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ENHANCEMENT OF NEURONAL REGENERATION BY OPTOGENETIC
CELLULAR ACTIVATION IN C. ELEGANS

by

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ENHANCEMENT OF NEURONAL REGENERATION BY OPTOGENETIC CELLULAR ACTIVATION IN C. ELEGANS

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ABSTRACT

Large numbers of people suffer from nervous system injuries and neurodegenerative diseases each year, with little success in regaining lost neural functions. Attempts to successfully regenerate nervous tissue in the mammalian Central Nervous System have meet with limited success. Simpler models have thus been useful in determining conserved mechanisms in the enhancement of neural regeneration. One such mechanism is intracellular calcium signaling. We used Caenorhabditis elegans as a model system to study the effects of optogenetic stimulation on regeneration. Using a femtosecond laser we cut individual C. elegans axons in vivo and then periodically stimulated the neurons by activating the genetically encoded light activated channel, Channelrodopsin-2. We found that periodic photo-activation could increase regeneration over 24h by at least 31%. We repeated these experiments with dantrolene treatment and in unc-68(e540) mutants to assess the effects from a lack of internal calcium ion
signaling in these worms. In both cases, we found a complete suppression of stimulated regeneration when calcium signaling was blocked. This indicates that intracellular calcium ion signaling is crucial in the initiation of neural regeneration in the first 24 hours and mediates the enhanced outgrowth we observe with periodic photo-activation. The importance of intracellular calcium ion signaling can lead to further studies to enhance the stimulation of neural regeneration, and improve therapies for patients with neural damage and loss of neural functions.
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Introduction:

In the United States, approximately 10 million people suffer from Central Nervous System (CNS) injuries and neurodegenerative diseases. Due to the unique nature and anatomy of neurons, physical trauma to the axons effect not only the local cell structures but also cell bodies that may be located large distances away from the injury site. Thus recovery may be particularly complex in these situations. While the Peripheral Nervous System (PNS) can recover from traumatic injuries, the mammalian CNS does not regenerate (Fig 1) (Case & Tessier, 2005).

Fig 1. Figure taken from Case & Tessier-Lavigne, 2005. Transection of the spinal cord of an adult mouse. Red arrow indicates transection location of
an adult mouse. Eight weeks after this spinal cord transection, there are still no axons that have grown past the lesion.

To address this deficiency of the mammalian nervous system, much research has been conducted in animal models on the topic of neural regeneration. Vertebrate models, such as mice, have been used to identify many molecular factors that influence and regulate regeneration of axons (Verhovshek et al., 2012). However, these experiments are typically time consuming and difficult to conduct. Additionally, the complexities of the vertebrate systems limit what can be studied in a highly controlled environment.

*Caenorhabditis elegans:*

In this study, we used the nematode *Caenorhabditis elegans* (*C. elegans*) to study the initiation of neural regeneration after traumatic injury. The *C. elegans* nervous system provides numerous advantages in regeneration studies. *C. elegans* has been extensively studied in various research efforts and has been well documented with respect to its development on a cellular and subcellular level (Berstein et al., 2012). The nervous system of *C. elegans* contains 302 neurons. These neurons are also well documented with respect to their location and time frame of development. Furthermore, the nervous system has also been well studied as a network of connections between different neurons (White et al., 1986). The simplicity and the already present knowledge available for the *C.*
_elegans_ nervous system model provide a powerful foundation for neural regeneration studies.

Although the nervous system of _C. elegans_ is simple in comparison to vertebrate nervous systems, there still exist significant similarities. Certain sensory neurons in the head and tail of _C. elegans_ do not have regenerative properties, much like neurons in the CNS of many vertebrate animals (Gabel, 2008). However, many motor neurons in _C. elegans_ do regenerate, resembling capabilities present in the vertebrate PNS. The presence of both regenerating and non-regenerating neurons in the same animal may provide opportunities for understanding the differences in these capabilities in more complex vertebrate animals.

**Calcium Signaling:**

Through studying the _C. elegans_ nervous system, much has been discovered regarding the mechanisms of axonal growth. During development, axonal growth is guided by structures called growth cones, which are fanlike cone shaped structures located at the growing ends of developing axons (Tojima et al., 2011). The growth cones respond to extracellular cues and steer the axon toward a specific target.

In order for the growth cones to function properly, many intracellular mechanisms must also be present. One of particular importance is the role of calcium ions (Ca$^{2+}$). Within a growth cone, an increase in the intracellular Ca$^{2+}$
concentration activates second messengers inside the cell, such as cyclic adenosine monophosphate (cAMP) molecules. Activation of cAMP decreases the intracellular concentration of cyclic guanosine monophosphate (cGMP) molecules through reciprocal inhibition (Tojima et al., 2011). Consequently, an increase in the ratio of cAMP to cGMP molecules is observed, leading to the activation of ryanodine receptors (RyRs). RyRs are receptor channels that facilitate further release of Ca\(^{2+}\) from intracellular stores in the endoplasmic reticulum (ER) in response to the intracellular increase in concentration of Ca\(^{2+}\), a process termed calcium-induced calcium release (CiCR). This additional increase in Ca\(^{2+}\) drives the formation of more cAMP (Fig 2), inducing axonal growth (Tojima et al., 2011).
Fig 2. Figure taken from Tojima et al., 2011. Diagram of the intracellular mechanisms of axonal steering. (a) Repulsive cues and attractive cues,
such as Netrin 1, SEMA3A, and NGF, stimulate receptors on the cell membrane to activate Ca$^{2+}$ pumps on the surface of the membrane to start pumping Ca$^{2+}$ into the cell. Depending on whether the signal is repulsive or attractive, either cGMP or cAMP is also activated by the cell surface receptors. Stimulating signals eventually activate the RyRs and increase intracellular Ca$^{2+}$ concentration. (b) cGMP and cAMP inhibit each other’s formation through reciprocal inhibition. An attractive cue will thus increase the ratio of cAMP to cGMP and a repulsive cue will decrease that ratio. Through this mechanism, repulsive cues can suppress the growth of axons and attractive cues can induce the growth of axons.

Through this mechanism, external cues can either stimulate axonal growth, or suppress it. By navigating through a matrix of these cues, axons are suppressed from growing in a wrong direction and guided toward growing to a proper target (Tojima et al., 2011).

Although the focus of our study is on the initiation of axonal growth, the stimulation of growth is part of the steering mechanism and is important in understanding the mechanisms for the initiation of regeneration after a physical damage to axons. In response to a physical damage, axons send out projections led by growth cones in search of the distal end of the cut axon (Gabel et al., 2008). These growth cones are similar in function to the growth cones present during development. The stimulation of axonal growth is facilitated by RyRs,
through releasing the intracellular stores of Ca\(^{2+}\) into the cytoplasm (Tojima et al., 2011).

Ca\(^{2+}\) signaling is also thought to be important in the initiation of regeneration. Pinan-Lucarre et al. studied the effects of the CED-3/CED-4 molecules of the caspase pathway. They concluded that these molecules are necessary in the initiation of axonal regeneration and the effectiveness of these molecules in the initiation mechanism is dependent on Ca\(^{2+}\) signaling. When Ca\(^{2+}\) signaling was disrupted through manipulation of the gene encoding calreticulin (which is necessary for Ca\(^{2+}\) homeostasis in the endoplasmic reticulum), there was suppression in the amount of regeneration and success rate of reconnection of axons (Pinan-Lucarre et al., 2012).

**Optogenetics:**

To aid in these studies, many tools have been recently developed and applied to neuroscience. One very versatile and useful set of tools involves the use of optogenetics, where light activated molecules are used to dynamically manipulated the cell. Optogenetic tools allow very high precision control of experiments through the use of light as a trigger (Berstein et al., 2012). Genes for light sensitive molecules can be incorporated into the *C. elegans* genome, and placed under the control of specific promoters so that they can be expressed only in targeted cells and cellular structures (Berstein et al., 2012).
The light sensitive molecule we used is a 7-transmembrane protein, called Channelrhodopsin-2 (ChR2). ChR2 translocates cations from one side of the membrane to the other as a response to blue light stimulation (Fig 3) (Berstein et al., 2012).

Fig 3. Figure taken from Berstein et al., 2012. Diagram of the ChR2 molecule. Upon stimulation by light, ChR2 transports cations, such as H\(^+\), Na\(^+\), K\(^+\), and Ca\(^{2+}\) from the extracellular environment into the cytoplasm.

The light intensity required to elicit a response from ChR2 is low enough that such light will not disrupt normal activity of neighboring cells, and is safe for the organism as a whole (Berstein et al. 2012).
Fig 4. Figure taken from Berstein et al., 2012. Physiological response to stimulation by light through ChR2. The depolarization of the cell membrane illustrates that ChR2 activates and depolarizes a cell immediately upon light stimulation (arrow). Furthermore, the depolarization is stopped almost immediately upon the termination of light.

Activation of ChR2 upon illumination (Fig 4) by blue light with a wavelength of 480 nm allows the depolarization of the cells that contain this molecule (Berstein et al., 2012). Once the light stimulation ceases, ChR2 inactivates, terminating depolarization as well. This system would also require the use of the chromophore all-trans-retinal (ATR), which could be supplemented in the diet of *C. elegans* easily (Berstein et al., 2012). When incorporated into neuronal axon membranes, ChR2 allows very specific temporally controlled electrical stimulation of neurons simply by shining blue light on the entire worm body.

**Femtosecond lasers:**

Another valuable tool used in this experiment are femtosecond lasers used to cut the axons of specific neurons. Traditional lasers produce continuous intensity beams to disrupt tissue, which could cause thermal damage affecting a large number of cells (Gabel, 2008). While recent developments have allowed the effective use of traditional lasers in producing focused injury to *C. elegans*
axons, femtosecond lasers remains as the standard in subcellular laser microsurgery (Williams et al., 2011).
Fig 5. Figure taken from Gabel, 2008. Diagram of a femtosecond laser system. (a) A continuous laser beam is emitted from the pump laser into a Ti-sapphire pulsed laser. The pulsed beam then travels through the pulse picker reducing the pulse rate to 1kHz. The laser beam is then directed into the compound microscope, which focuses it to a diffraction-limited spot at the target point. (b) The frequency spectrum of a continuous laser vs. pulsed laser. The broader spectrum of the pulsed laser generates distinct pulses of light. When focused to a small spot these extremely short pulses generate very high peak intensities with relatively low total pulse energy.

Femtosecond lasers produce very short, approximately 100 femtosecond pulses of light (Gabel, 2008). When focused to a very small spot, these pulses generate high intensities at that specific focal point, but allow for lower pulse energies during delivery compared to traditional laser systems (Fig 5). This system allows for the disruption of tissue only at the focal point, leaving the rest of the animal unharmed. The small focal point targeted by this system allows for the disruption of tissue on a submicron scale, allowing for much more refined and precise manipulations (Gabel, 2008).

In *C. elegans*, the application of such a system has tremendous implications. The branches of a neuron are approximately 300 nm in diameter (Gabel, 2008). The properties of femtosecond lasers not only allow for the cutting
of individual axons on such a scale, but also allows for the cutting to be performed through the tissue (Gabel, 2008).

Fig 6. Figure taken from Gabel, 2008. Femtosecond laser axotomy and subsequent regeneration. (i) Image of a C. elegans neuron with an axon projection from the cell body connecting to the ventral nerve cord. (ii) Using femtosecond laser, the neuron was cut without any damage to neighboring tissues, also leaving the rest of the neuron in tact at a precise
location on the axon. (iii) The remaining neuron sends out regenerated axonal projection in search of the ventral nerve cord.

Axons located greater than 50 µm from a tissue surface can still be cut with minimal damage to the surface tissue as well as the rest of the neuron and neighboring cells (Gabel, 2008). The properties of femtosecond lasers also allows for the continued monitoring of the neuron of interest throughout the entire procedure (Fig 6). The precise nature of such a system also allows for not only the cutting of individual axons, but also allows for the cutting at specific chosen locations (Gabel, 2008).

Genetics of *C. elegans*:

Another set of tools useful in these experiments is the genetics of *C. elegans*. *C. elegans* can be genetically modified relatively easily compared to vertebrate models (Fay, 2006). There are already numerous mutants with gene functions that have been suppressed, allowing the study of the effects of those genes. A specific set of mutants that is of particular importance to us is characterized by an uncoordinated movement in those worms (termed unc mutants) (Fay, 2006). We used the null mutant *unc-68*, which is lacking in the function of the allele *unc-68(e540)* encoding RyRs, the calcium release channel in the ER membrane involved in the facilitation of CiCR. These mutants allow us
to directly assess the effect of suppressing the internal release of Ca$^{2+}$ into the cytoplasm.

Another reason that the genetics of *C. elegans* is a useful tool is that foreign genes can also be introduced to the genome with relative ease. The genes for ChR2 can be incorporated into the genome driven by the promoter mec-4, for example, to allow the light stimulation of the ALM neuron at a specific stage of development of *C. elegans* (Suzuki et al., 2003). The gene for molecule mcherry, a red fluorescent protein, can also be introduced into the genome to allow us to visualize the neuron during surgery and re-imaging stages of this study.

**Current Study:**

The combination of optogenetics, femtosecond lasers, and the powerful *C. elegans* nervous system model including the genetic tools allow for very intricate study of the mechanisms behind neural regeneration.

With these tools and in the *C. elegans* model, we were able to study regeneration in the ALM neuron of young adults. The ALM is a mechanosensory neuron, with the cell body in the mid-body with an axon running toward the head of the animal in close proximity to the hypodermis and cuticle (Wormatlas, 2012). The ALM neuron has been documented as having regenerative properties after in the adult stage (Gabel et al., 2008). The location of the neuron also allows for
easy identification and precise cutting with minimal damage to the surrounding tissue and neurons.

The ChR2 protein was expressed in the ALM neurons to aid in electrical stimulation by optogenetics. The ChR2 gene was placed under the control of the MEC-4 promoter, which normally drives the expression of an ion channel gene targeted to the membrane of axons (Suzuki et al., 2003). This allowed the expression of the gene producing the ChR2 molecule in the ALM neurons and for the molecule to be targeted to the axonal membranes, giving us precise control of electrical stimulation over the course of 24 hours after the axon was cut. By measuring the growth of axons after this period, we set a baseline for the average regeneration rate in wild type animals.

To study the effects of Ca$^{2+}$, the worms were treated with dantrolene, a chemical agent that blocks RyRs (Kitigawa et al., 2003). By blocking the receptor that facilitates the release of internal stores of Ca$^{2+}$, we can suppress the large increase in the intracellular concentration of Ca$^{2+}$ after physical damage. We can then observe the effects this has on neural regeneration after 24 hours.

To further study the effects of Ca$^{2+}$ signaling, we also performed experiments with the mutant unc-68, which is deficient in the RyRs gene in the ALM neuron. By removing the receptor, we can block the release of Ca$^{2+}$ more robustly than with dantrolene, while at the same time removing any undetected side effects from the drug.
By targeting the RyRs and therefore suppressing the intracellular release of Ca\(^{2+}\) \textit{in vivo}, we hope to study Ca\(^{2+}\) signaling more directly than before. Following the theory that increases in the intracellular concentration of Ca\(^{2+}\) leads to axonal growth, the suppression of that intracellular concentration of Ca\(^{2+}\) should lead to a suppression of regeneration. Thus our hypothesis is that RyRs and the subsequent intracellular release of Ca\(^{2+}\) are necessary in the initiation of axonal regeneration. If the activity of the RyRs are suppressed and no intracellular release of Ca\(^{2+}\) occurs, then any observed successful stimulation of axonal regeneration should disappear.

Through comparisons of the different regenerative rates between the wildtype and mutant animals, we can assess the effects that Ca\(^{2+}\) signaling has on the initiation of regenerative capabilities in the ALM neurons in \textit{C. elegans}.

**Methods:**

\textit{C. elegans} with ChR2 expressed in the touch neurons were grown overnight on agar petri dishes seeded with \textit{Escherichia coli (E. coli)} OP50 and 10 \(\mu\text{M}\) ATR. Young adults were then isolated, making sure each worm did not contain more than 4 eggs. The worms were mounted onto 10% agarose gel, and then immobilized with polystyrene beads. Using femtosecond laser, one of the ALM axons was cut twice, once at 20 \(\mu\text{m}\) from the cell body and another time at 40 \(\mu\text{m}\) from the cell body (Fig 7). The axons were cut twice to reduce the rate of reconnection within the 24 hour period that we were observing these outgrowths.
After surgery, the worms are allowed to recover in NGM buffer, onto agar petri dishes with *E. coli* OP50 as the food source. These worms were placed in a light free environment for 90 minutes before the start of blue light stimulation.

Fig 7. Diagram of the cut points on an axon. Axon was cut using femtosecond laser at two points, one at 20 µm from the cell body and another at 40 µm from the cell body.

After 24 hours from the time of surgery, the worms were imaged to obtain pictures of the regenerated axons. At this time, worms were mounted on 2% agarose plate with 3 mM sodium azide to immobilize them. Worms were then imaged with a fluorescent microscope.

The outgrowths were measured using ImageJ software. Then subsequent calculations and statistical analysis were done with the Statistical Analysis Systems (SAS) software version 9.3. Using SAS, the ImageJ measurements in
pixels were converted to microns. Then all branches less than 5 µm were excluded from the analysis to exclude any small axonal projections that may be hard to visualize or that may not be due to stimulated regeneration. A total outgrowth for each worm was then obtained by calculating a total of all branch outgrowth measurements from one axon. A mean and standard error from the outgrowth measurements of all the worms with the same experimental condition were calculated. Means between different conditions were compared with an analysis of variance (ANOVA) test, with pair-wise comparisons tested using a Tukey adjustment.

This procedure was repeated with various control conditions: mCherry worms; ChR2 worms not grown with ATR and with no light stimulation; ChR2 worms not grown with ATR and with light stimulation; ChR2 worms grown with ATR but with no light stimulation. These control conditions were compared to the experimental conditions: ChR2 worms grown with ATR and with light stimulation.

For these control conditions and the experimental condition, blue light with a wavelength of 480 nm and an intensity of 175 µW/mm² was used for stimulation. Light stimulation pattern consisted of light delivery of 10 seconds out of one minute, while for the other 50 seconds of the minute no light was delivered. This 10-seconds-on/50-seconds-off pattern continued until 24 hours from the time of surgery.

Various light intensities were also used to observe varying amounts of outgrowths. These light intensities consisted of 125, 150, 175, and 200 µW/mm².
To further experiment with different stimulation patterns, light duration was also manipulated. Stimulation patterns used included light intensity at 175 µW/mm² shining for 20 seconds and off for 100 seconds, lights on for 10 seconds and off for 50 seconds, lights on for 2 seconds and off for 10 seconds, and constant light with intensity of 29 µW/mm² (175 µW/mm² / 6 = 29 µW/mm², so that the total amount of light over one minute was the same). These patterns were designed so that the total amount of light delivered to the worms is the same at the end of the 24-hour period, and the ratio of the duration of time that the lights were on to the duration of time that the lights were off was kept at 1 to 5.

To test the effect of Ca²⁺ signaling, we treated ChR2 worms grown on ATR with 10 µM dantrolene dissolved in dimethyl sulfoxide (DMSO). The illumination intensity and pattern of the light stimulation was kept at 175 µW/mm² with the 10-seconds-on/50-seconds-off pattern. The dantrolene experiments were also performed without light stimulation as controls. This experiment was also repeated with unc-68 mutant worms grown on ATR with the same illumination intensity and patterns as well as without any light stimulation for 24 hours.

We also performed behavioral assays on the ChR2 worms grown in ATR, ChR2 worms without ATR, ChR2 worms grown in ATR and dantrolene with DMSO, and ChR2 worms grown in ATR and DMSO without dantrolene. These behavioral assays were used to ensure the quality of the ATR and dantrolene, and that the intended effects on the worms are as expected. In the behavioral
assay, the worms were placed on a fluorescent microscope. If they showed a reversal of crawling direction within three seconds of the onset of illumination of light (7.25 mW/mm²), then that worm was considered as a positive response. Afterwards, a Fisher-Irwin test was performed to determine the statistical significance of the difference between the proportions of positive responses in the various conditions and their respective control groups.

The specific strains of *C. elegans* used in this study include: Pmec-4::mCherry; Pmec-4::mCherry, Pmec-4::ChR2:YFP; and Pmec-4::mCherry, *unc-68(e540)*.

**Results:**

The results from various experimental conditions were statistically analyzed in four different groups. The first group tested the growth rate of regenerated axons in ChR2 worms grown with ATR and light stimulation of 10 seconds of lights on and 50 seconds of lights off pattern against various controls (Fig 8). These controls included wild type mcherry worms without ChR2 molecules, ChR2 worms without ATR or light stimulation, ChR2 worms with light stimulation but no ATR, and ChR2 worms with ATR but no light stimulation. Images of the axons from these control and experimental conditions with light stimulation and without stimulation are shown in Fig 9.
Figure 8. Average outgrowths of regenerated axons. From left to right, the averages (and standard errors represented by vertical bars) were 150.93 µm (15.7), 136.78 µm (12.08), 166.32 µm (14.34), 155.02 µm (12.51), and 218.49 µm (13.88) for the conditions wild type mcherry, ChR2 only, ChR2 with light only, ChR2 with ATR only, and ChR2 with ATR and light, respectively. The numbers of worms in each condition (from left to right) were 23, 20, 25, 22, and 39. ANOVA test: alpha = 0.05; N = 129; Degrees of Freedom = 4, 124; F-value = 5.92; p-value = 0.0002.
Fig 9. Images of ALM neurons 24 hours after surgery. (a) Image of the ALM neuron 24 hours after surgery without light stimulated. (b) Image of the ALM neuron 24 hours after surgery. This worm contains ChR2, with ATR in the diet, and was light stimulated at 175 µW/mm² using the 10 seconds on with 50 seconds off stimulation pattern. The white arrow marks approximate cut points 20 µm from the cell bodies in both images.

The ANOVA test for this first group showed that the average growths of the axons under these different conditions were not all the same. The pair-wise test using the Tukey adjustment indicated that the experimental conditions (with ChR2, with ATR, and with light stimulation) showed average growths that were 67.57 µm (44.8% increase) greater than the average growths from the wild type mcherry condition, 81.71 µm (59.7% increase) greater than from the ChR2 only condition, 52.17 µm (31.4% increase) greater than from the ChR2 with light condition, and 63.48 µm (40.9% increase) greater than from the ChR2 with ATR condition. All of these differences were statistically significant at the alpha = 0.05 level. Additionally, all the growths from the control conditions were not statistically different from each other. Thus the various factors of light exposure, ATR, ChR2
expression did not appear to affect regeneration outgrowth. Only when ChR2 was activated was the outgrowth enhanced on average by at least 31%.

A second group of conditions tested how regeneration may respond to different intensities of light. This group included worms with the ChR2 molecule grown with ATR in their diet, and stimulated with light at various intensities (Fig 10). These intensities included 125, 150, 175, and 200 µW/mm². They were compared to ChR2 worms grown with ATR but without light stimulation.

![Figure 10](image.png)

Figure 10. Average outgrowths under various light intensities. The worms stimulated with no light, 125, 150, 175, 200 µW/mm² light intensities (left to right) showed growths (and standard errors) of 155.02 µm (12.51), 186.23 µm (18.01), 162.30 µm (10.53), 218.49 µm (13.88), and 217.46 µm (13.43) respectively. The numbers of worms for the conditions were (from
left to right) 22, 22, 27, 39, and 25. All conditions contained ChR2 and ATR. ANOVA test: alpha = 0.05; N = 135; Degrees of Freedom = 4, 130; F-value = 4.6; p-value = 0.0017.

The ANOVA test from this group showed that the growths from the different light intensities were not all the same. With a pair-wise test using the Tukey adjustment, only the outgrowths from the 175 and 200 µW/mm² intensities were significantly different from the control. In addition, the 175 - 150 µW/mm² and the 150 - 125 µW/mm² pairs were statistically different from each other at the alpha = 0.05 level. The average outgrowths from the 175 µW/mm² stimulations were 63.47 µm (40.9% increase) greater than the outgrowths from the control, and the average outgrowths from the 200 µW/mm² stimulations were 62.44 µm (40.3% increase) greater than the outgrowths from the control. The average outgrowths from the 175 µW/mm² intensity stimulation were 56.2 µm (35.6% increase) greater than the outgrowths from the 150 µW/mm² stimulation, and the average growths from the 125 µW/mm² stimulation were approximately 23.93 µm (14.7% increase) greater than the outgrowths from the 150 µW/mm² stimulation.

The third group of experiments explored the responses of outgrowths from regeneration in response to different stimulation patterns. To keep the total amount of light exposed to the worms constant at the end of the 24 hour period, various illumination patterns were used (Fig 11). A no-light control was also included as a comparison.
Figure 11. Average outgrowths under various stimulation patterns. From left to right, the different light stimulation patterns produced average outgrowths (and standard errors) of 155.02 µm (12.51), 141.48 µm (13.28), 173.19 µm (11.09), 218.49 µm (13.88), and 129.18 µm (10.73). The numbers of worms for the conditions from left to right were 22, 21, 23, 39, and 21. All conditions contained ChR2 and ATR. ANOVA test: alpha = 0.05; N = 126; Degrees of Freedom = 4, 121; F-value = 8.29; p-value < 0.0001.

The ANOVA test shows that the average outgrowths from these conditions are not all the same. Pair-wise tests using the Tukey adjustment showed that when compared to the outgrowths from the no-light control, only the outgrowths
from the 10s-on/50s-off (third bar from the left in Fig 11) stimulation pattern was significant different. The outgrowths from the 10s-on/50s-off pattern was also significantly different from the outgrowths of the constant light stimulation pattern (first bar from the left in Fig 11) and from the outgrowths of the 2s/10s stimulation pattern (fourth bar from the left in Fig 11) at the alpha = 0.05 level. Specifically, the outgrowths from the 10s-on/50s-off pattern were 63.47 µm (40.9% increase) greater than the outgrowths from the no-light control. The 10s-on/50s-off pattern also produced outgrowths that were 77.01 µm (54.4% increase) greater than from the constant light pattern, and the outgrowths from the 10s/50s pattern were also 89.31 µm (69.1% increase) longer than from the outgrowths from the 2s-on/10s-off stimulation pattern.

The fourth group of conditions explored the role that Ca\(^{2+}\) signaling may have in neural regeneration (Fig 12). The worms with ChR2 grown with ATR in their diet with and without light stimulation were compared to various conditions involving dantrolene treatment and unc-68 mutants with and without light stimulation.
Figure 12. Average outgrowths compared RyR-blocked worms. From left to right, these conditions generated outgrowths (and standard errors) of 155.02 µm (12.51), 218.49 µm (13.88), 144.3 µm (9.09), 138.39 µm (7.92), 129.15 µm (8.09), and 109.62 µm (8.74). The numbers of worms in each condition from left to right were 22, 39, 32, 23, 33, and 27. All worms contained ChR2 and ATR. Only the dantrolene treated worms were also exposed to DMSO, since DMSO use is necessary to dissolve dantrolene. ANOVA test: alpha = 0.05; N = 124; Degrees of Freedom = 5, 170; F-value = 13.98; p-value < 0.0001.
From the ANOVA test, the results show that the outgrowths from these conditions were not all the same. Pair-wise tests using the Tukey adjustment showed that the outgrowths from the light condition were significantly different from all the other conditions in Fig 12. The outgrowths from the light condition compared to the outgrowths of no-light control, dantrolene with DMSO, dantrolene with DMSO and light, \textit{unc-68}, and \textit{unc-68} with light were 63.48 µm (40.9% increase), 74.2 µm (51.4% increase), 80.1 µm (55.5% increase), 89.35 µm (64.6% increase), and 108.88 µm (84.31% increase), respectively. The outgrowths from the no-light control were also significantly different compared to the outgrowths from the \textit{unc-68} mutant with light stimulation. The average outgrowths from the no-light control worms were approximately 45.4 µm (41.4% increase) greater than the average outgrowths of the \textit{unc-68} worms with light stimulation.

Additionally, we also tested the behavioral responses of the worms under various conditions (fig 13). The behavioral responses of the ChR2 worms grown with ATR and ChR2 worms grown without ATR were compared to assess the efficacy of the ATR chromophore. The responses of the dantrolene treated worms with DMSO and ATR were compared to the responses of the worms grown in only DMSO and ATR. This comparison served to assess the efficacy of dantrolene.
Figure 13. Behavioral responses to light with various chemical agents. All worms contained the gene encoding ChR2 driven by the promoter mec-4. From left to right, the proportions of worms that responded to light under these various conditions were 0.75, 0.05, 0.28, and 0.64. The total numbers of worms in the categories from left to right were 20, 21, 53, and 22. Fisher-Irwin test: p-value testing the significance of the difference in proportion of positive responses from ChR2+ATR compared to ChR2 was < 0.0001; p-value testing the difference in proportion of positive responses from dantrolene treated worms compared to worms not treated with dantrolene was 0.0097; p-value testing the significance of the difference between the ChR2 + ATR and ChR2 + ATR + DMSO (2nd and 4th...
conditions from the left on the graph) was 0.65. The alpha levels for all statistical tests were set to 0.05.

The Fisher-Irwin statistical test for difference in proportions between two samples was performed to compare the proportions of positive responses between ChR2 with ATR worms and ChR2 without ATR worms. The p-value from the test between these two conditions was less than 0.0001. This was statistically significant since the p-value is less than the cut-off value of 0.05. The same test was also performed to compare the proportions of positive responses between the ChR2 worms treated with dantrolene with DMSO and ATR and worms with DMSO and ATR but without dantrolene. The p-value from this test was 0.0097. This was also statistically significant. One last Fisher-Irwin test was also performed to compare the proportions between the ChR2 with ATR worms and the worms treated with only DMSO and ATR. The p-value from this test was 0.65, indicating that this difference was not statistically significant since it is above the 0.05 cut-off.

These data indicate that a significantly larger proportion of ChR2 worms with ATR showed response to light than ChR2 worms without ATR, and a significantly smaller proportion of worms treated with dantrolene, DMSO, and ATR responded to light than worms treated with only DMSO and ATR. The proportion of responses from the DMSO + ATR worms were also statistically the same as the proportion of responses from the ChR2 + ATR worms.
Conclusions:

The development and application of optogenetics and femtosecond lasers allowed us to accomplish detailed procedures in search of the mechanisms of neural regeneration. To assess the validity and the effectiveness of the chemical agents used in these experiments, we first assessed the animals' behavioral responses to light stimulation. This difference in the proportion of positive responses to light in the ChR2 worms with ATR and without ATR in the diet was significant according to the Fisher-Irwin statistical test. The difference in the proportion of positive responses in these worms treated with dantrolene compared to worms not treated with dantrolene was also significant according to the Fisher-Irwin test. Adding ATR to the diet significantly increased the number of worms that responded positively to light stimulation. Treating the worms with dantrolene in the diet suppressed the number of positive responses to light stimulation, even in the presence of ATR. This was expected, since dantrolene blocks RyRs, which normally facilitates depolarizations and behavioral responses by increasing Ca\(^{2+}\) release from the ER. Furthermore, since dantrolene must be dissolved in DMSO, the effects of DMSO was also assessed by comparing DMSO and ATR treated worms to worms treated only with ATR. DMSO does seem to lower the proportion of positive responses slightly, but this difference was not statistically significant. This comparison allowed us to conclude that the presence of ATR increased the proportion of worms that responded positively to
light stimulation, and that dantrolene was responsible for the suppression of responses to light stimulation, as expected.

Our results indicate that initial stimulation of the axons during the first 24 hours significantly increases the lengths of axons that were regenerated. This increase in regeneration rate was not attributed to any of the components of the tools used (ChR2 molecule, ATR chromophore, or heat from the intensity light stimulation) individually. But the stimulated regeneration is a result of the electrical activity generated by the opening of the ChR2 channels allowing cations to cross the membranes of the axons (ChR2 is a general cation channel that opens and allows passive influx of cations including Na\(^+\) and Ca\(^{2+}\)).

To further investigate this mechanism, we also varied the amount of light intensity used for stimulation. There seems to be a peak intensity at which the stimulated outgrowth is maximized. This seems to be at 175 \(\mu\)W/mm\(^2\). Intensities above 175 \(\mu\)W/mm\(^2\) showed approximately the same amount of outgrowths, while intensities below 175 \(\mu\)W/mm\(^2\) generated outgrowths that were statistically the same as the outgrowths from the no-light control.

However, unexpected results were seen when outgrowths from the light intensity of 150 \(\mu\)W/mm\(^2\) were compared to outgrowths from intensity of 175 \(\mu\)W/mm\(^2\), as well as when the outgrowths from the intensity of 125 \(\mu\)W/mm\(^2\) were compared to the outgrowths from the intensity of 175 \(\mu\)W/mm\(^2\). The statistical insignificance of the latter comparison may be due to the slightly high standard error of the 125 \(\mu\)W/mm\(^2\) group at 18 compared to the other groups, which are all
below 14. This may be addressed in the future by increasing the number of worms in that group to increase the power of this statistical comparison, or this may point towards other variables not assessed in this study. In addition, the decrease in outgrowths from the intensity 150 µW/mm² was also unexpected. This may also involve statistical power, or other variables not assessed in this study. In either case, further manipulation of light intensity as a variable in determining regeneration may be needed to determine if intensities below 175 µW/mm² have any unexpected effects in the stimulated outgrowths.

Another area explored by our experiments is that of temporal stimulation patterns. Only the outgrowths from the 10s-on/50s-off pattern showed statistical difference when compared with the outgrowths from the no-light controls.

The relationship between axonal outgrowths and stimulation patterns may have a threshold at which point if the interval between stimulations are too short then axon outgrowths may be suppressed. This may be due to a recovery time needed if an axon is stimulated too often. This may be explored further in subsequent studies, where outgrowths in response to a more diverse array of stimulation patterns can be assessed.

Further experiments can calibrate stimulation to the ideal settings, such as the intensity at which outgrowths is the highest, but still minimize side effects such as responses to heat possibly generated by higher intensity light. Another area of calibration is the ideal stimulation pattern. So far we have kept a ratio of 1 to 5 in the amount of seconds of light stimulation to amount of seconds of no
stimulation. However, we can adjust this ratio to obtain an ideal pattern that produces the largest outgrowths in response to the various possible stimulation patterns.

The results also indicate that dantrolene treatment on the worms have a significant effect towards influencing the initiation of regeneration in the first 24 hours after physical damage. With the suppression of Ca\(^{2+}\) release from internal stores, the resulting stimulated regeneration response was statistically the same as the controls. The lack of any significant increase in outgrowths indicates that the internal release of Ca\(^{2+}\) is an important and crucial signaling mechanism for the initiation of regeneration in the first 24 hours.

This result is confirmed by a more robust method of blocking internal Ca\(^{2+}\) release, using the \textit{unc-68} mutant. The lack of any significant increase in stimulated outgrowths is also seen in these mutants, strongly supporting the theory that internal Ca\(^{2+}\) release through the RyR channels plays a crucial role in the initiation of axonal regeneration in the first 24 hours and mediates the enhanced outgrowth seen in ChR2 stimulated neurons.

In general the RyR-blocked worms (both dantrolene treated worms and \textit{unc-68} mutants) produced outgrowths that were statistically the same as the outgrowths from the no-light controls, with one exception. The \textit{unc-68} worms with light stimulation produced outgrowths that were statistically less than the outgrowths from the no-light controls. Further studies may be needed to either
conclude that this decrease in outgrowths is simply due to extreme variations in the sample or determine that this decrease is of scientific importance.

In addition to the previously proposed experiments that may serve to calibrate the ideal conditions for stimulating the most outgrowths from C. elegans ALM neurons, future directions may use more sensitive Channelrodopsin proteins. One potential problem in our studies may be that the ATR bleaches, rendering the chromophore ineffective, during the course of the 24 hours. Thus the ChR2 may only be stimulated over a few hours rather than the entire 24 h. More sensitive ChR2 variants (Berstein et al. 2012) will reduce the necessary light exposure and potentially increase the positive effect on regeneration outgrowth.

There are also Ca\(^{2+}\) specific Channelrodobsin variants that can be incorporated in the axons (Berstein et al., 2012). By using Ca\(^{2+}\) specific channels, we can further assess the role of Ca\(^{2+}\) signaling in regeneration. We may perhaps even explore the roles of other ions in these mechanisms by isolating Ca\(^{2+}\) as the only signaling molecule during stimulations.

Future directions would also include repeating these experiments in cell cultures of vertebrate neuronal cells. Once the mechanisms are clear in the C. elegans nervous system, a logical next step would be to assess the conservation of this mechanism across different species. The vertebrate nervous systems can be significantly more complex, so a cell culture may be a simpler method of initial experimentation.
Another future direction of great interest is the directionally specific stimulation of neural regeneration, made possible by the tools of optogenetics. By restricting light to a specific location in relationship to the cut site, we may observe the possibility of artificially guiding the outgrowths of an axon toward the illuminated spot. If the directional specific stimulation of regeneration is successful, methods may develop to reduce the time required for severed axons to reconnect. This may be an additional therapy to surgical reconnection of peripheral nerves to enhance the quality of the reconnection. This may also provide a controlled way to guide the reconnection of axons in the immensely complicated networks of neurons in the CNS.

Future applications of this research are hugely important in tackling the issues present in the injuries and diseases of the nervous system. Hopefully one day, we will be able to restore functions to people with injuries and diseases that deprive them of crucial motor and sensory functions.
References


Vita

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EDUCATION

Boston University Division of Graduate Medical Sciences  Boston, MA
Dual Degree Candidate in Master of Arts in Medical Science and Master of Public Health, January 2014
Dual Concentration in MPH: International Health and Biostatistics

Washington University in St. Louis  St. Louis, MO
Bachelor of Arts, May 2009
Double Major: Biology: Neuroscience and Music
Honors: Thomas Eliot Scholar, Dean’s List (2 semesters), Howard Hughes Medical Institutes Summer Research Fellowship, Hoopes Scholarship

RESEARCH

Boston University Graduate Medical Sciences  Boston, MA
Graduate Student, Gabel Lab  Summer 2011-current
• Research involves the study of the effect of electrical activity on neuroregeneration.
• Mounted C. elegans onto agar plates for immobilization during experiments and rescued them from those plates for further growth.
• Performed surgery on C. elegans while immobilized by using two-photon laser to cut neuronal axons.
• Performed behavioral assays by observing behavioral changes under fluororesent light to test for light sensitivity.
• Measured axon growth after surgery through the use of imageJ software.
• Performed various statistical analyses on the growth measurements through the use of the computer software Statistical Analyses System (SAS).

Washington University Medical School  St. Louis, MO
Full-time Lab Technician, Stein Lab  Summer 2009 – Summer 2010
• Performed surgery of turtles, consisting of transection of the spine at just below the cervical vertebras, dissection of numerous motor nerves enervating the knee and the hip of the turtle.
• Recorded electric neuronal signals simultaneously from all dissected nerves during various forms of scratch reflex exhibited by the turtles.
• Recorded electric neuronal signals from interneurons in the spinal cord during these reflexes.
• Analyzed data collected to study the various types of neurons that exist in the spinal cord, as well as the organization of these neurons and the interactions between them in order to determine how these reflexes are generated and controlled in the spinal cord.
• Performed routine lab maintenance activities.

Undergraduate Student, Nonet Lab  
Fall 2005 – Spring 2009
• Collected and analyzed DNA sequence data from online databases such as ZFIN (Zebrafish Information Network), and performed genomic DNA preps, plasmid DNA preps, PCR, gel electrophoresis, cloned gene promoters, and transformation of bacteria to create plasmid vectors.
• Performed injection of plasmid vectors into zebrafish embryo.
• Imaged embryonic zebrafish neurons and developing synapses using compound microscope as well as confocal microscope.
• Paper submitted with second authorship: Independently tractable Tol2 vectors for UAS GAL4 two component expression in zebrafish.
• Research work presented at Undergraduate Research Symposium at Washington University during summer of 2007: Imaging the Development of Rohon-Beard Neuron Synapses in Zebrafish.
• Earned Summer Undergraduate Research Fellowship (summer 2007) sponsored by Howard Hughes Medical Institute and Hoopes Scholarship.
• Wrote senior thesis detailing all my work in the Nonet Lab: Imaging the Development of Rohon-Beard Neuron Synapses in Zebrafish.

TEACHING
Washington University in St. Louis  
St. Louis, MO
Teaching Assistant for Laboratory of Neurophysiology  
Fall 2008
• Taught one of the six preparations to a group of three alternating students in weekly 9-hour long sessions and prepared lesson plans for teaching the students.
• Guided students through surgery of a live rat, data collection from electric potentials of the rat cochlea, data analysis, and data interpretation.
• Graded lab quizzes and 6 lab reports written in the form of research manuscripts.

Mentoring for January Intensive Program  
January 2008
• Assisted in teaching General Chemistry in a weeklong program.

Mentoring for Summer Intensive Program  
August 2007
• Taught Organic Chemistry during homework sessions and PLTL in a weeklong program.

Mentoring for Organic Chemistry  
Fall 2007 – Spring 2008
• Led 4 or 5 people study groups on a weekly basis. Helped students prepare for exams as well as go over weekly lecture material.
EXTRACURRICULAR EXPERIENCE

University Presidential Inaugural Conference  Washington, DC
University Presidential Inaugural Conference  January 17-January 21, 2009

- Selected as one of 5000 college students from around the world to participate in various events surrounding the inauguration of the 44th president of the United States, Barack Obama. These events include the following:
  - Attended motivational talks from General Colin Powell and former Vice President Al Gore.
  - Participated in panels with political strategists and prominent members of the press.
  - Attended “We are One: Opening Inaugural Celebration” concert on the steps of the Lincoln Memorial.
  - Witnessed in person, the swearing in to office of Barack Obama, Joe Biden, as well as the new cabinet members of the Obama administration.

Washington University  St. Louis, MO
Alpha Chi Sigma Chemistry Fraternity  Fall 2006 – Spring 2009

- Participated in events such as making ice cream with liquid nitrogen at Dance Marathon, and initiating new members into the fraternity each semester.

College Student Interest Group in Neurology (CO-SIGN)  Spring 2007 – 2008

- Participated in focus groups interacting with Neurologists, Neurosurgeons, MD/PhD’s to discuss different life styles of different fields related to neurology.

Club Soccer  Fall 2005 – Spring 2008

- Practice approximately 4-6 hours a week, with weekly games on the weekends during the fall semesters.
- Weekly indoor games during the spring semesters.

Medicine and Research Connections Program at WUMS  Summer 2006 & 2007

- Led groups of students through the one week long program, getting to know the students as well as answering questions they may have about college life, science classes, and research.

National Society of Collegiate Scholars (NSCS)  Spring 2006 – present

- Membership based on academic excellence.
- Provides opportunities for scholarships, internships, and various events.

Classical Piano  1990 – present

- Practice approximately 5-6 hours every week, with hourly lessons with a private teacher until 2009.
- Perform in recitals twice every semester from 2005-2009.
- Competed in various local and statewide competitions.