Regeneration of lost brain tissues by genetic manipulation of *nou-darake* in *Schmidtea mediterranea* planarians

By Carlos Gomez

Abstract

Neurodegenerative diseases, such as Alzheimer's, Parkinson's, and Huntington's disease, affect up to 40 million people worldwide. These diseases in humans work by slowly degrading neural structures, but some species, such as planarians, can combat such afflictions by their robust regenerative properties. While it is known that the progressive loss of neural structures are common in these diseases, it is not known whether regeneration of neural structures can restore the brain as it was. **Our objective is to inhibit *nou-darake* (*ndk*), a gene associated with ectopic brain formation, in genetically modified *Schmidtea mediterranea* planarians and analyze both partial and full neural regeneration.** Our general strategy starts by the development of three different planarian models utilizing RNA interference (RNAi): (1) *Smed-TOR(RNAi)* planarians which exhibit only wound-healing abilities similar to humans, (2) *Smed-apc(RNAi)* planarians which have disrupted anterior-posterior (A-P) polarity regenerating only posterior ends after amputation, and (3) *Smed-βcatenin-B(RNAi)* planarians which have disrupted A-P polarity regenerating only anterior ends after amputation. Afterwards, each planarian model will be given *Smed-ndk(RNAi)* and observed intact and after amputation. The proposed study will not only present detailed spatiotemporal images of neural regeneration in multiple planarian models, but propose the planarian as a model organism for future neurodegenerative research.

Our goal is to further the understanding of both partial and full neural regeneration in order to bring forth new treatments for neurodegenerative diseases.

Specific Aims

We hypothesize that inhibition of nou-darake in both amputated Smed-apc(RNAi) and Smed-TOR(RNAi) Schmidtea mediterranea planarians will cause full neural regeneration from their remaining central nervous system structures. We plan to test our hypothesis by the use of three specific aims:

Specific Aim 1: Develop *Smed-apc(RNAi)*, *Smed-βcatenin-B(RNAi)*, and *Smed-TOR(RNAi)* planarian models by RNA interference (RNAi) in *Schmidtea mediterranea* planarians. Development of these different planarian models has been successful in previous studies and has allowed us to analyze neural regeneration from different perspectives in the same organism. *Smed-apc(RNAi)* planarians, when transversely cut, are optimal for viewing the problem from a posterior perspective because they only regenerate tails. *Smed-βcatenin-B(RNAi)* planarians, when transversely cut, are optimal for viewing the problem from an anterior perspective because they only regenerate heads. *Smed-TOR(RNAi)* planarians are optimal for viewing the problem from a human perspective because when these are cut, they do not regenerate, but rather heal the injury. These models along with normal planarians will be used as controls for this study.

Specific Aim 2: Record the neural regeneration of intact, amputated, and *Smed-ndk(RNAi)* injected planarian models using fluorescent neural markers and NIS Elements 3.2 software. Planarian models made from **Specific Aim 1** will be used to record neural regeneration. To record the neural regeneration, we will fix planarians, utilize an array of neural markers, and observe them with fluorescent microscopy. Images will be taken every 12 hours and used to observe how neural structures in different models regenerate spatiotemporally.

Specific Aim 3: Induce the regeneration of brain tissue in both amputated *Smed-apc(RNAi)* and *Smed-TOR(RNAi)* planarians utilizing *Smed-ndk(RNAi)* and capture the process by fluorescent microscopy. Brain tissue regeneration will be induced by injecting *Smed-ndk(RNAi)* into genetically modified planarians. *Smed-apc(RNAi)* and *Smed-TOR(RNAi)* planarians are utilized because transversely amputated *Smed-apc(RNAi)* planarians contain no brains and longitudinally amputated *Smed-TOR(RNAi)* planarians contain a healed, but severed brain. Regeneration from transverse and longitudinal cuts will be observed by the use of neural markers and NIS Elements 3.2 software.

Introduction

Neurodegeneration is the progressive loss of neural structures that currently affects up to 40 million people worldwide. Individuals with this affliction develop as a result neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's disease. These diseases can affect an individual at any stage in life and progress at different rates; research into these diseases are becoming critical. Research in neurodegenerative diseases are currently investigated by the study of induced pluripotent stem cell, mouse, and rat models to understand how the development of these diseases occurs [1-3]. The use of different models to understand neurodegenerative diseases provides a distinct perspective into neurodegeneration by observing it from various evolutionary standpoints. For example, planarian models have also been utilized for investigation into neurodegenerative diseases not only because they contain an evolutionary primitive brain, but because they also contain robust regenerative properties not usually observed in evolutionary higher models [4]. Planarian studies are most noted for their contributions to the fields of aging, cancer, and wound healing, but Nishimura [4] utilized planarians to observe how Parkinson's disease affects an organism with strong regenerative capabilities. Few studies have utilized planarians specifically for neurodegenerative diseases, but many studies have utilized

planarians to investigate brain regeneration [5-18]. Studies on planarians have brought out many unique ideas: whether long-term memories can being contained in somatic cells, whether the physiological map of regeneration can be contained in muscle cells, and whether the regenerative properties of the brain are controlled by a unique set of genes [5-7]. One of the genes discovered for brain regeneration from these investigations, which also has a similar homolog in humans, is called *nou-durake (ndk)* [7]. When *ndk* is inhibited in planarians, the development of neural structures throughout the body occurs. This gene provides the information need to help stabilize or even reverse the effects of neurodegenerative diseases.

While medication and treatments have been developed to slow the progression of neurodegeneration, none have been developed to stop or reverse the loss of neural structures [8]. Our objective is to inhibit *nou-darake* (*ndk*) in a planarian model with human regenerative capabilities displaying characteristics of neurodegenerative diseases to analyze how neural regeneration of both a partial and absent brain occurs. The significance of this study is to propose the planarian as a model organism for future neurodegenerative research and to further the understanding of both partial and full neural regeneration in order to bring forth new treatments to stop the progression or potentially reverse the effects of neurodegenerative diseases.

Research Design and Methods

Specific Aim 1: Develop *Smed-apc(RNAi)*, *Smed-βcatenin-B(RNAi)*, and *Smed-TOR(RNAi)* planarian models by RNA interference (RNAi) in *Schmidtea mediterranea* planarian.

This aim focuses on developing *Smed-apc(RNAi)*, *Smed-βcatenin-B(RNAi)*, and *Smed-TOR(RNAi)* planarian models by RNAi to obtain different perspectives of the study.

Experimental design

Using *Schmidtea mediterranea* planarians and an injection set-up on the microscope, RNAi of either *apc*, *β-catenin,* or *TOR* will be inserted into the pharynx of the planarian. These injections will be done for 3 days consecutively and again on the 7th day.

Analysis

To analysis whether the injections worked, the planarians need to be amputated transversely. This protocol is fairly straightforward and we expect to see the development of double-tailed planarians from *Smed-apc(RNAi),* double-headed planarians from *Smed-βcatenin-B(RNAi),* and non-regenerating worms from *Smed-TOR(RNAi).* This experiment will help develop three different models in the same species allowing us to view the situation from different perspectives.

Specific Aim 2: Record the neural regeneration in intact, amputated, and *Smed-ndk(RNAi)* injected planarian models using fluorescent neural markers and NIS Elements 3.2 software.

This aim focuses on capturing how neural regeneration occurs in different planarian models.

Experimental design

Using planarian models developed in **Specific Aim 1** along with control planarians, we will fix planarians at key points of their regeneration process and every 24 hours afterwards. The fixed planarians will then be used to create a time course of the regeneration process until a stable image is formed. Afterwards, they will be washed in neural markers and analyzed under a fluorescent microscope using NIS Elements 3.2 software.

Analysis

First, we will have to create separate files for each planarian model. We will carefully label each image with the time after amputation and RNAi injection used. Second, we will compare the images to the control planarians to notice any differences. Lastly, we will compare the images

against all planarians models and look at them for any similarities. We expect to see extreme differences between each planarian model, especially in the *Smed-TOR(RNAi)* model. Seeing even slight variations between the models can give us an idea of how neural regeneration occurs and what we can further investigate.

Specific Aim 3: Induce the regeneration of brain tissue in both amputated *Smed-apc(RNAi)* and *Smed-TOR(RNAi)* planarians utilizing *Smed-ndk(RNAi)* and capture the process by fluorescent microscopy.

This aim focuses on discovering whether inducing regeneration on damaged or absent brains will cause it to regenerate to its original structure.

Experimental design

These worms will be given RNAi injections of either *apc* or *TOR* and then given an additional injection of *ndk(RNAi)*. We will then amputate specifically to create damaged brains and fix them at key points during their regeneration process as in **Specific Aim 2**. They will then be washed in neural markers and analyzed under a fluorescent microscope using NIS Elements 3.2 software.

Analysis

Double RNAi planarians will be analyzed as in **Specific Aim 2**. The difference being that we expect to see the neural regeneration of the damaged brains. The significance of this experiment will demonstrate whether neural regeneration of neurodegenerative-like brains is possible and that finding a mechanism for it can be proposed as a potential treatment for neurodegenerative diseases.

Schmidtea mediterranea

The planarians to be used in this study are *Schmidtea mediterranea* that will be stored in rectangular plastic containers, filled with Poland Springs natural spring water. These planarians

have a high tendency to undergo spontaneous fission so in order to prevent it, all worms will be stored in an incubator set at 10°C in continuous darkness and fed once or twice a week with organic beef liver.

Materials/Equipment

In this study, we will utilize refrigerated incubators (Cooler-Store) set at 10°C to create the environment for the *Schmidtea mediterranea* planarians (Oviedo Lab). These planarians will be stored in 40 oz. rectangular Ziploc Containers (SOAP). To feed the planarians, mashed beef liver (Grassland Beef), stored in a medical freezer (Living Direct), will be served using Research Plus 100 μL pipettes (Pipette). The Nanopure Water Purification system (Lab Depot, Inc.) will be utilized to clean the boxes after feedings. The Poland Springs natural spring water (Coffee for Less) will be stored in several Nalgene Autoclavable Lowboy containers (McQueen Labs) for ease of access. Two research assistants from the University of California, Merced will help out with the work.

When ready to inject the desired RNAi strand (Oviedo Lab), we will use a Nikon AZ100 microscope (Nikon Instruments) along with a Nanoject II injector (Drummond Sci) set-up. The stage of the microscope will be connected by bare copper wire (Amazon) to an AC/DC power source (Online Components) which will thereby chill the stage for the planarian. This cold effect will make the planarian less active. The stage will contain a fold sheet of Kimwipe (Uline) containing a black carbon mill finish paper fragment (Walmart) to act as a contrast against the flatworm. Using a squeeze bottle (Amazon) containing Poland Spring water, the stage will be doused to create a wet surface for the planarians. A LED light source (Fisher Scientific) can be used to get a better view of the planarian. Depending on which planarian group is being used, they will be either injected, amputated, or both. Using a surgical scalpel (Micromark), planarians

will be cut and placed into separate petri dishes (Sigma-Aldrich) containing Poland Springs water.

When the planarians are amputated, they will have to be fixed. To do this, chemicals from Sigma-Aldrich will be utilized and then the planarians will be washed in specific neural markers (Various Labs). Afterwards, they will be mounted on microscope slides (Amazon) and applied with Vectasheild (Vector Labs) to be preserved. Once completed, the planarians will be observed using a Zoom Stereomicroscope (Nixon) and images will be taken using a HP Pro desktop (CDW) connected to a Samsung monitor (Legends Micro).

Data Management

The data collected from this study will be digital images and numerical values calculated. To secure the data, each image will be backed up in a separate external hard drive and categorized based on the day and planarian model used. The numerical values calculated will be from the images created. Therefore, the calculations will be stored on an Excel sheet which will also be in the external hard drive as well. To allow others interested in looking at the images and numerical values produced, the results will be upload to a personal website.

Ethics

Research assistants in the lab will undergo Lab Safety training provided at the nearest research facility prior to doing experiments. To prevent any dishonest work in the lab, data will be checked using statistical methods on Excel and provided with each result to prove its validity. Since this study utilizes invertebrate animals, we do not need to submit applications for IRB/IACUC, but we are using guidelines set forth by the Mental Health Research Institute (1965) on planarian care [19]. This manual provide us with instructions on how to inject, graft, and feed planarians correctly.

Potential Pitfalls

Although this study starts by the optimization of protocols and refinement of techniques to increase the rate of success, plans always have the potential to go awry; therefore, certain precautions and strategies have been develop to ensure the maximum success of our experiments. The first potential pitfall could occur when developing the *Smed-apc(RNAi)*, *Smed-βcatenin-B(RNAi)*, and *Smed-TOR(RNAi)* planarian models. If the injections cannot be optimized or properly place, an alternative method of feeding the RNAi is available that produces the same results. The second potential pitfall could occur with the fluorescent makers and imaging software used. To avoid this situation, we have ordered specific neural markers indicating not only the brain, but other specific segments as well that could be identified by the imaging software. The imaging software could also fail, but if it does, we can contact the company on how to troubleshoot. If we still cannot see any results using the software, we could always utilize microscopes offered in nearby labs at the University of California, Merced. The final pitfall could occur by running out of planarians to test. To ensure we have a plentiful supply, we will be dividing normal planarians and growing them aside from the experiments.

Timeline

	Months															
	1				2				3				*4*			
	Weeks															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Aim 1	●	●	●	●	●	●	●	●								
Aim 2									●	●	●	●	●	●	●	●
Aim 3																
	Months															
	5				6				7				*8*			
	Weeks															
	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
Aim 1	●	●	●	●	●	●	●	●								
Aim 2									●	●	●	●	●	●	●	●
Aim 3	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●

Specific Aim 1: Develop *Smed-apc(RNAi)*, *Smed-βcatenin-B(RNAi)*, and *Smed-TOR(RNAi)* planarian models by RNA interference (RNAi) in the *Schmidtea mediterranea* planarian.

Specific Aim 2: Record the neural regeneration in intact, amputated, and *Smed-ndk(RNAi)* injected planarian models using fluorescent neural markers and imaging software.

Specific Aim 3: Induce the regeneration of brain tissue in both transversely amputated *Smed-apc(RNAi)* planarians and longitudinally amputated *Smed-TOR(RNAi)* planarians utilizing *Smed-ndk(RNAi)* and capture the process by fluorescent microscopy.

This study is estimated to be at maximum eight months. The **first specific aim** is a three-part process: (1) it requires the optimization of dosages and refinement of techniques [1 month], (2) the injection of the optimized dosages within all planarian models [2 weeks] and (3) an additional two weeks for the development of *Smed-apc(RNAi)* and *Smed-βcatenin-B(RNAi)* planarians. The **second specific aim** will take approximately 2-3 months due to time course imaging. Planarians will be fixed with specific neural markers at key times of regeneration (2, 4, 6, 8, 18, 24 hours) and then every 24 hours until a stable result is obtained. The time course of the **third specific aim** is identical to the second, but will be conducted separately instead of concurrently due to the amount of data being produced at once. The **first specific aim** will initiate again concurrently with the **third specific aim** to produce more planarian models to use with other neural markers. Once the **third specific aim** finishes, the worms created from the second round of the **first specific aim** will be utilized to repeat the procedure with different neural markers adding up to eight months.

Rationale:

We hypothesize that inhibition of nou-darake in both amputated Smed-apc(RNAi) and Smed-TOR(RNAi) Schmidtea mediterranea planarians will cause full neural regeneration from their remaining central nervous system structures. The rationale behind this study came from a paper published by Agata that discussed his findings about *nou-darake* (*ndk)* [7]. In this paper, he presented an image, seen below, that displayed the ectopic brain formation effects of *ndk* inhibition (Fig. 4b-d) versus a control (Fig. 4a).

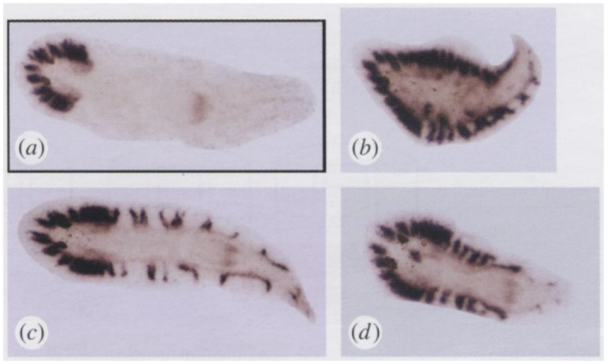

Fig. 4 of Agata paper [7]

In this image, *ndk(RNAi)* seems to cause ectopic brain formation along the ventral nerves of the planarian, which run alongside the body of the flatworm, and is heavily emphasized in the anterior region. No further research into this specific gene in planarians has been produced since its publication in 2003. So we developed a hypothesis based off this image that *ndk* inhibition will only cause ectopic brain formation if a central nervous system is present. To test this theory, planarians with special (*Smed-apc(RNAi))* or limited (*Smed-TOR(RNAi*) regenerative properties have to be utilized to cause incomplete regeneration of the brain and/or central nervous system. Being able to regenerate a fully functional brain from an incomplete brain would be instrumental in helping develop strategies to stabilize or even combat neurodegenerative diseases.

Budget:

The total cost for this 8 month study is $300,062.23. All experiments will be conducted from lab facilities available at the University of California, Merced in the Science and Engineering Building 1. To complete the protocols outlined in **Research Designs and Methods** the following materials will be necessary. Only the necessary equipment will be purchased. Remaining equipment will be donated to the University of California, Merced.

Category	Product Name	Amount	Price	Total	Company
Planarian Maintenance	*Schmidtea mediterranea* Culture	4 (200 worms)	$0.00	$0.00	Given by Oviedo lab
	Ziploc Container, Medium Rectangle-40 oz.	5	$4.25	$21.25	Soap
	Ethanol 200 proof, anhydrous, ≥99.5%	3	$74.50	$223.50	Sigma-Aldrich
	Chloroform anhydrous, contains amylenes as stabilizer, ≥99%	3	$34.60	$103.80	Sigma-Aldrich
	Methanol anhydrous, 99.8%	3	$53.60	$160.80	Sigma-Aldrich
	Glacial acetic acid ReagentPlus, >99%	3	$23.60	$70.80	Sigma-Aldrich
	Hydrogen Chloride 1.0 M in acetic acid	3	$105.00	$315.00	Sigma-Aldrich

	Reynolds Wrap Aluminum Foil 2 Pack - Includes: (2) 250 Square Feet Rolls	5	$24.00	$120.00	Amazon
	Poland Springs natural spring water 24 16.9oz Bottles	50	$36.00	$1,800.00	Coffee for Less
	Beef liver	20	$7.45	$149.00	Grassland Beef
	Nunc® petri dishes diam. × H 150 mm × 25 mm	50	$209.00	$10,450.	Sigma-Aldrich
	Vectashield	5	$125.00	$625.00	Vector Labs
	72 Blank Microscope Slides and 100 Square Cover Glass	20	$7.59	$151.80	Amazon
	Multi-Event Timer	1	$11.77	$11.77	Grainger
	Scintillation vials	5	$212.00	$1,060	Sigma-Aldrich
	Dj-apc	1	$0.00	$0.00	Given by Oviedo Lab
	Dj-βcatenin-B	1	$0.00	$0.00	
	Dj-TOR	1	$0.00	$0.00	
Neural Markers	*DjTH*	1	$0.00	$0.00	Requests from multiple labs
	DjAADCA	1	$0.00	$0.00	
	DjGAD	1	$0.00	$0.00	
	DjTBH	1	$0.00	$0.00	
	DjTPH	1	$0.00	$0.00	
	DjChAT	1	$0.00	$0.00	
	DjCHC	1	$0.00	$0.00	
	DjotxA	1	$0.00	$0.00	
	Djotp	1	$0.00	$0.00	
	DjotxB	1	$0.00	$0.00	
	DjFoxG	1	$0.00	$0.00	
	Djnetrin	1	$0.00	$0.00	

	Djndk	1	$0.00	$0.00	
Surgery Equipment	4.4 x 8.4" 1-Ply Low-Lint Kimwipes	5	$88	$440.00	Uline
	9-piece Surgical Scalpel and Blade Set	1	$10.45	$10.45	Micro-Mark
	Mead Black Carbon Mill Finish Paper, 10 Sheets/Pk, 4 Pack	1	$13.00	$13.00	Walmart
	Fisher Scientific™ LED Light Source	1	$453	$453	Fisher Scientific
	Bare Copper Wire	1	$27.61	$27.61	Amazon
	Amico Tattoo Wash Cleaning Green Soap Holder Clear White Plastic Squeeze Bottle 500mL	2	$5.72	$11.44	Amazon
Salaries	Research Assistants	2	$15/hr	$172,800	University of California, Merced
	Personal salary	1	$15/hr	$86,400	University of California, Merced
Lab Equipment	Nikon AZ100	1	$4,500	$4,500.00	Nikon Instrumentals
	Samsung 943SWX 19in LCD Monitor C-Grade	1	$99.01	$99.01	Legend Micro
	Zoom Stereo-microscope SMZ645/660	1	$1499	$1,499.00	Nikon
	AC/DC Power Supply Single-OUT 3V to 14V 20A	1	$339	$339.00	Online Components

	Nanoject II™ Auto-Nanoliter Injector	1	$1,686	$1,686.00	Drummond Scientific Company
	Nalgene Autoclavable Lowboy w/Spigot PP 15 L (Case of 4)	1	$683	$683	McQueen Labs
	MIR-254: 8.4 cu. ft. Refrigerated Incubators/ Environmental Testing Chambers	1	$8,784	$8,784	Cooler-Store
	Thermo Barnstead Nanopure Life Science UV/UF Water Purification System	1	$4,939	$4,939	Lab Depot, Inc.
	Research Plus 6 Pack (2.5/10/20/100/200/1000) plus Carousel Stand	1	$1,459	$1,459	Pipette
	HP Pro 3515 - A series A4-5300 3.4 GHz	1	$395	$395	CDW
	FS22L - Summit Medical Freezer with Lock	1	$261	$261	Living Direct

Lay Audience Abstract:

Neurodegeneration is the progressive loss of neural structures that currently affects up to 40 million people worldwide. Individuals with this affliction develop as a result neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's disease. In humans, the degradation of neural structures cannot be stopped, but some species, such as flatworms, can combat such afflictions by their robust regenerative properties. Flatworms, specifically planarians, have the capability of regenerating structures after amputation, injury, or even disease. Different types of planarian models have been developed to view the situation through different perspectives by the use of RNA interference (RNAi). RNAi is used in an organism to silence the expression of certain genes. **Our objective is to silence *nou-darake* (*ndk*), a gene associated with random planarian brain formation, in genetically modified planarians (*Schmidtea mediterranea)* designed to have the regenerative capabilities of a human and induce regeneration of a damaged brain.** Our general strategy starts by utilizing RNAi and developing three different planarian models: (1) *Smed-TOR(RNAi)* planarians which do not regenerate, but rather heal as humans do, (2) *Smed-apc(RNAi)* planarians which regenerate only tails, and (3) *Smed-βcatenin-B(RNAi)* planarians which regenerate only heads. Afterwards, each planarian model will be given *Smed-ndk(RNAi)* and observed intact and after amputation. This proposal will develop three different models for brain degradation useful for future research projects, investigate how regeneration occurs in these models, and whether it is possible to use genetic manipulations to create treatments for degraded neural structures. The proposed study will not only present information about how brain regeneration occurs in different planarian models, but propose the planarian as a model organism for future neurodegenerative research. Our goal is to further the understanding of both partial and full neural regeneration in order to bring forth new treatments for neurodegenerative diseases.

Public Health Impact Statement:

Neurodegenerative diseases, such as Alzheimer's, Parkinson's, and Huntington's disease are among the top leading causes of death and are noted to be affecting people at a younger age. Medications are currently used to slow the degeneration of neural structures, but none can stop the progression. Therefore, there is a vital need to develop treatments that will stop the progression at any stage of their disease. This research will provide information that could be used in developing a strategy to stabilize or potentially reverse the degradation of neural structures.

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