactions with others may lead to the inhibition of regeneration.

c-Jun seems to be prominently expressed in neurodegenerative diseases such as Alzheimer's, and Parkinson's. The involvement of dopaminergic neuronal cell death in the substantia nigra may be one of the causes leading up to Parkinson's disease. The dual expression of *c-jun* in cell death and cell regeneration has led to thorough studies of the effects caused by the effects of *c-jun* in neuronal cell apoptosis. A study by Crocker et al. (2001) demonstrates a correlation between *c-jun* expression and cell death of dopamine neurons. These studies have led to the consideration of the cellular pathways involved in activation of *c-jun* by the Jun-NH2-terminal kinase (JNK) (Besirli et al., 2005). Besirli et al. (2005) demonstrated that the phosphorylation of c-Jun by JNK is important to the promotion of apoptotic gene expression by c-Jun. Further studies may focus on finding ways to inhibit this phosphorylation with the goal of preventing the progression of dopaminergic cell death in the substantia nigra and therefore, aiding in the cure for Parkinson's.

Furthermore, Herdegen et al. (1997) demonstrated that c-Jun is expressed following hippocampal cell death due to hypoglycemia and optic nerve injury from increased pressure in the eye due to hyperglycemia in diabetes. Patients who suffer from glaucoma can directly benefit from research regarding genes expressed in optic nerve regeneration. Glaucoma is devastating to the patient because it is nearly asymptomatic in its early stages and is only irreversible due to the inability of humans to regenerate their optic nerve (Blodi, 1963). Blodi (1963) provides evidence that a direct relationship between increased ocular pressure and blindness has not been clearly determined, this leads to the belief that the degeneration of the optic nerve is a direct cause of blindness (Alward, 2003). Therefore, I propose that a genetic

basis for preventing axonal death of the optic nerve through gene therapy may be a more accurate form of blindness prevention in patients suffering from glaucoma.

A shortcoming in this experiment was the lack of comparison in *c-jun* expression between, control eye (no injury) sham operated, and optic nerve injury. However, we presume that there is a significant up-regulation of *c-jun* due to data reported by Veldman et al. (2007) using qRT-PCR from retina. Future studies could elaborate on the gene expression involved in bringing about cell body changes following a PNS injury and comparing them to those known to be absent consequent to CNS injury (Bernhardt, 1999) in mammals. Furthermore, the fish and amphibian gene expression following CNS injury should be compared to the mammalian gene expression following CNS injury. Comparing the two could lead to the isolation of genes solely involved in the regeneration of the CNS and thus be utilized in gene therapy for mammalian CNS regeneration.

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