Exploring Neurogenesis in Crustaceans

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Plasticity, learning and memory, and neurological disease are exciting topics for students. Discussion around these subjects often results in the consideration of the role of neurogenesis in development, or its involvement in a potential cure for some diseases. We have therefore designed a lab that allows students to experimentally examine how the rate of neurogenesis can be altered by environmental factors. Neuronal cell division in crayfish is identified with fluorescently-labeled BrdU and quantified using conventional or confocal microscopy.

Recent studies indicate a conservation of mechanisms that control neurogenesis from insects and crustaceans to mammals. Yet the use of invertebrate models such as crayfish or lobsters has advantages over mammalian models. Invertebrate nervous systems have a simpler organization and larger, identifiable neurons – qualities that make such preparations easier for students to manage.

This lab offers many opportunities for student designed experiments and discovery-oriented learning by exploring factors that regulate neurogenesis such as environment, hormones and light. This article illustrates our first experience with the lab, using an experiment designed by our students. We include ideas for expansion of this model and suggestions for avoiding potential pitfalls. It is written in the form of a scientific paper, reporting on a single student experiment, to aid as a teaching tool for future classes.

Key words: neuroscience, neurogenesis, crustacean, bromodeoxyuridine, BrdU

Neurogenesis, or the birth of new neuronal cells, was thought to occur only in developing organisms. However, recent research has demonstrated that neurogenesis does indeed continue into and throughout adult life in both vertebrate and invertebrate organisms. Examples of neurogenesis are found in the hippocampus of mammals (Altman and Das, 1965; Eriksson et al., 1998), song control nuclei of birds (Alvarez-Buylla et al., 1990), and the olfactory pathway of rodents (Lois and Alvarez, 1994), insects (Cayre et al., 1996) and crustaceans (Harzsch et al., 1999). On going neurogenesis is thought to be an important mechanism underlying neuronal plasticity, enabling organisms to adapt to environmental changes and influencing learning and memory throughout life (Gould et al., 1999; Harzsch et al., 1999; Sandeman and Sandeman, 2000, Schmidt, 2001). Examining neurogenesis in crayfish is an experimental system that can be easily modified for student labs.

A number of different factors have been identified that regulate neurogenesis. Physical activity and environmental conditions have been shown to affect proliferation and survival of neurons in vertebrates (Kemperman and Gage, 1999; Van Praag et al., 1999) as well as invertebrates. Sandeman and Sandeman (2000) found that crayfish in an "enriched" environment had increased neurogenesis and neuronal survival compared to siblings in an "impoverished" environment. Hormones have also been found to influence the rate of neurogenesis in vertebrates (testosterone - Rasika et al., 1994) and invertebrates (ecdysone - Harrison et al., 2001). Serotonin is believed to play a key role in neurogenesis in a variety of organisms (Gould, 1999; Benton and Beltz, 2001; Beltz et al. 2001). In lobsters, depletion of serotonin dramatically reduced the proliferation and survival of olfactory projection neurons (Beltz et al., 2001) and local interneurons (Benton and Beltz 2001). Most recently, neurogenesis was found to follow a circadian rhythm in the juvenile lobster (Goergen et al., 2002). Although neurons were born throughout the day, significantly more neurons were born at dusk, the most active time for lobsters. It was subsequently demonstrated that the period of neurogenesis is entrained by the light:dark cycle (Goergen et al. 2002).

These examples taken from a range of different species illustrate that mechanisms controlling neurogenesis have been phylogenetically conserved. In addition, the functional anatomy of the olfactory pathway, the region where neurogenesis occurs in most adult organisms, is conserved from invertebrates to vertebrates. Finally it is helpful for students to understand that many of the physiological mechanisms of neurons that were elucidated in invertebrates were found to hold true for vertebrates. While all of these aspects are similar between vertebrates and invertebrates, the use of an invertebrate model is especially advantageous for students because neurons are large and identifiable (the same neuron can be found in each animal).

In order to proceed with these experiments, students need to have an understanding of the crustacean brain. The brain is separated into three main divisions: the protocerebrum (concerned with vision, whose primary input is the eyes), the deutocerebrum (concerned with olfaction, having input from the 1st antennae) and the tritocerebrum (primarily mechanosensory and receiving input from the 2nd antennae; see Figure 1). The deutocerebrum, the "mid brain", includes the olfactory lobes (OL), accessory lobes (AL), and clusters 9/11 and 10.

Adult neurogenesis has been found deutocerebrum in clusters 9 and 11 (local interneurons) and cluster 10 (projection neurons that project to higher level processing areas; Sullivan and Beltz, 2001). The focus of these experiments is cluster 10 because neurogenesis is reliably found and easily quantified in this region.

This study was designed by students in the Developmental Neurobiology course (BISC 306) at Wellesley College. The goal of this experiment was to

determine whether gross differences in environment correlate with a change the level of neurogenesis.

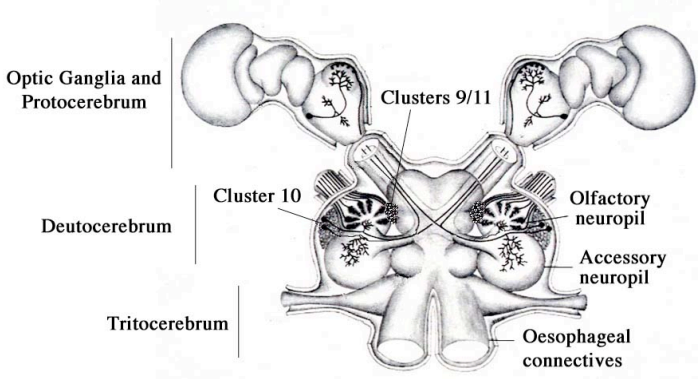

Figure 1. Drawing of the crayfish brain showing the three main divisions and the areas of life-long neurogenesis. Modified from drawing by David Sandeman; Mellon and Sandeman 1992.

MATERIALS AND METHODS.

Animals Australian crayfish (*Cherax destructor*) were used however, this protocol has also been successfully used in *Procambarus clarkii* and the American lobster, *Homarus americanus*. This protocol can be used for both adult and juvenile animals. If juvenile animals (defined as free from the mother) are used, staging is difficult unless each molt is observed (Sandeman and Sandeman, 1991). For this lab, it is suggested to use size as a criterion rather than stage. Animals between 2-4 centimeters are optimal.

Experimental Conditions For this experiment the animals used had been born into their experimental environments. However, if this is not possible, keep the animals in the experimental condition for at least two weeks. See appendix 3 for care of the animals.

Enriched environments contained: recirculating water, natural and artificial light, lots of toys/hiding places, regular external stimulation, frequent feeding (see appendix 3), deep water (depth 25 cm, volume 31 L), and living with both parents and siblings.

Impoverished environments contained: non-recirculating water, artificial light, no toys/hiding places, little external stimulation, twice a week feeding, shallow water (depth 4 cm, volume 4 L), and living with just the mother and siblings.

BrdU labeling for neurogenesis BrdU (5-Bromo-2-Deoxyuridine), a synthetic thymidine analog, is incorporated into single strand DNA during the S-phase of the cell cycle. Antibodies against BrdU conjugated to fluorescent markers can be used to label these cells, visually providing evidence of cell division. For the experiment presented here, five juvenile crayfish that had been raised in an enriched environment and five in an impoverished environment were immersed in 0.5 mg/ml BrdU (Roche) in tank water for 24 hours. Brains were then dissected (in crayfish saline) from the head capsule (see dissection videos below) and fixed in 4% paraformaldehyde for 24 hours. The juvenile brains were processed as whole mounts. If adult animals are used, the same concentration of BrdU is injected into the ventral hemolymph (1ml/100g body weight). Adult brains must be desheathed and sectioned using a Vibratome (100 μm thick), prior to immunocytochemical processing (see appendix 2).

Figure 3. Still images from the dissection of the juvenile crayfish brain video. This video may be found in JUNE's supplementary materials area (www.funjournal.org/movie.asp). a. removing the mandibles. b. viewing the brain after removing the anterior appendages. c. the dissected brain.

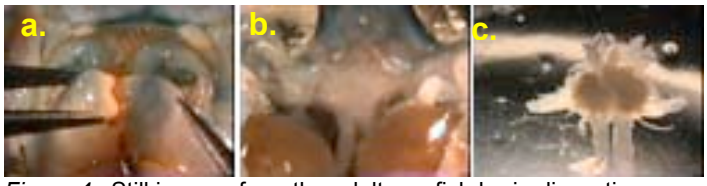

Figure 4. Still images from the adult crayfish brain dissection video. This video may be found in JUNE's supplementary materials area (www.funjournal.org/movie2.asp) a. removing the mandibles. b. viewing the brain after removing the anterior appendages. c. the dissected brain.

Serotonin Labeling Crayfish preparations were also labeled for serotonin to provide an outline and landmarks within the crustacean brain, however this step is optional. Both the olfactory and accessory lobes stain positively for serotonin and there are several large identifiable neurons (most notably the DGN) that are serotoninergic (Sandeman and Sandeman 1988). Using this second label also gives students experience with double labeling immunocytochemical techniques (Paul et al., 1997).

Immunocytochemical processing Brains were processed following immunocytochemical methods of Harzsch et al., 1999. See Appendix 1 for details.

Rinse 3 times for 20 minutes in 0.1 M PB
Rinse 1 time for 20 minutes in 2 N HCl
Rinse 3 times for 20 minutes in 0.1 M PBTx NGS
Incubate in rabbit anti-serotonin (Diasorin) overnight at 4 °C; diluted 1:1000 in 0.1 M PBTx
Rinse 3 times for 20 minutes in 0.1 M PBTx
Incubate in rat anti-BrdU (Accurate Chemical), for 2.5 hours at room temp; diluted 1:50 in 0.1 M PBTx
Rinse 3 times for 20 minutes in 0.1 M PBTx
Incubate in goat anti-rat Alexa 488 and goat anti-rabbit Alexa 594 (both from Molecular Probes), overnight at 4 °C; each at 1:50 dilution in 0.1 M PBTx (covered to protect from light).
Rinse 3 times for 20 minutes in 0.1 M PB
Mount in mounting medium and view preps with a fluorescence or confocal microscope

BrdU labeled cells were counted by stepping through serial scans made by a confocal microscope. Using a clear transparency sheet taped to the computer monitor, neurons were circled as they came into view and then counted. If a

fluorescent microscope is used, cells can be counted as they come into view when focusing through the brain.

If fluorescence microscopy is unavailable, the three step ABC peroxidase method may be used, which has the added advantage of being a permanent label (Beltz and Burd 1989). However, dissections must be more precise because nicking the brain may lead to a darkening of the entire prep when processed.

RESULTS

Neurogenesis was observed in all animals that were raised in the enriched environment (Figures 2a, 3). In contrast, no neurogenesis was observed in animals raised in the impoverished environment (Figure 2b, 3). Even though there was variation in the number of labeled neurons in the enriched treatment, there were still significantly more neurons born in the enriched than in the impoverished environment (Figure 3; Student t-test $p<0.001$). Serotonin labeling was similar in both preparations.

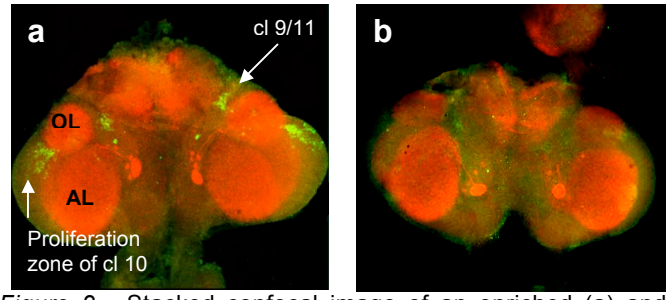

Figure 2. Stacked confocal image of an enriched (a) and an impoverished (b) juvenile crayfish brain. Note the labeling in the proliferation zone (cluster 10) and in clusters 9/11.

DISCUSSION

These data indicate that there are dramatically more neurons being born in cluster 10 of crayfish living in the enriched environment than in the impoverished environment. A number of different factors could contribute to these results, either individually or in combination. The animals in the enriched tank have more physical stimulation, both from the location of the tank (in a busy lab) and from the ability to swim more in a greater volume of tank water. This confirms prior findings in both crayfish and mice suggesting that increased physical activity and space increased neurogenesis (Van Praag et al., 1999; Sandeman and Sandeman, 2000).

Further, in mice, stress has been shown to negatively affect the level of neurogenesis (Tanapat et al., 1998). The enriched environment had more toys and hiding places for the juvenile crayfish, which decreases the potential confrontations among animals, and therefore decreases stress experienced by the animals. Increased neurogenesis in the enriched environment could also be influenced by the light conditions; enriched animals experience both artificial light, as well as a natural light cycle that has a more gradual transition to dawn and dusk. Light/dark regimes (Goergen et al., 2002) as well as photoperiod (Dawson et al., 2001; Huang et al., 1998) have been shown to influence neuronal proliferation or survival. Lastly, the enriched animals received more food and had recirculating water. Although the influence of these factors on neurogenesis has not been closely examined, they also may influence the level of neurogenesis.

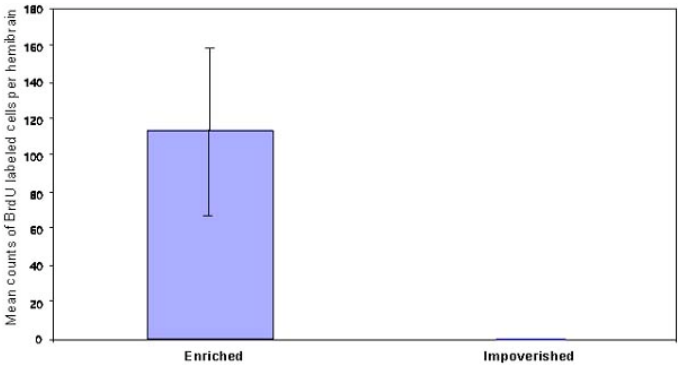

Figure 3. Graph of mean number of BrdU labeled neurons/hemibrain in enriched (n = 6) and impoverished (n = 8) environments.

Both sets of brains were processed in parallel to insure that they received identical treatments. Therefore, the dramatic difference in the results is likely to be due to one or more factors in the environment. It should also be noted that in prior experiments, a low level of neurogenesis was observed in animals in similar impoverished conditions (Paul and Goergen, unpublished observations, Sandeman and Sandeman, 2000). The lack of BrdU labeled neurons in the impoverished animals is therefore representative of the low end of the normal spectrum. Another factor that could help explain the great difference in proliferation between enriched and impoverished animals is molt stage. The level of neurogenesis of olfactory sensory neurons in the spiny lobster is dependant upon the stage of the molt cycle (Harrison et. al., 2001). Therefore, it is recommended that all animals be in intermolt to minimize variability. See Aiken (1973) for molt staging instructions.

Future experiments could test the level of neuronal cell division by separately varying environmental factors such as: 1) water depth, 2) density of similar aged juveniles, 3) density of other species of plants and animals, 4) the number of toys or hiding places, 5) lighting conditions, 7) amounts of food, 8) hormones, and 9) molt stage. These experiments could help to answer the question: what is causing more neurogenesis in animals living in the enriched environment? Such a question can easily be addressed by students trained in the immunocytochemical BrdU methodology of detecting neurogenesis. There are many lessons to be learned not only in trying to answer this interesting question of how environment influences neuronal cell division, but also in how to set up precise experiments with clearly defined controls and careful data analysis.

Finally it is important to make the point that is conveyed by the well-worn cliché that “the whole may be more than the sum of the parts”. It may turn out that when each of the environmental factors are analyzed individually, they do not add up to having the effect of a common active aquarium tank found in many labs, offices and homes. This is always an issue when doing behavioral research, and another critical lesson for students in this field. Of course the

ultimate experiment is to compare the animals from the enriched aquarium with crayfish taken from a wild population.

We feel that this experimental set-up is exciting for today's students due to the current interest in life long-neurogenesis. These methodologies offer the choice of many experiments for students and could provide important answers to these intriguing and seemingly straightforward questions.

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Appendix 1: Solutions *(all solutions used for immunocytochemistry should be filtered).*

1. Crayfish saline To 1 L double distilled water (ddH_20) add: 12.0 g NaCl, 0.4 g KCl, 1.5 g $CaCl_2$-$2H_2O$, 0.5 g $MgCl_2$-$6H_2O$, and 0.17 g $NaHCO_3$
2. 4% Paraformaldehye solution To make 200 ml: 50 ml dd H_20 at 60 °C, 8 g paraformaldehyde, 100 ml 0.2 M Phosphate Buffer (pH 7.4), and 50 ml ddH_20. Add paraformaldehyde to the 50 ml of 60 °C ddH_20 and stir solution for 10 mins. Clear solution with 1 M NaOH. Add remaining water and PB. Filter solution and adjust the pH to 7.4. Store the solution in an airtight container and in the fridge. Use within one week of making it. It works much better that way.
3. O.1 M PB Stock A: (pH 4.5) 26.7 g $NaH_2PO_4.H_2O$/1 L ddH_2O. Stock B: (pH 9) 28.4 g Na_2HPO_4 /1 L ddH_2O (53 g for heptahydrate). Mix one part stock A, 4 parts stock B, for a) 0.2 M solution; adjust pH to 7.3-7.4 with A or B. Mix 0.2 M buffer with an equal volume of ddH_2O for a O.1 M solution.
4. PBTx Add 400 µl Triton x-100 per 100 ml 0.1 M PB
5. PBTx NGS Add 5% non-immune goat serum (NGS) to PBTx.
6. Mounting Medium 80% glycerol and 20% 0.1 M PB; also commercially available from Sigma
7. Agar 5% in distilled water

Reagent suppliers

Labeling reagent: Roche #280 879
Amersham #RPN 201

1° antibody
Rabbit anti-serotonin (Diasorin # 20080)
Rat anti-BrdU (Accurate #OBTOO3O)
Mouse anti-BrdU (Amersham #RPN 202)

2° antibody
goat anti-rat Alexa 488 (Molecular Probes #A11006)
goat anti-rabbit Alexa 594 (Molecular Probes #A11037)
goat anti-mouse Alexa 488 (Molecular Probes #A11029)
goat anti-rabbit Texas Red (Jackson Immu #111075003)

Appendix 2: The Vibratome The Vibratome is an instrument that uses a single edged razor blade to cut sections through the tissue. The Vibratome gently vibrates the blade as it cuts through the tissue to ensure an even cut. Before cutting the tissue the sheath has to be removed from the brain and the brain is then embedded in agar, which gives the tissue more structure while it is being cut.

Desheathing the brain The sheath is a thin, "Saran wrap" type covering. It can be removed using a two pairs of very fine forceps. Under the dissecting microscope, gently grab the sheath at the edge of the brain and pull slowly while holding onto one of the connectives with the other pair of forceps. It should slide off the brain. Make sure that the brain is optimally lit so that the sheath can be clearly seen.

Agar Embedding

1. Place the brain on a small (10 cm x 10 cm) black rubber sheet.
2. Looking under the microscope, gently blot the brain dry with Kimwipes.
3. Using a Pasteur pipette, cover the brain with 0.01% poly-l-lysine solution (this will make it sticky so that the agar will adhere well).
4. Blot the brain dry again.
5. If the agar has been stored it will be solid. Heat the agar until melted (~60°C) by placing it in a beaker of water and heating it on a hot plate. It can also be microwaved but be careful that it doesn't boil over. Let it cool to just above the melting point.
6. Using a hot Pasteur pipette (heat it in the beaker of water containing the agar) put a drop of molten agar onto the black rubber sheet.
7. Pick up the brain on a small flat spatula. Carefully place the brain in the center of the agar drop, ventral surface down (bumps up). Do not push the brain to the bottom of the agar.
8. Put a second drop of agar on the top of the brain and let the agar cool and solidify.

Sectioning

1. With a single edged razor blade trim the agar around the brain with cuts that angle away from the brain, forming a trapezoid block slightly bigger than the brain. Trim a corner for orientation.
2. The Vibratome has a cork "chuck" where the tissues is adhered, to make it secure while the tissues is being sliced. Put a very small drop of superglue on the cork; pick up the agar block with a small flat spatula and place on the glue.
3. Slice the brain in 100 µm sections into a glass or ceramic depression slide plate or a well plate filled with PB. Make a note of the sequence that the sections are placed in the wells.

Appendix 3: Animal Care Animals can be kept in mouse or rat cages in approximately five cm of fresh water with a body sized PVC tube (or equivalent) for hiding. Aeration is not necessary but may aid in survival. Animals are fed shrimp pellets every three days. It has been our experience that combining a single male and a single female frequently results in production of a brood however timing is not predictable. Hatching time is approximately three months at room temperature.

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