

4-2012

## Effect of Exercise on Adult Neurogenesis in Crayfish

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Effect of Exercise on Adult Neurogenesis in Crayfish

Jennifer E. Klutts

A Thesis Submitted to the Graduate Faculty of

GRAND VALLEY STATE UNIVERSITY

In

Partial Fulfillment of the Requirements

For the Degree of

Master of Health Sciences

Biomedical Sciences

April 2012

## **ACKNOWLEDGEMENTS**

I want to thank my committee, Dr. Daniel Bergman, Dr. John Capodilupo, and Dr.

Merritt Taylor for their support and enthusiasm for my project. I also would like to thank

my friends and family for their support, especially my parents, Bradley Bourbina and

Farah Itani.

## ABSTRACT

Adult neurogenesis, formation of new neurons, has been determined to be a part of the normal physiology in all species of animals studied to date. There have been several factors observed to increase the number of newly formed cells; the most potent of these factors being exercise. Though exercise has a strong effect on neurogenesis by increasing proliferation and new cell survival it has not been extensively studied in many model organisms. This study is the first to look at the effect of exercise on neurogenesis in an invertebrate through the use of crayfish, *Orconectes propinquus*. Experimental designs, drying protocols and equipment troubleshooting developed here will guide future studies of invertebrate neurogenesis and its relationship to exercise.

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## STUDYING NEUROGENESIS

It was originally believed that the formation of new neurons, i.e. neurogenesis, was limited to embryonic development. However, in 1962 Joseph Altman made a discovery that began a line of evidence leading to the now supported concept that new neurons are born in adulthood as a part of the normal physiology. Altman was looking for gliogenesis following brain trauma using the marker for new cells, Thymidine- H3. He found, unexpectedly, that in regions not associated with brain lesions new cells were being generated (Altman, 1962). To date, neurogenesis has been established as a major part of the adult brains' plasticity in all organisms studied (reviewed in Ehninger, & Kemperman, 2007; Reviewed in Lindsey & Tropepe, 2006).

Though the concept of adult born neurons had the potential to be a groundbreaking and exciting area for research, the field of adult neurogenesis did flourish until the 1990's, when the technology became available to confirm the newly formed cells were, in fact, neurons (reviewed in Ma, Ming, Gage, & Song, 2008). Research in adult neurogenesis began to flourish when a synthetic thymine named 5-bromo-2-deoxyuridine (BrdU) entered the scene in 1989. BrdU is incorporated into the DNA in place of thymine during the S-phase of the cell cycle. This synthetic thymine is only found in cells which undergo mitosis while BrdU is available to the organism. Knowing the timeline of administration, and taking tissue samples at different times allow for the

determination of proliferation rates, cell survival, and the quantification of newly formed cells (Nowakowski, Lewin, & Miller, 1989).

To identify cells that uptake BrdU, a BrdU antibody (anti-BrdU) is administered to prepared tissue. With application of anti-BrdU newly formed cells will visually differ from the other cells in the sample (Schmidt, & Derby, 2011). Since the antibody is all that is used to determine the identity of the newly generated cells, it did not take researchers long to start adding a secondary antibody to determine cell type as they became available. Secondary glial and neuronal markers are used to observe differentiation of the proliferating cells since both lineages may be derived from one cell type (Lois, & Alvarez-Bully, 1993). The most commonly used markers for identification of cells that have become mature neurons include: neuron specific enolase (NSE), microtubule-associated protein (MAP-2) and neuronal specific nuclear protein (NeuN). For identification of glial fate, researchers commonly use 2,3, cyclic nucleotide (CNP), calcium binding proteins such as S100 $\beta$ , and glial fibrillary acid protein (GFAP) markers (reviewed in Abrous, Koehl, & Moal, 2005).

The use of BrdU has become standard practice for neurogenesis studies in the past 20 years. Currently, further advances in research methods have allowed the morphology of the newly formed cells to be observed by using a retroviral vector, expressing green fluorescent protein (GFP). GFP like BrdU will label only newly formed cells, in addition



GFP can fill the entire cell. Seeing the neurons branching allows researchers to make observations of its integration into the current circuitry (van Praag et al., 2002).

As the technology grows, so does the amount of obtainable information on adult born neurons. Each year, more research is compiled on how neurons are generated, neurogenic locations, and factors which affect rates of proliferation and survivability. Researchers continue to look for more markers that identify specific populations of neural progenitors, and ways to generate new neurons in non-neurogenic brain regions. It has yet to be determined how far clinical applications associated with adult born neurons will reach, but it is clear that the potential positive impacts are immense.

## **NEUROGENESIS IN ADULTHOOD VS DEVELOPMENT**

As researchers began to understand adult neurogenesis, it became clear that it is not just a continuation of embryonic neural development, but is its own separate process. Both adults and developing embryos have their own stem cell populations with different characteristics. Stem cells are characterized by their ability to generate many different cell types while maintaining the ability to self-renew through asymmetric division. Specific to the nervous system stem cells, formation of glia and neurons are possible from these cells (McKay, 1997).

As the central nervous system (CNS) develops in utero, the stem cell that gives rise to both glia and neurons are radial glial cells, which are located throughout what will become the brain and spinal cord (reviewed in Pollard, & Conti, 2007). The neuroblasts generated from the radial glia migrate to their destination, guided by either radial glia or axon fibers (Edmondson & Hatten, 1987). However, in adult neurogenesis both the stem cells and migration are different than in embryonic brain development. The stem cell for adult neurogenesis is a specialized astrocyte. This astrocyte-like stem cell is so similar to other astrocytes of the CNS, that there are no markers to date that can differentiate between the two (reviewed in Imayoshi, Sakamoto, Ohtsuka, & Kageyama, 2009). These adult stem cells, like the radial glia, can yield both neurons and glia (Lois, & Alvarez-Buylla, 1993). Neuroblasts arising from these specialized astrocytes reach their target through chain migration within a channel of similarly specialized astrocytes (Jankovski, & Sotelo, 1996).

In adult neurogenesis the rate of new cell formation is much slower than in utero, and continues to decline with age (Altman & Das, 1965; Kuhn, Dickinson-Anson, & Gage, 1996). One of the biggest differences between embryonic and adult neurogenesis is that adult neurogenesis is greatly limited in its anatomical location, and the types of neurons it can generate. Unlike embryonic development that occurs throughout the CNS, adult neurogenesis is compartmentalized into regions known as neurogenic niches, or

stem cell niches. Stem cell niches provide the surrounding support for stem cells. They function to protect and maintain stem cell populations throughout life, as well as provide signals to the stem cells located within their microenvironments (reviewed in Jones & Wagers, 2008; Morrison & Spradling, 2008).

Depending on the animal species, the number of stem cell niches varies. Evolutionary older organisms tend to have a greater number of proliferating zones, with the teleost fish possessing the most (reviewed in Lindsey, 2006). Mammals only have two zones that have the specialized conditions to generate adult born neurons; the subgranular and subventricular zones (reviewed in Zhao, Deng & Gage, 2008; reviewed in van Praag, 2006).

## **ADULT NEUROGENESIS IN MAMMALS**

### **Subgranular Zone**

The subgranular zone (SGZ) is located within the dentate gyrus, which is part of the hippocampus in the mammalian brain. Proliferation, the generation of new cells, in this zone can lead to either new glial cells or neurons destined to become hippocampal granular cells (Lois, & Alvarez-Bulla, 1993).

The stem cells of this zone have been identified as type 1 cells, which have a triangular shape, with their soma located below the granular layer, and possess an apical process that reaches into the molecular layer of the dentate gyrus [Figure 1] (reviewed in Ming, & Song, 2011; reviewed in Ehninger, & Kempermann, 2007). They are identified by their expression of nestin, an intermediate filament found in progenitors in association with astrocytic features. The type 1 cells have many similarities to adult astrocytes, and may even be a specialized subtype of these glial cells. Both astrocytes and type 1 cells are marked with glial fibrillary acidic protein (GFAP), and have a highly branching morphology. However, the type 1 cell does differ from the mature astrocyte by not forming an immune reaction with S100 $\beta$ , which is often used as a marker for astrocytes, indicating that the two groups of astrocytes are not identical (Filippov et al., 2003). However, astrocytes cultured from other areas not associated with the adult neurogenic niche can be manipulated by administration of transcription factors to behave as neuronal progenitor cells themselves (reviewed in Kriegstein, & Alvarez-Buylla, 2009).

Asymmetric division of type 1 cells gives rise to type 2 cells, which serves as the highly proliferative intermediate precursor cell. These intermediate precursors allow for a sufficient number of new cells to be formed with minimal divisions necessary from the original stem cells (Ehninger, & Kempermann, 2007). The type 2 cells are morphologically distinct from type 1; they lack long processes, have a smaller soma, and

possess a round to oval nuclei (Filippov et al., 2003). Type 2 cells can be arranged into two sub-categories based upon molecules expressed, type 2a and 2b. Type 2a cells still maintain glial markers; however they no longer have the morphology of a glial cell. Type 2b cells begin to express transcription factors, specific for developing granule cells, which will be marked using NeuroD1 (neuronal differentiation 1) and Prox1 (prospero homeobox 1) (reviewed in Kemperman, Song, & Gage, 2008).

From the type 2 cells, type 3 cells are derived, which are neuroblasts and immature neurons. To track these developing neurons Zhao et al. used retrovirus-mediated gene transduction in 2006 in the hippocampus of adult mice. They discovered four distinct morphological stages during their study. Stage A occurs during migration, when developing neurons begin to polarize and grow axons and dendrites. In the next stage of development, stage B, dendrites grow to reach into the molecular layer, and the axon of each immature neuron reaches into the CA3 area of the hippocampus. Stage C is where the spines are initially developed, which will only begin after the axon has integrated into the CA3. Stage D is the longest, taking place over several months, where spines are continually modified (Zhoa, Teng, Summers, Ming, & Gage, 2006).

Most new neurons will be eliminated before they form connections with target in the CA3 (reviewed in Kempermann, Song, & Gage, 2008). The neurons that survive are integrated into the current circuitry, and share many of the same properties as the

preexisting neighboring neurons. They have similar threshold and resting potentials, and input resistances (van Praag, Schme, Christie, Toni, Palmer, & Gage, 2002). Beyond the similar functional properties, newly generated cells are morphologically indistinguishable from the older cells surrounding it. Without mitotic markers researchers would have no way to identify preexisting neurons from adult born (Markakis, & Gage, 1999).

As the SGZ continues to generate new neurons, the volume of granular cells within the dentate gyrus increases. Researchers have inferred that since these granule cells are not created to replace old cells addition to the current circuitry is indicative of a functional role for adult neurogenesis (reviewed in Appleby, Kempermann, & Wiskott, 2011). There are several theories about the function of these new granular cells. The most prominent theory is that newly formed neurons are for increasing spatial memory, specifically retention and discrimination, since they are being integrated into the dentate gyrus, an area essential in the formation of spatial and episodic memories (reviewed in Eichenbaum, Dudchenko, Wood, Shapiro, & Tanila 1999).

An experiment in 2005 by Snyder et al. was the first to show the link between new neurons and long-term potentiation. To assess memory formation a Morris water maze was used (Morris, 1984). Some rats underwent irradiation to inhibit neurogenesis. Without newly formed granule cells the rats were still able to learn the spatial memory task with equal efficiency as their non-irradiated counterparts. However, when the rats

were re-tested at 2 weeks and 4 weeks post-learning, the irradiated group performed significantly worse. Results suggested that adult neurogenesis is required for the formation of long-term spatial memory (Snyder, Hong, McDonald, & Wojtowicz, 2005).

A study carried out four years later by Clelland et al. (2009) identified an association between spatial memory impairments resulting from ablated hippocampal neurogenesis with the distance of separation between targets. To impair hippocampal neurogenesis mice were exposed to low dose localized x-irradiation. Mice with hippocampal neurogenesis ablated performed significantly worse in spatial discrimination tasks than non-irradiated mice only when the targets were physically close together rather than far apart. Clelland et al. designed two tasks to test their hypothesis on pattern separation-dependent memory; one using a radial arm maze (RAM) with eight arms and external spatial cues, and the other using a nose stimulated touch screen grid. In the RAM test, when the target arm and the starting arm were in close proximity, the irradiated group made significantly more wrong arm choices than the non-irradiated group.

However, when the target arm was further away the two groups scored similarly. With the touch screen test, mice were trained to associate objects with a spatial location on a screen. When the image appeared at its correct location, the mouse would press its nose to it. In trials when the images were only separated by one unlit box, as opposed to three unlit boxes, the irradiated group was significantly impaired. These results indicate that

adult hippocampal neurogenesis is needed for memory tasks with a lower degree of spatial separation (Clelland et al., 2009).

### **Subventricular Zone**

The other neurogenic region within the mammalian brain, the subventricular zone, is located below the ependymal lining of the lateral ventricles [Figure 2]. This is the larger of the two niches and generates glial cells, either astrocytes or oligodendrocytes, and olfactory bulb interneurons (reviewed in Lim, Huang, & Alvarez-Buylla, 2008; and Lois, & Alvarez-Buylla, 1993). The olfactory bulb fate of the SVZ neuroblasts is still maintained, even for grafted cells, as long as they are placed in the SVZ or migratory stream extending from the SVZ to the olfactory bulb (Doetsch, & Alvarez-Buylla, 1996). Lois and Alvarez-Buylla transplanted SVZ stem cells from one mouse to the lateral ventricle of a host mouse. The transplanted SVZ cells migrated to the olfactory bulb to generate interneurons with the endogenous neuroblasts (Lois, & Alvarez-Buylla, 1994). It has also been found that neuron progenitor cells transplanted to the SVZ from the other proliferative zone, the SGZ, will form new neurons. However, the SGZ stem cells from the hippocampus will not generate neurons typically seen in the hippocampus, but those seen in the olfactory bulb. SGZ cells transplanted to the migratory stream move with the endogenous SVZ cells and adopt characteristics of the other newly generated olfactory



bulb interneurons. Characteristics which are not found in the hippocampal neurons became expressed in the cells grafted, such as production of tyrosine hydroxylase (reviewed in Gage, 2000). This indicates that the fate of these progenitors is not predetermined, there local extrinsic factors are at work to cause differentiation and formation of neuronal subtypes.

There are three major cell types making up the SVZ, similar to those seen in the SGZ. The neuronal stem cells here are labeled as B cells, which are GFAP-expressing astrocytes. To verify that the type B cells were the primary precursors of the SVZ adult mice were treated with an antimetabolic drug known as cytosine- $\beta$ -D-arabinofuranoside (Ara-C) to remove the migrating neuroblasts and intermediate precursors from this zone, leaving only the slow dividing type B astrocytes and ependymal cells. Half a day after completion of Ara-C treatments, mitosis continued throughout the SVZ, reestablishing a pool of intermediate precursors within two days, and neuroblasts two days following that. Through the use of retroviral labeling it was determined that it was the B cells, not the ependymal cells, from which these new cells derived (Doetsch, Caille, Lim, Garcia-Verdugo, & Alvarez-Buylla, 1999).

Type B cells can be subdivided into two types, B1 and B2 (Doetsch, Garcia-Verdugo, & Alvarez-Buylla, 1997). B1 cells have apical processes containing immobile cilia which reach into the lateral ventricle, possibly to receive signals from the cerebral

spinal fluid. Type B2 cells are located close to the lower striatal parenchyma (Mirzaden, Merkle, Soriano-Navarro, Garcia-Verdugo, & Alvarez-Buylla, 2008). The primary progenitors, B cells, are slow to divide and will give rise to transient amplifying secondary progenitors, known as type C cells, which have a more rapid cell division. When C cells divide they give rise to migrating neuroblasts, type A cells (Doetsch, Garcia-Verdugo, & Alvarez-Buylla, 1997). These neuroblasts migrate from the area below the lateral ventricle to the olfactory bulb through what is known as the rostral migratory stream [Figure 2] (reviewed in Ihire, & Alvarez-Buylla, 2011; reviewed in Kohwi, Galvao, & Alvarez-Buylla, 2006).

The rostral migratory stream (RMS) contains neuroblasts moving through chain migration within a sheath made of type B cells. The RMS begins as the polysialated glycoprotein neuronal cell adhesion molecule (PSA-NCAM)-positive chains of neuroblasts located in the SVZ start to arrange in parallel and converge along the longitudinal axis (Doetsch, & Alvarez-Buylla, 1996). NCAM deficient mutant mice still maintained the ability to form the RMS; however, there was a decrease in efficiency for migration of neuroblasts to the olfactory bulb without the adhesion molecules, resulting in smaller olfactory bulbs (Chazal, Durbec, Jankovski, Gougon, & Cremer, 2000).

Once in the RMS, neuroblast chain migration is directed through the use of chemorepulsion from the ventricular zone and chemoattraction from the olfactory bulb

(reviewed in Sun, Kim, & Moon, 2010). The primary repulsive factor for SVZ neuroblast migration is the protein Slit. There are three types of Slit proteins, two of which are expressed in the adult mammalian brain, Slit 1 and Slit 2. The choroid plexus will release Slit 2 while the septum, which is located caudally to the SVZ, will secrete both Slit 1 and Slit 2 (Wu et al., 1999; Hu, 1999). The receptors for Slit, roundabout (Robo), are located on migrating type A cells. Specifically, Robo 2 and Robo3/Rig-1 receptors can be found highly expressed on the membrane of these neuroblasts. To confirm slit-robo signaling is essential for chemorepulsion of type A cells, Nguyen-Ba-Charvet et al. (2004) developed Slit1/Slit2 null mutated mice. The mice lacked expression of Slit from the septum and choroid and, as a result, no longer possessed repulsive activity, resulting in abnormalities in migration of the neuroblasts (Nguyen-Ba-Charvet et al. 2004). Though Slit has an important role for migration of neuroblasts, it does not appear to be the only factor needed and may be only needed for initiating migration. Shortly after the neuroblasts begin their journey the cells will be out of the effective range for this protein (Wu et al., 1999).

Once migration is initiated other signals start to take effect on the migrating chain since a single signal directing the cells away from the SVZ is not specific enough to prevent these cells from entering other areas of the cerebral cortex (Hu, 1999). To account for cells being directed all the way to their target researchers believe that the

olfactory bulb is releasing attractant factors. Prokineticin-2 and Netrin-1 are some of the proposed molecular attractants being released from the olfactory bulb (reviewed in Kohwi, Galvao, & Alvarez-Buylla, 2006).

The rates at which type A cells travel through the RMS depends on maturation, with older neuroblast moving significantly faster than younger ones. Migratory speeds in rodents have been found to vary between 52-71 $\mu$ m (Nam et al., 2007). Rates observed in primates are significantly slower than in the rodent brain, even when accounting for the increase in distance the neuroblasts must travel (Kornack, & Rakic, 2001).

More than 30,000 type A cells leave the rodent RMS each day (reviewed in Lledo, Alonso, & Grubb, 2006). Of these thousands of cells making the trip the majority will not reach maturity, and out of those adult generated mature neurons only half will survive more than a month (reviewed in Lledo, Alonso, & Grubb, 2006). Survival rates can be increased, however, with the enhanced odor enrichment (Rocheffort, Gheusi, Vincent, & Lledo, 2002).

Upon reaching the olfactory bulb neuroblast must be separated from their chain mates and radially migrate toward their target areas. The molecule Reelin, which is expressed by olfactory bulb mitral cells, serves as the detachment signal which initiates the release of neighboring cells at the end of the RMS. However, Reelin has not been shown to give guidance cues for the radial migration observed in the olfactory bulb

(Hack, Bancila, Loulier, Carroll, & Cremer, 2002). To direct the detached neuroblasts to their correct positions on the outer layer of the olfactory bulb, Saghatelyn et al. (2004) determined that the molecule Tenascin-R was required. Tenascin-R is generated by granule cells in the deeper layers of the bulb. Its expression, or lack of, has no effect on proliferation rates, tangential chain migration orientation or speed, differentiation ratios, or cell apoptosis. However, what Tenascin-R deficiency did affect was the local migration of cells released from the RMS. Neuroblasts in mice without Tenascin-R expression travel normally through the migratory stream but upon reaching the olfactory bulb the cells would cluster together, forming an accumulation of new cells at that junction (Saghatelyan, Chevigny, Schachner, & Lledo, 2004).

Once the neuroblasts reach their target they will then differentiate into two types of interneurons: granule cells or periglomerular cells. These new cells enter into the current circuitry to replace old cells that underwent apoptosis (reviewed in Kohwi, Galvo, & Alvarez-Buylla, 2006). Both interneuron types develop receptors for the brain's primary inhibitory neurotransmitter GABA and function as local inhibitors due to this property. It has also been demonstrated that the periglomerular cell will sometimes respond to dopamine as well (reviewed in Ming, & Song, 2011).

It has been hypothesized that the turnover of new cells in the olfactory bulb may be to enhance odor differentiation through pattern separation since they are inhibitory

interneurons (reviewed in Shay, Wilson, & Hen, 2011). An experiment done by Gheusi et al. showed reduction in adult born olfactory interneurons is associated with a decrease in ability to discriminate odors (Gheusi, Cremer, McLean, Chazal, Vincent, & Lledo, 2000). The ability to change olfactory bulb interneurons provides extra plasticity, allowing organisms to make adjustments to ever-changing olfactory environment.

## **ADULT NEUROGENESIS IN OTHER ORGANISMS**

### **Birds**

As mentioned previously, adult neurogenesis is not unique to mammals; it has been found in all animals studied to date (reviewed in Ehninger, & Kemperman, 2007; reviewed in Lindsey & Tropepe, 2006). One of the most extensively studied organisms for adult neurogenesis is the canary (Alvarez-Buylla, Garcia-Verdugo, Mateo, & Merchant-Lorios, 1998). In birds the location receiving the greatest contribution of adult formed neurons is in the area for learned song production, the high vocal center or HVC (reviewed in Doetsch, & Scharff, 2001).

Canaries, in addition to other songbirds, will annually modify their song, with peaks and lulls in the number of sounds they can produce, called syllables. In 1967, Arnold and Nottenbohm identified a sex difference in the size of the HVC. Male canaries

have more complex songs and have a HVC three to four times larger than that of females. However, when females were given testosterone the volume of their HVC doubled and new cells formed connections with the robustus archistriatalis (RA) nucleus, mimicking their male counterparts (reviewed in Nottenbohm, 1989). A 12 month survey of the HVC size and song production by Kirn et al. (1994) showed that the fluctuations in syllables, which naturally occur throughout the year and number of newly formed neurons are closely correlated. The number of new neurons observed in the HVC and the syllable range fall substantially at the end of the breeding. These syllables will be regained before the next breeding season, which has been associated with the replenishment of HVC cells through adult neurogenesis (Kirn, Loughlin, Kasparin, & Nottenbohm, 1994).

There are many similarities between avian and mammalian adult neurogenesis. The primary neurogenic niche is still located within the walls of the lateral ventricle. However, in birds the progenitors are not located below the ependymal layer, but rather have direct interaction with the ventricle. Because of this spatial relationship the niche in birds is referred to as the ventricular zone (VZ), rather than the subventricular zone. Cells in the VZ are named similarly to their mammalian counterparts; progenitors are labeled as B cells, young migrating neurons are still identified as type A cells, and the niche is still surrounded by ependymal cells. Birds, however, lack the type C cells transient amplifying cells observed in mammals (Alvarez-Buylla, Garcia-Verdugo, Mateo, &

Merchant-Larios, 1998). Type C cells are not needed because the B cells are, themselves, capable of generating both glia, including radial glia, and neurons (Goldman, Zukhar, Barami, Mikawa, & Niedzwikecki, 1996). Another significant difference between the B cells seen in birds and those in mammals is that the primary progenitors in birds are more accurately described as radial glia that are preserved into adulthood, rather than differentiating into astrocytes (reviewed in Doetsch, & Scharff, 2001).

Many of the adult generated neurons, as previously mentioned, are added to the HVC to replace older neurons. To determine the role of adult neurogenesis in repair, an experiment was carried out by Scharff et al. (2000) with directed cell death. Through chromophore-targeted neuronal degeneration specific cell types were killed in the HVC. In one group of birds the cell death was targeted to a location which normally undergoes cell replacement, and in another group of birds cells were killed within an area that does not receive adult-born neurons. The cell death that was generated in the areas accustomed to gaining neurons throughout adulthood recruited replacement cells and recovered neuron volume and singing ability after two months. Birds with cell death in areas not normally associated with neurogenesis did not experience this recovery (Scharff, Kirn, Grossman, Macklis, and Nottebohm, 2000). Like mammals, neurogenesis in birds was determined to occur only in specific areas of the brain.



New neurons are also added to the hippocampus of the avian brain, and as with mammals, this addition has been tied to spatial memory. A study with 29 different species of bird showed that the birds which store and retrieve their food have larger hippocampuses, even when accounting for variations between each species brain size. Researchers determined that the increase in hippocampal size was associated with the spatial memory needs of the food storing birds (Krebs, Sherry, Healy, Perry, & Vaccarino, 1989). Later researchers would observe that birds, and other animals which store food, have an increase in hippocampal size during food storing seasons because of the spatial memory required for this task (van Praag, Christie, Sejnowski, & Gage, 1999).

### **Crustaceans**

Adult neurogenesis is not just isolated to vertebrates. Crustaceans are a commonly studied invertebrate model for the nervous system. The first observation of neurogenesis in decapod crustaceans occurring after embryological development was in 1996 using BrdU to look at new neuron formation in spider crabs from larval to juvenile stages of development. During the beginning stages of development neuroblasts are highly active but as the crab progresses through developmental stages proliferation decreases. By the time second metamorphosis is reached proliferation across the brain has terminated

leaving only one location to retain mitotic activity, the olfactory lobe (Harzsch & Dawirs, 1996).

To date, all crustaceans studied for neurogenesis have at least one proliferative zone throughout life [Figure 3] (reviewed in Schmidt, 2007). The proliferation zone common to all crustaceans is known as the lateral soma cluster, also known as cluster 10 (Schmidt, & Harzsch, 1999). Cluster 10 is located laterally to the olfactory and accessory lobes (Sandeman, & Sandeman, 2000). Cluster 10 contains the somata of projection neurons, which provide output to the neighboring accessory and olfactory lobes (Schmidt, & Derby, 2011; Sandeman, & Sandeman, 2000).

Some crustaceans, such as lobsters and crayfish, possess a secondary proliferative zone known as the medial cluster, or cluster 9 [Figure 4] (Schmidt, & Harzsch, 1999; and Sandeman, & Sandeman, 2000). Cluster 9 lies ventral and medial to the olfactory bulb with its soma belonging to local interneurons (Schmidt, & Harzsch, 1999; Sandeman, & Sandeman, 2000). Though cluster 9 is located on the opposite side of the olfactory bulb to cluster 10 they are connected by fibrous strands located on the ventral surface of the brain (Song, Johnstone, Edwards, Derby, & Schmidt, 2008).

Neurons generated from either of these neurogenic zones have a similar fate; they will become olfactory interneurons (Zhang, Allodi, Sandeman, & Beltz, 2009). The precursor cells which will derive these olfactory projection neurons are isolated inside a

clump of small glial-like cells which form the stem cell niches of clusters 9 and 10 (reviewed in Schmidt, 2007). In 2011 Schmidt and Derby proposed a model on the cell lineage within decapod crustaceans to summarize years of research. They proposed that the adult neuroblasts located within the stem cell niches divide asymmetrically with one daughter cell staying anchored to the niche and the other becoming a neuron progenitor. The progenitor cell migrates down the attached duct to a neighboring proliferation zone. These migrating daughter cells appear to be transit amplifying intermediate progenitors accounting for the continual replenishment of stem cells, known as ganglion mother cells, in the proliferation zones with minimal divisions of the adult neuroblasts within the niche (Schmidt, & Derby, 2011).

There are several similarities between neurogenesis in mammals and crustaceans. Each experiences lifelong neurogenesis accompanied by an age dependent decline in its rate (Kuhn, Dickinson-Anson, Gage, 1996; Hansen, Schmidt, 2004). There also exists an anatomical similarity in the arrangement of the stem cell niches. Both have progenitor cells surrounded by smaller glial cells with extended processes providing association with brain vasculature. The precursor identity however is varied for the two groups. In mammals, the precursor cells are specialized astrocytes; in crustaceans, they are neuroblasts with undifferentiated cell phenotypes. Precursors in each group will divide asymmetrically to form transit amplifying cells. The neuroblasts formed from the primary

division will travel through ducts formed from glia, similar to those around the niche, in both animal groups (Schmidt, & Derby, 2011; Sullivan, Benton, Sandeman, & Beltz, 2007). Such similarities are what allow researchers to extrapolate results discovered in these invertebrate models and apply them to mammals.

### **Insects**

Another invertebrate group which has been studied for life-long neurogenesis is hexapoda, insects. The proliferative zone has been identified within the corpora pedunculata, also known as the mushroom bodies. The mushroom bodies are the main associative center in insects. It is the apex of the mushroom body where neuroblasts generate new neurons. The newly formed neurons will become interneurons known as Kenyon cells. The Kenyon cells form connections with either the alpha or beta lobes of the association center (Cayre, Strambi, Charpin, Augier, Meyer, Edwards, & Strambi, 1996; reviewed in Cayre, Malaterre, Scotto-Lomassese, Strambi, & Strambi, 2002).

As shown by this small sampling of organisms, adult neurogenesis is a common theme across many different animal lineages (reviewed in Ehninger, & Kemperman, 2007; review in Lindsey & Tropepe, 2006). By looking at organisms of different phyla, researchers have found that there are many similarities in neurogenic niche arrangement,

types of cells generated, and effectors. These parallels observed may be indicative of either homology or convergent evolution (Sullivan, Benton, & Sandeman, 2007).

## **UPREGULATING ADULT NEUROGENESIS**

It has been observed that neurogenesis is not a static process; it can be manipulated through both extrinsic and intrinsic factors. Through increasing proliferation rates, promoting survival of new neurons, favoring adaptation of neural fate over glial, or a combination of these, organisms may increase circuitry within the hippocampus and olfactory bulbs. Further understanding of how to manipulate neurogenic rates and survival of newly formed neurons has great potential for future clinical applications particularly in areas associated with memory.

### **Extrinsic Factors**

The major external modifiers of neurogenesis are learning, enriched environment, and physical activity. All three factors result in an increase in new cell survival rates. However, only physical activity has shown to consistently increase proliferation (Gould, Beylin, Tanapat, Reeves, & Shors, 1999; Kempermann, Kuhn, & Gage 1998; Kim, Ko, Kim, Shin, & Cho, 2010; Sandeman, & Sandeman, 2000; van Praag, Schme, Christie, Tonl, Palmer, & Gage, 2002).

Experiments which used learning as the dependent variable showed that mice which learned a specific task have an associated increase in the number of new neurons in the dentate gyrus (reviewed in Luikart, Perederiy, & Westbrook, 2012). Work done by Gould et al. (1999) determined that general learning does not significantly increase new neuron survivability; instead, it is learning which relies on hippocampal activation leading to greater survival of neurons formed just prior to learning (Gould, Beylin, Tanapat, Reeves, & Shors, 1999). Increased new neuron survivability associated with hippocampal learning has been observed to be limited to one proliferative location, the subgranular zone (Kempermann, Song, & Gage, 2008; Gould, Beylin, Tanapat, Reeves, & Shors, 1999).

It should be noted, however, that tasks which utilize hippocampal memory also involve physical activity, which has shown to increase neurogenesis independently (van Praag, Kempermann, & Gage, 1999). Another factor which has been shown to increase cell survival, that also may have confounding effects from physical activity, is living in enriched environments. Enriched environments are composed of larger space with more obstacles to move around, increased possibilities for social interaction, and in the case of rodent based experiments, they are provided with a running wheel (van Praag, Kempermann, & Gage, 1999; Kempermann, & Gage 1999). Each of these factors may contribute to increased physical activity of the enriched group.

In experiments where mice were housed in enriched or impoverished environments, subjects in the enriched environment had a significant increase in newly formed neurons (Kempermann, Kuhn, & Gage, 1998). Similar results were found in experiments using crayfish. Crayfish housed in larger tanks with other crayfish experienced greater survival rates and increased proliferation when compared to crayfish housed in isolation (Sandeman & Sandeman, 2000). To further examine this relationship crayfish raised in a lab housed in laboratory aquaria for extended lengths of time were compared to wild crayfish. Those from large outdoor ponds had significantly larger clusters 9 and 10, even though they had the same carapace length (Sandeman & Sandeman, 2000).

The effects of both enriched environment and physical activity are specific to only one of the neurogenic zones, the subgranular zone. So far, neither has proven to be an effector for subventricular zone neurogenesis (Brown, et al., 2003). The only major factor observed to cause an up-regulation in subventricular zone neurogenesis is enriched odor exposure (Rochefort, Gheusi, Vincent, & Lledo, 2002).

When the effects that exercise and enriched environment have on neurogenesis were directly compared both yielded similar numbers of neurons and both significantly increased cell survival. However, only exercise significantly increases proliferation rates (reviewed in Olson, Eadie, Ernst, & Christie. 2006; van Praag, Kempermann, & Gage,

1999). Exercise is a strong stimulating factor for cell proliferation in the hippocampus, promoting neurogenesis and survival of new neurons (Kempermann, Song, & Gage, 2008; Lucassen, Oomen, van Dam, & Czeh, 2008). Exercise also increases spine densities and the complexity and length of dendrites, as well as affecting the cytoarchitecture surrounding the hippocampus (Eadie, Redila, & Christie, 2005).

Exercise has its most potent effect on neurogenesis when the activity is of a low intensity (Lou, Lin, Chang, & Chen, 2008). The lack of a linear relationship between activity intensity and neurogenesis may be more of a response of the exercise becoming a stressor with the greater intensity, and stress has been shown to be an antagonist for new neuron development in the adult CNS (Lucassen, Oomen, van Dam, & Czeh, 2008).

Stranahan et al. (2006) looked at the relationship between stressors and running-induced neurogenesis. They found that the stressor of daily BrdU injections caused a large decline in neurogenesis in the running group and when the experiment was carried out with only one injection, at the end of the trial, the decline was no longer present. They also found that when rats are housed together there is an associated increase in neurogenesis. When housed in social isolation the benefits of physical activity on neurogenesis was significantly delayed and the trials needed to be carried out longer to demonstrate an increase in new born cells (Stranahan, Khalil, & Gould, 2006).



Exercise has also been shown yield increased cognitive function and to facilitate recovery from brain injury (van Praag, Kemperman, and Gage, 1999). An experiment in 2010 by Sung-Eun Kim et al. looked at the effect of exercise with age-related memory loss in Sprague-Dawley rats. The rats in the old aged exercise group performed better in both short-term and spatial memory tasks than those in the old age control group without exercise; they also showed an increase in neurogenesis in the dentate gyrus not observed in the control (Kim et al., 2010).

Similar benefits between memory retention and exercise have been observed in humans. In a 31 year cohort study patients who exercised through midlife had a significant reduction in odds of dementia and Alzheimer's disease (Andel, Crowe, Pedersen, Fratiglioni, Johansson, & Gatz, 2008). Also, researchers are finding other associations between exercise-induced neurogenesis and depression. Continued exercise has shown to be a very potent antidepressant, working even better than medications at preventing depressive relapses (Bjornebekk, Mathe, & Brene, 2005). In the rodent model of depression it was discovered that one of the potential causes could be a decline in neurogenesis. Depressed rats had much lower proliferation rates in the hippocampus than their non-depressed counterparts (Bjornebekk, Mathe, & Brene, 2005).

### **Intrinsic Factors**

Intrinsic factors which have shown to enhance neurogenesis include several different growth factors, some selective signaling molecules, as well as a few proteins that influence differentiation. Growth factors which have been established to yield an increase in neurogenesis include: brain derived neurotrophic factor (BDNF), fibroblast growth factor 2 (FGF-2), insulin-like growth factor 1 (IGF-1), and vascular endothelial growth factor (VEGF).

Brain derived neurotrophic factor (BDNF) belongs to the neurotrophin family of growth factors. Barde et al. identified this growth factor in 1982 and observed that it increased cell survival within the dorsal root ganglion (reviewed in Binder & Scharfman, 2004). Infusion of BDNF into the ventricles results in an increase of new neurons within the olfactory bulb, parenchyma, septum, thalamus and hypothalamus (Pencea, Bingaman, Wiegand & Luskin, 2001; Zigova, Pencea, Wiegand & Luskin, 1998).

Recently it has been observed that with age there is a decline in circulating BDNF. This decrease in BDNF is accompanied by a similar decline in newly formed adult neurons. This may be one of the underlying factors for memory impairments associated with increased age (Hattiangady, Muddanna, Shetty & Shetty, 2005). Kim et al. in 2010 ran an experiment which looked at the association between exercise, BDNF expression and memory task performance with two age groups, young and old rats. They observed that the older rat group had less BDNF expression which was also associated with poorer performance on short term and spatial memory tasks compared to the young rat group. When they explored the effect exercise has on this relationship they found that BDNF was up-regulated in the rats subjected to treadmill running for 30 minutes each

day for 6 weeks. In the older rats this increase in BDNF was also accompanied by an increase in both short term and spatial memory task performance (Kim et al., 2010).

Other growth factors which effect neurogenesis have also been observed to increase in relationship to physical activity. Levels of both fibroblast growth factor 2 (FGF-2) and insulin-like growth factor 1 (IGF-1) elevate when subjects are exposed to physical activity (Gómez-Pinilla, Dao & So, 1997; reviewed in Kramer & Hillman, 2006). Exercise nearly doubles IGF-1 serum levels but does not result in significant changes in hippocampal levels. However, blocking the IGF-1 with antibodies does result in diminished hippocampal memory and exercise induced neurogenesis. Llorens- Martin et al. (2010) demonstrated that the dependence on IGF-1 for exercise induced neurogenesis varied by developmental stage of the cells. Serum levels of IGF-1 appears to effect only precursors and post-mitotic immature neurons but not pre-mitotic immature neurons, which are considered to be the intermediate stage in neuron development (Llorens-Martin, Torres-Aleman & Trejo, 2010). Blocking FGF-2 also has shown to reduce hippocampal neurogenesis. Through the administration of FGF-2 neutralizing antibodies, Tao et al. (1997) observed a 63% reduction hippocampal DNA synthesis in newborn rats indicating that this molecule is essential for hippocampal neurogenesis (Tao, Black & DiCicco-Bloom, 1997).

## **CONCLUSION**

There are several factors which have shown to increase adult neurogenesis. Intrinsic factors which promote new cell survival such as enriched environment, spatial learning, and exercise have a common underlying factor, they all involve physical

activity. Enriched environments are composed of larger space with more obstacles to move around, and in the case of rodent based experiments, they are provided with a running wheel, all of these factors are contributing to increased physical activity of the enriched group (van Praag, Kempermann, & Gage, 1999). Spatial memory is formed as organisms move around their environment also utilizing physical activity (Edie, Redila, & Christie, 2005; Holmes et al., 2004, Llorens, Torres-Aleman, & Trejo, 2010; Lou, Lin, Chang, & Chen, 2008; van Praag, Kempermann, & Gage, 1999). Therefore, the increase in survival observed through these two factors may be a result of exercise rather than the learning or the environment.

This important relationship between exercise and adult neurogenesis has only been examined in a few species to date, none of which were invertebrates. Looking at 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. It could also provide insight to the evolutionary significance of this relationship. Research on exercise induced neurogenesis using crayfish has begun at Grand Valley State University.

Exploring exercise and how it directly affects the nervous system in an invertebrate model will allow for greater understanding of how exercise may be used in a clinical setting. Also with increased knowledge of the molecular forces behind this relationship molecules that elicit proliferation could possibly be manipulated to induce neurogenesis. If neurogenesis continued to be stimulated and new neuron survivability improved, then some of the cognitive decline associated with age might be reduced, and there could potentially be a greater clinical application in treatment of neurodegenerative

diseases (van Praag, 2008; Zhang, Allodi, Sandeman, & Beltz, 2009; Zhao, Deng, & Gage, 2008).

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, Cameron, Encinas, Meltzer, Ming, & Overstreet-Wadiche, 2008; Gresbach, Hovda, Molteni, Wu, & Gomez-Pinilla, 2004; Teri, Logsdon, & McCurry, 2008; Wolf et al., 2006). Physical activity is already being looked at as a possible mode of treatment for patients with depression, dementia, schizophrenia, Alzheimer's disease, and alcoholism (Faulkner, & Biddle, 1999; Teri, Logsdon, & McCurry, 2008; Wolf et al., 2006).

Exploring exercise and how it directly affects neurogenesis in an invertebrate model will allow for greater understanding of this intricate relationship in humans. And greater understanding of the relationship between exercise and its associated physiological changes may allow for expansion of exercise as a treatment and possibly lead to more clinical applications than those that are currently being explored.

## INTRODUCTION

Neurogenesis, the formation of new neurons from neural stem cells, occurs throughout adulthood in a wide variety of animals, including humans (Ehninger, & Kempermann, 2007; Imayoshi, et al., 2009). Progenitor cells located in specific regions of the adult brain can generate not only glial cells but neurons which may become integrated into the current circuitry (Kornack, & Rakic, 2001; Lois, & Alvarez-Buylla, 1993; Rochefort, et al., 2002). While adult neurogenesis has great retentive potential, it is limited in location. There are only two locations in the mammalian central nervous system with the correct conditions for post-embryonic neurons to form, the subventricular zone (SVZ) and the subgranular zone (SGZ).

The SVZ lies beneath the ependymal cell lining of the lateral ventricles. This zone yields neuroblasts which will migrate through the rostral migratory stream to the olfactory bulb [Figure 2]. Once neuroblasts reach the olfactory bulb they will mature into new olfactory interneurons, resulting in an increased ability to recall and differentiate odors (Kornack, & Rakic, 2001; Lois, & Alvarez-Buylla, 1993; Rochefort et al., 2002; Zhao, Deng, & Gage, 2008). The other proliferative location, the SGZ, is located in the part of the hippocampus known as the dentate gyrus [Figure 1]. Neurogenesis in the SGZ is associated with spatial and short term memory (Altman, 1965; Gage, 2000; Snyder, et al., 2005).

There are several extrinsic factors that have shown to increase SVZ neurogenesis in adults; learning, enriched environment, and physical activity. When the effects each of these three factors has on neurogenesis are directly compared, it is voluntary exercise which yields the greatest impact (van Praag, Kempermann, & Gage, 1999). The finding

that exercise greatly enhances cell proliferation in mammals has been verified by several experiments over the last 10 years (Eadie et al., 2005, van Praag, Kempermann, & Gage, 1999; Kempermann, 2008; van Praag 2006). Exercises that last longer and are of a lower intensity tend to generate the greatest increase in proliferation (Holmes et al., 2004; Kempermann, 2008; Lou et al., 2008; Lucassen, et al., 2008).

Beyond enhancing proliferation, exercise has been shown to increase spine density and increase dendritic length and complexity on neurons within the dentate gyrus (Eadie, Redila, & Christie, 2005). 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, Redila, & Christie, 2005; Ernst et al., 2005; Kim et al., 2010).

The benefits derived from exercise maybe due to a combination of several molecular factors. Factors such as: brain derived neurotropic factor (BDNF), N-methyl-D-aspartate receptor (NMDAR), vascular endothelial growth factor (VEGF), Insulin-like growth factor-1 (IGF-1), serotonin (5-HT) and  $\beta$ -endorphin are up-regulated with exercise and have shown to have a positive effect on neurogenesis in several model organisms (Ernst et al., 2005; Kemperman, 2008; Llorens-Martin, et al., 2010; Lou et al., 2008; Olson, et al., 2006). Since the nervous system operates under common rules and themes in both vertebrates and invertebrates, information can be extrapolated from 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 (Cayre, et al., 2002).

An invertebrate model which 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 antenna bulb to become interneurons (Zhang et al., 2009). Similarities between crayfish and vertebrates with regards to neurogenesis include: progenitor cells having both glial and neural properties, the use of a migratory stream to direct the proliferated cells, as well as having similar features and arrangements within the neurogenic niches (Sullivan, et al., 2007).

Beyond the cellular similarities, factors that affect neurogenesis in vertebrates have also shown the same correlation in crayfish. Sandeman and Sandeman (2000) showed that enriched environments will 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 which increase neurogenesis, physical activity, has yet to be tested in crayfish, or any invertebrate model to our knowledge.

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). Physical activity is already being looked at as a possible mode of treatment for patients with depression, dementia, schizophrenia, and Alzheimer's disease, and alcoholism (Faulkner, & Biddle, 1999; Teri, et al., 2008; Wolf et al., 2006). Exploring exercise and how it directly



affects neurogenesis in an invertebrate model will allow for greater understanding of this intricate relationship in humans. And with greater understanding of this relationship and its associated physiological changes a larger scope of clinical application may become apparent.

## **MATERIALS AND METHODS**

### **Subjects**

Forty crayfish of the species *Orconectes propinquus* were used in the initial experiment, 20 for the experimental group, and 20 for the control. Following initial trials five additional male crayfish were used to improve sectioning procedures. Ages of the crayfish were estimated from length to be between 1-2 years. Control crayfish were on average 6.69 grams and 3.85 centimeters from rostrum to tail (reflects all but one subject). The experimental group had an average weight of 6.37 grams and was 6.86 cm long (averages reflect all but two subjects) [Table 1].

Subjects were broken into eight groups. Each group consisted of four males and one female. Experimental Group One and Control Group One were sacrificed following the first week of trials. Experimental Group Two and Control Group Two were sacrificed following the second week of trials. Experimental and Control Groups Three and Four were sacrificed following week 3 and week 4 [Table 2]. Staggering the time when samples were taken from subjects allows for observation of migration and survival rates throughout the experiment.

Times of molting were also recorded in addition to any loss of subjects [Table 2]. All subjects were housed in isolation to diminish olfactory stimulation since olfactory enrichment alone has shown to increase survival of newly formed cells (Rocheffort, et al., 2002). Isolation tanks contained 1L of fresh water, an air stone, and its tubing. Tanks were cleaned at the end of each week. All crayfish were fed with the same feeding schedule, receiving one rabbit pellet (average size 0.12g) three times a week. Also, subjects were housed in the same room with light controlled day and night cycles to mimic natural conditions in Michigan ensure similar circadian rhythms and tank temperatures.

### **BrdU Administration**

In order to quantify cell division within the crayfish brain, specifically clusters 9 and 10, a synthetic thymine, 5-bromo-2-deoxyuridine (BrdU), was used. Any new cell formed while BrdU is available inserts the BrdU into its DNA during mitosis. Cells with this synthetic thymine can later be detected through the use of an anti-BrdU marker which will attach to the BrdU and stain the cell brown.

The BrdU was purchased through Invitrogen and stored at 4°C. Crayfish were exposed to BrdU three days prior to experimental trials. The BrdU ordered from invitrogen was an aqueous solution concentration consisting of 5-bromo-2-deoxyuridine and 5-fluoro-2-deoxyuridine in a 10:1 ratio. Subjects were bathed in a BrdU solution containing 0.1L BrdU and 1L water within their isolation tanks. The crayfish were exposed to the BrdU solution for 24 hours allowing adequate time for the BrdU to

become integrated into newly formed cells. Following the BrdU bath the solution was removed and replaced with fresh water.

### **Experimental Trials**

Trials were carried out around dawn each day since there has been shown to be a link between circadian rhythm and neurogenesis in crustaceans with activity lulling during day time (Goergen, Bagay, Rehm, Benton, & Beltz, 2002). Trials were carried out for four consecutive days, with three days off between, for up to four weeks.

Crayfish assigned to the exercise group were placed in a water flume for 30 minutes. The flume consisted of five separate lanes to prevent interaction and smells from other crayfish. Each lane was 36 cm. long, 13 cm. wide, with a water depth of 3.2 cm. Flow was constant for the 30 minutes trial to entice the crayfish to move around and explore their lane. For consistency, the order each group was placed into their trials was rotated each day as well as lane assignment.

The control group crayfish were handled with the same schedule as the exercise group to allow for the elimination of stress as a variable between groups. They were removed from their isolation tanks and placed in a separate isolation tank containing 1L of fresh water for 30 minutes instead of the flume. It is important to expose both groups to similar stressors because stress has been shown to greatly diminish neurogenesis (Lucassen, et al., 2008). Behavioral observations were also taken throughout the trials to monitor activity levels for both groups.

### **Tissue Preparation**

The next day following the last trial day, each week, brain samples were taken from five crayfish from each group; four males, one female. Brains were removed keeping eye stalks intact to help with orientation. In order to preserve the tissue, a 4% paraformaldehyde (PFA) fixative was created using a 7.4 pH phosphate buffer, and applied to brains. Each brain was placed into its own test tube with 4mL PFA for six hours and placed in 4°C. Later trials utilized a longer fixative time of 51 hours. Following fixation brains were rinsed with a phosphate buffer for 20 minutes three consecutive times.

Brains were then dried following the protocol on table 3. The first time through, samples were placed in wax prior to drying, de-waxed then dried. Following samples were taken from additional crayfish not in the study to improve upon preparation techniques. The improved drying protocol is provided on table 4. Following drying brains were embedded in paraffin blocks and stored at 4°C until sectioning.

Using a microtome, 60 µm sections were prepared working from the dorsal aspect of the brain. To troubleshoot rolling waxed sections a 40 degree water bath grid was created to allow multiple samples to unroll and be placed onto a glass slide. On average about 70 sections were obtained from each brain and adhered to slides using heat and stored in slide boxes at room temperature.

### **Analysis**

In order to quantify BrdU labeled cells a BrdU antibody is used to stain the cells with BrdU. In order to apply the antibody samples must first be de-waxed. Sections from

this first trial with the new equipment were consistently tearing. The most likely cause for the shredding of the tissue observed is the use of a dull microtome blade. Due to the sections on the slides being fragile the samples from the experiment will be saved until the appropriate de-waxing procedure can be determined.

Once brains are de-waxed with then the antibodies may be applied to mark BrdU cells. According to the procedure listed by Paul, Goergen, and Beltz (2002) the brains will need to be incubated in rabbit anti-serotonin and rinsed with phosphate buffer prior to the administration of the primary antibody. Rat anti-BrdU has been established as a primary antibody that will work in crustacean brains to detect BrdU. It would be administered to the brains for 2.5 hours in a 1:50 dilution with a 0.1M phosphate buffer. Following three 20 minute rinses with the phosphate buffer a secondary antibody, goat anti-rat, will be applied overnight, also with a 1:50 dilution with 0.1M phosphate buffer. After three more 20 minute rinses of the buffer the brains can then be mounted in a medium and observed using a confocal microscope. Areas of the brain which underwent neurogenesis will be determined based on amount of BrdU labeling. If exercise did cause increased proliferation and survivability, as hypothesized, these brains will have greater antibody staining (Paul, Georgen, & Beltz, 2002).

## **RESULTS AND CONCLUSION**

The results on neurogenesis in association with physical activity in crayfish are pending. However, procedural protocols were refined, laboratory techniques were improved and new equipment was acquired to allow future work on adult crustacean neurogenesis at Grand Valley State University. There are very few labs looking a

neurogenesis in invertebrates and no other labs to date looking at the effect exercise has on invertebrate neurogenesis. The experimental design developed here has potential application for future branches of study. With slight modification future experiments can look at aspects such as ideal exercise durations, intensities, time of day of physical activity, gender differences, and molecular fluctuations with exercise.

Looking at this association in the more simplistic invertebrate model will help to establish if there is a direct relationship between exercise and neurogenesis and look more closely at factors which may optimize its effect. Since exercise is the most potent enhancer of neurogenesis it shows the most potential for development of treatments for the reduction of cognitive decline associated with age and potentially for some neurodegenerative diseases as well (van Praag, 2008; Zhang, Allodi, Sandeman, & Beltz, 2009; Zhao, Deng, & Gage, 2008).

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## **LIST OF FIGURES**

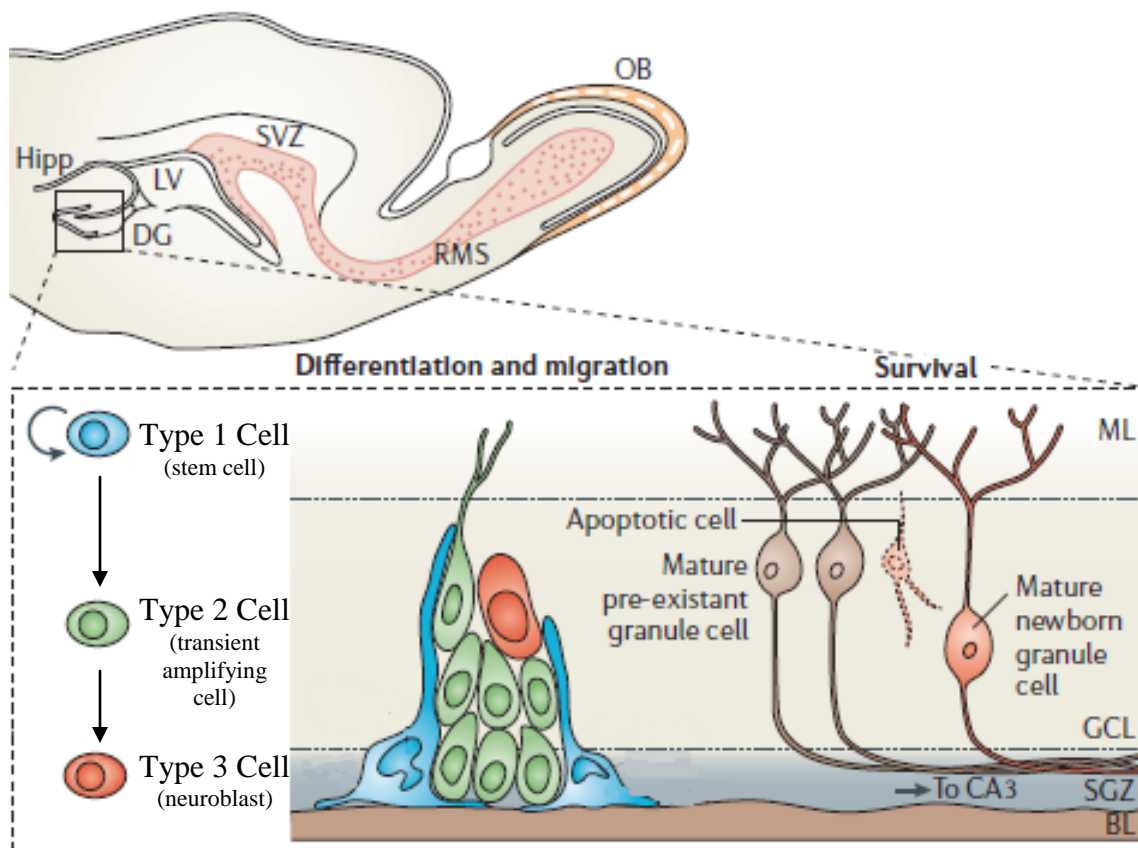
**Figure 1:** Subgranular Zone

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**Figure 3:** Neurogenesis in Decopod Crustaceans

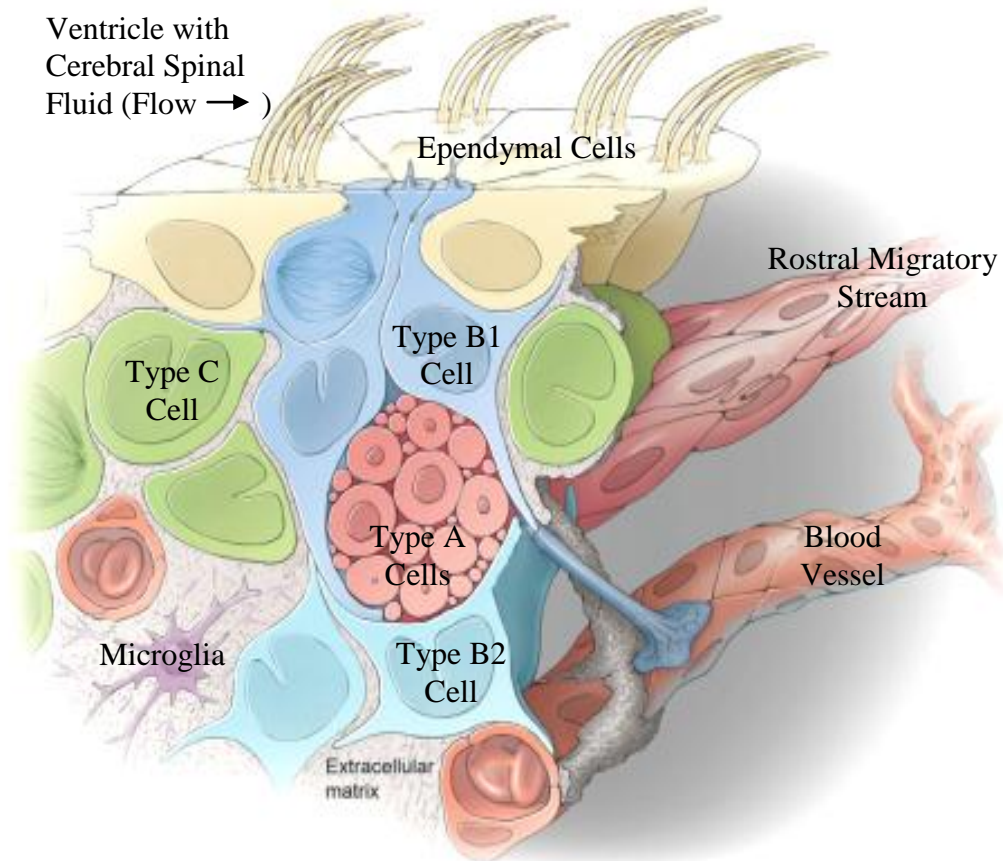
**Figure 4:** The Crayfish Brain





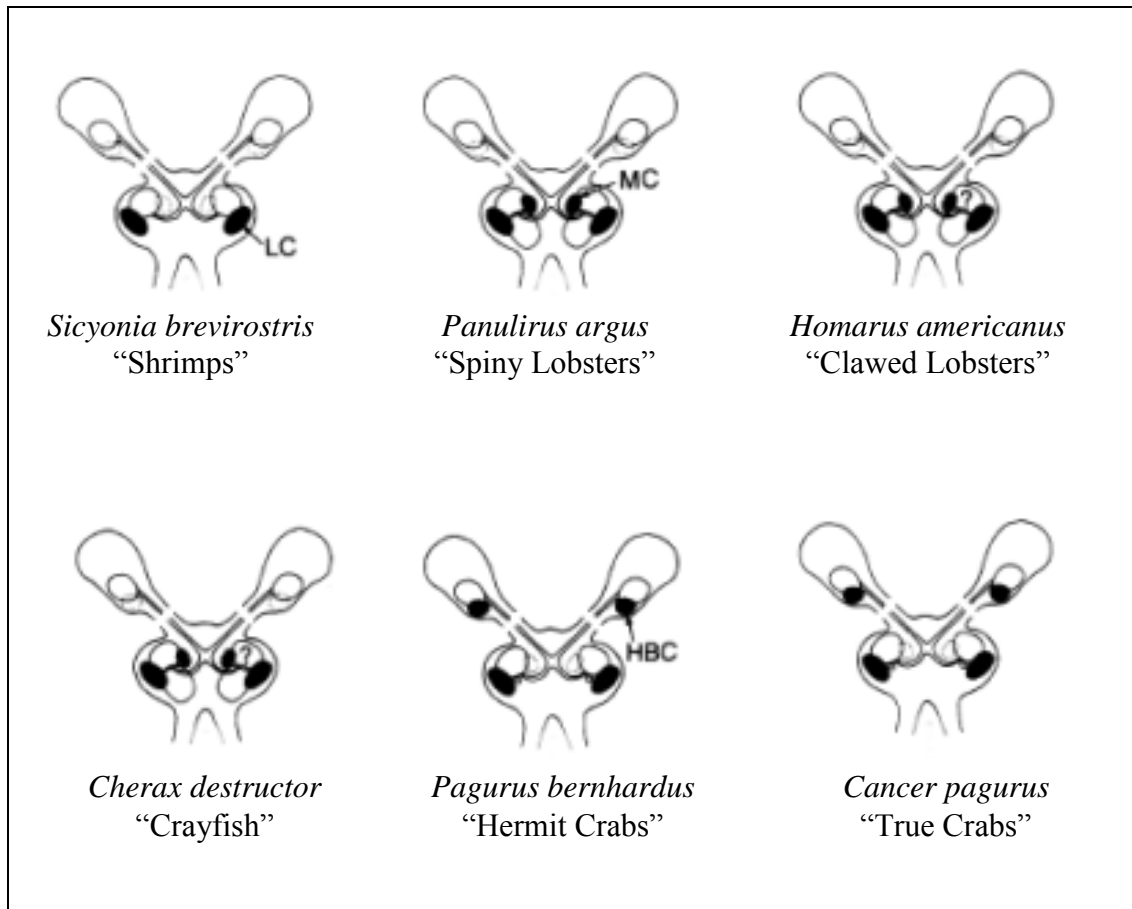
**Figure 1:** Subgranular Zone

The figure above adapted from Lledo, Alonso, and Grubb (2006) depicts the cell lineage of the subgranular zone in mammals. The stem cells of the subgranular zone are referred to as type 1 cells. The type 1 cells are triangular in shape and have their cell bodies below the granular cell layer (GCL) and their apical processes reaching to the molecular layer (ML). Type 1 cells will give rise to type 2 cells (green) which serve as transient amplifying cells. The division of the type 2 cells led to the formation of neuroblasts, type 3 cells (red) that integrate into the current circuitry. Brain abbreviations: DG- dentate gyrus; LV- lateral ventricle; Hipp- hippocampus; OB- olfactory bulb; RMS- rostral migratory stream; SVZ- subventricular zone.



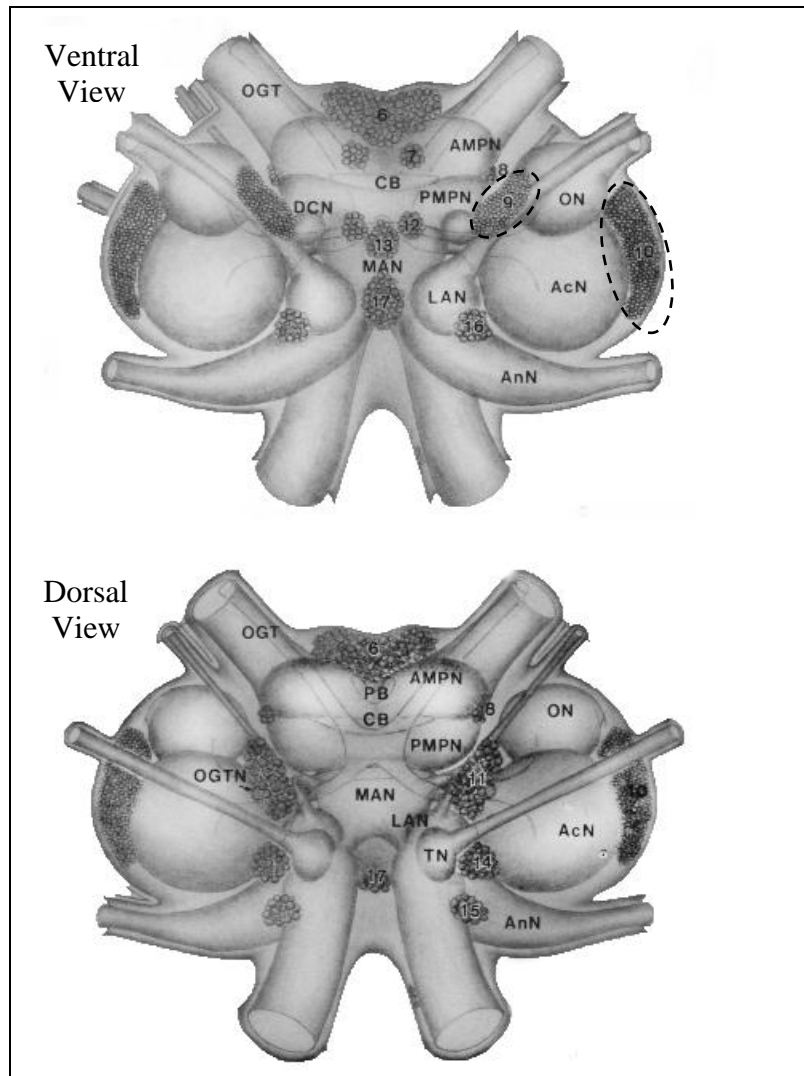
**Figure 2:** Subventricular Zone

The diagram above adapted from Ihrie and Alvarez-Buylla (2011) shows the lineage of the adult born neurons of the subventricular zone. The Type B cells (in blue) are the primary progenitors which are subdivided to those with a primary cilium in the ventricle (B1) and those without (B2). The B cell gives rise to the transient amplifying type C cell (in green). The C cells will then divide to form neuroblasts, type A cells, which migrate to the olfactory bulb through the rostral migratory stream.



**Figure 3:** Neurogenesis in Decapod Crustaceans

Location of newly formed adult cells (BrdU positive cells) in various species of decapod crustaceans are shown in this image adapted from Schmidt and Harzsch (1999). Species names as well as common names are provided. The medial cluster (MC) represents newly formed olfactory deutocerebral interneurons which are consistently observed in spiny lobsters, clawed lobsters, and crayfish. Common to all decapod crustaceans is the presence of new olfactory projection neurons in the lateral cluster (LC). Adult born interneurons have also been discovered in the hemiellipsoid body cluster (HBC) of crabs.



**Figure 4:** The Crayfish Brain

Graphic representation of the crayfish brain adapted from Sandeman et al. (1992).

Dashed circles denote location of neurogenesis, clusters 9 and 10. Abbreviations used:

AcN – Accessory lobe; AMPN- Anterior medial protocerebral neuropil; AnN- Antenna II Neuropil; CB- Central Body; LAN- Lateral antenna I neuropil; MAN- Median antenna I neuropil; OGT – Olfactory globular tract; OGT N- Olfactory globular tract neuropil; ON- Olfactory Lobe; PB- Protocerebral Bridge; PMPN- Posterior medial protocerebral neuropil; TN- Tegmentary neuropil.

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Control Crayfish				Exercise/Experimental Crayfish					
Crayfish	Gender	Weight (g)	Length (cm)	Crayfish	Gender	Weight (g)	Length (cm)		
1	Con. Group 1	male	measurments not taken	1	Ex. Group 1	male	measurments not taken		
2		male	6.40	4.509		2	male	measurments not taken	
3		male	8.22	2.794		3	male	5.54	2.54
4		male	9.59	3.099		4	male	5.64	4.06
5		female	4.89	2.718		5	female	4.91	2.57
Group Avg.		7.28	3.280	Group Avg.		5.36	3.06		
6	Con. Group 2	male	6.11	2.794	6	Ex. Group 2	male	8.76	2.96
7		male	4.35	2.413	7		male	7.23	2.73
8		male	8.66	3.523	8		male	5.60	3.03
9		male	7.81	2.962	9		male	8.17	3.13
10		female	6.28	2.657	10		female	4.60	2.67
Group Avg.		6.64	2.870	Group Avg.		6.87	2.91		
11	Con. Group 3	male	4.81	2.288	11	Ex. Group 3	male	5.47	2.68
12		male	6.94	2.860	12		male	6.44	2.70
13		male	5.96	2.692	13		male	4.84	2.43
14		male	9.91	2.878	14		male	7.79	3.02
15		female	6.14	2.598	15		female	5.33	2.54
Group Avg.		6.75	2.663	Group Avg.		5.97	2.77		
16	Con. Group 4	male	8.28	3.287	16	Ex. Group 4	male	9.61	3.06
17		male	7.83	3.025	17		male	Died mid. of week 1	
18		male	8.42	2.865	18		male	Died end of week 3	
19		male	Died start of week 2		19		male	Died end of week 2	
20		female	6.47	2.629	20		female	5.65	2.75
Group Avg.		7.75	2.951	Group Avg.		7.63	2.90		
Average		6.69	2.768	Average		6.37	2.86		

**Table 1:** Subject Measurements

Table 1 contains the weight and length of crayfish in control and experimental groups.



<u>Time</u>	<u>Solution</u>
1 hour	70% ethanol
1 hour	70% ethanol
1 hour	80% ethanol
1 hour	95% ethanol
1.5 hours	100% ethanol
1.5 hours	100% ethanol
1.5 hours	100% ethanol
1 hour	xylene
1 hour	xylene
1.5 hours	paraffin bath
1.5 hours	paraffin bath

**Table 3:** Tissue Preparation Procedure #1

The drying procedure provided here was carried out on the original 36 samples. The increased concentrations of ethanol were used to dry the tissue, and xylene was used as a clearing agent.



<u>Time</u>	<u>Solution</u>
1 hour	70% alcohol
1 hour	70% alcohol
1 hour	80% alcohol
1 hour	95% alcohol
12.5 hours	100% alcohol
3.5 hours	100 % alcohol
1 hour	xylene
1 hour	xylene
1.5 hours	paraffin bath
1.5 hours	paraffin bath

**Table 4:** Tissue Preparation Procedure #2

This tissue preparation procedure was used on follow-up brains in hopes to find a more suitable protocol to obtain better sections. The alcohol used was 95% methanol + ethanol, and 5% isopropyl alcohol. The drying steps were lengthened compared to tissue preparation procedure #1 (Table 3).