Regulation of Adult Neurogenesis in Decapod Crustaceans

Barbara S. Beltz[1], Jeannie L. Benton[1], Maria C. Genco[1],
DeForest Mellon[2], David C. Sandeman[1] and Jeremy M. Sullivan[1]

[1]Department of Biological Sciences, Wellesley College, Wellesley MA 02481
[2]Department of Biology, University of Virginia, Charlottesville, VA 22901

The primary goal of our experiments at MDIBL was to test the feasibility of using different crustacean species in our research. For many years, laboratory-reared juvenile lobsters (*Homarus americanus)* have been the primary subject of our research on the regulation of life-long neurogenesis[1-5,12]. However, during the summer of 2004, we wanted to investigate whether other crustacean species indigenous to Mt. Desert Island might be useful models for studying neurogenesis. Adult neurogenesis has been documented in *Carcinus maenas*, for example[9], but environmental factors that regulate this process have not been examined in this organism. Our initial hypothesis was that mechanisms regulating neurogenesis would be very similar among decapod crustacean species, and that we could therefore use lobsters, crabs or crayfish to answer our questions and expect to get similar answers. *The very important take-home message from our summer's work is that environmental factors regulating neurogenesis, as well as the cell cycle dynamics leading to the production of new neurons, can be very different even in closely related species.* Characteristics of the cell cycle and regulatory mechanisms must therefore be evaluated independently in each organism. We believe these differences may be related to the varied lifestyles and life spans of these species.

Most of our efforts at MDIBL were directed at exploring aspects of neurogenesis in the shore crab, *Carcinus maenas*. We asked two primary questions: (1) Do tidal and circadian influences regulate the timing of neurogenesis? This study was based on research in the lobster that demonstrated circadian control of neurogenesis[4]; the idea that tides might also play a regulatory role in crabs emerged from their lifestyle as an intertidal species. (2) Can we estimate the length of the cell cycle resulting in the production of new neurons? Our interest in this question grew from our desire to understand circadian influences on neurogenesis; in order to study these phenomena, ideally we need a model organism where the S phase is relatively short and the complete cell cycle is less than 24 hours. To address these questions we utilized bromodeoxyuridine (BrdU) labeling of cells in S phase, in the cluster of olfactory projection neurons (cluster 10) in the midbrain of the crab. To examine tidal and circadian influences, we collected crabs on days when sunset and high tide (SSHT), sunrise and high tide (SRHT), sunset and low tide (SSLT), or sunrise and low tide (SRLT) were coincident. The idea here was to take advantage of a presumed circadian rhythm in neurogenesis and look at the peak (dusk) and trough (dawn) periods in neurogenesis that were observed in lobsters[4], and examine this circadian rhythm with an overlay of tidal influences; we therefore hoped to observe the levels of neurogenesis for this species under natural conditions.

Animals were collected from Seawall and the rate of neurogenesis was assessed over the subsequent 24-hour period. Groups of crabs (n=5-10 per group) were placed in BrdU in seawater (2mg/ml) for 4 hours at 4 hour intervals. Following the 4-hour incubation in BrdU, crabs were killed and their brains dissected and fixed. Immunocytochemical methods were used to detect the presence of BrdU in cells in cluster 10[1]. The presence of BrdU labeling indicated those cells that were in S phase during the period of BrdU incubation. Labeled cells were counted for each time point, statistical measures applied, and means and standard errors were graphed (Fig.1). Results indicate that there may be a subtle influence of tides on the period of neurogenesis, but there were no statistically significant differences between the peaks and troughs in the rate of neurogenesis throughout the 24-hour periods sampled. There was no indication that the day/night cycle influenced the rate of neurogenesis.

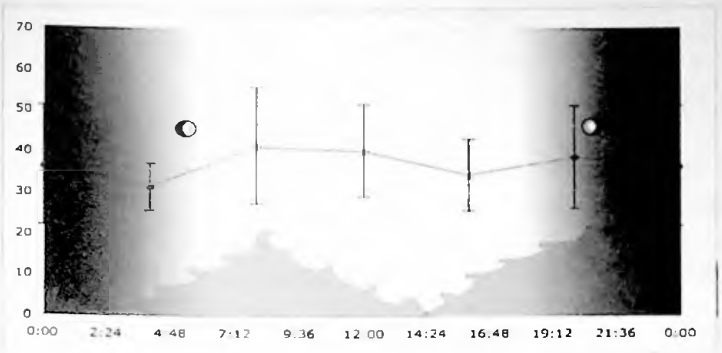

Fig. 1. Crabs (*Carcinus maenas*) were incubated in BrdU for 4 hours at six time points during a 24-hour cycle from June 29-30, 2004. n=4 for 12:00 and 0400 samples; n=5 for all other points. Sunset and high tide (SSHT) were coincident on the day of this study (sunset, 20:21; sunrise, 04:51; high tide, 08:18 and 20:37; low tide, 02:09 and 14:21). The tidal cycle is indicated by the wave patterns, and the light conditions by the shading on the graph. The sun/moon symbols mark sunrise and sunset, on the sampling day.

Since returning to our lab at Wellesley College where we have controlled lighting in the animal care facilities, we have tested whether neurogenesis in *Carcinus maenas* is regulated by the light/dark cycle. We found that there is no difference in the rate of neurogenesis relative to time of day, when the crabs are removed from their tidal influences and maintained in laboratory tanks for at least 1 month with a 12/12 L/D cycle. This finding, therefore, points out a critical difference in regulatory mechanisms controlling neurogenesis between *C. maenas* and *H. americanus*, where the light cycle entrains the period of neurogenesis[4]. With this in mind, we therefore will re-examine tidal influences next summer from a different perspective. Instead of examining days when tides, sunset and sunrise are coincident, we plan to examine neurogenesis during 24-hour periods when tides are at their extremes (e.g., during a full moon).

While working with *C. maenas*, we also became curious about the duration of the S phase and cell cycle, because it appeared that regardless of the length of the BrdU incubation period (3 to >24 hours), approximately the same numbers of neurons were labeled. This was not consistent with our experience using juvenile lobsters, where we observed differences in the numbers of cells labeled as a result of relatively minor changes in BrdU incubation times. We therefore did a comparative study using both crabs and lobsters, to ask how many neurons would label with BrdU over 3, 6, 9 and 12-hour incubation periods. The results of this study demonstrate a critical difference in the dynamics of neurogenesis between these two species (Fig. 2). While increases in the numbers of labeled neurons are seen with increasing length of BrdU incubation in lobsters, there are no differences seen in the numbers of labeled neurons in crabs over these time periods. This suggests that the S phase is comparatively long in *C. maenas* relative to *H. americanus*. These data and the finding that neurogenesis in crabs is not under circadian control demonstrate that there are significant differences in cell cycle dynamics and regulatory controls between these species. Further, these findings indicate that *Carcinus* would not be a favorable model for examining circadian control mechanisms.

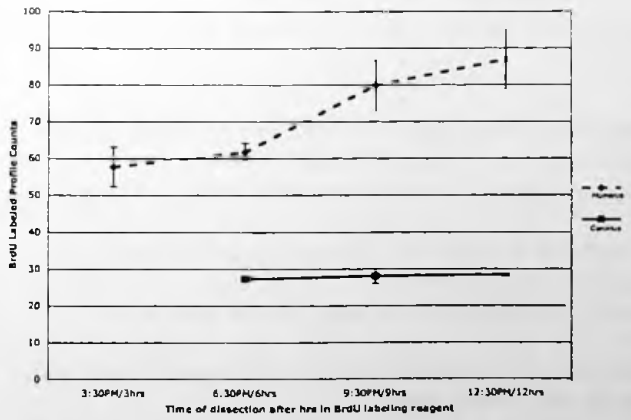

Fig. 2. Graph of the numbers of BrdU-labeled cells in the brains of lobsters (blue broken line) and crabs (pink solid line) during a 12-hour period. The numbers of labeled cells were assessed after incubation in BrdU for 3, 6, 9 and 12 hours. Labeling in the crab brains following a 3-hour BrdU incubation resulted in weak labeling in cluster 10 neurons; these faint cells could not be counted accurately, and therefore this time point is not included in the graph for *C. maenas*. This observation is consistent with the suggestion that the cell cycle is longer in *C. maenas* than in *H. americanus*.

In addition, while at MDIBL, we pursued three other lines of research related to our goal of understanding neurogenesis and its regulation:

DeForest Mellon explored neurogenesis in the fiddler crab, *Uca pugilator*, another intertidal species indigenous to Frenchman's Bay. Its behavior is strongly influenced by the tides; periods of locomotory activity are entrained by the tidal cycle and are maintained for several weeks even when crabs are placed in laboratory conditions without tidal signals[8]. This persistent locomotory rhythm is unlike the situation in *Carcinus maenas*, which lose their tidal rhythmicity after only a few days in laboratory conditions[7]. We therefore hypothesized that comparing neurogenesis among these species might provide an opportunity to examine tidal regulation of this process in organisms (*U. pugilator*, *C. maenas* and *H. americanus*) that have very different life styles relative to the tides. We therefore conducted pilot studies in adult fiddler crabs, to determine the extent of BrdU labeling over various incubation periods. The principal result of these experiments is that very few (~4-6) olfactory projection neurons label even over a 12-hour BrdU incubation period, as compared to 35-50 in *C. maenas* and >100 in *H. americanus*. This result is not encouraging in terms of using *U. pugilator* as a model for exploring circadian or tidal regulation of neurogenesis, as the phenomenon is not robust enough for experimental manipulation over the course of a 24-hour period.

Jeannie Benton and Jeremy Sullivan developed immunocytochemical methods for detection of proliferating nuclear cell antigen (PCNA). Although the use of the BrdU labeling method in our lab has been highly successful as a marker for proliferating cells in crustacean species, this technique has the disadvantage of requiring the incorporation of this thymidine analogue over a period of hours. Therefore, while the label is incorporated during the S phase, the stage of the cell cycle when the label is observed is not restricted to the S phase because many of the labeled cells will have advanced through G_2 and M phases by the time the tissue is fixed. PCNA is an enzyme expressed only in mitotically active (S-phase) cells and its presence can therefore be used to define cells in S phase at a specific time[6]. Utilization of the antibody against PCNA requires antigen retrieval techniques that have been used in only a few non-mammalian species[10]. A novel version of a high temperature, low pH pretreatment method to recover the antigenicity of tissue sections that had been masked by formalin fixation was successful in whole brains of *H. americanus*, *C. maenas* and *Cragnon cragnon*.

Jeremy Sullivan also localized pigment dispersing hormone (PDH) in the brains of larval, juvenile and adult lobsters using immunocytochemical methods. These studies were done in order to identify neurons potentially involved in regulating the circadian rhythm of neurogenesis observed in the lobster brain. PDH is a neuropeptide identified as an important component of the circadian pacemaker in the brains of insects[11]. These studies identified an extensive network of PDH-immunoreactive neurons in the brain of *H. americanus,* a number of which have arbors that overlap with those of extraretinal photoreceptors which are thought to be important inputs to the circadian pacemaker. In addition, the distribution of PDH-immunoreactivity was found to differ between larval, juvenile and adult lobsters providing evidence of substantial changes in the organization of the brain circadian pacemaker during the development of the lobster.

1. Beltz, B. S., J. L. Benton, and J. M. Sullivan. Transient uptake of serotonin by newborn olfactory projection neurons may mediate their survival. *Proceedings of the National Academy of Science* 98: 12730-12735, 2001.
2. Beltz, B. S. and D. C. Sandeman. Regulation of life-long neurogenesis in the decapod crustacean brain. *Arthropod Structure and Development* 32: 39-60, 2003.
3. Benton, J. L. and B.S. Beltz. Patterns of neurogenesis in the midbrain of embryonic lobsters are different from proliferation in the insect and crustacean ventral nerve cord. *Journal of Neurobiology* 53: 57-67, 2002.
4. Goergen, E., L. A. Bagay, K. Reh, J. L. Benton, and B. S. Beltz. Circadian control of neurogenesis. *Journal of Neurobiology* 53: 90-95, 2002.
5. Harzsch, S., J. Miller, J. Benton, and B. Beltz. From embryo to adult: Persistent neurogenesis and apoptotic cell death shape the crustacean deutocerebrum. *Journal of Neuroscience* 19: 3472-3485, 1999.

6. Kisielewska, J., P. Lu, and M. Whitaker. GFP-PCNA as an S-phase marker in embryos during the first and subsequent cell cycles. *Biology of the Cell* [electronic publication prior to paper], December, 2004.
7. Naylor, E. Crab clockwork: the case for interactive circatidal and circadian oscillators controlling rhythmic locomotor activity of *Carcinus maenas*. *Chronobiology International* 13: 153-61, 1996.
8. Palmer, J. D. Contributions made to chronobiology by studies of fiddler crab rhythms. *Chronobiology International* 8: 110-130, 1991.
9. Schmidt, M. Continuous neurogenesis in the olfactory brain of adult shore crabs, *Carcinus maenas*. *Brain Research* 762: 131-43, 1997.
10. Shi, S-R, R. J. Cote, and C.R. Taylor. Antigen retrieval techniques: Current perspectives. *Journal of Histochemistry and Cytochemistry* 49: 931-937, 2001.
11. Stanewsky, R. Clock mechanisms in *Drosophila*. *Cell and Tissue Research* 309: 11-26, 2002.
12. Sullivan, J. M., J. L. Benton, and B. S. Beltz. Serotonin depletion *in vivo* inhibits the branching of olfactory projection neurons in the lobster deutocerebrum. *Journal of Neuroscience* 20: 7716-7721, 2000.

Sponsored by National Science Foundation Grant numbers IBN-0344448 (BSB) and DBI-0139190 (MDIBL), Fiske and Staley Awards from Wellesley College, and the Maren Foundation (MDIBL).