Hematopoietic-Derived Cells As Potential Neural Precursors in Adult Neurogenesis: An Astakine-1 Mediated Pathway?

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Hematopoietic-Derived Cells As Potential Neural Precursors in Adult Neurogenesis: an Astakine-1 Mediated Pathway?

Rachel Kery

Submitted in Partial Fulfillment of the Prerequisite for Honors in Neuroscience

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ABSTRACT

Adult neurogenesis, the production of new neurons in the adult brain, occurs in the olfactory pathways of many vertebrate and invertebrate species, including decapod crustaceans. In Procambarus clarkii, new neurons are integrated into interneuronal cell clusters 9 and 10. The 1st-generation precursor cells reside in a niche, where they divide symmetrically, with both daughter cells migrating along glial strands to clusters 9 and 10, where additional divisions and neuronal differentiation occur. Although divisions of existing niche cells do not replenish the niche, the niche increases in size over an animal’s lifetime. Niche precursor cells must, therefore, originate from an extrinsic source. It has been hypothesized that circulating stem cells of possible hematopoietic origin are migrating into the niche from the hemolymph. Previous lines of inquiry in crayfish have suggested that semi-granular cells circulating in the blood are attracted to the niche. Astakine-1, a homolog of vertebrate prokineticins, has been shown in prior studies to increase release of semi-granular cells from the hematopoietic tissue; in parallel, astakine increases the number of cells in the neurogenic niche. Astakine-1 release from hematopoietic tissues has a circadian rhythm, but it was not previously known whether or not astakine expression in the brain changes in accordance with this rhythm. Astakine-1 labeling was found in cell clusters 11, 14, 16 and 17, medial giant neurons, and fibers on the dorsal surface of the brain. A large astakine-labeled cell in cluster 16 extends a fiber which may contact the niche, and the dorsal giant neuron.

In this study, ablations of hematopoietic tissue were done in an attempt to better define the relationship between the hematopoietic system and adult neurogenesis. Only the most dorsal regions of the tissue were removed, leaving a small region on the ventral surface. 10 days post-surgery, ablated animals had significantly fewer niche cells relative to sham and control animals (p<0.001). There were no significant differences in numbers of dividing (BrdU-labeled) cells in the niche, or in the interneuron clusters 9 and 10. If astakine-1 is injected into ablated animals 48 hours prior to sacrifice, this deficit in niche cells is ameliorated.

These data demonstrate that astakine-1 is correlated with increases in niche cell numbers and restores the number of cells in the niche following the ablation of hematopoietic tissues. Astakine labeling in neural tissues is distributed among several brain cell clusters, which is consistent from morning to evening sampling times. The intensity of labeling of neuronal fibers varies with time of day. Taken together, these data confirm the potential importance of astakine-1 in adult neurogenesis in the crayfish brain, and demonstrate that the number of cells in the neurogenic niche is closely tied to the hematopoietic system.
INTRODUCTION

Adult neurogenesis occurs throughout life in a broad range of vertebrate and invertebrate species. In humans and other mammals, neurogenesis occurs in the subventricular and subgranular zones, providing new neurons to the olfactory bulbs and the hippocampus, respectively. In the freshwater crayfish *Procambarus clarkii*, cells with glial properties that reside in a neurogenic niche divide symmetrically, and both daughter cells migrate away along glial strands to cell clusters 9 and 10 (Fig. 1) (Zhang et al., 2009; Benton et al., 2011). Following migration, further divisions take place in the cell clusters. Neurons in these clusters become local (cluster 9) and projection (cluster 10) neurons in the olfactory pathway, innervating the olfactory and accessory lobes (Fig. 1A). Cells in the niche play roles as both 1st-generation neuronal precursors and as support cells (Beltz et al., 2011); it is unclear, however, if all niche cells are capable of becoming neural progenitors. Unlike mammalian systems where several precursor cell generations coexist in the neurogenic niche, the cell lineages in crustaceans are spatially segregated (Figure 1B, C), allowing for easier quantitative assessment of changes in the precursor cell generations (Beltz et al., 2011).

The migratory streams, which receive all of the daughters of the niche cell divisions, rarely contain more than 10 to 12 labeled cells (Beltz et al., 2011); it is also known that these 2nd-generation cells require 5 to 7 days to migrate to the proliferation zones in the cell clusters (Sullivan et al., 2007a). Thus, niche cells require a minimum of 48 hours to complete the cell cycle (Benton et al., 2011). Pulse-chase experiments using modified synthetic thymidine analogs 5-bromo-2’-dioxoyuridine (BrdU) and 5-ethynyl-2’-deoxyuridine (EdU), where the animals were maintained in pond water for 3.5 days between BrdU and later EdU treatments, resulted in BrdU-labeled cells confined to the migratory streams, with only EdU labeling present in the niche (Benton et al., 2011). As the BrdU clearing time following a 24-hr incubation is known to be 42-48 hr (Benton et al., 2011) and the cell-cycle time of the niche precursors is at least 48 hrs, it is highly unlikely that BrdU labeling in niche cells could have been diluted below detection levels by mitosis (Benton et al 2011). These studies therefore directly demonstrate that the niche cell divisions are not self-renewing.
As both daughters of each niche cell division exit the niche, migrating away along the niche cell fibers to the clusters, divisions of cells within the niche itself are not responsible for maintaining the pool of 1st-generation neuronal precursor cells in the niche (Benton et al., 2011). However, the niche is not depleted by age; rather, the number of cells increase over an animal’s lifetime (Zhang et al., 2009). The niche therefore cannot be a closed system, but must be replenished from a source extrinsic to the niche. Further, cells in the crayfish niche do not adhere to the basic definition of a ‘stem cell’ because they are not self-renewing. Nevertheless, the 1st-generation precursor cells in the niche are functionally equivalent to
neuronal stem cells in mammalian systems (Beltz et al., 2011). A major goal of current studies is to identify the source of the 1st generation neuronal precursors that reside in the niche.

**I. What is the source of 1st-generation neuronal precursors in the crustacean brain?**

As a discrete, relatively isolated organ, there are relatively few potential sources of niche cells. A cavity runs through the center of the crustacean niche, and dextran-ruby dye injections have demonstrated that this cavity is confluent with the vasculature (Fig. 1D) ([*Procambarus*]: Benton et al., 2010, 2011; Sullivan et al., 2007a, 2005; [*Cherax destructor*]: Sandeman et al., 2009; the vasculature is also closely associated with the niche in the lobster [*Panulirus argus*], although direct communication between the vasculature and the niche has not been demonstrated: Schmidt, 2007). The close association between the niche and the vasculature is seen across a broad phyletic range, and is a common feature of all stem cell niches, including those in the nervous system (Tavazoie et al., 2008). The vasculature is emerging as a critical element in neuronal stem-cell niches, serving as conduits for circulating hormones and cytokines (Lenlington et al., 2003), regulating stem cell self-renewal and neurogenesis via a number of secreted factors (Riquelme et al., 2008; Shen et al., 2004, 2008), and through the associated extracellular matrix, providing means of cell anchoring (Shen et al., 2008) and creating a supportive microenvironment and architecture (Riquelme et al., 2008). While it is not clear at present how the vasculature interacts with the crustacean niche, the hypothesis that cells migrate into the niche from the vasculature is being explored (Benton et al., 2011).

Crustacean blood cells (hemocytes), especially hemocytes of the semi-granular subtype, are strongly attracted to the niche, much more so than cells from other tissue types (e.g. hepatopancreas, green gland) (Benton et al., 2010, 2011). There is a rich precedent for this finding in the mammalian literature: mammalian bone marrow cells tend to migrate to the brain when infused into a host animal (Eglitis & Mezey, 1997; Kopen et al., 1999; Brazelton et al., 2000). Bone marrow cells grafted into the lateral ventricle migrate throughout the brain, including areas undergoing active postnatal neurogenesis, and are capable of producing

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1 It should be noted that the stem cell definition in mammalian neurogenic systems is based primarily on *in vitro* and non-vertebrate studies (Zhao et al., 2008).
descendants that express a variety of glial and neuronal markers (Eglitis & Mezey, 1997; Mezey et al., 2000; Chen et al., 2001; Mahmood et al., 2001). In turn, adult forebrain neuronal stem cells can be induced in vivo to become hematopoietic cells by transplanting them into animals with damaged hematopoietic systems (Vescovi et al., 2001). A multipotent adult progenitor cell type, capable of differentiating into mesenchymal, as well as endothelial and endodermal lineages has been isolated from rodent and human bone marrow (Hermann et al., 2006). The potential importance of these parallel findings in mammalian systems has been obscured by in vitro studies suggesting that stem cells from bone marrow can fuse with other cell types (Wang et al., 2003; Weimann et al., 2003), and by a strong bias that neuronal stem cells are self-renewing, and thus long-lived or immortal. An objective of this study is to examine the relationship between the hematopoietic system and the neurogenic niche in the crustacean brain by performing ablations of hematopoietic tissues. These studies test whether or not the numbers of cells in the niche are depleted when hematopoietic output is reduced.

In mammalian neurogenic niches, some of the astroglia are neurogenic cells, while others appear to be quiescent (Ma et al., 2008; Riquelme et al., 2008; Kriegstein & Alvarez-Buylla, 2009) and potentially capable of becoming neurogenic in the presence of appropriate signals (Nyfeler et al., 2005). It is unknown if niche cells in the crustacean brain have differential capacities to become neuronal precursors. It is possible that cells in the niche act primarily as nurse cells, providing the appropriate signals for precursor cells coming into the niche to divide and migrate out (the ‘pack n ship’ model). Alternatively, the niche may be a reservoir of potential neural precursors, and newly arriving cells may join the waiting pool (the ‘inventory’ model). At present, much of the data suggests the latter may apply to adult neurogenesis in the crustacean niche. For example, studies using MCM2-7 (a cell cycle marker for the G1 phase) indicate that all niche cells are in G1 phase of the cell cycle, excluding those processing through S through M phase (Sullivan et al., 2007a), suggesting that niche cells are not terminally differentiated (Beltz et al., 2011). Second, regardless of the phase in the cell cycle, all niche cells label immunocytochemically for glutamine synthetase (GS), a glial marker, and the vast majority have identical cellular characteristics (Zhang et al., 2009). Third, external factors (environmental enrichment) have been shown to regulate the numbers of S-phase precursors (Ayub et al., 2011). In contrast, attempts to ablate hematopoietic tissues (Kery, unpublished data) have provided preliminary evidence for the
‘pack n ship’ hypothesis; however additional experiments are needed to confirm these findings. The results of the hematopoietic tissue ablations to date are included in this thesis.

II. Astakine-1, a crustacean cytokine, provides a second approach to understanding the relationship between the hematopoietic system and the crustacean niche.

Crustaceans have a simplified blood-cell system relative to vertebrate species. They rely on an innate immune defense, rather than the combined innate and adaptive responses seen in vertebrates. For this reason, the lymphatic and blood systems are not separate—and are referred to as the ‘hemolymph’. The hemolymph carries large quantities of dissolved oxygen, as invertebrates do not have erythrocytes. Crustacean blood cells (hemocytes) are divided into three classes: hyaline, granular, and semi-granular. Figure 2 shows the three subtypes awaiting release from the hematopoietic tissue. Granular cells have large characteristic vesicles, giving the cells a bubble-like appearance. Hyaline cells are defined by the absence of these vesicles. Semi-granular cells have vesicles, but in far fewer numbers than granular hemocytes. The semi-granular subtype has been posited by our collaborator Dr. Irene Söderhäll (Uppsala, Sweden) to be a potential stem cell type, and also has been implicated as one type of hemocyte attracted to the niche in vitro (Benton et al., 2011). The maturation and release of semi-granular hemocytes is regulated by the cytokine astakine-1 (Watthanasurorot et al., 2011). A second astakine isoform, astakine-2, is also present and is involved in regulating the maturation and release of granular hemocytes (Lin et al., 2010).

![Fig.2 Crayfish Hemocytes. Nomarski image of the three classes of hemocytes awaiting release from the hematopoietic tissue. Granular hemocytes have large distinctive vesicles. These are also present in semi-granular hemocytes, but in fewer numbers. Hyaline hemocytes do not have these vesicles.](image-url)
Invertebrates rely solely on innate immune mechanisms (phagocytosis/cellular encapsulation, clotting/coagulation, lectins and pattern recognition proteins, and a prophenoloxidase-activating signaling cascade) in their host defense (Söderhäll et al., 2005). Cytokines such as astakine-1 play a vital role in the regulation of this innate immunity, as they do in vertebrates (Söderhäll et al., 2005). Astakine-1 is expressed primarily by semi-granular hemocytes, but is also expressed by hematopoietic tissue, granular hemocytes, and by neural tissue (Lin et al., 2010). The precise mechanisms of the signaling cascade in which astakine-1 triggers rapid release of semi-granular cells is still being determined, though it is likely that semi-granular cells release astakine-1 into the hemolymph if they become damaged (Lin et al., 2010).

Astakine-1 is a prokineticin homologue, a cytokine family that is highly conserved across vertebrate and non-vertebrate species (Söderhäll et al., 2005). Cytokines are proteins that are generally involved in immune and inflammation reactions, but may also act as general intercellular communication signals (Kruger et al., 1990). Prokineticin signaling pathways are important in in angiogenesis, neurogenesis, reproduction, cancer pathogenesis, hematopoiesis, and in regulating various physiological functions that underlie circadian rhythms (e.g. sleep/wake cycle, hormone secretion, ingestive behaviors) (Negri et al., 2007).

Astakine-1 expression in hematopoietic tissue (HPT) and hemocytes cycles in a circadian manner, peaking in the HPT about an hour after dawn and decreasing over the course of the day (Watthanasurorot et al., 2011). This result has also been reported for prokineticin homologues in mammals, in both nocturnal (Negri et al., 2004, Cheng et al., 2002) and diurnal species (Lambert et al., 2005; Smaaland et al., 2002), suggesting that the timing of this rhythm is not dictated by a photosensitive circadian clock alone, as these molecules peak just after dawn regardless of whether the species is diurnal or nocturnal (Negri et al., 2007). It is possible, if the semi-granular cells are being attracted to the niche as proposed by Benton et al. (2011), that integration of hematopoietic-derived cells into the niche may also have a strong circadian component.

A growing body of literature suggests that cytokines have important regulatory functions in neurogenesis in mammalian systems (Ming & Song, 2011). In the crayfish brain, by 48 hours after astakine-1 injection, the numbers of cells in the niche increases; this effect is in parallel with an increase in BrdU incorporation and a significantly greater chance of
visualizing cells within the vascular cavity (Benton, unpublished results). In the intact system, regulation of neurogenesis could be triggered by circulating levels of astakine-1. However, recent work also has shown that a number of cell clusters and large neurons in the crayfish brain label with astakine-1 antibody (Fig. 3) (Benton 2011, unpublished data), suggesting that there are also likely to be influences due to neuronal astakine-1. In addition to the cell clusters which label with astakine-1 on the dorsal and ventral surface (Fig. 3), there are also astakine-1-labeled fibers which run along and between the connectives and eyestalks. Given the circadian control of astakine-1 expression in hemocytes and hematopoietic tissue found by Watthanasurorot et al. (2011), another important question is whether this astakine-1 labeling in the brain varies with time of day. Because astakine-1 plays a potentially important role in adult neurogenesis in the crayfish brain, a second goal of this research has been to determine whether there are circadian changes in astakine-1 localization or intensity.

Fig. 3 Astakine-1 mapping in Procambarus clarkii—cell cluster labeling pattern. Diagrams of the crayfish brain adapted from Sandeman et.al. 1992. (A) Preliminary localization pattern of astakine-1 in the cell clusters on the dorsal surface. Astakine-1 immunoreactivity was observed in cell clusters 6, 11 and 14. (B) Preliminary localization pattern for astakine-1 in cell clusters on the ventral surface. Astakine-1 immunoreactivity was found in cell clusters 6, 16 and 17, and in the medial giant neurons.

A final series of experiments merges these efforts to understand the influences of hematopoietic tissues and astakine-1 on adult neurogenesis in the crayfish brain. The hypothesis was that if hematopoietic ablations deplete the niche cells (see Results), then astakine-1 injection 48-72 hours prior to sacrifice will “rescue” this phenotype by releasing semi-granular cells from remaining hematopoietic tissues. By combining these manipulations of niche cell numbers using hematopoietic ablations (cell depletion) and astakine-1 injection (cell addition), my goal was to test the dependency of the niche cell population on the vascular system.
MATERIALS AND METHODS

Animals

*Procambarus clarkii* and *Procambarus acutus*, two closely related crayfish species, were used in these experiments; these were obtained from a commercial supplier (Carolina Biological Supply Company, Burlington, NC). Animals were housed and maintained in artificial freshwater at room temperature in the Wellesley College Animal Care Facility, and were kept in a light:dark cycle of 12:12 hours unless otherwise specified.

Astakine Circadian Regulation Studies

In Experiment 1, crayfish were held in the 12:12 hour light cycle for 5 days prior to sacrifice. Watthanasurorot et al. (2011) found that astakine-1 expression levels in hemocytes and the hepatopoietic tissue peaked around dawn, and were lowest after dusk. Thus, in the initial 12:12 circadian experiment, three time points were selected: two in the morning (5:30 and 7:30, a half-hour and an hour and a half after dawn respectively), and an hour and a half after dusk (18:30) (Fig. 4).

In Experiment 2, crayfish were placed in a modified light:dark cycle of 9:15 for two weeks prior to sacrifice, to replicate the lighting conditions used in a related study by Watthanasurorot et al. (2011). In this experiment, two time points were sampled: a half hour before dawn (8:30) and an hour and a half after dusk (18:30) (Fig 4).
Fig. 4 Light/dark cycles for astakine expression circadian experiments. In the first circadian experiment, a 12:12 light/dark cycle was used with three sampling times: thirty minutes after lights on, 90 minutes after lights on, and 90 minutes after lights out. A 9:15 light/dark cycle was used for the second experiment, based on a study of circadian rhythmicity of astakine expression by Watthanasuorrot et al. (2011). There were two sampling times used in this experiment: thirty minutes after lights on, and 90 minutes after lights out.

**Hematopoietic Ablations**

Crayfish in the experimental and sham groups were anesthetized on ice for twenty minutes prior to, as well as during, the procedure. A variable speed rotary drill (Dremel model 300 with 225-01 Flex Shaft Attachment, Racine, Wisconsin) with an engraving cutter bit (model 105, Dremel, Racine, Wisconsin) was used to make a circular incision dorsally in the rostral part of the cephalothorax, just above the cephalic groove (Fig. 5A). The outer layer of carapace was removed. The underlying newly forming carapace, which is immediately dorsal to and often adhering to the hematopoietic tissue (HPT), was then excised by gently tugging at the tissue with forceps (Fine Science Tools, Foster City, CA) (Fig. 5B). Once the HPT was exposed, it was easier to visualize the ophthalmic artery (OA), which is located medially in the tissue, running rostral to caudal (Fig. 5B-C). The HPT stretches over and then wraps around the ossicle located above the cardiac stomach (Fig. 5B-C).

For the experimental animals, two incisions were made one mm from each side of the OA using dissecting scissors (Fine Science Tools). Forceps were then used to tease away each side of the HPT, removing as much of the tissue as possible from the lateral boundaries without damaging other tissues (Fig. 5B). In the “full ablation” experiments, most of the
visible tissue was removed but much of the tissue wrapping around and beneath the ossicle remained, as well as one mm on either side of the OA. For the sham group, the animals were left open (but HPT was untouched) for approximately the same amount of time it took to remove the HPT in the experimental animals.

Following HPT ablation, the outer carapace was reattached by melting dental wax over the incision site (Fig. 1D). A small dot of nail polish was painted at the most caudal part of the cephalothorax as means of identification (each group—sham, control, and experimental—had its own color). Animals were housed individually for the first 24 hours after the procedure, and were then held together in a circulating tank. Control animals were not marked with nail polish, and did not spend 24 hours housed individually. A video of this process is available online: http://www.youtube.com/watch?v=t2ZvwnF8-Fg

The animals were injected with BrdU 8 days after surgery, 48 hours prior to sacrifice, and killed on day 10 after surgery. The 10-day time course was derived from previous attempts at this experiment that tested various time periods following hematopoietic ablations. By day 10, the HPT has already begun to regenerate (Rachel Kery 2011, unpublished result), thus allowing more of the HPT to recover over a longer survival time could occlude being able to see changes in niche cell counts.

**Astakine Rescue Experiment**

There were four groups of crayfish: control, sham, and two half-ablation (HA) groups. The procedures were identical to those described above, except that for both half-ablation groups, only one side of the HPT was removed around the OA. 48 hours prior to sacrifice, 0.5mL of a 5mg/ml solution of BrdU was injected into the control, sham, and one of the half-ablation groups (HA-only). For the other half-ablation group (HA+ASK), this injection was 0.5mL of 5mg/ml BrdU and 0.05mg/ml Astakine-1 (a gift from Dr. Irene Söderhäll). The animals were sacrificed on day 7 after surgery.
Fig. 5 Hematopoietic Ablation. (A) A circular incision was made in the rostral part of the cephalothorax. (B) New carapace forms below the old, immediately dorsal to and often adhered to the hematopoietic tissue (HPT). Red lines indicate the approximate margins where the HPT will be cut. (C) An HPT that has been dissected out. Arrow indicates the ophthalmic artery. (D) An animal recovering, the outer carapace sealed with dental wax.

Immunohistochemical Labeling

Brains were dissected, desheathed, and fixed overnight in 4% paraformaldehyde in 0.1M phosphate buffer (PB). Fixative was flushed out using several rises with 0.1M PB containing 0.3% Triton X-100 (PBTx). For the ablation experiment, the brains were incubated for 40 minutes in 0.2N HCl, rinsed multiple times with PBTx, and then incubated for 4 hours at room temperature in rat α-BrdU (1:50, Sigma) before undergoing additional rinses. Preps were then incubated overnight at 4°C in primary antibodies, with the exception of the second circadian experiment in which preps were incubated for 48-hours in anti-astakine primary antibody, followed by a 24 hour anti-GS incubation. This longer incubation was attempted to increase the signal-to-noise and to decrease background labeling, both of which improved compared with the prior experiment. For ablation experiments, mouse α-GS (1:100, Becton Dickenson) was used, and for circadian experiments, mouse α-GS (1:100, BD) and rabbit α-Astakine-1 (1:200, a gift from Dr. Irene Söderhäll). Following PBTx rinses to remove
unbound primary antibodies, brains were incubated overnight at 4°C with secondary antibodies obtained from Jackson Imunoresearch: CY2-conjugated goat α-rat IgG (1:100) and CY5-conjugated goat α-mouse IgG (1:100) for the ablation experiment, and Alexa 488-conjugated goat α-rabbit IgG (1:100) and CY5-conjugated goat α-mouse IgG (1:100) for the circadian study. Brains were rinsed with PBTx, incubated in the nuclear marker propidium iodide (1:100, Sigma) for 10 minutes, rinsed again in PB, and mounted using Gel/Mount™ (ElectroMicroscopy Sciences, Hatfield, PA).

Total Hemocyte Counts

Total hemocyte counts were taken twenty-four hours prior to sacrifice. 0.01 mL of hemolymph was drawn from the dorsal sinus using a 1-ml plastic syringe, placed in an Eppendorf tube, and diluted 1:2 with an anti-coagulant solution of citrate buffer/EDTA (NaCl 0.14M, glucose 0.1M, trisodium citrate 30mM, citric acid 26mM, EDTA 10mM, pH 4.6) stored at 4°C. The blood sample was then mixed with 0.4% Typan Blue solution (Sigma, 1:1 dilution) in order to distinguish between living and dead cells, and then transferred to the hemocytometer. The cover slip was placed horizontally on the chambers and pressed gently on the sides. The hemolymph filled the chamber via capillary motion. The slide was analyzed using light microscopy (Nikon, 20x objective). One of the hemocytometer’s 4x4 grids was selected at random, and all cells present on that grid were counted. This was repeated three more times to a total of four grids (two each from the upper and lower chambers of the hemocytometer). The hemocyte count/mL of hemolymph was calculated for each hemocytometer count and recorded separately. All four counts were then averaged together. A total hemocyte count represents the total numbers of circulating cells (we were unable to differentiate between subtypes).

Confocal Microscopy

Brains were imaged using a Leica TCS SP laser-scanning confocal microscope equipped with Argon 488nm, Krypton 561nm, and Helium-Neon-633nm lasers. Serial optical 1 µm sections were saved as two-dimensional stacks.
**Counting Hepatopancreas Cells**

As shown in figure 6, the tips of the hepatopancreas were severed, processed for BrdU and PI, and then sliced into 100µm sections using a Vibratome®. Each piece of hepatopancreas is comprised of multiple bunches of hair-like fronds. BrdU labeling is confined to the outside, and anterior edges of these fronds (Fig. 6A). Because these fronds vary in size, the ratio between proliferating (BrdU and PI-labeled) and non-proliferating (PI-labeled only) cells was used. To determine the x- and y-axis counting limits, a line was drawn beneath the furthest posterior BrdU-labeled cell, as close to possible to a 90° angle from the tip of the frond. The z-axis counting limits were determined by looking for the very first and last faint traces of the BrdU label.

**Counting Niche and Astakine-1 labeled Cells**

All cells expressing both GS and PI were counted in the niche as ‘niche cells’. In each of the interneuronal cell clusters described, the total numbers of cells that labeled with the astakine-1 antibody were counted.

**Data Analysis and Statistics**

Comparisons between different groups of animals were calculated via one-way ANOVA analysis followed by Tukey multiple comparisons using SPSS 17.0 software.
Fig. 6 Counting BrdU-labeled cells in the hepatopancreas. (A) BrdU labeling in a hepatopancreas frond. (B) PI-labeled hepatopancreas cells. (C) BrdU-labeled cells are present at the tips of the hepatopancreas frond. To determine what proportion of cells were dividing, a line was drawn below the furthest posterior dividing cell at as close as possible to a 90° angle from the tip of the frond. All cells within that space were counted. (D) A diagram of the hepatopancreas, detailing the severed piece and the fronds. Scale bars denote 20µm.
RESULTS

The experiments in this thesis address two main issues to characterizing the relationship between the neurogenic niche and hematopoiesis: (i) localization of astakine-1 in the crayfish brain, and whether this distribution is influenced by the circadian cycle, and (ii) testing whether ablation of hematopoietic tissue, which decreases the numbers of circulating cells in the hemolymph, is correlated with changes in the numbers of cells in the neurogenic niche, and whether injection of astakine-1 can rescue this phenotype.

All figures referenced in the results section can be found in a separate section immediately following the results.

I. Astakine: distribution and circadian regulation

Watthanasurorot et al. (2011) found that astakine expression levels in hemocytes peak around dawn and fall after dusk in the crayfish *Pacifastacus leniusculus*. Based on these findings, time points were selected in the present studies in order to explore whether astakine labeling in the brain varies with time of day. (Experiment 1-12:12 light cycle, time points at 5:30 (one half-hour after dawn), 7:30 (an hour and a half after dawn), and 18:30 (an hour and a half after dusk); Experiment 2-9:15 light cycle, time points at 8:30 (one half-hour before dawn) and 18:30 (one half hour after dusk).

In Experiment 1, astakine labeling of cell clusters was faint at all time points. Therefore, in the second experiment, brains were incubated in anti-astakine antibody for 48 hours, rather than overnight as in the first experiment. This resulted in more intense labeling for astakine; in addition, particulate background labeling that was observed in the first experiment was eliminated in the second study. Although specific localization patterns were similar in the two experiments, the following results focus primarily on the second study because the quality of labeling was superior. Note that brain structures, including the numerical identification of the various cell clusters in the brain, uses the nomenclature introduced by Sandeman et al., 1992.
Astakine labeling on the Ventral Surface of the Brain. There were no major differences seen between dawn and dusk time points in astakine labeling of the ventral surface of the brain. Fibers near clusters 16 and 17 were observed (Fig. 6C, F). Astakine-labeling of the distinctive medial giant neurons (MGNs) was confined to the nucleus (Figs. 6E-F, 7).

Cluster 6. Cluster 6 is a large cell cluster that is located anteriorly in the crayfish brain (protocerebrum), and penetrates through from ventral to dorsal surfaces (Fig. 3). Astakine-labeled cells in cluster 6 are found near both the ventral and dorsal surfaces, but not in the center of the cluster. Cell labeling in this cluster is confined to the nucleus (Fig. 8A, B), similar to the medial giant neurons (Fig. 6E, F). Small astakine-labeled processes extend toward (or originate from) these cells (Fig. 8A, B). At the 8:30 time point, there were more astakine-labeled cells near the ventral surface of cluster 6 than the dorsal surface (p<0.05, ANOVA with Tukey comparisons) (Fig. 8C); this was a trend for the 18:30 time point (p<0.07, ANOVA with Tukey comparisons) (Fig. 8C), suggesting that there tend to be more astakine-labeled cells on the ventral surface of cluster 6 than the dorsal. The overall difference in the number of cluster 6 cells labeled (both ventral and dorsal) between 8:30 and 18:30 time points had a p-value of less than 0.06 (ANOVA, with Tukey comparisons), indicating a trend towards more astakine-labeled cluster 6 cells in the morning compared to the evening (Fig. 8D).

Cluster 16. Astakine labeling in neurons located in cluster 16 was also confined to the nucleus (Fig. 9A). There were no differences in overall intensity or numbers of cells stained for the morning and evening sampling times (Fig. 9B).

Cluster 17. Astakine labels the cytoplasm of cluster 17 cells, rather than the nucleus, as in the medial giants and neurons in cluster 16 (Fig. 10A). There were no statistical differences in the numbers of labeled cells between the two time points, but there was a trend (p<0.07, ANOVA, Tukey-comparisons) towards more cells labeled in the morning (Fig. 10B).
Astakine Labeling of the Dorsal Surface. Astakine-labeled fibers running in the anterior-posterior direction were found on the dorsal surface of the brain (Figs. 7D, 11). These were intensely labeled at the 8:30 time point, and only weakly labeled or unlabeled at 18:30 (Figs. 11A,C,E,F). Several astakine-labeled fibers run along the connectives and towards (or from) the anterior part of the brain; these cross near the deutocerebral bridge (Fig. 11A,E). What appears to be either a large blood vessel or a particularly thick nerve fiber runs along the eyestalks (Fig. 14A, E). Another fiber runs near to clusters 9 and 11 (cluster 9 is immediately ventral to cluster 11), where a cell on the edge of cluster 11 has intense cytoplasmic labeling (Fig. 11E, F).

Clusters 11 and 14. Visible on the dorsal surface, cell clusters 11 and 14 contain astakine-immunoreactive cells, the vast majority of which have labeling confined to the nucleus (Fig. 12). There is at least one cell at the proximal edge of cluster 11 that labels cytoplasmically (Fig. 11E, F). In cluster 14, there appear to be a large number of astakine-labeled processes that may innervate these cells (Fig. 12B). Astakine labeling of cluster 14 cells is confined to the nucleus. Although it is possible that these fibers emerge from cluster 14 cells, given that they do not appear to manufacture the cytokine, this seems unlikely. There were no qualitative differences in the numbers of labeled cells in clusters 11 and 14 between the morning and evening time points.

Astakine Labeling of Hemocytes and Blood Vessels. In tissues representing both time points, astakine-labeled hemocytes were present on the ventral and dorsal surfaces, but not often found in deeper tissue layers. Hemocytes were identified on the basis of morphology seen in previous light microscopy work, as well as the astakine-labeling patterns seen in hematopoietic cells by Söderhäll et al. (2005). Astakine labels what appears to be inclusions (vesicles or granules) in the cytoplasm of hemocytes, but not the nucleus (Fig. 13). Hyaline hemocytes contained cytoplasmic labeling and these cells were often seen in a row, possibly indicating the presence of a small blood vessel (Fig. 14B). Semi-granular and granular cells were more commonly seen individually (Fig. 14A), had no astakine labeling in the nucleus, and contained varying numbers of astakine-labeled vesicles in the surrounding cytoplasm.
Semi-granular and especially granular hemocytes were visually more obvious than hyaline cells, which probably contributed to the observed differences in the subtypes’ localizations.

Posteriorly, near the connectives, there were often rows of hemocytes on both surfaces of the brain, which are likely within blood vessels, although the walls of the vessels do not label (Fig. 14A). Crustaceans have a partially open, partially closed circulatory system: there are blood vessels within the brain that bring hemolymph from the heart, and fine capillaries then release blood into intercellular spaces. The hemolymph then percolates through brain tissues to the surface, where it collects in blood sinuses and is drawn back into the heart. Punctate astakine labeling of ‘tunnels’ within clusters 6, 9, 10 and 11 are likely to be blood vessels or spaces within the tissue where hemolymph has collected, as astakine circulates not only in hemocytes but also freely in hemolymph (Fig. 14A). There are small nuclei present within these tracts that are likely the nuclei of hemocytes within the vessel (Fig. 14A).

At first glance, the thick astakine-labeled tracts on the surface of the brain near cluster 6 (towards the eyestalks) appeared to be blood vessels (Fig. 11A, E). However, upon closer inspection, these astakine-labeled tracts are only 3 µm thick, while hemocytes tend to be at least 5 µm in diameter. Further, while there were nuclei on top of and beneath these astakine-labeled tracts, no nuclei were present within the astakine label (Fig. 15B), suggesting that there are no blood cells lying within the structure. Therefore, based on their diameter and the absence of nuclei within them, it seems highly probable that these are large astakine-labeled neuronal fibers, not blood vessels. Their origin and targets were not identified.

**Astakine Labeling of the Niche and Vascular Cavity.** Earlier investigations of astakine labeling in the brain (Benton, unpublished results) focused on labeling in the cell clusters and in fibers. The current studies are the first to examine astakine labeling in the neurogenic niche located on the ventral side of the brain.

The niche cells label with an antibody against glutamine synthetase (GS), which was used as a marker in all of these experiments (Fig. 16A, D, F). In some preparations, this antibody also appeared to label occasional astakine-immunoreactive granular hemocytes (Fig. 16A, D, F). However, if special blocking protocols are not used, the goat-anti-mouse
secondary antibody can bind non-specifically to hemocytes (see Ayub et al. 2011); therefore this GS label may be non-specific and needs to be further characterized.

The niche is made up of several layers of cells surrounding a central cavity (Figs. 1D, 16, 17). This vascular cavity is not a shallow, uniform opening, but a longer passage that tapers, bends, or bifurcates as it reaches its dorsal limits (Fig. 17A-B). Bifurcation of the cavity was more commonly seen at 5:30 and 7:30 than at 18:30, suggesting that the niche may change shape or position over the course of the day. In some preparations, astakine- and GS-labeled material was observed in the vascular cavity. In only one example, from the 18:30 time point of the initial circadian experiment, an astakine-labeled cell was found to contact a GS-labeled fiber extending toward (or from) the niche, possibly indicating that this cell is attempting to integrate itself into the niche (Fig. 17D, arrow). Astakine labeling in this cell is confined to the nucleus, rather than the cytoplasm as seen in hemocytes.

In all time points from both experiments, the vascular cavity was devoid of particulate background labeling (Fig. 16A-B, F). However, in some preparations a granular or ‘bubble-like’ substance was observed in the cavity, similar to the substance previously reported by Zhang et al. (2009) (Fig. 17C). The niche cells themselves are not immunoreactive for astakine.

An astakine-labeled fiber was seen running underneath the niches in the evening time point (Fig. 18A). This fiber, which was immediately dorsal to and extended across (anterior to posterior) the niche, belongs to a large astakine-labeled cell (Fig. 17B, C; 18C,D) that I have named ‘cell F’. Astakine antibody labels the cytoplasm and nucleus of cell F, but not in the densely packed DNA (Fig. 18 B, C).

Cell F is generally located at the anterior edge of cluster 16, slightly dorsal to the rest of the cluster (Fig. 19A,B) and near the niche (Fig. 17 B, C; 19A). However, cell F has also been found in a variety of locations in the space between cell clusters 9, 10 and 16, where the niche is usually found (Fig. 20). Cell F’s position did not seem to vary in any consistent way between the two time points sampled. The astakine-labeled fiber of cell F projects towards cluster 9, where it appears to travel through the cluster, turn dorsally, and finally contact a large astakine-labeled cell in cluster 11.
II. Hematopoietic ablations

Given the multiple lines of evidence from earlier studies that suggested a close relationship between cells in the niche and hemocytes circulating in the blood—that these cells might be entering the niche and transforming into niche cells—ablations of the hematopoietic tissue (HPT) were performed to further characterize this relationship. If, through ablation of hematopoietic tissues, the numbers of circulating cells in the hemolymph were decreased, it was hypothesized that the numbers of cells in the niche would also decrease.

Although a series of experiments (n=13) have been conducted, animal mortality (in all groups, including controls) has complicated the data analysis and reduced the statistical power in the data sets. In a final experiment these issues were resolved, and the ‘n’ was adequate for a complete statistical analysis. These data are provided in figures 21-23.

Ablation of hematopoietic tissues consistently decreased the total number of PI-labeled cells in the niche relative to sham and control preparations (Fig. 21A), but the numbers of BrdU-labeled niche cells were not altered (Fig. 21B). No differences existed for the experimental (FA) BrdU counts in clusters 9 (Fig. 22B) and 10 (Fig. 22A) and the streams (Fig. 22C) relative to sham and control. However, the control animals had fewer BrdU cells in cluster 10 and in the streams relative to sham controls (Fig. 22A, C); the differences in cluster 9 (Fig. 22B) were not statistically significant.

In previous attempts at this same experiment, the differences in BrdU counts for cluster 10 varied wildly (in one experiment, the experimental group had significantly fewer cells relative to sham and control, and in three others, there were no differences) (see data, Appendix A). In all previous attempts, however, the decrease seen in the niche PI cell count for the experimental group relative to sham and control was consistently found, although sample sizes were small. The final rendition of this study had a substantial sample size in one single experiment.

In prior attempts at this experiment, a total hemocyte count was used to judge the effect of hematopoietic tissue ablations on blood cell numbers. However, this assay was not effective due to tremendous variability within all groups. Crustacean hemocyte counts can vary tremendously with counting methods, molt stage, many forms of injury and infection,
and on an individual-by-individual basis (Matozzo & Marin 2010, Cheng & Chen 2001, Thornqvist & Soderhall 1993, Hose et al. 1992, Fontaine & Lightner 1974). Even drawing a hemolymph sample on one day influences counts over several subsequent days. For this reason, reliable counts could not be acquired in a longitudinal fashion within the time course of this experiment.

Throughout the crayfish’s life, cells in the hepatopancreas divide at the distal tubule tips (Davis & Burnett 1964). This tissue was therefore used as a control for animal health following the hematopoietic tissue ablations. Infection and a variety of environmental insults can cause inflammation and necrosis of the hepatopancreas (Lee et al. 1985). It would seem likely, therefore, that the numbers of dividing cells in the hepatopancreas would be affected, should an animal’s health deteriorate. Cell proliferation in the hepatopancreas resembles cell proliferation in the crypts of Lieberkuhn in mammals (Vogt 2008), which has been used as a positive control for mitotic activity in the rat brain (Bryans 1959). The numbers of dividing cells in the tips of distal tubules were therefore used as a general assay of animal health. As the tubule tips varied tremendously in size and shape, to normalize these comparisons, the ratio between dividing and non-dividing cells in the tubule tip was used. There were no differences between the three groups in this ratio (Fig. 23), suggesting that differences in BrdU and PI cell counts in the brain between groups were not due to a general decline in the health of the animals. Rather, this suggests a relationship between the proliferative capacity of hematopoietic tissues on the cell numbers in the neurogenic niche.

Astakine rescue experiment. Astakine is known to promote differentiation and release of semi-granular cells from hematopoietic tissue (Lin and Soderhall, 2011). As described in the introduction, astakine injection into crayfish results in an increase in the number of niche cells by 48 hours following injection. Therefore, in a final experiment, the influence of injected astakine on niche cell numbers following hematopoietic tissue ablation was tested. The hypothesis was that if the proliferative capacity of hematopoietic tissues is indeed correlated to niche cell numbers, then astakine injection and subsequent release of semi-granular cells from remaining hematopoietic tissues should “rescue” the niche cell depletion following hematopoietic tissue ablation seen previously. In this experiment, only half of the
hematopoietic tissue was ablated so that there would be adequate tissue remaining on which the astakine could act.

Ablated animals were injected with astakine-1 5 days after hematopoietic tissue ablation, and 48 hours prior to sacrifice. Results show that the astakine-injected crayfish had increased numbers of niche cells relative to ablated animals that received a saline injection (Fig. 24A). Animals that received sham surgeries—in which no tissue was removed—had substantially greater numbers of niche cells than control and ablated animals without astakine treatment, but not ablated animals with astakine treatment (Fig. 24A). There were no differences in the numbers of BrdU cells in the niche between the groups (Fig. 24B).

Niche cells and total hemocyte count. In an earlier ablation experiment, animals were sacrificed fourteen days after surgery. Just prior to ablation, total hemocyte counts and niche cell counts were assessed (Fig. 25). There is a strong positive correlation between these counts ($R^2=0.925$), further demonstrating the close linkage between the hematopoietic system and the neurogenic niche.
Fig. 6 Astakine-1 labeling on the ventral surface. Propidium iodide (PI)-labeling is in red, Astakine-1 (ASK-1)-labeling in green, stacked image. (A) ASK-1 labeling, 830. (B) PI labeling, 830. Relevant cell clusters, cluster 16 (CL 16), cluster 17 (CL 17), cluster 6 (CL 6), and the medial giant neurons (MGN) are indicated. (C) ASK-1 labeling, 1830. There is a slight difference in labeling intensity between (A) and (C) because the posterior portion of the brain in (A) is tilted backwards slightly relative to the brain in (B). (D) PI labeling, 1830 with relevant cell clusters indicated. One of the MGNs is missing from this prep. (E) Overlay of PI and ASK-1 labeling, 830 (merger of A and B). ASK-1 labeling is evident in the nucleus of the MGNs, cells in CL 6, and CL 16. The cytoplasm of cells in CL 17 was astakine-1-immunoreactive. (F) Overlay of PI and ASK labeling, 1830 (merger of C and D). The ASK-1 labeling pattern seen is similar to that shown in (E). A speckled astakine-1 labeling pattern (likely fibers from the antennal II neuropil) is present near CL 16 and CL 17 (indicated by arrowheads). Size bars denote 100µm.
Fig. 7 Astakine-1 mapping in Procambarus clarkii—initial results from first circadian experiment. Propidium iodide (PI)-labeling is in red, Astakine-1 (ASK-1)-labeling in green, stacked images. (A) Ventral view, image from 530 time-point. The nucleus and possibly the surrounding cytoplasm of the medial giant neurons (indicated by white arrow) were astakine-1-labeled. (B) Cluster 6 astakine-1 labeling was found only on the ventral surface, and was largely confined to the nucleus of two large cells. Image from 1830. (C) Cluster 17 (ventral) astakine-1 labeling was confined to the cytoplasm, and not present in the nucleus. Image from 530. (D) Astakine-1-labeled fibers were present on the dorsal surface. Image from 730. Size bars: (A), (B) and (C) 100 µm, (D) 200 µm.
Fig. 8 Astakine-1 labeling in Cluster 6. Propidium iodide (PI)-labeling is in red, Astakine-1 (ASK-1)-labeling in green, stacked images from 830. Labeling both ventrally (A) and dorsally (B) was confined to the nucleus, though there was slight astakine-1 labeling in the cytoplasm on the dorsal side. Cluster 6 had more astakine-1-labeled cells ventrally than dorsally *(p<0.05) at 830, but not 1830 (C). There were no differences in cluster 6 labeling ventrally or dorsally between the two time points (D). Size bars denote 50µm.
Fig. 9 Astakine-1 labeling in Cluster 16. Propidium iodide (PI)-labeling is in red, Astakine-1 (ASK-1)-labeling in green, stacked image from 1830. Astakine-1 is present in the nucleus of cluster 16 cells (A). There were no differences in the numbers of cells labeled between the two time points (B). Size bar denotes 50µm.

Fig. 10 Astakine-1 labeling in Cluster 17. Propidium iodide (PI)-labeling is in red, Astakine-1 (ASK-1)-labeling in green, stacked image from 1830. Astakine-1 is present in the cytoplasm, but not the nucleus of cluster 17 cells (A). There were no differences in the numbers of cells labeled between the two time points (B). Size bar denotes 50µm.
Fig. 11 Astakine-1 labeling on the dorsal surface. Propidium iodide (PI)-labeling is in red, Astakine-1 (ASK-1)-labeling in green. (A) Astakine-1 labeling, 830. Astakine-1-labeled fibers are present medially emerging from the connectives up towards the eyestalks. (B) PI labeling, 830, with relevant cell clusters indicated: cluster 6 (CL 6), cluster 11 (CL 11), cluster 17 (CL 17), and cluster 14 (CL 14). (C) Astakine-1 labeling, 1830. There are far fewer astakine-1-labeled fibers—the only clearly labeled ones are near the connectives posteriorly. (D) PI labeling, 1830. (E) Overlay of PI and ASK labeling, 830 (Merger of A and B). Astakine-1-labeled cells are present in CL 6, CL 11, and CL 14. An astakine-1-labeled fiber runs proximal to cluster 11 (indicated by arrow). (F) Overlay of PI and ASK labeling, 1830 (Merger of C and D). Cell clusters 11 and 14 appear to have astakine-1-labeled cells. Though not nearly as distinct as in the 830 image, there appears to be a similar astakine-1-labeled fiber proximal to cluster 9/11 (indicated by arrow). Size bars denote 200 µm.
Fig. 12 Astakine-1 labeling in Clusters 11 and 14. Propidium iodide (PI)-labeling is in red, Astakine-1 (ASK-1)-labeling in green, stacked images from 830. Astakine-1 is present in the nucleus, but not the surrounding cytoplasm of cluster 11 cells (A) and cluster 14 cells (B), similar to clusters 6 and 16. In cluster 14, small astakine-1-immunoreactive fibers are present near and within the cluster (indicated by arrows), possibly innervating these cells. There did not appear to be differences in astakine-1 labeling between the two time points in cluster 11 or cluster 14. Size bars: (A) 50 µm, (B) 20 µm.

Fig. 13 Astakine-1 labeling of hemocytes. Small numbers of granular and semi-granular hemocytes were present on the ventral and dorsal surfaces of brains from both time points. (A) Granular cells on the ventral surface of cluster 16. (B) Granular cells have more astakine-1-labeled vesicles than semi-granular cells. Propidium iodide (PI)-labeling is in red, Astakine-1 (ASK-1)-labeling in green, stacked images from 830. Size bars (A) 10µm, (B) 5 µm.
Fig. 14 Astakine-1 labeling of blood vessels. (A) There are small blood vessels on the surface of cluster 10 that label with astakine-1. The orthogonal slicer function of the Leica software was used to obtain sagittal and coronal at the level of the white reference lines. The nucleus at the intersection of the reference lines is completely surrounded by astakine-1 labeling indicating that the nucleus is within a blood vessel. Punctate labeling may be astakine-1 present in the hemolymph. (B) A series of mostly granular hemocytes positioned right in a row on the ventral surface of the brain (indicated by white dotted line), near the connectives are likely in a blood vessel. Propidium iodide (PI)-labeling is in red, Astakine-1 (ASK-1)-labeling in green, stacked images from 830. Size bars denote 10 μm.
Fig. 15 Thick fiber near eyestalks. This thick astakine-1-labeled tract, which was found on the dorsal surface, and is indicated by arrow in (A), was found at both time points (though it was more brightly labeled at 830). This tract does not appear to be a blood vessel, because it is less than 3 μm thick, and none of the nuclei present are either above or below the astakine-1 label. Propidium iodide (PI)-labeling is in red, Astakine-1 (ASK-1)-labeling in green, images from 830. Size bars (A) 200 μm, (B) 20 μm.
Fig. 16 Astakine-1 mapping—the niche and vascular cavity. Propidium iodide (PI)-labeling is in red, Astakine-1 (ASK-1)-labeling in green, stacked images from 830. (A) Astakine-1 labeling of the niche. Astakine-1 produces a higher background for the niche then for surrounding tissues. Astakine-1 background is not present in the vascular cavity (indicated by arrow) (B) A higher-magnification image of astakine-1 labeling of the vascular cavity. Astakine-1 background labeling (present virtually everywhere in the brain) is not present in the vascular cavity. (C) Overlay image of GS, PI and Astakine-1 labeling of the vascular cavity. The niche appears to be multi-layered, with the vascular cavity tapering or turning dorsally. (D) GS labeling of the niche, vascular cavity indicated with arrow (E) PI labeling of the niche, vascular cavity indicated with arrow. (F) Overlay image of (A), (D) and (E) A thick rim of astakine-1 and GS labeling surrounds the vascular cavity, which appears to be turning dorsally. An astakine-1 and GS-labeled hemocyte is located near the vascular cavity. Size bars (C) 10µm, (F) 20 µm.
Fig. 17 Astakine-1 mapping—the vascular cavity. Propidium iodide (PI)-labeling is in red, astakine-1 (ASK-1)-labeling in green, glutamine synthetase (GS)-labeling in blue. (A) This stacked image shows the most dorsal sections of a niche from 1830. The layers cells surrounding the vascular cavity indicate that the niche is at least several cell layers thick. The vascular cavity appears to taper or turn dorsally. (B) The vascular cavity often bifurcates dorsally. Single section image from 730. (C) Many of the vascular cavities from the morning time points (530 or 730) had astakine-1 and GS (but not PI) labeled material (indicated by arrow), which could indicate that the cells surrounding the vascular cavity are engaged in some form of uptake or secretory activity. Single section image from 730. (D) This astakine-1-labeled cell (indicated by arrow) appears to contact a GS process extending from or toward the niche, possibly indicating that this cell is attempting to integrate itself into the niche. Stacked image from 1830. Size bars (A), (B) and (D) 20µm, (C) 10 µm.
Fig. 18 An Astakine-1-labeled fiber runs underneath the niche at 1830. Propidium iodide (PI)-labeling is in red, astakine-1 (ASK-1)-labeling in green, glutamine synthetase (GS)-labeling in blue. Stacked images from 1830. (A) The orthogonal slicer function of the Leica software was used to obtain sagittal and coronal at the level of the white reference lines. The intersection of the reference lines was placed at the edge of the vascular cavity to visualize the point at which the astakine-1-labeled fiber crosses paths with this part of the niche, which can be seen in the Z-axis on the right side (yz axis, fiber indicated by arrow) of this figure. The fiber passes directly underneath some portion of the niche, and occasionally directly underneath the vascular cavity. This fiber, which is almost always present at 1830, is seen only rarely at 830. (B) The astakine-1-labeled fiber that runs underneath the niche emerges from a large, astakine-1-labeled cell found near the niche. (C) This large astakine-1-labeled cell (with astakine-1-labeling confined to the cytoplasm) was found near the niche in every time point sampled. Size bars denote 20µm.
Fig. 19 Characterizing large astakine-1-labeled cell (cell F). Propidium iodide (PI)-labeling is in red, astakine-1 (ASK-1)-labeling in green, glutamine synthetase (GS)-labeling in blue, images from 1830. (A) Cell F is dorsal to the niche and to cluster 16, stacked image. (B) The same cell, at higher magnification. Astakine-1 labels the cytoplasm and nucleus of cell F (single section image). (C) The astakine-1-labeled fiber emerges from cell F dorsally. (D) Another image of a fiber emerging from cell F. Size bars (A) 10µm, (B) (C) and (D) 5µm.
Fig. 20 Localization of cell F in relation to the niche. Propidium iodide (PI)-labeling is in red, astakine-1 (ASK-1)-labeling in green, glutamine synthetase (GS)-labeling in blue. (A) Cell F is usually located at the anterior and dorsal edge of cluster 16. Stacked image from 1830. (B) Cell F has also been found in a variety of positions within the space between clusters 9, 10, and 16. Stacked image from 830. For both time points, cell F was most commonly found in cluster 16. Size bars denote 100 µm.
Fig. 21 Hematopoietic ablation—niche results. (A) Full-ablation (FA) animals had fewer niche PI cells relative to sham and control (p<0.001, ANOVA with Tukey comparisons). There were no differences in niche PI cell counts between sham and control. (B) There were no differences in the numbers of BrdU-labeled cells in the niche between the groups.
Fig. 22 Hematopoietic ablation—Streams and Clusters 9 and 10. (A) Cluster 10 BrdU cell counts. Controls had fewer BrdU cells in cluster 10 relative to sham (p<0.03, ANOVA with Tukey comparisons), but not full-ablation (FA), which was not different from either sham or control. (B) Cluster 9 BrdU cell counts. There were no differences in cluster 9 counts between any of the groups. (C) Mean numbers of BrdU cells in the streams. Similar to cluster 10, Controls had fewer BrdU cells in the streams relative to sham (p<0.02, ANOVA with Tukey comparisons), but not full-ablation (FA), which was not different from either sham or control.
Fig. 23 Hematopoietic ablation does not affect the numbers of dividing cells in the hepatopancreas. There were no differences between the three groups in the ratio of dividing (BrdU-labeled) cells to total (PI-labeled) cells.
Fig. 24 Hematopoietic half-ablation—niche results.  (A) Ablated animals that received astakine-1 injection (HA+ASK) had more niche PI cells than ablated animals that were injected with saline (HA) (p<0.02, ANOVA with Tukey comparisons). Animals in the sham group had more niche cells than control (p<0.02, ANOVA with Tukey comparisons) and HA animals (p<0.001, ANOVA with Tukey comparisons)). There were no differences between the sham and HA+ASK groups, or between control and either HA group. (B) There were no differences in the numbers of BrdU-labeled cells in the niche between the groups (p<0.10 in all comparisons, ANOVA with Tukey comparisons).
Fig. 25 The mean number of niche PI-labeled cells is positively correlated with total blood cell count. Blood counts were taken twenty-four hours prior to sacrifice (at the time of BrdU injection). Each data point represents an individual animal.
DISCUSSION

Astakine-1, a crustacean cytokine in the prokineticin family, regulates the renewal of circulating hemocytes from crustacean hematopoietic tissues. Specifically, astakine-1 induces the proliferation and differentiation of hematopoietic cells along the semi-granular lineage and causes the release of semi-granular cells from hematopoietic tissues (Söderhäll et al., 2003, 2005). The semi-granular cells have been proposed as hematopoietic stem cells in crustaceans (Söderhäll et al., 2003). Astakine expression in hemocytes is regulated in a circadian fashion, with a peak just after dawn and a gradual drop throughout the daytime hours to a low just before dusk (Watthanasurot et al., 2011). There is a corresponding increase in total hemocyte counts, which peak within an hour of the astakine high. For these reasons, this molecule is of major interest in our studies of adult neurogenesis in the crayfish brain, and may provide a critical link to discovering the source of 1st-generation neuronal precursors that reside in the niche.

The experiments described in this thesis address two themes relating to adult neurogenesis in the crayfish brain and the potential role of astakine-1 in this process. Studies in the Söderhäll lab demonstrated astakine-1 expression in neural tissues, and subsequent work in the Beltz lab showed that astakine-1 immunoreactivity is localized in specific neuronal cell clusters in the brain. Therefore, the first group of studies tested whether astakine localization in the brain is under circadian regulation. The second set of experiments tested whether quantifiable aspects of niche cell function are dependent on the hematopoietic system. Hematopoietic tissues were ablated and the number and cell cycle activity of niche cells were assessed, demonstrating that niche cell numbers are depleted following hematopoietic tissue ablation. The final experiment asked whether the depletion of niche cells could be “rescued” by injection of astakine-1 into crayfish after hematopoietic tissue ablation. Overall, these studies contribute to our understanding of the role of astakine-1 and the importance of hematopoietic tissues in sustaining the neurogenic niche in the crayfish brain.

I: Astakine-1 localization and circadian regulation in Procambarus clarkii brain

The major aim of this study was to investigate whether the pattern of astakine-localization was under circadian influence. There were no differences observed between the time points in
the localization of astakine in cell clusters. However, the intensity of labeling in neuronal fibers was much greater in the morning time points; thus more labeled fibers were observed in the morning than in the evening samples.

**A. Astakine immunoreactivity in neuronal cell bodies and fibers**

The Medial Giant Neurons

The medial giant neurons (MGNs) are large interneurons that integrate sensory information from the paraolfactory (olfaction), and antennal lobes (proprioception) (Glantz & Viacour 1983) and are involved in a number of important motor functions, including the tail flip escape reaction (El Manira & Clarac 1994), serotonin (5-HT) signaling to motor pathways (Heinrich et al., 2000), and circadian motor responses (Gordon et al., 1977). These neurons label with astakine-1 in the nucleus only (Fig. 6, 7a) suggesting that astakine is playing a role as a transcription factor that somehow regulates the functioning of these cells. The MGN’s dendritic arches are quite large and distinctive (Glantz & Kirk 1980, Horuichi et al., 1971), and do not appear to label with astakine-1 antibodies. Astakine is present in low concentrations in the surrounding hemolymph, which could be the source of astakine for the MGNs, but the mechanism involved in its synthesis and/or accumulation in the nuclei of these neurons is not known.

The role of astakine-1 as a transcription factor in these cells also is not understood. In vertebrates, cytokines are important mediators of short and long-term stress responses. Stimulated by central catecholaminergic pathways, in mammals cytokines have activating effects on the hypothalamic-pituitary-adrenal (HPA) axis (Tsigos & Chrousos 2002). Bv8, another prokineticin homologue, has been shown to stimulate the release of corticosterone and increase blood glucose (Negri et al., 2004). It is possible, given the involvement of the MGNs in the tail flip escape reaction—a behavioral response to avoid injury or predation—that long-term elevations in astakine concentrations in the hemolymph brought about by extended periods of stress or injury could lead to stress-induced changes in the tail-flip reaction. Exogenous stress has been shown to alter tail-flip response behavior (Kellie et al., 2001). How astakine in the MGN nucleus may relate to these functions has not been examined.
Cluster 6

Similar to MGN labeling, astakine-immunoreactive cells in cluster 6 label only in the nucleus (Fig. 7 b, 8 a-b), once again suggesting that astakine is operating as a transcription factor. At present, it is not clear what effects astakine-1 might have. Cells in cluster 6 are involved in a wide variety of regulatory functions. Among these roles, Cluster 6 contains photoreceptor cells that serve as core-pacemaker components in the central circadian system (Sullivan et al., 2009). Octopamine- (the phenol analog of norepinephrine) and dopamine-containing neurons are also found in cluster 6 (Tinikul et al., 2009). In crustaceans, octopamine (OA) and dopamine (DA) are involved in a wide range of behaviors, including motor activity, aggression, escape and tail-flipping behavior, feeding behavior, and sexual behavior and reproduction (Livingstone et al., 1980; Sarojini et al., 1995; Fingerman, 1997; Beltz, 1999; Antonsen & Paul, 2001).

A large population (one of the largest in the crustacean brain) of gonadotropin-releasing hormone (GnRH)-immunoreactive neurons and associated fibers (Tinikul et al., 2011) that express estrogen and androgen receptors (ER and AR, respectively) (Ye, 2008) are found in cluster 6. These findings suggest that cluster 6 neurons play a key role in the neuroregulation of reproduction, possibly by maintaining a circadian rhythm for reproduction, which is under photoperiodic control (Castanon-Cervantes et al., 1995).

One of the key ways in which cytokines mediate long-term stress response is through bidirectional interactions with the gonadal axis, to suppress reproductive function (Tsigos & Chrousos, 2002). Astakine could potentially play a role in this suppression, and it would therefore be interesting to ask whether astakine-1 co-localizes with GnRH or ER/AR expression.

In mammals, cytokines—particularly prokineticin (PK) cytokines (Negri et al., 2007)—mediate circadian control by the suprachiasmatic nucleus (SCN). Studies have demonstrated circadian regulation of locomotor activity (Kravez & Weitz 2006), sleep (Opp et al., 1991), body temperature (Huitron-Resendiz et al., 2007) and age-related circadian dysfunction (Godbout & Johnson 2006)—particularly circadian dysregulation associated with Alzheimer’s disease (Hatfield et al., 2004; Coogan & Weitz 2008). PK knockout mutants have essentially normal oscillations of clockwork genes in the SCN, but circadian control of a
number of physiological processes and behaviors, including thermoregulation, locomotion, and sleep/wake regulation is compromised (Li et al., 2006; Hu et al., 2007). This suggests that PK does not affect the core cellular timekeeping mechanism, but nevertheless regulates circadian control over behavioral and physiological processes (Negri et al., 2007). Astakine may play a similar role in mediating crustacean circadian rhythms. Astakine-labeled cells in cluster 6 are quite large and do not have the clustered distribution typical of photoreceptor cells (Fig. 8 a-b); a co-localization experiment examining the distributions of astakine and pigment-dispersing hormone (PDH), which labels the photoreceptor cells (Sullivan et al., 2009) could make this relationship clearer. Given the size of the labeled cells, astakine could be labeling the interneurons in this cluster, which tend to be larger than primary sensory neurons (Glantz et al., 1981).

Astakine may be acting as a transcription factor in these cells, mediating how circadian information from photoreceptor cells is conveyed to other cells. Serotonin is a key regulator of a number of important functions in crustaceans (Beltz & Kravitz, 2003). On the anterior margins of cluster 6, there are two very large bilateral pairs of cells whose nucleoli stain brightly for both 5-HT and astakine-1 (Benton, unpublished result). Astakine and 5-HT label the nucleolus in discrete, non-overlapping packets. The purpose of this co-labeling within these cells is unknown, but perhaps the two factors could be interacting together with in these cells in order to regulate circadian control of various physiological processes and behaviors.

Additionally, astakine’s action as a transcription factor in cluster 6 neurons may be a source of regulation for diurnal oscillations in astakine levels. Although diurnal oscillation in prokineticin expression is largely driven by endogenous circadian clocks in mammals, inputs from retinal ganglion neurons can directly regulate prokineticin mRNA expression in the SCN (Cheng et al., 2002; Cheng et al., 2005; Zhou & Cheng, 2005; Zhou, 2006; Negri et al., 2007). It is possible that through some sort of feedback loop, astakine is influencing its own expression levels in a similar manner. Serotonin has been shown to regulate astakine-1 expression (Irene Söderhäll, unpublished result). Thus, interactions between these two factors could regulate circadian astakine-1 expression in a rather circular manner.

There were more astakine-labeled cells on the ventral than dorsal surface for the morning (8:30) group (although the 1830 group showed a similar trend) (Fig. 8 c). In the crab
Cancer borealis, cells on the ventral surface of cluster 6 are much more likely to project axons posteriorly into the connectives than cells on the dorsal surface (Kirby & Nusbaum, 2007). It is possible that labeled cells on the ventral surface have different functions from those on the dorsal surface. The differences between the two time points was not significant (p<0.06), but rather suggested a trend (Fig. 8 d); an increased sample size might clarify this relationship.

Cluster 16

Similar to the pattern of labeling in cluster 6 neurons and the MGNs, the nuclei (rather than the cytoplasm) of cells in cluster 16 labels with astakine (Fig. 9a), suggesting that astakine-1 may be a transcription factor in these cells. Cluster 16 cells may be receiving inputs from astakine-labeled fibers present in the antennal II neuropil (Fig. 6e, 9a).

Large cells in cluster 16 are immunoreactive for cystathionine β-synthase, an enzyme that produces hydrogen sulfide (Kotsyuba, 2011). In mammalian systems, hydrogen sulfide is an important, though relatively newly-recognized neuromodulator, that regulates the formation of potentiated circuits in the hippocampus (i.e., crucial for learning and memory) (Kimura, 2002), the release of corticotropin-releasing hormone from the hypothalamus (Russo et al., 2000), the microglial and monocyte release of pro-inflammatory cytokines (TNF-α, and IL-1β) (Qu et al., 2008, Zhi et al., 2007), and the manufacture and release of nitric oxide (NO) (Gadalla & Snyder 2010, Whiteman & Moore 2009). It would be interesting to see if cystathionine β-synthase labels neurons in the crustacean brain, and if so, how these cells might be related to those that are astakine-immunoreactive.

Cluster 17

The cytoplasm in the somata, but not nuclei, of cluster 17 cells is astakine-immunoreactive (Fig. 7 c, 10 a), perhaps indicating that cluster 17 cells synthesize astakine. The statistical trend suggesting more astakine in cluster 17 cells in the morning than evening (Fig. 13) may reflect a systemic increase in astakine production around dawn, as this is the time when hemocyte expression of astakine-1 is at its peak. Cells in cluster 17 have been shown to be involved in a variety of neural processes including rhythmic motor patterns
Invertebrate homologues of IL-1α, IL-1β, IL-2, IL6, and TNF-α have been found (Ottaviani et al., 1993). Vertebrate-derived IL-1α, IL-2 and TNF-α have been shown to induce nitric oxide synthase, stimulate hemocyte motility, and increase phagocytic activity in the mollusk, suggesting that cytokines are highly conserved (Ottaviani et al., 1995). It is possible that the same cells in cluster 17 that are immunoreactive for nitric oxide synthetase in response to injury (Kotsyuba et al., 2010), also label for astakine-1, and could be part of a larger neural response to injury. If this is the case, then astakine production in cluster 17 cells could be a means for astakine to mediate the stress response axis. Although it has been shown that cluster 6 cells have dendritic branches in the vicinity of 17 (Glantz et al., 1981), it is not currently clear if labeled cells in cluster 6 and in 17 have any physical connection.

Clusters 11 and 14

Astakine-immunoreactive cells in cluster 11 label in the nucleus (Fig. 11, 12 a), except for one large cell at the proximal edge which labels cytoplasmically (Fig. 11 e-f); this cell is discussed in more detail below. Similar to cluster 6 neurons, cells in cluster 11 are immunoreactive for ER, AR, and GnRH, suggesting that these cells are involved in regulation of reproduction (Tinikul et al., 2011, Tinikul et al., 2009, Ye et al., 2008). Cluster 11 also contains the cell bodies of the dorsal giant neuron (DGN) and a few smaller serotonergic interneurons, all of which innervate the olfactory and accessory lobes (Wildt et al., 2004). Overall, however, cluster 11 had relatively few astakine-labeled cells compared to the other clusters. Given the connection found between astakine expression and 5-HT, it would be interesting to see if 5-HT and astakine immunoreactivity colocalize. The serotonergic DGN is known to directly regulate adult neurogenesis in the crayfish brain (Sandeman et al., 2009), and so astakine labeling in this neuron could provide a critical link between serotonin and astakine regulation of neurogenesis. In serotonin and astakine co-localization experiments conducted in younger animals, the nucleolus of the DGN was found to label with both astakine and serotonin, in discrete packets (Benton 2012, unpublished result). Immediately
ventral to the DGN was one or more smaller cells that labeled cytoplasmically for astakine-1 (Benton 2012, unpublished result).

Nuclei of several cells in cluster 14 are immunoreactive for astakine-1 (Fig. 12 b), and the cells that label for astakine in this cluster also may be innervated by astakine-labeled fibers (Fig. 11, 12 b). There is relatively little information available in the literature about cluster 14, which contains the somata of a heterogeneous population of olfactory interneurons (Sandeman et al., 1990, Derby & Blaustein 1988, Arbas et al., 1987).

Astakine-Labeled Fibers

Ventrally, astakine-immunoreactive fibers were found running along the antenna II neuropil near clusters 16 and 17 (Fig. 6 e). Astakine fibers on the dorsal surface were seen primarily in the morning time points (Fig. 7 d, 11 a,c,e-f), and labeled in a pattern quite similar to fibers immunoreactive to pigment-dispersing hormone (PDH; an arthropod circadian signaling molecule) (Sullivan et al., 2009). The large fibers on either side of cluster 6 (Fig. 11, 15) were particularly reminiscent of fiber labeling for PDH. Astakine-labeled fibers were present along the connectives running to the most posterior part of the ventral nerve cord, and crossed to the contralateral side near the deutocerebral bridge (Fig. 7 d, 11). Cells in cluster 17, a single cell in clusters 16 and at least one cell in cluster 11 appear to be the only cells that label cytoplasmically for astakine, and thus are likely to be producing this molecule. Therefore, if the astakine-labeled fibers are originating from neurons in the brain, they are likely from one or more of these cells. It is also possible that these fibers originate from cells in other ganglia, which have not been examined for astakine immunoreactivity localization; astakine expression in the ventral nerve cord has, however, been documented (Lin et al., 2010).

**B: Astakine in the neurogenic niche and cell ‘F’**

The Niche

Astakine-1 immunoreactivity in hemocytes (Fig. 13-14), and in the cell clusters (Fig. 6-10, 12) was quite vibrant, while the niche cells appear to be devoid of specific labeling (Fig.
Semi-granular hemocytes are weakly astakine-labeled (Fig. 13-14), containing small numbers of astakine-labeled vesicles (Fig. 13). Cells in the niche do not label with astakine unlike cells in the blood.

Adult human bone-marrow mesodermal stromal cells (hMSCs) undergo a series of protein expression changes as they differentiate into neuroectodermal cell types in vitro (Hermann et al., 2006). Undifferentiated hMCSs express, in low levels, the major markers commonly used to characterize early and mature neuroectodermal cells (Corti et al., 2003, Tondreau et al., 2004). In vitro protocols to convert undifferentiated hMSCs into a neural phenotype require a multi-step process in which hMCSs must first loose expression of mesodermal markers, and express neuroectodermal marker levels (Hermann et al., 2006, Hermann et al., 2004), suggesting that the in vivo process might operate similarly. Given the highly conserved nature of hematopoietic mechanisms (Evans et al., 2003), it is likely that crayfish hemocytes would have to undergo a similar transformation if they become niche cells as we have hypothesized. This transformation could include sharply decreased astakine-1 expression, and so the lack of niche cell astakine labeling does not by itself rule out blood cells as niche cell precursors.

The cells immediately surrounding the vascular cavity have many small, finger-like processes projecting into the cavity (Sandeman et al., 2011, Zhang et al., 2009), and a network of septate (equivalent to tight junctions in vertebrates) and adherens junctions, reminiscent of the absorptive surface of the intestines and the blood-brain barrier of the lateral ventricles in mammalian systems (Chaves da Silva et al., 2012 in press). These cells also contain densely packed Golgi apparatus and ribosomes, and when injected with intracellular dyes, leak these dyes into the cavity. It is likely, therefore, that the niche cells lining the vascular cavity have some sort of absorptive (microvillar border and junctional complexes) and secretive (organelles and dye leakage) functions. Although immunocytochemical labeling suggests that the niche cells and the vascular cavity do not contain astakine-1, in situ hybridization analysis of niches at different times of day would confirm whether or not astakine-1 is expressed by niche cells. However, the results of this study suggest that astakine-1 may be taken up by niche cells, but it is unlikely that it is being secreted from cells in the niche.

The brain and niche are surrounded by a protective sheath, which is generally removed during the dissections used for immunocytochemical studies of the niche. Recently, J. Benton
has designed an approach that allows more careful removal of this sheath, and in these preparations astakine- and serotonin-labeled fibers appear to contact the niche on its dorsal surface (Benton, unpublished result). Additional studies are therefore necessary to understand the relationship of astakine to niche cells.

The vascular cavity, and by extension, the niche, is a dynamic, multi-layered opening that appears to change shape over the course of the day (Fig. 17 a-b). This could reflect time-dependent changes in the vascular cavity itself or in the position of the niche. The niche can change position over the course of an animal’s lifetime (Zhang et al., 2009), though it is not known if the niche changes position over the course of the day. The vascular cavity may serve as the entryway for substances and cells (e.g., hemocytes) into the niche; however, it is possible that hemocytes could enter the niche from the surrounding hemolymph. In an in vitro experiment, where hemocytes labeled with Cell-Tracker Green (Molecular Probes) were placed in a dish with dissected, desheathed brains, some of the hemocytes that had integrated themselves into the niche appeared to have done so from the sides, rather than coming through the vascular cavity (Benton et al., 2011). It is not clear whether or not the cell seen contacting a glial fiber in Fig. 17 d is integrating into the niche, however this might be another line of evidence to suggest that hemocytes may have multiple entry points into the niche.

Injection of astakine-1 was found to greatly increase the numbers of PI-labeled niche cells after 48 hours, with increases in the numbers of BrdU-labeled cells after 72 hours (Benton, unpublished result). Cytokines are important regulators of neural progenitor proliferation, with varying effects: Elevated levels of cytokines IL-6 (Vallieres et al., 2002; Monje et al., 2003), IL-1β (Song & Wang 2011), IL-2 (Beck et al., 2005), transforming growth factor (TGF)-β1 (Buckwalter et al., 2006), interferon (IFN)-α (by increasing IL-1 production) (Kaneko et al., 2006) have negative effects on neurogenesis. In contrast, epidermal growth factor (Craig et al., 1996), and IFN-γ (Baron et al., 2008) increased neurogenesis. TNF-1α has been found to be both a positive and a negative regulator of progenitor proliferation, depending on which TNF receptor is activated (Wu et al., 2000; Iosif et al., 2006).

It is also possible that astakine could be affecting niche cell recruitment and proliferation indirectly: IL-2 likely modulates proliferation via androgen synthesis (Beck et al., 2005). Androgens and estrogens have been implicated in the regulation of adult
neurogenesis (Gould et al., 2000; Perez-Martin et al., 2004), and IL-2 is known to inhibit steroidogenesis in Leydig cells (Guo et al., 1990). IL-1β stimulates the HPA axis to release stress hormones, and glucocorticoids, both of which are thought to have adverse effects on neural progenitor proliferation (Song & Wang, 2011).

In mammals, prokineticins have been reported to affect later generations of neural precursors. Prokineticin PK-2 is secreted in the olfactory bulb, and functions as a chemottractant for migrating neural progenitors from the subventricular zone (Negri et al., 2007), as well as promoting neuronal differentiation (Zhang et al., 2007). Astakine immunoreactivity does not appear to be present in clusters 9 and 10 in the crayfish brain, where new adult-born neurons are integrated.

**Cell F and its Associated Fiber**

A large neuron containing astakine-immunoreactivity in the cytoplasm, ‘cell F’, was consistently found slightly dorsal to the niche and at the anterior edge of cluster 16 (Fig. 18-20). A medium-sized fiber from this cell runs underneath the niches from the evening (18:30) time-point (Fig. 18), projecting towards and through cluster 9 and terminating dorsally in cluster 11, where it appears to contact one or more cells that label cytoplasmically for astakine-1 (this study and Benton, unpublished results).

There are a number of important serotonergic cells located at the edges of cluster 11, none more so than the dorsal giant neuron (DGN), which has been found to regulate divisions of neural progenitor cells in clusters 9 and 10 (Sandeman et al., 2009, Benton et al., 2008). A small fiber from the DGN has also been proposed to be the source of serotonergic labeling seen around the rim of the vascular cavity (Chavez de Silva 2012 in press), which may be involved in signaling hemocytes to come into the niche (Benton et al., 2011). Therefore looking for co-localization between 5-HT and astakine in cluster 11 was of immediate interest.

Jeannie Benton (2012, unpublished result) has found that the fiber contacts one or more cytoplasmically astakine-immunoreactive cells, which are immediately ventral to the DGN (Benton 2012 unpublished result). These cells appear to have processes that extend ventrally into cluster 9, as well as processes that follow the DGN fiber pathway—indicating
that astakine-1 fibers could possibly be innervating the olfactory globular tract neuropils (OGTNs). Olfactory interneurons synapse onto the DGN in this region (Benton & Beltz 2001). Astakine-1 labeling does not co-localize with 5-HT labeling in fibers (Benton 2012, unpublished result). DGN labels with astakine quite strongly in the nucleus, but does not appear to be manufacturing astakine. The DGN fibers do not appear to label with astakine. As serotonin is known to regulate astakine expression (Irene Söderhäll 2012, unpublished results), it is possible that astakine-1 is acting as a transcription factor in the DGN as a form of auto-regulation (regulating the regulator). Determining whether cell F interacts with the DGN is of immediate importance, given that the DGN is already known to regulate neurogenesis (Sandeman et al., 2009, Benton et al., 2008).

While it is not presently clear whether or not the fiber extending from cell F directly or indirectly interacts with the niche. Cell F is always found in the general vicinity of the niche, and while it may vary in position relative to the niche (Fig. 20), its fiber, if labeled, is always seen extending underneath the niche or streams. The proximity of these elements may simply be a coincidence, or could indicate a relationship. Attempting to inject cell F with dye and trace its connections will be a critically important experiment. Further characterization of cell F is needed, for example to determine if cell F is the single dopamine-producing neuron of cluster 16 (Tierney et al., 2003, Wood & Derby 1996) and to have a clear understanding of its anatomy and target cells.

II. Hematopoietic Ablations

Previous work by Benton et al. (2011) demonstrated that hemocytes had a strong attraction to the niche—more so than cells extracted other tissues. The present study found that hematopoietic tissue (HPT) ablation, which would lead to decreases in the numbers of circulating hemocytes, resulted in a decrease in the numbers of niche cells (Fig. 21A), without significant adverse effects on the animals’ health (Fig. 23), or differences in numbers of BrdU-labeled cells in the niche, streams or clusters 9 and 10 (Fig. 21B, 22, 24B).

Furthermore, when ablated animals were injected with astakine-1, a cytokine that mediates the release of semi-granular cells (Soderhall et al., 2005), this decrease is “rescued” (Fig. 24A). We have previously proposed that the semi-granular cells may be the hemocytes that
are preferentially attracted to the niche, and that may be transformed into niche cells (Benton et al., 2011).

Animals in the sham group from the astakine-rescue experiment had higher total niche cell counts than animals in the control group, and in the ablation group that did not receive astakine (Fig. 24A). Animals in the ablation group that did receive astakine had niche PI cell counts that were between control and sham levels, not differing significantly from either (Fig. 24A). As physical or septic injury dramatically increases astakine-1 expression, triggering the release of semi-granular cells from the HPT (Söderhäll et al., 2005), it is likely that the sham surgery led to an increase in astakine-1 expression and this would have let to an increase in circulating semi-granular cell numbers; such an increase could be responsible for the increased numbers of niche cells seen in the sham controls in this experiment.

In contrast, in prior experiments utilizing a 10-day survival time, animals that underwent sham surgeries tended to have a similar or somewhat lower total niche cell count relative to controls (Fig. 21A; Appendix A). This may suggest that the total numbers of cells in the niche are regulated by some homeostatic mechanism and that observing changes in niche cell counts will be time-sensitive. Additional experiments are required to test this idea.

In the 10-day survival time study (Figs. 21-23), increases were seen in the sham animals in BrdU-labeled cells in cluster 10 and in the streams relative to the controls (Fig. 22A, C). This effect also was not seen consistently in prior studies (Appendix A), although the sample size was small in those studies. If the BrdU-labeled cell increases seen in shams in recent experiments is replicated, this also could be due to factors released due to surgical damage, the upregulate the cell cycle in the niche, streams and cell clusters.

Astakine-1 is expressed in both the HPT and in circulating hemocytes; the partial removal of the HPT decreases plasma levels of astakine-1 (Lin et al., 2010). Over the course of the week following surgeries, it is presumed that the total hemocyte counts in the hemolymph gradually decreases, as the damaged HPT is not able to fully replenish their numbers; obtaining counts to verify this decrease in hemocytes has, however, been problematic for reasons described earlier. Our hypothesis is that fewer cells are available to manufacture astakine, astakine-1 plasma levels further diminish, further limiting the release of hemocytes from the HPT in a cyclical fashion.
When ablated animals are injected with astakine-1, the remaining HPT is given a strong signal to release hemocytes, increasing the circulating hemocyte numbers. However, because the HPT is damaged, fewer cells are likely to be released than from intact tissue. Thus while there was an increase in the numbers of niche cells of ablated animals receiving astakine, this increase was less substantial than that seen among animals in the sham group.

Astakine-1 injection increases the numbers of BrdU-labeled cells in the niche by almost two-fold after forty-eight hours, (Benton 2011, unpublished data). This effect was not seen in the ablated animals that received astakine treatment, suggesting that this increase in niche BrdU-labeled cells in intact animals is not mediated by astakine-1 alone.

The results of some ablation experiments, in which the numbers of BrdU cells in cluster 10 decreased in ablated animals relative to sham and control, suggested that the precursor cells coming into the niche immediately divided and migrated out (the ‘pack n ship’ model). However this result has not been consistently seen. It remains unclear if niche cells have differential capacities to become neural precursors.

The incredibly close, positive relationship between the numbers of circulating cells in the hemolymph, and the numbers of cells in the niche (Fig. 25), indicates that the niche and the hematopoietic system have a far more dynamic and tightly regulated relationship than had previously been appreciated.

Overall, these results indicate a relationship between the niche and the hematopoietic system, as removing hematopoietic tissues also appears to eliminate the source of niche precursor cells in this neurogenic system, and the number of hemocytes present in the blood predicts, with high accuracy, the numbers of cells present in the niche.
Conclusions

There is a growing appreciation for the dynamic and critical role the blood system plays in neurogenesis. This thesis has examined the labeling patterns and discussed potential regulatory mechanisms of a crustacean prokineticin cytokine, astakine-1, in the crayfish brain. Data presented in this thesis suggest possible roles this factor might play in neurogenesis, as well as providing compelling evidence for a relationship between circulating hemocytes and niche precursor cells. In many respects, however, this is a preliminary study that has raised many more questions than answers. Additional experiments are required to substantiate the conclusions of these studies.
REFERENCES


Appendix 1: Hematopoietic ablation experiments leading to the final thesis trials

The following summary provides historical background and additional data from a series of prior experiments.

Hematopoietic tissue ablations reduce the numbers of niche cells. In previous experiments, sample sizes were relatively low due to high mortality across all groups, reducing the statistical power in the data sets. However, in every experiment in which more than three animals survived in each group (n=4), animals in which the hematopoietic tissues were ablated consistently had significantly fewer niche cells relative to sham and control crayfish (see A in accompanying figure). Because these studies spanned all seasons (August, 2010; February, September and November, 2011; and April, 2012 [see Figs. 21-22, 24]), and used freshly caught wild animals from multiple vendors, this finding does not depend appear to depend on seasonality, issues related to animal shipment [source, temperature], molt-cycle stage, or reproductive cycle stage.—all of which could potentially affect the numbers of cells in the niche. Although only the final experiment (Fig. 21-22) had, by itself, adequate statistical strength, the consistency of prior experiments regarding the influence of hematopoietic ablations on niche cell numbers does strengthen this point. As a group, these studies provide compelling evidence that hematopoietic ablation results in a profound decrease in the numbers of cells in the niche.

Shams tend to have greater numbers of BrdU-labeled cells in the niche and in cluster 10 relative to ablated animals. The relative numbers of BrdU cells between groups varied considerably from experiment to experiment, likely because I was unable to ensure that all of the animals were in the same stages of the molting or reproductive cycles. If, in an attempt to remove some of this variability, I examine only differences in BrdU labeling between ablated animals and sham controls, a pattern emerges: the shams tend to have greater numbers of BrdU cells in the niche and in cluster 10 relative to ablated animals. This difference is not seen in cluster 9 or in the streams (it can be very difficult to establish where the stream ends and cluster 9 begins in animals of this size, thus counts of cluster 9 and of the streams are not as accurate as for the niche and cluster 10). It seems possible that, due to ablation of the tissue decreasing the numbers of cells in the niche, fewer cells are dividing and migrating away. As this deficit accumulates over the time of the experiment, this could lead to differences in numbers of cells in cluster 10.
The increase in BrdU labeling in shams relative to controls in all parts of the neurogenic pathway (niches, streams and cell clusters) that was seen in recent experiments (Figs. X and X) was not observed consistently in early studies. This could be because the sample sizes were small and variability relatively high in these experiments. It may also be that my technical skills improved; for example, the surgical time decreased significantly from early to later experiments, as I became more proficient. Additional experiments will be required to confirm the influence of sham manipulations on niche cell numbers, however, the increase seen is consistent with the release of hematopoietic cells after damage (sham surgery) (ref.) ---and thus, possible incorporation of some released cells into the niche.
BrdU counts for the control group have been lightened relative to sham and full ablation in order to make the relationship between the two groups clearer. The experimental sequence is identical for all three groups.