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**CRAYFISH CEREBRAL GANGLIA PRESERVATION AND SECTIONING FOR AN ASSESSMENT  
OF EXERCISE INDUCED NEUROGENESIS**

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THE FOLLOWING IS A REPORT FOR THE STUDENT SUMMER SCHOLARS PROGRAM

**Abstract**

Neurogenesis is the formation of new neurons from neural stem cells that occurs throughout adulthood in a variety of animals, including humans. Exercise enhances cell proliferation in mammals, and has been linked to ameliorating age associated declines in memory. Since the nervous system operates under common rules and themes in both vertebrates and invertebrates, our experiment aimed to observe the effects of exercise on the simpler nervous system of invertebrates using BrdU, which labels newly synthesized DNA and indicates cell proliferation. Multiple factors involved in sample preparation, preservation in paraffin, and sectioning via microtome created various challenges early on. Our fundamental focus has centered on mastering these techniques, as it is crucial to eliminate any variability that might affect results. After much practice and troubleshooting, we were able to obtain viable brain tissue sections and are now able to progress toward the exercise trials of the experiment.

## **Introduction**

Age associated declines in memory occur in both vertebrates and invertebrates (Altman & Das, 1965; Kuhn et al., 1996). Studies have found that neurons are formed during adulthood as well as during embryonic development; however, the ways in which neurons form during these two stages in life differ significantly. Clusters of stem cells produce many other types of cells; during embryonic development, nervous system stem cell clusters are located in many different parts of the mammalian brain (Ehninger & Kempermann, 2008; Imayoshi et al., 2009). In adult mammals, nervous system stem cells actively produce neurons in only two locations (Kornack & Rakic, 2001; Rochefort et al., 2002; reviewed in Zhao et al., 2008), which limits the rate at which adult neurogenesis occurs.

One location where newly generated adult neurons grow in the vertebrate brain is the dentate gyrus. This part of the brain is associated with both spatial and episodic recollection; one of the most supported theories concerning the function of newly formed adult neurons involves spatial memory (reviewed by Eichenbaum et al., 1999). Results from recent studies indicate that adult neurogenesis is necessary for long-term spatial memory (Snyder, et al., 2005) and that adult hippocampal neurogenesis is necessary for tasks with low degrees of spatial separation (Clelland et al., 2009). If factors that increase adult neurogenesis are identified, they could eventually be utilized research that would link them at a molecular level to neuron production. This research could eventually be applied in a clinical setting to assist patients of diseases associated with memory loss, such as Alzheimer's disease and dementia.

There are several extrinsic factors that have shown to increase neurogenesis in adults; memory and learning tasks, enriched environment, and physical activity. When directly comparing the effect each of these three factors has on neurogenesis, it was demonstrated that voluntary exercise had the greatest impact (van Praag et al., 1999). The finding that exercise greatly enhances cell proliferation in mammals has been verified by several experiments over the last 10 years (Kempermann, 2008; van Praag 2006). Conditions that have generated the greatest increase in proliferation are when exercises last longer and are of a lower intensity (Holmes et al., 2004; Lucassen et al., 2008). Physical activity's cardiovascular effect extends to the brain by increasing blood flow in the cerebrum and inducing angiogenesis, formation of new capillaries, near the motor cortex (Swain et al., 2003). Exercise has also been linked to a reduction in stress and depression as well as ameliorating age associated declines in memory (Eadie et al., 2005; Ernst et al., 2005; Kim et al., 2010). Exercise in human subjects throughout midlife has shown to significantly reduce the odds of dementia and Alzheimer's disease (Andel et al., 2008) and is being considered as a treatment for both. In a 2010 study conducted by Sung-Eun Kim et al., rats that exercised had performed much better in short-term and spatial memory tasks than those who did not exercise. It is important that the link between exercise and adult neurogenesis be explored so that future application can be made in a clinical environment.

Since the nervous system operates under common rules and themes in both vertebrates and invertebrates, information can be extrapolated from these model organisms and applied to humans. The benefits to using an invertebrate model specifically is that they provide a more simplified nervous system and show part of the

evolutionary significance to traits which have been preserved and refined. An invertebrate model that has been commonly used to study the nervous system is the crayfish. As in mammals, the adult born crayfish neurons will travel to the olfactory bulb and become interneurons (Zhang et al., 2008). Similarities between crayfish and vertebrates with regards to neurogenesis also include: progenitor cells having both glial and neural properties, the use of a migratory stream to direct the growth of proliferated cells, as well as having similar features of their neurons (Sullivan et al., 2007).

Beyond the cellular similarities, factors that affect neurogenesis in vertebrates have also showed the same correlation in crayfish. Sandeman and Sandeman (2000) showed that enriched environments increase neurogenesis in crayfish as it does in the mammalian models. Also, there has been a link established between olfactory stimulus and rate of neurogenesis (Cayre et al., 2002). However, one of the major factors affecting neurogenesis, physical activity, has yet to be tested in crayfish. Examining this association in the more simplistic invertebrate model will help to establish if there is a direct relationship between exercise and neurogenesis, or if there is something else leading to this association in the more complicated mammalian system. Though exercise has a strong effect on neurogenesis by increasing proliferation and new cell survival, it has not been studied extensively in many model organisms. The purpose of this study will be to determine whether exercise will increase neurogenesis in the adult crayfish.

## **Materials and Methods**

### *Model Organism*

Crayfish were collected from the Little Rio Grande River in Patterson Park, Muskegon County, MI. The species utilized in this experiment was *Orconectes propinquus*. Both adult male and female subjects were used.

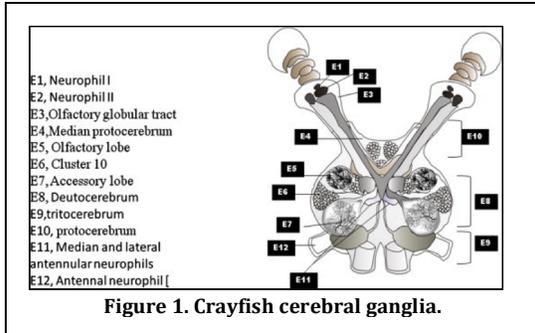
### *Initial Experimental Proposal*

The experimental procedure involves 80 male crayfish of the species *Orconectes propinquus*, 40 for experimental group, 40 for control group. They will be housed in isolation to diminish olfactory stimulation since olfactory enrichment alone has shown to increase survival of newly formed cells (Rocheffort et al., 2002). The experimental group will be exercised using a water flume forcing the crayfish to walk during exercise testing. The flume is not used for the control group; however the crayfish are handled with the same schedule as the exercise group for three weeks to allow for the elimination of stress as a variable between groups. It is important to account for similar stressors because stress has been shown to greatly diminish neurogenesis (Lucassen et al., 2008).

The experiment is carried out over a six-week time frame. The experimental group is subjected to thirty minutes of physical activity for five consecutive days. The control group is placed in a different isolation chamber with no water flow for thirty minutes. Each group will experience similar feeding schedules and day/night schedules.

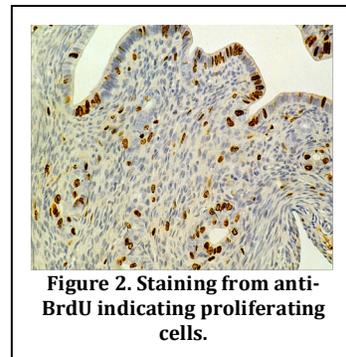
At the beginning of week one, crayfish from both groups will be immersed in a one-liter solution containing 10mL of the tag bromodeoxyuridine (BrdU) over night (Sandeman & Sandeman, 2000). BrdU is a synthetic thymine that inserts into newly

synthesized DNA and can be detected by administration of an anti-BrdU marker (Nowakowski, et al., 1989). Crayfish are then exercised for two weeks and starting week three, five crayfish from each group will be sacrificed. This will be done each week for four weeks to quantify the number of new cells that proliferate and survive through each week. The brains of these crayfish are dissected, preserved, dried, and embedded into



paraffin blocks. Twenty-five micrometer sections of the preserved paraffin brains are placed on slides to show the proliferating zones, soma clusters nine and ten, as well as the olfactory migratory stream (Fig 1). Cells

that are formed after administration of BrdU will be marked with the Anti-BrdU antibody and will appear dark brown on the prepared slides (Figure 2; Sandeman & Sandeman, 2000). If exercise affects neurogenesis in crayfish similar to what is seen in mammals, the number of BrdU positive cells containing neuronal markers should be significantly greater in the exercise group than the controls due to increased proliferation rate and/or survival. Additional cellular antibodies maybe used based on results of initial BrdU exposures.



### *Progress Report*

Crayfish were dissected using a common dissection kit and tray. The cerebral ganglia were initially retrieved with eyestalks intact. Eyestalks were kept to help with orientation of samples during paraffin encasement and slide preparation. Following

dissection, tissue samples were kept in a solution of 4% paraformaldehyde (PFA) in 10X phosphate buffer solution (PBS) for a period of 48 hours. Neural tissue ended up deteriorating by the end of this period, so the 48 hours was reduced to 24 hours with an addition of three washes in 1X PBS solution. After this adjustment, neural tissue still exhibited deterioration, leading an adjustment of the pH of the PBS. The solution was adjusted to slightly basic by adding sodium hydroxide until the pH was 6.2.

After 24 hours, samples were dehydrated in preparation for paraffin encasement. Neural tissue was exposed to a 70% ethanol solution for one wash, which was twenty minutes in duration. Tissue was subsequently washed in a 95% and the 100% ethanol solutions, to be followed by two xylene washes. Each wash lasted twenty minutes. An 80% ethanol wash was eliminated so that tissue was not over dehydrated, and trials were reduced to one per solution type. Neural tissue was then placed in a heated paraffin bath for one hour to allow for permeation of paraffin into the samples. A Micron® paraffin embedding station was used to embed the tissue for later sectioning. Embedded tissues were refrigerated for twenty four hours in order to solidify, then cut with a box cutter blade and fitted into a Microm® microtome for sectioning.

Sections were cut at a thickness of 12.5  $\mu\text{m}$  then placed in a 36° C water bath to unfurl. Sections were then placed on slides and melted over a hotplate at 65° C. Inspection was then done under microscope to determine if brain tissue was adequately preserved.

## **Results**

Over the course of the experiment, several adjustments were made to the process of crayfish dissection, tissue preservation, and sectioning. The changes made to these processes were imperative to the overall project goal of running exercise trials with crayfish. Without properly establishing the techniques of dissection and cerebral ganglia tissue sectioning, we could not progress to the next phase of the project without having prodigious inconsistencies in our results due to deterioration of neural tissue during preservation phase and dehydration when exposed to ethanol washes.

Reducing the forty-eight hour preservation phase by 50% and adding the pH monitored 1X PBS wash prevented tissue from deteriorating before sectioning. Reducing the amount of washes per ethanol solution allowed for tissue dehydration at the desired level without yielding brittle sections. Samples run under these adjustments produced viable tissue sections. Thus far, the preservation and sectioning procedures have been refined and we are now continuing on to the exercise portion the proposed work.

## **Discussion**

It was critical to perfect the techniques for dissection, tissue preservation, paraffin encasement, and sectioning. The initial techniques had previously been established by a graduate student working the lab, yet when we attempted her protocols, we found the neural tissue to either be too wet from the tissue deterioration or too brittle from being over dehydrated. The prior work helped us in creating tissue preservation protocols that we have refined to now allow us to move onto the exercise portion of the experiment.

Exercise has been shown to delay onset and progression of dementia and Alzheimer 's disease, serves as a potent antidepressant, leads to greater improvement in cognition following brain injury and reduces age-related decline of neurogenesis (Eisch et al., 2008; Gresbach et al., 2004; Teri et al., 2008; Wolf et al., 2006). Exploring exercise and how it directly affects neurogenesis in an invertebrate model will allow for greater flexibility in testing exercise's viability. And greater understanding of the relationship between exercise and its associated physiological changes in crayfish may allow for expansion of physical activity as a treatment for neurological disorders and possibly lead to more clinical applications than those that are currently being explored.

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