Response of the Larval Zebrafish to Spinal Cord Injury: Labeled Lesions, Two-Photon Axotomy and Recovery of Visuomotor Behaviors

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Abstract

Spinal cord injuries can result in near total loss of function below the spinal-level of damage, due to the interruption of descending neural commands. A number of approaches are being tested to enhance axonal regeneration and assess the recovery of spinal cord function although, to date, no effective regeneration has been observed following complete spinal transection. Given the limited functional recovery seen in a variety of studies, and a lack of understanding of underlying molecular mechanisms, a system that enables faster and more precise analyses is needed. We attempt here to lay a foundation for such studies in larval zebrafish. Three distinct strategies were employed to assess restoration of the zebrafish descending motor control system after spinal injuries. First, we followed anatomical regeneration using the labeled-lesion technique. Second, we assessed functional recovery based on a suite of larval locomotor behaviors. Third, we used in vivo two-photon microscopy to observe axonal behaviors immediately after precision axotomies. The suite of visuomotor assays used documented a descending motor control system that proved fairly robust, even in the presence of substantial axonal injuries. In contrast, however, the motility and elongation of individual severed axons proved limited as did the larger scale regeneration of major brainstem nuclei into spinal cord. While this fits with an emerging view of limited regeneration in lower vertebrates, it is surprising that these limitations were so substantial in robustly developing young larval zebrafish.
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# Table of Contents

Abstract................................................................................................................................. 2  
Acknowledgements............................................................................................................. 3  
List of Tables......................................................................................................................... 7  
List of Figures....................................................................................................................... 8  

1. Introduction ....................................................................................................................... 10  
   1.1 General Problem of Spinal Cord Injury ................................................................. 10  
   1.2 Role of Neurogenesis and Axonal Regeneration .................................................. 12  
   1.3 Lower Vertebrate Models ....................................................................................... 13  
      1.3.1 Studies on Larval Lamprey ........................................................................... 13  
      1.3.2 Adult Lamprey .............................................................................................. 16  
      1.3.3 Adult Zebrafish and Goldfish ...................................................................... 17  
      1.3.4 The Larval Zebrafish Model ....................................................................... 20  
   1.4 Central Aims ............................................................................................................. 23  

2. Cell-Specific Regeneration of Larval Zebrafish Descending Neurons ....................... 29  
   2.1 Introduction ............................................................................................................. 29  
   2.2 Methods ................................................................................................................ 32  
      2.2.1 Fish Husbandry ............................................................................................. 32  
      2.2.2 Proximal Micropipette Axotomy (Labeled Lesion) .................................... 33  
      2.2.3 Distal Spinal Labeling .................................................................................. 33  
      2.2.4 High-Resolution Confocal Imaging and Analysis .................................... 34  
   2.3 Results ................................................................................................................... 34  
      2.3.1 Regeneration Patterns of Reticulospinal Neurons ....................................... 34  
      2.3.2 Regenerative Rates of Ro & Mi Cells ........................................................... 35  
      2.3.3 Regenerative Capacity of the nMLF ............................................................. 36  
      2.3.4 Morphological Observations ..................................................................... 37  
   2.4 Discussion .............................................................................................................. 38  
      2.4.1 Limits to Determination of Regeneration .................................................... 38  
      2.4.2 Actual Time Course and Extent of Regeneration ....................................... 41
3. Dynamics of Injured Axon Endings in the Descending Motor Control System ........................................54

3.1 Introduction .................................................................................................................................54

3.2 Methods .....................................................................................................................................57

3.2.1 Retrograde Labeling of Descending Neurons .......................................................................57
3.2.2 Two-Photon Laser Scanning Microscope and Axotomy ........................................................58
3.2.3 Calcium Imaging .....................................................................................................................60
3.2.4 Data Analysis ..........................................................................................................................60

3.3 Results .......................................................................................................................................61

3.3.1 Response to 2-Photon Axotomy ............................................................................................61
3.3.2 Fluorescence and Calcium Responses to Axotomy ...............................................................61
3.3.3 Shapes of Axon Terminals Post-Axotomy ............................................................................62
3.3.4 Dynamic Responses of Axon Terminals ..............................................................................64
3.3.5 Mauthner Cell Response to Axotomy ....................................................................................67

3.4 Discussion ...................................................................................................................................68

3.4.1 Acute and Time-Lapse Response to Focal Axotomy ............................................................68
3.4.2 Efficacy of Axonal Regeneration ...........................................................................................70
3.4.3 Conclusions ............................................................................................................................71

4. Disturbances of Visuomotor Functioning after Rostral Spinal Axotomy ........................................87

4.1 Introduction ...................................................................................................................................87

4.2 Methods .......................................................................................................................................90

4.2.1 High Speed Imaging ...............................................................................................................90
4.2.2 Behavioral Assays ..................................................................................................................91
4.2.3 Behavioral Analysis .................................................................................................................93

4.3 Results .......................................................................................................................................95

4.3.1 Dark-Evoked Responses .........................................................................................................96
4.3.2 Light-Evoked Responses ........................................................................................................98
4.3.3 Relative Performance of DET- Impaired Fish on Visuomotor Tasks ....................................99
4.3.4 Tail-beat Frequencies of Visually-Evoked Responses .........................................................100
4.3.5 Analysis of Posture, Yaw and Swim Parameters Associated with Visuomotor Behaviors .................................................................101
4.3.6 Behavioral Testing of Zebrafish Larvae Following Laser Axotomy ....................................103

4.4 Discussion ..................................................................................................................................105

4.4.1 Primary Deficits and Recovery of Visually-Evoked Behaviors ...........................................105
4.4.2 Conclusions ............................................................................................................................108

5. Conclusions and Future Directions ..............................................................................................126

References ..........................................................................................................................................130
### List of Tables

1.1  Partial summary of studies performed in lower vertebrate model systems………………..25

2.1  Regenerating Cell Types…………………………………………………………………43

2.2  Strong positive correlation in the observed numbers of regenerated neurons………………48

4.1  Descending neurons axotomized via labeled lesion technique…………………………125
List of Figures

1.1  Schematic view of spinal cord…………………………………………………………………………………26
1.2  Spinal cord injury scale as defined by the American Spinal Injury Association………27
1.3  The larval zebrafish has ~300 descending neurons……………………………………………………………28
2.1  Reticulospinal neurons of the larval zebrafish, goldfish and lamprey…………………44
2.2  Segmental homologies conserved in the hindbrain of lamprey and zebrafish……45
2.3  Time course of regeneration of descending neurons (DN) following transection at the brainstem-spinal cord (BSC) juncture………………………………………………………………46
2.4  Regeneration of nMLF, Ro and Mi cells at 2-10 days post transection……………47
2.5  Differential regeneration rates of descending neurons classes……………………………49
2.6  Regeneration of distinct nMLF cell clusters, in 21 larvae………………………………50
2.7  Mi cell response to axotomy…………………………………………………………………………………51
2.8  Response in nMLF to axotomy………………………………………………………………………………52
2.9  Bilateral responses in nMLF post-axotomy……………………………………………………………53
3.1  Schematic of the two-photon microscopy configuration…………………………………72
3.2  Method of two-photon laser axotomy……………………………………………………………………73
3.3  Axotomy Induced calcium responses……………………………………………………………………74
3.4  Examples of axon terminal morphologies following axotomy……………………………75
3.5  Quantitative analysis of axon ending morphology as determined by time-lapse imaging……………………………………………………………………………………………………76
3.6  Rapid retraction of axons following rostral axotomy………………………………………77
3.7  Example of laser axotomy of multiple reticulospinal neurons at a single focal plane….78
3.8  Response of Axon 1 to axotomy with subsequent movements over 4 hours…………79
3.9  Response of Axon 2 to axotomy with subsequent movements over 6.75 hrs…………80
3.10 Axonal branch dynamics between 255 and 585 minutes post axotomy………………81
3.11 Axonal dynamics of proximal branch in relation to a second distal branch between 165 and 495 minutes post axotomy……………………………………………………………………………...82
3.12 Cross-sections of severed axons from 2-photon z-stacks following axotomy………83
3.13 Continuation of Fig. 3.12, showing montages at 24 hours post-axotomy .................84
3.14 Differential axon migration followed post-axotomy over 12 hours.......................85
3.15 Response of Mauthner cell axon post axotomy.............................................86
4.1 Possible pathways of light entering the eye.....................................................109
4.2 Behavioral configuration for high-speed imaging..........................................110
4.3 Labeled-Lesion Method..................................................................................111
4.4 Latencies of touch-evoked escape responses...............................................112
4.5 Latency of Dark-Evoked Turns (DET) .........................................................113
4.6 Distinct DET deficits in 3 groups of lesioned larvae......................................114
4.7 Latency of light-evoked swims.................................................................115
4.8 Range of LE latency responses following transection...............................116
4.9 Comparison of light and dark evoked responses in select groups of experimental animals.................................................................117
4.10 Transected reticulospinal cells of a fish imaged 9 dpt.................................118
4.11 Tail-Beat Frequency (TBF) following visually-evoked responses...............119
4.12 Example of exaggerated yaw following labeled lesion................................120
4.13 Analysis of larval head and tail velocity profiles after light-evoked stimuli....121
4.14 Analysis of larval head and tail velocity profiles following dark-evoked stimuli....122
4.15 Behavioral deficits following 2-photon axotomy.....................................123
4.16 Examples of spontaneous slow swim and prey capture of fish following 2-photon axotomy.................................................................124
5.1 Variable regenerative responses in vertebrates (adult and larvae)..............129
Chapter 1: Introduction

1.1 General Problem of Spinal Cord Injury

Spinal cord and brain injuries resulting from trauma or disease pose challenging medical issues that are often insurmountable, often leading to chronic disabilities and death. Major goals of the medical community aims are to minimize the neural damage (Figure 1.1) that occurs in these types of injuries and to repair the damage by induction of neurogenesis, self-repair mechanisms and other means. However, there are formidable barriers to repairing damage in the mammalian central nervous system (CNS) because of: 1) scar tissue formation after injury; 2) gaps in the tissue due to phagocytosis; 3) inhibitory factors that impede axonal growth in the mature CNS; and 4) the failure of most neurons to initiate axonal extension [Kwon et al., 2001; Silver and Miller, 2004; Thuret et al., 2006].

Spinal Cord Injuries (SCIs) (Figure 1.2) are unique in a number of respects, including their immediate impact on the victims, sometimes leaving them completely paralyzed. This sudden loss of behavioral function following spinal cord injury (SCI) has two different functional states: an initial acute phase and a subsequent chronic condition. Current therapies are primarily aimed at minimizing the spread of damage during the acute phase and it appears that there is currently little that can be done in the chronic phase to promote meaningful recovery. They are also unique in that the damage is typically localized to a narrow segment of spinal cord, involving in severe cases a complete crush or severing of the connections between brain and spinal cord below the lesion site. Extensive research indicates that substantial reorganization of the lesion site and surrounding nervous tissue occurs, as found both by analysis of pathology and in experimental studies. Whether this occurs as a result of 1) alterations to pre-existing circuitry
along the lines of synaptic plasticity or 2) the formation of essentially new circuits as a result of more extensive anatomical reorganization [Raineteau and Schwab, 2001], there appears to be substantial opportunity for modification of the motor control system in mammals. For example, a study in cats in which descending fibers to spinal cord were cut by spinal transection showed that these cats were capable of limited locomotion on a treadmill at one month post injury [de Leon et al. 1998]. In these types of animals afferent feedback, or information directed from peripheral nerves to the brain and spinal cord, is fundamental in the process of recovery and correction of stepping movements. This type of functional recovery was observed in peripheral nerve lesioning experiments performed on cats following denervation of the hind leg muscles [Pearson and Misiaszek, 2000].

Because of the focal nature of SCI, it offers an attractive target for interventions seeking to repair neural damage or work around a lesion site. Regeneration or recovery of normal locomotor control, in cases of complete transection, requires that descending axons from the brainstem can traverse the lesion and restore control over spinal locomotor networks below the lesion. Much of this work has been focused on studies in rodent models which are intended to provide results that can be translated into human treatments. Many studies have used growth factors to try and stimulate regeneration of axons from brain into spinal cord [Giger et al., 2010; Kasai et al., 2010; Yang et al., 2010]. Other studies have sought to counteract inhibitory factors such as Nogo [Harvey et al., 2009; Wang et al., 2010; Wu et al., 2010]. A third strategy is to use glial cells, such as olfactory ensheathing cells, to promote axonal regeneration [Kocsis, 2009; Lindsay et al., 2010; Raisman et al., 2010]. Alternatively, engineering approaches can be employed to route neural signals around the site of injury e.g. using bridging grafts [Tabesh et al., 2008; Wong et al., 2008; Zurita et al., 2010], perhaps one day using neural implants as are
being used now in the case of cochlear implants and being tested in cortical and retinal implants. But despite the efforts of many research groups, there has been, to date, no effective progress in restoring locomotor function in humans following complete spinal-cord transection. The best results to date have involved rehabilitative treatments and neuroprotective/growth factors, but these with only limited success [Thuret et al., 2006]. This is a formidable problem, not simply because of the biological barriers to axonal regeneration, but also because we are lacking basic knowledge about the numbers and types of descending connections required either for normal locomotor functions or to restore function after SCI. Contributions from simpler model systems are potentially beneficial in both regards.

1.2 Role of Neurogenesis and Axonal Regeneration

In the mammalian CNS, functional neurogenesis is restricted primarily to the olfactory system via the rostral migratory stream, along with an apparently more limited neurogenic process in the hippocampus [Eriksson et al., 1998; van Praag et al., 2002; Ghashghaei et al., 2007]. In contrast, many lower vertebrates display a much greater capacity to continually produce neurons through adulthood. We should note that adult neurogenesis and axonal regeneration are present in many anamniotes, which include fishes and amphibians, and that this clade of organisms has provided a variety of insights into the regenerative processes of the CNS [Windle, 1956; Bernstein and Gelderd, 1970; Rovainen, 1976; Davis et al., 1990; Becker et al., 1997; Chernoff et al., 2003; Chevallier et al., 2004; Zupanc and Zupanc, 2006; Kaslin et al., 2008; Kragl et al., 2009]. Studies in lower vertebrates are thus of potential benefit, both in terms of understanding the genesis and reorganization of neural circuits following SCI and in terms of understanding molecular differences that enable them to naturally respond to injury. Of particular interest are vertebrate models shown to have axonal regrowth and functional recovery,
as they may be useful in determining the mechanisms necessary for functionally-relevant regeneration. In the next section we summarize some of the results obtained with certain models and the extent of functional recovery attained. Our focus is on the collective pathways from brain to spinal cord-- the descending motor control system (DMCS). We are particularly interested in the regenerative potential of individually-identified descending neurons that make up the reticulospinal, vestibulospinal and other pathways that project from brain into spinal cord.

1.3 Lower Vertebrate Models

It is commonly assumed that lower vertebrate animals “regenerate” damaged spinal cords and recover locomotor functions. An accompanying notion is the idea that the regenerated descending axons reconnect to their normal spinal targets. But a careful read of the results obtained after spinal cord transection in lower vertebrates reveals that these ideas are often overstatements and may be incorrect in several important respects. While amphibians and fishes clearly show greater regeneration than mammals, all vertebrate animals have some limited capability to respond to neural damage by such means as neurogenesis, axonal sprouting, axonal regeneration and functional reorganization [Raineteau and Schwab, 2001; Thuret, Moon and Gage, 2006]. But all vertebrate animals are also limited in their ability to regenerate descending spinal axons past a glial scar [Silver and Miller, 2004] into undamaged lower spinal cord. To clarify this situation, we summarize below a number of key findings on the regenerative potential of several well-studied model organisms.

1.3.1 Studies on Larval Lamprey

Recovery of descending motor control, in the case of spinal transection, requires that axons descending from the brainstem traverse the spinal lesion and restore control over caudal
spinal locomotor networks. Among lower vertebrates, the lamprey has the best documented regenerative capabilities (Table 1.1A) wherein a variety of locomotor functions are recovered following complete spinal cord transection [Windle, 1956; Ayers, 1989; Jin et al., 2009]. Larval lamprey in particular were reported to recover a variety of locomotor functions, including swimming, coiling and crawling [Rovainen, 1976]. They exhibit several stages of recovery in which they obtain progressively better-coordinated swimming and other behaviors [Ayers, 1989]. For example, functional regeneration of the L1 “fin-command” neuron was reported in that strong stimulation of L1 could control fin posture after 80 days of regeneration [Currie and Ayers, 1987]. McClellan’s group reported the first regeneration of “command” neurons in lamprey (in 1988, and again in 1990) in that stimulation of brainstem could evoke fictive locomotor activity in the in vitro brain/spinal cord preparation and this appeared to be mediated by a lateral swim-command pathway. Accompanying these behavioral recoveries, numerous anatomical studies have shown varying degrees of regeneration of descending axons [Rovainen, 1976; Selzer, 1978; Wood and Cohen, 1979; Davis and McClellan, 1994]. Double-label studies indicate that 70% of brainstem neurons regenerate their axons at least 10% of the body length beyond a rostral-spinal lesion site [Zhang and McClellan, 1999]. Roughly 90% of those axons that do regenerate after spinal transection are observed projecting in the correct direction, including both large descending axons and spinal interneurons [Mackler et al., 1986].

There are, however, substantial limits to lamprey regeneration. Most notable is the frequent failure of neurons, including the Mauthner and Muller neurons, to properly regenerate: their regenerating “neurites” may instead branch and project aberrantly by e.g. looping backwards [Yin and Selzer, 1983; Becker et al., 1997]. Yin and Selzer noted that after spinal transections “all cell types examined thus far…have a limited capacity for axonal regeneration.”
In this study, the regenerating axons failed to regenerate significant distances into spinal cord, projecting on average much less than 1 mm beyond the site of the spinal transection, as estimated from intracellular HRP fills. Moreover, the number of branches was reported to drop late in the regeneration process [Yin and Selzer, 1983]. In other studies, however, longer-range regeneration was reported, reaching 40% of body length for 27% of regenerating axons, and in 5% of axons regenerating up to 60% of body length [Davis and McClellan, 1994]. In particular, B1, B3, B4 (bulbar), and I1 (isthmic) neurons of the lamprey, located in rhombomere 1-5 comparable to the zebrafish, have been shown to have regeneration rates lower than 20% [Jacobs et al., 1997; Davis & McClellan, 1994]. The Ro cells in the larval zebrafish occupy similar positions near I1 were observed to have more frequent double-labeling (personal observation) in contrast to other RNs of the hindbrain. The mth' cell in the lamprey, which has features similar to the MiD cells, is reported to have 68% regeneration rates [Jacobs et al., 1997; Davis & McClellan, 1994], which are unlike data to be presented below which indicates Mi cell regeneration rates lower than 20% (see Chapter 2). Giant reticulospinal neurons, M1 and M4 neurons that are positioned most rostral within the mesencephalon, were observed to have regeneration rates of ~50% [Jacobs et al., 1997, Davis & McClellan, 1994] as did the cells in the nMLF in larval zebrafish. In particular, M2 cells in the lamprey midbrain which have dendrites that cross the midline [Martin, 1979] were shown to have low regenerative rates near 13%. Overall, isthmic, bulbar, and giant reticulospinal neurons indicated similar patterns of heterogeneity in their ability to regenerate. But these regenerated axons failed to re-establish normal patterns of synaptic connections in spinal cord, as indicated in ultrastructural studies where the number of presynaptic boutons was greatly reduced [Oliphint, et al., 2010]. Based on these and other studies we can conclude that the “regeneration” does not entail a normal degree
of reconnection of descending axons with their normal spinal targets, especially since the normal targets typically are much further down spinal cord than the regeneration distances observed. Regeneration of correct intraspinal synapses below the lesion site has been reported [Mackler and Selzer, 1987] and this might lead to functional recovery via the “propriospinal” system—the long-range intraspinal projection neurons. Such pathways are suggested to account for locomotor recovery beyond the anatomical reach of regenerating axons [Davis, et al., 1993]. Thus some combination of axonal regeneration and rearrangement of connectivity, possibly utilizing propriospinal neurons, may contribute to a reorganizational mechanism of locomotor recovery, as is also believed to occur in mammals, albeit to a much more limited degree [Raineteau and Schwab, 2001; Thuret et al., 2006].

1.3.2 Adult Lamprey

The repertoire of adult lamprey behaviors is more extensive and has been assessed in a spinal regeneration context [Ayers, 1989]. Kinematic analyses that focused on parameters central to the flexion waves that characterize undulatory swimming were able to distinguish a broader range of behaviors than those exhibited by the filter-feeding larvae. These adult behaviors included escape and normal (navigational) swimming and other locomotor behaviors such as “terrestrial” swimming, crawling, burrowing, struggling and aversive withdrawal. Adult lamprey appear to have similar regenerative capabilities as larval lamprey in that substantial numbers of descending neurons regenerate axons past the lesion site into the distal spinal cord, with most neurites oriented in the correct direction and running along ipsilateral spinal cord [Lurie and Selzer, 1991].
In summary, both larval and adult lampreys have substantial limits to regeneration, but also substantial regenerative capabilities. There are some striking similarities between the rat and lamprey models, as e.g. a recently documented distinction between growth-cone mediated and growth-cone independent regenerative mechanisms in lamprey [Jin et al., 2009; Bhatt et al. 2004] and a “2 mm limit” for regrowth of axons into healthy tissue [Yin and Selzer, 1983], both of which may apply to lamprey and higher vertebrates alike. It also seems clear that cAMP facilitates regeneration and that this proceeds via a later developmental mechanism that may be independent of growth-cone actin dynamics, relying instead upon propulsive forces probably involving intermediate filaments [Jin et al., 2009]. The lamprey model has thus been a rich source of information with implications for regeneration in mammals, teleost fishes and other higher vertebrates. Complementary to this is the quite recent emphasis being placed on understanding the precise neural circuits and behaviors of the larval zebrafish. After reviewing some of the historical adult zebrafish and goldfish regeneration literature, certain benefits of examining regeneration in larval zebrafish are proposed.

1.3.3 Adult Zebrafish and Goldfish

While lampreys exhibit a variety of swimming and postural behaviors, teleost fishes have a richer locomotor and behavioral repertoire, although there has not been to our knowledge an effort to describe a complete “ethogram” of behaviors for teleost fishes or higher vertebrates. Historically, the next best studied model has been the goldfish, *Carassius auratus*. To the extent characterized, teleost fishes appear to have similar capacities and limits to their regeneration as lamprey (Table 1.1A). “Normal” swimming appears 25 days after spinal transection of adult goldfish, and by 60 days, up to 50% of descending fibers have regenerated 2 cm into the distal spinal cord [Bernstein and Gelderd, 1970]. Curiously, the regenerating fibers were quite
swollen, between 4 and 8 times their normal size, which is in contrast to the abnormally thinner diameters of regenerating lamprey fibers [Oliphint et al., 2010]. The regenerating neurons included RS, vestibulospinal and nMLF neurons [Coggeshall et al., 1982], and an HRP labeling study showed that some regenerating axons could extend almost the length of the spinal cord [Coggeshall and Youngblood, 1983]. A recent study suggests that regenerating nMLF axons re-establish synapses to “correct” spinal targets after a partial spinal cord lesion [Takeda et al., 2007], although the specific spinal targets of these nMLF neurons are not known [Gahtan and O’Malley, 2003].

There are however, varying reports on the extent of regeneration, with one study noting great variability in regeneration of different fiber tracts [Bunt and Fill-Moebs, 1984]. The degree and specificity of axonal targeting by descending neurons following an insult to the system also remains unknown. Early work showed that after spinal transection, many of the connections from goldfish RS neurons onto spinal neurons were lost and were replaced by synapses from spinal interneurons [Bernstein and Gelderd, 1973]. A more recent study of adult goldfish reported that only 11 of the 17 brainstem nuclei that normally project into spinal cord are able to do so after spinal transection [Sharma et al., 1993], and the degree of functional recovery is also incomplete, and limited by incorrect choices of pathway by fibers that are regenerating [Bentley and Zottoli, 1993]. More detailed behavioral analysis showed frequent failure to recover normal posture and absent or abnormal C-start escape behaviors [Zottoli and Freemer, 2003]. These findings are in accord with studies in lamprey, where there is limited axonal regeneration and even more limited re-establishment of synaptic connections to the original spinal targets. In both cases, engagement of intraspinal networks appears to be important for the limited recovery of the locomotor repertoire.
As the zebrafish has gained traction as a model organism, a number of studies have examined axonal regeneration in adult zebrafish [Becker and Becker, 2008]. Becker et al. [1997] reported that most but not all of the 20 different descending nuclei are able to regenerate axons into distal spinal cord after spinal transection. These tracts re-route to gray matter below the lesion site, while the adjacent white matter showed extensive myelin debris [Becker and Becker, 2001]. As befitting the suitability of zebrafish for molecular studies [Goldman et al., 2001], the Becker group has also explored a number of molecular aspects of spinal regeneration, showing for example that some descending neurons upregulated their expression of the growth promoting genes GAP-43 and L1.1 and regenerated after spinal transection, whereas ascending spinal-brainstem neurons showed no such molecular response and concomitantly failed to regenerate [Becker et al., 2005]. Morpholino knockdown of L1.1 impaired both axonal regeneration and locomotor recovery [Becker et al., 2004]. In addition, a zebrafish homolog of mammalian contactin1 was found to be upregulated in both axotomized descending neurons and spinal white matter glial cells [Schweitzer et al., 2007]. Expression of other cell recognition molecules such as NCAM and L1.2 were examined in several brain regions after distal spinal lesions, but they showed no responses associated with spinal regeneration [Becker et al., 1998].

Because zebrafish and goldfish are closely related otophysan species, the cumulative anatomical results on regeneration are understandably similar, but they also are complementary to and reinforce conclusions from lamprey regarding the limits of spinal regeneration. We have, however, less information on the “locomotor recovery” of adult otophysan swimming, as this has been described mainly to the extent of the claim that swimming is “normal”, but without any definitive high-speed kinematic analyses that might distinguish different types of locomotor behavior. One clear conclusion from the adult studies is that the swimming fatigues very easily,
suggesting that the muscles are used in a less-efficient manner which might reflect subtle abnormalities to the swimming pattern that are not detectable from visual or video-rate observations. Zebrafish larvae are known to have a surprisingly large and specialized locomotor repertoire, and it seems only reasonable that adult zebrafish will have an even larger and more sophisticated repertoire. While this has been documented for several forms of social-locomotor behaviors [Larson et al., 2006; Imada et al., 2010], the adult locomotor repertoire has not been examined in any kinematic detail, beyond the much examined escape behavior [Foreman and Eaton, 1994; Zottoli and Freemer, 2003].

1.3.4 The Larval Zebrafish Model

The transparency of larval zebrafish and identifiability of the descending motor neurons makes this animal model a candidate for a more comprehensive investigation into neural regeneration and repair processes. In order for efforts along these lines to be successful, a good deal of foundational work is required. The locomotor repertoire of larval zebrafish has been intensively studied, demonstrating a great variety of distinct behaviors, which can serve as benchmarks for studies of regeneration. These include the escape behavior [Kimmel et al., 1974], routine turning and swimming behaviors [Budick and O’Malley, 2000; Thorsen et al., 2004], predatory behaviors [Borla et al., 2002; McElligott and O’Malley, 2005] and navigational behaviors [Burgess and Granato, 2007a; Orger et al., 2000; Sankrithi and O’Malley, 2010]. There has been a corresponding emphasis on understanding the neural basis of many of these behaviors [O’Malley et al., 1996; Liu and Fetcho, 1999; Ritter et al., 2001; Gahtan et al., 2002, 2005; Burgess and Granato, 2007b; Orger et al., 2009], which include calcium imaging and laser-ablation experiments directed at individually-identified neurons. The anatomical basis for
these behaviors was described many years ago [Kimmel et al., 1982; 1985] and has recently been summarized [O’Malley et al., 2003] and is shown in Figure 1.3. These reticulospinal neurons (RN) of the larval zebrafish which are organized into 7 different groups (Ro1 & Ro2, Mi1, Mi2 & Mi3, Ca) identified in Metcalfe et al. (1986) have described in detail the projections of these longitudinal fiber tracts descending into spinal cord which indicate that the axons of reticulospinal neurons separate into two pathways: the medial longitudinal fascicle (mlf), originating from the midbrain at the nMLF, extends fibers into the spinal cord as does the lateral longitudinal fascicle (llf). The caudal-most projections [Metcalfe et al., 1986] of the primary axons of these RNs have been identified which indicate the axon paths of taken by these descend as far into spinal cord as myotome 23 in the larval zebrafish. Anatomical data from these studies show that few types of Mi cells project beyond myotome 15 along these axon paths, while RoM3 are found to reach as far as myotome 25.

While there have been few studies of spinal regeneration in larval zebrafish, the targeted regeneration of the axon of the Mauthner cell was examined in what was perhaps the first study to visualize the process of axonal regeneration in an intact, living vertebrate animal [Bhatt et al., 2004]. From the lamprey and teleost literature it has long been known that large, specialized descending axons, including the Mauthner and Muller cells, often follow incorrect paths during regeneration and this is also the case for larval zebrafish. Bhatt and coworkers showed that the Mauthner cell could be induced to regenerate and restore escape-like behaviors by directly applying cAMP to the Mauthner cell. This presaged a study showing strong stimulating effects of cAMP on axonal regeneration in lamprey [Jin et al., 2009]. However, we still have only limited understanding of the regenerative capabilities of the larval zebrafish in terms of both
anatomy and behavioral recovery, despite the increasing palate of genetic and optogenetic tools, and its increasingly well characterized set of neural systems.

Surprisingly, we also do not know the normal pattern of innervation of descending neurons onto spinal interneurons—even though these two populations are better defined in larval zebrafish than in any other vertebrate animal. There are at least 40 distinct classes or types of descending neuron, many of them individually identified (Figure 1.3) and at least 15 distinct types of spinal interneuron, along with 2 classes of spinal motoneuron including the large primary motoneurons RoP, MiP and CaP which are individually identified [Liu and Westerfield, 1988; Bernhardt et al., 1990; Hale et al. 2001]. While spinal axonal trajectories have been visualized for many of the identified descending neuron types [Gahtan and O’Malley, 2003], there has not yet been any determination of their spinal targets, aside from the exceptional case of the Mauthner cell [Fetcho and Faber, 1988]. While this may seem a straightforward experiment, this data has not been acquired despite the many groups studying zebrafish brainstem and spinal cord. This is not a “zebrafish” problem, because as noted above, the connectivity of the DMCS has not been mapped for any vertebrate animal. Indeed, the cells that make up the most fundamental unit of the spinal cord, the swimming/walking CPG, have not been conclusively identified in any vertebrate animal despite many years of effort [Buchanan, 2001; Gosgnach et al., 2006; Fetcho and McLean, 2010; Kiehn, 2006]. The identities of the neurons that descend into the spinal cord are even more poorly defined, and absent. Without an understanding of which neurons descend from brainstem and what specific sets of spinal neurons they synapse upon, it is impossible to construct a lower-motor system connectome.

Aside from Bhatt et al. (2004), we are not aware of other studies where the regeneration of individually-identified neurons has been followed in intact vertebrate animals. Careful
observations of the critical steps to regeneration, based on identified populations of axotomized descending neurons, will be important for evaluating neuroanatomical regeneration of spinal circuits and relating these anatomical deficits to functional deficits.

1.4 Central aims of dissertation

AIM 1: Determine the degree to which the CNS of the larval zebrafish provides an environment that allows regeneration of identified brainstem neurons following labeled-lesion experiments.

Hypothesis: There may be variability in the number and fraction of regenerating descending neurons.

Though previous studies [Bhatt, et. al., 2004; Becker et. al., 1997] have provided evidence in zebrafish of spontaneous neuroregenerative processes, there is a limited understanding of the extent and functional impact of spontaneous regeneration. In particular, we hope to understand how particular subsets of descending fibers respond to injury and how they may be able to reinnervate appropriate spinal target regions. By examining the regenerative responses of individually-identified neurons we also hope to learn more about the variability of the regenerative process.

AIM 2: Visualize the time-course and extent of regeneration of labeled descending axons following 2-photon axotomy producing small precise lesions of either single axons or small axon bundles descending from brainstem with minimal damage to the surrounding tissue.

Hypothesis: During the process of regeneration, severed axon endings will form distinct shapes which will retract, extend forward and undergo fragmentation.
Two-photon laser axotomy has been demonstrated in several animal models [Villegas et al., 2012; O’Brien et al., 2009; Hammurlund et al., 2009; De-Miguel et al., 2002] to sever single axons after which axonal regeneration can be observed. We expect to observe fine details of the regenerative process including retraction, regrowth and the path taken by the regrowing axon/neurite.

AIM 3: Perform labeled-lesions 2-photon and laser axotomies on zebrafish larvae and assess their functional consequences using the locomotor battery. Identify the degree of locomotor impairments. Perform appropriate controls (naive and spinal-only (muscle and interneuron lesions).

Hypothesis: Both small and substantial perturbations of the descending motor control system will result in minimal disruption of locomotor performance, suggesting possible organizational redundancy within the larval zebrafish DMCS.

Larval motor patterns that are readily elicited include escape behaviors, light-evoked swims, dark-evoked turns, rheotaxis and the optomotor response (OMR). We will establish kinematic performance measures from control animals (naïve and spinal-only) that can then be used in characterizing both locomotor deficits and the subsequent recovery of function after labeled-lesion and 2-photon axon cutting experiments.
Table 1.1 Partial summary of studies performed in lower vertebrate model systems.
Surveys of work described in (A) larval and adult lamprey, (B) goldfish and (C) adult and larval zebrafish on axonal regeneration of brainstem neurons the level of functional recovery and expression of intrinsic factors.
Figure 1.1 Schematic view of spinal cord [Figure from Thuret et. al., 2006] (A) Intact view of brainstem neurons (white matter) projecting to human spinal cord (grey matter) and motor neurons. (B) Features found in a region of cervical spinal cord injury (SCI).
Figure 1.2 Spinal cord injury scale as defined by the American Spinal Injury Association.  
[Figure from Thuret et. al., 2006]
Figure 1.3 The larval zebrafish has ~300 descending neurons. [Figure from O’Malley et al., 2003] (A) A confocal montage of the larval zebrafish neurons in the brain which includes reticulospinal and vestibulospinal neurons, neurons of the nucleus MLF (nMLF) and other neurons projecting into spinal cord. Identified in original HRP studies from the Kimmel lab [Kimmel et al., 1982, 1985; Metcalfe et al., 1986] (B) The Descending Motor Control System (DMCS) template which includes ~150 DNs on each side of the brain, and can be subdivided into 40 distinct cell types. Each box in the template represents an individual neuron, and many types are comprised of a single neuron, such as the Mauthner cell, MiD2cm and MiD3cm, and so are identifiable as exact individuals.
Chapter 2: Cell-Specific Regeneration of Larval Zebrafish Descending Neurons

2.1 Introduction:

Cell-specific regeneration of descending neurons following an injury to the spinal cord has long been pursued in various model systems where a spinal transection of ascending and descending tracts allows investigation of the regenerative capacity of a variety of neuronal cell types. Most of our current understanding relating to the regeneration of transected axonal tracts has primarily been achieved from the larval lamprey [Yin & Selzer, 1983; Davis & McClellan, 1994a; Jacobs et al., 1997; Zhang & McClellan, 1999]. The differential regenerative response of reticulospinal neurons whose axons project into spinal cord (Table 2.1, Figure 2.1) indicates the importance of investigating the relationship between regenerative potential and such attributes as cell type, size and cell intrinsic factors.

Long-term axonal regeneration studies in the lamprey, using wheat-germ HRP, have indicated the potential of reticulospinal neurons to regenerate over varying distances as well as the directional pathfinding trajectories that are observed in the proximal tip. Early studies in lamprey have shown that the proximal tip extended as far as 71 mm past the transection site [Davis & McClellan, 1994a] and 90% of growing fibers that extended towards the transected site followed a directional specificity [Mackler, et al., 1986]. Additionally, unlike the inhibitory factors which limit the regeneration of CNS axons through the injury site in mammals, regenerating axons in both the larval and adult lamprey are able to navigate through the lesion site while maintaining directional specificity [Lurie & Selzer, 1991a]. Their studies suggested that the directional specificity of the severed axons was defined early following the injury, before the axonal processes took on the elongated neurite feature.
A comparison of the varying regenerative capabilities of organisms within the chordata phylum indicates that there are groups of brain nuclei in each organism that are better able to regenerate than others. Studies in adult and larval lamprey have demonstrated that approximately 50%-57% of the 36 giant reticulospinal neurons (RN) regenerate with some reliability [Lurie & Selzer, 1991a; Yin & Selzer, 1983]. In particular, of the 1000 classified brainstem neurons (Figure 2.1C), isthmic reticulospinal, middle reticulospinal, medial superior reticulospinal and posterior mesencephalic groups (Table 2.1) [Jacobs et al., 1997; Davis & McClellan, 1994b] were confirmed to substantially regenerate. In comparison, the regeneration rates of neurons in the medial diencephalic and dorsal inferior reticulospinal groups [Jacobs et al., 1997; Davis & McClellan, 1994b] were low. These findings are similar to that observed in salamanders where at 8.5 weeks post-transection nearly 40% of the brainstem neurons [Davis et al., 2004] were found to reconnect to the spinal cord. These limbed vertebrates of the urodela order were found to have regenerated axons from the red nucleus, medullary reticular neurons, mesencephalic reticular neurons, and the interstitial nucleus of the fasciculus longitudinalis medialis (nFLM) [Davis et al., 1990; Duffy et al., 1990]. The longer recovery times seen in these studies, wherein axonal regeneration seems to coincide with “relatively normal” locomotor function, suggests that the functional reconnection of descending brainstem axons with spinal targets is a slow process.

Studies in teleost fishes have provided further insights regarding the extent of neuroregeneration possible within the population of descending motor control neurons. In adult goldfish, subsets of descending axons retrogradely labeled with HRP showed robust regenerative capability. These included the nucleus ventromedialis, nucleus reticularis medialis, nucleus reticularis superior, nucleus reticularis inferior, anterior octaval nucleus, magnocellular octaval nucleus, descending octaval nucleus, and the nucleus of the medial longitudinal fasciculus.
(nMLF) \textbf{(Table 2.1, Figure 2.1B)} \cite{Sharma et al., 1993}. It appears though, that while 90% of these descending axons are able to cross the lesion site only 35-49% of the transected descending neurons \cite{Bernstein and Gelderd, 1970} are able to reestablish appropriate targets in the spinal cord. Behavioral movements, such as swimming and tail fin flexion observed in these animals also suggested a restoration of normal locomotor patterns within 20-25 days post-transection. Interestingly, the regenerated nerve fibers of these descending neurons were found to be 4-8 times larger in diameter than those of normal axons. Observations in the adult zebrafish, which has 20 distinct classes of descending neurons, also provide evidence for axonal regeneration of specific groups of brain nuclei following a transection. Double-tracing studies performed by Becker et al., 1997, indicate that while there was no axotomy induced neuronal death, 7-15 weeks post injury only 40% of descending neurons were able to extend past the transection site. Tracings of retrograde labeled HRP animals demonstrated that the neurons of inferior reticular formation, superior reticular formation, intermediate reticular formation, magnocellular octaval nucleus, and the nMLFs were the most favorable regenerators of the varying descending neurons. Behavioral recovery (“normal swimming”) in these animals was found to be limited even at 15 weeks post-transection and animals with low numbers of retrogradely-labeled cells tended to have little recovery of swimming.

Theoretically then, similar numbers of transected axons of descending neurons are expected to regenerate in the larval zebrafish, especially given the conservation of descending pathways \textbf{(Figure 2.2)}. Though previous studies \cite{Bhatt, et. al., 2004; Becker et. al., 1997} have provided evidence in zebrafish of some spontaneous neuroregenerative processes, there is a limited understanding of the extent and functional impact of spontaneous regeneration. The anatomical results presented here suggest differential regenerative capability of descending
neurons as has been shown in adult zebrafish [Becker et al., 1997]. In particular, these findings indicate that there are limitations of particular subsets of descending fibers, which induces variability in their response to injury and reinnervation of appropriate spinal target regions.

2.2 Methods

2.2.1 Fish Husbandry

Adult zebrafish (wild type and nacre) were maintained for breeding purposes and housed in groups of 2 or less in 10 gallon tanks. The zebrafish were fed live nauplii of brine shrimp two times per day and TetraMin flakes (Melle, Germany). The water conditions of tanks were kept at pH 6.6 -6.8 range and temperature of 28 -29 °C. To prevent accumulation of feces and maintain proper levels of nitrates and nitrites 20% water changes were performed weekly. Zebrafish strains used in this study included AB or Ekkwill (Ekkwill Fish Farms, Gibsontown, FL) and nacre [missing melanophores (black pigment cells)] [Moore, 1995; Lister 1999]. Embryos were produced by pairwise matings and raised at 28.5 °C in 12% Instant Ocean (IO) salt solution. Fertilized eggs were collected from colonies of adult zebrafish, and maintained in an incubator at 28.1 -28.5 °C in IO water (proper pH and 2.4g Instant Ocean). Larvae were used (4 -9 days post fertilization (dpf)) and raised on a 14-10 hour light-dark cycle. The 60 - 90% of the water was replaced daily and were fed paramecium from 5 dpf and naupilii of brine shrimp from 10 days post fertilization. All animal studies were approved by Northeastern University Animal Care and Use Committee.
2.2.2 Proximal Micropipette Axotomy (Labeled Lesion)

Larvae (4-5 days post fertilization) are anesthetized with 0.02% 3-aminobenzoic acid ethyl ester (MS222; Sigma, St. Louis, MO) prior to an initial spinal injection of a 10% solution of 10,000 MW Alexa 488 dextran (Molecular Probes, Eugene, OR) to retrogradely label reticulospinal cell bodies in the hindbrain. The spinal injection procedure has been described previously [O’Malley et al., 1996; Gahtan and O’Malley, 2003]. A glass micropipette with tip broken to 15-20 µm was lowered into the brainstem to cause a localized axonal disruption and a small amount (2.0nl - 4.0nl) of fluorescent tracer was pressure-ejected at approximately the 3rd myotome (brainstem-spinal cord juncture) fish (Figure 2.3C). This axotomy is referred to as a labeled lesion, since the injection of fluorescent dextran into rostral spinal cord simultaneously axotomizes descending neurons, and labels their cell bodies in brainstem. Larvae are then placed in individual circular wells in 6-well tissue culture plates containing IO salt solution and placed in the incubator for 18 -24 hours. This period provides time for recovery and retrograde transport of the dye from the spinal axons to the somas of the reticulospinal neurons.

2.2.3 Distal Spinal Labeling

Similar to proximal micropipette axotomy, a glass micropipette with tip broken to 15-20 µm was gently tapped into the spinal cord through the lateral axial muscle at approximately the level of myotome 23rd or slightly more rostral. A small amount (approximately 1.0nl -2.0nl) of fluorescent tracer is pressure-ejected [Gahtan & O’Malley, 2003] into the fish at this location (Figure 2.3C). Larvae are then returned to individual circular wells in 6-well tissue culture plates containing IO salt solution and placed in the incubator for another 18-24 hours. They are then placed in a small chamber cut out of solidified agarose and restrained by a droplet of molten agarose.
agarose, dorsal surface down in a 35mm glass bottom petri dish in preparation for confocal imaging. The anesthetic was removed and replaced with IO salt solution.

2.2.4 High-Resolution Confocal Imaging and Analysis

A Zeiss Axiovert microscope with a Olympus LUM PlanFI 60 X, 0.9 NA or Apo/340 40X, 1.15 NA objective and BioRad MRC600 laser scanning confocal microscope is also used for imaging reticulospinal neurons and descending axons in the intact larvae. Image stacks of each fish were acquired synchronously in both channels (519nm - 567nm, or 488-568nm) with 1μm increments. Images collected from the confocal microscope were analyzed using ImageJ (NIH software). Reticulospinal and other descending neurons were identified morphologically based on soma location and other anatomical features [Kimmel et al. 1985; Metcalfe et al. 1986].

2.3 Results

2.3.1 Regeneration Patterns of Reticulospinal Neurons

The basic paradigm of this study was to double-label reticulospinal neurons that were able to regenerate past the transection site in order to examine the time course and cell-specificity of regeneration (Figure 2.3). Spinal transections were performed by disconnecting large subsets of descending neurons using the labeled-lesion method (Figure 2.3C) where a micropipette severs axons and simultaneously labels the damaged cells with fluorescent tracer retrogradely at or near the brainstem-spinal cord juncture (BSC) [Gahtan and O’Malley, 2003]. Subsequently, a second fluorescent tracer was injected at approximately the level of the 23rd myotome to label both regenerated and intact descending axons at times between 2 – 10 days post transection. Double labeled cells from each of three groups of reticulospinal cells (nMLF, Ro, Mi) were counted to calculate the percentage of regeneration observed. Several other cell
types were labeled, but no distal regeneration was observed, including the Mauthner cell and its segmental homologues, MiD2 and MiD3.

**Figure 2.3A and 2.3B** show example distributions of labeled neurons observed at two time points following the rostral transection. Though on average we transected 24 descending neurons (n=15 fish, total number transected = 480, total number regenerated = 95), each animal had varying subsets of labeled neurons. Note that this technique provides just a lower limit of the number of regenerating neurons. The fraction of neurons regenerating (from the three cell groups studied) is plotted for successive days up to 8 days post-axotomy (**Figure 2.3D**). Because of noise inherent in the double-labeling method, there is no clear trend in the fraction of cells that are double-labeled, given that fewer cells were labeled on days 2 and 8 and a higher fraction labeled between days 3 and 7. Note also that poor spinal morphology precluded us from seeing clear axonal regeneration on day 1, and while there may have been some, it was not included with this data.

### 2.3.2 Regenerative Rates of Ro & Mi Cells

Despite the overall variability of regeneration, we wanted to assess the regenerative capacity of different cell groups, namely nMLF cells, the Ro cluster in rostral hindbrain segments and the Mi cells in mid-hindbrain (**Figure 2.4A**). Varying numbers of cells are labeled in individual larvae and so the regeneration is plotted in relation to the number of labeled neurons using stacked bar plots, where the darker color indicates regenerated cells and the lighter color the total number of that cell type labeled in that fish. The largest fraction of regenerated axons were from nMLF (62 of 230 cells; 95% CI [21.33, 33.18]), while fewer Ro cells (34 of 146 cells; 95% CI [16.70, 30.99]) and Mi cells (10 of 126 cells; 95% CI [3.87, 14.11]) showed
regeneration (Figure 2.4B-D). Summarizing and comparing the regeneration between the three groups of descending neurons that we measured indicated that there was a normal distribution of cells that were axotomized using the labeled lesion method. Though nonparametric tests (Pearson and Spearman) indicate positive correlation between the numbers of descending neurons severed and the number of regenerated cells in each group, we observed no statistical significance in comparing the regenerative rates.

Figure 2.5 plots the fraction of each cell type’s regeneration observed between 2 and 8 days with the bars indicating the range of values and averages on each day. While the variability and the few data points from each day preclude statistical conclusions, the Ro cells showed modest regeneration, e.g. 17% and 15% (Figure 2.5B), while the Mi cells had the lowest apparent regeneration rates across the 2-8 days studied (5% - 9%) (Figure 2.5C).

In comparison, in the nMLF cluster, where we tended to transect the highest numbers of descending neurons (n=19, 229 cells), an average of 3 cells were observed to regenerate (n=65 total) over the course of 10 days following transection (Figure 2.5A). Larger numbers of nMLF cells were observed to regenerate on days 6, 7 and 10 (Figure 2.4A), but this cannot be statistically distinguished from those individuals with good regeneration between days 2 and 4. While these data suggests that the nMLF cluster can more effectively regenerate than the Ro and Mi cell groups, overall what is clear is that all three cell groups show modest regeneration within the limits of our techniques.

2.3.3 Regenerative Capacity of the nMLF

To further explore the regenerative potential of the midbrain cells, we identified two populations of cells within the nMLF that were transected, namely the MeL and MeM cell types
(Figure 2.6A). Though the total number of cells in each population (MeL n=146 transected, 46 regenerated; MeM n=80 transected, 19 regenerated) differ somewhat due to the nature of the labeled-lesion technique, 30% of MeL cells were observed to regenerate in comparison to 23% of MeM cells (Figure 2.6B, C) which suggests that the MeLs might be a more competent cell type in restoring connections to the spinal cord within the 2-10 day period.

2.3.4 Morphological Observations

The condition of regenerating neurons was examined by imaging neuronal cell bodies and axons in the brainstem. Morphological features associated with axotomy (recall that all labeled neurons had been axotomized in spinal cord) included bulbous axons, fragmentation and other abnormalities. An example of reactive mid-hindbrain Mi cells in a well-labeled larva (Figure 2.7) is examined at higher resolution in Figure 2.7B and C. One-day post axotomy, two Mi cells (arrow in B; asterisk in C), show abnormal trajectories and evidence of fragmentation. While structures appearing as possible collaterals are seen in these images, the 3D spatial resolution was not sufficient to determine this. Six days post-lesion, the cell from (C) has an altered appearance (Figure 2.7D).

For the nMLF neurons, confocal imaging revealed further punctuate labeling and bulbous swellings as in Figure 2.8 where swellings (possibly terminals) are seen on processes near the cell body of an MeL cell. It could not be determined if the apparent endings were the ends of axon collaterals or perhaps dendrites of nearby, labeled nMLF cells—quite a few cells were labeled in this experiment and there was a variety of punctate structures throughout the nMLF. Figure 2.9A shows bilateral nMLF labeling one-day post axotomy with descending axons as well as rostrally and medially oriented dendrites. Two days post-axotomy there is a variety of
abnormalities bilaterally (animal is tilted slightly upon re-embedding; Figure 2.9B). There are bulbous structures on the axons running into the right nMLF, while a zoom of the left axon bundle (Figure 2.9C) shows a number of dark, swollen axons. There are quite a variety of responses to axotomy and in the following chapter, 2-photon microscopy is employed to visualize axon dynamics in vivo immediately after laser-axotomy in restrained animals.

2.4 Discussion

The findings presented here document axonal regeneration into distal spinal cord of multiple classes of larval reticulospinal neurons, with the most regeneration observed in nMLF cells. This pattern is in agreement with earlier work on larval lamprey and adult zebrafish. Heterogeneous patterns of regeneration among RNs have previously been reported in larval lamprey [Davis & McClellan, 1994a,b; Jacobs, 1997] adult goldfish [Sharma et al., 1993] and adult zebrafish [Becker et al., 1997]. It seems in larval zebrafish that cell types other than nMLF have a lower propensity to regenerate, but with our current methods it is not possible to conclude this with certainty, despite the large numbers of fish studied. There is obvious fish to fish variability in the number and fraction of cells regenerating, including the nMLF, Ro and Mi cells (Figure 2.4). Moreover, this variability persists across the 8 days of regeneration studied. To aid in interpreting this data, we should consider the extent to which our approach and other methods directly capture the true degree of regeneration.

2.4.1 Limits to Determination of Regeneration

In regards to the total number of neurons regenerating, our results establish just the minimum degree of regeneration. The most prominent reason why we might not have double-labeled at the distal injection site the regenerated axons of all neurons initially transected/labeled
at the proximal injection site in myotome 3 (Figure 2.3C) is that we did not “hit” all of the regenerated axons. Double-labeling, which does not provide a conditioning effect, requires that axons hit (transected) in myotome 3 are struck again in myotome 23, after sufficient time has passed for those axons to regenerate to the distal lesion site. There is variability in this approach even in terms of hitting the descending fibers with the initial injection (e.g. Figure 2.4) perhaps due to the requirement that the severed axon be immersed in the viscous dextran solution. This approach is necessitated by the fragility of larvae, especially in cases where subsequent behavior is to be studied. It is notable in other studies that variability in observed regeneration occurs, even when equivalent experiments are done by different research groups, as e.g. in the lamprey literature. To the extent that our experiments validly report “variable regeneration” they are consistent with the broader literature. One interpretation is that there is naturally a variable but limited regeneration as considered further below.

Another possible reason for the limited regeneration observed is that for some cell types, the proximal tip of the axon may have extended past the transection site into proximal spinal cord but stopped prematurely, i.e. before reaching the distal site where our second tracer was introduced. Varying ability to regenerate deep into spinal cord might explain the overall low fractions of regeneration and might produce differential effects between the nMLF and Ro/Mi cells. This fits with e.g. the inability of the Mauthner to regenerate into distal cord without the assistance of cAMP treatment [Bhatt et al., 2004]. Yet a third reason for the limited regeneration is the amount of time required to regenerate down to myotome 23. But because substantial distal regeneration was seen in some fish at days 2, 3, 4 and 6, while in other fish sparse regeneration is often seen on days 7 and 8, it seems that we were equally likely to see regenerated axons in early days as in later days (Figures 2.4 and 2.5). One possibility is that axonal regeneration occurs
relatively quickly in these young, growing zebrafish and that little additional regeneration would be seen after 8 days. The direct live observations of axonal regeneration reported in the next chapter are intended to further explore regeneration time course.

A last reason why some rostrally labeled neurons cannot be labeled distally is that some neurons’ axons do not project to the distal labeling site. Work by Metcalfe et al. (1986) quantifies the numbers of different RN cell types projecting deep into spinal cord and a surprisingly large fraction of many cell types project nearly the complete length of spinal cord. For the Ro and nMLF cell types a large majority project well into distal cord and were accessible to double-labeling by our technique had they regenerated. In the case of the Mi cell types, however, only about 1/3rd project into distal spinal cord and this makes the low numbers of regenerated Mi cells more comparable to the other regenerating classes. Thus, while we see the largest fraction of regenerating neurons in the nMLF, the variability in regeneration prevents us from concluding a statistical difference in regenerative capability of the different descending cell groups examined here.

While the fraction of descending cell types regenerated, i.e. double-labeled may be small due to technical reasons, our results are in rough agreement with regeneration in adult fishes, and we do not see a regeneration pattern that would suggest that substantially greater regeneration would emerge were much longer time points to be studied. On the whole, we did not observe near complete regeneration of substantial numbers of injured neurons that one might expect for young zebrafish larvae, given the robust activity of developmental processes in this time window. However, this variable regenerative response of the CNS is observed in other organisms. For example, molecular responses to injury in the mature C. elegans model indicates a significant fraction of neurons, including peripheral motor neurons are not able to regenerate in
even wild type animals [El Bejjani and Hammarlund, 2012]. In the Xenopus, functional and anatomical regeneration is observed to occur although this does not fully arise from the original population of neural crest derivatives [Lin et al., 2007], suggesting a modification of the normal development.

2.4.2 Actual Time Course and Extent of Regeneration

The time course of larval regeneration is expected to be faster than adult axonal regrowth. In larval lamprey (which are immensely larger than larval zebrafish), the time course of recovery of regenerating descending axons is seen to reach the center of the transection by 20 days, though coordinated swimming patterns were not found till 6-7 weeks post transection [Yin & Selzer, 1983]. In the case of adult zebrafish, they recover dorsal fin movement within 3-4 weeks, while retrograde tracing studies show that by 6 weeks, neurons of the nMLF, superior and intermediate reticular formation, anterior, descending and magnocellular octaval nucleus and inferior reticular formation are (Table 2.1) able to reach the location of the second tracer injection (13th vertebra) [Becker et al., 1997]. Molecular responses to injury indicate that differential regulation of various growth promoting molecules, rather than intrinsic cell differences may also play a role in the injury response [Becker et al., 1998; Goldshmit et al., 2012]. Based on the present studies on larval zebrafish, what we can say with certainty is that only a small fraction of axotomized cells is labeled in our experiments and this fraction does not change substantially over the 2 to 8 day window examined.

Because the highest instances of regeneration achieved in our experiments agree with those seen in lamprey and in adult zebrafish, this suggests that we have been achieving comparable success in detecting the regenerated neurons and that regeneration in these different
experimental paradigms occurs to a roughly similar extent. Given the variability in our studies, one interpretation is that there is some variable aspect to regeneration in lower vertebrates, although experimental influences cannot be ruled out. The modest degree of regeneration in the larval zebrafish is unexpected given the small distances required to regenerate and the robust developmental state of the larval. It seems that once the sensorimotor control system in zebrafish is “up and running” (5-day old larvae have an extensive locomotor repertoire: Budick and O’Malley, 2000; Borla et al., 2002; Burgess and Granato, 2007; Orger et al., 2008), the regeneration of axotomized fibers occurs to only a limited degree. Bhatt et al. (2004), showed serious deficits in the regeneration of Mauthner axons, but since this publication there has been no further results published on zebrafish larvae. To better understand the nature and effectiveness of axonal regeneration we went on to visualize live regeneration via time-lapse 2-photon imaging (Chapter 3) and accessed behavioral recovery of labeled-lesion larvae (Chapter 4).
Table 2.1 Regenerating cell types. Summary of regenerated cell types observed in the larval lamprey [Jacobs et al., 1997; Davis & McClellan, 1994(b)], salamander [Davis et al., 1990; Duffy et al., 1990], adult goldfish [Sharma et al., 1993], and adult zebrafish [Becker et. al., 1997].
Figure 2.1 Reticulospinal neurons of the larval zebrafish, goldfish and lamprey. (A) 7 cell groups of the larval zebrafish hindbrain. Cells of the nMLF located in the midbrain rostral to the Ro cells are not shown here. [from Metcalfe et al., 1986]. The 2 major fiber tracts (mlf, llf), through which the descending axon of each cell projects are shown. Scale: 25µm (B) Segmental rhombomeres in the goldfish indicating the some of the Mauthner cell at RS4 and the tracts of the nMLF. [from Korn & Faber, 2005] Scale= 200µm. (C) Diagram of lamprey brain indicating the mesencephalic cells (M1-M3), isthmic cells (I1-I4) and bulbar cells (B1-B5). [from Davis & McClellan, 1994a].
Figure 2.2 Segmental homologies conserved in the hindbrain of lamprey and zebrafish [from Murakami, 2004]. Note the position of the Mauthner cell (Mth) in rhombomere 4. Nomenclature of lamprey [Rovainen, 1976; Swain et al., 1993] is as follows: M, mesencephalic; I, isthmic; B, bulbar; Mth, Mauthner; mth, secondary Mauthner. Nomenclature in zebrafish [Kimmel et al., 1982, Metcalf et al., 1986] are as follows: Ro, rostral; Mth, Mauthner; Mi, middle.
Figure 2.3 Time course of regeneration of descending neurons (DN) following transection at the brainstem-spinal cord (BSC) juncture. (A-B) Representative animals with double-labeled cells at different time points: 2 days post-lesion (A), 8 days post-lesion (B). Scale bar: 25µm. (C) Schematic diagram of retrograde labeling of DN via the labeled-lesion method, where axons are (1) labeled and severed at the BSC juncture (myotome 3) and subsequently (2) double-labeled with a second tracer (~myotome 23) for those axons that have regenerated to that site. (D) The number of double-labeled (yellow) DN colocalized, i.e. containing both Alexa 488 (green) and Texas Red (red) is plotted as a percentage of the total number of cells initially labeled with Alexa 488, (n=15 fish, 480 transected cells, 95 regenerated cells). Error bars are shown with minimum and maximum fractions of cells regenerated for given days post transection.
Figure 2.4 Regeneration of nMLF, Ro and Mi cells 2-10 days post transection. (A) Sketch of descending neurons of the larval zebrafish (adapted from O’Malley et al., 2003) indicating the groups of nMLF in the midbrain and Ro, Mi cells in the medulla. Scale: 25µm. (B, C, D) Bar charts indicate the number of descending axons transected (lighter colors) and the number of axons regenerated (darker bar color overlay). Numbers of neurons: nMLF (n=19, 230 transected, 62 cells regenerated = 26.9%), Ro (n=20, 146 transected, 34 cells regenerated= 23.3%) and Mi (n=20, 126 transected, 10 cells regenerated = 7.9%).
Table 2.2 Strong positive correlation in the observed numbers of regenerated neurons.
Comparison of regeneration rates of the descending neurons (nMLF (MeL), Ro and Mi) based on Pearson Product-Moment vs. Spearman Rank correlation coefficients. The value of $\rho$ indicates the strength of relationship between these variables, where if two variables are perfectly monotonically related, $\rho = 1$.

<table>
<thead>
<tr>
<th></th>
<th>NucMLF</th>
<th>MeL</th>
<th>Ro</th>
<th>Mi</th>
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<td></td>
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<td>$\rho$</td>
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<td>0.0591</td>
<td>0.3029</td>
<td>0.0068</td>
</tr>
</tbody>
</table>
Figure 2.5 Differential regeneration rates of descending neurons classes following transection at the BSC juncture. (A-C) Graphs indicate the percentage of regenerated cells over the period of 2-8 days post transection for each cell type. The error bars shown define the minimum and maximum fractions of cells regenerated for each day post-transection.
Figure 2.6 Regeneration of distinct nMLF cell clusters, in 21 larvae. (A) Confocal image of nMLF cluster indicating the position of MeL and MeM cells. Scale Bar: 25µm. (B) Total number of labeled MeL cells (lighter shading) = 149, with 43 regenerated cells indicated by darker shading. (C) 80 MeM cells (lighter shading) were labeled/transected cells, of which 15 were determined to have regenerated (darker shading).
Figure 2.7 Mi cell responses to axotomy. A) Transected descending neurons in the midbrain 1-day post-transection. Zoomed regions: (B) Proximal axon of Mi cell shows punctate labeling and a possible collateral 1 day post-transection; (C) the lower Mi cell has a curved, punctate axon. In (D) the same soma (*) is rounded at 6 days post-transection. Scale Bars: 25µm (A, D), Scale: 10µm (B-C).
Figure 2.8 Responses in nMLF to axotomy. (A) Arrow indicates a possible bulbous end of an axon or dendrite near the soma of an MeM cell 1 day post-transection. (B) At 3 days post-transection different structures are visible. 25µm.
Figure 2.9 Bilateral responses in nMLF post-axotomy. (A) Confocal image of the nMLF cluster, at 1 day post-transection shows labeled MeL and MeM cells. (B-C) Severed axons at 2 days post-transection show swellings: box in (B), right arrow in (C). Bulbous endings are indicated by the right arrow in (B) and left arrow in (C). Scale bars: 25µm (A), 10µm (B-C).
Chapter 3: Dynamics of Injured Axon Endings in the Descending Motor Control System

3.1 Introduction:

Inhibitory factors, such as myelin proteins, extracellular matrix proteins, as well as axon guidance molecules have been indicated as the primary inhibitors to neuronal regeneration in higher chordates, including humans. In contrast to mammals, the fish CNS provides a positive environment for pathfinding and reinnervation of injured axons. Retinal axons in particular have been shown to grow and elongate indicating that the myelin in the adult teleost fish [Bastmeyer et. al., 1991; Wanner et. al., 1995] and amphibians such as Xenopus [Lang, 1995] and urodeles [Mitashov, 1996] permits axonal regrowth. Regeneration in the spinal cord however, has proved a greater challenge to investigators, and work remains in understanding the mechanisms necessary for functional recovery. Ultimately, the reconnection of appropriate spinal contacts is critical for the recovery process, and this in turn relies on the mechanism of axon pathfinding, including the characteristic behavior of severed axonal endings.

Numerous studies have investigated the axonal pathfinding choices of regenerating CNS axons. In developing axons, the propagation that leads to axon collaterals has been described to occur by one of three possibilities: splitting, delayed and interstitial (see below, Fig. 3.4a) [Acebes and Ferrus, 2000]. Di-I-labeling of regenerating axons in adult goldfish optic tectum shows within 4-5 days post-injury that while there are some incorrect paths, individual fiber tracts can be found heading towards the appropriate brachium of the optic tract [Bernhardt, 1989]. These axon endings were categorized into two classes: simple and complex. Simple endings were described to be bulbous without specializations and were prevalent mainly in the optic nerve and tectal layer. Complex endings were observed to be flattened with differentiated
extensions, prevalent mainly in the optic tract and its brachia [Bernhardt, 1989]. Time-lapse microscopy from isolated preparations of the retinal pathway in embryonic mice has further shown that retinal axons have periods of extension and pauses that are dependent on position in varying locales along its developing path [Mason and Wang, 1997]. Similar growth cones have been described in *Xenopus* [Harris et. al., 1987] as having elongated bodies with torpedo-like endings that rapidly move in straight-line paths while during pauses more complex growth cones exhibit irregular shape with filopodia. In primary cultures, pauses of interstitial axon branches were followed by the emergence of newly formed tips [Szebenyi et al., 1998], which was coupled by the movement of microtubules into these newly forming branches. Reorganization of microtubules entering into the new branches are described in Dent et al. (1999) as a process of splaying, which extends the axon and allows microtubules to move forward or backward during branch dynamics.

Landmark studies in the adult mammalian CNS have attempted to document the fate of severed axons and have shown similar morphological features as those seen in other preparations. Simple unbranched axon endings of the regenerating corticospinal tract, labeled with biotin dextran were observed at early time points (6 days post-injury) while at longer time points (3 months post-injury) the cut ends of regenerating axons showed terminal sprouting [Li et al., 1998]. The bulb shaped dystrophic ends of severed axons, originally described by Cajal [1928], have been shown to advance short distances before rounding up and retracting *in vitro* [Tom et al., 2004]. These types of endings typically contained vesicles that were formed at the leading tip of the axon and were resorbed at the shaft and showed an abnormal cytoskeletal arrangement. In these different studies, axonal regeneration was substantially limited.
In contrast to the regeneration of central neurons, Kerschensteiner et al. (2005) published promising findings from long-term in vivo imaging of dorsal root ganglia (DRG) neurons. Using a transgenic mouse with GFP-labeled DRG neurons, they were observed via confocal imaging the behavior of axons in vivo following a spinal transection. Following axonal fragmentation of the distal ending of the central process, forward progress of the proximal segment occurred within 6-24 hours post-axotomy. They described this early growth as having phases of elongation where the axon moves forward rapidly in a straight path, and branching where elongation stalls and numerous lateral outgrowths are observed to form. As in studies of mammalian CNS neurons, however, this pattern of growth in not prevalent for a long period of time and over 24 hours does not allow the transected axons to approach or reach their original spinal targets.

Despite the limits of regeneration, the above studies illustrate the value of different imaging modalities in the investigation of spinal cord injury. Recently, two-photon (2P) fluorescence microscopy has become a powerful tool both for observing neural circuitry and for in vivo optical surgery. This imaging mode allows for deeper tissue penetration compared to confocal microscopy, with better deep-tissue spatial resolution, and reduced photo-damage. With conventional fluorescence and confocal fluorescence microscopy, the excitation light is increasingly absorbed with depth and contrast is also lost due to the scattering of both the incoming and emitted light. In comparison, the two-photon absorption process takes place in only a small volume at the focus of the laser beam and so provides three-dimensional resolution often at depths much deeper than what confocal can achieve [reviewed in O’Malley, 2008].

In the past few years, 2-photon laser axotomy has been utilized in several animal models [O’Brien et. al., 2009; Hammarlund et. al., 2009; De-Miguel et. al., 2002]. In particular, in the
nematode *C. elegans* model, femtosecond pulsed lasers have been used to sever single axons, after which axonal regeneration can be observed [Bejjani and Hammarlund, 2012; Byrne et al., 2011; Yanik et al., 2004; Wu et al., 2007]. Following this approach, we have used the 2-photon laser on the Keck 3D Fusion Microscope (CenSSIS Laboratory, Northeastern University, Boston, MA), to cut either small bundles of axons or single-axons, in living zebrafish, depending on the amount of cellular labeling and the positioning of the cutting laser. This approach also allows us, in some cases, to trace the cut axons back to the reticulospinal cell bodies in hindbrain, enabling us to individually identify the neurons whose spinal axons were severed. After laser axotomy, the 2-photon microscope is then used in time-lapse mode to study the regenerative process.

3.2 Methods:

3.2.1 *Retrograde Labeling of Descending Neurons*

Larvae (4-5 days post fertilization) were anesthetized with 0.02% 3-aminobenzoic acid ethyl ester (MS222; Sigma, St. Louis, MO) prior to an initial spinal injection of a 10% solution of 10,000 MW Alexa 488 dextran (Molecular Probes, Eugene, OR) to retrogradely label reticulospinal cell bodies in the hindbrain. The spinal injection procedure has been described previously [O’Malley and Fetcho, 2000; Gahtan and O’Malley, 2001]. A glass micropipette with tip broken to 15-20 µm was gently tapped into the spinal cord through the lateral axial muscle at approximately the level of the 23rd myotome (90% of body length) and a small amount (2.0nl - 4.0nl) of fluorescent tracer was pressure-ejected into the fish. Larvae are then placed in individual circular wells in 6-well tissue culture plates containing Instant Ocean salt solution and placed in the incubator for 18 -24 hours. This period provides time for recovery and retrograde transport of the dye from the spinal axons to the somas of the reticulospinal neurons.
3.2.2 Two-Photon Laser Scanning Microscope and Axotomy

The 2-photon axotomy methodology was first established as a collaborative effort with J. Kerimo in Prof. Chuck DiMarzio’s imaging facility (at Northeastern University). Later imaging sessions on the instrument were collected with the help of Z. Lai, who also provided the information detailed in Figure 3.1. The 2-photon instrument setup is described below.

A custom-built laser scanning microscope (Figure 3.1A) was used to image and perform the axotomy on the samples. In preparation, retrogradely-labeled larvae were anesthetized and embedded in agarose as described in Chapter 2. A detailed description of the imaging system can be found elsewhere [Warger et. al., 2007], but the relevant components are described here. The system is based on an inverted Nikon microscope with a fast polygonal-galvanometric laser scanning system. The polygonal scanner consists of a mirror with 36 facets that spins at high speed and operates together with a galvo-based scanning system to scan a rectangular area on the sample. The galvo scanner can also be turned off so that the polygonal scanner performs continuous line-scanning at a select location on the sample. In this configuration the line is scanned continuously across the sample. This line-scanning mode of scanning is used for the 2-photon axotomy since laser power levels can be delivered continuously to a single-line with this method. Also, there is less collateral damage with this method than scanning a large area with high laser power. The extent of the line was further limited by placing a mask or iris at one of the intermediate image planes.

A 2-photon laser was used to image the samples and perform the axotomy. The laser consisted of a mode-locked Titanium-sapphire pumped laser system (Tsunami) from Spectra Physics operating with 80 MHz pulse repetition rate. The pulses of the laser were about 100 fs
The laser could be tuned from 700 nm to about 980 nm. This laser was used to image the sample by two-photon excitation and the filtered emission was detected by a photomultiplier tube (Hamamatsu, HC124-02). Typically, 20 mW average power (at the sample) was used to image the samples. After the samples were imaged, the axon was positioned and the scanner was switched to line-scanning mode and the laser intensity was increased to 30-50 mW average power (at the sample). It is important to note that the laser delivers much more energy to the sample in line-scanning than in imaging mode. In one second each pixel is visited 6000 times in line-scanning but only 10 times in 2D imaging mode and so efficient laser axotomy is possible.

2-photon imaging is used to acquire anatomical details of the descending neurons and determine the desired site of axotomy. The region of interest is located on the two-photon microscope by imaging the labeled axons under low, non-damaging power. The iris is then closed down to reduce the size of the line to about 20 microns for the axotomy and a single line was scanned at a 6 kHz repetition rate across the axon using between 700-1000mW of laser power. A CCD camera was used to detect the emitted fluorescence (**Figure 3.1B, 3.2B**) during the axotomy and track the procedure’s progress. The camera (Diagnostic Instruments, Inc., SPOT RT900) was cooled to -28 degrees Celsius and monitored the site of the axotomy. Most of the imaging and axotomy was done using a 20X Plan Apo 0.75NA Nikon microscope objective. This objective had high throughput to the near-infrared laser and could resolve most of the fine axons. We also used a 40X Plan Apo 1.45NA Nikon microscope objective to image more details and cut smaller axons. This objective required more laser power since its near-infrared throughput is not as good as the 20X; it also has a shorter working distance (160 microns).
3.2.3 Calcium Imaging

For calcium imaging, larval zebrafish were retrogradely injected as described previously, but using the fluorescent calcium indicator Oregon Green BAPTA. Descending neurons were identified and the axon of an individual cell then traced when possible to a position in caudal brainstem where laser axotomy was performed. The axotomy was performed as described above but using 600 mW laser power. Calcium responses were recorded using a 20X Plan Apo 0.75NA or 40X Nikon microscope objective with a SPOT RT900 camera operating at 132 frames/sec, using a mercury lamp to collect fluorescent images of the entire field of view. Calcium recording included up to several seconds of baseline prior to axotomy and were analyzed using the ImageJ software (NIH). Brainstem neurons were individually identified after the recording and regions of interest were selected and analyzed to calculate the fluorescence/calcium response, $\Delta F/F$.

3.2.4 Data Analysis

Image stacks were analyzed using MatLab (MathWorks) based software from the C. DiMarzio lab and modified for image analysis. The software allows users to select points (x,y) in each z-plane of an image stack and calculates the change in angle over each frame (see Results). The average of both the positional displacement and angular change were then calculated.

Image Surfer 2 [Feng et al., 2007], a Java based software was used to produce 3D images from the z-stacks collected to produce surface outlines of the imaged planes based on the pixels applied to each point on the x,y, z (volume) grid. The values in this grid are mapped based on a grayscale map. We used the slice rendering method to select a plane to generate a cross-section of interest. This method produces a surface map of the axons in an image stack in a 2D format.
We generate surface maps of varied planes in both the –y, and +y directions from the axotomy (0 axis) based on serial imaging datasets that were collected for each fish. Additionally, Image J and Adobe © Photoshop CS2 were used to crop, and adjust brightness or contrast of images.

3.3 Results:

3.3.1 Response to 2-Photon Axotomy

Traumatic physical injury to the spinal cord leads to the mechanical disruption and degeneration of both ascending and descending axons. In order to monitor the regenerative responses of these severed descending axons we determined the power necessary to precisely sever one or more axons after retrograde labeling with Alexa 488 (Figure 3.1B). This data was collected on the 2-photon instrument in collaboration with J. Kerimo who helped determine the initial parameters for the experiment. Z. Lai, also performed several axotomies and produced the final version of Figure 3.1. We sometimes noted the formation of a bubble on the skin of the fish at the location of the laser beam, which typically receded within 30 minutes. Time-lapse imaging of CNS axons allowed us to monitor laser axotomy and subsequently follow the regenerative response of individual axons in vivo. The structural events visualized and described below provide details of the anatomical response of axons to an injury near the brainstem-spinal cord juncture in a developing larval zebrafish.

3.3.2 Fluorescence and Calcium Responses to Axotomy

After retrograde labeling (Figure 3.2A), axons can be targeted for laser axotomy. When larger axons were cut (Figure 3.2B) it was possible to see an increase in fluorescence due to the apparent release of fluorophores into the surrounding area. This typically occurred within 1-3 min. of the axotomy. For smaller axons we saw a loss of fluorescence signal when the axon was
cut (Figure 3.2C). This was likely due to the retraction of the two ends and diffuse removal of fluorophores from the focal region. The two cut ends of the axon, proximal and distal endings, as we will refer to them here, are typically observed to retract, resulting in a gap following the axotomy.

In some experiments, intracellular calcium responses of neurons were recorded in the course of the laser axotomy. Typically an axotomy is followed by membrane depolarization at the severed end which results in the propagation of action potentials to the soma and a subsequent influx of calcium [Mandolesi et al., 2004]. We examined somatic calcium responses in the soma after laser axotomy of axons projecting into spinal cord (Figure 3.3). The location of an example laser axotomy, performed in caudal hindbrain, is indicated in Figure 3.3A. We were able to observe successive spikes in calcium accumulating in the cell body in cases where we could clearly trace the cut axon to a cell body, as illustrated in Figures 3.3B, C. Note in this example that the Mauthner cell, whose axon was not severed, shows no fluorescence response. This process of electrical signaling to the cell body likely generates numerous calcium-dependent molecular events that could be involved in both the degeneration of the lesioned tracts and also regenerative responses. In a number of cases we did not observe somatic calcium responses after axotomy, but in all such cases the cut axon could not be conclusively traced back to a specific cell body in hindbrain.

3.3.3 Shapes of Axon Terminals Post-Axotomy

The distinct fates of severed axonal endings (Figure 3.4A, B; Acebes and Ferrus, 2000) may depend on the molecular signals which lead to the changes in shape trajectory and motility of the proximal and distal endings. Two processes to be examined here include elongation of the
stem axon and the sprouting of new collaterals from the proximal or distal stumps. While “neuronal sprouting” is often used to describe processes emerging from uninjured neurons, we did not study sprouting from uninjured neurons or axons as they were unlabeled and thus not able to be seen. Our emphasis is thus on the responses of severed axons and we observed that the axoplasm first begins to have a dark appearance (as also observed in Chapter 2), which might result from the breakdown of structural components of the cytoskeleton [Lieberman, 1971]. There also may be swelling or shrinkage of axons as has been observed in the goldfish [Oliphint, 2010]. Though our focus primarily lay in observing the changes in the severed proximal end, we also made observations of the distal endings; in all such cases only clearly identifiable endings were analyzed.

To characterize the dynamics structural events following axotomy, we examined the morphology of different axon endings (Figure 3.4C, D). Proximal endings of severed axons most typically formed rounded, retracted terminations immediately post-axotomy (Figure 3.4Ca). Serial, time-lapse imaging subsequently showed an evolving morphology where conical and bifurcated shapes sometimes formed as with the examples in Figure 3.4Cb and 3.4Cc. Outlines of these endings are shown in Figure 3.4D to illustrate the different morphologies. In a summary of multiple, severed axonal terminals from four fish (Figure 3.5), proximal rounded endings were always seen between 5-15 minutes following axotomy (Figure 3.5A), while proximal conical shaped endings were observed to form as early as 45-60 minutes (Figure 3.5B, C) post-axotomy (note the different imaging time scales for the different larvae that showed regenerative responses). Quantitative analysis revealed over time a preponderance of rounded endings in some fish, while others showed an increase in conical endings over the duration of the
time-lapse imaging. The ends of the distal axon segments were also generally rounded (yellow stacked bars).

3.3.4 Dynamic Responses of Axon Terminals

An example of axonal retraction is shown in Figure 3.6, where the endings of two neurons are observed to retract a considerable distance. The brainstem pre-axotomy is shown in Figure 3.6A, with the location of the axotomy site indicated. After lasing, two axons are seen to retract distances of up to 17µm within 15 minutes post-lesion (Figure 3.6B) one of which has a rounded ending and the other a sharp ending at this early time point (Figure 3.6Cb1). Over the ensuing 6 hour period these two axons were observed to move minor incremental distances (Figure 3.6B, Cb2). In Figure 3.6C, images taken immediately post-axotomy are contrasted with images taken a number of hours post-axotomy. This is shown for the Mauthner cell whose axon has retracted and shriveled (Figure 3.6C a1, and at 8 hours a2) and for the 2 small axons plotted in Figure 3.6B (Figure 3.6C b1 and at 5.5 hours b2). In other larvae, proximal conical or bifurcated endings were observed to have longer trajectories of movement, unlike the bulbar endings that tended to remain fairly immobile or static.

Displacement and angular measurements were made to illustrate retraction and angular movements of severed axons as illustrated in Figure 3.7 and presented in Figures 3.8 to 3.11. The pre-axotomy anatomy of this example dataset is shown in Figure 3.7A, with the region of analysis zoomed in Figure 3.7B. The total distance retracted (between the two tips) and angular displacement between the cut tips are calculated as specified in the figure legend based on the root-mean-square calculation (Figure 3.7B inset). These measurements were intended to quantify axon retraction and elongation distances and to reveal any pathfinding strategies that
might be seen in regenerating axons. **Figure 3.7C** shows several severed axons shortly after laser axotomy, including a thin one (Axon 1) to the left of the thick Mauthner axon and a second thin axon (Axon 2) that appears to run along the top of the Mauthner axon. The dynamics of these severed axons and ensuing collaterals are quantified in the next several figures.

**Figure 3.8** shows the severed endings of Axon 1 (from Figure 3.7) which retracted only a short distance as compared with some other larval axotomies. In this set of displacement plots, the length of the red arrow indicates the total retraction distance while its vertical orientation in the initial plot indicates that proximal and distal segments are oriented on a straight line. Minimal axonal movement distance and angular direction changes are seen over the course of this 4 hour period; the upper dashed arrow indicates the tip of the proximal segments, while solid arrow notes the lower, distal axon tip. After several hours, the two tips are quite close together, raising the possibility that the proximal tip might rejoin the distal tip, but our spatial resolution was insufficient to document any such occurrence.

**Figure 3.9** shows the dynamics of Axon 2. Initially, there was a small movement of about 5 um immediately post-transection, but by 15 minutes the retraction distance had increased to 28 um, after which it showed further slight increase over the next six hours of imaging. These zoomed images show the anatomy of the conical proximal and distal tips; while the distal segment is out of view in several images it is present in the raw images and the distance accurately quantified. The gap between them reaches a maximum of 35 um at 225 minutes. The conical endings of these axon terminals appeared to give rise to fine branches that are quantified in **Figures 3.10 and 3.11**. These figures show one proximal process to the right of Axon 2 that is in the vicinity of two branches that emanate from the distal axonal segment. These processes appear similar to fine filopodia extending from growth cones and show greater angular
orientation than the stem axon segments. The yellow circle in each figure highlights branches from the distal axon stump (near branch in Figure 3.10 and far branch in Figure 3.11) which appear to emanate from a forked branch near the stem axon. While this distal stump was noted to be elongating rostrally, no other instances of this was observed in other distal endings.

Because it was difficult to discern the precise topology of the proximal and distal axon segments as well as their fine protrusions, 3D image and volume analysis was used to attempt better visualization of axon morphology and dynamics. Figures 3.12 and 3.13 show Axon 1 and Axon 2 from the previous dataset with successive time points at roughly 5 and 10 hours post axotomy. Part (A) shows the original data set in reversed contrast, while (B) presents a segmented volume rendering. At particular distances rostral (+) and caudal (-) to the site of the axotomy cross sectional cuts through the rendered volumes were generated as indicated in (C) and shown in (D). Though the axons in this location were tightly intertwined, the 3D renderings provided further visualization of axon endings as illustrated in Figure 3.12A2, B2 where two bulbous distal endings are visible in close proximity to one another. At later time points (Figure 3.13; 24 hours) there appeared to be regeneration of axons distal to the axotomy (A and B form a montage in the rostral-caudal direction); movement of the fish before 24 hours, however, prevented a conclusive identification of the labeled axons.

Another example of regeneration was observed over a 12 hour time window, where one severed reticulospinal axon was clearly descending towards the injury site while an adjacent axon showed no forward movement but rather long pauses with some gradual retraction (Figure 3.14), as indicated by the red and blue arrows/axons respectively (Figure 3.14B, C). The static blue axon had a bulbous ending while the elongating red axon had a conical shape. By the end of this recording (12 hours), the blue axon tip is showing a conical tip but no elongation. In
contrast to the proximal tips, the distal endings primarily retained a bulbar ending, with some undergoing clear fragmentation as illustrated in Figure 3.14C. The results presented in the foregoing examples are representative of the axonal dynamics observed in N=30 fish. While Figure 3.13 was suggestive of axonal regeneration past the injection site, in no other fish were there compelling instances of axonal regeneration into distal spinal cord. Out of 15 proximal endings observed, about 40% showed significant elongation (>3μm) towards the site of laser axotomy. In regards to conical and bulbous we observed 26% percent of conical elongating and 13% percent of bulbous.

3.3.5 Mauthner Cell Response to Axotomy

Given their prominence, we in some cases severed the large myelinated axon of the Mauthner cell, which has been reported in lamprey, goldfish as well as larval zebrafish systems to have a poor regenerative response after a spinal lesion [Becker et al., 1998; Becker et al., 1997; Zottoli and Freemer, 2003]. The well-characterized escape behavior initiated by this neuron, has also been the subject of investigation, wherein cAMP treatment enhanced Mauthner cell regeneration in vivo, and recovery of the escape behavior [Bhatt et al., 2004]. Our observations of the Mauthner axon responses via 2-photon time-lapse imaging reveals a characteristic conical ending of the proximal axon (Figure 3.15) which is capable of extending fine processes within 45 minutes post axotomy. Twenty-four hours post axotomy, this proximal ending was observed to nearly meet the severed ending of the distal axon (Figure 3.15B). However, in most cases when the Mauthner axon was severed, we observed extensive retractions (Figure 3.6Ca1-a2) of both ends of the axon. In these cases, the distal segment was observed to begin fragmentation within 1 hour post axotomy. Following axotomy the cell body of the Mauthner cell was also observed to undergo morphological changes. Previous studies in other
vertebrates indicated that this type of morphological change can occur within a few days to several weeks via the process of chromatolysis [Lieberman AR, 1971].

3.4 Discussion:

These studies provide the first live imaging of axonal and somatic responses to two-photon axotomy in the zebrafish central nervous system. By enabling precise cutting of one or a few axons, 2P-microscopy allowed us to visualize aspects of the response to axotomy that might reflect a basal, intrinsic injury response, given the minimally invasive axotomy technique. This contrasts with other methods that can cause much larger scale damage including damage to vasculature and surrounding tissues. While the 2-photon technique causes damage of its own, the minimal nature of the damage allowed us to see a diversity of injury responses of individual axons (e.g. nMLF in Figure 3.15) that arise from defined populations of descending brainstem neurons. We consider below the implications of the observed injury responses for CNS axonal regeneration and also compare the 2P-axotomy responses observed here with the response to the larger-scale axotomies associated with the labeled-lesion technique in Chapter 2.

3.4.1 Acute and Time-Lapse Response to Focal Axotomy

The aim of this study was to visualize the axonal responses to injury in the teleost descending motor control system (DMCS). What are the dynamic structural events of reticulospinal axons of larval zebrafish following axotomy? These could be: (1) regeneration of axons across the injury site to approach spinal targets; (2) pathfinding of the injured axons to circumvent the injury site and reach appropriate spinal targets; or (3) sprouting of branches from uninjured axons to achieve functional reinnervation. The data presented here documents the
variety and course of initial axonal responses that may precede axonal regeneration into distal spinal cord of the larval zebrafish.

Our serial imaging showed that post-axotomy the proximal axon segments often retract rapidly to become either static or dynamic axon endings, some of which are capable of substantial elongation towards or past the lesion site. The axon terminals took on a variety of shapes including bulbous, sharp and conical endings, as well as branching endings that looked similar to filopodia that emanate from growth cones. The conical shapes formed appeared similar to those observed in regenerating axonal endings in lamprey [Jin et al., 2009]. In some cases these formed soon after axotomy, following an initial rapid retraction. Fine filopodia-like extensions could be seen emanating from the proximal conical endings and might lead to the instances of axonal regeneration observed in Chapter 2. Over time, the endings could go from conical to rounded or vice versa (Figure 3.5), indicating a dynamical flexibility in the proximal stump. We also observed several instances of close apposition between severed descending axons and their distal segments, but whether these could fuse with the distal segment to form a continuous axon, while also avoiding distal fragmentation is not known.

While our data suggest greater regenerative capacity in the conical endings, we are unsure of the intracellular events associated with the “molecular decision” to become static vs. elongating, or the intraxonial mechanisms that lead to the diversity of morphological responses. It is known that cytoskeletal mechanisms, including microtubules and other proteins are critical to growth cone motility, as well as axon elongation and arborization and retraction, as has been studied in a variety of regenerating systems [Ahmad et al., 2000; Kerschensteiner et al., 2005], although there are species differences between e.g. teleosts and lamprey [Roederer et al., 1983; Marsh et al., 1984, Letourneau, 1987; Jin et al., 2009]. One possibility is that there are cell-type
specific regenerative responses, but our limited ability to trace individual axons hinders our ability to make strong inferences on this point. The labeled-lesion data in Chapter 2 also did not provide strong support for cell-specific responses. While the axotomy-induced somatic calcium responses we observed likely contribute to the axonal responses, the small number of neurons we could image again precluded us from making cell-specific conclusions. More work is needed to characterize the molecular and morphological responses of identified, severed descending axons in the living zebrafish.

3.4.2 Efficacy of Axonal Regeneration

Out of many severed axons observed for periods of 6 hours, 12 hours and up to 24 hours, a small number (36%) showed elongation into or beyond the lesion site. This is surprising given the robust developmental state of these larvae. It also seems at odds with the results from Chapter 2, where some instances of regeneration deep into spinal cord were observed within 48 hours. This might be reconciled by noting that there were few instances of early distal regeneration at 2 days, with the preponderance of double-labeling (distal regeneration) being seen in the range of 3 to 7 days, in conjunction with the essentially sparse sampling of descending axons with the 2-photon technique. But there is still the issue of regeneration length: a maximum elongation of about 50 microns was seen after 2-photon axotomy which contrasts sharply with the distance of approximately 2000 um needed to reach distal spinal cord in Chapter 2.

The limited regeneration observed could potentially be explained by an early slow regenerative process, including pauses, that was followed by more robust growth, perhaps once the lesion site has been crossed. In the case of the Mauthner cell, it regenerates quite robustly,
but only after application of cAMP [Bhatt et al., 2004]. It is thus possible that 2P-axotomy does not induce the conditions necessary to stimulate regeneration to the extent seen with large cuts into spinal cord. Nonetheless, the retraction, elongation and branchings seen in the 2P-experiments indicate lively molecular activity within the proximal axon tips. Moreover, there are conditioning effects, where a prior injury enhances the response to a subsequent axotomy [Redshaw and Bisby, 1987; Neumann and Woolf, 1999] which might in principle occur here, where after an initial caudal axotomy (to label the descending axons), the two-photon cut is made rostrally. In the double-label experiments, there was no conditioning effect, but the impact of the large initial lesion might produce an ultimately greater impact on the regenerative processes. A final and (in our opinion) least likely possibility is the resealing of the proximal and distal segments, which could in theory account for early double-labeling in Chapter 2 in the absence of any long-distance growth into caudal spinal cord.

3.4.3 Conclusions

We observed a diversity of axonal responses to focal laser axotomy, which included substantial retraction as well as such regenerative processes as branching and axon elongation. The modest speed and extent of regeneration observed here does not seem sufficient to account for the regeneration observed in Chapter 2 which raises questions about the regenerative process. This is more surprising given the active developmental state of the zebrafish larvae studied here. A further approach to studying larval regeneration is to examine behavioral recovery after rostral axotomy and to characterize it over the time spans of these studies. Behavioral results along these lines are therefore presented in Chapter 4.
Figure 3.1 Schematic of the two-photon microscope configuration. (A) The entire setup is positioned on a vibration-isolated optical table. Major components of the multimodal microscope include: a Nikon inverted TE2000U microscope, a polygonal-galvanometric laser scanner, titanium-sapphire laser, PMT (HC1024-02, Hamamatsu), and a CCD (SPOT RT900) camera. This microscope is interfaced with various optical, electrical and other hardware and software components for optimal image capture. (B) Graph shows the output intensity of the fluorescence signal at peak wavelength of 720nm with increasing laser power. These data were obtained using Alexa 488 dextran, a bright fluorophore with a good 2-photon cross section for these experiments.
Figure 3.2 Method of two-photon laser axotomy. (A) Reticulospinal neurons were first retrogradely labeled with a fluorescent tracer (1). After allowing 24 hours or longer for transport, axons were visualized and small, precise axotomies were produced using a two-photon pulsed laser. Focal lasing severed targeted axons or small axon bundles at or near the brainstem-spinal juncture (2). (B) The position of the laser on the axon is outlined by the box and the outline of one large axon is highlighted to illustrate the magnification and lasing configuration. With larger axons, local leakage of fluorophore can be observed immediately after the lasing process. (C) Plot shows the decay of the fluorescence signal when a smaller axons were severed. The dashed arrow indicates the position where a sharp decay in signal is observed, which is a useful indicator of the time when the axon is severed. Imaging of this location was delayed minimally by 3-4 mins as modifications to the experimental setup was required.
Figure 3.3 Axotomy-induced calcium responses. (A) Confocal image stack in brainstem from which a calcium movie was recorded. The left Ro cell (circled ROI) underwent laser axotomy. Red arrow indicates the position of the laser, where the Ro cell axon was cut. Scale bar is 50 µm. (B) Black line on the graph represents relative fluorescence ∆F/F of the Ro cell over the course of the axotomy which began approximately 100 frames into the recording. The red line indicates the response of the left Mauthner cell, which shows no change, indicating a select effect on the targeted axon. (C) Select frames during the calcium recording of the Ro cell are shown for time points capturing the rise of calcium between frames 117 – 229 and 425 – 536, with an elevated calcium level between frames 600 – 800. 132 frames per second were acquired, which was thus approximately 7.5 msec per frame.
Figure 3.4 Examples of axon terminal morphologies following axotomy. Previous work by Acebes & Ferrus is shown in panels (A) and Jin et al. (B). (A) Axon collaterals: splitting (a bifurcation), delayed (a branching from a previous growth cone) and interstitial (a club-like extension from an axon shaft) (from Acebes & Ferrus 2000). (B) Contour sketch of axon ending (retracting (a), static (b) and regenerating* (c)) observed in larval lamprey [from Jin et. al., 2009]. Our examples from larval zebrafish are shown in (C) and (D). (C) Within 5-15 minutes following two-photon laser axotomy, severed axon endings tended to retract and form bulbous ends (a). Subsequently (1-11.5hrs), some proximal axons formed conical shapes (b), or began to bifurcate (c) as they begin descending towards the injury site; other endings appear completely immobile. (D) Schematic drawings of the 3 axon contours from (C).
Figure 3.5 Quantitative analysis of axon ending morphologies as determined by time-lapse imaging. Axon endings were classified at varying time points ranging from 15 minutes to 15 hours post axotomy (N=4 fish). (A-D) Each plot shows all severed axonal endings from a single fish collected at 15 minute increments and classified in stacked bar charts with yellow indicating distal segments and green proximal segments, which were classified as either round or conical. Branching of axon tips led to increased numbers of terminals in some fish, involving both proximal and distal endings. Conversely resorption of branches and distal segment fragmentation can reduce axon endings. In (D) imaging of distal tips was not possible after 1.5 hours.
Figure 3.6 Rapid retraction of axons following rostral axotomy. (A) Labeling of reticulospinal neurons (prior to axotomy) by caudal injection with Alexa 488 dextran is primarily on the left side of brainstem in this larva. Red arrow indicates the position of the axotomy; inset image of the axon bundles when the iris is closed down to reduce the size of the line to about 20 microns for the axotomy. A single line was scanned at a 6 kHz repetition rate across the axon.

(B) Two small axons retract rapidly within the first 15 minutes following axotomy. Over the next 5 hours there is only minor movement: slight further retraction for axon 1 and elongation for axon 2. Inset image: bulbous terminals are seen at both axon tips at 2 hrs post axotomy. (C) Maximum projection images immediately following axotomy (a1, b1). The Mauthner axon retracted back towards the soma by 8 hrs post axotomy (a2). The two small axons that were severed in the same plane (b1), one with a bulbous end and the other with a thin sharp terminal, are shown again at 5.5 hrs post axotomy (b2).
Figure 3.7 Example laser axotomy of multiple reticulospinal axons at a single focal plane. (A) 2-photon image of reticulospinal cells labeled with Alexa 488 dextran prior to laser axotomy. Circle indicates location of axotomy. (B) Zoom of region before axotomy with inset diagram of distance and angle measurements: (1) Displacement between distal and proximal endings: $\sqrt{\Delta x^2 + \Delta y^2}$. (2) Angle measurement: $\theta = \tan^{-1}(\Delta x/\Delta y)$. (C) Immediately following axotomy multiple axons are severed. Indicated measurements were performed on Axon 1 (positioned to the left of the Mauthner axon) and Axon 2 (positioned on top of the Mauthner axon) in Figures 3.8-3.11. Scale bar = 20µm.
Figure 3.8 Response of Axon 1 to axotomy with subsequent movements over 4 hours. Serial 2-photon images of the severed axon #1 (within yellow oval) are shown. The displacement plots indicate the distance and angular orientation between the proximal (dashed arrow) and distal (solid arrow) axonal segments. The directional heading of the arrow and size denote the angular offset and distance, i.e. movement, of the axon endings relative to one another over 10 successive time points. Scale = 20μm.
Figure 3.9 Response of Axon 2 to axotomy with subsequent movements over 6.75 hrs. This set of displacement plots tracks the movements of Axon 2’s proximal and distal segments, again within the yellow oval with proximal (dashed arrow) and distal (solid arrow) segments indicated in these reverse contrast images. Images were collected at intervals from 15 to 405 minutes post axotomy. In some images the distal ending is outside the zoomed region shown, but was still measured in the original images. Scale bar = 20μm. Note that the zoom factor of the images and the magnitudes associated with the red arrow indicator in the displacement plots are different from Figure 3.8.
Figure 3.10 Axonal branch dynamics between 255 and 585 minutes post axotomy. Serial 2-photon images (yellow oval) with its corresponding angular plot indicate the change in distance between the branched proximal (dashed arrow) and a branched distal (solid arrow) ending. The directional vector (arrow) from the vertical (0º) denotes the relative position (net movement) of the axon endings observed at each time point shown. Scale bar = 20 μm.
Figure 3.11 Axonal dynamics of proximal branch in relation to a second distal branch between 165 and 495 minutes post axotomy. Serial 2-photon images with its corresponding angular plot indicate the change in distance between a proximal branch (dashed arrow) and a second distal branch (solid arrow), inside yellow oval. The displacement plot again indicates orientation between the two branch endings over the time course of the recording. Scale bar = 20 μm.
Figure 3.12 Cross-sections of severed axons from 2-photon z-stacks following axotomy. (A1-A3) Same axons as shown in Figure 3.7. 3D Z-stacks were collected at different time points (0-585 minutes), from which 3D renderings (B1-B3) and cross-sections of different planes (C1-C3; D1-D3) were rendered (see Methods). The distance of the cross section planes are indicated relative to the locus of the axotomy. Dashed arrows indicate the proximal endings and solid arrows the distal endings) of Axon 1 and Axon 2 (A2). C and D: Axonal surface maps and cut planes (A1) of each cross-section are shown with −y (distal to axotomy) and +y (proximal to axotomy) indicated. Scale bar = 20 µm.
Chapter 4: Disturbances of Visuomotor Functioning after Rostral Spinal Axotomy

4.1 Introduction:

The larval zebrafish, for which many genetic tools have been developed, has an elegant suite of visually-guided behaviors, and so provides an excellent model to study disruption of visuomotor behaviors and their functional recovery. The convex shape of the eyes in fish enables them to effectively focus light on the retina underwater. The line of sight in the natural environment of fish, which may include both clear and turbid regions, can influence the path of light entering the eye (Figure 4.1) and so visual processing mechanisms must compensate. The dimension of their pigmented eyes, in comparison to the body of the larval zebrafish, indicates the importance of the visual system in this model vertebrate organism. Strikingly, the zebrafish retina contains the same classes of cells as, and is organized along the lines of, the human retina. Behavioral experiments by Easter and Nicola (1996) showed that the first responses to changes in light intensity in zebrafish larvae are present at 68 hours post fertilization. At 72 hours, larvae are capable of performing eye tracking movements evoked by a striped rotating drum (OKR) as well as compensatory eye movements evoked by rotation of the head (VOR) [Easter & Nicola, 1997a, b]. By 5 to 7 days post-fertilization, zebrafish larvae have a well-developed visual system [Burrill and Easter, 1994; Biehlmaier et al., 2003], one which has proven to be sufficiently functional to meet a variety of pressing ethological needs.

Larval zebrafish have a surprising diversity of visual-evoked behaviors. In addition to OKR and VOR responses, optical patterns elicit important locomotor behaviors such as the optomotor response (OMR), which is important for maintaining orientation and location in the environment [Neuhauss et al., 1999; Orger et al., 2008], visually-guided prey tracking [Gahtan et
al., 2005; McElligott and O’Malley, 2005; Westphal, 2011; Bianco et al., 2011] and whole-field dark- and light-evoked responses, which appear to constitute additional navigational behaviors [O’Malley et al., 2004; Day, 2006, 2008; Burgess and Granato, 2007; Sankrithi and O’Malley, 2010; Mueller and Neuhauss, 2010]. Larval zebrafish also appear to have a visual startle response [Portugues and Engert, 2009], similar to the startle responses in teleost fish that are mediated by reticulospinal neurons [Nissanov et al., 1990; Faber and Korn, 1978; Fetcho, 1992]. The escape responses are initiated by a very fast C-shaped bend away from the stimulus that is followed by a large, rapid counter-bend in the opposite direction [Kimmel et al., 1980; Foreman and Eaton, 1993; Liu and Fetcho, 1999, Budick and O’Malley, 2000].

Because each type of behavior is a kinematically distinct response to a visual stimulus, the larval CNS contains visual processing systems that are able to transform each class of input into the appropriate motor response. These sensorimotor transformations are mediated by 10 retinal afferent fields [Burrill and Easter, 1994], which in turn control the descending motor systems to produce the correct behaviors. While we are only beginning to understand the neuronal mechanisms underlying these transformations [Orger et al., 2008; Severi, 2011], the distinctiveness of each behavior enables efforts to perturb associated neural circuits and to subsequently monitor behavioral recovery.

Light-evoked swimming is a response to sudden whole-field illumination that entails mild caudal bending yielding a forward “slow swim” pattern that can be observed in freely swimming larvae [Day, 2006, 2008; Burgess and Granato, 2007] or partially restrained larvae [O’Malley et al., 2004]. Slow swims, including light-evoked, spontaneous, OMR slow swims and those used during prey-tracking, have low tail-beat frequencies that fit the original range for slow swimming of 25 to 40 Hz [Budick and O’Malley, 2000]. In a fictive swimming
preparation, light-evoked tail beat frequencies of 20-63 Hz were observed [Masino and Fetcho, 2005] but the fastest frequencies have never been seen in any natural behavior other than escape or strike behaviors.

A second behavior, the dark-evoked turn, occurs after a sudden drop in illumination and is striking in that the dark stimulus results in a very large, low angular velocity turn that has been termed the O-bend; the larva’s head can sometimes touch its tail [Burgess and Granato, 2007; Day, 2006; 2008]. These navigational behaviors are easily distinguished based on simple kinematic parameters, and they in turn can be distinguished from the OMR, wherein larvae swim in intermittent bouts along the axis of a large-field moving visual stimulus, such as moving stripes. What all of these behaviors have in common is their relatively slow nature, in which a “slow” swim CPG is engaged, sometimes following a slow (i.e. low angular velocity) turn [Budick and O’Malley, 2000]. Larval prey tracking also uses a slow swim pattern, although the strike or capture swim involves a ramping up of CPG frequency into the fast motor regime (Borla et al., 2002) that underlies the fast bending and burst swimming epitomized by the startle or escape behavior [Kimmel et al., 1974; Gahtan et al., 2002].

The particular kinematics of these different behaviors are thus suitable for (1) disruption via axotomy, (2) assessment of deficits and (3) monitoring of functional recovery. Other kinematic details are important as in slow swimming where the fish alternates its pectoral fins at 18-28Hz [Thorsen et. al, 2004]. Burst swims are very different in that the pectoral fins are tucked against the side and much faster tail beat frequencies (45 to 75 Hz) are observed with larger-amplitude bending of the trunk and much greater yaw (side-to-side motion of the head) as compared to slow swims [Budick & O'Malley, 2000]; Thorsen et. al [2004] reported burst swim tail beat frequencies of 36-67 Hz. Based on these well-characterized details of turning and
swimming behaviors, and the varied responses to photic stimulation, this chapter explores the consequences of brainstem-spinal cord transections of descending motor pathways. In particular, we focus on quantifying behavioral deficits and monitoring recovery of behavior over the course of several days following the injury.

4.2 Methods

Methods for animal husbandry and the labeled-lesion technique were described in Chapter 2, while the 2-photon laser axotomy technique is described in Chapter 3. Additional methods for acquiring and analyzing larval behaviors using a high-speed camera are detailed below.

4.2.1 High Speed Imaging

Each larva was placed in a 35mm petri dish filled with 28°C IO solution up to a 2mm depth. A fluorescent ring light was used to illuminate the field within which the larva was placed depending on the behavioral battery being tested (Figure 4.2). Larvae were allowed to acclimate to light / dark conditions for 10-15 minutes for each video collected (more details below), and the water temperature in the Petri dish was carefully monitored. High-speed videos were acquired using a Redlake Motionscope (PCI-500) circuit interfaced with a charge coupled device camera mounted on a Zeiss dissecting microscope, at acquisition rates of 600 frames per second and imaging area of 658 X 496 pixels (7.4 µm = pixel). Videos were saved as AVI files and analyzed with ImageJ (NIH) software.
4.2.2 Behavioral Assays

Behavioral analysis was performed on larvae beginning on day 5 and up to day 13 post-fertilization (dpf). Larva of this age have a relatively fixed locomotor repertoire [O’Malley et al. 2004], as well as a reliable response to the optomotor (OMR) stimulus [Orger et al. 2000]. The locomotor assays consisted of recording mechanical and visually elicited responses. In addition, we collected any spontaneous behaviors that occurred during these recording sessions to compare with the lesioned animals’ evoked behaviors. The four components of the locomotor battery are as follows: (1) touch-elicited escapes, (2) light-evoked responses, (3) dark-evoked responses and (4) OMR. All behavioral testing was performed between 12 and 48 hours post injury to determine behavioral deficits with subsequent monitoring of recovery up to 9 days post injury. To avoid any perturbation to the circadian rhythm all behavioral assays were performed during the daylight hours between 9AM and 5PM.

The experimental setup was as illustrated in Figure 4.2. Larval zebrafish (4 -10 days post fertilization) were transferred into transparent 35mm circular dishes and experiments carried out at 26 -28°C. An infrared LED (880nm peak) was placed below the larvae, in view of the high-speed camera. The entire apparatus is isolated in a heavy black shroud to prevent any stray light from penetrating the setup. As previous work indicates, larval sensitivity to light drops above 620nm [Brockerhoff et. al., 1995] and therefore should not produce any significant visual stimulation [McElligott, 2005]. Details of the different behavioral assays are as follows:

(1) **Spontaneous Slow Swims**: After acclimation to the lighting for 10 minutes, each spontaneous swimming behavior that consisted of at least three consecutive tail-beat cycles was recorded. The great majority of spontaneous swims are of the “slow” variant [Budick and O’Malley,
2000; Thorsen et al., 2004], but infrequently an apparently “spontaneous” swim bout may occur of the burst or escape type.

(2) **Touch Elicited Escape:** Larval zebrafish (4-10 dpf) were transferred into transparent 35mm circular dishes and exposed to a bright white LED ring light (intensity, approximately 8 W/m², 1000 lux) for 10 min. After the larvae was allowed to acclimate in the dish for 10 minutes, an escape response is elicited with a light tap on the right or left side of the head with a fine, smooth fire-polished glass probe. An interval of at least 2 min and at most 10 min passed between trials.

(3) **Light / Dark Elicited Behavior:** The larva is allowed to acclimate for 10-15 minutes to the swimming arena illuminated with only the IR light (CS 100, Advanced Illumination) from below. To trigger light-evoked responses, the ring light is turned on and the latency to respond measured. Bona fide responses reliably occur in less than one second and any responses at latencies greater than 2 seconds were excluded. Once the arena is illuminated, it remains lit for 10 minutes, after which it is turned off to elicit a dark-evoked response. High-speed video recordings were initialized 300ms before each stimulus is presented and frames are collected for several seconds more at 500 frames/sec.

(4) **Optomotor Response.** A pattern of light and dark bars was projected outside the arena and moved at a speed that reliably elicits the OMR.

(5) **Prey Capture:** Fish were acclimated to the dish for 10 minutes with the arena illuminated before Paramecium culture was added. Videos of prey capture were collected and reviewed later using *Image J*.
4.2.3 Behavioral Analysis

Quantitative measurements are based on kinematic features that characterize the performance of each behavior. Listed below are measurements that were used to analyze behaviors from our experimental and control groups of animals. All behaviors acquired as components of the locomotor battery were analyzed either using NIH Image J, and/or by direct, manual measurements. The measurements of these behaviors are based on those previously used to characterize zebrafish behaviors [Budick and O’Malley, 2000; Day, 2008].

(1) Spontaneous Slow Swim:

a. Tail Beat Frequency- The tail-beat cycle is the time required for one complete cycle of bending of the larvae’s right and left sides.

(2) Touch-Elicited Escape:

a. Latency was measured based on the time difference (msec) of the frame indicating the light tap to the head to the frame when the fish begins to move away from the probe for the escape behavior.

(3) Visually-Evoked Swim:

a. Latency was measured based on the time difference (msec) of the frame indicating the light was turned ON to the frame when the fish begins to move either its head or tail.

b. Tail Beat Frequency- The tail-beat cycle is the time required for one complete cycle of bending of the larvae’s right and left sides.
c. Yaw- The side to side movement of the head during forward swimming is measured as the angle between the direction of travel of the larva’s center of mass and the maximum deflection from that angle, on each half cycle of swimming, of a line drawn between midpoint of the swim bladder and a point on the midline between the two eyes. X-Y coordinates of the center location between the eyes, midpoint of the swim bladder, an arbitrary location on the trunk and the tail tip of the fish were first determined using the “Manual Tracking” macro [http://fiji.sc/wiki/index.php/Manual_Tracking, by Fabrice P. Cordelieres] in ImageJ. A linear fit through the movement of the swim bladder was first determined. The yaw angle was then measured between the fit line and the direction of the larva’s head for each half of the swim cycle using a code written in Matlab®. Manual visual analyses of videos were performed to confirm the accuracy of the automated measurements.

d. Swim velocity- X-Y coordinates of the center position between the eyes and the tail tip were first determined using “manual tracker” macro in ImageJ. The velocity of both the head and the tail tip movements were plotted, which provides information about both forward swimming and turning, as presented in the Results section.
(4) Dark-Evoked Turn:

a. Latency was measured based on the time difference (msec) of the frame indicating the light was turned OFF to the frame when the fish first begins to move either its head or tail.

b. Tail Beat Frequency- measured as in (3).

c. Swim velocity- measured as in (3).

4.3 Results

Our primary objective was to evaluate specific behaviors of the larval zebrafish that could be analyzed for functional deficits. Using high-speed video (Figure 4.2), we recorded touch-evoked escapes, spontaneous swims and visuomotor behaviors in fish following “labeled lesions” whereby varying numbers and subsets of axons of reticulospinal neurons (RNs) were severed. This transection method (Figure 4.3A) serves to retrogradely label and sever axons and was done near the brainstem-spinal cord (BSC) juncture so as to disconnect descending axons from their spinal targets (Figure 4.3B). In some fish, descending axons are not transected, and so we use such fish as “spinal-only” control fish (Figure 4.3C); having labeling of only spinal neurons near the injection site, they serve as a control for the damage ensuing from the spinal injection. A second control group was naïve, age-matched fish that had never been injected. To assess the performance of lesioned animals performing visuomotor behaviors, response latency and the tail beat frequency were quantified.

To first ascertain the overall responsiveness of the experimental animals we recorded touch elicited escape responses (Figure 4.4) which is characterized by a high angular velocity
followed by a burst swim [Budick and O’Malley, 2000]. We noted that a subset of fish (N=6 of 25 total experimental fish; Group A) with descending axotomies where the pooled (L and R) escape latencies, ranging from 5-127 ms for the group, showed at least one escape latency that was far above the naïve control average (4.43 ms). The majority of experimental animals tested (N=19) showed latencies more similar to the controls, including the spinal-only control animals, which showed a range of escape latencies from 9.5-16.1 ms. There were several animals (as in Figure 4.3B) in which the Mauthner cell’s axon had been severed and which may have contributed to the long latency responses, but there was not a simple one-to-one correlation between cut Mauthner axon and escape latency on that side of the fish. Long latencies were observed in fish with both Mauthner axons cut but ranged in values from 10-fold longer than normal to near-normal latencies. In other fish, long latencies were observed despite both Mauthner axons being intact. Overall, despite the long latencies in some fish, the experimental fish executed well-formed escape responses. We next examined responses to photic stimulation.

4.3.1 Dark-Evoked Responses

An example labeled-lesion larva with axons from multiple cell groups severed is shown in Figure 4.5A. This was one of a group of larvae that did not show dark-evoked turns, but most lesioned animals responded to the dark-evoked stimulus. We will first consider behaviors of those fish that did respond by producing dark-evoked turns. For these larvae there was an overall distribution of dark-response latencies (Figure 4.5B) that appeared relatively similar to that of the two control groups, naïve and spinal-only (Figure 4.5C). The control groups had an overall range of latencies between 45-790 ms, while the experimental group showed a similar range of values and all fit well with previous latency measurements reported by Burgess and Granato [2007] and Day [2008]. We did statistical comparisons of latencies between the 3
groups and, for the experimental group showing responses, between different time epochs, Days 0 – 1 post-lesion vs. potentially recovered animals between days 2 – 9 post-lesion, but found no significant differences amongst those fish showing appropriate dark-evoked responses.

The principal deficit found was a complete absence of dark-evoked responses for 5 fish, and an initial failure to respond by an additional 12 fish. To further investigate these potential deficits in the dark-evoked turn (DET) response, we averaged all trials for each individual animal performed per day and sorted the data points (columns) from earliest to latest days post transection, shown from back to front in the column plots (Figure 4.6A). We displayed this data by grouping fish, from left to right as: no dark-evoked responses (**, 5/33 fish), consistent dark-evoked responses (16/33 fish) and those with recovered dark-evoked responses (*, 12/33), i.e. that showed no response between 0-1 day post axotomy but then recovered a normal response, with this latter group showing latencies ranging from 67 to 837 msec. Of the 5 fish with no response, 1 died and was excluded from further analysis. The other 4 fish were capable of other behaviors including escape behaviors and light-evoked swims, so the failure to respond to dimming illumination was a selective failure to respond to a particular visual stimulus. The most normal of the lesioned fish, those consistently responding to a dark stimulus, had latencies of 53 to 708 ms, again overlapping the controls. While we cannot statistically quantify the differences between no-response and those fish/epochs with measured response latencies, both of these two lesioned groups (recovery, no recovery) were conclusively different from the control groups, because both the naïve and spinal controls consistently responded to the dark stimulus.
4.3.2 Light-Evoked Responses

The same group of labeled lesion animals was tested for light-evoked (LE) responses (N=31). Figure 4.7A shows a labeled-lesion from a fish that showed decreasing (~improving) latency to a whole-field illumination increase. In comparing the latency of light-evoked responses between lesioned fish and controls, there were no marked differences. There was substantial overlap in the latencies for fish with transected axons (30 to 450 ms on day 1; 30-642 ms on later days) (Figure 4.7B) and the control groups (30-520 ms, naïve; 30 – 313 ms, spinal-only) (Figure 4.7C). A comparison of the aggregate latencies of the lesioned fish with the spinal controls indicated shorter latencies in the controls (*p<0.02, two-tailed test, assuming unequal variances), but the overlap between each of these groups and the naïve controls is substantial, and so substantive conclusions cannot be drawn from this.

As was done for the dark-response dataset, we averaged the light-evoked responses collected on each day for each animal, and sorted these averages according to days post transection in the column plot in Figure 4.8A. Only two (N230, N174) of the 31 lesioned fish were noted to be initially unresponsive (day 1) to light-on following the axotomy. A total of 5 fish were unresponsive on days 6 or 7, though several of these animals responded to trials up to 9 days post-transection (Figure 4.8A). Figure 4.8B shows the responses of the two control groups and they had similar response profiles, with a number of fish showing consistent short latencies in both groups. Note also the difference in scale between the experimental animals (A) and the control groups (B). While a few experimental animals had quite long latencies, this was not sufficient to produce a statistical difference that held for both control groups.
4.3.3 Relative Performance of DET- Impaired Fish on Visuomotor Tasks

To further evaluate the visuomotor responses of larvae after spinal axotomies, we compared the DET and LE responses of fish that were noted to have a transient DET deficit (0-1dpt) (Figure 4.9). Figure 4.9A shows the dark-evoked responses sorted into groups with decreasing latency (a) and increasing latency (b) of DET. To more directly illustrate the relative performance of the two behaviors, we plot in Figure 4.9B the LE responses of fish with either a total absence of DETs (left columns) or those with an initially missing DET response: same a, b groups as in (A). Comparison of these two kinematically different behaviors indicates that animals impaired in producing DETs were quite capable of performing LE responses. A variety of individual patterns were observed, e.g. fish N166 initially lacked DETs, and then showed long-latency dark responses, yet showed consistently short-latency LE responses over 4 days of testing. In Figure 4.9C, the LE responses of animals with a transient DET deficit are replotted and sorted between animals with consistently short latencies (a) and those with variable latencies (b). It is clear that a subset of fish with DET deficits have consistently good light-evoked responses, indicating a differential deficit.

Figure 4.10 shows an anatomical example of a larva with a large number of disconnected reticulospinal cells (imaged 9 days post transection). This larva had an initially absent DET followed by decreasing latency in the DET; it also showed moderately short latencies on 3 of the 4 days in its LE responses. Overall, it was not possible to unambiguously correlate behavioral deficits with specific neuroanatomical details because of difficulty in obtaining good anatomical images in the course of behavioral testing across the recovery period.
Optomotor responses (OMR) were also examined in a number of fish in the overall labeled-lesion group, and some abnormal OMR behaviors were observed. Control fish and most experimental fish performed the OMR in a normal fashion, turning and swimming along the motion axis of the moving stripe pattern. A small number of experimental fish swam along the wall of the dish, with a few swimming along the wall in the opposite direction, an aberrant behavior not seen in the control groups. Proximity to the wall made it difficult to obtain quality measurement of kinematic details, so the parameters of yaw, velocity and tail-beat frequency analyzed for the LE and DET behaviors were not measured for the OMR behaviors.

4.3.4 Tail-beat Frequencies of Visually-Evoked Responses

Swim patterns were examined for control and experimental larvae after eliciting dark- and light-evoked responses. Tail-beat frequency is a defining measure of the fundamental forward swim patterns, burst and slow. All swim bouts observed after photic stimulation were of the slow variety, as illustrated in Figure 4.11. We compared the tail-beat frequencies of the DET and LE responses of experimental animals (Figure 4.11A) with those recorded from the two control groups (Figure 4.11B). While the labeled-lesion fish showed a fairly normal slow swim pattern, some larvae had unusually low tail-beat frequencies (overall ranges: 13-35 ±5.99Hz LE, 17-27 ±1.52Hz DET) in comparison to controls (spinal only: 20-29 ±3.05Hz LE; 20-33 ±6.97Hz DET; naïve age-matched: 20-33 ±3.16Hz (LE); 21-31 ±6.02 Hz (DET). Over the course of 2 – 9 days post transection, these animals were observed to have tail-beat frequencies (20-38 ±3.90Hz LE, 20-35 ±3.49Hz DET) that matched the controls, suggesting a recovery of normal swimming patterns.
Other behavioral abnormalities were observed in some experimental larvae, for example at rest 40% of fish with labeled-lesions were observed to be leaning to one side (N= 47) unlike spinal-only controls and naïve larvae which did not exhibit this behavior. Interestingly, within 24 hours of the rostral axotomy, this leaning was no longer observed. We also observed swim bouts with unusually large yaw, as illustrated in Figure 4.12. Yaw, a side-to-side movement of the head, is a generally unavoidable byproduct of forward swimming and quite pronounced in the case of larval burst swims. In contrast, in the much gentler slow swim, yaw nearly disappears.

To quantify the side to side movement of the head in experimental animals we calculated the forward trajectory of the larvae and measure displacements of the head to either side (see Methods and Figure 4.12 for details). Successive movements of the larval fish, as part of a light-evoked response, are illustrated in Figure 4.12A. The darker lines indicate three successive orientations after two small turns (pre-turn1, post-turn1, post-turn2). The thinner lines indicate side-side movements during the forward movements in between the discrete turns, which are larger than in the control fish. In Figure 4.12B, vectors were drawn based upon the current heading of the fish, i.e. based on a line drawn between the midpoint of the swim bladder and the midpoint between the two eyes. Yaw is the deviation of current heading from the general direction of motion of the fish (measured only during forward swimming). The offset between these two lines is plotted in Figure 4.12C and shows peaks representing successive peak displacements from the direction of travel as the head moves to the left, to the right and back to the left again. The degree of yaw ranged from $0.2138^\circ$ - $10.31^\circ$ with an average yaw of $4.3^\circ$ over this swim bout. These yaw angles are quite large in comparison to those observed during normal
slow swimming (0.56°) [Budick and O’Malley, 2000] and were seen in 13 out of 33 fish, many of which showed the transient postural deficit.

Velocity profiles of the head and tail of the fish provide another measure of swim performance. **Figures 4.13 and 4.14** illustrate these parameters for experimental and control fish during light and dark evoked turns. Light-evoked responses are shown in **Figure 4.13**, with (A) and (B) showing the swim pattern of a larva one and two days post-lesion respectively. The normal, brief rhythmic bouts of movement seen in (B) are less prominent in (A), although in both cases the tail movement (red line) is quite pronounced in relation to the head movement (blue line), which fits with the larger movement of the tail in relation to the minimal yaw of the head during forward slow swims. **Figure 4.13 C and D** show an even more pronounced difference for another fish with an extended bout of trunk movement on day 0 (an abnormality seen in previous labeled lesion studies [Day et al., 2005] while on day 7 there were 3 well formed swim bouts. Both of the experimental fish showed dark-evoked turn deficits. The control animals shown in **Figure 4.13 E and F** (naïve, spinal-only) show a well-formed pattern in (E) and a modestly extended swim bout in (F).

**Figure 4.14** illustrates the head and tail velocities of fish performing dark-evoked turns. In these cases the head movement (blue), relative to the tail movement (red) is much more pronounced as compared with **Figure 4.13**. This is because the dark evoked turn consists of an initially very large but low angular velocity turn that reorients the fish, presumably to avoid the darkened environment. The two experimental fish in (A) and (B) show well-formed O-turns, but (A) is somewhat unusual because of the succession of them. An experimental fish that showed no obvious deficits performed DETs well on days 1 and 7 (C and D, respectively). The naïve (E) and spinal-only (F) controls showed similar dynamics as the experimental animals: there is
an initial blue peak (O-turn) followed by a series of tail-movements (red), as the O-turn is typically followed by a slow swim.

The varying subsets of transected cells in this series of experiments were imaged on the confocal microscope (Table 4.1), generally near the end of a behavioral testing period, so as to reduce the mortality of fish that accompanies the stress of confocal imaging. Because of frequent mortality and other issues impeding imaging, we were in many cases unable to obtain the morphology corresponding to a specific larva’s behavioral deficit. A number of confocal brainstem images are shown for different example larvae, as in Figures 4.3B, 4.5A, 4.7A, and 4.10 but the limited number of images precluded assignment of specific behavioral deficits to specific sets of identified neurons or specific cell clusters. The labeled-lesion method produced unilaterally or bilaterally, varying numbers and sets of transected cells in the midbrain (nMLF) and as well in the hindbrain (Mauthner array, Ro, Mi and T-reticular) and while we cannot assign them to specific behavioral deficits, we can conclude that all of the spared or normal behaviors could be performed in the absence of descending commands from these cells.

4.3.6 Behavioral Testing of Zebrafish Larvae Following Laser Axotomy

In concert with analyzing the behavior of labeled-lesion fish, we also examined behaviors of larvae that had undergone 2-photon laser axotomy. While many fewer axons are severed with this technique, it may still be possible to produce observable deficits, given the deficits associated with laser ablation of small numbers of brainstem neurons [Liu and Fetcho, 1999; Gahtan et al., 2005; Orger et al., 2008]. Two-photon laser axotomy has the additional benefit of being minimally invasive. Using high-speed video, we recorded touch-evoked escapes, and spontaneous slow swims of fish following a 2-photon laser axotomy where we severed varying
numbers axons of reticulospinal neurons (RNs), ranging in number from a single axon to approximately 5 axons (Figure 4.15A, B). The intent was to detect deficits and monitor recovery of these two distinct behaviors that spans the range from simple to high-performance locomotor behaviors. Larvae (4-12dpf) were studied after small subsets of descending neurons were disrupted. Behavioral testing on these fish was performed starting the day of the axotomy (day 0) and up to 72 hours post injury (N=12 transected fish).

Behavioral analysis of the escape behavior focused on latency, an important aspect of the startle response. The latency of each escape response captured was averaged and plotted in relationship to the number of days post transection. In comparing touch-evoked escape responses of fish that had transected axons (Figure 4.15C) we observed a small number of latencies with 48 hours of the axotomy that were considerably longer than observed in wild-type fish, where latencies are often <5 ms [Liu and Fetcho et al., 1999]. Though we could not identify with certainty all reticulospinal cells that were transected in these animals, we were able to identify that either one or both the Mauthner cells had been transected in these larvae. Secondly, in terms of the slow swimming behavior, a modest difference appeared between the day of axotomy (day 0) and 24 hours post axotomy. In comparison to tail-beat frequencies of normal fish at this age (18-28Hz), the tail-beat frequencies of transected fish (Figure 4.15D and 4.16A) were slightly smaller ranging from 16.6 Hz to 21.7 Hz. In addition several of these axotomized fish had noticeable yaw, (n=4) within the 24 hours post axotomy period, complementing the yaw findings from the labeled lesion fish.

Lastly, we attempted to evaluate the prey capture capability of the 2-photon lased animals (Figure 4.16B). This behavior shows an interesting maturation over the first few weeks of development [Westphal, 2011]. The kinematics of prey capture has been previously described in
6-8 days post-fertilization larvae as having the following observed characteristics: (1) an orienting J-turn and forward swimming (2) a capture swim followed by a halt before proceeding to another prey item [Borla et al. 2002, McElligott & O’Malley, 2005]. The orienting turns are separated by intermittent pauses, constituting discrete tracking maneuvers, and these were observed in the axotomized animals as was the capture swim (Figure 4.16B), so no clear deficits were observed. As bilateral ablations of nMLF neurons (MeLr and MeLc), had previously been shown to affect prey capture [Gahtan et al., 2005], we wanted to also assess whether or not transecting larger subsets of axons from the nMLF using labeled-lesions would yield substantial deficits. But it proved difficult to obtain prey capture events within the time frame available to collect the visuomotor and other data on impaired and recovering animals, so we did not pursue this aim further.

4.4 Discussion

The work presented here characterizes the impairment and recovery of visuomotor kinematics of larval zebrafish following a transection at or near the brainstem-spinal cord juncture. Analysis of individual fish from high-speed recordings allowed us to observe fine aspects of motor control that might otherwise have been missed. Comparing axotomized animals to two control groups, which included spinal-neuron limited injured animals, revealed that after transection of varying subsets of descending axons, subtle yet significant motor deficits occurred primarily in the 24 hour period following the injury.

4.4.1 Primary Deficits and Recovery of Visually-Evoked Behaviors

Zebrafish larvae exhibit acute motor responses as a result of an abrupt change in illumination. When lights are turned off, larvae may turn directly towards an extinguished light
or execute an O-bend in response to whole-field dimming. Kinematic analysis of the dark-evoked turn revealed slow, large-angle turns [Burgess and Granato, 2007; Day, 2006; Day, 2008]. In contrast, after lights are turned on, larvae perform a slow swim that is sometimes initiated by a routine turn—two basic motor patterns [Budick and O’Malley, 2000] that are now known to be used in a variety of different behaviors. While the underlying mechanisms of these visuomotor transformations remain undetermined, our first aim was to understand how the severing of subsets of reticulospinal axons affected the larva’s ability to generate appropriate maneuvers given a specific visual stimulus.

We observed normal large O-bends triggered by a dark-stimulus (light OFF) in 48% of larvae within one day of axotomy. However, 51% of transected animals indicated a DET deficit, showing no responses within the 24 hours following the transection. These animals subsequently recovered within 1-2 days of the axonal transections. Light-evoked motor responses were, in contrast, minimally impacted following the labeled lesion. Unlike the significant numbers of animals that were unresponsive to DE trials, animals responded with either slow swims or bends to abrupt increase in light (LE), though 38% of these responses were delayed by more than 150ms. Visuomotor behaviors depend upon visual information relayed via a multisynaptic pathway which involves ON-center and OFF-center bipolar cells and a variety of amacrine and retinal ganglion cell types [Schiller et al. 1986; Dowling, 1987; Rockhill et al., 2002; MacNeil et al., 2004], which collectively provide the information needed to produce specific behaviors in response to different patterns of light and contrast. Recent work has shown that the MeL (medial-lateral) cells of the nMLF respond to a whole-field illumination increase [Sankrithi and O’Malley, 2010]. Given the nMLF’s involvement in other visuomotor behaviors [Gahtan et al., 2005], it may also play a role in the dark-evoked response. Based on the observed responses to
visual stimuli following transection of varying subsets of DNs we hypothesize that there are differing subsets of neurons within a common neural circuit which mediates the responses to both dark and light stimuli.

The swimming and turning maneuvers underlying visually-evoked responses have been previously assigned to slow and fast categories, in agreement with the suggestion that there are two functionally distinct central pattern generators (CPGs) in the larval spinal cord [Budick & O’Malley, 2000], which could be controlled by separate descending motor pathways. This is in agreement with the tail-beat frequencies observed for dark-evoked and light-evoked responses, even in spinally-transected animals. However, low tail-beat frequencies of these animals during the 24 hours following transection also indicates a perturbed state of the spinal cord circuitry, which was not observed in our spinal-only controls. This was also evidenced in animals with smaller numbers of descending axons transected following the more precise 2-photon axotomy. The sequence of alternating motor patterns resulting in undulatory swim movements [Fetcho, 1992; Hale et al., 2001] of the trunk has been explained previously by the increased firing of active and the recruitment of new motor neurons [Henneman, 1957]. Studies describing patterns of activity observed in identified interneuron types [Sillar and Roberts, 1993; Ritter et al., 2001; Bhatt et al., 2007; McLean et al., 2007; McLean et al., 2008] presents mounting evidence towards the differentiation of slow vs. fast movements. The diminished tail-beat frequency we observed as a part of the early injury response may in part be explained by disruption of the neural pathways controlling the recruitment of MCoD interneurons, which have been demonstrated to coordinate the movement of slow swims [McLean et al. 2007].

Finally the postural defects and enhanced yaw evident in a group of these spinal-axotomized animals also suggests the impairment of descending pathways including
vestibulospinal neurons and the premotor neurons controlling the pectoral fins which act as stabilizers, rudders and brakes in the context of different locomotor behaviors. Altered yaw has previously been observed in labeled-lesion studies of the prey capture behavior [Day et al., 2005] where it seems that maintaining a precise heading is computationally demanding in the context of producing a rapid strike. More generally, minimization of yaw is desirable because it makes forward swimming less efficient; it appears that our lesions impaired descending commands that contribute to this function.

4.4.2 Conclusion

In this study, we presented data which suggests that there may be different groups of cells involved in the neural circuits that control motor responses to different visual stimuli (Table 4.1). These circuits likely have common components which allow activation of slow swim patterns but they also require distinct subsets of descending neurons to produce selective deficits in different visuomotor responses, such as OMR, LE swimming and DETs. Further detailed examination of these altered behavioral patterns following transection of DNs is necessary to help understand the underlying circuitry involved in the visuomotor control of this important vertebrate model organism. The base of knowledge that we continue to acquire with regards to the neural control of various locomotor behaviors in zebrafish system may allow us to better identify the patterns of cells recruited to illicit a visuomotor response.
Figure 4.1 Possible pathways of light entering the eye. From Guthrie DM and Muntz WRA (2nd Ed. 1993 Chapman & Hall) this diagram illustrates different sources of light entering the eye, including effects of light scattering and absorption. Shown are: a) light overhead; b) scattered light from below; c) scattered light from particles; d) scattered light from object within the water; e) same as (d), but light is absorbed; f) same as (c) with longer light path; g) same as (b) with shorter path; h) same as (b and g) with reflectance from the water. Zebrafish larvae integrate a wide variety of light sources in an often complex environment in order to perform a range of distinct visuomotor behaviors.
Figure 4.2 Behavioral configuration for high-speed imaging. Schematic diagram of visuomotor recording setup with high-speed imaging camera. A fluorescent ring light was used to illuminate the field within the dark box to test visuomotor responses. This light could be turned on or off, while a continuous infrared light source below was able to capture videos of larval behaviors in both the light and the dark. The elicitation of distinct light- and dark-evoked behaviors indicates that the infrared light source did not impair the sensory stimulation of these larvae.
Figure 4.3 Labeled-Lesion Method. (A) Schematic diagram of retrograde labeling of descending neurons via the labeled lesion method where axons are severed at or near the brainstem-spinal cord juncture (myotome 3) and the axons simultaneously labeled. (B) Example of fish with numerous labeled cells in the reticulospinal system (nMLF, Ro, Mi and T-reticular) after injection with Alexa 488 dextran. A Mauthner axon stump is visible (arrow) in this 9 days post transection animal. (C) In some fish, only spinal neurons are labeled in a localized region bracketing the injection site. These larvae serve as “spinal only” controls because the descending axons are not labeled. The position of the micropipette injection is evident in labeling seen in muscle myotomes through which the pipette passed before hitting the spinal cord, as indicated by the arrow.
Figure 4.4 Latencies of touch-evoked escape responses. Responses of fish (N=25) 0-8 days following axotomy showing latencies for taps to the right and left sides of the fish. Group (a) shows larvae that exhibit very long latencies, up to 127ms. Group (b) shows fish with shorter latencies, ranging from 4-19ms, but even some of these are long by normal larval escape standards, and possibly involve non-Mauthner pathways. For example, fish N224 had both Mauthner axons severed, yet showed escapes of under 20 ms latency. In fish N212 (group a), both Mauthner axons appeared intact (unlabeled), so the lesion interfered with the escape by some other means.
Figure 4.5 Latency of Dark-Evoked Turns (DET). (A) Axotomized cells of fish that lacked any dark-evoked response, imaged at 9 days post-transection (dpt). Anatomy is shown in Figure 4.3B. In (B) the responses of axotomized animals (N=33) are sorted into two time periods of 0-1 dpt and 2-9 dpt. Response latencies were 53-552ms and 60-606ms for the two groups respectively, though there were several animals that were not responsive to trials between 0-1 dpt (N=12). (C) Control groups: naïve (6-15 dpf, N=10) and spinal only (1-4 days post lesion, N=9). Responses of both naïve (45-788ms) and spinal only (45-790ms) animals showed a similarly broad range of latencies.
Figure 4.6 Distinct DET deficits in 3 groups of lesioned larvae. (A) Example of the dark-evoked turn by a 7dpf naïve control showing the O-bend, with the head nearly touching the tip of the tail. (B) Injected animals were grouped according to latency response. Each bar indicates the average of trials for each dpt ranging from (0-9 dpt). A single * indicates animals with a transient absence of DET at 0-1 dpt, but that subsequently recovers. Larvae indicated by a double ** never responded to the dark stimulus. The remaining larvae exhibited similar DET responses as controls. (C) Control groups: naïve (5-15 dpf) and spinal only animals (0-4 days post-lesion), with each bar representing the average response for each day.
Figure 4.7 Latency of light-evoked swims. (A) Brainstem image of axotomized cells in a fish with a decreasing latency (30ms - 400ms) to light-ON. Larva was imaged at 6 dpt. (B) Responses of experimental animals (N=33) were sorted into two time periods of 0-1 dpt and 2-9 dpt. Response latencies ranged from 30-450 ms and 30-642 ms respectively. Some animals with no responses on 0-1 dpt (N=3) are indicated. (C) Control groups: naïve (6-15 DPF) and spinal only (1-4 DPL) animals indicated a similar range of short to long latencies.
Figure 4.8 Range of LE latency responses following transection. (A) Example of light-evoked swim that is preceded by a small routine turn (7dpf naïve control). Light-ON typically elicits a forward slow swim that is sometimes preceded by a routine turn. (B) Experimental animals are grouped according to response latency. Each bar indicates the average of trials for each dpt ranging from (0-9 dpt). Asterisks indicate animals that were grouped based on DET responses. (C) Control groups: naïve (5-15 dpf) and spinal only animals (0-4 dpl), with each bar representing the average response for each day. Latency of response for both control groups indicates a range of latency period for each animal. Note that fish N174 showed no light-evoked response, but did show a dark-evoked turn, but died before further data could be collected. N230 initially failed to respond but subsequently responded; this same larva also showed an initial absence of dark-evoked response but later performed one.
Figure 4.9 Comparison of light and dark evoked responses in select groups of experimental animals. (A) Larvae are shown with a transient absence of dark-evoked turns. Upon recovery, DETs were observed; they are sorted into two groups: a) increasing latencies over the (2-9) days post axotomy, b) those with decreasing latencies. (B) Light evoked responses are shown for the same groups as sorted in (A), as well as for four additional larvae that failed to show any DETs. (C) Light-evoked responses sorted by performance. Two groups of behavioral patterns over the 0-9 trial period are seen: a) relative stable latency periods, b) decreasing latencies. Arrows indicate an example fish with transected axons, presented in Figure 4.10.
Figure 4.10 Transected reticulospinal cells of a fish imaged 9 dpt. (A) Numerous cell groups (nMLF, Ro, Mi and T- reticular) of the reticulospinal system were axotomized and labeled. (B) Z-stack of the right nMLF (MeL & MeM cells) group showing punctate labeling in a variety of structures. (C) Punctate labeling of severed axons (bracket) is seen near the crossing of Mauthner cell axons. The nature of the structures with punctate labeling is often difficult to determine.
Figure 4.11 Tail-Beat Frequency (TBF) following visually-evoked responses. TBFs are shown for the swims accompanying dark-evoked turns and light-evoked responses. (A) TBFs for experimental animals (N=20). (B) TBFs for control animals: Naïve (N=14) and Spinal Only (N=9).
**Figure 4.12 Example of exaggerated yaw following labeled lesion.** (A) Movement of the fish initiated by a light-evoked stimuli, indicating two turns and the yaw of the swim bout following turn 2. (B) Angular measurements made based on the best fit line (dashed) drawn through the middle of the swim bladder (red circles) to the position of the head (arrowhead). X-Y coordinates indicate the movements based on the original position of the fish prior to the presentation of the stimulus. (C) Yaw of swim bout 2, average yaw = 4.3082°. The peaks in yaw represent maximum deflections in the side-to-side movement of the head.
Figure 4.13 Analysis of larval head and tail velocity profiles after light-evoked stimuli. Red indicates tail movement while blue tracks movement of the head. The swim bouts and/or turns are interspersed with periods of rest. (A-B) Velocity profiles of swim bouts of fish N200 are plotted for 1 and 2 days post transection. (C-D) Velocity profiles of swim bouts of fish N223 plotted for 0 and 7 days post transection, where an abnormally extended bout of swimming is evident at day 0. (E-F) Velocity profile of swim bouts for a naïve control (7 dpf) (E) and a spinal only control (1 dpt) (F). Peak velocity responses to LE stimuli in control animals ranged from (naïve: 6-7dpf: head 65.3-43.8ms; tail 142.5-152.2ms, spinal-only: head 29.9-36.9; tail 87.6-120.3ms), while the range of peak velocities for transected animals indicated larger variations (0-1dpt: head 20.6-186.7ms; tail 116.2-389.4ms, 2-7dpt: head 38.1-76.1ms; tail 137.2-284.8ms).
Figure 4.14 Analysis of larval head and tail velocity profiles following dark-evoked stimuli. Large-angle, low velocity O-turns are evident here as an initial spike in the blue trace. Typically a single movement bout is elicited with the red peaks indicating the rhythmic tail movements of slow swimming. (A-B) Velocity profiles of swim bouts plotted at 6 days (fish N200) and 7 days (fish N223) post-injection, for fish that had no DET on days 0-1 dpt. (C-D) Velocity profiles of swim bouts (N226) plotted for 1 and 7 days post transection. (E-F) Velocity profiles of swim bouts for a naïve control (7dpf: head 264.0 ms; tail 302.7 ms, peak velocities) (E) and a spinal only control (1 dpt: head 65.3 ms; tail 140.6 ms, peak velocities) (F). The velocity of transected animals’ ranges from (0-1dpt: head 112.7-no response time (ms); tail 204.0-no response (ms); 6-7 dpt head 26.9-138.7ms; tail 47.5-213.1ms), including those which initially indicated an infinite response time.
Figure 4.15 Behavioral deficits following 2-photon axotomy. (A-B) Projections of descending neurons in fish prior to laser axotomy. The box indicates the approximate location of the axotomy. (A1) Image of severed axon endings of other neurons that are bulbous at 1 day post axotomy. (B1) Retracted proximal ending of Mauthner cell ~1-2 hrs following the axotomy. (C) Latency responses of right and left escapes. At 0-2 days following axotomy shorter latencies ranged from 8-12ms and longer latencies from 22-75ms. (D) Slow swim tail-beat frequencies of fish between 0-4 days following axotomy ranged from 16-21 Hz. The data point at 60 Hz is probably a burst swim, perhaps caused by a vibration or other stimulus not evident to us.
Figure 4.16 Examples of spontaneous slow swim and prey capture of fish following 2-photon axotomy. (A) An example of the altered spontaneous slow swim episodes observed in transected fish, observed between the day of axotomy to 4 days post axotomy. This kinematically well-formed but slow tail-beat frequency was captured ~1-2 hours following the axotomy. (B) Prey capture of transected fish 2 days following axotomy. Asterisk indicates position of paramecium that was captured. Arrow shows pectoral movement to orient towards target. J-turns used to orient towards the Paramecium are seen twice in this episode at 367ms and 610ms, while a strike (capture swim) is in progress at 825ms and engulfment of the paramecium complete at 898ms.
Table 4.1 Descending neurons axotomized via labeled lesion technique. Numerous cell groups were axotomized and imaged using confocal microscopy 8-9 days post transection. Larvae are grouped according to their dark-evoked response following axotomy (A) transient DET deficit with subsequent recovery (B) consistent DET response (C) no DET response.

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Table 4.1 Descending neurons axotomized via labeled lesion technique. Numerous cell groups were axotomized and imaged using confocal microscopy 8-9 days post transection. Larvae are grouped according to their dark-evoked response following axotomy (A) transient DET deficit with subsequent recovery (B) consistent DET response (C) no DET response.
Chapter 5: Conclusions and Future Directions

Our overall findings of a limited regenerative capability, one that involves many different descending pathways, with possible cell type specific differences, are in general accord with much research on regeneration in lower vertebrates. In particular, we see modest regeneration of Ro, nMLF and Mi cell types, which largely fits with response of neurons located in potentially homologous brain regions in the lamprey. Another prominent aspect of our findings was the variability in regenerative response, which was true for individual axonal responses after 2-photon axotomy, and for the regeneration of individually identified neurons and clusters in the labeled lesion experiments. While the variable regeneration seen between larvae and at different time points could reflect uncontrolled aspects of the experiments, innate variability is perhaps suggested by some work in lamprey where different groups report widely different outcomes as e.g. Yin and Selzer [1983] vs. Davis and McClellan [1994]. There are many stochastic processes inside living cells, e.g. the states of transcriptional regulators, and if such processes result in variable injury response, they might be good candidates for therapeutic interventions. The ability to individually identify neurons inside living animals, while not fully realized in the present experiments, offers an opportunity to study with single-cell precision the regenerative capabilities of the roughly 40 types of descending neurons in the larval zebrafish.

Perhaps the most surprising outcome was the limited and (generally) slow axonal regeneration seen in young, seemingly healthy lesioned animals. While the anatomical data in Chapter 2 was acquired from different cohorts of larvae than the behavioral studies in Chapter 4, we find a majority of fish tolerating the rostral axotomy well in terms of survival and in their performance of a broad locomotor battery. The least damage occurs during 2-photon imaging experiments (Chapter 3) and it was in these experiments that larvae showed the most limited
regenerative responses. As discussed in Chapter 3, there are unresolved issues regarding the
time course of regenerative processes, as well as the conditions needed to elicit robust
regeneration, but the anatomical data as a whole present a limited and slow regenerative process,
especially given the active and rapid developmental state of these young larvae.

Outside the regeneration field it is widely held that lower vertebrates regenerate well, a
perception perhaps fueled by the remarkable regenerative capabilities of the salamander
[Chernoff, 1996], but our data indicate that once central neural pathways are formed and
functioning, there are major barriers to regeneration that affect all vertebrate animals, young and
old alike. Even though anurans (larval frogs) and reptiles have been shown to have functional
regeneration, the limitation and imperfections of spinal cord regeneration within these organisms
[Egar et al., 1970; Filoni and Bosco, 1981; Russo et al., 2004; Lin et al., 2007; Rehermann et al.,
2009] also indicates the variable regenerative response of descending axons within the spinal
cord across species. In addition, the injury paradigm and location within the CNS (Table 5.1)
may be an influential factor that suggests a variable response to injury [Font et al., 1991].

While we expect molecular differences between the larval and adult CNS environments,
the larval zebrafish seems to provide an important opportunity for examining identified neuron
regeneration in the face of both molecular level and tissue level barriers. DNA microarrays
could prove useful by revealing between-cell differences if gene expression profiles can be
compared between identified neurons. Similarly, comparisons between species (salamander vs.
zebrafish) and between tissues (glial scar vs. healthy neuropil) could further bolster efforts to
enhance axonal regeneration in larval zebrafish and in other model organisms. The small size
and transparency of the larval CNS would be especially useful in terms of observing immediate
consequences of molecular manipulations.
The complexity of descending motor commands in the larval zebrafish is suggested by both its diversity of cell types and its diversity of behaviors. In many cases, conserved locomotor maneuvers are reused in a variety of behaviors, e.g. routine turns occur spontaneously and in the context of OMR and light-evoked turns. This is especially true of the slow-swim pattern which is used in at least 5 distinct behaviors. We observed only mild deficits of these basic motor patterns, namely a slowing of the slow-swim CPG, which is potentially a non-specific effect in our experiments in that it may not have involved specific descending neurons, but reflected instead the condition of individual larvae. This is in contrast to effects on behaviors produced by distinct sensorimotor transformations, namely the loss of dark-evoked turns by larvae that could still produce light-evoked swims. While technical issues precluded anatomical determination of the exact cells involved, it must be the case that distinct visually-triggered commands (via light or dark carrying pathways) must be relayed to the appropriate descending neurons and the correct behavior implemented. Our lesions must have interfered in these instances with the O-turn control pathway. Lesion studies are, however, limited in their ability to reveal precise roles or even the general computational strategy involved, but future studies involving optogenetic manipulation will allow cells to be turned on and off at will, especially in transparent larval zebrafish! A better understanding of the basic mechanisms by which distinct larval behaviors are implemented would complement studies aimed at finding the molecular determinants of axonal regeneration.
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<th>Adult regeneration</th>
<th>Ependymal tube formation</th>
<th>Successful neurogenesis</th>
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*Tail/caudal spinal cord regeneration is profiled across different vertebrate species. In birds and mammals, information from spinal cord lesion was used in lieu of the caudal spinal cord regeneration. ? denotes no known information.

Figure 5.1 Variable regenerative responses in vertebrates (adult and larvae). From Tanaka and Ferretti 2009.
References


