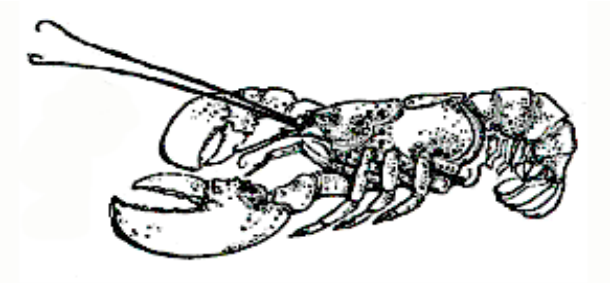
Abteilung Neurobiologie der Universität Ulm

The impact of day, night and other external synchronizers on brain serotonin levels in the American lobster *Homarus americanus*.

Dissertation zur Erlangung des Doktorgrades Dr. rer. Nat. der Fakultät für Naturwissenschaften der Universität Ulm



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The universe is full of magical things patiently waiting for our wits to grow sharper.

(Eden Phillpotts 1862-1960)

Meinen Eltern

The american lobster

***Homarus americanus* Milne Edwards, 1837**

(Malacostraca, Decapoda, Reptantia, Homarida)

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1. Introduction

1.1 The brain of decapod crustaceans

The decapod crustacean brain is subdivided into three segmental units: the lateral and median protocerebrum with the optic ganglia, the deutocerebrum and tritocerebrum. The two optic ganglia, lamina ganglionaris and medulla externa, are located distal to the lateral protocerebrum (Figure 1). The lateral protocerebrum, which is thought to be involved in higher-order integration of multimodal sensory inputs (Hanström, 1924, 1925; Blaustein et al., 1988; Sandeman et al., 1993; Sullivan & Beltz, 2001) is composed of two main regions: the lobula (formerly called medulla interna) and the medulla terminalis with the hemiellipsoid body, the eight glomeruli centrals, and the diamedullary neuropil. The median protocerebrum holds the cell soma clusters 6, 7 and 8 (terminology from Sandeman et al., 1992), the anterior and posterior medial protocerebral neuropils and the central complex, and is connected to the lateral protocerebrum via the protocerebral tract. The central complex, which is believed to be involved in visual integration and the control of motor activities (Utting et al., 2000) is composed of the lateral lobes, the central body and the protocerebral bridge (Sandeman et al., 1992; Harzsch et al., 2004).

The deutocerebrum is largely composed of two prominent regions, the accessory lobes (ALs; only present in some Decapoda; compare Sandeman et al., 1992, 1993) and the olfactory lobes (OLs) (Figure 2). The olfactory processing region is innervated by first-order sensory neurons projecting from the chemosensory receptor cells located on the first pair of antennae (antennulae) to the olfactory lobe (OL) (Figure 3). The accessory lobe, when present lies caudal to the olfactory lobe and does not receive terminals of primary afferent axons but input from higher order neurons via the deutocerebral commissure (DC) (Figure 3). In addition the AL is innervated by local interneurons in soma cluster 9 which project also to the olfactory lobe. Soma cluster 11 is also arranged close to the accessory lobe. It contains interneurons that have their axons in the deutocerebral commissure and which terminate in the olfactory lobe and the accessory lobe. Soma cluster 11 also holds the cell body of the dorsal giant neuron (DGN). The dorsal giant neuron receives input from the olfactory globular tract neuropil (OGTN), deutocerebral commissure interneurons and via the olfactory lobe. Lateral

to the olfactory lobe and the accessory lobe lie soma cluster 10 which contains the cell bodies of the projection neurons and which is the site of life-long neurogenesis (see chapter 1.3). The projection neurons have dendrites in the olfactory lobe and the accessory lobe, and their axons project towards the lateral protocerebrum via the olfactory globular tract (OGT) (Sandeman et al., 1992; Harzsch et al., 2004).

The most caudal brain unit is the tritocerebrum. It is composed of the antenna II neuropil with cell soma clusters 14 and 15 and the tegumentary neuropil with the tegumentary nerve. The interneurons and motorneurons of the antenna II neuropil receive input from the second antennae. Mechanosensory information from the carapace reaches the tegumentary neuropil via the tegumentary nerve.

Because of the extensive studies on the architecture of the decapod crustacean brain (e.g. Bethe, 1897a,b, 1898; Helm, 1928; Holmgren, 1916; Hanström, 1924, 1925, 1928, 1931, 1933; Abbott, 1971; Sandeman & Luff, 1973; Strausfeld & Nässel, 1981, Titova, 1985; Tsileneva & Titova, 1985; Tsileneva et al., 1985; Blaustein, 1988; Sandeman et al., 1992; Sandeman & Scholtz, 1995; Sullivan & Beltz, 2001; McKinzie et al., 2003), its structural plasticity, and the fact that single neurons or small clusters of cells can easily be identified it serves as an elegant model and provides the fundament for all studies conducted for this thesis.

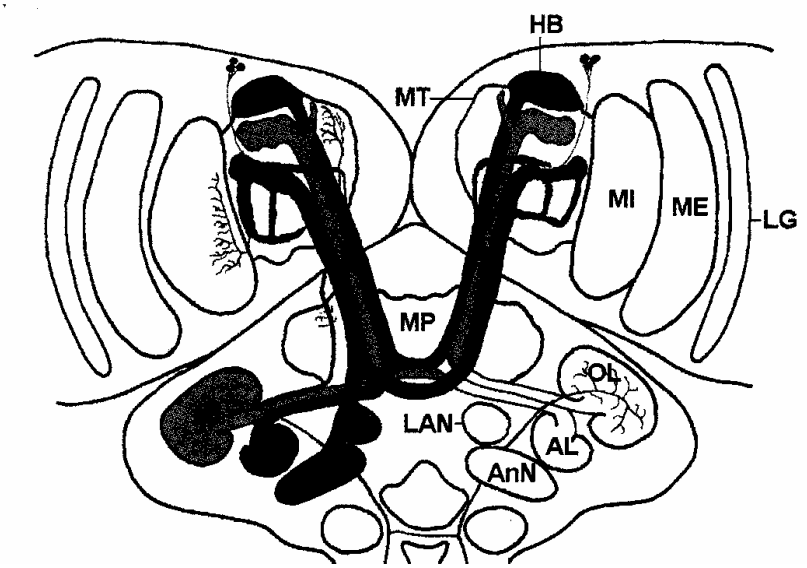


Figure 1. Schematic diagram of the decapod crustacean brain showing the optic ganglia (lamina ganglionaris (LG), medulla externa (ME)), the lateral protocerebrum (lobula/medulla interna (MI), medulla terminalis (MT), hemiellipsoid body (HB)), the median protocerebrum (MP), the

deutocerebrum (accessory lobe (AL), olfactory lobe (OL) and lateral antenna I neuropil (LAN)) and the tritocerebrum (antenna II neuropil (AnN). Highlighted region indicates the patterns of connectivity of the medulla terminalis and the deutocerebrum. (Figure modified from Sullivan & Beltz, 2001).

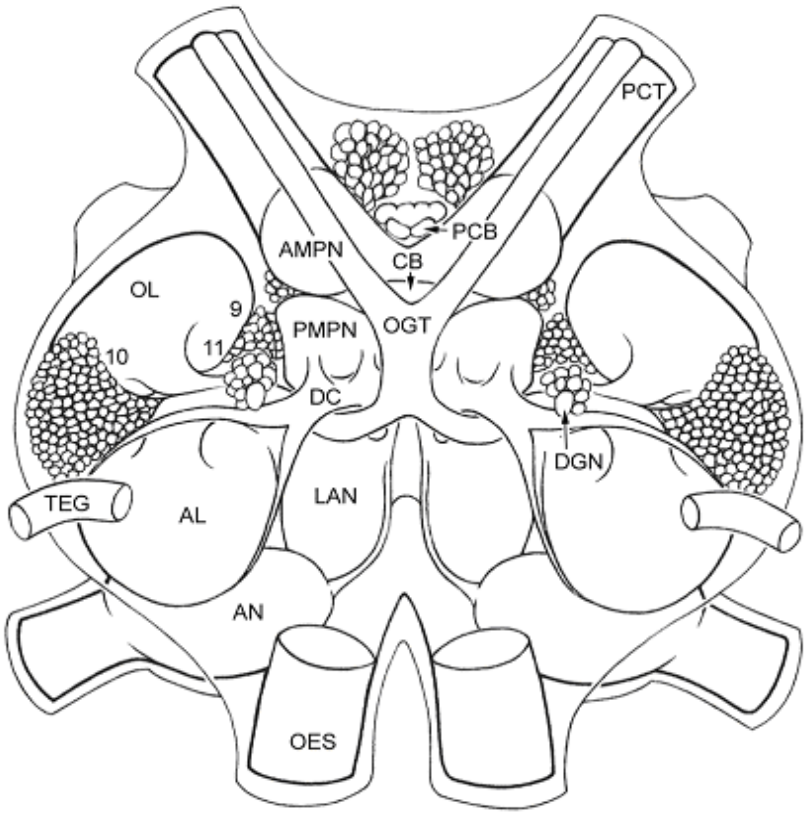


Figure 2. Diagram of the lobster brain reconstructed from serial sections which shows the location of the neuropils and cell somata relevant to this study. Accessory lobe (AL), anterior median protocerebral neuropil (AMPN), antenna II neuropil (AN), central body (CB), deutocerebral commissure (DC), dorsal giant neuron (DGN), lateral antennular neuropil (LAN), oesophagael connectives (OES), olfactory globular tract (OGT), olfactory lobe (OL), protocerebral bridge (PB), protocerebral tract (PCT), posterior median protocerebral neuropil (PMPN), tegumentary nerves (TEG), soma cluster 9 (9), soma cluster 10 (10), soma cluster 11 (11). (Figure from Wildt et al., 2004).

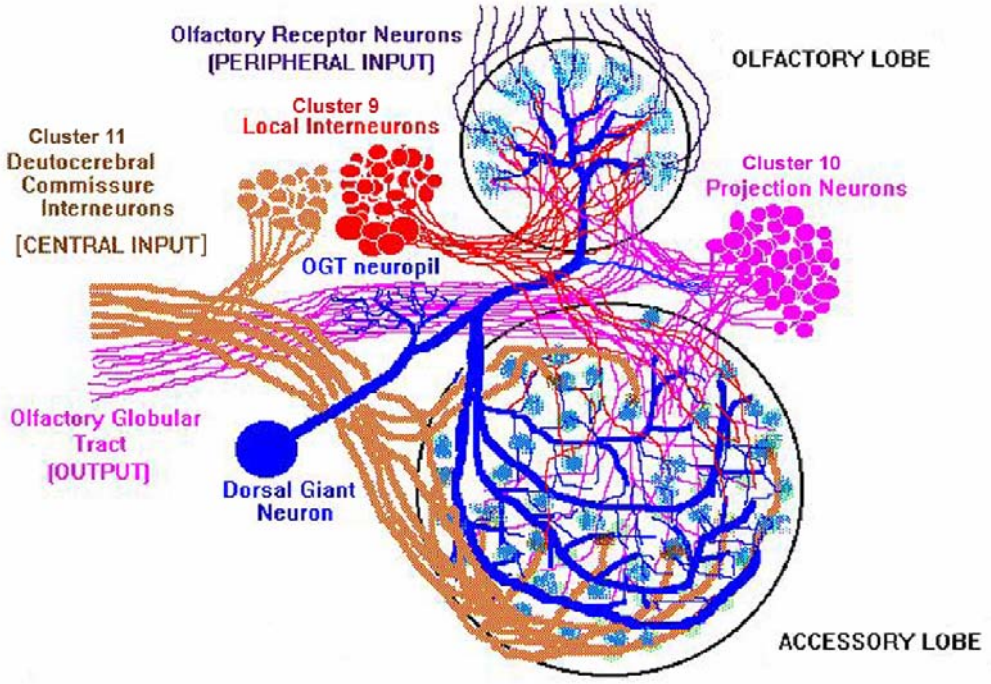


Figure 3. Schematic diagram of the circuits associated with the accessory and the olfactory lobe in the crayfish brain. Projection neurons: pink, local interneurons: red, deutocerebral commissure neurons (DCNs): orange, DGN : blue. OGT (olfactory globular tract). (Figure from Beltz & Sandeman, 2003).

1.2 Serotonin

Serotonin is a prominent aminergic neurotransmitter in the brain of the American lobster, *Homarus americanus*, and its biochemical pathway involves the conversion from L-tryptophan to 5-hydroxytryptophan via the rate limiting enzyme L-tryptophan hydroxylase. In lobsters, serotonin was first detected by Florey & Florey in 1954 (Florey & Florey, 1954; Florey 1954) and its distribution has since been extensively studied (Beltz & Kravitz, 1983; Beltz & Kravitz, 1987; Beltz et al., 1990; Beltz, 1999; Figure 4). About 70 immunoreactive neurons have been localized in the ventral nerve cord (Beltz & Kravitz, 1983) and an additional ~100 in the cell soma cluster 6, 9 and 11 of the brain (Beltz, 1999). Functionally, serotonin is known to regulate transmitter release (for review see Beltz & Kravitz, 2003), the contractility of muscle fibers (Marmot & Wiersma, 1938; Florey & Florey, 1954; Dudel, 1965; Glusman & Kravitz, 1982, Ma et al., 1992; Horner et al., 1997), the sensitivity of

sensory neurons (Pasztor & Bush, 1987; Pasztor & Macmillan, 1990), the frequency and intensity of the heartbeat (Battelle & Kravitz, 1978; Florey & Rathmayer, 1978, Berlind, 2001), posture (Livingstone et al., 1981; Harris-Warrick & Kravitz, 1984, Ma et al., 1992, Weiger & Ma, 1993) and agonistic and aggressive behavior (Huber & Kravitz, 1995; Kravitz, 2000).

In embryonic lobsters serotonergic neurons are among the first neurons during development that can be labeled immunocytochemically. In the brain, serotonin can already be seen by 10% of embryonic development and each one of the serotonergic neurons is fully developed by mid-embryonic life (Beltz et al., 1990). Because of their precocious appearance, serotonin is also thought to function as a "developmental architect", shaping growing neurons and regulating their pattern of connectivity (Lauder, 1991; Whitaker-Azmita et al., 1996). Pharmacological depletion of serotonin using the neurotoxic serotonin analogue 5,7-Hydroxytryptamine (5,7-DHT) demonstrated a reduction of the volume of the olfactory lobes (OLs) and accessory lobes (ALs) (Benton et al., 1997; Benton & Beltz, 2001) and an inhibition of the branching of olfactory projection neurons (Sullivan et al., 2000), but no difference in the organization and structure of the glomeruli within these regions (Benton & Beltz, 2001). Also, the size, morphology and pattern of innervation of the DGN cell body showed no significant changes. Subsequent studies using the proliferation marker bromodeoxyuredine (BrdU) showed that the number of newly born cells among the local interneurons (cluster 9/11 neurons) are lower when serotonin levels are reduced by 5,7-DHT (Benton & Beltz, 2001) suggesting that serotonin is involved in regulating cell proliferation and survival in this region. In the projection neuron cluster 10, serotonergic fibers have been demonstrated to terminate blindly at the site of life-long neurogenesis (Beltz et al., 2001, Figure 5), hence supporting that serotonin has a role in the process of shaping new neurons. Also, newly born neurons in cluster 10 could be demonstrated to transiently take up serotonin in embryonic and juvenile lobsters, thus extending the functional role of this amine to shaping and maintaining the olfactory system (Beltz et al., 2001).

In vertebrates and other invertebrates than crustacea, serotonin is well known to influences the proliferation of neuronal stem cells (Lauder et al., 1991; Brezun & Daszuta, 1999, 2000) and to modulate synaptogenesis (Lipton & Kater, 1989) as well as to modulate axonal growth (Haydon et al., 1984; Chubakov et al., 1986; McCobb et al., 1988; Goldberg, 1998). In mature vertebrates, it is further known to maintain synapses (Okado et al., 1993) and to cause glomerular atrophy (Moriizumi et al., 1994).

Given the broad array of functions that serotonin is associated with it is important to elucidate elements that synchronize the factors that regulate serotonin levels and in turn neurogenesis.

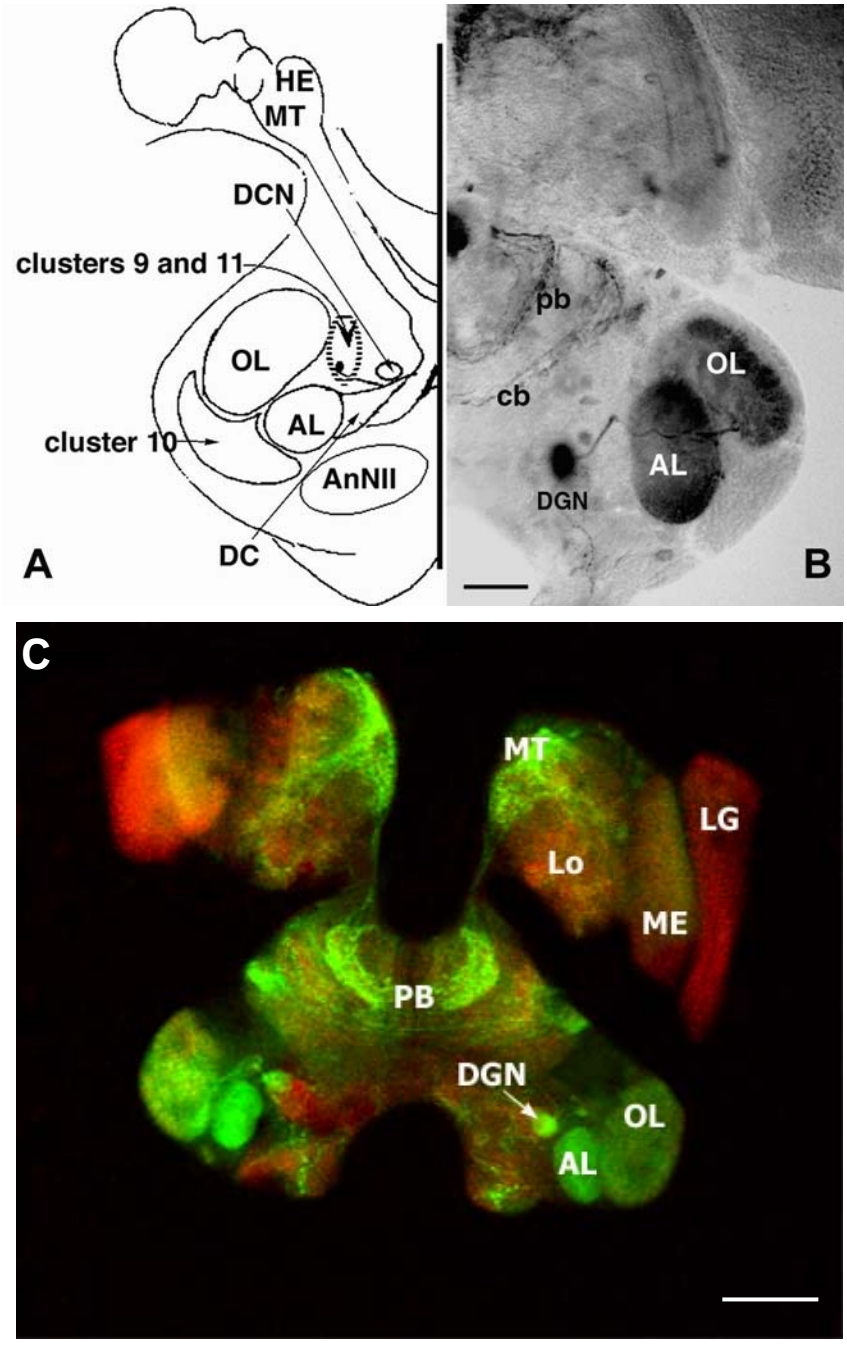


Figure 4. A) Schematic diagram of serotonin of the left hemisphere of a juvenile lobster brain, B) a photomicrograph of the right hemisphere labeled against serotonin, C) and a confocal image which include the eyestalks (green= anti-serotonin; red= anti-synapsin). The structures stained for serotonin are: accessory lobe (AL), central body (cb), dorsal giant neuron (DGN), lobula,

(Lo), medulla externa (ME), medulla terminalis (MT), olfactory lobe (OL), and protocerebral bridge (PB). AnNII (antenna II neuropil), deutocerebral commissure (DC), deutocerebral commissure neuropil (DCN), hemiellipsoid body (HE), medulla terminalis (MT), soma cluster 9, 10, 11. Scale bars: B= 20µm, C= 70µm. (Figure A, B taken from Benton & Beltz, 2001; C taken from Wildt et al., 2002).

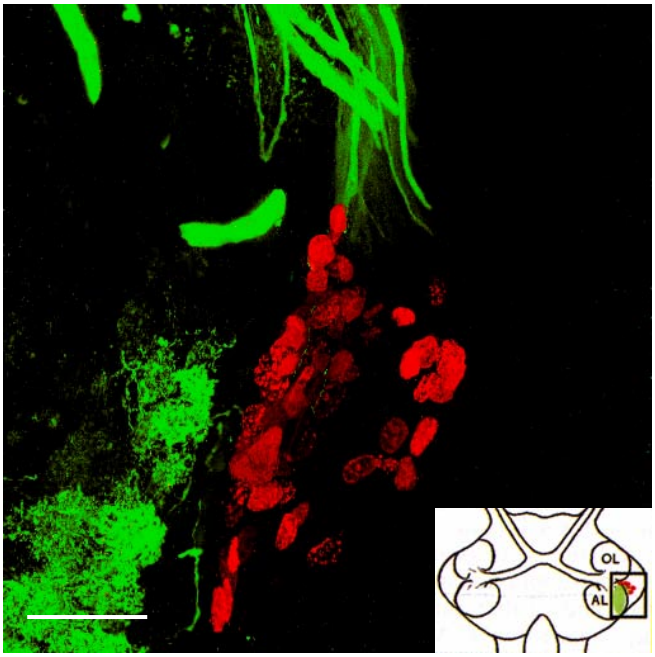


Figure 5. Confocal images of the brain of a juvenile lobster labeled immunocytochemically against BrdU (red) and serotonin (green). Serotonergic fibers terminate blindly into the region of life-long neurogenesis. Scale bar: 50µm. (Figure taken from Beltz et al., 2001).

1.3 Neurogenesis

As we now know, neurogenesis (the generation of new neurons) is a life-long process in many animals which takes place within certain regions of the vertebrate and invertebrate brain. Examples of life-long neurogenesis in the central nervous system have been revealed in crustaceans (Harzsch & Dawirs, 1996; Schmidt, 1997; Sandeman et al., 1998; Harzsch et al., 1999; Schmidt, 2001), insects (Booker & Truman, 1987; Booker et al., 1996; Cayre et al., 1996, 2002; Scotto-Lomassese et al., 2002), fish (Kranz & Richter, 1975; Byrd & Brunjes,

1998, 2001; Zupanc, 1999; Zikopoulos et al., 2000), rodents (Lois & Alvarez-Buylla, 1994), birds (Alvarez-Buylla & Kirn, 1997), and mammals (Altman & Das, 1965; Eriksson et al., 1998; Gage et al, 1998).

Lobsters grow throughout life. This constant increase in body size is accompanied by a linear increase in volume of cluster 9 and 10 (Helluy et al., 1995). However the size and packing density of these cell clusters does not change and subsequent experiments showed that new cells are added to these regions throughout the lives of the animals (Harzsch et al., 1999; Schmidt & Harzsch, 1999). Studies using the terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling method (TUNEL) in conjunction with the mitosis marker anti-phospho-Histone H3 further demonstrate that apoptosis (programmed cell death) occurs in parallel with proliferation among the interneurons, thus indicating a select incorporation of new neurons into the region of life-long neurogenesis (Harzsch et al., 1999; Mahoney, 2002).

Recent studies on the proliferation sequence engaged in neurogenesis show the involvement of three distinct cell types (large precursor cells, intermediate precursor cells and neurons; based on size and shape) with multiple divisions of the intermediate precursor cells (Benton & Beltz, 2002). These findings stay in contrast to the single division of the ganglion mother cells of insects and the crustacean ventral nerve cord (Doe et al., 1991, 1998; Campos-Ortega, 1995; Doe& Skeath, 1996; Matsuzaki, 2000; Scholtz & Dohle, 1996; Dohle & Scholtz, 1997; Harzsch, 2001). However, to this day it is still undetermined whether the divisions of the large precursor cells are also asymmetric (Benton & Beltz, 2002) as described for neuroblasts in both insects (Matsazukai, 2000; Ceron et al., 2001) and the crustacean ventral nerve cord (Scholtz, 1992; Harzsch & Dawirs, 1994; Harzsch et al., 1998). Minor differences in the counts of BrdU labeled large and intermediate precursor cells suggest that the large precursor cells may be dividing only once (Benton & Beltz, 2002; Beltz & Sandeman, 2003).

Exogenous factors have been identified to modulate the structural plasticity that life-long neurogenesis induces. In vertebrates and invertebrates, living conditions (Kempermann & Gage, 1999; Scotto-Lomassese et al., 2000; Sandeman & Sandeman, 2000), locomotor activity (van Praag et al., 1999a,b), the process of forming a hierarchic relationship (Pelz, 2001; Mazzarella, 2003), dietary restrictions (Lee et al., 2000; Lee et al., 2002) and the day and night cycle (Goergen et al., 2002) have been shown to influence the rate of neurogenesis.

1.4 Circadian rhythms

Circadian rhythms (that is, rhythms of near 24-hours) are important synchronizers for life. A synchrony between the organism and its external environment is critical to the organism's well-being and survival. Biological "clocks", thus allow the organism to anticipate and prepare for the changes in its internal environment (physiology) which are associated with the changes in its external environment. Although the circadian clock in many organisms has not been localized, many factors that influence the organisms' rhythmicity have been determined. The most well known and best studied zeitgeber (external synchronizer) for the possible signals for circadian rhythm synchronization is the daily light-dark cycle. Other cues such as the cycling changes in temperature (Aschoff & Tokura, 1986; Brown et al., 2002; Rensing & Ruoff, 2002), tidal-and lunar rhythm (Naylor, 1996; Palmer, 1997), social factors (Menaker & Eskin, 1966; Davis et al., 1987) and the availability of food have also been reported to function as time signals across species. Because of the existence of several zeitgebers it is not surprising that many entraining signals have the ability to function in concert. However, the multiocilatory structure of the circadian system differs in its entrainability by zeitgeber and often times "masking", the obscuring of the "true" state of a rhythm by multiple time cues, makes it difficult to obtain clear information on a single zeitgeber. Constant conditions such as constant darkness (D/D) help to eliminate conflicting zeitgebers and distinguish whether a signal is capable of self-sustaining oscillations (endogenous rhythm) and therefore runs free in the absence of the time signal, or whether it can only persist in the presence of the synchronizing biological unit (exogenous rhythm). Constant conditions are, therefore, a powerful tool to determine the mechanisms underlying the biological timekeeping system. Given the broad array of factors capable of synchronizing circadian rhythms it is important to explore the power and interdependency of coexisting zeitgeber.

In lobsters, the day and night cycle causes rhythmic changes in neurogenesis over 24-hours (Goergen et al., 2002). Under normal 12:12 light/dark (L/D) conditions, a clear diurnal rhythm with a peak surrounding dusk and a trough surrounding dawn becomes evident. This circadian rhythm can also be maintained under constant light conditions. Animals that were entrained to 12:12 L/D light cycle followed by three days in constant darkness (D/D) showed the same rhythmic fluctuations of neurogenesis as under L/D conditions, therefore, indicating that this rhythm is endogenous. Additional phase-shifts in the entraining light regime of the L/D cycle showed that the characteristic peaks and troughs shifted accordingly, thus

providing the first example of a light entrained circadian rhythm of neurogenesis in any species (Goergen et al., 2002).

Because light was hypothesized to provide a dominant coordinating signal for the many factors, including serotonin levels, that influence the persistent generation of new neurons (Goergen et al., 2002), it was speculated for the matter of the research demonstrated here, whether serotonin levels also cycle diurnally, be entrainable by light, and show a fixed correlation to the neurogenic rhythm.

1.5 Food and the circadian rhythm of neurogenesis and serotonin

The diversity of circadian rhythms within an organism is almost as broad as there are variations in cell types. Coordinating mechanisms are therefore critical to an organism's well-being and survival. The daily light dark cycle is the most well known external synchronizer, or zeitgeber, and the discovery of circadian clock genes (e.g. *period, timeless, Clock*) has contributed to the localization of a light-entrainable oscillator in mammals (Stephan & Zucker, 1972; Moore & Eichler, 1972). Interestingly, homologous of these clock genes have also been identified in insects (Konopka & Benzer, 1971; Sehgal et al., 1994; Allada et al., 1998) and have hitherto enabled the localization of a circadian pacemaker center, or master clock, in cockroaches and crickets (Sokolove, 1975; Page, 1985). Subsequent experiments in *Drosophila melanogaster* have further demonstrated a co-localization of the neuropeptide pigment-dispersing factor (PDF) in neurons expressing the gene product *period* and *timeless* suggesting that this molecule functions as a critical relay between the pacemaker neurons and the neural circuits controlling circadian activities (Helfrich-Förster et al., 2000; Helfrich-Förster, 2002; Stanewsky, 2002). Although our understanding on the underlying mechanisms synchronizing an organism's internal environment with the external environment has been greatly enriched by the many studies on mammals and insects other species, including Crustacea, are still lacking the localization of their circadian clock(s). In contrast, many factors are known to function as entraining signals across species (Aschoff & Tokura, 1986; Brown et al., 2002; Davis et al, 1987; Menaker & Eskin, 1966; Naylor, 1996; Palmer, 1997; Rensing & Ruoff, 2002). The entraining capabilities of food are of particular interest because,

circadian clock genes have also been localized in other areas of the central nervous system and in peripheral tissues (Sun et al, 1997; Sherman et al., 1997), and interestingly have recently been shown to be autonomously or semiautonomously entrainable by food (Damiola et al., 2000; Stokkan et al., 2001) suggesting the existence of a separate food-entrainable oscillator (Coleman et al., 1992).

In the American lobster, *Homarus americanus*, feeding time has been shown to alter the endogenous rhythm of life-long neurogenesis (Goergen et al., 2002). As discussed above, circadian studies on the endogenous rhythm of life-long neurogenesis have indicated that the rate of neurogenesis is highest surrounding dusk, the normal time of foraging and feeding, and lowest surrounding dawn, the normal period of rest. However, when food is given at dawn rather than at dusk, the rate of neurogenesis increases at dawn. Nevertheless, this food-induced dawn peak disappeared under constant darkness and in the extended absence of the food stimulus, and it was then concluded that feeding has a direct effect on the rate of neurogenesis, rather than to entrain an endogenous rhythm (Goergen et al., 2002).

Serotonin levels have also been demonstrated to regulate life-long neurogenesis (Brezun & Daszuta, 1999; Brezun & Daszuta, 2000; Benton & Beltz, 2001; Beltz et al., 2001) and to undergo circadian variations (Castanon-Cervantes et al., 1999). In addition feeding has been shown to change the levels of brain serotonin in mammals (Tachibana et al., 2001; De Fanti et al., 2001) either indirectly through altered levels in blood glucose and changes in plasma amino acid concentrations (Blundell & Hill, 1987; Blundell, 1992; Cangiano et al., 1992; Hall & van Ham, 1998) or directly through serotonergic neurons (Fernstrom & Wurtman, 1973; Wurtman & Fernstrom, 1973) and serotonergic receptors (5-HT-1A, 5-HT-1B) (Curzon, 1991; Bouwknecht et al., 2001). Serotonin, therefore, is an important mediator in the regulatory mechanisms, including neurogenesis, underlying circadian rhythm variations.

1.6 Melatonin

The indoleamide melatonin is a biosynthetic product of serotonin which can be found across species and which is generally associated with photosensitive tissue (for review see Vivien-Roels & Pévet, 1986; Arendt, 1995; Binkley, 1997; Gorbert & Steel, 2003; Hardeland

& Poeggeler, 2003). In vertebrates, melatonin is produced in the pineal gland and in the retina but in invertebrates the site of melatonin synthesis and release has not been detected. Among crustaceans, melatonin has been found in eyestalks of the crab *Carcinus maenas* (Vivien-Roels & Pévet, 1986), the prawn *Macrobrachium rosenbergii* (Withyachumnarnkul et al., 1992), the giant tiger shrimp *Penaeus monodon* (Withyachumnarnkul et al., 1995), the crayfish *Procambarus clarkii* (Agapito et al., 1995; Balzar et al., 1997), and the fiddler crab *Uca pugilator* (Tilden et al., 1997; Tilden et al., 2001a, b; Tilden et al., 2003). However, circadian rhythm of melatonin could so far only be detected in the eyestalks of *P. clarkii* (Agapito et al., 1995; Balzar et al., 1997) and *U. pugilator* (Tilden et al., 1997; Tilden et al., 2001b; Tilden et al., 2003). In *C. maenas*, solely seasonal differences between May and November can be observed (Vivien-Roels & Pévet, 1986). These variations reflect findings in other invertebrates such as dinoflagellata, molluscs and insects (for review see Hardeland & Poeggeler, 2003). Since we are still lacking the site of melatonin synthesis and release and because the availability of melatonin does not seem to follow a consistent and therefore conclusive pattern within invertebrates, a number of potential functions have been proposed. For those species that show circadian or seasonal fluctuations, melatonin may function as a signal for chronobiological regulation (Linn et al., 1995; Agapito et al., 1995; Balzar et al., 1997; Tilden et al., 2003; Blanc et al., 2003; Hardeland & Poeggeler, 2003). In addition, melatonin might have a protective role. In dinoflagellates, melatonin has been shown to protect the organism from damage of free radicals and other oxidants, such as hydrogen peroxide, paraquat (a herbicide), and buthionine sulfoximine (Antolín et al., 1997; Antolín & Hardeland, 1997; Hardeland et al., 1999; Hardeland & Coto-Montes, 2000). In another report on *D. melanogaster*, the application of melatonin substantially prolonged the animals' lives (Izmaylov & Obukhova, 1999). Two interesting studies using immunocytochemical labeling techniques and radioimmunoassay in the anthozoan *Renilla köllikeri* (Mechawar & Anctil, 1997) and the mollusc *Helix aspersa maxima* (Blanc et al., 2003) localized melatonin within neuronal tissue, thus suggesting that melatonin may function as a neurotransmitter or neurohormone. This is especially interesting in the light of several studies indicating a direct correlation between the fluctuating levels of serotonin due to the synthesis of melatonin (Sudgen, 1990; Olcese & Munker, 1994; Miguez et al., 1997).

Because melatonin is a biosynthetic product of serotonin which is thought to regulate serotonin synthesis and availability, and has been demonstrated to fluctuate on a circadian rhythm, as well as to act as a neurotransmitter in many other species, analysis of its levels and

distribution in the lobster brain might provide additional insight into the nature directing rhythmic changes of serotonin levels in this species.

1.7 Serotonin Transporter (SERT)

Serotonin, as described earlier, is a versatile neurotransmitter and its regulatory functions range from sensory sensitivity (Pasztor & Bush, 1987) to cell growth (Lipton & Kater, 1989) and, direct or indirectly, neurogenesis (Gould, 1999; Beltz et al., 2001; Whitaker-Azmitia, 2001). The serotonin transporter (SERT), which allows serotonin to travel out of, into, within or between cells and which co-transports Na^+ and Cl^-, might play a key role in these mechanisms.

The SERT, which was first cloned in rat (Blakely et al., 1991) and later in humans (Ramamoorthy et al., 1993) and mouse (Chang et al., 1996), is known to be a member of a large neurotransmitter transporter superfamily (for review see Amara & Kuhar, 1993; Worall & Williams, 1994, Schloss et al., 1994). It is characterized by 12 putative transmembrane domains, cytoplasmic N- and C-termini and N-glycosylation sites (Mayser et al., 1991; Hoffman et al., 1991). Genetic studies have identified a single SERT gene in rodents (Blakely et al., 1991), humans (Austin et al., 1994; Lesch et al., 1994; Ramamoorthy et al., 1993) and flies (Corey et al., 1994; Demchyshyn et al., 1994) with no known coding splicing variants, thus suggesting the presence of a single SERT across species. In addition, Bruns and colleagues (1993) showed in leech that SERT functions as a rapid transmitter processing system.

Recent studies using immonocytochemical labeling techniques in mammals, could localize the SERT in the hippocampus as well as the olfactory bulb, cerebellum, amygdale, raphe nuclei (Sur et al., 1996) and suprachiasmatic nucleus (Legutko & Gannon, 2001). These findings are especially intriguing knowing that the SERT plays a key role in the pharmacological regulation of serotonin which in turn has been shown to modulate persistent neurogenesis in mammals (Gould, 1999; Brezun & Daszuta, 1999; Brezun & Daszuta, 2000; Jacobs et al., 2002) and lobsters (Benton et al., 1997). The SERT is the site of action of the selective serotonin transporter inhibitor fluoxetine which prevents the transduction of serotonergic information. Fluoxetine (Prozac) is widely used to treat patients suffering from

depression. Interestingly, depression is also thought to be the consequence of a shrinking hippocampus (Sapolsky, 2001), which is the site of persistent neurogenesis in mammals and the site of immunocytochemical SERT labeling in rats (Sur et al., 1996). Similarly, in lobsters pharmacological depletion of serotonin inhibits the generation of new neurons at the site of life-long neurogenesis (Benton et al., 1997) and serotonergic fibers have been shown to terminate blindly at the site of life-long neurogenesis, indicating that serotonergic release might be involved in the process of shaping new neurons (Beltz et al., 2001). In addition, a select number of newly born cells has been identified to transiently take up serotonin at the site of life-long neurogenesis thus suggesting that the SERT is only active for a short period after birth in this system (Beltz et al., 2001).

Because serotonin has been indicated to play a critical role in the regulatory pathway modulating life-long neurogenesis it is important to study the mechanisms that accompany serotonins' action. Immunocytochemical double labeling techniques introducing the anti-SERT antibody and an antibody against a mitosis marker in lobsters are, therefore, anticipated to provide further insight.

1.8 Motivation

The motivation of this thesis lies in the wealth of studies suggesting that serotonin has a functional role in the process of life-long neurogenesis but providing little information on the exogenous and endogenous mechanisms regulating serotonin synthesis, release and action.

- We (the "Beltz lab") initially hypothesized that a coupling of environmental influences and endogenous factors is important in controlling the timing and rate of neurogenesis in the lobster brain, and that light and/or feeding may provide a dominant coordinating signal for the many factors, including serotonin, that influence the persistent generation of new neurons. If this hypothesis is correct, then serotonin levels should cycle diurnally, be entrainable by light and/or feeding, and show a fixed correlation with the neurogenic rhythm. *
- Subsequent experiments then used immunocytochemical methods to verify the most striking changes (peak vs. trough) of the light entrainable circadian rhythm of brain serotonin levels. *

- Because primary experiments described here demonstrated that the circadian rhythms of serotonin in functionally distinct regions of the lobster brain are out of phase, subsequent studies used electro- and immunocytochemical techniques to test for melatonin (the biosynthetic product of serotonin) as a potential source for the contrasting rhythm of serotonin in these regions.
- In addition, immunocytochemical methods were established to employ the first commercially available serotonin transporter (SERT) antibody to analyze for the SERT in lobster. ‡
- Finally, immunohistochemistry was used to localize SERT at the site and during the process of life-long neurogenesis. ‡

* These experiments have been presented, published or are currently in preparation for publication (see 7.3 “List of Publications and Posters”).

‡ These experiments have been presented at the 2004 Forum of European Neuroscience (see 7.3 “List of Publications and Posters”).

2. Material & Methods

2.1 Animal rearing and maintenance

Juvenile american lobsters, *Homarus americanus* Milne Edwards, 1837 (Malacostraca, Decapoda, Reptantia, Homarida) (2-4 cm total body length) were obtained from the New England Aquarium (Boston, MA, USA). Animals were kept in individual containers (5x5 cm) (Figure 6) that floated freely on the surface of a re-circulating artificial seawater tank (14°C) (Figure 7). Holes in the floating containers allowed free exchange of water between the animals and the tank water. Because the rate of neurogenesis and endogenous levels of various hormones fluctuate during the molt cycle (Harrison et al., 2001; Gorissen, 2002), the lobsters were molt staged (Aiken, 1973) and only intermolt animals were included in the experimental groups. Animals were fed frozen brine shrimp, *Artemia salina*, three times a week 1 hour before dusk (unless otherwise noted); food was always supplied on the same days of the week. Prior to all experiments animals were entrained to a 12:12 L/D cycle for a minimum of 2 weeks. The light intensity at the surface of the water was 6-11 µ Einsteins/meter^{2}s. Animals used for serotonin measurements were transferred to constant darkness (D/D) for three days prior to the experiment in order to reveal the endogenous rhythm in serotonin levels, and to avoid masking effects of sudden intensity changes in light on serotonin release (Wildt et. al, 2004). During this time the room was neither entered for maintenance purpose nor for feeding. Feeding as a direct stimulus could consequently be excluded.

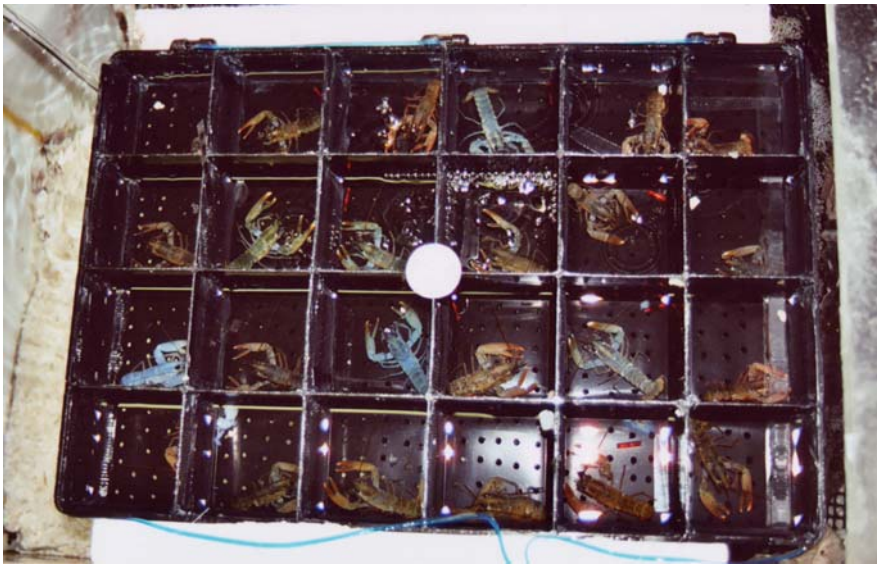


Figure 6. Individual living space (5x5 cm) of juvenile lobsters. Each container houses 24 animals. Note that the container is perforated at the bottom and along each side to allow free water exchange. Centered white circle represents a 25 cent coin for size comparison.

Figure 7. The container floats freely at the surface of re-circulating artificial but enriched seawater.

2.2 Dissection

Animals were retrieved from their containers using night vision goggles (Bushnell Corporation, Overland Park, KS, USA) (excluding sampling time points for melatonin analysis during light exposure in L/D), and placed on ice in the dark. The eyes and lateral protocerebrum were quickly removed in dim light in order to minimize light activation of serotonergic pathways. The length of each specimen was then measured from rostrum to telson to determine the animals total body size for further comparison. Using a stereomicroscope and fiber light source, brains were then dissected in cold lobster saline [46mM NaCl, 16mM KCl, 26mM $CaCl_2 \bullet 2H_2O$ dihydrate, 8mM $MgCl_2 \bullet 2H_2O$, α-D (+)-glucose (2g/L), and HEPES buffer (2.38g/L; pH 3.25); Sigma].

2.3 Electrochemical detection of brain substances using high pressure liquid chromatography (HPLC)

2.3.1 Electrochemical fundamentals (HPLC)

High pressure liquid chromatography (HPLC) is an elegant method to detect electrochemical gradients based on the difference in the surface interactions of the analyte and molecules of the mobile phase. The mobile phase functions as a carrier which will transfer the analyte to the stationary phase (column), the site of chemical reaction. Depending on the binding activity of the substance to be detected, an analyte-specific retention time will result. This retention time can than be compared to the retention time of the external standard. Electrochemical analysis of a dilution series of the external standard will ultimately allow the mathematical calculation of the solute content detected.

For serotonin detection reverse-phase HPLC was used. Here, the polar mobile phase will help to bind the amino group of serotonin to the non-polar C18 column (using a column that features this many carbon compounds demonstrates high binding activities). The mobile phase consists of heptane which provides the non-polar end that binds to the C18 column and sulfonic acid which offers the negatively charged ending for serotonin to bind. To assure that the negatively charged endings of sulfonic acid will not bind with other amino acids of the lobster brain sample, the pH was set to 3.25. In order for serotonin to bind properly to the binding sites of sulfonic acid, serotonins' amines need to be dissociated using 0.1M perchloric acid. This guarantees a greater stability of the amines and safeguardes them for spontaneous oxidations.

Because the virtue of HPLC lies in its simplicity, it is also this simplicity that makes it susceptible to the variability of a magnitude of exogenous factors and thus a challenge for any kind of circadian rhythm detection (and such dynamics have been experienced in great detail). As mentioned earlier, spontaneous oxidation should be avoided. This can be achieved by either degassing freshly made-up mobile phase using a degasser or by "recycling" the mobile phase by running the eluent several times through the system. The later however, bears the risk of contaminating the mobile phase. Of equal importance is the stability of the ambient temperature. Any discrepancies of the temperature of the sample, the solvent solution, and the ambient temperature will inevitably cause a false reading with the detector. Dead sample volume is also a key feature that needs to be avoided. Air entering the system will cause a

delay in the retention time and thus mislead the data analysis. Finally, vibrations of any kind, including floor vibrations, will be transmitted onto the system and result in a peak reading.

Because HPLC was used to detect circadian variations at a very responsive current it became evident that above mentioned factors had to be avoided under all circumstances.

2.3.2 Analysis of circadian brain serotonin levels – experimental set-up

To test whether brain serotonin levels fluctuate in the same circadian fashion as neurogenesis, all animals were entrained to a 12:12 L/D cycle for a minimum of 2 weeks with feeding ~1 hour before dusk (even when animals were subjected to a shifted or reversed light cycle, or unless stated otherwise). The light intensity at the surface of the water was 6-11 μ Einsteins/meter^{2}s. Over the course of this study, groups of lobsters were exposed to four different experimental protocols, all based on a 12:12 L/D schedule.

- Group 1 (n=24) was exposed to the 12:12 L/D cycle and whole brain levels of serotonin assessed by high pressure liquid chromatography (HPLC).
- Group 2 (n=24) was exposed to the same 12:12 L/D cycle as Group 1, but following the 12:12 L/D exposure (conditioning light regime) animals were transferred to constant darkness for three days without food (D/D conditions).
- Group 3 (n=24) was exposed to an 12:12 L/D regime for two weeks that was phase-shifted by 4 hours relative to Group 2, followed by D/D for 3 days prior to HPLC measurements of brain serotonin levels.
- A fourth group of animals (Group 4; n=24) was subjected to the same light regime as Group 2, but in these animals the OLs, ALs and the remainder of the brain were separated from one another and the individual areas then assayed for serotonin content.

2.3.3 Analysis of circadian brain serotonin levels and neurogenesis after feeding – experimental set-up

Surprise feeding. The 'surprise feeding' experiment was performed to test whether feeding has a direct effect on the levels of brain serotonin. For this purpose, animals were 'surprise' fed 8 hours into their dark time (=15 hours prior to entrained feeding) instead of 1 hour before dusk (compare to 'Animal rearing and maintenance'). Determinations of the rate of neurogenesis were not included into this experiment because the rate of the cell cycle was not expected to show an immediate change upon spontaneous feeding.

For this experiment, animals (n=36) were fed frozen *Artemia* every other day 1.5 hours prior to dusk for two weeks (Group 1*). On the day of the experiment food was supplied 15 hours prior to the expected feeding time. This time was chosen because serotonin is then at its lowest level. In order to determine the level of brain serotonin before feeding (baseline), one sample was taken directly before feeding. After the feeding stimulus had been provided samples (n=4/timepoint) were taken 30 minutes, 1 hour, 2 hours, 4 hours and then in order to allow comparison with previous data every 4 hours.

Fictive feeding. The components, such as chemosensory stimulation, caloric intake, and physical activity that contribute to the complex activity of feeding were analyzed and the influence of each of these on serotonin levels and the rate of neurogenesis was tested. For both, serotonin and neurogenesis analysis, animals were fed frozen *Artemia* every other day 5 hours prior to dawn for 2 weeks. This time was chosen to test whether regular feeding at a time when serotonin and neurogenesis are low would alter the endogenous rhythms of serotonin and also the rate of neurogenesis. In lobsters, physical activity related to feeding can be separated from food ingestion/chemosensory stimulation by simply substituting food with a plastic bead, which will be manipulated by hungry lobsters in the same way as ingestible food. This allowed us to test whether physical activity or the actual intake of food alters the endogenous rhythm of serotonin and neurogenesis in lobsters. On the day of the experiment, animals were either

- excluded from a food or bead stimulus ("No Stimulus"; Group 2*; serotonin: n=24, neurogenesis: n=36),
- fed *Artemia* as usual ("*Artemia*"; Group 3*; serotonin: n=24, neurogenesis: n=35), or
- given a bead ("Bead"; Group 4*; serotonin: n=24, neurogenesis: n=35).

To prevent food odorants from influencing the "Bead" and "No Stimulus" animals, animals from Group 3* were housed in a separate tank of equal water quality and temperature in the

same room as animals from Group 2*+4*. For serotonin measurements, the first sample (n=4) was taken prior to giving the stimulus and then every 4 hours over the 24-hour period. Electrochemical detections of circadian peaks and troughs of serotonin may vary in their amplitude when measured on separate days (see serotonin chronograms as shown in 7. Appendix). Prior detections of characteristic features are, therefore, a valuable tool to determine variations within the circadian rhythm of serotonin.

To determine the number of proliferating neurons in cell soma cluster 10 at various time intervals over a 24 hour period an incubation time of 3 hours per time point was chosen (Goergen et al., 2002) (compare 2.5). The first 3 hours of BrdU (Bromodeoxyuredine) incubation occurred at the same time that the stimulus was given and then every 4 hours over the 24-hour period, as in Goergen et al. (2002). In addition, previous studies had shown that neurogenesis shows the same circadian rhythm when measured under D/D conditions and L/D conditions. Animals for neurogenesis studies were therefore taken from an L/D light regime exclusively.

2.3.3.1 Observational methods- physical activity in response to food and bead – experimental set-up

To asses the animals' physical activity in response to feeding, observational studies were performed in parallel with BrdU and electrochemical analysis. This method was included in order to determine the duration of physical activity after feeding and, therefore, to have a better understanding on the proportional input of calorie intake vs. altered physical activity. Since lobsters show an immediate response to an observer leaning over the tank, night vision goggles (Bushnell Corporation, Overland Park, KS, USA) were used to monitor the animals' physical activity from a distance. Animals in Group 2*-4* were observed and ranked according to their locomotion or whether they were manipulating the plastic bead given with their mandibles, maxillae, maxillipeds and extremities. Specimen were monitored and ranked on a scale from 1 (not active) to 3 (very active) animals were monitored every 30 minutes for 2 minutes for the first 4 hours after providing the stimulus and then every 4 hours to parallel the immunocytochemical and electrochemical sampling.

2.3.4 HPLC method to detect serotonin

For all serotonin measurements, whole brains were dissected out of the animals and transferred to microcentrifuge tubes containing 50µl of 0.1N perchloric acid (Sigma). Samples were diluted with 100µl mobile phase (described below) and manually homogenized with a pestle. Homogenates were transferred into Eppendorf tubes containing a 0.45µm inserted filter (VWR, West Chester, PA, USA) and centrifuged (Beckman centrifuge) for 15 minutes at 20,000 g and 21°C. The clear supernatants were transferred into BAS autosampler microvials (Bioanalytical Systems Inc., West Lafayette, IN, USA and sealed with teflon caps. 10µl samples were applied to a C18 reverse phase column (Alltech Associates Inc., Deerfield, IL, USA; 3µm, 100 x 4.6mm) via a BAS autosampler (Samplesentinel). Eluted compounds were detected electrochemically with a Bioanalytical Systems (BAS) liquid chromatography system consisting of a CC-5 liquid chromatography module, a PM 80 solvent delivery system and a LC 4C amperometric detector. The mobile phase contained 20mM sodium phosphate (2.4g/L monobasic anhydrous), 1.85 mM heptanesulfonic acid (375 mg/L sodium salt), 0.27 mM ethylenediaminetetraacetic acid (EDTA) (80 mg/L anhydrous), and vol/vol 16%MeOH and 4% acetonitrile as organic modifiers. The final solvent buffer was adjusted to pH 3.25 with concentrated phosphoric acid. A/D converters (A/D Instruments Inc., Colorado Springs, CO, USA) and a strip-chart program with integrated chromatography software (A/D Instruments PowerChrom version 2.2.4) were used to analyze the peaks (Figure 9). The detector potential was set at 625mV and the detection limit was in the range of 2nA. Recovery rates were close to 100% and no further corrections were applied. Differences among sampling times were assessed using ANOVA statistics with *post-hoc* analysis (SPSS, Inc., Chicago, IL, USA).

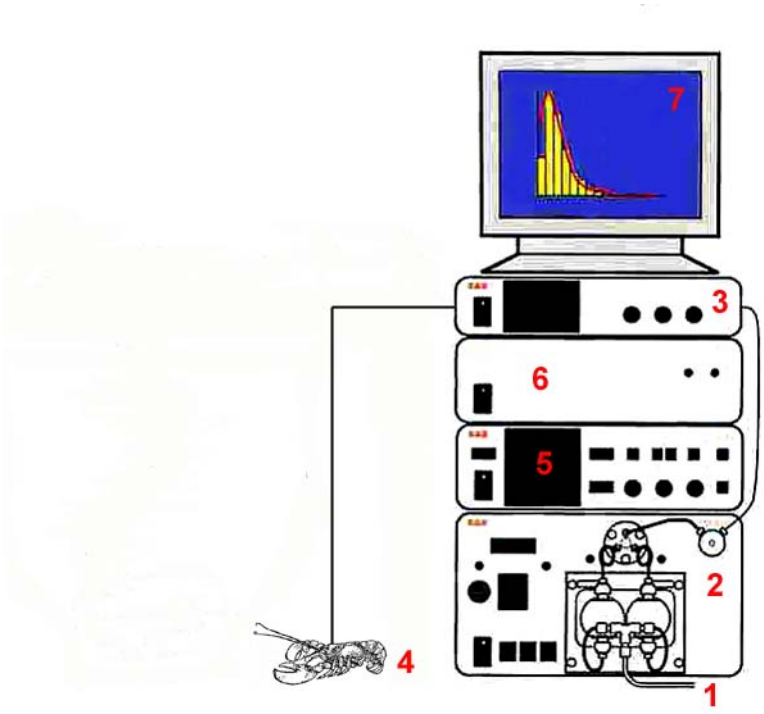


Figure 8. HPLC set-up showing the devices involved to read the analyte. (1) mobile phase enters the system via the pumps (2) and is pumped to the column (3). Here the sample (4) is injected onto the column and reads according to the detector settings (5) and via an A/D converter (6) on the computer (7).

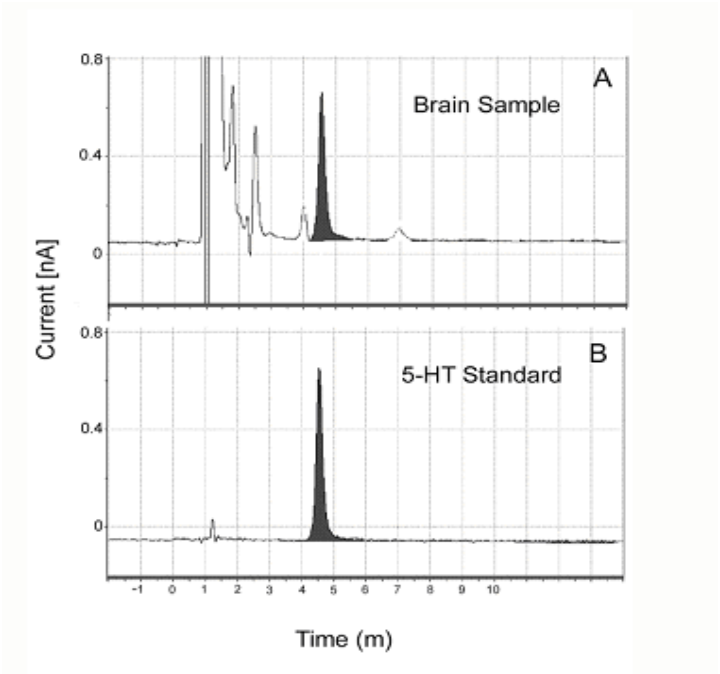


Figure 9. HPLC chromatograms (A/D Instruments Inc.; PowerChrom version 2.2.4) showing the peaks contained in a brain sample (A) compared with a serotonin (5-HT) external standard (B). (also see 2.3.8 for additional external standards tried).

2.3.5 HPLC method to detect melatonin

Classically melatonin, a biosynthetic product of serotonin, is considered to function as a hormone that also fluctuates on a circadian rhythm (Arendt, 1995; Binkley, 1997; Tilden et al., 2003; Gorbert & Steel, 2003). However, data provided by Mechawar & Anctil (1997) in a primitive metazoan *Renilla köllikeri* suggests that melatonin also acts as a neurotransmitter or neurohormone. In addition, several authors promote a direct correlation between the fluctuating levels of serotonin due to the synthesis of melatonin (Sudgen, 1990; Olcese & Munker, 1994; Miguez et al., 1997). This and the fact that the levels of serotonin in the brains of juvenile lobsters fluctuate on a strong circadian rhythm, gave rise to the speculation that part of the fluctuations of the serotonergic rhythm might be due to melatonin activation. In order to make the data on melatonin comparable to the already acquired data on serotonin in lobsters, two 24-hour experiments were performed using HPLC to test for circadian fluctuations of melatonin in the brains of juvenile lobsters. One was to sample for melatonin under normal L/D conditions (light on at zeitgeber time (ZT) 6:00 and light off at ZT 18:00) and one to sample after the animals had been exposed to normal L/D conditions followed by three days in constant darkness (D/D).

For all melatonin measurements, eyestalks as a potential source of melatonin synthesis (Arendt, 1995; Binkley, 1997; Hardeland & Poeggeler, 2003), were separated from the head in the dark using night vision goggles (Bushnell Corp.). Whole brains were then dissected out of the animals under dim red light and transferred to microcentrifuge tubes containing 50µl of 0.1N perchloric acid (Sigma). Samples were diluted with 100µl mobile phase (Fadool et al., 1988) and manually homogenized with a pestle. Homogenates were transferred into Eppendorf tubes containing a 0.45µm inserted filter (VWR) and centrifuged (Beckman centrifuge) for 15 minutes at 20,000 g and 21°C. The clear supernatants were transferred into BAS autosampler microvials and sealed with teflon caps. 10µl samples were applied to a C18 reverse phase column (Alltech Inc.; 3µm, 100 x 4.6mm) via a BAS autosampler (Samplesentinel). Eluted compounds were detected electrochemically with a Bioanalytical Systems (BAS) liquid chromatography system consisting of a CC-5 liquid chromatography module, a PM 80 solvent delivery system and a LC 4C amperometric detector. The mobile phase contained 0.02M Trichloric acid, 1.5 µm EDTA, 2.2mM Sodium dodecylsulphate (SDS), 0.07M dibasic sodium phosphate and vol/vol 12%MeOH and 20% acetonitrile as organic modifiers (Fadool et al, 1988). The final solvent buffer was adjusted to pH 7.4 using acidic acid (50%). The flow rate was set at 0.8mL/min. A/D converters and a strip-chart program with integrated chromatography software (A/D Instruments PowerChrom version

2.2.4) were used to analyze the peaks. The detector potential was set at 900mV and the detection limit was in the range of 0.5nA. Recovery rates were close to 100% and no further corrections were applied. Differences among sampling times were assessed using ANOVA statistics with *post-hoc* analysis (SPSS, Inc).

2.3.6 Data analysis for serotonin measurements performed under D/D conditions

The data for the chronograms were obtained from serially independent measurements of the serotonin levels in groups of animals killed and assayed at each time interval. A requirement for serially independent measurements of this nature is that the individuals in the groups are as similar to one another, and to those in the other groups, as possible. To ensure this equality, only animals were used that had been reared under the same conditions of temperature and nourishment and were the same size. A comparison of the total body length (rostrum to telson) of all animals that were used in the results presented here confirmed that there was no statistical difference between them (n=70; mean, 4.49mm; SE, 0.056). Furthermore, in a previous study Helluy et al. (1995) found that the volume of the lobster brain is linearly correlated with body volume for the size range that was used here. Given that the individual animals, and therefore their brains, were very close to the same size, it was possible to simply use the serotonin content in picomoles per brain in the chronograms without normalizing these values to brain weight or dimensions, which were very difficult to assess for these small tissue volumes.

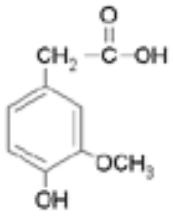
2.3.7 Statistical analysis

The HPLC data analyzing for circadian changes of serotonin (2.3.2 and 2.3.3) were subjected to a three-factor ANOVA after the data of Groups 2 and 3 had been adjusted to zeitgeber time (i.e. light on/off) with factors being Group 2 vs. Group 3, light on vs. off and hours since zeitgeber change (SPSS Inc., Chicago, IL, USA). *Post-hoc* analyses were

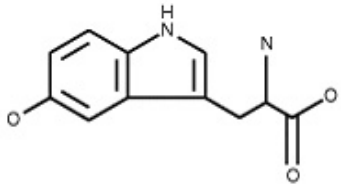
performed (Bonferroni and Scheffe tests) for AL, OL, rest of brain data (SPSS Inc., Chicago, IL, USA). All other data (immunocytochemical analysis of circadian fluctuations of serotonin, feeding data and HPLC analysis of melatonin) were analyzed using *post-hoc* analysis only.

2.3.8 List of standards tried for HPLC analysis

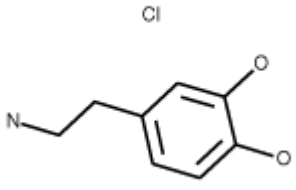
1. 4-Hydroxy-3-methoxyphenylacetic Acid (HVA; homovanillic acid; Sigma)



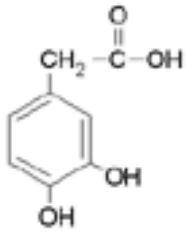
2. 5-Hydroxy-L-tryptophan (5-HTP; Sigma)



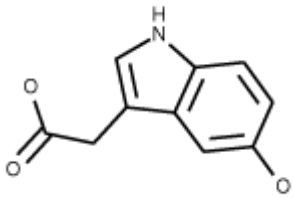
3. Dopamine hydrochloride (DA; Sigma)



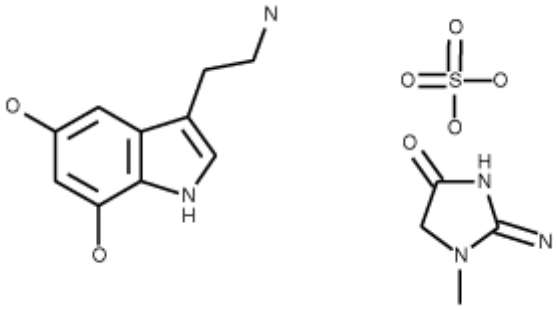
4. 3,4-Dihydroxyphenylacetic acid (DOPAC; Sigma)



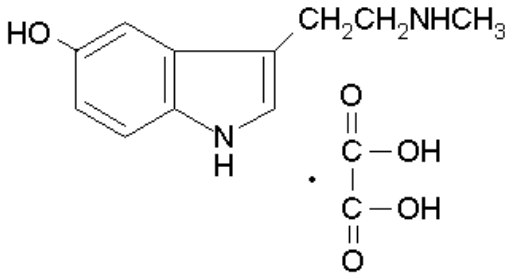
5. 5-Hydroxyindole-3-acetic acid (5-HIAA; Sigma)



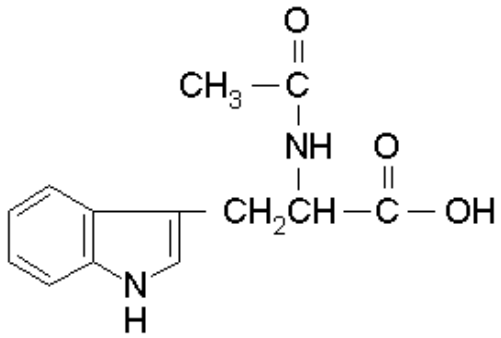
6. 5,7 Hydroxytryptamine (5,7 DHT; Sigma)



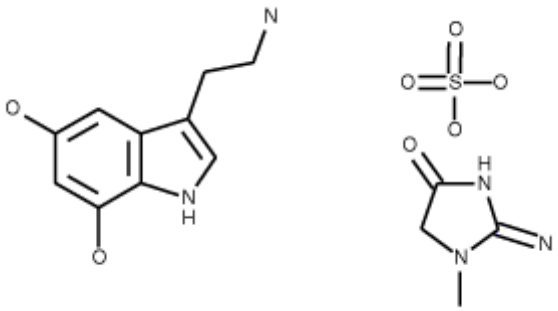
8. Nω-Methyl-5-hydroxy-trypt-amine (N-m-5-HT; Sigma)



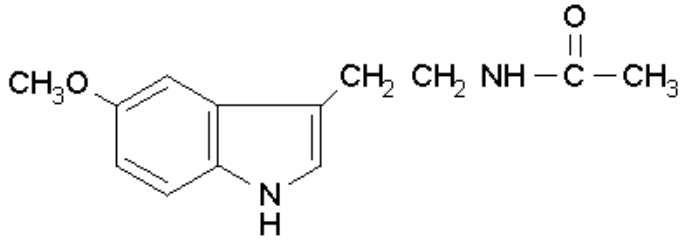
9. Tryptophan (Sigma)



10. N-Acetyl-5-hydroxytryptamine creatinine sulfate salt (5-HT; Sigma)



11. Melatonin (MP Biomedicals Inc., formerly ICN Biomedicals, Inc., Irvine, CA- USA)



* images of all structural formulas taken off:

http://www.sigmaaldrich.com/Area_of_Interest/The_Americas/United_States.html

2.3.9 Sequential listing of all electrochemical experiments performed

	experimental light condition	**lights on***	**body size:** rostrum to telson in cm	**range**
Experiment 1	normal L/D	6:00-18:00	-	10nA
Experiment 2	normal L/D	6:00-18:00	-	10nA
Experiment 3	normal L/D	6:00-18:00	~ 2.0	0.5nA
Experiment 4	normal L/D	6:00-18:00	~ 2.2	0.5nA
Experiment 5	normal L/D + reverse L/D*	6:00-18:00	~1.8	0.5nA
Experiment 6	normal L/D + reverse L/D*	6:30-18:30	~2.2	0.5nA
Experiment 7	normal L/D		~2.5	5.0nA
Experiment 8	normal L/D + reverse L/D*		~2.1	5.0nA
Experiment 9	normal L/D + reverse L/D*		~0.9	5.0nA
Experiment 10	normal L/D + reverse L/D*		~4.4	2.0nA
Experiment 11	D/D for 3 days prior to exp		~2.3	5.0nA
Experiment 12	normal L/D		~3.2	2.0nA
Experiment 13	normal L/D with sudden lights off (13:00)		~2.4	2.0nA
Experiment 14	reverse D/D AL/OL only		~3.4	1.0nA
Experiment 15	normal D/D AL/OL		~4.5	1.0nA
Experiment 16	shifted DD whole brain	2:00-14:00	~4.5	2.0nA
Experiment 17	normal D/D AL/OL/rest of brain		~4.4	1.0nA
Experiment 18	normal D/D AL/OL/rest of brain		~4.5	1.0nA
Experiment 19	shortened photoperiod followed by D/D	6:00-12:00	~4.6	2.0nA
Experiment 20	fictive feeding- normal D/D		~2.0	2.0nA
Experiment 21	surprise feeding at 5:30		~2.3	2.0nA
Experiment 22	Melatonin whole brain normal L/D		~2.2	0.5nA
Experiment 23	Melatonin whole brain D/D		~2.0	0.5nA
Experiment 24	fictive feeding II- normal D/D		~2.1	2.0nA
Experiment 25	surprise feeding II		~2.2	2.0nA
Experiment 26	fictive feeding III		~4.0	5.0nA

* lights on for reverse L/D = 18:00-6:00

Table 1. Sequential listing of all electrochemical experiments that have been performed during the time course of this research project. The respective chronograms are either shown in 3. Results or in 7. Appendix.

n	date	known problems
	27/11/2001	Injected air bubbles
	13/01/2002	
36	18/03/2002	Eppendorf centrifuge did not cool
36	25/04/2002	room temp. changes
18	09/05/2002	
18	07/06/2002	
24	01/07/2002	used internal standard (N-m-5-HT) that reacts with natural 5-HT.
24	31/07/2002	
24	20/09/2002	
24	10/10/2002	
24	22/10/2002	
24	22/11/2002	power failure
24	01/12/2002	column broke
24	13/02/2003	
24	03/03/2003	
24	04/04/2003	
24	11/04/2003	
24	27/05/2003	
24	29/05/2003	
24	12/08/2003	autosampler broke
36	15/08/2003	
24	21/08/2003	
24	28/08/2003	
24	29/09/2003	
36	13/10/2003	
24	05/03/2003	

comments
Analyzed for peak area
Analyzed for peak area
peak height; with autosampler
peak height; started using reverse room to prevent daytime disruption of the nocturnal animals.
from now on all samples centrifuged at room temp. to avoid temp. gradient.
same light bulbs throughout room. Thus same light intensity but still different light adsorption due to room size.
from here on 5-HT as external standard used; all siblings
Installed degasser, Maclab
used external chiller to keep water temp. at 15ºC
starting PowerChrom use
only been in changed photoperiod for 2 weeks
whole brain
whole brain
hand injections

2.4 Immunocytochemistry

2.4.1 Immunocytochemical fundamentals

The detection of antigens in tissue using antibodies, that either carry or offer binding sites for chromophores, is known as immunocytochemistry. Antibodies (immunoglobulins) are proteins which are produced by differentiated B cells on exposure to an antigen. Each antigen stimulates the secretion of an exact antibody, thus demonstrates an antigen-specific binding machinery. However, distinctions can be made in the "genetic purity" of the antibody. Once a host has been immunized by a specific antigen either monoclonal or polyclonal antibodies can be generated. Monoclonal antibodies involve the subsequent generation of a hybrid cell by joining an activated B cell with a myeloma B cell which consequently will be "immortal" and, therefore, continually produce antibodies of one cell type. Polyclonal antibodies, in contrast, are obtained by extracting a product of different immunoglobulins from different antibody-producing cells.

In general, three main immunocytochemical methods are available but each one of these techniques can widely be modified and, therefore, be tailored to the specific need (Figure 10).

The direct method applies labeled primary antibody which will picture the antigen directly (Figure 10A). Labeling reagents can be fluorescent compounds, or enzymes such as horseradish peroxidase. Although this technique is fast and the quality of the staining entirely relies on the specificity and affinity of the primary antibody, its preparation is time consuming (labeling every single primary antibody), produces a low signal level, allows little flexibility when diluting the primary antibody, no flexibility in the choice of chromophore (color) and overall bears the risk that the binding sites of the primary antibody might have undergone some conformational changes due to the labeling procedure and thus "sacrificed" their stability.

The two-step method uses a primary and a secondary antibody which carries the chromophore (Figure 10B). The primary antibody will bind to the tissue antigen and the secondary antibody will bind to the primary antibody. Because the key of each antibody lies in its specificity, the application of the secondary antibody will depend upon the host animal (in which the primary antibody was raised) of the primary antibody. For example, the antigen to be detected is serotonin. The antibody (primary antibody) against serotonin however was

generated in rabbit. The secondary antibody, consequently, will have to be rabbit-specific, thus anti-rabbit. Since the visualization marker is attached to the secondary antibody this method offers a wide usage of different chromophores.

The three-step method requires a third layer that carries the label (Figures 10C, D). The secondary antibody serves as a bridge between the primary antibody which is bound to the tissue antigen and the peroxidase anti-peroxidase complex (PAP) (Figure 10C). It is therefore important that the primary antibody and the anti-peroxidase are raised in the same host. The virtue of the "sandwich" technique is that extra binding sites are created which allow more binding activity and, hence, an amplification of the signal. However, the PAP molecule is considerably large and thus might cause problems when the complex tries to enter the sample tissue.

Alternatively, the avidin-biotin-complex (ABC) can be used (Figure 10D). Here a biotin-labeled secondary antibody (which again is directed against the host of the primary antibody) will bind to the primary antibody. As a last step, an avidin-biotin-complex (peroxidase or fluorochrome) will bind to the secondary antibody which is already bound to the primary antibody and thus offers an antigen-specific visualization. Even though, this technique amplifies the peroxidase signal (because many binding sites are provided on avidin for biotin and, hence, for the avidin-biotin-peroxidase complex), the complex is large, and thus, might block the penetration.

In general, the specificity of antibodies and the ease by which they can be employed provides a sensitive technique to visualize molecular structures at the site of storage or action.

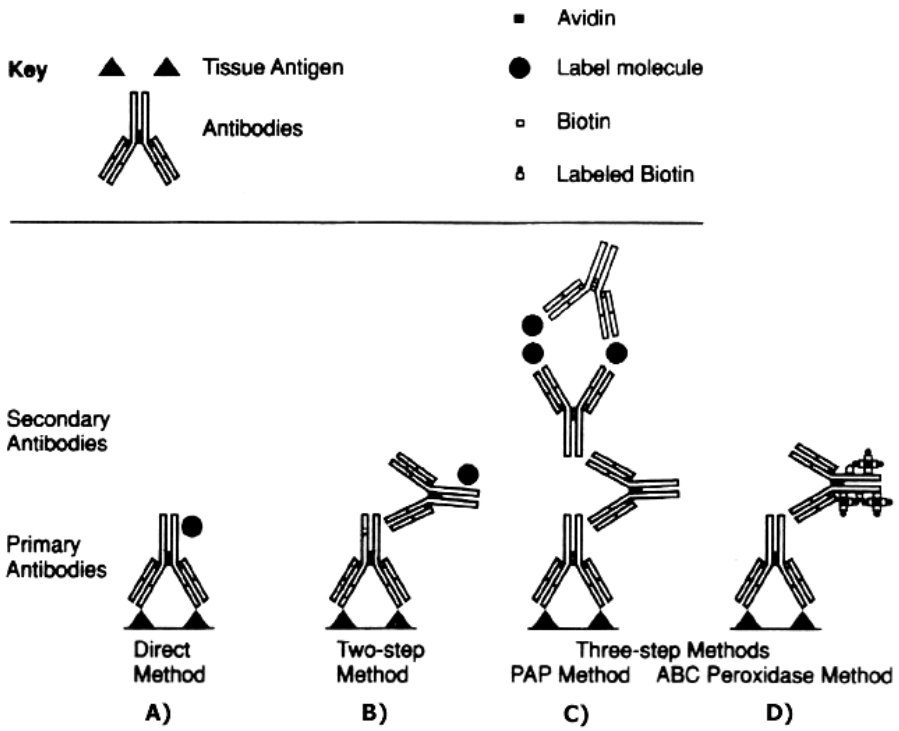


Figure 10. Four fundamental immunocytochemical methods that can be tailored to ones needs: A) One-step (direct) method; B) two-step method, and three step-method: C) peroxidase anti-peroxidase (PAP) complex and avidin-biotin complex (ABC). For further explanation see text. (Diagram taken from Beltz & Burd, 1989).

2.4.2 Immunocytochemical labeling against serotonin

Methods for processing brains as whole mounts for serotonin immunocytochemistry were taken from Beltz & Burd (1989). Juvenile lobsters were maintained either on a 12:12 L/D cycle for at least two weeks (see Group 1 protocol, Material & Methods 2.3), or on a 12:12 L/D conditioning regime followed by 3 days in D/D (see Group 2 protocol, Material & Methods 2.3). Animals were retrieved from their chambers, placed on ice in the dark, and the eyestalks removed in dim light. Brains were then dissected at 3 hours prior to subjective dawn (n=4) and 3 hours prior to subjective dusk (n=4), and fixed in cold 4% paraformaldehyde for ~18 hours. Whole mounts (n=8) were then rinsed 5 times for 15 minutes in 0.1M phosphate buffer (PB; pH 7.4) and incubated in 0.1M PB with 0.3% Triton X-100 (PBTx) for 45 minutes, followed by incubation in rabbit anti-serotonin antibody (1:1000; DiaSorin, Stillwater, MN, USA) for 48 hours at 4°C. Following 6 rinses in PBTx, goat anti-rabbit Alexa 488 (1:50; Molecular Probes, Eugene, OR, USA) was applied for 36 hours, after which

samples were rinsed 6 more times in PB. Preparations were mounted in Gel Mount (Biømeda Corp., Foster City, CA, USA) and viewed using a Leica TCS SP confocal microscope (Leica Inc., Bannockburn, IL, USA).

In order to assess relative levels of serotonin immunoreactivity in the samples, all brains were screened during a single session and the initial laser and filter settings on the confocal microscope were maintained at the same levels throughout the entire analysis. Leica confocal quantitative software (Leica Microsystems, Germany; version 2.0) was used for semi-quantitative analyses of the intensity of serotonin labeling in the OLs and ALs. These measurements were done by comparing the intensity of fluorescence (serotonin immunoreactivity) in the OLs and ALs for each time point, and then scoring this intensity on a scale relative to background levels in the brain. Statistical analyses were performed using ANOVA with Bonferroni and Scheffe post-*hoc* analyses (SPSS Inc., Chicago, IL, USA).

2.4.3 Immunocytochemical labeling against melatonin

To test whether melatonin, a hormone associated with photosensitive tissue (Vivien-Roels & Pévet, 1986; Binkley, 1997; Hardeland & Poeggeler, 2003; Tilden et al., 2003), can be found in the brain and thus support electrochemical findings standard immunocytochemical techniques for fluorescence confocal microscopy were employed. Juvenile lobster brains were dissected out at zeitgeber time 7:00, fixed in 4% PFA overnight and thoroughly rinsed in 0.1M PB before sectioning (100 μm) using a vibratome (Technical Products International (TPI), St. Louis, MI, USA). Subsequently, preparations were rinsed in 0.3% PBTx before incubation in sheep anti-melatonin primary antibody (1:1000; Stockgrand LTD, Guildford, Surrey, UK) for 17 hours. Secondary antibody used was donkey anti-sheep Alexa 488 (1:50; Molecular Probes).

To test whether temporal fluctuations in the level of melatonin could also be measured using immunocytochemical techniques, animals were sacrificed every 4 hours over a period of 24-hours (zeitgeber time 22:00, 2:00, 6:00, 10:00, 14:00, 18:00). Brains were processed as laid out above. The relative intensity of immunocytochemical labeling was analyzed using a Leica confocal microscope. Samples were screened for the most intense labeling for confocal microscope adjustment. All samples were analyzed within one session and without changing

the initial setting on the microscope. Final images were quantified using Leica's confocal software.

Also, double labeling techniques were employed to test for a close spatial correlation between the site of melatonin synthesis and the site of SERT expression. Antibodies used were: sheep anti-melatonin (1:1000; Stockgrand LTD) and mouse anti-SERT (1:1000; Advanced Targeting Systems). Secondary antibodies were from Molecular Probes: donkey anti-sheep Alexa 488 (1:50) and goat anti-mouse Alexa 594 (1:50).

2.4.4 Immunocytochemical labeling against the Serotonin Transporter (SERT)

Standard immunocytochemical techniques for fluorescence confocal microscopy were employed according to Beltz & Burd (1989). Juvenile and embryonic (E 84%; staged according to the Perkins eye index, which correlates eye width and length to the developmental stage of the animal; see Helluy & Beltz, 1991) lobsters were maintained on a 12:12 L/D cycle for at least two weeks. Brains were dissected out in cold lobster saline at zeitgeber time 14:00 (it was speculated that SERT is numerous at that time of day because brain serotonin levels had previously been shown to be highest), fixed in 4% paraformaldehyde (PFA) overnight and thoroughly rinsed in 0.3% phosphate buffer + Triton X-100 (PBTx) before processed as whole mounts or sections (100µm). Primary antibody used was mouse (monoclonal) anti-SERT (1:1000; Advanced Targeting System, San Diego, CA, USA). Incubation time for anti-SERT was 36 hours. Incubation in secondary antibodies (goat anti-mouse Alexa 488 and goat anti-rabbit Alexa 594 (1:50); Molecular Probes) was carried out overnight. All preparations were analyzed using a Leica TCS SP confocal microscope.

2.4.4.1 Preadsorption control of SERT

To test for antibody specificity of the newly acquired SERT antibody a preadsortion control was performed by preincubating the antibody (1:1000) in 10^{-5} M of the antigen (SERT

peptide, mouse; Advanced Targeting Systems) at 4ºC overnight (Beltz & Burd, 1989). The precipitating antigen-antibody complex was separated using an ultracentrifuge (Sorvall 90SE) and spun at 100,000g for 30 minutes. The supernatant (preadsorbed serum) was separated and used in place of the primary antibody as described earlier.

2.4.4.2 CellTracker CM-DiI for membrane double-labeling with anti-SERT immunohistochemistry

The lipophilic carbocyanine derivative CM-DiI (Molecular Probes) was used to track SERT labeling on membranes. Dissected brains of juvenile lobsters were first sectioned (100µm) and than incubated in CM-DiI (2.0µM) for 5 minutes at 37ºC followed by 15 minutes at 4ºC and slightly agitated. Subsequently, preparations were rinsed thoroughly in PBTx and fixed in 4% PFA overnight. Standard immunocytochemical techniques to label for SERT were further employed using mouse anti-SERT (1:1000; Advanced Targeting Systems) as primary antibody and goat anti-mouse Alexa 488 as secondary antibody (1:50; Molecular Probes). Preparations were then embedded in Gel Mount (Biømeda Corp.) and viewed using a Nikon compound fluorescence microscope equipped with a FITC filter (Nikon Instruments Inc., Melville, NY, USA).

2.4.4.3 Immunocytochemical co-labeling of SERT and the mitosis markers BrdU or phospho-Histone H3

For BrdU (Bromodeoxyuredine) labeling juvenile and embryonic (E 84%) lobsters were incubated in BrdU (5mg/mL seawater; Sigma; Harzsch et al., 1999) for 4 hours and subsequently dissected out at zeitgeber time 14:00 and processed as whole mounts or sections (100 µm). Antibodies used were mouse anti-SERT (1:1000; Advanced Targeting System, San Diego, CA, USA) and either rat anti-BrdU (1:50; Accurate Chemicals, Westbury, NY, USA) or rabbit anti-phospho-Histone H3 (1:200; Upstate Biotechnology, Chicago, IL, USA). Incubation time for anti-phospho-Histone H3 and anti-BrdU was 12 hours and 36 hours for

anti-SERT. Secondary antibodies were from Molecular Probes: goat anti-mouse Alexa 488 and goat anti-rabbit Alexa 594. All preparations were analyzed using a Leica TCS SP confocal microscope.

It is important to note that for the purpose of BrdU incorporation into embryonic lobsters, the egg shell of the animal had to be opened gently to allow the BrdU to enter the tissue. This procedure, however, bore the risk of killing the animal during the 4 hour process of BrdU incubation because the exogenous milieu (tried physiological lobster saline, PB and Leibovitz cell medium) inevitably infiltrates the endogenous milieu. All experiments, therefore, paid particular attention to the "well-being" of the embryos after termination of the 4 hour BrdU incubation time by carefully screening for a heartbeat of the translucent animals and for reflexes such as tail flipping upon touch.

2.4.4.4 Serotonin upregulation: Immunocytochemical labeling against SERT and serotonin (5-HT)

Beltz et al. (2001) have shown that in the brain of juvenile lobsters a small number of cells transiently take up serotonin and have suggested that this uptake is due to a transporter mechanism. To test whether SERT is part of this uptake mechanism, dissected brains were incubated for 3 hours at 10-12ºC in the dark in 10^{-5}M serotonin in Leibovitz culture medium with salts adjusted to the concentrations of physiological lobster saline (NaCl (27g/L), KCl (1.19g/L), $CaCl_2*H_2O$ (3.82g/L), Cl_2*6H_2O (1.63g/L), HEPES (2.38g/L)). Brains were then rinsed in saline and fixed in 4% PFA overnight. Standard immunocytochemical techniques were applied using mouse anti-SERT (1:1000; Advanced Targeting Systems) and rabbit anti-serotonin (1:1000; DiaSorin). Secondary antibodies used were goat anti-rabbit Alexa 488 (1:50; Molecular Probes) and goat anti-mouse Alexa 594 (1:50; Molecular Probes). All preparations were analyzed using a Leica TCS SP confocal microscope.

2.5 BrdU labeling and feeding experiment*

This set of experiments is part of the 'feeding experiment' (see 2.3.3) and was performed in parallel to electrochemical studies presented here. To determine the number of proliferating neurons for each of the six sampling times, three juvenile animals were incubated in 5 mg BrdU/ml (Sigma) seawater for 3 hours. This protocol therefore sampled proliferative activity for 18 hours out of a 24-hour period. Prior work (Harzsch & Dawirs, 1996; Harzsch et al, 1999; Benton & Beltz, 2002) established that the BrdU concentration used here results in intense labeling of a maximum number of cells. The 3 hour incubation time was chosen based on the necessity to resolve the BrdU incorporation at various time intervals during a 24-hour period. Studies in other crustaceans have also shown that the cell cycle of postembryonic neuroblasts is 2-3 hours (Harzsch & Dawirs, 1996). Thus, the chosen incubation time most likely was sufficient to capture a complete cell cycle.

Following BrdU incubation, brains were immediately dissected out and immersed in 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4 for 12-17 hours. Brains were processed as whole mounts by standard immunocytochemical techniques for detection of BrdU using the fluorescent marker Alexa 488 for visualization of the nucleotide label (Harzsch et al., 1999). Brains were examined using a Leica confocal microscope (Leica Inc.), and labeled cells in cluster 10 were counted blindly by stepping through a serial scan of each preparation and marking neurons as they came into focus. Differences among sampling times were assessed using ANOVA statistics with *post-hoc* analysis (SPSS, Inc.).

* Even though I participated in the experiment, Erin Goergen (formerly Wellesley College; now University of Nevada at Reno) was the person responsible for planning and conducting the experiment. Both data (serotonin and neurogenesis) are currently in preparation for publication (M.Wildt, E. M. Goergen & B.S. Beltz "The modulatory effect of feeding on serotonin levels and neurogenesis in the brain of the American lobster, *Homarus americanus*"; see 7.3).

2.6 General immunocytochemical protocol

0. Incubate life animal in BrdU (5mg/mL seawater) for 4h*
1. Dissect out brain in cold lobster saline
2. Fix brain in 4% PFA; overnight
3. rinse 6x/15 min in 0.1M PBTx
4. rinse in 2N HCL 20 min*
5. rinse 6x/15 min in 0.1M PBTx*
6. 1stAB; incubation time may vary to achieve best staining
7. rinse 6x/15 min in 0.1M PBTx
8. 2nd AB; overnight
9. rinse 4x/20min in PB
10. mount and view

* only apply when labeling against BrdU

2.7 Sequential listing of all immunocytochemical experiments performed

labeling against	**aim** (focuses on AL, OL and cluster 10 only)	**results**
whole brain L/D serotonin	verify HPLC data	no statistical difference within the labeling intensity
whole brain D/D serotonin	verify HPLC data	due to two outliers no statistical difference within the labeling intensity
SERT	1) establish anti-SERT antibody in Crustacea (lobster) 2) elucidate whether SERT presence at the site of life long-neurogenesis?	Good labeling in cortex of AL, Antenna II neuropil, soma cluster (cl) 9/11, lateral antennular neuropil, protocerebrum and cl 10.
SERT peptide	is immunoreactivity specific to the SERT antigen?	Anti-SERT labeling is eliminated using the SERT peptide
SERT + 5-HT	is SERT expressed at the site of serotonin labeling?	extensive SERT labeling can be found throughout the glomeruli of the AL.
SERT + 5-HT upregulation	Beltz et al. (2001) have shown that in the brain of juvenile lobsters a small number of neurons transiently take up serotonin after these neurons have been born, thus suggesting the activity of a serotonin transporter mechanism. If the newly established anti-SERT antibody labels those neurons, further evidence would be provided that the serotonin transporter most likely is involved in the uptake mechanism.	single anti-serotonin labeled cells in cluster 10 but anti-SERT labeling is difficult to localize (faint labeling).
SERT + BrdU	can SERT be found among the site of mitotically active cells?	SERT labeling can be found in close proximity to BrdU labeled cells. However, since anti-SERT most likely labels on the outer cell membrane and anti-BrdU labels DNA, it is extremely difficult to distinguish positive labeling on the membrane of mitotically active cells.
SERT + phospho-Histone H3	does a different mitose marker give more pronounced results?	ring of anti-SERT labeled cells around mitotically active cells (8-12µm) within the region of life-long neurogenesis.
melatonin	does the intensity of melatonin labeling vary over 24-hours?	no significant variation can be noticed. Anti-melatonin labels within the medulla terminalis, AL, OL and median protocerebrum
melatonin + SERT	is there a spatial correlation between melatonin and SERT labeling?	both anti-melatonin and anti-SERT label the cortex of the AL. However no double labeling, thus spatial overlap, could be noticed. Goat anti-sheep secondary antibody (Molecular Probes) cross reacts (precipitates) with goat anti-mouse (Molecular Probes). Therefore, use donkey anti-sheep (Molecular Probes) as secondary antibody for melatonin.
melatonin + BrdU	is melatonin involved in the process of life-long neurogenesis?	no double labeling could be noticed within the proliferation zone of cluster 10

Table 2. Sequential listing of all immunocytochemical experiments that have been performed during the time course of this research project.

3. Results

3.1 HPLC measurements of circadian brain serotonin levels

Serotonin levels under L/D conditions. Rhythmic changes in brain serotonin levels could be observed in all of the animals subjected to an entraining 12:12 L/D light cycle (Group 1; Figures 27-39; see 7. Appendix). The overall common feature in all experiments conducted is a pronounced decrease in the levels of brain serotonin at the beginning of the light phase (Figures 28-35). Additional circadian characteristics (peaks and troughs) appear more diverse. Analysis performed after the animals had been entrained to a 12:12 L/D light cycle with lights on at 6:00 and lights off at 18:00 (normal L/D) show high levels of serotonin surrounding dusk (Figures 28, 29A, 32A, 33). Contrasting to these findings, however, is the demonstration that the characteristic peaks vary when exposed to an entraining light cycle that had been reversed from the normal L/D rhythm (lights on at 18:00 and off at 6:00) (Figures 29 and 33). Not only do they diverge from the normal L/D experiment performed in parallel (compare Figures 29A and B, 33 A and B; see 7. Appendix) but also do they deviate from other reversed L/D experiments (compare Figures 29B, 32B, 33B; see 7. Appendix). Because of the multiplicity of technical difficulties such as variations in ambient temperature and hence in the sample, humidity, floor vibrations, column break-down and power failure, standard errors for most experiments are large (excluding Figure 32; see 7. Appendix) thus, eliminating any statistical differences.

Serotonin levels under D/D conditions. A consistent pattern in brain serotonin measurements, with the major peak of serotonin occurring prior to subjective dusk and a trough in serotonin before subjective dawn (Figure 11) can be observed in the brains of lobsters subjected to D/D conditions for 3 days following maintenance in L/D conditions for at least 2 weeks (Group 2). Imposing a shift in the L/D cycle before entering 3 days in D/D (Group 3) results in a phase shift of the peak and trough (Figure 11B, C), indicating that brain serotonin levels are light entrainable. Despite the variability of whole brain serotonin levels among the individuals in each group, visual inspection of the chronograms suggest that in constant darkness the changes in serotonin levels precede the time at which the light changed

during the conditioning L/D regime. Using a 3-way ANOVA to test this possibility, a significant correlation (p=0.039) between an "anticipatory" rise or fall in serotonin level and the occurrence of the light-off or light-on, respectively (Figures 11A, 12) was found when D/D data from the normal and time-shifted Groups (2 and 3) were pooled. These data confirm the existence of a pre-dusk maximum and pre-dawn minimum in the chronograms.

Serotonin Levels in the OLs and ALs. Experiments also were conducted to determine whether the OLs and ALs, two midbrain regions with distinct functions and intense serotonergic innervation, individually exhibit rhythmic changes in serotonin levels. In Group 2 animals, the OLs show a clear rhythm in serotonin levels (Figure 13A-D) with a peak before dusk, and a sharp decline at dusk to a level that is maintained throughout the subjective night phase and into the subjective daytime hours. The most distinctive aspect of the OL serotonin rhythm is the peak that occurs prior to subjective dusk (paired t-test comparing the immediate levels preceding the peak and the levels at the peak itself; OL, p=0.016), a feature that is reminiscent of the whole-brain fluctuations in serotonin content (Figure 11). A similar pattern in serotonin levels was measured in the rest of the brain (brain remainder, p=0.0002) (Figure 13B, E), which was comprised of the protocerebral and tritocerebral areas, and medial deutocerebral regions such as soma clusters 9 and 11, the lateral antennular neuropil and the olfactory globular tract (see Figure 1; 1. Introduction). The ALs, however, show a rhythm in serotonin levels that is distinct from that of the OLs and the rest of the brain. High levels in serotonin were measured in the ALs beginning at the hours around dusk, as in the OLs; however, high levels in serotonin were measured throughout the night, with a drop at subjective dawn (paired t-test; AL, p=0.242) (Figure 13C, F).

If the levels of serotonin measured in the OLs, ALs and the rest of the brain are added together for each of the 6 time points, the resulting graph (Figure 14) shows the relative proportions of serotonin found in the different brain regions. These histograms illustrate that the serotonin content in the OLs and ALs accounts for ~70% of total brain levels. As in the whole brain studies (Figure 11), the pre-dusk peak is the most striking feature of Figure 14. Examination of the components of this pre-dusk peak show that serotonin levels in the ALs rise by 18%, the OLs by greater than two-fold (123%), and the rest of the brain by roughly three-fold (197%) over the pre-dawn values.

The critical role of the dorsal giant neuron (DGN) when dissecting. The DGN is a prominent serotonergic neuron on each side of the brain. Its massive cell soma lies on the

dorsal surface of the brain and its neurite projects into each and every glomerulus of the adjacent accessory lobe (AL) and olfactory lobe (OL) (Figure 15; Sandeman and Sandeman, 1987; 1994; Sandeman et al., 1995; Beltz, 1999; Benton and Beltz, 2001). Because of the close position of the serotonergic cell soma of the DGN to the AL and OL, it is important to verify that this cell soma is consistently attached to either the AL or the OL when dissecting. Mixing the DGN with any of the lobes during a 24-hour experiment will result in false serotonin measurements (compare Figure 13 to Figure 37 and 39 in 7. Appendix). Differential interference contrast microscopy (DIC; Nomarski) and immunocytochemical labeling against serotonin, however, demonstrated that the cell body of the DGN did not repeatedly stay with either lobe (data not shown). In order to interpret the true serotonin content per lobe, the DGN was, henceforth, consistently dissected out with the rest of the brain composed of the protocerebral and tritocerebral areas, the medial deutocerebral regions, the lateral antennular neuropil, the medial antennular neuropil, the olfactory globular tract and the antenna II neuropil using DIC (experiment 18).

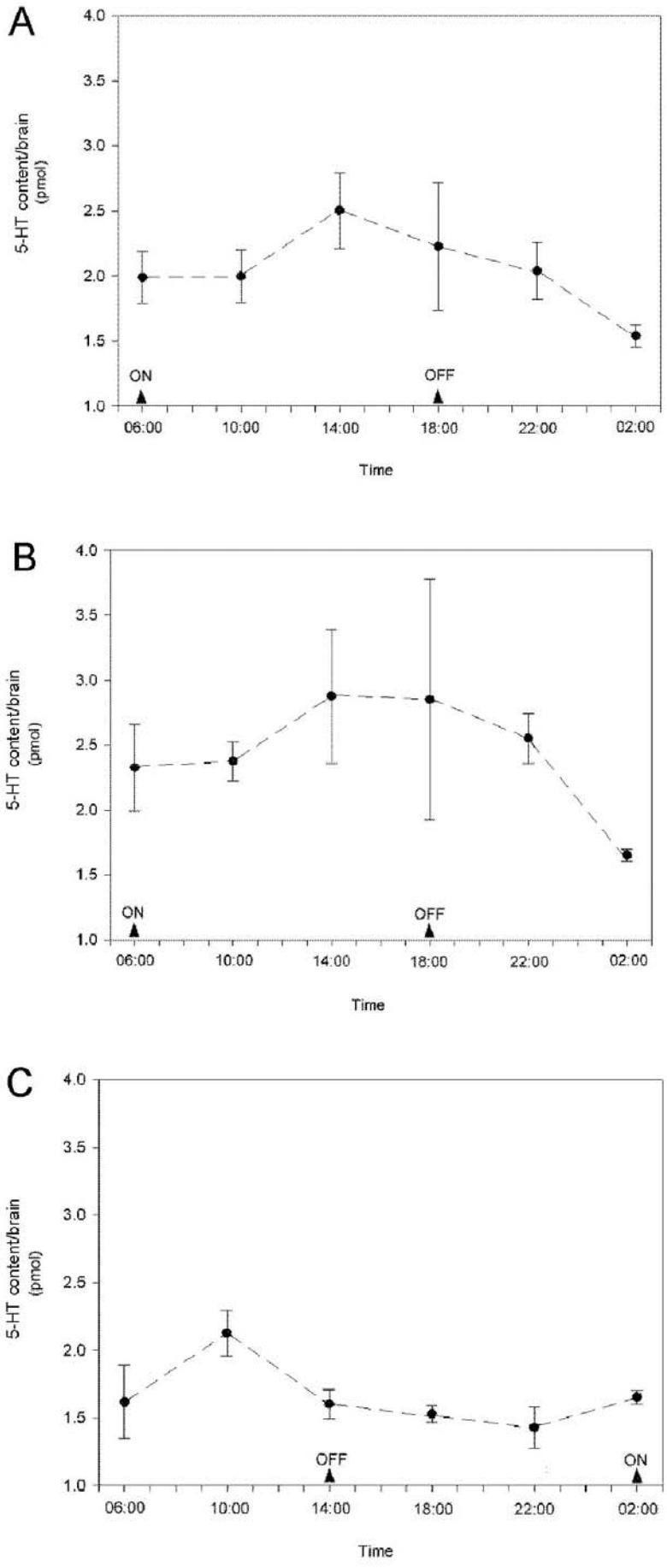


Figure 11. Chronograms in which the serotonin (5-HT) levels in whole lobster brains measured by HPLC are shown over a 24-hour period, plotted as means ± SEM. All three graphs show the measurements taken after the animals had been in D/D for 3 days without food. The timing of light-on and light-off experienced by the animals during their entraining L/D periods are marked by the black triangles. Note that in 3C light-off has been phase shifted by 4 hours in time, and occurred at 14:00. A) Pooled data from B) juvenile lobsters that had been maintained for 2 weeks on an entraining 12:12 L/D cycle and C) brains of animals that had been subjected to a phase shifted light cycle. In matching subjective light-on and light-off of the two groups, the pooled data (A) show a pre-dusk peak and pre-dawn trough (the peak prior to subjective dusk is significantly different (p=0.039) from the trough prior to subjective dawn). B), C) Plotting the data for

the two groups separately, illustrates the phase shift of the peak and trough in the B) animals that had been subjected to the 4 hour shifted light regime. B), C) n=4/time point; A) n=8/time point; these data represent experiments 11 and 16 (see Table 1 in 2. Material & Methods).

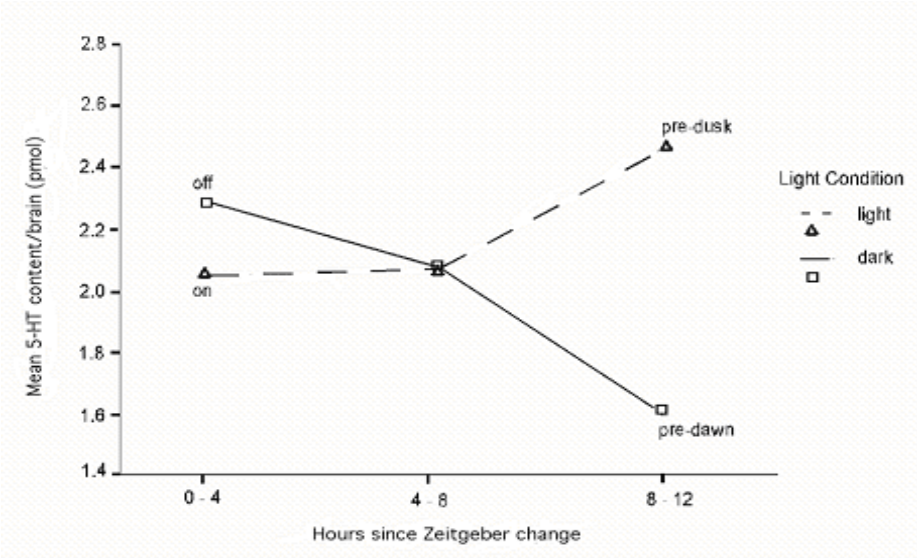


Figure 12. An "anticipatory" rise or fall in the level of serotonin in whole lobster brains is revealed when the means of serotonin (5HT) content (data from Figure 11A) is pooled and plotted against the hours since light-on or light-off in a 3-way ANOVA (SPSS Inc., Chicago, IL, USA). Little change in serotonin levels follows light-on or light-off either in the 0-4 or 4-8 hour time periods after they have occurred. In the 8-12 hour period however, the levels in the animals approaching dusk rise, and those approaching dawn decrease. These changes occur well *before* the light-on or light-off transition.

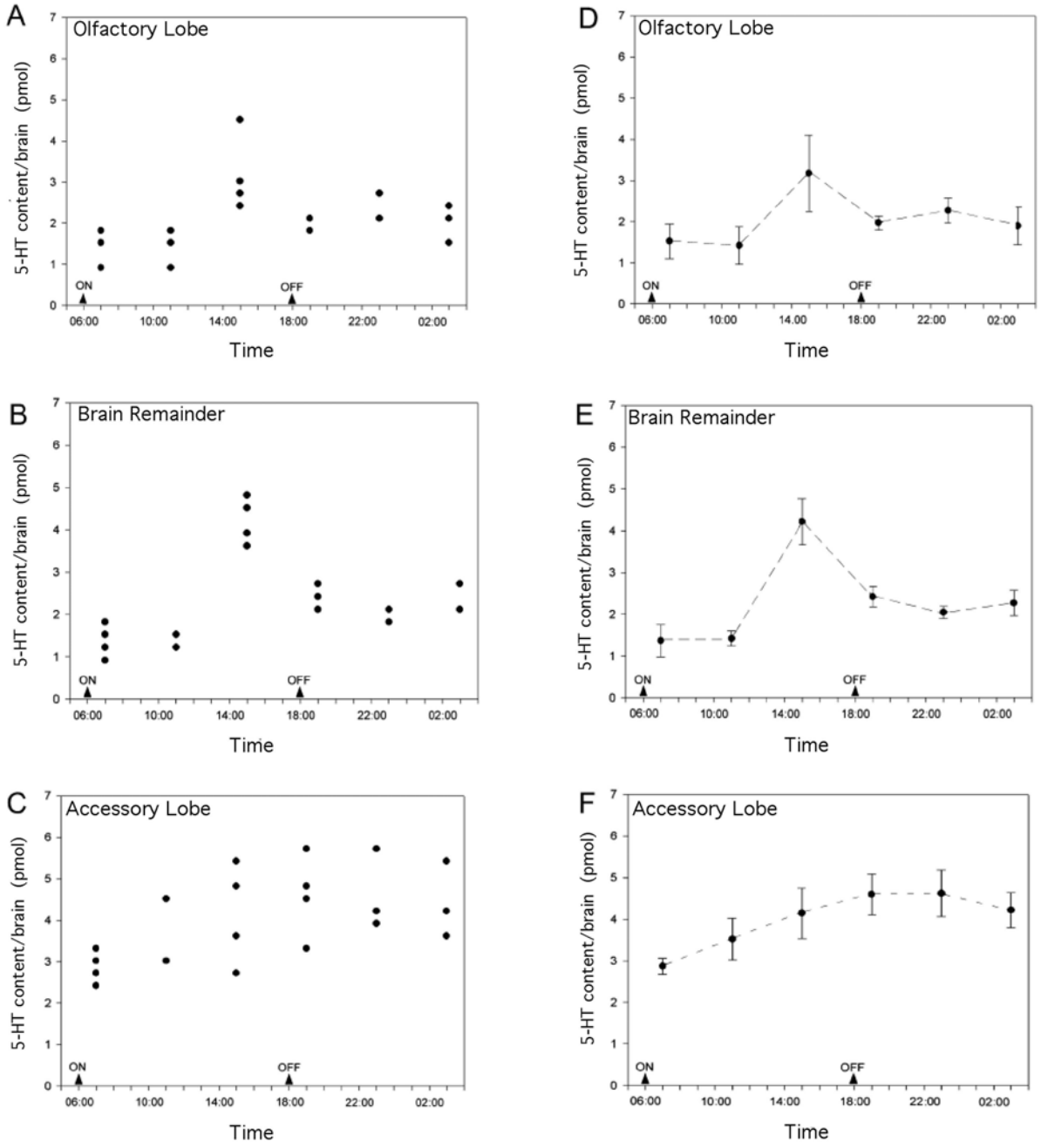


Figure 13. Separate analyses of serotonin levels in individual regions of the brains of juvenile lobsters that were entrained to a 12:12 L/D light regime followed by 3 days in D/D. Chronograms on the left show the raw data collected from 4 individuals at each time point (values for some measurements were the same and so appear as a single point). Chronograms on the right show the arithmetic means (± SEM) of the data. The most distinctive aspect of the serotonin levels of the OL and the brain remainder (consisting of the protocerebral and tritocerebral areas, the medial deutocerebral regions, the lateral antennular neuropil, the medial antennular neuropil, the olfactory globular tract, and the antenna II neuropil), is the abrupt rise to a peak that occurs prior to subjective dusk followed by an exponential-like decay (A, B, D, E). In contrast, serotonin levels in the ALs rise during the subjective day to a high around dusk,

are sustained throughout the night hours, and drop at subjective dawn (C,F). T-tests between the peaks (AL: 19:00; OL: 15:00; BR: 15:00) and troughs (AL: 7:00; OL: 11:00; BR: 11:00) in serotonin levels show significant differences in each of these brain regions (AL, *p*=0.016; OL, *p*=0.029; BR, *p*=0.00034). n=4 for each time point. These data represent experiments 14, 16 and 18 (see Table 1 in 2. Material & Methods).

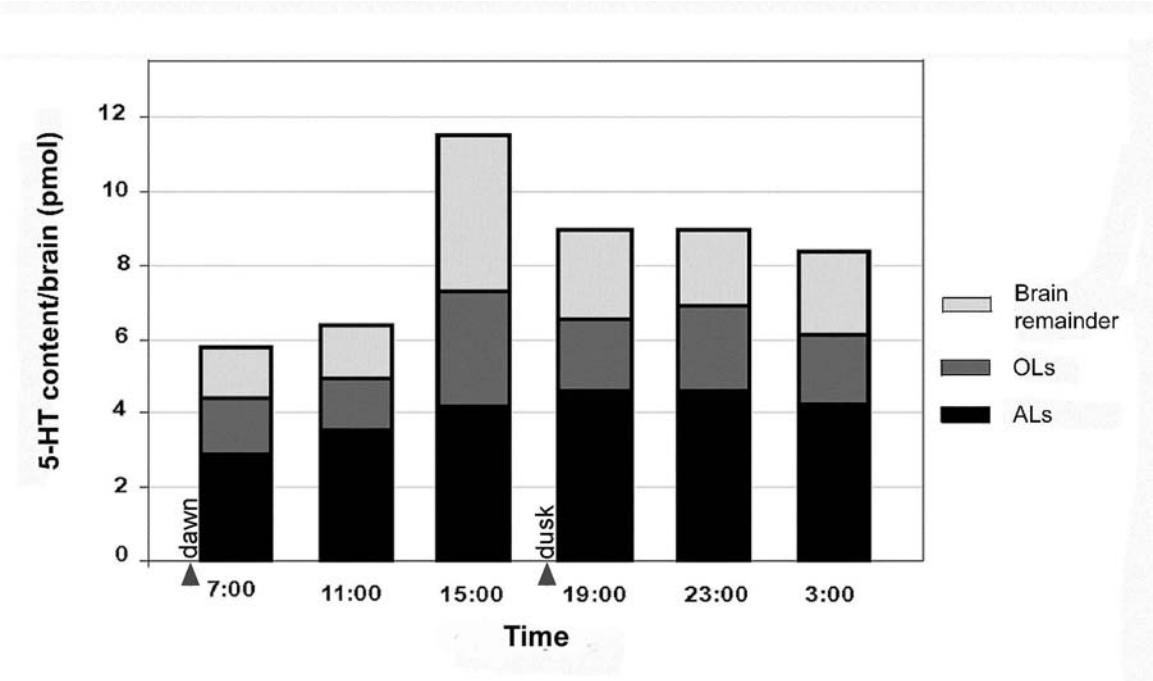


Figure 14. Combined serotonin levels in various areas in the brains of juvenile lobsters. The levels of serotonin in all the ALs (black), OLs (dark gray) and brain remainders (light gray) are added together for each of the 6 time points (data from Figure 13A-C). As in the whole brain studies (Figure 11), the pre-dusk peak is the most pronounced feature. Comparisons of the changes in serotonin content in individual brain areas at the pre-dusk peak (11:00 compared to 15:00) show that serotonin levels in the ALs rise by 18%, the OLs by 123% and the brain remainder by 197% during this period.

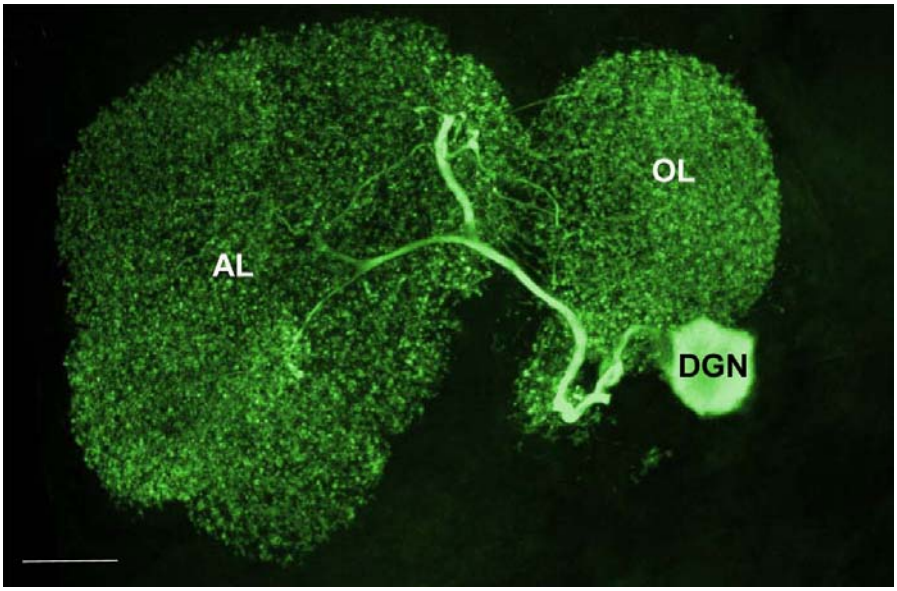


Figure 15. Serotonergic dorsal giant neuron (DGN) innervating each and every glomerulus of the accessory lobe (AL) and the olfactory lobe (OL). Scale bar: 50 µm. (Image taken from Benton & Beltz, 2001).

3.2 HPLC measurements to determine the effect of feeding on circadian brain serotonin levels and neurogenesis

Surprise feeding and the impact on serotonin levels. To avoid masking effects, 36 lobsters (Group 1*) that had been entrained to a 12:12 L/D cycle for 2 weeks (the conditioning light regime), were introduced to D/D conditions for 3 days prior to the experiment with food being supplied every other day 1 hour before dusk. Prior D/D experiments which excluded feeding during the time of constant conditions indicated a trough in brain serotonin levels prior to dawn and a peak prior to dusk (Figure 11). When animals were fed at the time when brain serotonin levels had previously been demonstrated to be low, brain serotonin levels show a tendency to immediately increase (Figure 16). But because the standard errors are large the differences are not statistically significant ($p>0.05$). Further analysis indicates a second peak 12 hours after feeding. This coincides with the endogenous peak of serotonin demonstrated earlier (compare to Figure 11).

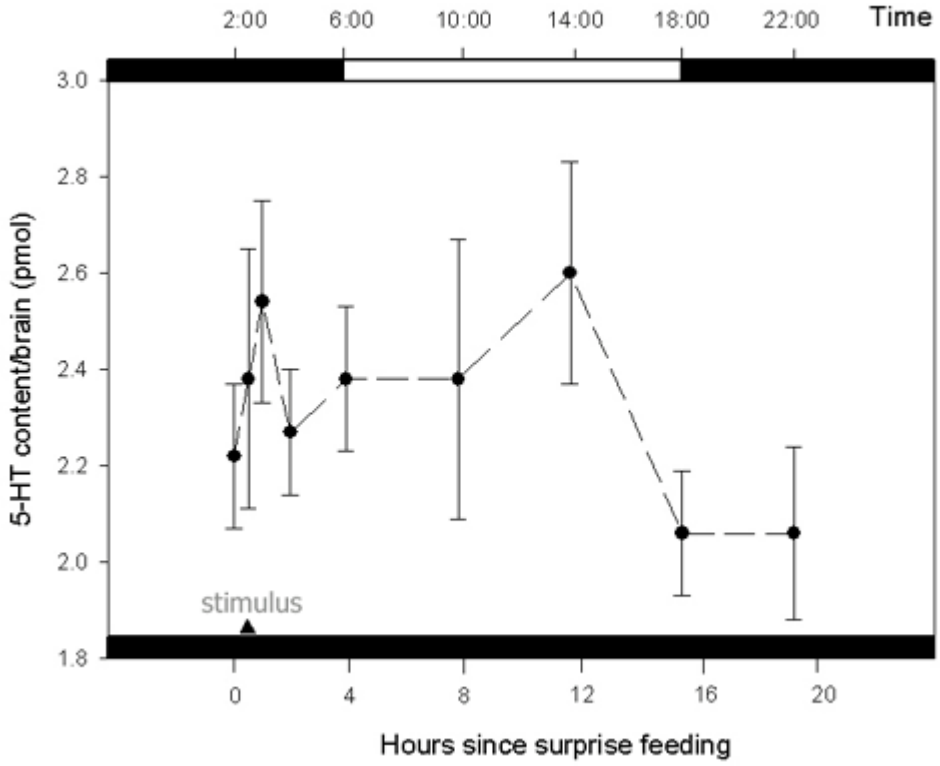


Figure 16. Chronogram in which the serotonin (5-HT) levels in lobster brains measured by HPLC after surprise feeding are shown over a 24-hour period, plotted as means ± SEM. Food was supplied 15 hours prior to entrained feeding (ZT 2:00 as compared to ZT 17:00). The graph shows a tendency of elevated levels immediately after feeding and again at pre-dusk. Measurements were taken instantaneously before feeding (0), 30 minutes, 1 hour, 2 hours, 4 hours (4), 8 hours (8), 12 hours (12), 16 hours (16) and 20 hours (20) after the initial stimulus was given. The dark bar at the bottom of the graph indicates illumination 3 days prior to sampling and the light/dark bar on top of the graph indicates illumination during the entrainment period. Triangle indicates time of feeding. n=4/time point. This experiment represents experiments 21 and 25 (see Table 1 in 2. Material & Methods).

Beads induce greater physical activity than feeding. Physical activity is prolonged in lobsters when given a bead (Group* 4; Figure 17). Animals given frozen *Artemia* show a short-term increase in activity while gathering food but 30 min after the induced feeding no further physical activity could be monitored. Because lobsters that were excluded from any food stimulus were housed in the same room but in a different tank of equal water quality as animals given a bead, an immediate response in their physical activity was observed when the "feeder" entered the room. About 2 minutes into the appearance of the "feeder", the animals

settled into a more quiescent state. Only animals that were given a plastic bead as a stimulus showed heightened physical activity for ~13 hours after the stimulus had been provided. Also for each of the 4 time points (18:00, 22:00, 2:00, 6:00) after the bead stimulus had been given, escape responses such as tail flipping could be monitored while capturing these animals.

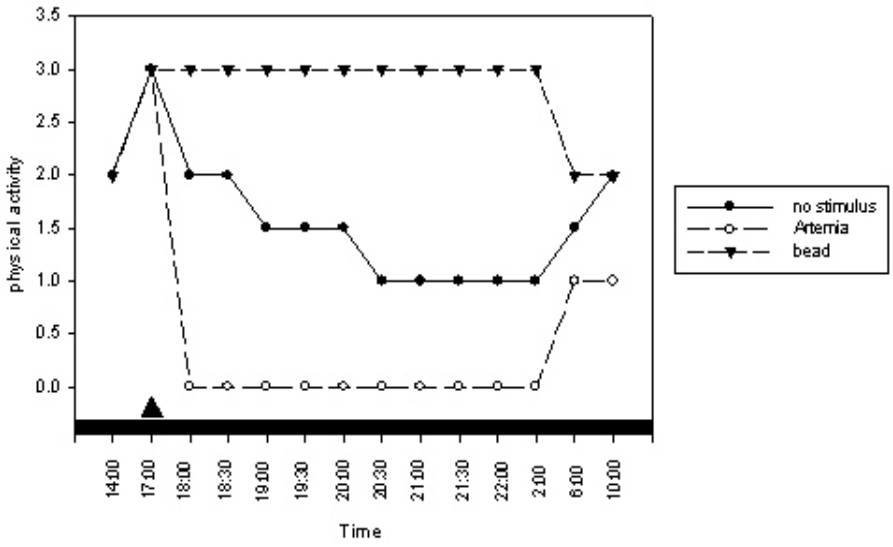


Figure 17. Observational analysis of animals that were either given "no stimulus", "*Artemia*", or a "bead" show substantial differences after the time of "feeding" at ZT 17:00 over 24-hours. Animals physical activity was scored on a scale from 0 (not active) to 3 (very active). Dark bar at the bottom of the graph indicates illumination 3 days prior to the experiment. Triangle indicates time of "feeding". n=4/time point. This observational experiment was performed in parallel to experiments 24 and is not listed as a separate experiment in Table 1 (see 2. Material & Methods).

Food or physical activity as a modulator of brain serotonin levels and neurogenesis? To determine the effect of some of the underlying components of feeding on the endogenous rhythms of serotonin and neurogenesis, animals in a 12:12 L/D cycle were entrained to pre-dawn feeding for 2 weeks, and then either given a bead (to test for the effect of physical activity), *Artemia* (to test for the effect of chemosensory stimulation and caloric intake), or no stimulus (control) the day of the experiment. Animals that received no immediate stimulus showed increased levels of both serotonin and neurogenesis surrounding dawn, the time of entrained feeding, when compared to the corresponding time point of pre-dusk fed animals (compare Figure 18 and 19A-C). The most distinct features of the serotonergic rhythm in this

set of experiments is a peak prior to the conditioning feeding time point and a second peak prior to subjective dusk (Figure 19A) suggesting that brain serotonin levels can be entrained to *both* feeding time and the light dark cycle. In contrast, the peak level of neurogenesis was shifted from the normal dusk peak to a pre-dusk peak (data E. Goergen, Wellesley College), coinciding with the serotonin peak (Figure 19A). Animals that received *Artemia,* on the other hand, show elevated levels of serotonin and neurogenesis for 5 (serotonin) to 9 hours (neurogenesis) post stimulus (Figure 19B). Serotonin levels thereafter show a tendency to collapse to immediately recover again; however, levels of neurogenesis decrease and stay low for 8 hours before they begin to increase again after dusk. Likewise, animals that were given a bead show a significant immediate ($p<0.05$) increase in neuronal birth and a tendency of an increase for serotonin levels (Figure 19C). Again, serotonin levels in these animals stayed high for the first 5 hours and drop to show an absolute trough 17 hours after feeding. The rate of neurogenesis in animals fed with a bead in comparison dropped significantly after the first post-feeding sampling and peaked again 13 hours later at pre-dusk. Overall, the dampened rhythm for serotonin of Group 3*+4* animals suggest that food consumption (Figure 19B) and heightened physical activity (Figure 19C) may individually balance out the characteristic pattern of the circadian rhythm of brain serotonin levels. In addition, when comparing the number of newly born cells within the three treatments over a 24-hour period, animals that were given a bead produce fewer neurons than those that received *Artemia* or nothing at all, however this effect is not significantly different. Interestingly, statistical analysis using ANOVA with post-*hoc* analysis (Bonferroni and Scheffe) show that the regulatory effect of light ($p=0.911$) on the circadian rhythm of neurogenesis is suppressed when feeding ($p\geq0.001$) comes into play, supporting Stephan's (2002) conclusion "when food competes with light, food usually wins". Taken together, our data demonstrates that the time of feeding alters the endogenous rhythms of brain serotonin levels and neurogenesis. Further, physical activity in the absence of caloric intake has a tendency of a negative effect on neuronal production and feeding suggests to be a stronger entraining signal than light.

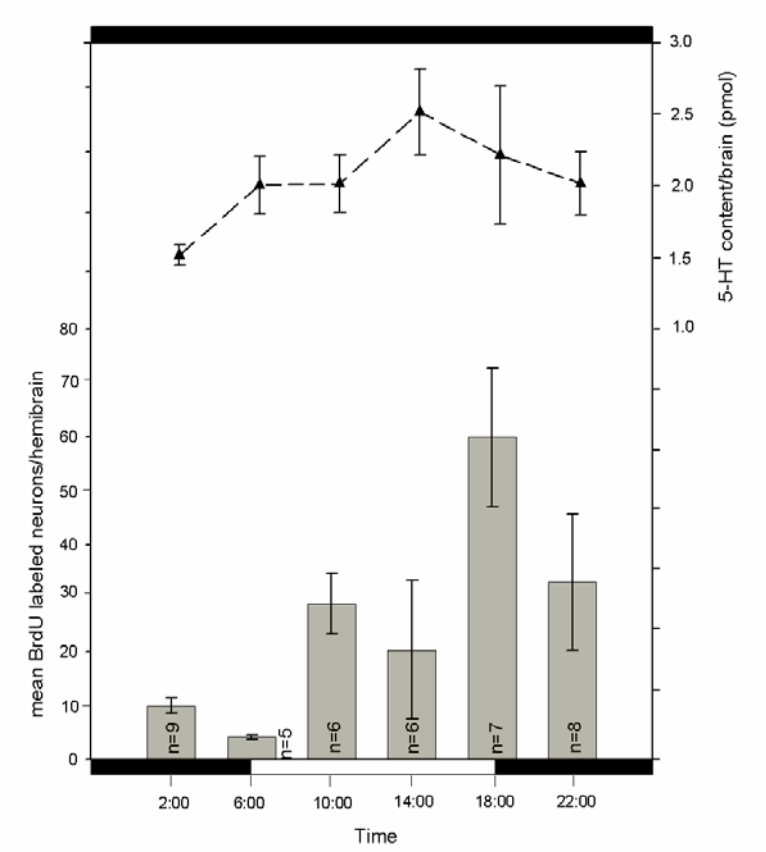
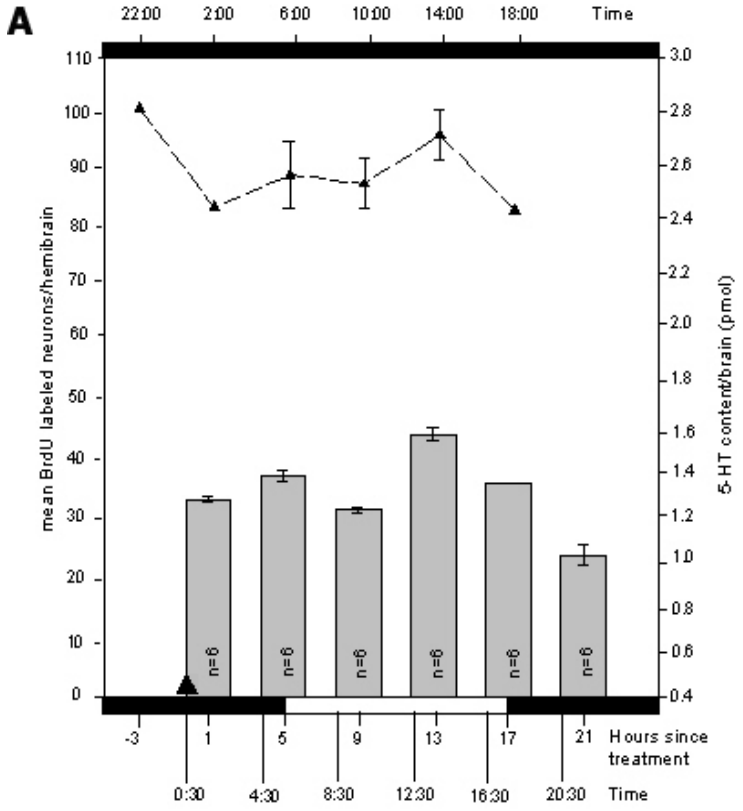


Figure 18. Chronogram shows the endogenous circadian rhythm of brain serotonin (line graph) when measured after a 12:12 L/D followed by 3 days in constant darkness as indicated by the dark bar on top of the graph. Vertical bars indicate the mean counts of BrdU labeled neurons in the projection neuron cluster relative to time of day for lobsters maintained on the normal L/D light/dark cycle as indicated by the light/dark bar at the bottom of the graph. Note that animals presented in the BrdU study were given food 1 hour before dusk at the day of the experiment whereas animals participating in the serotonin study did not get fed the day of the experiment. Both trial groups were entrained to pre-dusk feeding as indicated by the dark triangle. The numbers of samples assessed for each time point are indicated within the respective vertical bars. n=4/time point for serotonin measurements. (Graphs taken and combined from Figure 11 and Goergen et al., 2002).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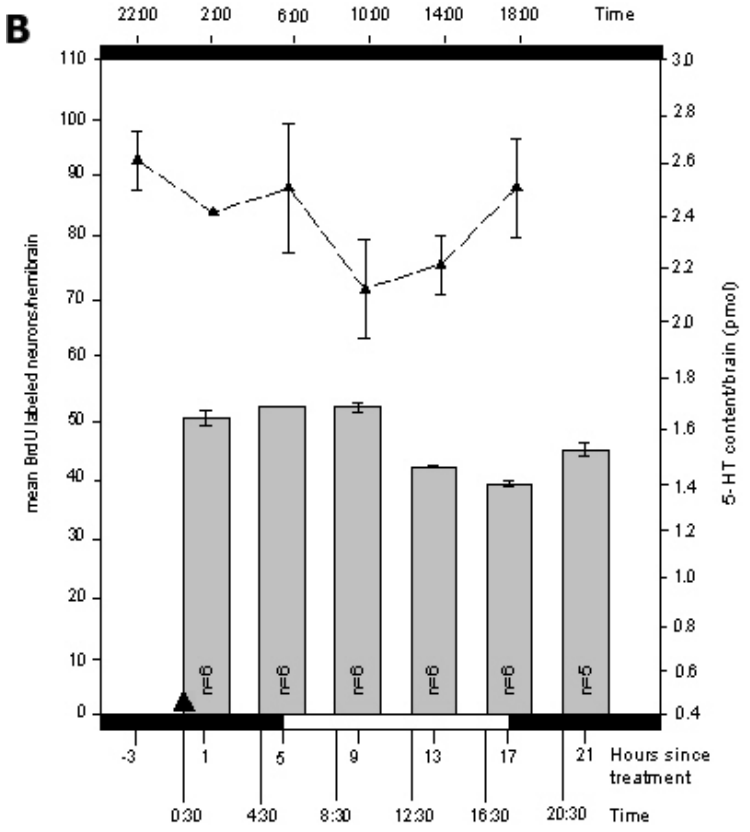
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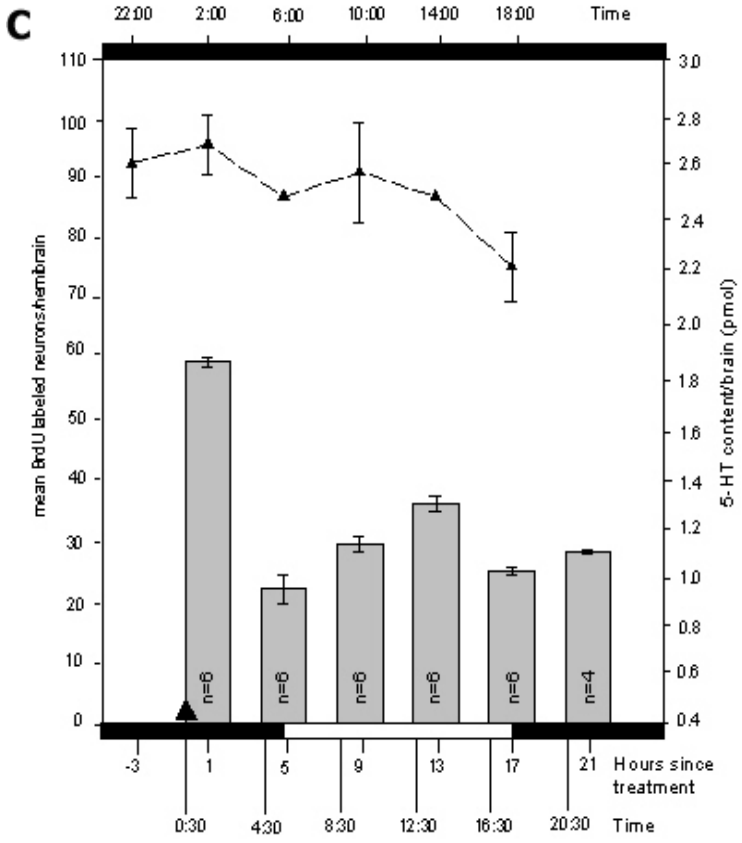


Figure 19. Chronograms in which the serotonin (5-HT) levels in lobster brains measured by HPLC (line graph) and the mean number of BrdU labeled neurons per hemibrain (bar graph) after fictive feeding are shown over a 24-hour period, plotted as means ± SEM. To avoid masking effects by light all three graphs show the measurements taken for serotonin after the animals had been in D/D for 3 days; indicated by dark bar at the top of the graph. Because masking effects by light are not known for neurogenesis specimen used for BrdU labeling remained under the entraining 12:12 L/D light cycle as indicated by the light/dark bar at the bottom of the graph. Note that serotonin measurements were taken 4 hours prior to feeding to establish a baseline for HPLC analysis. A) "no stimulus": serotonin levels are high prior to entrained feeding and then again before subjective dusk. BrdU labeled neurons shown an increase in mean numbers surrounding dawn and an absolute increase before dusk, thus being similar to serotonin levels. B) "*Artemia*": serotonin levels are elevated 5 hours post *Artemia* feeding. After a short decrease serotonin levels peak again at subjective dusk. Numbers of BrdU profiles are elevated 9 hours post stimulus and then decrease. A second peak can be noted again during the early night. C) "Bead": animals that were given a bead show elevated levels of serotonin until subjective dawn. An absolute trough in serotonin levels shows at subjective dusk. Mean number of BrdU labeled neurons is highest at the time of "feeding" and than increases again before dusk. Triangle indicates the time of "feeding". The numbers of samples assessed for each time point of BrdU profiles are indicated within the respective vertical bar. n=4/time point

for serotonin measurements. This experiment represents experiment 24 (see Table 1 in 2. Material & Methods).

3.3 HPLC measurements of brain melatonin content

Circadian changes in the levels of brain melatonin are observed in juvenile lobsters. Under normal L/D conditions brain melatonin levels show a dramatic increase during the first hours of light (~ 330%) and a potential but not significant (p>0.05) second peak after dusk (Figure 20A). Experiments performed after the animals had been entrained to a 12:12 L/D light cycle followed by for 3 days in constant darkness (D/D), in contrast, show little rhythmic changes of brain melatonin during the first half of the day and a tendency towards elevated levels shortly after dusk (Figure 20B). The persistence of the melatonin rhythm under D/D conditions, although the circadian characteristics are reduced, indicates that this rhythm is endogenous. In addition, the dramatic increase in melatonin shortly after the on-set of light, suggest that this rise is stimulated by light.

Because of the minimal *endogenous* whole brain melatonin content detected, which required maximal sensitivity of the HPLC system, and the fact that this set of experiments was performed to analyze for correlations in the rhythmic fluctuations of melatonin in respect to *endogenous* serotonin (thus requiring D/D conditions), no further experiments were attempted to analyze melatonin levels within functionally distinct brain region, such as the ALs, OLs vs. brain remainder.

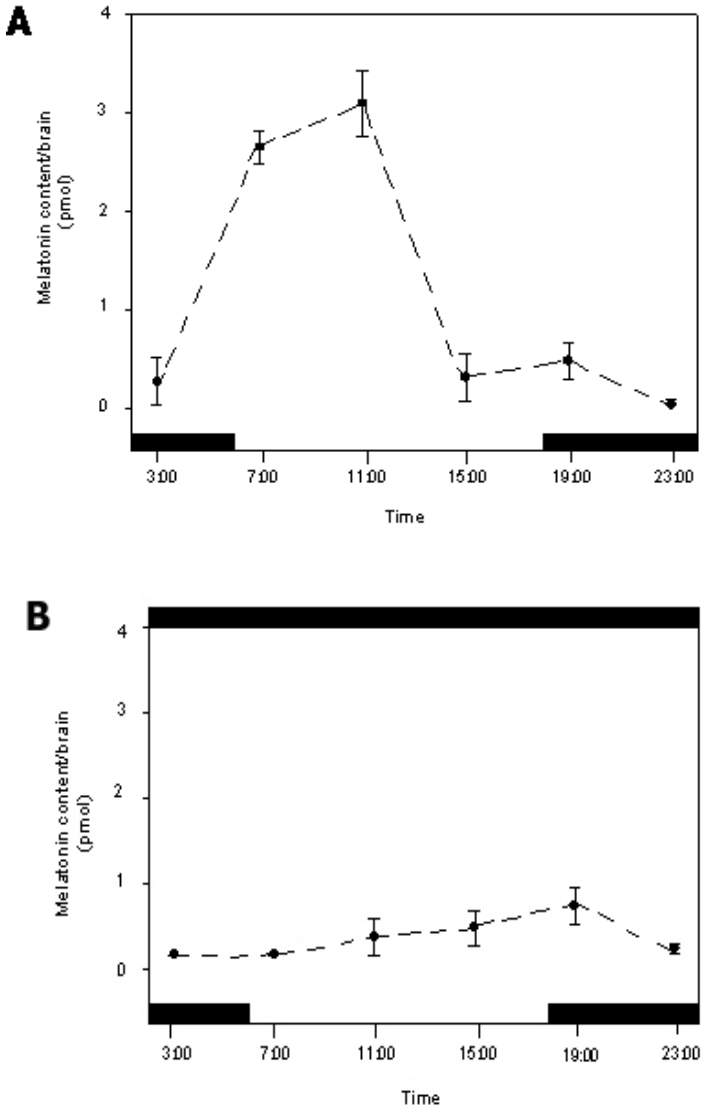


Figure 20. Chronograms show the levels of brain melatonin measured by HPLC over a 24-hour period. A) Brain melatonin levels are elevated during the first hours of light when measured under L/D conditions as indicated by the light/dark bar at the bottom of the graph and a tendency of a second smaller peak after dusk. B) The endogenous rhythm of brain melatonin, in comparison, shows high levels after dusk only. Light/dark bar at the bottom of the graph indicates the entraining light regime whereas the dark bar on top of the graph indicates illumination conditions three days prior to sampling. Note that animals in both experimental conditions were fed every other day before dusk. n=4/time point. These experiments represent experiments 22 and 23 (see Table 1 in 2. Material & Methods).

3.4 Immunocytochemistry

3.4.1 Immunocytochemistry to verify HPLC findings on circadian rhythms of brain serotonin levels

To test whether the observed differences in brain serotonin levels of Group 1 and 2 animals using HPLC are also detectable using alternative techniques, immunocytochemical labeling methods were applied. Whole mounts of brains dissected from animals reared in the same conditions as Groups 1 and 2 (see 2.3.1 in 2. Material & Methods) and killed three hours before subjective dawn and dusk were used for these studies. Labeling was measured semi-quantitatively in the OLs and ALs, regions that receive a massive serotonergic innervation. Differences in the intensity of immunocytochemical labeling for serotonin were seen between the time points in the brains of both Group 1 and Group 2 lobsters (Table 3, Figure 21). However, the intensity of labeling in the Group 1 brains was highly variable. Therefore, although the means for pre-dawn and pre-dusk brains are different, the standard errors are large and the differences are not statistically significant (see Table 1 L/D). For the Group 2 (D/D) brains dissected at pre-dusk (15:00; n=4), brains show intense labeling of the OLs, ALs, DGNs, protocerebral bridge and central body, while the brains of pre-dawn (3:00; n=4) lobsters show reduced labeling in these same regions. These qualitative observations were confirmed by densitometry measurements and statistical analyses of the ALs (p=0.050) and OLs (p=0.042) at pre-dawn and pre-dusk time points. These immunocytochemical results support the HPLC findings.

L/D Group 1	**Staining intensity (15:00)**	**Staining intensity (3:00)**	***p-value***
ALs	78.62 ± 97.70	12.38 ± 4.70	0.158
OLs	64.87 ± 78.50	14.82 ± 7.13	0.180

D/D Group 2	**Staining intensity (15:00)**	**Staining intensity (3:00)**	***p-value***
ALs	178.21 ± 48.54	124.39 ± 51.74	0.050
OLs	169.62 ± 55.94	106.48 ± 52.03	0.042

Table 3. The results of densitometric measurements of immunofluorescence in the accessory lobe (AL) and the olfactory lobe (OL). Labeling in both lobes was more intense at pre-dusk (15:00) than at pre-dawn (3:00) after the animals had been exposed to constant darkness for 3 days (D/D). No significant difference was detected between pre-dusk (15:00) and pre-dawn (3:00) immunofluorescence in the AL and OL of animals living under 12:12 L/D conditions. n=4 per time point.

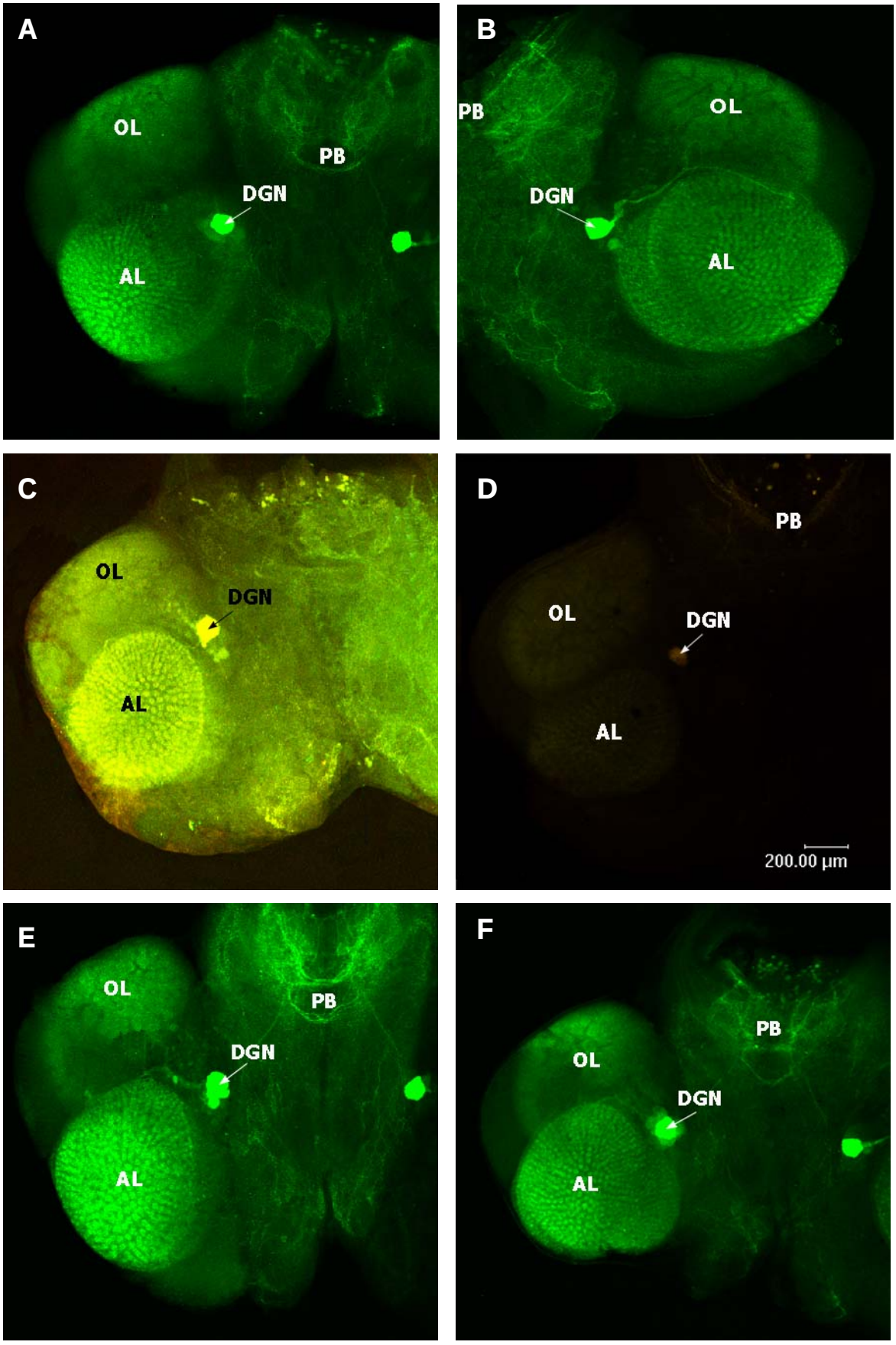
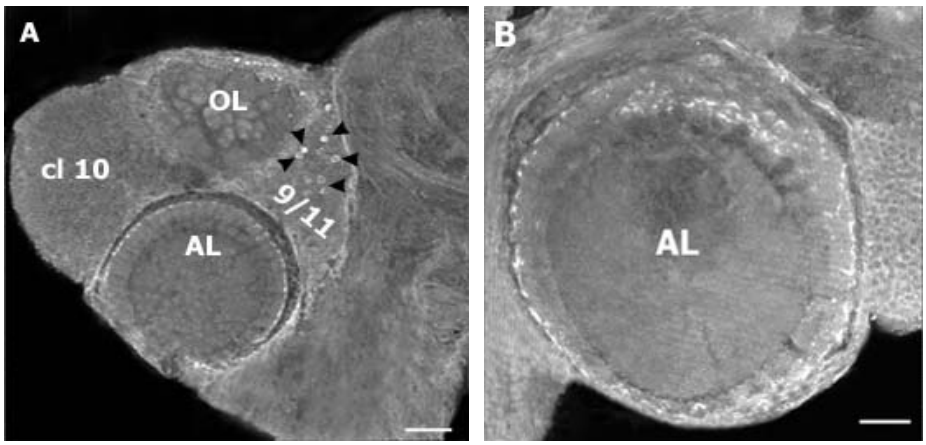


Figure 21. A-D) Immunocytochemical labeling for serotonin of whole juvenile lobster brains entrained to a 12:12 L/D regime A, C) sacrificed at pre-dusk (15:00) and B, D) at pre-dawn

(3:00). Because of outliers (C) at the pre-dusk sampling time the standard deviation is large and therefore no significant difference between pre-dusk and pre-dawn can be attributed (see Table 3). E, F) Animals entrained to a 12:12 L/D regime followed by three days in constant darkness and than sacrificed at E) pre-dusk (15:00) and F) pre-dawn (3:00) show a significant difference in the intensity of serotonin labeling (compare to Table 3). Accessory lobe (AL); dorsal giant neuron (DGN); olfactory lobe (OL), protocerebral bridge (PB). Scale bar as seen in D) accounts for all six images.

3.4.2 Immunocytochemical localization of melatonin in the brain

These experiments were conducted to determine whether anti-melatonin labeling can be found in the olfactory processing area of the brain of lobsters and whether this labeling appears in spatial correlation to anti-serotonin labeling. For this purpose, the only commercially available anti-melatonin antibody was tested and successfully established using a working concentration of 1:1000. Anti-melatonin labeling could be demonstrated within a select number of cells in cluster 9/11, as well as throughout the cortex of the olfactory lobe (OL), and along the outer cortex of the accessory lobe (AL) (Figure 22 A-C). Experiments performed over 24-hours to assess relative levels of brain melatonin immunocytochemically did not show any variations in staining intensity (data not shown).



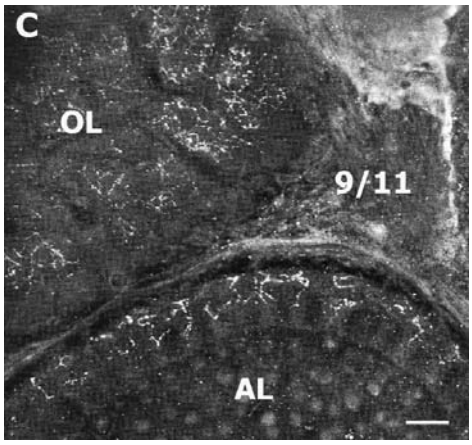


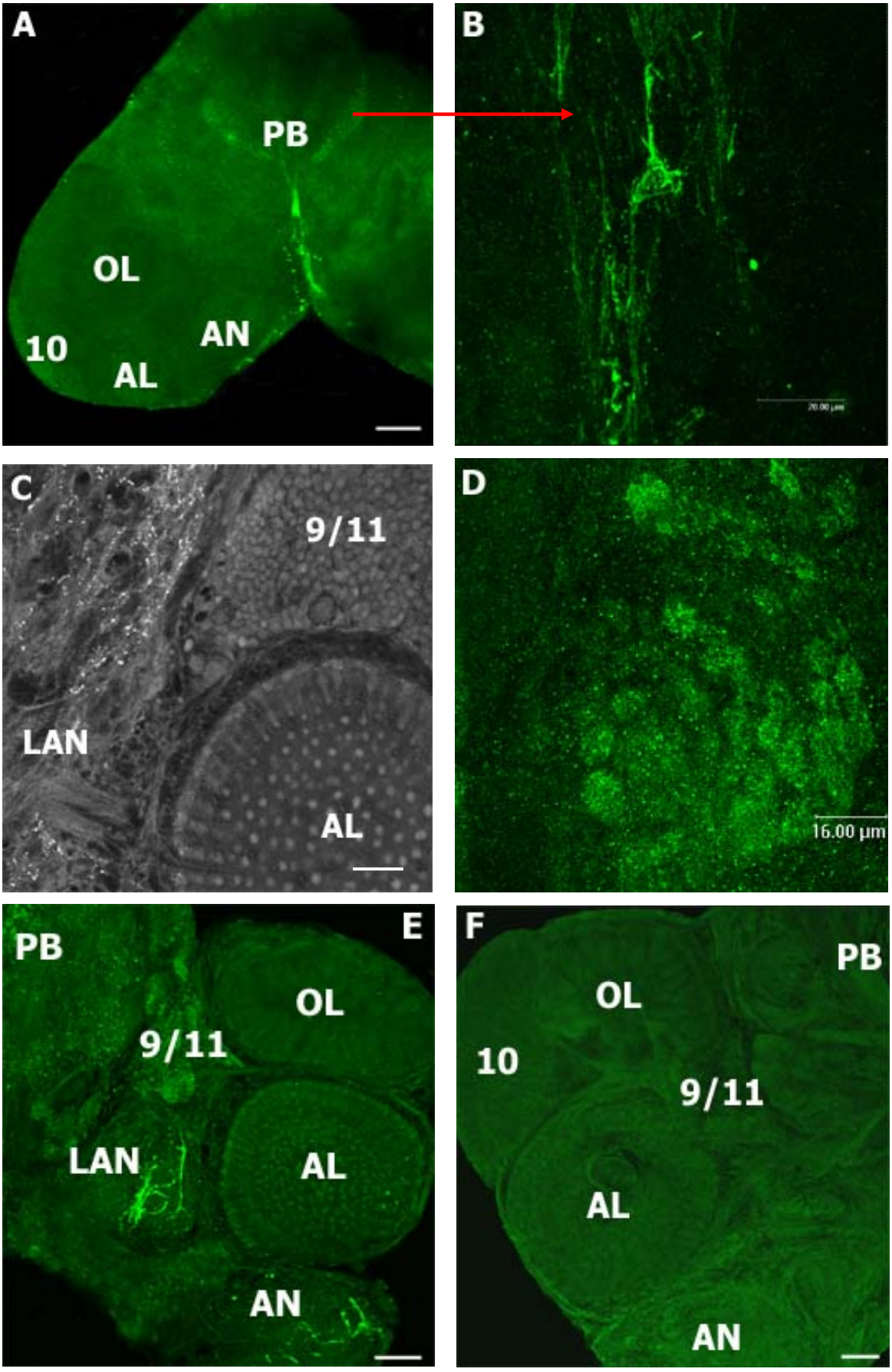
Figure 22. A-C) Sections (100µm) of juvenile lobster brains show anti-melatonin labeling within the cortex of the accessory lobe (AL), the olfactory lobe and within a few cells of cluster 9/11, arrow heads. B) accessory lobe (AL). C) olfactory lobe. 10 (projection neuron cluster 10); 9/11 (local interneuron cluster 9/11). C) Courtesy of J.M. Sullivan, Wellesley College. Scale bars: A=200 µm; B= 100µm; C=70µm.

3.4.3 Serotonin Transporter SERT

Immunocytochemical labeling with the mouse monoclonal antibody to the serotonin transporter (SERT) could be established for *Homarus americanus* using a working dilution at 1:1000. Screening of juvenile and embryonic brains (E 84%) show extensive labeling in the outer cortex of the accessory lobe (AL), the antenna II neuropil (AN), soma cluster 9/soma cluster 11 (cl 9/11), lateral antennular neuropil (LAN) and along fibers of the protocerebrum (PB) (Figure 23A-D). Further in depth analysis of 100µm sections using a 100x lens, show scarce labeling within the proliferation zone of life-long neurogenesis in cluster 10 (Figure 23D).

3.4.3.1 Preadsorption control of SERT

To test the specificity of the newly established anti-SERT antibody a preadsorption control was performed alongside with a positive control (sample incubated in the antigen). No labeling could be demonstrated in the brain of juvenile and embryonic lobsters using the preadsorbed serum (Figure 23F). Familiar labeling in all above mentioned regions of the brain (see 3.4.3) could be shown for the positive control (Figure 23E). The loss of all immunoreactivity specific to the SERT antigen was, therefore, interpreted as a valid confirmation for the specificity of the antigen.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AN
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10
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AL
AN

Figure 23. A) Confocal images of anti-SERT labeling in embryonic lobster brains (E 84%; whole mount). B) Fibers within the protocerebrum label extensively for anti-SERT. C) In juvenile lobsters brains sections (100 µm) anti-SERT labeling can also be localized in the lateral antennular neuropil (LAN) and D) within the projection neuron cluster 10. The specificity of the SERT antibody was tested performing a preadsoption assay which indicates F) no labeling when using the SERT peptide and extensive SERT labeling in E) the cortex of the accessory lobe (AL), the antenna II neuropil (AN), local interneuron cluster 9/11 (9/11), lateral antennular neuropil (LAN), and the protocerebrum (PB) and in contrast is eliminated using the SERT peptide AL (accessory lobe); AN (antenna II neuropil); OL (olfactory lobe); PB (protocerebrum); 10 (projection neuron cluster 10). Scale bar A= 100µm; B=20µm; C=100µm; D=16µm; E+F= 200µm.

3.4.3.2 Dual labeling techniques show spatial separation of melatonin and SERT

Because in parallel conducted immunocytochemical experiments labeling against melatonin and the serotonin transporter SERT (compare 3.4.3) seemed to show a similar spatial pattern, especially within the cortex of the accessory lobe which had previously been demonstrated to show a distinct circadian rhythm of serotonin (see Figure 13), additional dual labeling experiments against SERT and melatonin were performed to verify whether labeling can be seen within the same regions (Figure 24A). Analysis using high resolution (100x lens plus zoom) indicate that anti-melatonin labels the more proximal region of the cortex whereas anti-SERT labels the distal region (Figure 24B), thus providing no evidence that SERT may be involved in the biosynthetic pathway converting serotonin to melatonin.

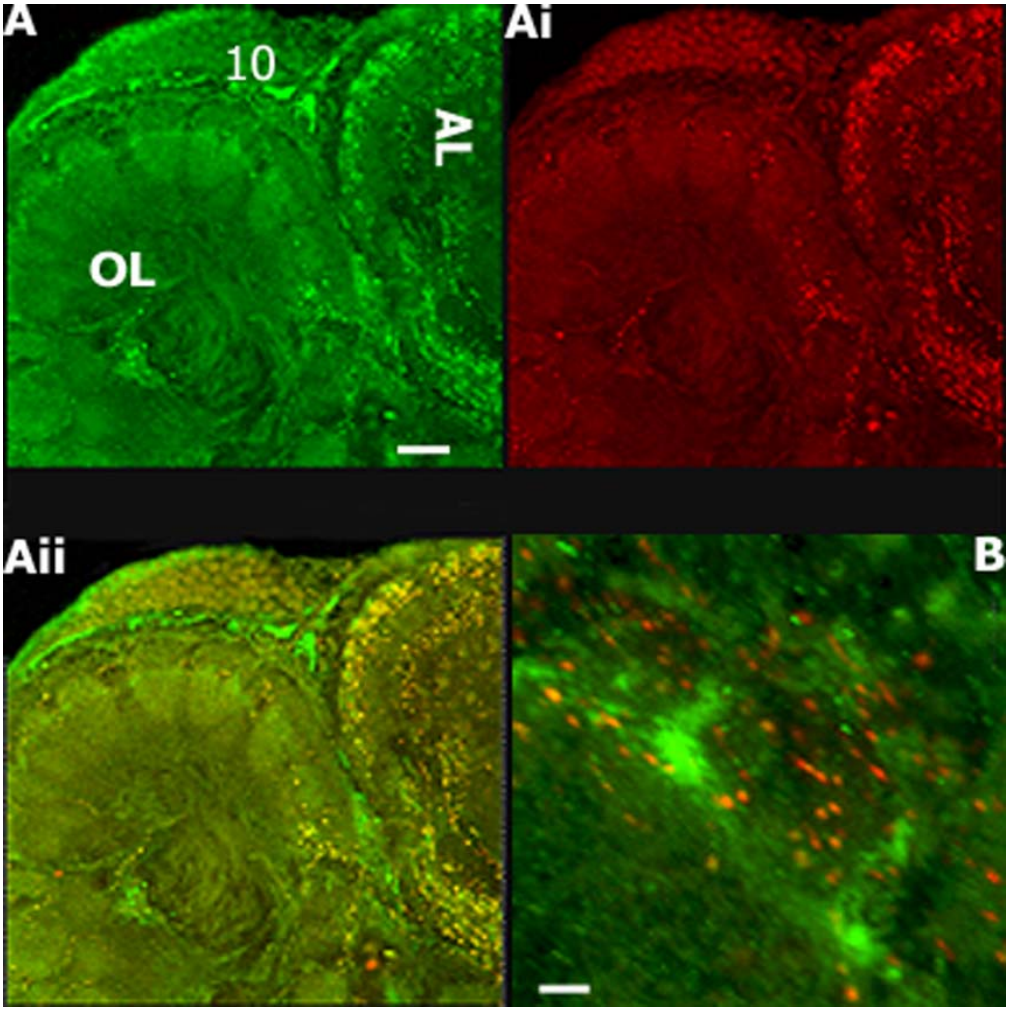


Figure 24. Confocal images of anti-melatonin (green) and anti-SERT (red) show a close spatial appearance within the accessory lobe (AL). A-Aii) shows sequential scans for anti-melatonin (green), anti-SERT (red) and the final overlay of the two scans (green + red). B) the cortex of the accessory lobe (AL) reveals no co-localization of anti-melatonin and anti-SERT at higher resolution. Scale bars: A-Aii= 100 µm; B=20 µm.

3.4.3.3 Fixation does not preserve CellTracker CM-DiI labeling

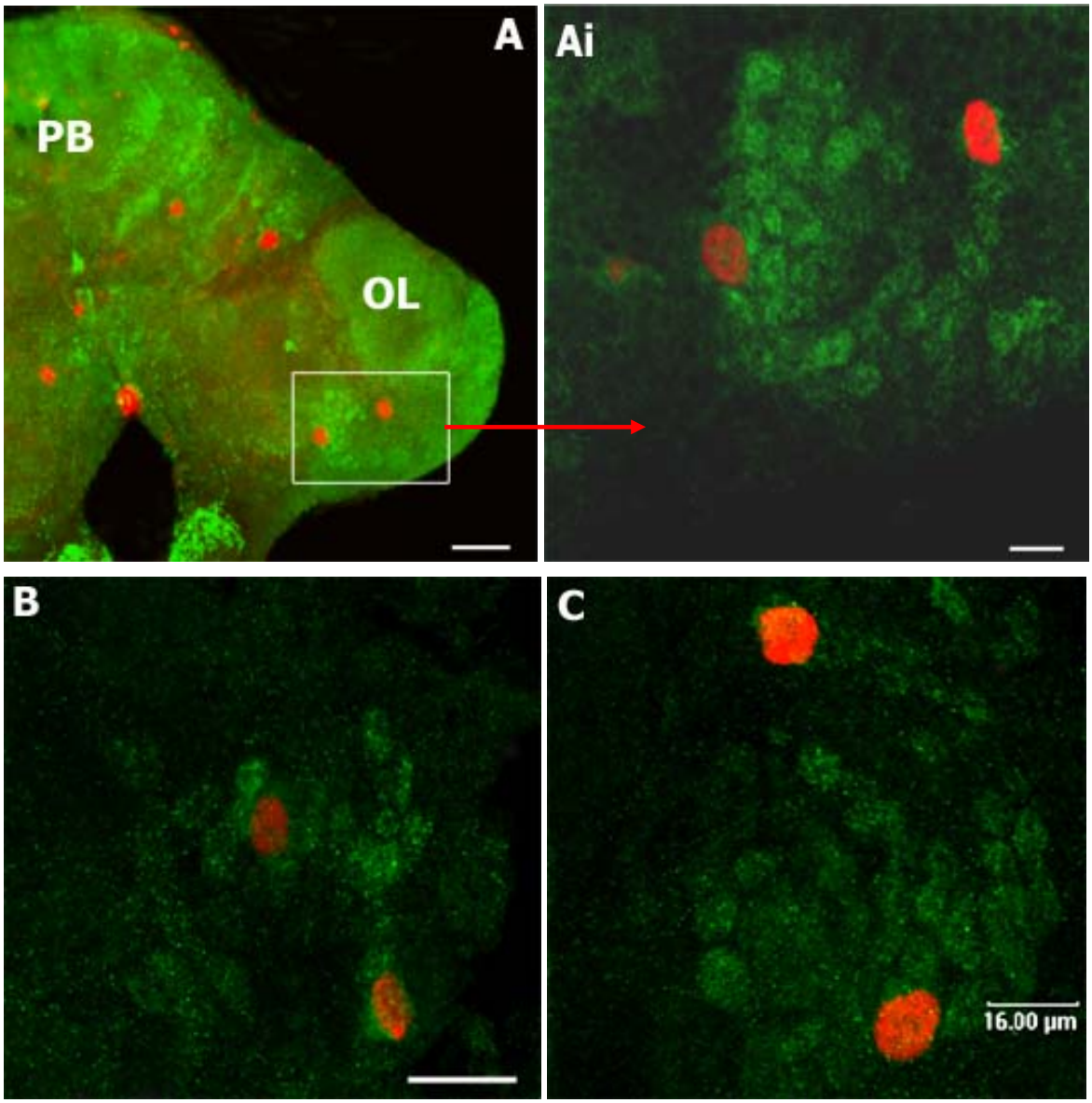
The CellTracker CM-DiI incorporates into cell membranes. SERT transports serotonin across pre-synaptic terminals. Immunocytochemical double labeling using CM-DiI and anti-SERT was therefore intended to localize anti-SERT labeling on or in cell membranes as an additional approach to confirm the specificity and labeling properties of anti-SERT. Although,

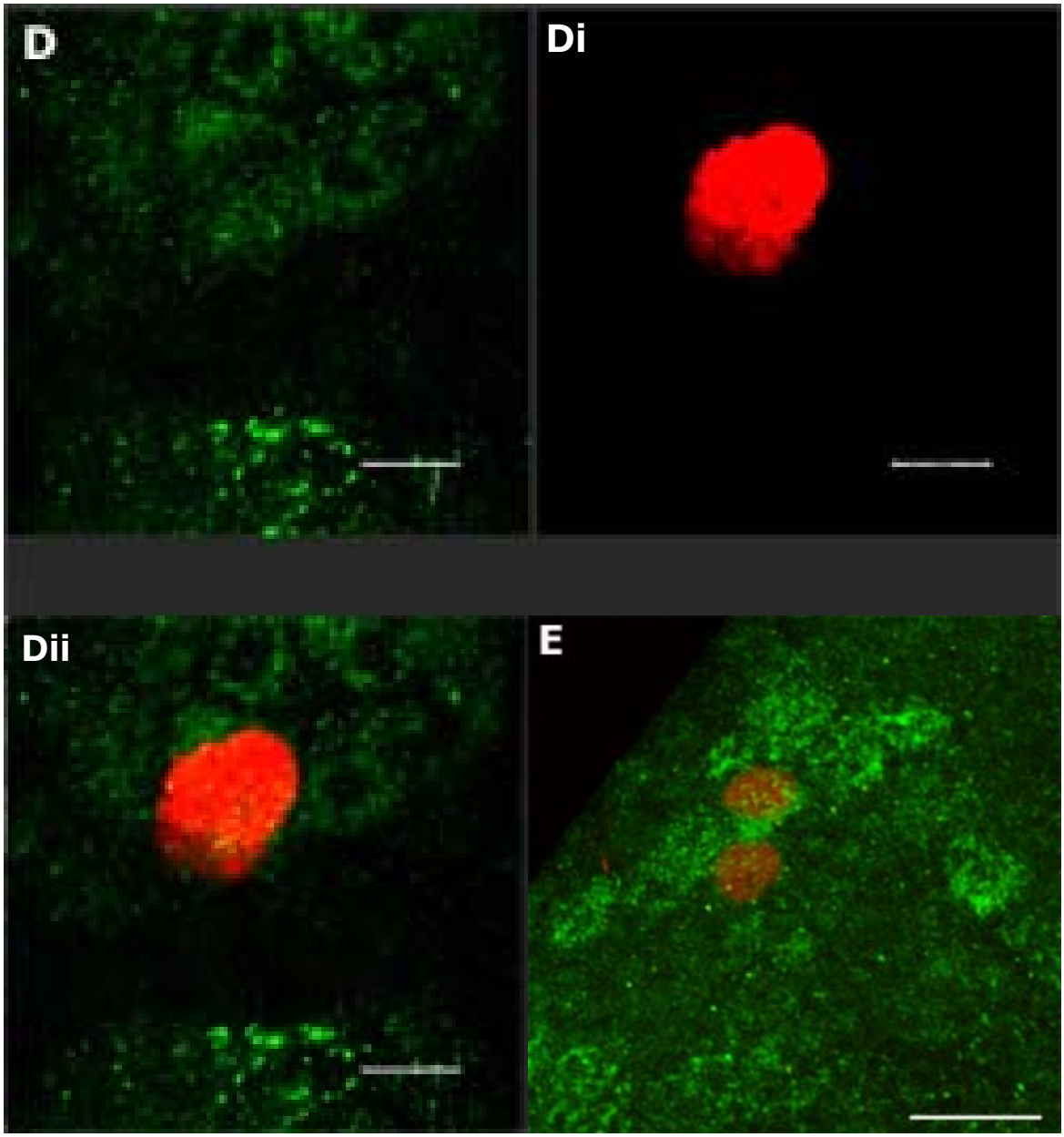
CM-DiI was pointed out as a valid dye to be applied to methods using standard tissue fixation (personal communication with Molecular Probes) no CM-DiI labeling could be retained after rinses with 0.1M PB of freshly fixed cells. Combining the properties of CM-DiI with an immunocytochemical standard protocol was therefore not beneficial.

3.4.3.4 Mitotically active cells at the site of life-long neurogenesis

In lobsters serotonin is involved in regulating the rate of life-long neurogenesis (Benton & Beltz, 2001). Work by Savner (2002) showed that the enzyme involved in the serotonin synthesis is not present in these newly born neurons, thus suggesting that serotonin's action on neurogenesis is via the serotonin transporter (Savner, 2002). Immunocytochemical studies in juvenile lobsters using the mitose markers anti-BrdU in conjunction with anti-SERT show mitotically active cells in the proliferation zone of cluster 10. However, an identification of anti-SERT labeled cells within that region was not possible due to technical problems with the confocal microscope (the 40x lens was broken and the 100x lens needed professional cleaning). Subsequent experiments did have a proper 100x lens available but had to use embryonic lobsters (E84 %), since the availability of juvenile lobsters underlies seasonal changes. Studies in embryonic lobsters (E 84 %) using anti-BrdU and anti-SERT labeling with a 100x lens at hand nevertheless demonstrated no labeling for anti-BrdU throughout the brain but intensive labeling of anti-SERT in the accessory lobe (AL), antenna II neuropil (AN), local interneuron cluster 9/11 (9/11), lateral antennular neuropil (LAN), the protocerebrum (PB) and projection neuron cluster 10 (10). Because no anti-BrdU labeling could be recognized, a failure of incorporating BrdU into the DNA or problems with using the rat anti-BrdU primary antibody were assumed. Subsequent studies, therefore, used the M-phase mitosis marker anti-phospho-Histone H3 (which does not require a perforation of the egg shell and thus avoids early lethality; see 2.4.3.3 in Material & Methods) in place of anti-BrdU. Dual labeling for anti-phospho-Histone H3 and anti-SERT in embryonic lobsters (E 84%) show mitotically active cells in cluster 10 that are surrounded by SERT (Figure 25A-H). Measurements of the diameter of the anti-phospho-Histone H3 labeled nucleus within the proliferation zone of cluster 10 indicate two cell sizes: A) 12 µm (Figure 25B-E) and B) 8 µm

(Figure 25F-H), suggesting the detection of ganglion mother cells (12 µm) and neurons (8 µm) (compare with Benton and Beltz, 2002).

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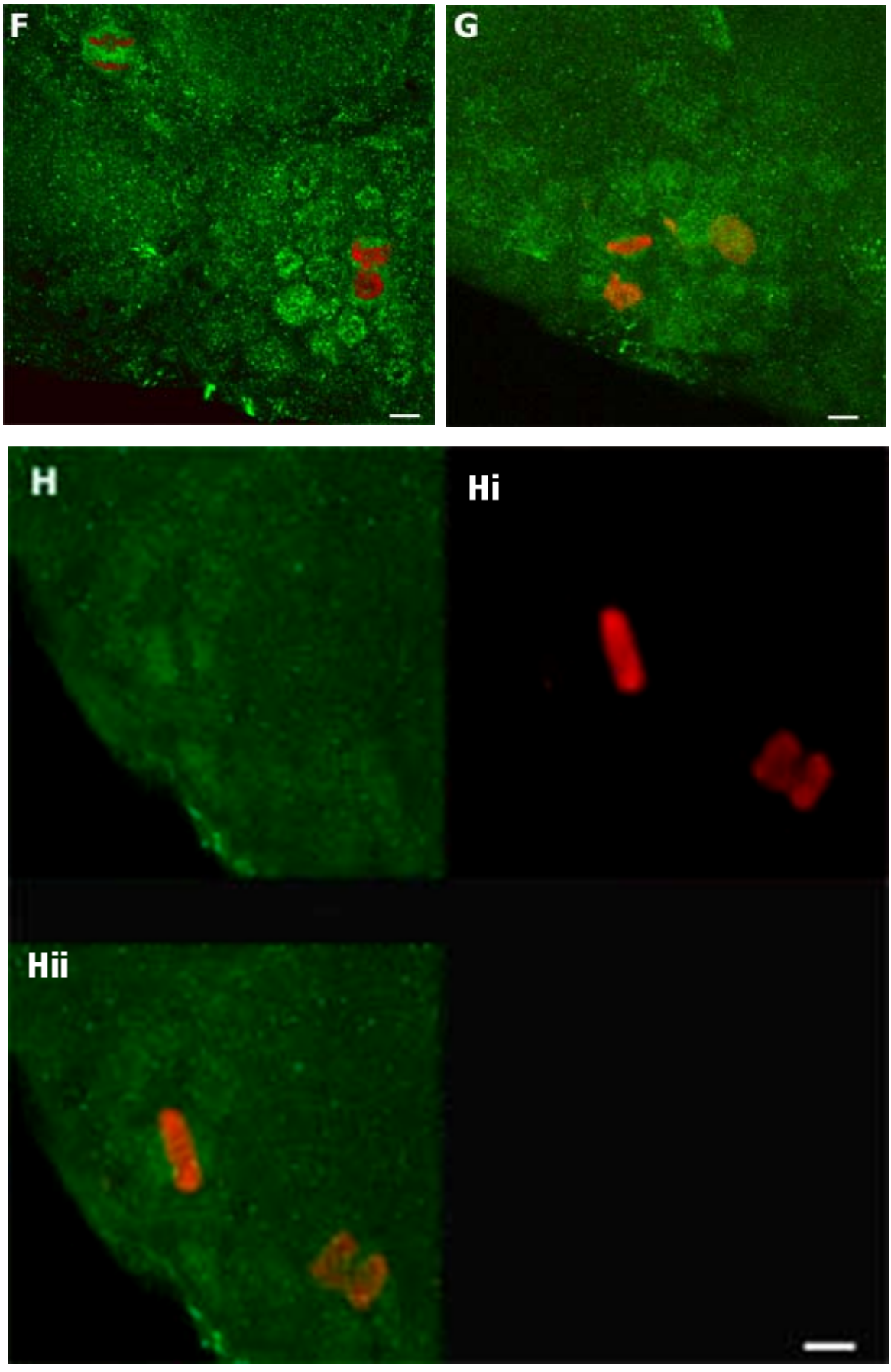
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Figure 25. A. Confocal images of anti-SERT labeling (green) and the mitosis marker anti-phospho-Histone H3 (red) in embryonic brains (E 84%; whole mount). Ai-E) Note the size (~12 µm = ± size of a ganglion mother cell) of the anti-phospho-Histone H3 labeled nuclei within the region of life-long neurogenesis and the arrangement of adjacent SERT expressing cells within that region. D + H) show sequential scans for anti-SERT (D, H; green), anti-phospho-Histone H3 (Di, Hi; red) and the final overlay of the two scans (Dii, Hii; green + red). F-H) The size of the anti-phospho-Histone H3 labeled nuclei corresponds to the size of neurons (Benton & Beltz, 2002). Also note the "twin" appearance of those mitotic figures measuring ~ 8 µm. Scale bars: A= 100 µm; Ai= 20 µm; B-C= 16 µm; D= 8 µm; E-H= 8 µm.

3.4.3.5 Transient uptake of serotonin within the region of life-long neurogenesis: dual labeling of serotonin and SERT

A repeat of the experiments carried out by Beltz et al. (2001) showing transient uptake of serotonin into newborn olfactory projection neurons was performed. One concluding proposal of their work is that the serotonin transporter may only be active for a limited time after these neurons are born. If this hypothesis is correct anti-SERT labeling should then be seen at the site of transient serotonin uptake. Immunocytochemical studies using the anti-SERT antibody and the anti-serotonin antibody post serotonin incubation, show single cells that label for serotonin at the posterior margin of cluster 10 as well as faint SERT labeling within that region (Figure 26A, B). A convincing co-localization of the two chromophores, however, could not be identified, thus providing no supportive evidence for a transporter mediated mechanism at the site of transient serotonin uptake using this method.

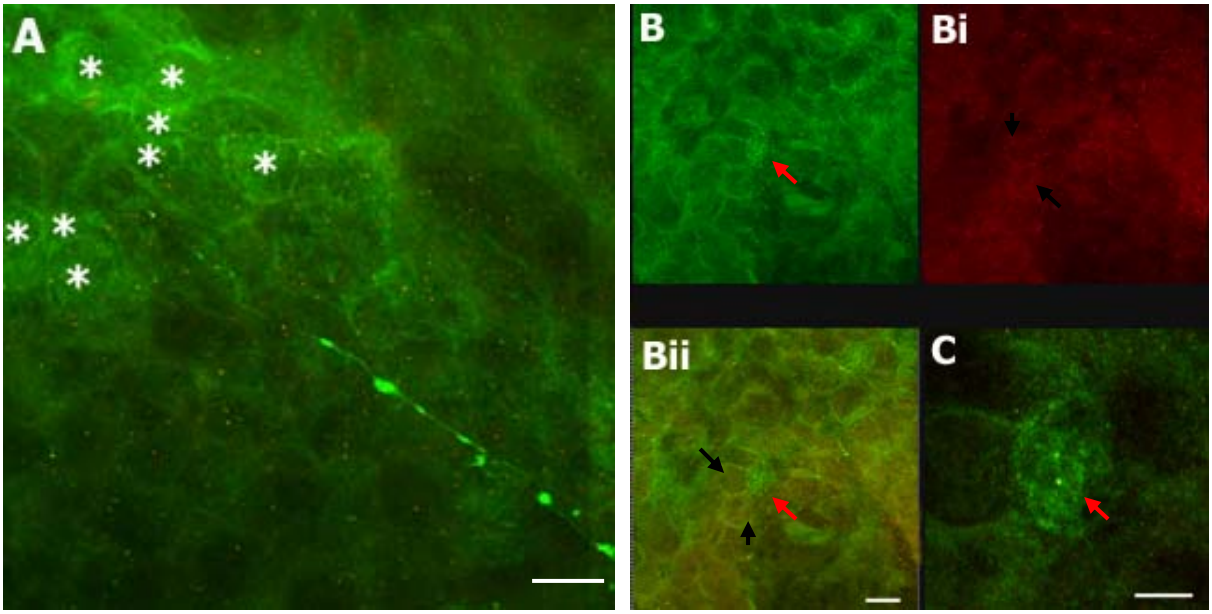

Figure 26. Confocal images of juvenile lobster brains show a select number of cells that transiently take up serotonin within the posterior margin of the proliferation zone in projection neuron cluster 10. A) A group of cells label against serotonin as indicated by the asterisks. Note the fiber that labels against serotonin which projects into the region of transient serotonin uptake. B-Bii) Sequential scans for anti-serotonin (green) and anti-SERT (red) and the final overlay of the two scans (green + red; "orange"). C) Shows a higher magnification of Bii. Note the red arrow indicates the cell that labels against serotonin; black arrows indicate two adjacent cells that label against SERT. Scale bars A-C= 8µm

4. Discussion

The present study was undertaken to learn whether serotonin levels in the lobster brain are under circadian control, and to understand these findings in the context of serotonins' actions in the crustacean nervous system. Particular focus was set on the serotonergic regulation of neurogenesis in the lobster brain. Beltz et al. (2001) and Benton & Beltz (2001) have shown that serotonin levels regulate the rate of newly born neurons in this system, and that neurogenesis is also under circadian control (Goergen et al., 2002). It was hypothesized that if serotonin is involved in the circadian pathway that regulates neurogenesis, brain levels of this molecule should also cycle diurnally, be light entrainable, and show a fixed correlation to the neurogenic rhythm. In keeping with this model, this thesis demonstrates that serotonin levels in the brains of lobsters have diurnal fluctuations that are sustained by a light entrainable endogenous rhythm, and that functionally different regions in the brain show distinct circadian rhythms. It was further anticipated to learn more about the functional role of melatonin in the brain of the lobster and whether the fluctuating levels of brain serotonin could be, in part, due to rhythmic changes of its biosynthetic product melatonin.

Having explored that serotonin levels show a similar light entrainable circadian rhythm as demonstrated for neurogenesis, we further analyzed whether other exogenous factors such as feeding have an effect on both the rate of neurogenesis and the levels of serotonin. Therefore, we monitored two primary factors, actual food intake (chemosensory stimulation/caloric intake) and physical activity, as modulating factors on the levels of both serotonin and neurogenesis. Data presented here show that the circadian rhythms of both serotonin and neurogenesis can be modified by feeding time, that both circadian rhythm fluctuate in a close temporal fashion, and that factors that change the circadian rhythm of serotonin also change the circadian rhythm of neurogenesis.

Additional studies, using immunocytochemical techniques, then focused on the distribution of the serotonin transporter (SERT) at the site of life-long neurogenesis. As reported here, anti-SERT immunocytochemical labeling appears in close spatial relationship to the newly born neurons, thus providing further evidence that serotonin might have a functional role in the generation of new neurons. Because SERT could not be found to co-

localize with newly generated neurons caution needs to be exercised when attributing the serotonin transporter a direct role in this process.

4.1 The circadian rhythm of serotonin in the brain

In other crustaceans such as the fiddler crab *Uca pugilator* and the crayfish *Procambarus clarkii* fluctuating serotonin levels have mostly been described for eyestalks (Fingerman & Fingerman, 1977; Fingerman et al., 1978, Escamilla-Chimal et al., 2001; Fanjul-Moles & Prieto-Sagredo, 2003). Castanon-Cervantes and colleagues (1999) are the only authors to my knowledge who have assessed serotonin levels over 24-hours in the brain of another crustacean. These authors have shown that in post-embryonic crayfish, *P. clarkii,* brain serotonin levels show a bimodal rhythm with a major peak at night and a minor peak during the day when held in constant light. A trimodal rhythm becomes evident in adults, reflecting an endogenous rhythm peaking every 8 hours (Castanon-Cervantes et al., 1999).

The lack of a *clear* circadian rhythm in serotonin levels in lobsters maintained in 12:12 L/D conditions (see 3.1 “Serotonin levels under L/D conditions”) is contrary to the reports cited above. Masking, a situation where an endogenous rhythm is obscured by transient changes in the molecule being measured due to direct stimulation by light-activated neural pathways, might be a potential cause for this result. Alternatively, serotonin content in different parts of the brain may cycle at different times and thus obscure a distinct single rhythm. This is one possible interpretation of the trimodal rhythm reported in adult crayfish by Castanon-Cervantes et al. (1999).

Constant darkness reveals an endogenous, light entrainable, diurnal serotonergic rhythm. To exclude the influence of masking from the study presented here, lobsters were entrained to a 12:12 L/D cycle for at least 2 weeks (the entraining light regime), and then introduced to D/D conditions where no direct light stimulation or feeding occurred. At the end of the third day in D/D, serotonin levels were measured at 6 time points during a 24-hour period. Using this D/D protocol, an endogenous diurnal serotonergic rhythm was revealed. The most reliable characteristics of this whole-brain rhythm are a pre-dusk serotonin peak,

with a decrease in serotonin levels to a pre-dawn serotonin trough. Altering the light cycle for a group of lobsters so that light-off was shifted by 4 hours, had the effect of shifting the serotonin peak and trough accordingly, showing that the characteristic components of the whole-brain rhythm are light entrainable (Figure 11C).-

A visual inspection of the chronograms in Figure 11C showed that they are not sinusoidal and hence analysis with the single cosinor is inappropriate (Nelson et al., 1979; Reinberg & Smolensky 1983; Minors & Waterhouse 1988; De Prins & Waldura 1993), excluding a mathematical dissection of the chronograms to reveal sub-rhythms hidden within them. However, due to the highly modular construction of the crustacean brain, we could physically dissect the brains into three regions, the olfactory lobes, accessory lobes and the brain remainder (medial regions of the protocerebrum, deutocerebrum and the tritocerebrum), all of which contain measurable levels of serotonin (Beltz et al., 1990).

Serotonergic rhythms in the olfactory and accessory lobes. Regional separation of the brain into the OL, AL and the brain remainder and assaying these individually reveals that not only are the changes in serotonin levels in these areas out of phase with one another, but that the nature of the specific patterns of these changes are characteristically different. The changes in the serotonin levels in the OL and the brain remainder, for example, are abrupt. That is, the levels of serotonin in these areas of the brain during the interval preceding the peak are clearly different from the levels at the peak itself. Serotonin levels then decline in an exponential fashion (Figure 13D and E). In contrast, a comparison of these two measurements in the ALs shows that they change slowly and monotonically (Figure 13F). The combination of the gradual rise and extended plateau of serotonin level in the ALs with the peaks and troughs of the serotonin levels in the OL and brain remainder, explain the relative flatness of the whole-brain chronogram and histogram (Figure 11A-C; Figure 14). If one assumes that changes in serotonin levels in the brain are indicative of changes in the releasable pool of this transmitter, then availability of serotonin in the OL and the brain remainder changes in a pulse-like fashion in comparison with the AL.

The contrasting diurnal rhythms in serotonin levels that were measured in the OLs, ALs and brain remainder (Figure 13) are of particular interest because these regions are functionally distinct. The paired ALs in crustaceans receive no primary sensory input, but rather receive projections of local interneurons in clusters 9 and 11 that carry higher-order visual, mechanosensory and olfactory information (Sandeman et al., 1995; Wachowiak et al., 1996; Sullivan & Beltz, 2004). The AL output is carried by the axons of the cluster 10

projection neurons, which continue to proliferate throughout the animals' life (Harzsch et al., 1999), and which project to the hemiellipsoid bodies located in the lateral protocerebrum (Sullivan & Beltz, 2001). This connectivity pattern and the fact that multimodal inputs project to this region, suggest that the ALs are involved in higher-order integration (Sandeman et al., 1995; Sullivan & Beltz, 2001). The OLs, on the other hand, are innervated by olfactory receptor neurons from the first antennae (antennules). Their output, like that of the ALs, is carried by the axons of cluster 10 neurons that project to the lateral protocerebrum via the olfactory globular tract (Figure 3). However, the olfactory projection neurons are different from those that innervate the ALs in that they target neuropil regions of the medulla terminalis. Therefore, the output pathways from the OLs and ALs project to separate, largely non-overlapping regions of the lateral protocerebrum (Sullivan & Beltz, 2001), further evidence of the distinctive functions of these regions.

While the connectivity and functions of the OLs and ALs are distinct, both regions nevertheless receive a massive serotonergic innervation from *the same neuron* ---the ipsilateral dorsal giant neuron (DGN; Sandeman & Sandeman, 1987; Benton & Beltz, 2001). The input-output relationship of the DGN in these regions is not known, but in both regions this neuron innervates each and every glomerulus (Benton & Beltz, 2001). The OLs and ALs are also innervated by relatively few, smaller serotonergic interneurons whose cell bodies are located in clusters 9 and 11 (Beltz, 1999). However, as the predominant serotonergic input to both areas is from the DGN, our results suggest that the contrasting rhythms in serotonin content measured in these areas may reflect a differential regulation of serotonin metabolism in the OL and AL arbors of *the same neuron*. Some years ago Sandeman and colleagues (1993) proposed that the DGNs, by virtue of their massive axonal arbors projecting to functionally distinct areas, were likely to engage in localized signaling within discrete areas of the OLs and ALs, thereby "multitasking". This concept takes on new meaning if neuronal activity is able to regulate serotonin levels in these areas.

The logical extension of this idea is that local activity patterns imposed by differential inputs to the OLs and ALs may be able to influence the synthesis, degradation, uptake and release of serotonin in these regions. Serotonin levels in the OL arbors of the DGNs could thereby be altered by chemosensory stimulation when lobsters become aroused and begin to forage during the hours just prior to dusk (Weiss, 1970; Ennis, 1983; Cooper & Uzmann, 1980; Arechiga et al., 1993; Watson et al., 2001). Zimmer-Faust et al. (1996) have shown, using behavioral tests, that the activity state does influence the sensitivity of spiny lobsters and crabs to food odorants in the water and that such sensitivity is much higher during periods

when physical activity is high. The implication from this work is that the change in the responsiveness is centrally and not peripherally determined. If responsiveness of lobsters also increases abruptly during pre-dusk/dusk arousal, then the OLs may be strongly activated during this period. It is therefore intriguing that we consistently see the highest serotonin levels in the OLs during this pre-dusk period.

In contrast, serotonin levels in the ALs rise throughout subjective day to a peak at dusk and are sustained at a high level throughout subjective night, rather than dropping precipitously as in the OLs. In this context, it is interesting that activity patterns in the ALs will be sensitive not only to chemosensory activation, but also to visual and mechanosensory stimulation. It is possible that the sustained high levels of serotonin in these regions during subjective night reflects a heightened sensitivity, and corresponding increased activity, in the variety of sensory systems involved in nocturnal behaviors. The decrease in serotonin levels during the early subjective morning hours coincides with the onset of a low activity period in lobsters (Ennis, 1983; Watson et al., 2001). The fact that in mammals and insects serotonin has been repeatedly associated with learning and memory mechanisms (Harvey, 2003; Meneses, 2003; Orsetti et al., 2003; Wolff et al., 2003; Blenau & Baumann, 2001) also may be relevant to the rhythms measured in these brain regions. Interestingly, fluctuations in the levels of serotonin have also been suggested to drive melatonin synthesis at night in rats, which in turn is correlated to visual processing areas (Sugden, 1990; Olcese & Munker, 1994; Miguez et al., 1997). Certainly the fact that serotonin is differentially regulated in specific brain regions, may reflect the potential importance of time-of-day performance of those areas.

Multiple functional, entrainable circadian rhythms also have been found in the mammalian brain. Nuclei in the olfactory bulb and the ventral hypothalamus of rats are rhythmic with peak expression of the *period* gene product *Per* at night, while other brain areas were only weakly rhythmic, or arrhythmic (Abe et al., 2002). It is believed that cells within the mitral cell layer of the olfactory bulb are competent circadian pacemakers, regulating their own gene expression and membrane excitability (Granados-Fuentes et al., 2004a, b). Such decentralized circadian pacemakers are also known in insects indicating that multiple pacemaking tissues exist across species, which function semi-autonomously from each other. In *Drosophila melanogaster* cycling of the *period* and *timeless* gene was demonstrated in the Malpigian tubules (Hege et al., 1997; Giebultowicz et al., 2000), the alimentary canal, the fat body and within the reproductive system of males and females, respectively (reviewed by Hall, 1998; Giebultowicz, 1999, 2000; Helfrich-Förster, 2002). Further, the observation of the circadian cycling of the *period* gene in chemosensory cells of the antennae (Krishnan et al.,

1999) suggests the presence of a brain-independent *olfactory* circadian oscillator across species. These data, in combination with the fact that circadian modulation of olfaction has been reported in mammals (Amir et al., 1999; Funk & Amir, 2000) and insects (Tanoue et al., 2001; Miller & Stelinski, 2004), suggest that the presence of an independent pacemaker in the olfactory bulb may be related to the need for local regulation of olfactory processing. However, how such rhythms in transcriptional or electrical activity in the bulb relate to olfaction is not known. Therefore, in contrast to the traditional view of a single pacemaker driving multiple rhythms, the presence of independent pacemakers in functionally distinct brain regions whose activities are coordinated by master clocks such as the SCN in mammals or the circadian pacemaker center in cockroaches and crickets, appears to be the standard in many tissues.

4.2 Feeding has a modulatory effect on both brain serotonin levels and neurogenesis

The present study was also undertaken to learn whether feeding has an immediate effect on the levels of brain serotonin. In addition it was determined whether feeding time alters the endogenous circadian rhythm of both serotonin and neurogenesis. My colleague Erin Goergen and I also attempted to distinguish whether the actual intake of food (caloric intake/chemosensory stimulation) or physical activity as a result of foraging and feeding has a modulatory effect on the levels of both serotonin and neurogenesis in the brain of the lobster. We were interested in this question because previous studies have implicated light as one common zeitgeber on both neurogenesis and serotonin (Goergen et al., 2002; Wildt et al., 2004) and in addition have reported a feeding induced increase in brain serotonin (De Fanti et al., 2001; Tachibana et al., 2001) as well as changes in the rate of newborn cells due to dietary restrictions (Lee et al, 2000; Lee et al., 2002). In addition heightened physical activity in mice has been associated with increases in levels of both serotonin and neurogenesis, suggesting that activity drives serotonin levels, which in turn may drive neurogenesis (Jacobs et al., 2002).

Of additional interest to us is the serotonergic regulation of neurogenesis in the lobster brain and the mechanisms that modulate it. As described earlier, Beltz et al. (2001) and

Benton & Beltz (2001) have shown that serotonin levels regulate the rate of newly generated cells in this system. E. Goergen (Goergen et al., 2002) and myself (Wildt et al, 2004; and 3.1 "HPLC measurements of circadian brain serotonin levels") have further reported that both neurogenesis and brain serotonin levels are under circadian control with light as one common zeitgeber. Feeding affects the levels of both serotonin and neurogenesis in vertebrates (Lee et al., 2004; Bondolfi et al., 2004; Akman et al., 2004) and invertebrates (Novak & Rowley, 1994; Goergen et al., 2002). These experiments extend our understanding of factors that modulate serotonin and neurogenesis by showing that feeding also functions as a modulator on serotonin and neurogenesis. Further we were able to assess the contribution of both chemosensory and physical stimulation. Our results demonstrate that entrained feeding at a different time of day increases the overall rate of neurogenesis over 24-hours and that the levels of serotonin precedes the rate of neurogenesis in a close temporal fashion when fed.

Surprise feeding and food anticipation alter the levels of serotonin. When fed before dusk, the endogenous rhythm of serotonin in lobsters peak prior to dusk and trough prior to dawn (see 3.1 "HPLC measurements of circadian brain serotonin levels" and Wildt et al., 2004). Surprise feeding (Group 1*) results in prompt increases in brain serotonin levels in lobsters, indicating that feeding functions as a modifier. This has also been observed on brain serotonin levels in mammals (De Fanti et al., 2001; Tachibana et al., 2001). However, feeding is a complex system of chemosensory stimulation/caloric intake and physical activity. We therefore aimed on the isolation of physical activity from chemosensory stimulation/caloric intake by substituting food with a bead (Group 2*-4*) (which is manipulated by hungry lobsters the same way as food, however, providing physical activity only), in addition to phase-shifting the conditioning feeding time in order to elucidate the entraining power of feeding on neurogenesis and serotonin.

Entrained feeding (Group 2*-4*; Figure 19A-C) at a time when serotonin levels had previously been demonstrated to be low (Figure 18; Wildt et al., 2004) does result in increased levels of this amine but also demonstrates increased brain serotonin levels prior to this feeding, suggesting an anticipatory effect (Group 2*; Figure 19A). This food anticipatory response in serotonin levels occurs in mammals (Richter, 1922) and is suggested to also exist in marine Crustacea (Zimmer-Faust, 1987). Interestingly, in lobsters, peak levels in brain serotonin coincide with high rates of foraging (Wildt et al., 2004; 3.1 "HPLC measurements of circadian brain serotonin levels"). The high levels of pre-dusk serotonin suggests that this

peak might be a combination of food anticipation and the changing light condition. One could argue that if entrained pre-dusk feeding leads to an anticipatory response for food *and* a response to light then the endogenous peak prior to the light change should decrease when feeding is uncoupled from pre-dusk feeding. However, this distinction is difficult to obtain because serotonin levels in lobsters show little rhythmic changes other than a peak and trough, so feeding at a time of day other than prior to the trough might fall into the noise ratio and thus not show a distinguishable effect.

Serotonin rhythms are differentially expressed in distinct regions of the lobster brain when measured over 24-hours (Figure 13; Wildt et al., 2004). In the accessory lobe, a region which is thought to be involved in higher order integration serotonin levels rise gradually throughout the day and into the early night. In contrast, the serotonergic rise and fall of the olfactory lobe and the remaining regions of the brain only show one sharp increase prior to dusk (see Figure 13 D and E). These findings suggest that altered stimulations of one processing area at other times of the day might change the serotonergic rhythm of the area involved which in turn would modify the overall brain serotonin rhythm. The little rhythmic changes observed for *Artemia* (Group 3*; Figure 19B) and bead (Group 4*; Figure 19C) might demonstrate such powerful modifying abilities of one lobe on the whole brain rhythm. Individual sampling of the distinct regions would provide more explicit information.

Further analysis on the rhythmic changes of serotonin after *Artemia* feeding (Group 3*; Figure 19B) show increasing levels at the end of the sampling period. Work done by Orosco & Nicolaidis (1992) suggests that increased serotonin levels not only indicate satiety but that the fluctuating levels of serotonin reflect the state of satiety. In addition, pharmacological changes in the levels of serotonin can also stimulate feeding (Blundell, 1992). It is therefore likely that the slight rise in serotonin levels at the end of the *Artemia* sampling period is due to a signaling process indicating the end of the satiation period and the beginning of a new foraging period.

Physical activity modulates the rate of neurogenesis. Entrained feeding modifies the endogenous pattern of neurogenesis over 24-hours. Short-term odor exposure to food (*Artemia*; Group 3*; Figure 19B) significantly increase neurogenesis in the region of life-long neurogenesis. Those newly generated cells are part of the neuronal projection cluster 10 which is in part innervated by projections of the olfactory processing area. This stands in contrast to mitotic studies using BrdU in mice. Data presented by Rochefort et al. (2002) have

shown that the number of newly generated neurons in the olfactory bulb does not change after 4 hours of BrdU incubation but does when tested 20 days after the initial incubation, suggesting that enriched olfactory conditions have no influence on the proliferative activity of progenitor cells in this region but rather on the overall survival of cells. However, studies on the circadian rhythm of neurogenesis by Goergen et al., (2002) suggest that due to the changing dynamic of peaks and troughs every 12 hours one full cell cycle of the most rapidly dividing cell type(s) most likely completes within that time span. This might hint why the rate of neurogenesis of animals that had been fed *Artemia*, redirects from one plateau into a slightly lower one exactly 12 hours after the onset of peak rates of neurogenesis.

Food consumption during the day of the experiment further alters the circadian rhythm of neurogenesis (Group 3*; Figure 19B) and physical activity without caloric intake has a tendency to decrease neurogenesis over 24-hours (Group 4*; Figure 19C). Studies in mammals have demonstrated that physical activity is one stimulating factor for increased neurogenesis (van Praag et al., 1999a, b; Trejo et al., 2001; Kempermann et al., 2002). Time of day has recently been shown to influence physical activity-induced neurogenesis in mice (Holmes et al., 2004). In support of these findings is our demonstration of high rates in neurogenesis surrounding the time of bead-feeding when animals are at the end of their circadian activity phase. However, compared to the control groups that were fed *Artemia* or not fed at all, neurogenesis increases only drastically in response to newly induced physical activity but then decreases possibly due to high energy requirements that remain unsaturated (compare Figure 19B and C). Contrasting is our overall analysis of the number of newly born cells over 24-hours indicating that heightened physical activity (induced by bead feeding: Group 4*; Figure 19C) in lobsters seems to decrease neurogenesis by 15% over a 24-hour period. However our data displays the short-term effect (24-hours) of physical activity whereas data presented by others show conditioning time spans of a minimum of 6 days (van Praag et al., 1999a,b; Kempermann et al., 1997; Sandeman & Sandeman, 2000; Kempermann et al., 2002). Further, in these experiments animals exposed to such long-term conditions are usually fed on a regular basis, thus fulfilling the animals' extra metabolic requirements induced by heightened physical activity, whereas animals in our experiment did not receive any food but a bead the day of the experiment. We, therefore, believe that heightened physical activity accompanied by a lack of nutritional supply may attribute to the decrease in neurogenesis.

Serotonin and neurogenesis – how are they regulated? In both vertebrates and invertebrates serotonin is an effective regulator of neurogenesis (Brezun & Daszuta, 1999, 2000; Benton & Beltz, 2001, Beltz et al., 2001; Jacobs, 2002; Radley & Jacobs, 2002; Malberg & Duman, 2003). In the brain of the American lobster, reduced serotonin levels result in decreased neurogenesis (Benton & Beltz, 2001; Beltz et al., 2001), while elevated serotonin levels result in an increased rates of neurogenesis (Benton, Goergen & Beltz, unpublished results). It is also known that serotonin levels fluctuate in close temporal correlation with the rates of neurogenesis and that both serotonin levels and the rate of neurogenesis are light entrainable (Goergen et al., 2002; Wildt et al., 2004). Feeding depends on physical activity and in mice physical activity has been associated with increases in levels of both serotonin and neurogenesis, suggesting that activity drives serotonin levels, which in turn may drive neurogenesis (Jacobs, 2002). Our findings that the circadian rhythm of brain serotonin levels and the rate of neurogenesis can be altered according to the time of day feeding takes place, that both serotonin levels and the rate of neurogenesis fluctuate in a close temporal fashion when measured over 24-hours and that factors that change the circadian rhythm of serotonin also change the circadian rhythm of neurogenesis suggest that feeding serves as an essential modifier and that it might either influences serotonin and neurogenesis via parallel secondary regulatory mechanisms, or that serotonin may be an element in the direct pathway by which feeding regulates neurogenesis in the lobster brain.

In addition, when feeding and light are both available as potential modifiers on circadian rhythm, feeding seems to alter the characteristic peaks and troughs of the light entrainable rhythm of both serotonin and neurogenesis, suggesting that “when food competes with light, food usually wins” (Stephan, 2002).

4.3 Circadian rhythm of melatonin in the brain

Data presented here indicate that melatonin, a biosynthetic product of serotonin, is present in the brain of juvenile lobsters and that these levels fluctuate in a circadian fashion with an endogenous peak at night and a second, much greater peak during the day when exposed to light.

The biosynthetic pathway for melatonin is from tryptophan, via 5-hydroxytryptophan (5-HTP), serotonin, serotonin-N-acetyltransferase (SNAT, the rate limiting enzyme which converts serotonin to N-acetylserotonin) and hydroxyindole-O-methyltransferase (HIOMT). In lobsters, both endogenous tryptophan and endogenous 5-HTP can be seen when analyzing for serotonin, however, concentrations of endogenous tryptophan are within the limits of detection using the mobile phase and setting mentioned in Materials & Methods, thus showing no significant circadian fluctuations. Endogenous 5-HTP in contrast shows pronounced circadian variations with high levels before dusk (peak content >6 pmol/brain) (Figure 38, see 7. Appendix). As described earlier, elevated levels of endogenous serotonin can also be seen before dusk (peak content >2 pmol/brain). No data is available on the levels of SNAT or HIOMT in lobsters. The only study known on endogenous SNAT in crustaceans has been performed on the fiddler crab *Uca pugilator* and there endogenous SNAT peaks just before dusk (peak content < 2 pg/mg) or when measured under L/D conditions at the end of the dark phase (peak content > 1.5 pg/mg) (Tilden et al., 2001b). Brain melatonin levels in lobsters are elevated at the beginning of the dark phase; 4 hours after endogenous SNAT and 4 hours after light-stimulated SNAT in the crab. These data, therefore, suggest that both endogenous melatonin and light-stimulated melatonin are rapidly formed from serotonin but not immediately, as known from other species (e.g. Linn et al., 1995, Ganguly et al., 2002). However, caution needs to be exercised when using the data of the fiddler crab (Tilden et al., 2001b) for direct comparison since these data have been acquired in eyestalks.

Also, the pulse-like increase of melatonin measured under L/D conditions shortly after dawn and into mid-day is especially interesting in the light of serotonin measurements presented earlier. Despite the strong variablity of brain serotonin levels of animals that had been exposed to an entraining L/D light regime only, the overall common feature in the circadian rhythm of all preparations sampled is a pronounced decrease in the levels of serotonin at the beginning of the light phase, thus preceeding the distinct decrease in brain melatonin levels by ~ 4 hours (see Figures 27-35 in 7. Appendix). This close temporal time frame of light-induced circadian fluctuations of brain melatonin and brain serotonin plays in concert with the changes of endogenous serotonin- and melatonin levels discussed earlier, thus further suggesting that melatonin is rapidly converted from serotonin in lobsters.

Circadian measurements of melatonin in other crustaceans have only been described for eyestalks (Agapito et al., 1995; Balzar et al., 1997; Withyachumnarnkul et al., 1992; Tilden et al., 1997; Tilden et al., 2001b; Tilden et al., 2003). In the eyestalks of *Macrobrachium rosenberii* and *Uca pugilator* melatonin has been reported to peak during the day

(Withyachumnarnkul et al., 1992; Tilden et al., 1997; Tilden et al., 2001b; Tilden et al., 2003). Melatonin levels in *Procambarus clarkii* have been demonstrated to peak during the day (Agipato et al., 1995) and during the night (Balzar et al., 1997). The later authors attribute the differences to either two different patterns of melatonin production because the animals used for each study represent different habitats (Spain vs. Mexico), or to methodical differences using radioimmunoassay. Also Agipato and colleagues (1995) used entraining light regimes apart the 12:12 L/D light regime (14L:10D, 8L:16D). Despite the differences in circadian peaks and troughs all before mentioned authors attribute melatonin a role in conveying photoperiodic information, hence indicating light as an important regulator on brain melatonin levels.

Melatonin could also be reported to fluctuate rhythmically over a 24-hour period in cerebral tissue of two other invertebrates. In the mollusc *Helix aspera maxima* melatonin shows elevated levels during the night in the cerebroid ganglion (Blanc et al., 2003). High levels of brain melatonin during the night could also be demonstrated for the cabbage looper moth *Trichoplusia ni* (Linn et al., 1995). In *T. ni* circadian fluctuations of melatonin also persist under D/D conditions suggesting that the circadian rhythm of brain melatonin is endogenous. However, the levels of brain melatonin in the protocerebrum when measured under constant darkness do not phase-shift when compared to L/D conditions. The discrepancy in the characteristic peaks and troughs of the light-induced circadian rhythm and the endogenous circadian rhythm of brain melatonin levels in lobsters, therefore, suggest a strong modulatory effect of light.

Taken together it is intriguing to see that light not only has a profound effect on brain serotonin levels and neurogenesis but also on melatonin and other elements involved in the biosynthetic pathway to melatonin. In addition, the sheer presence of melatonin in the brain, which is commonly presented as a photoperiodic transductor located in the visual system and its associated tissues across species, might suggest alternative or at least additional functions of this indoleamide.

Immunocytochemical labeling of melatonin in the brain. In vertebrates the principal site of melatonin synthesis is the pineal gland; a neural pathway involved in processing visual information (Reiter, 1991). In invertebrates the site of melatonin synthesis has not been identified yet, but following what is known from vertebrates much effort has been put into the

examination of photosensitive tissues (see above mentioned studies). So far, melatonin has *inter alia* been localized in the eyes of the locust *Locusta micratoria*, the sea slug *Aplysia californica* (Abran et al., 1994), the dinoflagellata *Lingolodinium polyedrum* (Hardeland et al. 1995) and various crustaceans such as the crabs *Carcinus maenus* and *Uca pugilator* (Vivien-Roels & Pévet, 1986; Tilden et al., 1997; Tilden et al., 2001), the giant freshwater prawn *Machrobrachium rosenbergii* (Withyachumnarnkul et al. 1992; Tilden et al., 2001b) and the crayfish *Procambarus clarkii* (Agapito et al., 1995: Balzar et al., 1997). Because of the extensive presence of melatonin in photoreceptive tissue it is believed to be synthesized there. In the lobster, however, extra-retinal photoreceptors, which are superficially located within a rostral cluster of cell somata (cluster 6) within the brain (J. M. Sullivan and B. S. Beltz, personal communication), extend the potential sites of melatonin synthesis. Interestingly, J. M. Sullivan (unpublished results) also localized melatonin in neuronal somata adjacent to the extra-retinal brain photoreceptors, thus suggesting that melatonin is not solely synthesized at one specific site in the eyestalks of lobsters but rather at multiple sites - also within the brain.

In the crayfish *Cherax destructor* extra-retinal photoreceptors are part of an extensive connection between the compound eyes and the accessory lobes (Sandeman et al., 1990; Utting et al., 2000). Projections of the extra-retinal photoreceptors synapse with second order neurons in the protocerebral bridge (Sandeman et al., 1990) which in turn has connections to the medulla terminalis and via the central body to the accessory lobe (Utting et al., 2000). In this thesis, using immunocytochemical labeling techniques melatonin could be localized in the cortex of the olfactory lobe, but most interestingly also in the accessory lobe (see results; Figure 22B) which receives higher order visual information, including input from the compound eyes and the extra-retinal photoreceptors. However, one discrepancy arises when associating potential sites of melatonin synthesis in the brain with the expression of melatonin in the accessory lobe. Melatonin could so far only be localized within those few cells adjacent to the extra-retinal photoreceptors, selectively within cluster 9/11, and within the cortex of the olfactory lobe and the accessory lobe, but nowhere in between these sites. Assuming that the region of the extra-retinal photoreceptors are one site of melatonin synthesis, one would expect to see some sign of melatonin “along the way” when “traveling” the relative distance between the extra-retinal photoreceptors and compound eyes to the two lobes. However, no sign of evidence could so far be provided. Alternatively, melatonin in the olfactory and accessory lobe could also derive from direct conversion of serotonin in these lobes. This thinking is especially attractive considering that the circadian rhythm of serotonin, in the accessory lobe displays a gradual increase throughout the day and into the early night; just

like melatonin. Immunocytochemical studies using double labeling techniques against serotonin and melatonin might give further insight on whether melatonin is actually converted directly at the site of serotonin expression in the AL. However, these experiments have yet to be performed.

In any case, even though the origin of melatonin in the brain of juvenile lobsters still remains uncertain and no conclusive answer could be discovered whether the fluctuating serotonin levels within the individual lobes might be due to the conversion into melatonin (in part because melatonin content within functionally distinct lobes appears to be below electrochemical detection), new aspects on the function of melatonin in lobsters might have evolved: because of the sheer presence of melatonin in neuronal tissue melatonin might not solely act as a daily photoperiodic messenger but also as a neurotransmitter or neurohormone (also see Mechawar & Anctil, 1997).

4.4 Serotonin transporter (SERT)

The present study demonstrates that anti-SERT, an antibody which has been generated to recognize proteins with close sequence homology to the mouse SERT, can also be used in lobsters. Subsequent experiments, which tried to elucidate the functional role of serotonin in the process of life-long neurogenesis, could then show positive labeling for the SERT among newly born cells with nuclei of 8 µm and 12 µm in the olfactory neuron cluster (cluster 10).

Using the preadsorption assay, the SERT antibody was found to be specific for the serotonin re-uptake transporter. However, antibodies can cross react with more than one antigen; e.g. the tested antigen binds to the antibody which then binds to a similar antigen in the tissue. In support of the specificity of the SERT antibody is the finding that the antenna II neuropil (AN) in embryos does not label for SERT. This structure had previously been shown not to be serotonin immunoreactive during embryonic life (Langworthy et al., 1997). In addition, no SERT-positive labeling could be found in the cell body of the dorsal giant neuron but within the site of DGN projection- the ALs, suggesting that the SERT is a sensitive presynaptic marker of serotonergic terminals.

Although, the SERT was localized in many regions of serotonin immunoreactivity, a few discrepancies could be observed. Most striking is the negative labeling of anti-SERT within

the central region of the olfactory lobe (OL). The olfactory lobe is innervated by olfactory receptor neurons as well as by local interneurons (cluster 9 neurons) and projection neurons (cluster 10 neurons), most of which are serotonin immunoreactive. Based on these observations a number of possible explanations arise for the negative SERT labeling in the central region of the olfactory lobe: SERT may only be expressed at certain times of the day, information is processed within the OLs also on a receptor based mechanism, or SERT localization indicates the transduction of information in only one direction (input vs. output), thus indicating a functional partitioning of neurons within one processing area. The former case seems less likely considering that HPLC data presented above show high levels of serotonin within the OLs at a time when animals were sacrificed for SERT sampling (ZT 14:00). On the other hand, even though no data is yet available on the distribution of serotonin receptors in the brain of lobsters or any other crustacean brain, a wealth of information is available on the distribution of various serotonin receptor subtypes in the olfactory system in vertebrates (Waeber et al., 1994; Canton et al., 1996; Mijnster et al., 1997; Morales et al., 1998; Roberts et al., 2002), suggesting that serotonin- mediated transduction of olfactory information is mainly due to serotonin receptors. In addition, considering that the SERT expression might only indicate the presence of release sites of serotonin, the lack of the serotonin transporter in the OLs might indicate that the DGN projections in the OLs are receiving inputs instead of releasing serotonin and providing output. This thinking is supported by recent findings of Sullivan & Beltz (2004), indicating that the ALs have a functional architecture wherein the different sensory modalities are partitioned into different anatomical regions. Alternatively, serotonin could be converted to melatonin in these regions. This speculation would be supported by immunocytochemical labeling of anti-melatonin in the *cortex* of the OL only (see 3.4.2).

Consequently, all possible interpretations presented here would in turn underline the "multitasking" abilities of the DGN suggested earlier (see 4.1 discussion on "serotonergic rhythms in the olfactory and accessory lobes"; Sandeman et al., 1993). The DGN might be a site of signal input and output, respectively, and could function via a transporter- as well as receptor mediated mechanism. In addition, the incorporation of a putative role of melatonin as a cause for the negative labeling of SERT in the OLs would broaden the possible regulatory roles of the DGN even further.

The role of SERT at the site of transient serotonin uptake within the region of life-long neurogenesis. Serotonin can be transiently taken up by newborn olfactory projection neurons in the brain of lobsters (Beltz et al., 2001). Based on these findings it could be speculated whether the transient serotonin uptake is due to a serotonin synthesis within the newly proliferated neurons or whether it is the result of a transporter mechanism as had been described previously for vertebrates (Cases et al, 1998; Lebrand et al., 1998; Hansson et al., 1998). More recent work could provide no evidence for a transient serotonin synthesis in the newborn projection neurons, or in the associated stem cells (Savner, 2003), thus giving-way to a transporter based mechanism. Results demonstrated here could trace this select group of cells that transiently label for serotonin (compare to Beltz et al., 2001), however, could not trace any co-labeling between anti-SERT labeled cells and those select cells. The serotonin transporter does therefore not seem to be directly involved in the transient uptake of serotonin within the region of life-long neurogenesis.

Numerous reports have indicated a multitude of different serotonin receptors and how their activity contributes to the many facets serotonin exhibits. More interestingly, in vertebrates some of these studies have also indicated the involvement of serotonin receptors on cell proliferation and neurogenesis, even within the olfactory pathway (Dooley et al., 1997; Gould et al., 1999; Radley & Jacobs, 2002; De Lucchini et al., 2003; Gaspar et al., 2003; Banasr et al., 2004). Unfortunately, to this day, no data is available on serotonin receptors in lobster brains or any other crustacean brain. The recent generation of two antibodies against crustacean serotonin receptors (5-HT $1A_{crust}$ and 5-HT $1B_{crust}$; D. Baro, Georgia State University, Georgia, USA) will help to determine the role of serotonin receptor in the process of neurogenesis in lobsters and hence help to elucidate the mechanisms by which serotonin acts.

Although, the many functional roles of the multiple serotonin receptors are currently discovered, recent data in mice already indicate that different developmental processes such as neurogenesis and axon branching are modulated by the different activities of serotonin receptors (Gaspar et al., 2003).

SERT can also be found at the site of newly born cells within cluster 10. Anti-BrdU labeling in the proliferation zone of cluster 10 in embryos had previously been demonstrated by Harzsch et al. (1999). However, consistent anti-BrdU labeling could not be achieved in the experiments performed here. There are two possible explanations why BrdU did not label in

embryonic brains this time: the BrdU labeling reagent did not incorporate into the DNA because embryos did not survive the egg shell perforation procedure, or the primary anti-BrdU antibody was a different one than the one used by Harzsch et al. (1999). The premature death, and thus "failure" of BrdU incorporation into the DNA, of embryos whose egg shell had been perforated was a known problem right from the beginning. So additional attention was paid to the reflexes and heartbeat of the animals at the end of the 4 hour incubation time and only those animals were processed who still showed a heartbeat and tail-flipping reflexes. The need for a different primary anti-BrdU than employed by Harzsch et al. (1999) was necessary because the anti-SERT antibody is raised in the same host as the primary antibody used by Harzsch and colleagues. Although, the simultaneous usage of two antibodies raised in the same host can be applied within one experiment (because ideally primary antibody 1 is only specific against antigen one and primary antibody 2 only specific against antigen two), caution should still be exercised. Mainly because primary antibodies that are raised in the same host might still bind to the same tissue antigen, thus giving a misleading result (Beltz & Burd, 1989). The anti-BrdU primary antibody raised in rat was therefore preferred. However, the unreliability of the rat anti-BrdU primary antibody used had previously been experienced by other members of the "Beltz lab". Further experiments used the M-phase mitosis marker anti-phospho-Histone H3. The advantage hereby lies in the direct application of the antibody: since no labeling reagent is required egg shells do not need to be perforated.

Anti-phospho-Histone H3 labeling showed *inter alia* mitotically active cells within the region of life-long neurogenesis in cluster 10. When simultaneously labeled against the SERT, a close spatial localization, but no co-localization, of anti-SERT labeled cells and cells within the M-Phase of mitosis could further be demonstrated (see Figure 25), thus suggesting that the SERT does not play a direct role in generating new neurons. However, the close spatial relation of the SERT to newborn cells might suggest some regulatory function on neurogenesis. The SERT could, therefore, fine tune or refine brain connections whereas different serotonin receptors are involved in cell proliferation and neurogenesis (Dooley et al., 1997; Gould et al., 1999; Benton & Beltz, 2001; Beltz et al., 2001; Radley & Jacobs, 2002; Gaspar et al., 2003; Banasr et al., 2004). This would also explain the finding of the SERT apart the proliferation zone and be in accordance to unpublished data by J. Benton (Wellesley College, Massachusetts, USA), E. Goergen (University of Nevada at Reno, Nevada, USA) and B. Beltz (Wellesley College, Massachusetts, USA). They have shown that 1) incubating brains in a 10^{-4}M solution of serotonin increases neurogenesis compared to brains that were incubated in BrdU alone, and 2) that fluoxetine incubation and fluoxetine and serotonin

incubation causes similar increases in the rate of neurogenesis within the olfactory projection neurons (cell cluster 10). Because blocking the serotonin transporter results in an increase in the rate of neurogenesis Benton, Geoergen & Beltz concluded that the pace of the cell cycle does not depend upon serotonin uptake but rather be receptor mediated (Benton, Goergen & Beltz, personal communication).
Additional experiments will be needed to further examine the many mechanisms involved in growing and shaping new neurons in this system.

Serotonin as a regulator of neurogenesis. In both vertebrate and invertebrate species, serotonin is a potent regulator of neurogenesis (Brezun & Daszuta, 1999, 2000; Benton & Beltz, 2001, Beltz et al., 2001; Jacobs, 2002; Radley & Jacobs, 2002; Malberg & Duman, 2003). In the brain of the American lobster, reduced serotonin levels result in a decrease in neurogenesis among the deutocerebral local and projection neurons (clusters 9 and 10) (Benton & Beltz, 2001; Beltz et al., 2001), while elevated serotonin levels result in an increased rate of neurogenesis (Benton, Goergen and Beltz, unpublished results). It is also known that a bundle of fine serotonergic fibers from the DGN terminate blindly into the region where new projection neurons are born in cluster 10 (Beltz et al., 2001). Serotonin is therefore thought to be important in regulating the cell cycle period of progenitor cells that produce neurons in the lobster. Hence, it was of particular interest in the context of the current study on circadian regulation of serotonin levels, that the rate of neurogenesis in the lobster brain follows a diurnal rhythm with the lowest rate of neurogenesis at dawn and a peak rate at dusk and that this rhythm is due to a light entrainable, endogenous circadian oscillation mechanism (Goergen et al., 2002).

Data presented here demonstrate that serotonin levels in the brain also follow an endogenous circadian rhythm that is light entrainable. Electrochemical (Figure 11) and immunocytochemical (Table 3) analyses show that serotonin levels are at their highest in whole lobster brains prior to dusk, and at their lowest in the pre-dawn period. Therefore, the peak and trough in serotonin levels in the lobster brain precede the peak and trough in the rate of neurogenesis among the projection neurons. Rhythmic changes in brain serotonin levels also precede the rhythmic changes in neurogenesis when feeding is uncoupled from the dark stimulus of the day and night cycle (Figure 19). Interestingly, the characteristic peaks and troughs of the light entrainable circadian rhythm of both brain serotonin levels and the rate of neurogenesis appear less pronounced when feeding appends as a second stimulus. Studies on

the rate of neurogenesis even indicate that the mean rate of neurogenesis over 24-hours increases when compared to animals that were fed at dusk, the animals' onset in activity. Analysis of some of the factors that underlay feeding, such as chemosensory stimulation/caloric intake and physical activity, indicate that feeding-induced physical activity has an inhibitory effect on the mean rate of neurogenesis over 24-hours. This effect however reverses when feeding-induced physical activity is accompanied by caloric intake (compare Figure 19B and C).

Concluding, the circadian regulation of neurogenesis and serotonin seem to be part of a complex mechanism which is modulated on several levels. Two possible interpretations of the fact that exogenous factors such as feeding and light entrain both brain serotonin levels *and* the timing of neurogenesis arise: either exogenous factors such as feeding and the day/night cycle influence these two processes via parallel regulatory mechanisms, or serotonin may be an element in the direct pathway by which endogenous factors regulates neurogenesis in the lobster brain. However, as we now know that serotonin levels are regulated independently in different brain regions, it is not possible at this stage to relate endogenous fluctuations in brain serotonin levels to the rate of neurogenesis. Local serotonin levels in the proliferation zone of cluster 10 cannot be directly measured, because this region is very small and serotonin levels would be below the limits of detection. Nevertheless, data presented here promote the current understanding that serotonin and neurogenesis are closely linked-factors and that the serotonin transporter SERT, a determinant of serotonins' action, can be found adjacent to newborn cell within the region of life-long neurogenesis.

4.5 Prospective experiments

The data presented here, have given exciting new insights into the regulatory mechanisms of serotonin levels and its role on neurogenesis. A listing of prospective experiments might help to elucidate the many mechanisms that regulate serotonin even further and hence extend our knowledge on neurogenesis.

1. **HPLC:**
    - ***Feeding:*** Individual sampling of the functionally distinct regions (ALs, OLs, brain remainder) of the brain might provide more explicit information whether one lobe

might have powerful modifying abilities on the whole brain rhythm when feeding comes into play (see 4.2).

- Establishing a method that would support enhancement of small serotonin samples to be used for HPLC analysis. This would allow the analysis of local serotonin levels in the proliferation zone of cluster 10 and, therefore, might help to relate endogenous fluctuations in brain serotonin levels to the rate of neurogenesis (see 4.1 and 4.4).
- Such method would also enable the electrochemical analysis of melatonin in the functionally distinct lobes, which might in turn help to elucidate the activity of melatonin as a potential factor for the contrasting rhythm observed in the ALs, OLs and the brain remainder (see 4.3).

2. **ICC:**

- ***Melatonin:*** Immunocytochemical studies using double labeling techniques against serotonin and melatonin might give further insight on whether melatonin is actually converted directly at the site of serotonin expression in the ALs (see 4.3).
- ***SERT:*** The recent generation of two new antibodies against **crustacean** serotonin receptors (5-HT $1A_{crust}$ and 5-HT $1B_{crust}$; D. Baro, Georgia State University, Georgia, USA) will help to determine the role of serotonin receptor in the process of neurogenesis in lobsters and hence help to elucidate the mechanisms by which serotonin acts (see 4.4).

5. Zusammenfassung

Serotonerge Neuronen gehören mit zu den ersten Neuronen, die sich während der Ontogenese im Nervensystem sowohl der Vertebraten als auch der Invertebraten entwickeln (Lauder & Bloom, 1974; Taghert & Goodman, 1984; Fujuminya et al, 1986; Glover et al, 1987; Beltz et al, 1990). Die frühe Entstehung führte zu der Hypothese, dass Serotonin eine wichtige Rolle in der Differenzierung wachsender Neurone spielt, so z.B. in der Regulierung des neuritischen Wachstums und des Aufbaus von Konnektivitäten (Lauder, 1991). Es ist bekannt, dass Serotonin die Teilungsaktivität (Proliferation) neuronaler Stammzellen beeinflusst (Lauder et al, 1981; Brezun & Daszuta, 1999; Benton & Beltz, 2001; Beltz et al, 2001), die Synaptogenese (Lipton & Kater, 1989) moduliert und die Aktivität der Wachstumszentren sowie axonales Wachstum (Sullivan et al, 2000) unterbinden (Haydon et al, 1984) oder fördern (Chubakov et al, 1986) kann. Untersuchungen am Amerikanischen Hummer, *Homarus americanus*, lassen vermuten, dass Serotonin die Proliferation und das Überleben der Projektionsneuronen, die lebenslang im zentralen olfaktorischen System neu generiert werden, beeinflusst (Benton & Beltz, 2001; Beltz et al, 2001). Die aufgeführten Erkenntnisse lassen eine Schlüsselrolle des Serotonins im Kontrollsignalweg, der die mitotische Aktivität der adulten neuronalen Stammzellen im Hummergehirn moduliert, vermuten. Die Arbeiten zur vorgelegte Dissertation sollten daher die Rolle des Serotonins und möglicher, die Neurogenese kontrollierenden exogenen Faktoren im Gehirn juveniler Amerikanischer Hummer untersuchen, insbesondere im Hinblick auf mögliche circadiane Rhythmen des Serotoninspiegels.

Die hier präsentierten Daten zeigen:

1. Der Serotoninspiegel im Gehirn juveniler Hummer unterliegt circadianen (tageszeitlichen) Schwankungen.
2. Licht fungiert als exogener Synchronisator (Zeitgeber) auf den circadianen Rhythmus des Serotonins.
3. Der akzessorische Lobus und der olfaktorische Lobus weisen unterschiedliche circadiane Rhythmen im Serotoninspiegel auf.
4. Futter fungiert ebenfalls als Zeitgeber und kann mit Licht als Zeitgeber koexistieren und komkurrieren.
5. Spontane Nahrungsaufnahme bewirkt eine unmittelbare Erhöhung des endogenen Serotoninspiegels („überraschendes Füttern“).

6. Nahrung *per se* hat einen modulierenden Effekt auf den circadianen Rhythmus von *Serotonin* und auf die *Neurogeneserate.*
7. Physische Aktivität hat einen modulierenden Effekt auf den circadianen *Serotoninrhythmus* und die *Neurogeneserate* („fikitves Füttern").
8. Physische Aktivität ohne Nahrungsaufnahme scheint einen negativen Effekt auf die Entstehung neuer Nervenzellen zu haben.
9. Im akzessorische Lobus liegen melatonerge Strukturen in unmittelbarer Nähe zu serotonergen, wie durch immunohistochemische Doppelmarkierungen gezeigt.
10. Der Melatoninspiegel, als biosynthetisches Produkt von Serotonin, schwankt ebenfalls tageszeitlich.
11. Der circadiane Rhythmus des Melatoninspiegels ist endogen.
12. Serotonin-Transporter (SERT) expremierende Zellen können u.a. im olfaktorischen Projektionscluster 10 von Embryonen gefunden werden.
13. Diese SERT expremierenden Zellen befinden sich u.a. in unmittelbarere Nähe mitotisch aktiver Zellen (Neurone).
14. Diese sich teilenden Zellen sind aufgrund ihrer Größe vermutlich Ganglionmutterzellen.
15. SERT Markierungen konnten in Gegenwart von transient Serotonin aufnehmenden Zellen im olfaktorischen Projektionscluster 10 nachgewiesen werden, allerdings konnten keine Co-Lokalisationen nachgewiesen werden.

Die hier aufgeführten Daten belegen, dass es zwischen den Änderungen des Serotoninspiegels und der Neurogenese funktionale Zusammenhänge geben könnte und dass SERT, als ein Determinant für den Wirkungsort von Serotonin, zudem in unmittelbarer Nähe zu mitotisch aktiven Zellen vorgefunden werden kann. Dies unterstützt die Hypothese, dass Serotonin als *ein* Signal (unter anderen) in der die Neurogenesrate modulierenden Kaskade aggiert.

6. Literature

Abbott N.J. (1971) The organization of the cerebral ganglion in the shore crab, *Carcinus maenas. Z. Zellforsch.* **120**, 401-419.

Abe M., Herzog E.D., Yamazaki S., Straume M., Tei H., Sakaki Y., Menaker M., Block G.D. (2002) Circadian rhythms in isolated brain regions. *J. Neurosci.* **22,** 350-356.

Abran D., Anctil M., Ali M.A. (1994) Melatonin activity rhythms in eyes and cerebral ganglia of Aplysia californica. *Gen. Comp. Endocrinol*. **96**, 215-222.

Agapito M.T., Herrero B., Pablos M.I., Miguel J.L., Recio J.M. (1995) Circadian rhythms of melatonin and serotonin-N-acetyltransferase acitivity in *Procambarus clarkii. Comp. Biochem. Physiol.* **112A**, 179-185.

Aiken D.E. (1973) Proecdysis, setal development, and molt prediction in the American lobster (*Homarus americanus*). *J. Fish. Res. Board. Can.* **30**, 1337-1344.

Akman C., Zhao Q., Liu X., Holmes G.L. (2004) Effect of food deprivation during early development on cognition and neurogenesis in the rat. *Epilepsy Behav.* **5**, 446-454.

Allada R., White N.E., So W.V., Hall J.C., Rosbash M. (1998) A mutant Drosophila homolog of mammalian Clock disrupts circadian rhythms and transcription of period and timeless. *Cell*. **29**, 791-804.

Altman J. and Das G.D. (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J. Comp. Neurol.* **124**, 319-335.

Alvarez-Buylla A. and Kirn J.R. (1997) Birth, migration, incorporation, and death of vocal control neurons in adult songbirds. *J. Neurobiol.* **33**, 585-601.

Amara S.G. and Kuhar M.J. (1993) Neurotransmitter transporters: recent progress. *Annu. Rev. Neurosci.* **16**, 73-93.

Amir S., Cain S., Sullivan J., Robinson B., Stewart J. (1999) In rats, odor-induced Fos in the olfactory pathways depends on the phase of the circadian clock. *Neurosci. Lett.*. **272,** 175-178.

Antolín I., Obst B., Burkhardt S., Hardeland R. (1997) Anti-oxidative protection in a high-melatonin organism: the dinoflagellata *Gongyaulax polyedra* is rescued from lethal oxidative stress by strongly elevated, but physiologically possible concentrations of melatonin. *J. Pineal Res.* **23**, 182-190.

Antolín I. and Hardeland R. (1997) Suppression of the *Gongyaulax polyedra* peak by paraquat and its restoration by melatonin. In: Biological Rhythms and Antioxidative Protection. Hardeland R. ed., Cuvillier, Göttingen, pp. 86-97.

Arechiga H., Fernandez-Quiroz F., Fernandez de Miguel F., Rodriguez-Sosa L. (1993) The circadian system of crustaceans. *Chronobiol. Int.* **10**, 1-19.

Arendt J. (1998) Melatonin and the pineal gland: influence on mammalian seasonal and circadian physiology. *Rev. Reprod.* **3**, 13-22.

Aschoff J. and Tokura H. (1986). Circadian activity rhythms in squirrel monkeys: entrainment by temperature cycles. *J. Biol. Rhythms* **1**, 91-99.

Austin M.C., Bradley C.C., Mann J.J., Blakely R.D. (1994) Expression of serotonin transporter messenger RNA in the human brain. *J. Neurochem.* **62**, 2362-2367.

Balzer I., Espinola I.R., Fuentes-Pardo R. (1997) Daily variations of immunoreactive melatonin in the visual system of the crayfish. *Biol. Cell*. **89**, 539-543.

Banasr M., Hery M., Printemps R., Daszuta A. (2004) Serotonin-induced increases in adult cell proliferation and neurogenesis are mediated through different and common 5-HT receptor subtypes in the dentate gyrus and the subventricular zone. *Neuropsychopharmacology.* **29**, 450-460.

Battelle B.A. and Kravitz E.A. (1978) Targets of octopamine action in the lobster: cyclic nucleotide changes and physiological effects in haemolymph, heart and exoskeletal muscle. *J. Pharmacol. Exp. Ther.* **205**, 438-448.

Beltz B.S. and Kravitz E.A. (1983) Mapping of serotonin-like immunoreactivity in the lobster nervous system. *J. Neurosci.* **3**, 585-602.

Beltz B.S. and Kravitz E.A. (1987) Physiological identification, morphological analysis, and development of identified serotonin-proctolin containing neurons in the lobster ventral nerve cord. *J. Neurosci.* **7**, 533-546.

Beltz B.S. and Burd G.D. (1989) *Immunocytochemical Techniques: Principles and practice,* Cambridge, MA: Blackwell Scientific Publications.

Beltz B.S., Pontes M., Helluy S.M., Kravitz E.A. (1990) Patterns of appearance of serotonin and proctolin immunoreactivities in the developing nervous system of the American lobster. *J. Neurobiol.* **21**, 521-542.

Beltz B.S. (1999) Distribution and functional anatomy of amine-containing neurons in decapod crustaceans. *Microsc. Res. Tech*. **44**, 105-120.

Beltz B.S., Benton J.L., Sullivan J.M. (2001) Transient uptake of serotonin by newborn olfactory projection neurons. *Proc. Natl. Acad. Sci. USA.* **98**, 12730-12735.

Beltz B.S. and Kravitz E.A. (2003) Serotonin in crustacean systems: more than a half century of fundamental discoveries. In *Crustacean Experimental Systems in Neurobiology,* Wiese K. ed, pp. 141-163. Springer-Verlag, Berlin.

Beltz B. and Sandeman D. (2003) Regulation of life-long neurogenesis in the decapod crustacean brain. *Arthropod. Struct. Dev.* **32**, 39-60.

Benton J., Helluy S., Huber R., Ruchhoeft M., Beltz B.S. (1997) Serotonin depletion by 5,7-dihydroxytryptamine alters deutocerebral development in the lobster, *Homarus americanus. J. Neurobiol.* **33**, 357-373.

Benton J. and Beltz B.S. (2001) Effects of embryonic serotonin depletion on local interneurons in the olfactory pathway of lobsters. *J. Neurobiol.* **46**, 193-205.

Benton J.L. and Beltz B.S. (2002) Patterns of neurogenesis in the midbrain of embryonic lobsters differ from proliferation in the insect and the crustacean ventral nerve cord. *J. Neurobiol.* **53**, 57-67.

Berlind A. (2001) Monoamine pharmacology of the lobster cardiac ganglion. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **128**, 377-90.

Bethe A. (1897a) Das Nervensystem von *Carcinus maenas*, ein anatomisch-physiologischer Versuch. Theil, I. *Mittheil. Arch. Mikr. Anat.* **50**, 460-546.

Bethe A. (1897b) Das Centralnervensystem von *Carcinus maenas*. Ein anatomisch-physiologischer Versuch , I, Theil II *Mittheil. Arch. Mikr. Anal.* **50**, 589-639.

Bethe A. (1998) Das Centralnervensystem von *Carcinus maenas*. Ein anatomisch-physiologischer Versuch. II. Theil. *Arch. Mikr. Anal.* **51**, 382-452.

Binkley S. (1997) *Biological clocks.* London, UK: CRC Press.

Blakely R.D., Berson H.E., Fremeau R.T. Jr., Caron M.G., Peek M.M., Prince H.K., Bradley C.C. (1991) Cloning and expression of a functional serotonin transporter from rat brain. *Nature*. **354**, 66-70.

Blanc A., Vivien-Roels B., Pevet P., Attia J., Buisson B. (2003) Melatonin and 5-methoxytryptophol (5-ML) in nervous and/or neurosensory structures of a gastropod mollusc (Helix aspersa maxima): synthesis and diurnal rhythms. *Gen Comp Endocrinol.* **131**, 168-175.

Blaustein D.N., Derby C.D., Simmons R.B., Beall A.C. (1988) Structure of the brain and medulla terminalis of the spiny lobster *Panulirus argus* and the crayfish *Procambarus clarkii*, with an emphasis on olfactory centers. *J. Crustacean Biol.* **8**, 493-519.

Blenau W. and Baumann A. (2001) Molecular and pharmacological properties of insect biogenic amine receptors: lessons from *Drosophila melanogaster* and *Apis mellifera*. *Arch. Insect Biochem. Physiol.* **48**, 13-38.

Blundell J.E. (1992) Serotonin and the biology of feeding. *Am. J. Clin. Nutr.* **56**, 155-159.

Blundell J.E. and Hill A.J. (1987) Serotonergic modulation of the pattern of eating and the profile of hunger-satiety in humans. *Int. J. Obes.* **11**, 141-155.

Bondolfi L., Ermini F., Long J.M., Ingram D.K., Jucker M. (2004) Impact of age and caloric restriction on neurogenesis in the dentate gyrus of C57BL/6 mice. *Neurobiol. Aging.* **25**, 333-340.

Booker R., Babashak J., Kim J.B. (1996) Postembryonic neurogenesis in the central nervous system of the tobacco hornworm, Manduca sexta. III. Spatial and temporal patterns of proliferation. *J. Neurobiol.* **29**,233-248.

Booker R. and Truman J.W. (1987) Postembryonic neurogenesis in the CNS of the tobacco hornworm, Manduca sexta. I. Neuroblast arrays and the fate of their progeny during metamorphosis. *J. Comp. Neurol.* **22**, 548-559.

Bouwknecht J.A., van der Gugten J., Hijzen T.H., Maes R.A., Hen R., Oliver B. (2001) Male and female 5-HT(1B) receptor knockout mice have higher body weights than wildtypes. *Physiol. Behav.* **74**, 507-516.

Brezun J.M. and Daszuta A. (1999) Depletion in serotonin decreases neurogenesis in the dentate gyrus and the subventricular zone of adult rats. *Neuroscience* **89**, 999-1002.

Brezun J.M. and Daszuta A. (2000) Serotonin may stimulate granule cell proliferation in the adult hippocampus, as observed in rats grafted with foetal raphe neurons. *Eur. J. Neurosci.* **12**, 391-396.

Brown S.A., Zumbrunn G., Fleury-Olela F., Preitner N., Schibler U. (2002) Rhythms of mammalian body temperature can sustain peripheral circadian clocks. *Curr. Biol.* **12**, 574-583.

Bruns D., Engert F., Lux H.D. (1993) A fast activating presynaptic reuptake current during serotonergic transmission in identified neurons of Hirudo.*Neuron.* **10**, 559-572.

Byrd C.A. and Brunjes P.C. (2001) Neurogenesis in the olfactory bulb of adult zebrafish. *Neuroscience*. **105**, 793-801.

Byrd C.A. and Brunjes P.C. (1998) Addition of new cells to the olfactory bulb of adult zebrafish. *Ann. N Y Acad. Sci.* **855**, 274-276.

Campos-Ortega J.A. (1995) Genetic mechanisms of early neurogenesis in Drosophila melanogaster. *Mol. Neurobiol.* **10**, 75-89.

Cangiano C., Ceci F., Casciano A., Del Ben M., Laviano A., Muscaritoli M., Antonucci F., Rossi-Fanelli F. (1992) Eating behavior and adherence to dietary prescriptions in obese adult subjects treated with 5-hydroxytryptophan. *Am. J. Clin. Nutr.* **56**, 863-867.

Canton H., Emeson R.B., Barker E.L., Backstrom J.R., Lu J.T., Chang M.S., Sanders-Bush E. (1996) Identification, molecular cloning, and distribution of a short variant of the 5-hydroxytryptamine2C receptor produced by alternative splicing. *Mol. Pharmacol.* **50**, 799-807.

Cases O., Lebrand C., Giros B., Vitalis T., De Maeyer E., Caron M.G., Price D.J., Gaspar P., Seif I. (1998) Plasma membrane transporters of serotonin, dopamine, and norepinephrine mediate serotonin accumulation in atypical locations in the developing brain of monoamine oxidase A knock-outs. *J. Neurosci.* **18**, 6914-6927.

Castanon-Cervantes O., Battelle B., Fanjul-Moles M.L. (1999) Rhythmic changes in the serotonin content of the brain and eyestalk of crayfish during development. *J. Exp. Biol.* **202**, 2823-2830.

Cayre M., Malaterre J., Scotto-Lomassese S., Strambi C., Strambi A. (2002) The common properties of neurogenesis in the adult brain: from invertebrates to vertebrates. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **132**, 1-15.

Cayre M., Strambi C., Charpin P., Augier R., Meyer M.R., Edwards J.S., Strambi A. (1996) Neurogenesis in adult insect mushroom bodies. *J. Comp. Neurol.* **371**, 300-310.

Ceron J., González C., Tejedor F.J. (2001) Patterns of cell division and expression of asymmetric cell fate determinants in postembryonic neuroblast lineage of *Drosophila. Dev. Biol.* **230**, 125-138.

Chang A.S., Chang S.M., Starnes D.M., Schroeter S., Bauman A.L., Blakely R.D. (1996) Cloning and expression of the mouse serotonin transporter. *Brain Res. Mol. Brain. Res.* **43**, 185-192.

Chubakov A.R., Gromova E.A., Konolov G.V., Sarkisova E.F., Chumasov E.I. (1986) The effects of serotonin on the morpho-functional development of rat cerebral cortex in tissue culture. *Brain Res.* **369,** 285-297.

Coleman G.J., Harper S., Clarke J.D., Armstrong S. (1992) Evidence for a separate meal-induced oscillator in the rat. *Physiol. Behav.* **29**, 107-115.

Cooper R.A. and Uzmann J.R. (1980) Ecology of juvenile and adult *Homarus*. In *The Biology and Management of Lobsters* (eds. J. S. Cobb and B.F. Philipps), pp. 97-142. New York: Academic Press.

Corey J.L., Quick M.W., Davidson N., Lester H.A., Guastella J. (1994) A cocaine-sensitive Drosophila serotonin transporter: cloning, expression, and electrophysiological characterization. *Proc. Natl. Acad. Sci. USA.* **91**, 1188-1192.

Curzon G. (1991). Effects of tryptophan and 5-hydroxytryptamine receptor subtype agonists on feeding. *Adv. Exp. Med. Biol.* **294**, 377-388.

Damiola F., Le Minh N., Preitner N., Korman B., Fleiry-Olela F., Schibler U. (2000) Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev.* **14**, 2950-2961.

Davis F.C., Stice S., Menaker M. (1987) Activity and reproductive state in the hamster: independent control by social stimuli and a circadian pacemaker. *Physiol. Behav.* **40**, 583-590.

De Fanti B.A., Hamilton J.S., Horwitz B.A. (2001) Meal-induced changes in extracellular 5-HT in medial hypothalamus of lean (Fa/Fa) and obese (fa/fa) Zucker rats. *Brain Res.* **902**, 164-170.

De Lucchini S., Ori M., Nardini M., Marracci S., Nardi I. (2003) Expression of 5-HT2B and 5-HT2C receptor genes is associated with proliferative regions of Xenopus developing brain and eye. *Brain Res. Mol. Brain Res.* **115**, 196-201.

Demchyshyn L.L., Pristupa Z.B., Sugamori K.S., Barker E.L., Blakely R.D., Wolfgang W.J., Forte M.A., Niznik H.B. (1994) Cloning, expression, and localization of a chloride-facilitated, cocaine-sensitive serotonin transporter from Drosophila melanogaster. *Proc. Natl. Acad. Sci. USA*. **91**, 5158-5162.

De Prins J. and Waldura J. (1993) Sightseeing around the single cosinor. *Chronobiol. Int.* **10**, 395-400.

Doe C.Q., Chu-LaGraff Q., Wright D.M., Scott M.P. (1991) The prospero gene specifies cell fates in the Drosophila central nervous system. *Cell.* **65**, 451-464.

Doe C.Q. and Skeath J.B. (1996) Neurogenesis in the insect central nervous system. *Curr. Opin. Neurobiol.* **6**, 18-24.

Doe C.Q., Fuerstenberg S., Peng C.Y. (1998) Neural stem cells: from fly to vertebrates. *J. Neurobiol.* **36**, 111-127.

Dohle W. and Scholtz G. (1997) How far does cell lineage influence cell fate specification in crustacean embryos? *Semin. Cell. Dev. Biol.* **8**, 379-390.

Dooley A.E., Pappas I.S., Parnavelas J.G. (1997) Serotonin promotes the survival of cortical glutamatergic neurons in vitro. *Exp. Neurol.* **148**, 205-214.

Dudel J. (1965) Facilitatory effects of 5-hydroxytryptamine on the crayfish neuromuscular junction. *Naunyn Schmiedebergs Arch. Pharmacol.* **249**, 515-528.

Ennis G.P. (1983) Observation on the behaviour and activity of lobsters, *Homarus americanus,* in nature. *Canadian Technical Report of Fisheries and Aquatic Sciences*, **1165**, 1-26.

Eriksson P.S., Perfilieva E., Bjork-Eriksson T., Alborn A.M., Nordborg C., Peterson D.A., Gage F.H. (1998) Neurogenesis in the adult human hippocampus. *Nat. Med.* **4**, 1313-1317.

Escamilla-Chimal E.G., Van Herp F., Fanjul-Moles M.L. (2001) Variations in crustacean hyperglycaemic hormone and serotonin immunoreactivity during the development of crayfish. *J. Exp. Biol.* **204**, 1073-1081.

Fadool D.A., Brown P.R., Cobb J.S., Kass-Simon G. (1988) HPLC analysis of lobster haemolymph over the molt cycle. *Comp. Biochem. Physiol*. **93C**, 225-230.

Fanjul-Moles M.L. and Prieto-Sagredo J. (2003) The circadian system of crayfish: a developmental approach. *Microsc. Res. Tech.* **60**, 291-301.

Fernstrom J.D. and Wurtman R.J. (1973) Control of brain 5-HT content by dietary carbohydrates. In Serotonin and behavior, J Barchas, E. Usdin (eds.), pp 121-128. Academic Press, NY.

Fingerman S.W. and Fingerman M. (1977) Circadian variation in the levels of red pigment-dispersing hormone and 5-hydroxytryptamine in the eyestalks of the fiddler crab, *Uca pugilator*. *Comp. Biochem. Physiol. C.* **56**, 5-8.

Fingerman M., Schultz R.E., Bordlee B.P., Dalton D.P. (1978) Twenty-four-hour variation of 5-hydroxytryptophan decarboxylase and monoamine oxidase activities in the eyestalks of the fiddler crab, *Uca pugilator*. *Comp. Biochem. Physiol. C.* **61C**, 171-175.

Florey E. and Florey E. (1954) Über die mögliche Bedeutung von Enteramin (5-Hydroxytryptamine) als nervöse Aktionssubstanz bei cephalopoden und dekapoden Crustaceen. *Z. Naturfosch.* **9B**, 58-68.

Florey E. (1954) Über die Wirkung von 5-Oxytryptamine (Enteramin) in der Krebsschere. *Z. Naturforsch.* **9B**, 540-547.

Florey E. and Rathmayer M. (1978) The effects of octopamine and other amines on the heart and on neuromuscular transmission in decapod crustacean: further evidence for a role as a neurohormone. *Comp. Biochem. Physiol.* **61C**, 229-237.

Funk D. and Amir S. (2000) Circadian modulation of fos responses to odor of the red fox, a rodent predator, in the rat olfactory system. *Brain Res.* **866,** 262-267.

Gage F.H., Kempermann G., Palmer T.D., Peterson D.A., Ray J. (1998) Multipotent progenitor cells in the adult dentate gyrus. *J. Neurobiol.* **36**, 249-266.

Ganguly S., Coon S.L., Klein D.C. (2002) Control of melatonin synthesis in the mammalian pineal gland: the critical role of serotonin acetylation. *Cell Tissue Res.* **309**, 127-37.

Gaspar P., Cases O., Maroteaux L. (2003) The developmental role of serotonin: news from mouse molecular genetics. *Nat. Rev. Neurosci.* **4**, 1002-1012.

Giebultowicz J.M. Stanewsky R., Hall J.C., Hege D.M. (2000) Transplanted *Drosophila* excretory tubulus maintain circadian clock cycling out of phase with the host. *Curr. Biol.* **10**, 107-110.

Giebultowicz J.M. (2000) Molecular mechanism and cellular distribution of insect circadian clocks. *Annu. Rev. Entomol.* **45**, 769-793.

Giebultowicz J.M. (1999) Insect circadian clocks: is it all in their heads? *J. Insect Physiol.* **45**, 791-800.

Glusman S. and Kravitz E.A. (1982) The action of serotonin and excitatory nerve terminals in lobster- muscle preparations. *J. Physiol.* **325**, 223-241.

Goldberg J.I. (1998) Serotonin regulation of neurite outgrowth in identified neurons from mature and embryonic *Helisoma trivolvis*. *Perspect. Dev. Neurobiol.* **5**, 373-387.

Gorbet D.J. and Steel C.G.H. (2003) A miniature radioimmunoassay for melatonin for use with small samples from invertebrates. *Gen. and Comp. Endocrinology*. **134**,193-197.

Goergen E.M., Bagay L.A., Rehm K., Benton J.L., Beltz B.S. (2002) Circadian control of neurogenesis. *J. Neurobiol.* **53**, 90-95.

Gorissen S. (2002) Endogenous control of neurogenesis in the juvenile crustacean brain. Honors Thesis, University of New South Wales, Sydney, Australia.

Gould E. (1999) Serotonin and hippocampal neurogenesis. *Neuropsychopharmacology*. **21**, 46-51.

Granados-Fuentes D., Saxeena M.T., Prolo L.M., Aton S.J., Herzog E.D. (2004a) Olfactory bulb neurons express functional, entrainable circadian rhythms. *Eur. J. Neurosci.* **19**, 898-906.

Granados-Fuentes D., Prolo L.M., Abraham U., Herzog E.D. (2004b) The suprachiasmatic nucleus entrains, but does not sustain, circadian rhythmicity in the olfactory bulb. *J. Neurosci.* **24**, 615-619.

Hall J.C. (1998) Molecular neurogenetics of biological rhythms. *J. Neurogenet.* **12**, 115-181.

Hall M.R. and Van Ham E.H. (1998) Diurnal variation and effects of feeding on blood glucose in the giant tiger prawn, *Penaeus monodon. Physiol. Zool.* **71**, 574-583.

Hansson S.R., Mezey E., Hoffman B.J. (1998) Serotonin transporter messenger RNA in the developing rat brain: early expression in serotonergic neurons and transient expression in non-serotonergic neurons. *Neuroscience.* **83**, 1185-1201.

Hanström B. (1924) Untersuchungen über das Gehirn, insbesodnere die Sehganglion der Crustaceen. *Ark. Zool.* **16**, 1-117.

Hanström B. (1925) The olfactory centers in crustaceans. *J. Comp. Neurol.* **38,** 221-250.

Hanström B. (1928) Vergleichende Anatomie des Nervensystems der Wirbellosen Tiere. Springer Verlag, Berlin.

Hanström B. (1931) Neue Untersuchungen über die Sinnesorgane und Nervensystem der Crustaceen. I. *Z. Morphol. Oekol. Tiere* **23**, 80-236.

Hanström B. (1933) Neue Untersuchungen über Sinnesorgane und Nervensystem der Crustaceen. II. *Zool. Jb. Abt. Anat.* **56**, 387-520.

Hardeland R. and Poeggeler B. (2003) Non-vertebrate melatonin. *J. Pineal Res.* **34**, 233-241.

Hardeland R., Burkhardt S., Antolin I., Fuhrberg B., Coto-Montes A. (1999) Melatonin and 5-methoxytryptamine in the bioluminescent dinoflagellate *Gongyaulax polyedra*: restoration of the circadian glow peak after suppression of indoleamine biosynthesis and oxidative stress. *Adv. Exp. Med. Biol.* **460**, 387-390.

Hardeland R. and Coto-Montes A. (2000) Chronobiology of oxidative stress and antioxidative defense mechanisms. In: Recent Research Developments in Comperative

Biochemistry and Physiology, Vol. 1. Pandalai S.G. ed., Transworld Research Network, Trivandrum, pp. 123-137.

Hardeland R. (1997) New actions of melatonin and their relevance to biometeorology. *Int. J. Biometerol.* **41**, 47-57.

Hardeland R., Balzer I., Poeggeler B., Fuhrberg B., Uria H., Behrmann G., Wolf R., Meyer T.J., Reiter R.J. (1995) On the primary functions of melatonin in evolution: mediation of photoperiodic signals in a unicell, photooxidation, and scavenging of free radicals. *J. Pineal Res.* **18,**104-111.

Harris-Warrick R.M. and Kravitz E.A. (1984) Cellular mechanisms for modulation of posture by octopamine and serotonin in the lobster. *J. Neurosci.* **4**, 1976-1993.

Harrison P., Cate H., Swanson E., Derby C. (2001) Postembryonic proliferation in the spiny lobster antennular epithelium: rate of genesis of olfactory receptor neurons is dependent on molt stage. *J. Neurobiol.* **47**, 51-66.

Harvey J.A. (2003) Role of the serotonin 5-HT(2A) receptor in learning. *Learn. Mem.* **10**, 355-362.

Harzsch S. and Dawirs R.R. (1994) Neurogenesis in larval stages of the spider crab *Hyas araneus* L. (Decapoda, Brachura): proliferation of neuroblasts in the ventral nerve cord. *Roux's Arch. Dev. Biol.* **204**, 92-100.

Harzsch S. and Dawirs R.R. (1996) Neurogenesis in the developing crab brain: postembryonic generation of neurons persists beyond metamorphosis. *J. Neurobiol.* **29**, 384-398.

Harzsch S., Miller J., Benton J., Dawirs R.R., Beltz B. (1998) Neurogenesis in the thoracic neuromeres of two crustaceans with different types of metamorphic development. *J. Exp. Biol.* **201**, 2465-2479.

Harzsch S., Miller J., Benton J., Beltz B. (1999) From embryo to adult: persistent neurogenesis and apoptotic cell death shape the lobster deutocerebrum. *J. Neurosci.* **19**, 3472-3485.

Harzsch S. (2001) Neurogenesis in the crustacean ventral nerve cord: homology of neuronal stem cells in Malacostraca and Branchiopoda? *Evol. Dev.* **3**, 154-169.

Harzsch S., Sandeman D, Chaigneau J. (2004) Morphology and development of the central nervous system. In: *Treatise on Zoology – Crustacea*. Forest J. and von Vaupel Klein J.C. eds., Koninklijke Brill Academic Publishers, Leiden, Netherlands, pp. 4-84.

Haydon P.G., McCobb D.P., Kater S.B. (1984) Serotonin selectively inhibits growth cone motility and synaptogenesis of specific identified neurons. *Science*. **226**, 561-564.

Hege D.M., Stanewsky R., Hall J.C., Giebultowicz J.M. (1997) Rhythmic expression of a PER-reporter in the Malpigian tubulus of decapitated *Drosophila*: evidence for a brain-independent clock. *J. Biol. Rhythms* **12**, 300-308.

Helfrich-Förster C. (2002) the circadian system of *Drosophila melanogaster* and its light input pathways. *Zoology.* **105**, 297-312.

Helfrich-Förster C., Tauber M., Park J.H., Muhlig-Versen M., Schneuwly S., Hofbauer A. (2000) Ectopic expression of the neuropeptide pigment-dispersing factor alters behavioral rhythms in Drosophila melanogaster. *J. Neurosci.* **20**, 3339-3353.

Helm F. (1928) Vergleichend-anatomische Untersuchungen über das Gehirn, insbesondere der Antennalganglion der Decapoden. *Z. Morphol. Oekol. der Tiere* **12**, 70-134.

Helluy S.M. and Beltz B.S. (1991) Embryonic development of the American lobster: quantitative staging and characterization of an embryonic molt cycle. *Biol. Bull.* **180**, 355-371.

Helluy S., Ruchhoeft M., Beltz B. (1995). Development of the olfactory and accessory lobes in the American lobster: An allometric analysis and its implications for the deutocerebral structure of decapods. *J. Comp. Neurol.* **358**, 1-13.

Hoffman B.J., Mezey E., Brownstein M.J. (1991) Cloning of a serotonin transporter affected by antidepressants. *Science.* **254**, 579-580.

Holmes M.M., Galea L.A., Mistlberger R.E., Kempermann G. (2004) Adult hippocampal neurogenesis and voluntary running activity: circadian ad dose-dependent effects. *J. Neurosci. Res.* **76**, 216-222.

Holmgren N. (1916) Zur Anatomie des Gehirns von Polychaeten, Onychophoren, Xiphosuren, Arachniden, Crustaceen, Myriapoden und Insekten. *Kunglig Svenska Vetenkapsakademiens Handlingar* **56**, 1-303.

Horner M., Weiger W.A., Edwards D.H., Kravitz E.A. (1997) Excitation of identified serotonergic neurons by escape command neurons in lobsters. *J. Exp. Biol.* **200**, 2017-2033.

Huber R. and Kravitz E.A. (1995) A quantitative analysis of agonistic behavior in juvenile American lobsters (*Homarus americanus* L). *Brain Behav. Evol.* **46**, 72-83.

Izmaylov D.M. and Obukhova, L.K. (1999) Geroprotector effectiveness of melatonin: investigation of lifespan of *Drosophila melanogaster*. *Mech. Ageing Dev.* **106**, 233-240.

Jacobs B.L. (2002) Adult brain neurogenesis and depression. *Brain Behav. Immun.* **16**, 602-609.

Kempermann G., Kuhn H.G., Gage F.H. (1997) More hippocampal neurons in adult mice living in an enriched environment. *Nature* **386**, 493-495.

Kempermann G. and Gage F.H. (1999) New nerve cells for the adult brain. *Sci. Am.* **280,** 48-53.

Kempermann G., Gast D., Gage F.H. (2002) Neuroplasticity in old age: sustained fivefold induction of hippocampal neurogenesis by long-term environmental enrichment. *Ann. Neurol.* **52**, 135-143.

Konopka R.J. and Benzer S. (1971) Clock mutants of Drosophila melanogaster. Proc. Natl. Acad. Sci. USA. **68**, 2112-2116.

Kranz V.D. and Richter W. (1975) [Neurogenesis and regeneration in the brain of teleosts in relation to age. (Autoradiographic studies)] *Z. Alternsforsch.* **30**, 371-382.

Kravitz E.A. (2000) Serotonin and aggression: insights gained from a lobster model system and speculations on the role of amine neurons in a complex behavior. *J. Comp. Physiol. A.* **186**, 221-238.

Krishnan B., Dryer S.E., Hardin P.E. (1999) Circadian rhythms in olfactory responses of *Drosophila melanogaster. Nature.* **400**, 375-378.

Langworthy K., Helluy S., Benton J., Beltz B. (1997) Amines and peptides in the brain of the American lobster: immunocytochemical localization patterns and implications for brain function. *Cell Tissue Res.* **288**, 191-206.

Lauder J.M. (1991) Ontogeny of serotonergic system in the rat: serotonin as a developmental signal. *Ann NY Acad. Sci.* **600**, 297-314.

Lebrand C., Cases O., Wehrle R., Blakely R.D., Edwards R.H., Gaspar P. (1998) Transient developmental expression of monoamine transporters in the rodent forebrain. *J. Comp. Neurol.* **401**, 506-524.

Lee J., Seroogy K.B., Mattson M.P. (2002) Dietary restriction enhances neurotrophin expression and neurogenesis in the hippocampus of adult mice. *J. Neurochem.* **80**, 539-547.

Lee J., Duan W., Long J.M., Ingram D.K., Mattson M.P. (2000) Dietary restriction increases the number of newly generated cells, and induces BNDF expression, in the dentate gyrus of rats. *J. Mol. Neurosci.* **15**, 99-108.

Legutko R. and Gannon R.L. (2001) Serotonin transporter localization in the hamster suprachiasmatic nucleus. *Brain Res.* **893**, 77-83.

Lesch K.P., Balling U., Gross J., Strauss K., Wolozin B.L., Murphy D.L., Riederer P. (1994) Organization of the human serotonin transporter gene. *J. Neural. Transm. Gen. Sect.* **95**, 157-162.

Lipton S.A. and Kater S.B. (1989) Neurotransmitter regulation of neuronal outgrowth, plasticity and survival. *Trends Neurosci*. **12**, 265-270.

Linn C.E., Poole K.P., Wu W.Q., Roelofs W.L. (1995) Circadian changes in melatonin in the nervous system and haemolymph of the cabbage looper moth, *Trichoplusia ni*. *J. Comp. Physiol. A.* **176**, 761-771.

Livingstone M.S., Schaeffer S.F., Kravitz, E.A. (1981) Biochemistry and ultrastructure of serotonergic nerve endings in the lobster: serotonin and octopamine are contained in different nerve endings. *J. Neurobiol.* **12**, 27-54.

Lois C. and Alvarez-Buylla A. (1994) Long distance neuronal migration in the adult mammalian brain. *Science.* **264**, 1145-1148.

Ma P.M., Beltz B.S., Kravitz E.A. (1992) Serotonin-containing neurons in lobsters: their role as gain-setters in postural control mechanisms. *J. Neurophysiol.* **68**, 36-54.

Malberg J.E. and Duman R.S. (2003) Cell proliferation in adult hippocampus is decreased by inescapable stress: reversal by fluoxetine treatment. *Neuropsychopharm*. **28**, 1562-1571.

Mayser W., Betz H., Schloss P. (1991) Isolation of cDNAs encoding a novel member of the neurotransmitter transporter gene family. *FEBS Lett.* **295**, 203-206.

Mahoney J.A. (2002) Apoptosis in the lobster brain: regulation of cell number or a mechanism of neuronal replacement. Honors Thesis, Wellesley College, Wellesley, MA, USA.

Matsuzaki F. (2000) Asymmetric division of Drosophila neural stem cells: a basis for neural diversity. *Curr. Opin. Neurobiol.* **10**, 38-44.

Marmot G. and Wiersma C.A.G. (1938) On the mechanism of inhibition and excitation of crayfish muscle. *J. Physiol.* **93**, 173-193.

Mazarella E. (2003) The influence of social status on neurogenesis in juvenile lobsters, *Homarus americanus.* Honors Thesis, Wellesley College, Wellesley, MA, USA.

McCobb D.P., Haydon P.G., Kater S.B. (1988) Dopamine and serotonin inhibition of neurite elongation of different identified neurons. *J. Neurosci. Res.* **19,** 19-26.

McKinzie M.E., Benton J.L., Beltz B.S., Mellon D. (2003) Parasol cells of the hemiellipsoid body in the crayfish Procambarus clarkii: dendritic branching patterns and functional implications. *J. Comp. Neurol.* **462**, 168-79.

Mechawar N. and Anctil M. (1997) Melatonin in a primitive metazoan: seasonal changes of levels and immunohistochemical visualization in neurons. *J. Comp. Neurol.* **387**, 243-254.

Menaker M. and Eskin A. (1966) Entrainment of circadian rhythms by sound in *Passer domesticus*. *Science* **154**, 1579-1581.

Meneses A. (2003) A pharmacological analysis of an associative learning task: 5-HT(1) to 5-HT(7) receptor subtypes function on a Pavlovian/instrumental autoshaped memory. *Learn. Mem.* **10**, 363-372.

Miguez J.M., Simonneaux V. Pévet P. (1997) The role of the intracellular and extracellular serotonin in the regulation of melatonin production in rat pinealocytes. *J. Pineal Res.* **23**, 63-71.

Mijnster M.J., Raimundo A.G., Koskuba K., Klop H., Docter G.J., Groenewegen H.J., Voorn P. (1997) Regional and cellular distribution of serotonin 5-hydroxytryptamine 2A receptor mRNA in the nucleus accumbens, olfactory tubercle, and caudate putamen of the rat. *J. Comp. Neurol.* **389**, 1-11.

Miller J.R. and Stelinski L.L. (2004) Letter to the editors: does cockroach sensitivity to odors vary ten fold with circadian rhythm? *J. Insect Physiol.* **50**, 1-4.

Minors D.S. and Waterhouse J.M. (1988) Mathematical and statistical analysis of circadian rhythms. *Psychoneuroendocrinol.* **13**, 443-464.

Moore R.Y. and Eichler V.B. (1972) Loss of circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. *Brain Res.* **42**, 201-206.

Morales M., Battenberg E., Bloom F.E. (1998) Distribution of neurons expressing immunoreactivity fort he 5-HT3 receptor subtype in the rat brain and spinal cord. *J. Comp. Neurol.* **402**, 385-401.

Moriizumi T., Tsukatani T., Sakashita H., Miwa T. (1994) Olfactory disturbance induced by deafferentiation of serotonergic fibers in the olfactory bulb. *Neurosci.* **61**, 733-738.

Naylor E. (1996) Crab clockwork: the case for interactive circatidal and circadian oscillators controlling rhythmic locomotor activity of *Carcinus maenas*. *Chronobiol. Int*. **13**, 153-161.

Nelson W., Tong Y.L., Lee J.-K., Halberg F. (1979) Methods for cosinor-rhythmometry. *Chronobiologia* **6**, 305-323.

Novak M.G. and Rowley W.A. (1994) Serotonin depletion affects blood-feeding but not host-seeking ability in Aedes triseriatus (Diptera: Culicidae). *J. Med. Entomol.* **31**, 600-606.

Okado N., Cheng L., Tanatsugu Y., Hamada S., Hamaguchi K. (1993) Synaptic loss following removal of serotoninergic fibers in newly hatched and adult chickens. J. Neurobiol. **24**, 687-698.

Olcese J. and Munker M. (1994) Extracellular serotonin promotes melatonin release from cultured rat pinealocytes: evidence for an S2-type receptor-mediated autocrine feedback. *Brain Res.* **643**, 150-154.

Orosco M. and Nicolaidis S. (1992) Spontaneous feeding-related monoaminergic changes in the rostromedial hypothalamus revealed by microdialysis. *Physiol. Behav.* **52**, 1015-1019.

Orsetti M., Dellarole A., Ferri S., Ghi P. (2003) Acquisition, retention, and recall of memory after injection of RS67333, a 5-HT(4) receptor agonist, into the nucleus basalis magnocellularis of the rat. *Learn. Mem.* **10**, 420-426.

Page T.L. (1985) Clocks and circadian rhythms. *Comp. Insect Physiol. Biochem. Pharmacol.* **6**, 557-653.

Palmer J.D. (1997) Dueling hypotheses: circatidal versus circalunidian battle basics--second engagement. *Chronobiol. Int.* **14**, 431-433.

Pasztor V.M. and Bush B.M. (1987) Peripheral modulation of mechano-sensitivity in primary afferent neurons. *Nature*. **326**, 793-795.

Pasztor V.M. and Macmillan D.L. (1990) The actions of proctolin, octopamine and serotonin on crustacean proprioceptors show species and neurone specificity. *J. Exp. Biol.* **152**, 485-504.

Pelz D. (2001) Der Einfluss verschiedener Umweltfaktoren und sozialer Stellung auf die Neurogenese von Hirnneuronen beim australischen Flusskrebs, Diploma Thesis, Rheinische Friederich-Wilhelm Universität Bonn, Germany.

Radley J.J. and Jacobs B.L. (2002) 5-HT1A receptor antagonist administration decreases cell proliferation in the dentate gyrus. *Brain Res.* **955**, 264-267.

Ramamoorthy S., Bauman A.L., Moore K.R., Han H., Yang-Feng T., Chang A.S., Ganapathy V., Blakely R.D. (1993) Antidepressant- and cocaine-sensitive human serotonin transporter: molecular cloning, expression, and chromosomal localization. *Proc. Natl. Acad. Sci. USA.* **90**, 2542-2546.

Reinberg A. and Smolensky M.H. (1983) Investigative methodology for chronobiology. In *Biological Rhythms in Medicine.* A. (eds A. Reinberg and M. H. Smolensky), pp.23-46. New York: Springer Verlag.

Reiter R.J. (1991) Neuroendocrine effects of light. *Int. J. Biometeorol*. **35**, 169-175.

Rensing L. and Ruoff P. (2002) Temperature effect on entrainment, phase shifting, and amplitude of circadian clocks and its molecular bases. *Chronobiol. Int.* **19**, 807-864.

Richter C.P. (1922) A behavioristic study of the activity of the rat. *Comp. Psychol. Monogr.* **1**, 1-54.

Rochefort C., Gheusi G., Vincent J.D., Lledo P.M. (2002) Enriched odor exposure increases the number of newborn neurons in the adult olfactory bulb and improves odor memory. J *Neurosci.* **22**, 2679-2689.

Roberts J.C., Reavill C., East S.Z., Harrison P.J., Patel S., Routledge C., Leslie R.A. (2002) The distribution of 5-HT(6) receptors in rat brain: an autoradiographic binding study using the radiolabelled 5-HT(6) receptor antagonist. *Brain Res.* **934**, 49-57.

Sandeman D.C. and Luff S.E. (1973) The structural organization of glomerular neuropil in the olfactory and accessory lobes of an Australian freshwater crayfish, *Cherax destructor. Z. Zellforsch.* **142**, 31-61.

Sandeman R.E. and Sandeman D.C. (1987) Serotonin-like immunoreactivity of giant olfactory interneurons in the crayfish brain. *Brain Res.* **403**, 371-374.

Sandeman D.C., Sandeman R.E., de Couet H.G. (1990) Extraretinal photoreceptors in the brain of the crayfish Cherax destructor. *J. Neurobiol.* **21**, 619-629.

Sandeman D.C., Sandeman R.E., Derby C., Schmidt M. (1992) Morphology of the brain of crayfish, crabs, and spiny lobsters: a common nomenclature for homologous structures. *Biol. Bull.* **183**, 304-326.

Sandeman, D.C., Beltz, B.S., Sandeman, R.E. (1993) Inputs to multi-tasking neurons in the crayfish cortex. *Soc. Neurosci. Abstract* **19**, 338.

Sandeman D.C. and Sandeman R.E. (1994) Electrical responses and synaptic connections of giant serotonin-immunoreactive neurons in crayfish olfactory and accessory lobes. *J. Comp. Neurol.* **341**, 130-144.

Sandeman D., Beltz B., Sandeman R. (1995a) Crayfish brain interneurons that converge with serotonin giant cells in accessory lobe glomeruli. *J. Comp. Neurol.* **352**, 263-279.

Sandeman R.E., Watson A.H., Sandeman D.C. (1995b) Ultrastructure of the synaptic terminals of the dorsal giant serotonin-IR neuron and deutocerebral commissure interneurons in the accessory and olfactory lobes of the crayfish. *J. Comp. Neurol.* **361**, 617-632.

Sandeman D.C. and Scholtz G. (1995) Ground plans, evolutionary changes and homologies in decapod Crustacea. In. Breidbach O. and Kutsch W. eds, The Nervous system of invertebrates: an evolutionary and comperative approach. Birkhäuser, Basel.

Sandeman R., Clarke D. Sandeman D., Manly M. (1998) Growth-related and antennular amputation-induced changes in the olfactory centers of crayfish brain. *J. Neurosci.* **18**, 6195-6206.

Sandeman R. and Sandeman D. (2000) "Impoverished" and "enriched" living conditions influence the proliferation and survival of neurons in crayfish brain. *J. Neurobiol.* **45,** 215-226.

Sapolsky R.M. (2001) Depression, antidepressants, and the shrinking hippocampus. *Proc. Natl. Acad. Sci. USA.* **98**, 12320-12322.

Savner, E. (2003) Serotonin synthesis and neurogenesis in the american lobster. Honors Thesis, Wellesley College, Wellesley, MA, USA.

Schloss P., Puschel A.W., Betz H. (1994) Neurotransmitter transporters: new members of known families. *Curr. Opin. Cell Biol.* **6**, 595-599.

Schmidt M. (1997) Continuous neurogenesis in the olfactory brain of adult shore crabs, Carcinus maenas. *Brain Res.* 762, 131-143.

Schmidt M. and Harzsch S. (1999) Comperative analysis of neurogeneis in the central olfactory pathway of adult decapod crustaceans by in vivo BrdU-labeling. *Biol. Bull.* **196**, 127-136.

Schmidt M. (2001) Neuronal differentiation and long-term survival of newly generated cells in the olfactory midbrain of the adult spiny lobster, Panulirus argus. *J. Neurobiol.* **48**, 181-203.

Scholtz G. (1992) Cell lineage studies in the crayfish *Cherax destructor* (Crustacea, Decapoda): germ band formation, segmentation and early neurogenesis. *Roux's Arch. Dev. Biol.* **202**, 36-48.

Scholtz G. and Dohle W. (1996) Cell lineage and cell fate in crustacean embryos--a comparative approach. *Int. J. Dev. Biol.* **40**, 211-220.

Scotto-Lomassese S., Strambi C., Aouane A., Strambi A., Cayre M. (2002) Sensory inputs stimulate progenitor cell proliferation in an adult insect brain. *Curr. Biol.* **25**, 1001-1005.

Scotto-Lomassese S., Strambi C., Strambi A., Charpin P., Augier R., Aouane A., Cayre M. (2000) Influence of environmental stimulation on neurogenesis in the adult insect brain. *Neurobiol.* **45**, 162-171.

Sehgal A., Price J.L., Man B., Young M.W. (1994) Loss of circadian behavioral rhythms and per RNA oscillations in the Drosophila mutant timeless. *Science*. **263**, 1603-1606.

Sherman L.P., Zylka M.J., Weaver D.R., Kalakowski L.F. Jr., Reppert S.M. (1997) Two period homologs: Circadian expression and photic regulation in the suprachiasmatic nuclei. *Neuron.* **19**, 1261-1269.

Sokolove P.G. (1975) Localization of the cockroach optic lobe circadian pacemaker with microlesions. *Brain Res.* **87**, 13-21.

Stanewsky R. (2002) Clock mechanisms in Drosophila. *Cell Tissue Res.* **309**, 11-26.

Stephan F.K. (2002). The "other" circadian system: food as a zeitgeber. *J. Biol. Rhythms* **17**, 284-292.

Stephan F.K. and Zucker, I. (1972) Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. *Proc. Nat. Acad. Sci. USA.* **69**, 1583-1586.

Stokkan K., Yamazaki S., Tei H., Sakaki Y., Menaker M. 2001. Entrainment of the circadian clock in the liver by feeding. *Science.* **291,** 490-493.

Strausfeld N.J. & Nässel D.R. (1981) Neuroarchitecture of brain regions that subserve the compound eyes of Crustacea and insects. In: Autrum H. ed., Handbook of Sensory Physiology. Vol. VII/6B, Invertebrate Visual Center and Behaviors I. Springer-Verlag, Berlin, pp. 1-132.

Sudgen D. (1990) 5-Hydroxytrptamine amplifies beta-adrenergic stimulation of *N*-acetyltransferase activity in rat pinealocytes. *J. Neurochem.* **55**, 1655-1659.

Sullivan J.M., Benton J.L., Beltz B.S. (2000) Serotonin depletion in vivo inhibits the branching of olfactory projection neurons in the lobster deutocerebrum. *J. Neurosci.* **20**, 7716-7721.

Sullivan J.M. and Beltz B.S. (2001) Neural pathways connecting the deutocerebrum and lateral protocerebrum in the brains of decapod crustaceans. *J. Comp. Neurol.* **441**, 9-22.

Sullivan J.M. and Beltz B. S. (2004) Integration and segregation of inputs to higher-order neuropils of the crayfish brain. *J. Comp. Neurol.* In press.

Sun Z.S., Albrecht U., Zhuchenko O., Bailey J., Eichele G., Lee C.C. (1997) RIGUI, a putative mammalian ortholog of the *Drosophila* period gene. *Cell.* **90**, 1003-1011.

Sur C., Betz H., Schloss P. (1996) Localization of the serotonin transporter in rat spinal cord. *Eur. J. Neurosci.* **8**, 2753-2757.

Tachibana T., Tazawa M., Sugahara K. (2001) Feeding increases 5-hydroxytryptamine and norepinephrine within the hypothalamus of chicks. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **130**, 715-722.

Tanoue S., Sumida S., Suetsugu T., Endo Y., Nishioka T. (2001) Identification of a receptor type guanylyl cyclase in the antennal lobe and antennal sensory neurons of the silkmoth, Bombyx mori. *Insect Biochem. Mol. Biol.* **31,** 971-979.

Tilden A.R., Shanahan J.K., Khilji Z.S., Owen J.G., Sterio T.W., Thurston K.T. (2003) Melatonin and locomotor activity in the fiddler crab *Uca pugilator*. *J. Exp. Zool.* **297**, 80-87.

Tilden A., McGann L., Schwartz J., Bowe A., Salazar C. (2001a) Effect of melatonin on hemolymph glucose and lactate levels in the fiddler crab *Uca pugilator*. *J. Exp. Zool.* **290**, 379-383.

Tilden A.R., Alt J., Brummer K., Groth R., Herwig K., Wilson A., Wilson S. (2001b) Influence of photoperiod on N-acetyltransferase activity and melatonin in the fiddler crab *Uca pugilator. Gen. Comp. Endocrinol.* **122**, 233-237.

Tilden A.R., Rasmussen P., Awantang R.M., Furlan S., Goldstein J., Palsgrove M., Sauer A. (1997) Melatonin cycle in the fiddler crab Uca pugilator and influence of melatonin on limb regeneration. *J. Pineal. Res.* **23**, 142-147.

Titova V.A. (1985) Neuropil topography in the cerebral ganglion of the crayfish *Astacus leptodactylus. J. Evol. Biochem. Physiol.* **21**, 256-263.

Trejo J.L., Carro E., Torres-Aleman I. (2001) Circulating insulin-like growth factor I mediates exercise-induced increases in the number of new neurons in the adult hippocampus. *J. Neurosci.* **21**, 1628-1634.

Tsvileneva V.A. and Titova V.A. (1985) On the brain structures of decapods. *Zool. Jb. Anat.* **113**, 217-266.

Tsvileneva V.A., Titova V.A., Kvashina T.V. (1985) Brain topography of the shore crab *Hemigraspus sanguineus. J. Evol. Biochem. Physiol.* **21**, 394-400.

Utting M., Agricola H., Sandeman R., Sandeman D. (2000) Central complex in the brain of crayfish and its possible homology with that of insects. *J. Comp. Neurol.* **10**, 245-261.

van Praag H., Kempermann G., Gage F.H. (1999a) Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nat. Neurosci.* **2**, 266-270.

van Praag H., Christie B.R., Sejnowski T.J., Gage F.H. (1999b) Running enhances neurogenesis, learning, and long-term potentiation in mice. *Proc. Natl. Acad. Sci. USA* **96**, 13427-13431.

Vivien-Roels B. and Pévet P. (1986) Is melatonin an evolutionary conservative molecule involved in the transduction of photoperiodic information in living organisms? *Adv. Pineal Res*. **1**, 61-68.

Waeber C. and Palacios J.M. (1994) Binding sites for 5-hydroxytryptamine-2 receptor agonists are predominantly located in striosomes in the human basal ganglia. *Brain Res. Mol. Brain. Res.* **24**, 199-209.

Wachowiak, M., Diebel, C. E., Ache, B. W. (1996) Functional organization of olfactory processing in the accessory lobe of the spiny lobster. *J. Comp. Physiol. A.* **178**, 211-226.

Watson W., Jury J., Howell H., Schreiber S., O'Grady D., Chabot C. (2001) Daily and seasonal patterns of locomotion expressed by the American lobster, in both the field and the laboratory. *Sixth International Conference and Workshop on Lobster Biology and Management*. 2000, Halifax, Nova Scotia, Canada.

Weiger W.A. and Ma P.M. (1993) Serotonin-containing neurons in lobsters: origins and characterization of inhibitory postsynaptic potentials. *J. Neurophysiol.* **69**, 2003-2014.

Weiss H.M. (1970) The diet and feeding behavior of the lobster, *Homarus americanus*, in Long Island. Ph.D. Thesis, University of Connecticut, Storrs, 800pp.

Whitaker-Azmitia P.M., Druse M., Walker P., Lauder J.M. (1996) Serotonin as a developmental signal. *Behav. Brain Res.* **73**, 19-29.

Wildt M., Goergen E.M., Benton J.L., Sandeman D.C., Beltz B.S. (2004) Regulation of serotonin levels by multiple light-entrainable endogenous rhythm. *J. Exp. Biol.* **207**, 3765-3774.

Wildt M. Kohler S.J., Benton J.L., Beltz B.S. (2002) Circadian variation in brain serotonin levels in juvenile lobsters. *Society for Neuroscience Abstracts.* **28,** 23.17.

Wurtman R.J. and Fernstrom J. D. (1973) Effects of diet on brain neurotransmitters. *Nutr. Rev.* **32**, 193-200.

Withyachumnarnkul B., Pongsa-Asawapaiboon A., Ajpru S., Siamwalla P., Trakulrungsi W., Samritthong C. (1992) Continuous light increases *N*-acetyltransferanse activity in the optic lobe of the giant freshwater prawn *Macrobrachium rosenbergii* de Man (Crustacea Decapoda). *Life Sci.* **54**, 1479-1484.

Withyachumnarnkul B., Pongtippatee P., Ajpru S. (1995). *N*-acetyltransferase, hydroxyindole-O-methyltransferase and melatonin in the optic lobes of the giant tiger shrimp *Penaeus monodon. J. Pineal Res.* **18,** 217-221.

Wolff M., Savova M., Malleret G., Hen R., Segu L., Buhot M.C. (2003) Serotonin 1B knockout mice exhibit a task-dependent selective learning facilitation. *Neurosci. Lett*. **338**, 1-4.

Worall D.M. and Williams D.C. (1994) Sodium-ion dependent transporters for neurotransmitters: a review of recent developments. *Biochem. J.* **297**, 425-436.

Zikopoulos B., Kentouri M., Dermon C.R. (2000) Proliferation zones in the adult brain of a sequential hermaphrodite teleost species (Sparus aurata). *Brain Behav. Evol*. **56**, 310-322.

Zimmer-Faust R.K., O'Neill P.B., Schar D.W. (1996) The relationship between predator activity state and sensitivity to prey odor. *Biol. Bull.* **190**, 82-87.

Zimmer-Faust R.K. (1987) Crustacean chemical reception: towards a theory on optimal chemoreception. *Biol. Bull.* **172**, 10-29.

Zupanc G.K. (1999) Neurogenesis, cell death and regeneration in the adult gymnotiform brain. *J. Exp. Biol.* **202**, 1435-1446.

7. Appendix

7.1 Sequential listing of all chronograms

Note, all experiments listed here have been subjected to an entraining L/D light cycle only: the light/dark bar at the bottom of each graph indicates illumination.

*** Experiments 1 and 2 used peak area (compared to peak height) for analysis and can therefore not be presented as a valid approach.**

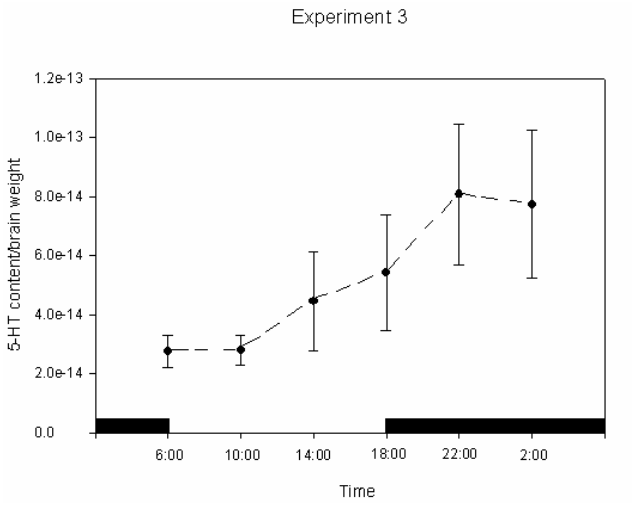

Figure 27. The first chronogram analyzing for peak height shows low levels of brain serotonin at dawn and high levels after dusk. Note 5-HT content is normalized to brain weight. n=36.

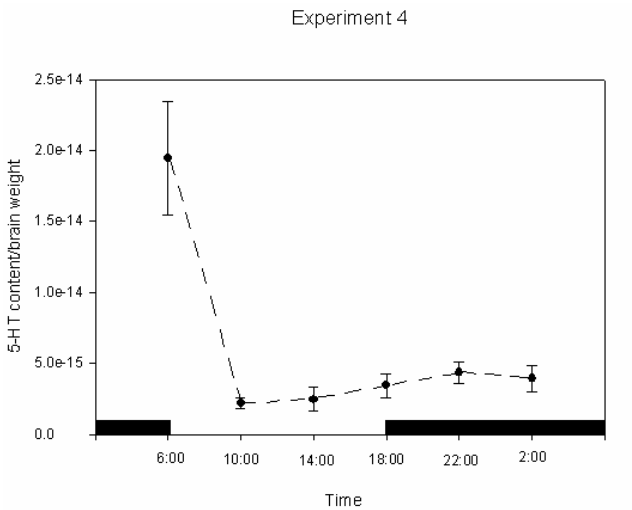

Figure 28. High levels of brain serotonin are measured at dawn thus not confirming data acquired during experiment 3. Note 5-HT content is normalized to brain weight. n=36.

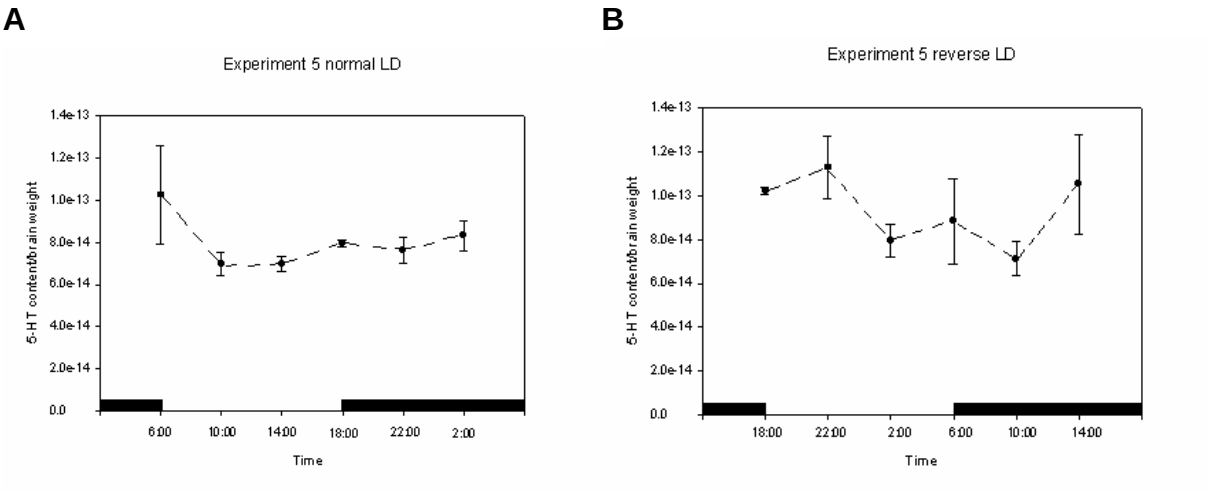

Figure 29. Animals were either entrained for two weeks to A) a normal 12:12 L/D light cycle (lights on at 6:00; off at 18:00) or B) to a reversed 12:12 L/D light cycle (light on at 18:00; off at 6:00). Because light was speculated to be the entraining factor B) should show the same circadian rhythm as A). Note this set of experiments was performed in parallel. Note also that 5-HT content is normalized to brain weight. n= 18/experiment.

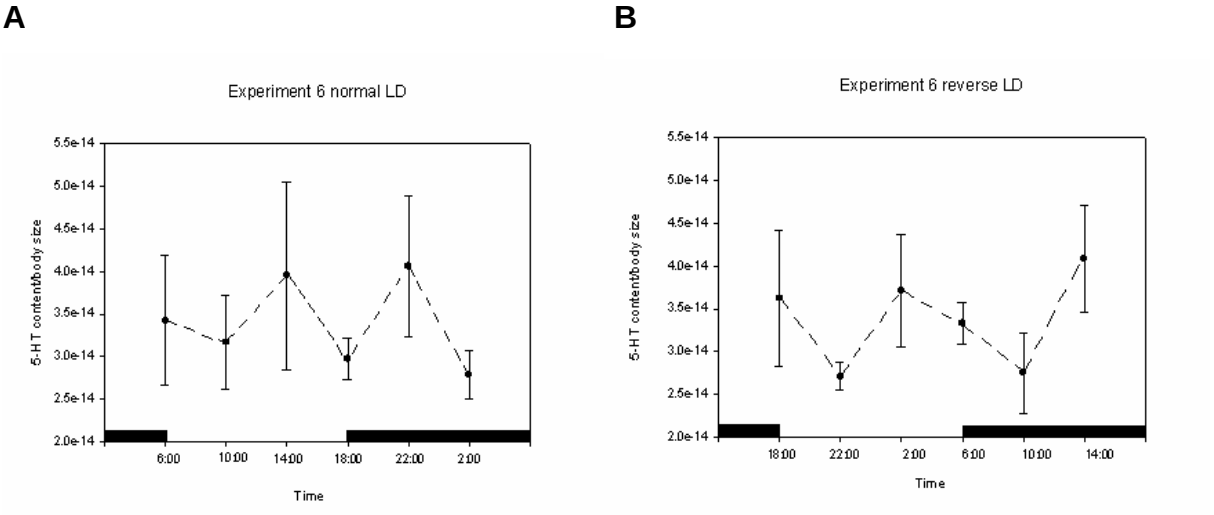

Figure 30. Because previous experiments sampled right at the change of illumination (dawn/dusk), these samples were taken 30 minutes after the onset of light (dawn) and then every 4 hours to allow longer light/dark adaptation. Again, standard errors are overlapping and no

consistent circadian rhythm can be analyzed. Note 5-HT content is normalized to body size. n= 18/experiment.

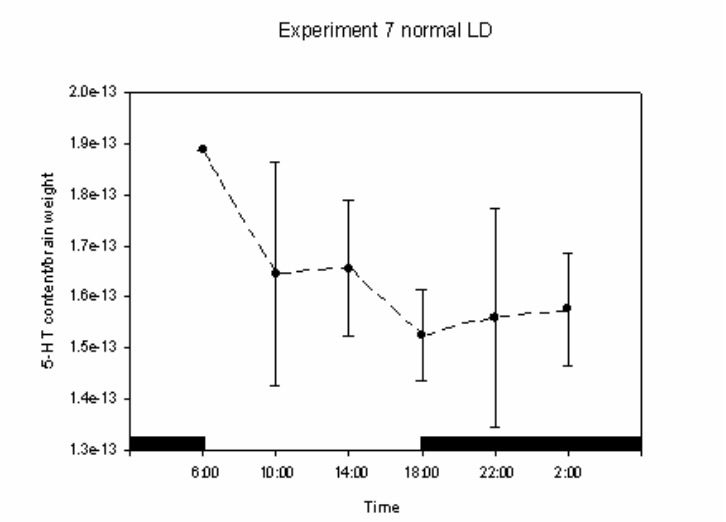

Figure 31. To allow better statistical analysis n was increased (n=4/time point). Also all samples were centrifuged at room temperature to avoid critical temperature changes throughout the system. This technical advancement was maintained for all subsequent experiments. Note 5-HT content is normalized to brain weight. n=24.

A **B**

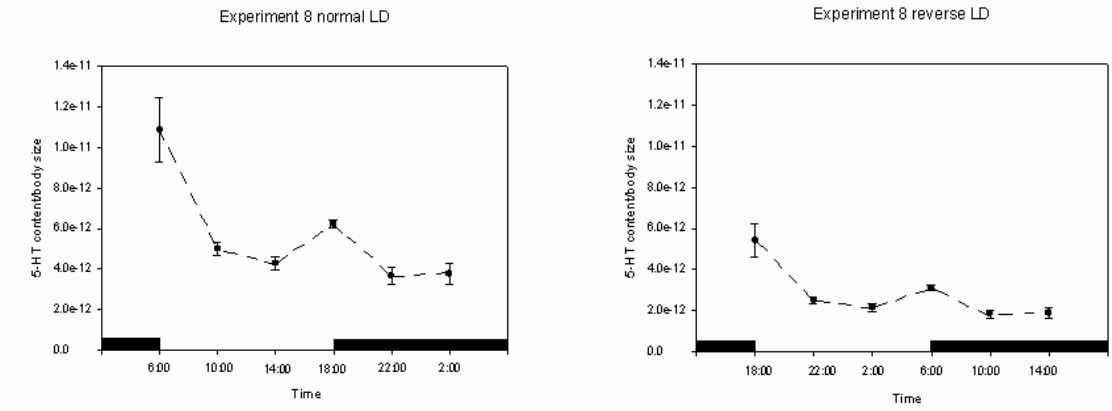

Figure 32. The light bulbs were adjusted in both rooms to ensure minimal changes within the light spectra. Again, animals were entrained to either A) a normal 12:12 L/D light cycle or B) a reversed 12:12 L/D light cycle. For the first time the standard errors are not overlapping and the "reversed" chromatogram matches the "normal" one. However, variations in the serotonin content measured call for attention. Note 5-HT content is normalized to body size. n=24/experiment.

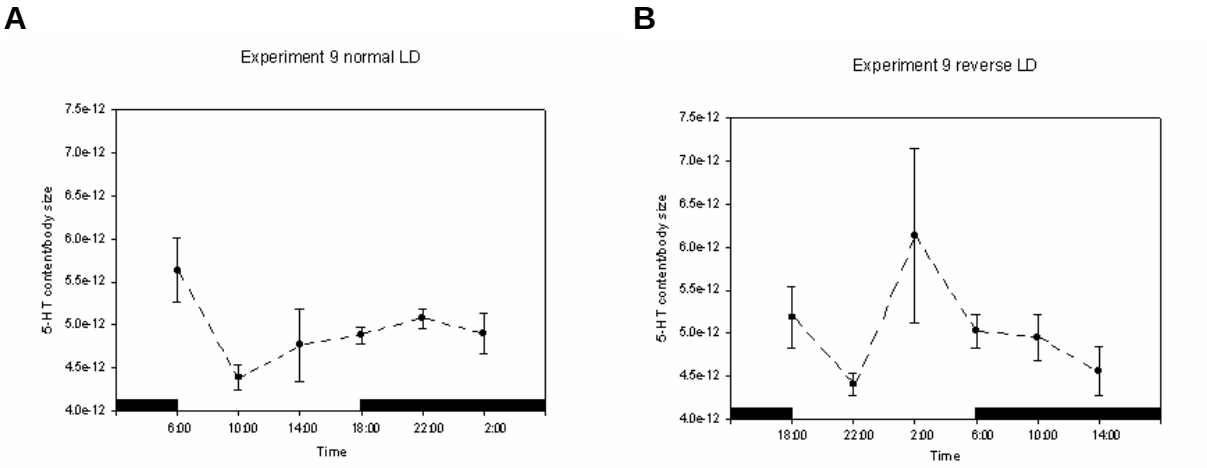

Figure 33. This set of experiments was conducted to verify the data acquired during experiment 8. Unfortunately, this set does not resemble the chromatogram of experiment 8 nor does the "reversed" circadian rhythm B) match the pattern seen in A). Note 5-HT content is normalized to body size. n=24/experiment.

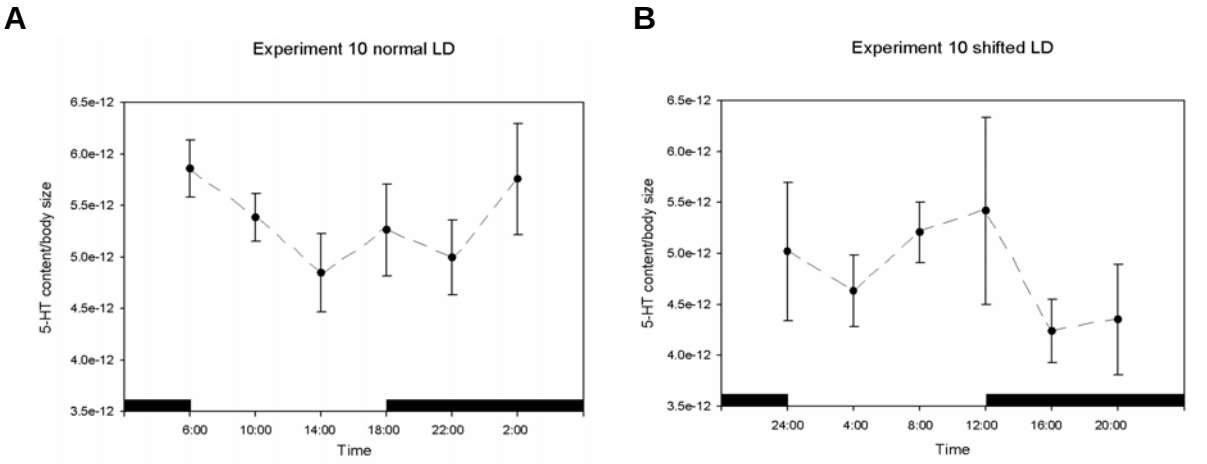

Figure 34. This set of experiments features the technical advancement of a mobile phase degasser. Again, standard errors are large and B) does not show a reversal of the normal entraining 12:12 L/D chromatogram as shown in A). Note 5-HT content is normalized to body size. n=24/experiment.

*** Experiment 11 is reported in Figure 11A (see 3.1).**

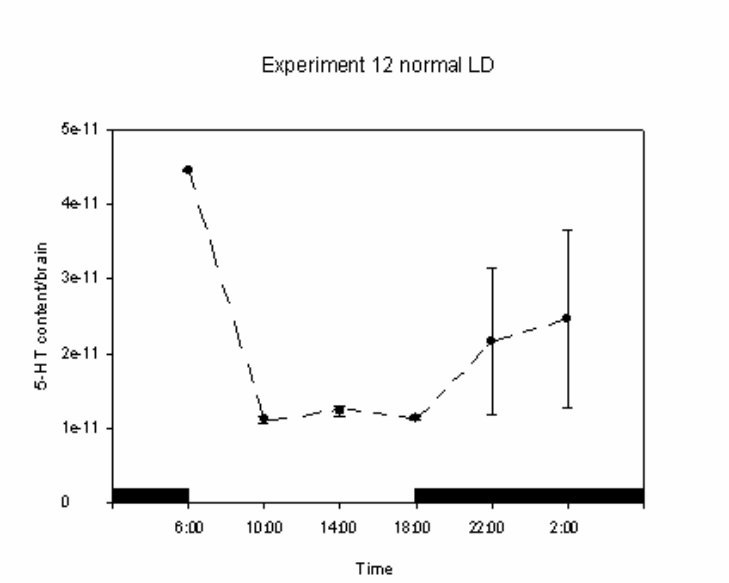

Figure 35. A power failure was experienced during the first sampling time point. Consequently, the HPLC system shut down causing a critical delay in the sample analysis process.

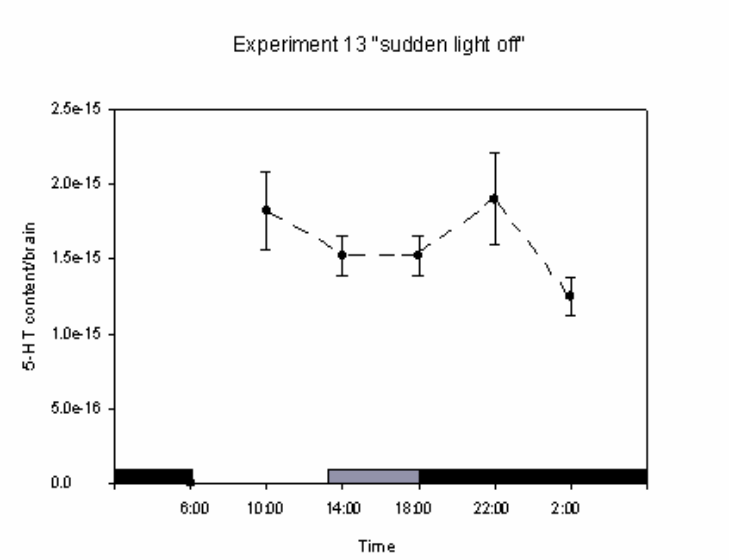

Figure 36. This experiment was to test whether a sudden change in the light regime (indicated by gray bar) has an immediate effect on the circadian rhythm of brain serotonin. Unfortunately, the column broke during the first sampling period which inevitably caused a complete loss of the dawn time point. n=24 (initially).

*** Experiments 14 and 18 are represented in Figure 13A-F. Experiment 16 is shown in Figure 11C (see 3.1).**

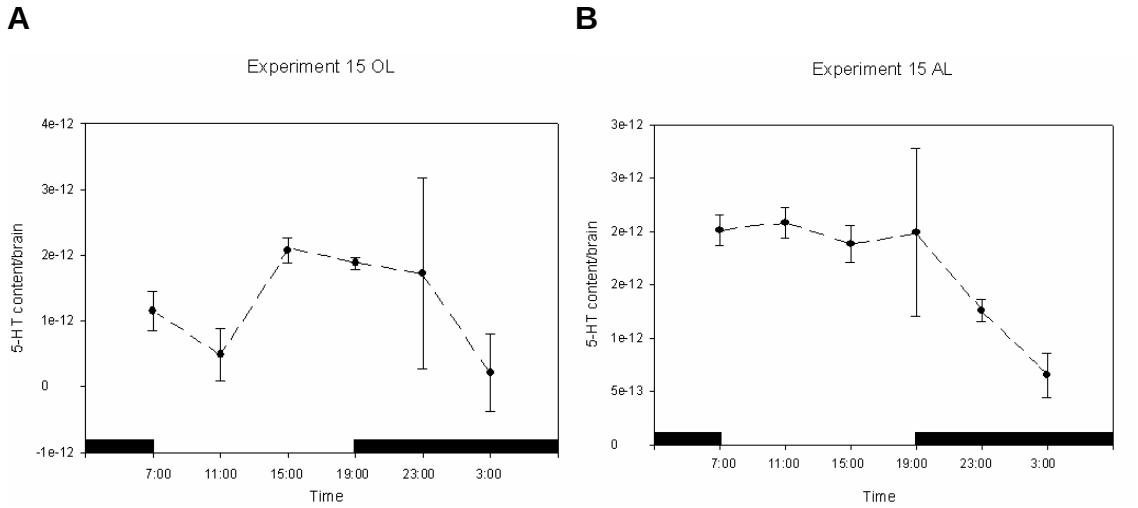

Figure 37. Separate analysis of functionally distinct areas of the brain show contrasting serotonin rhythm. As for ALs serotonin levels are high during the day and decrease into the early night A). The serotonergic rhythm of the OLs in contrast shows elevated levels surrounding dusk B). Because of one respective outlier no statistical difference can be seen in either experiment. n=24

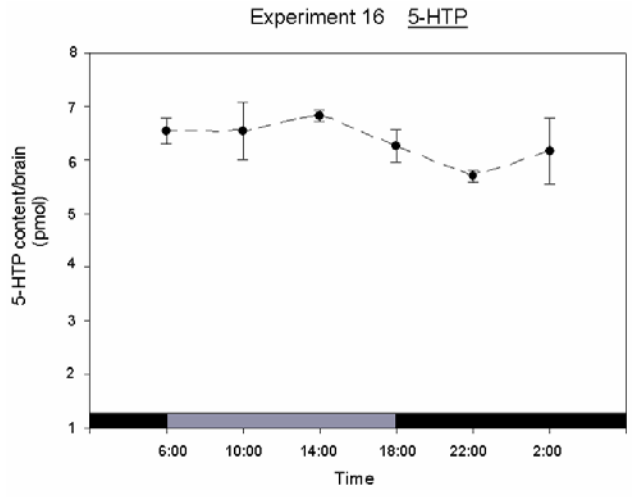

Figure 38. Chronogram showing the circadian rhythm of the immediate serotonin precursor 5-hydroxy-L-tryptophan (5-HTP) with high levels 4 hours before dusk and a trough 4 hours after dusk. The black bars at the bottom of the graph indicate the entraining light regime. The gray bar indicates constant conditions (D/D) three days prior to the experiment. This experiment was performed in parallel to Figure 11C. n=24.

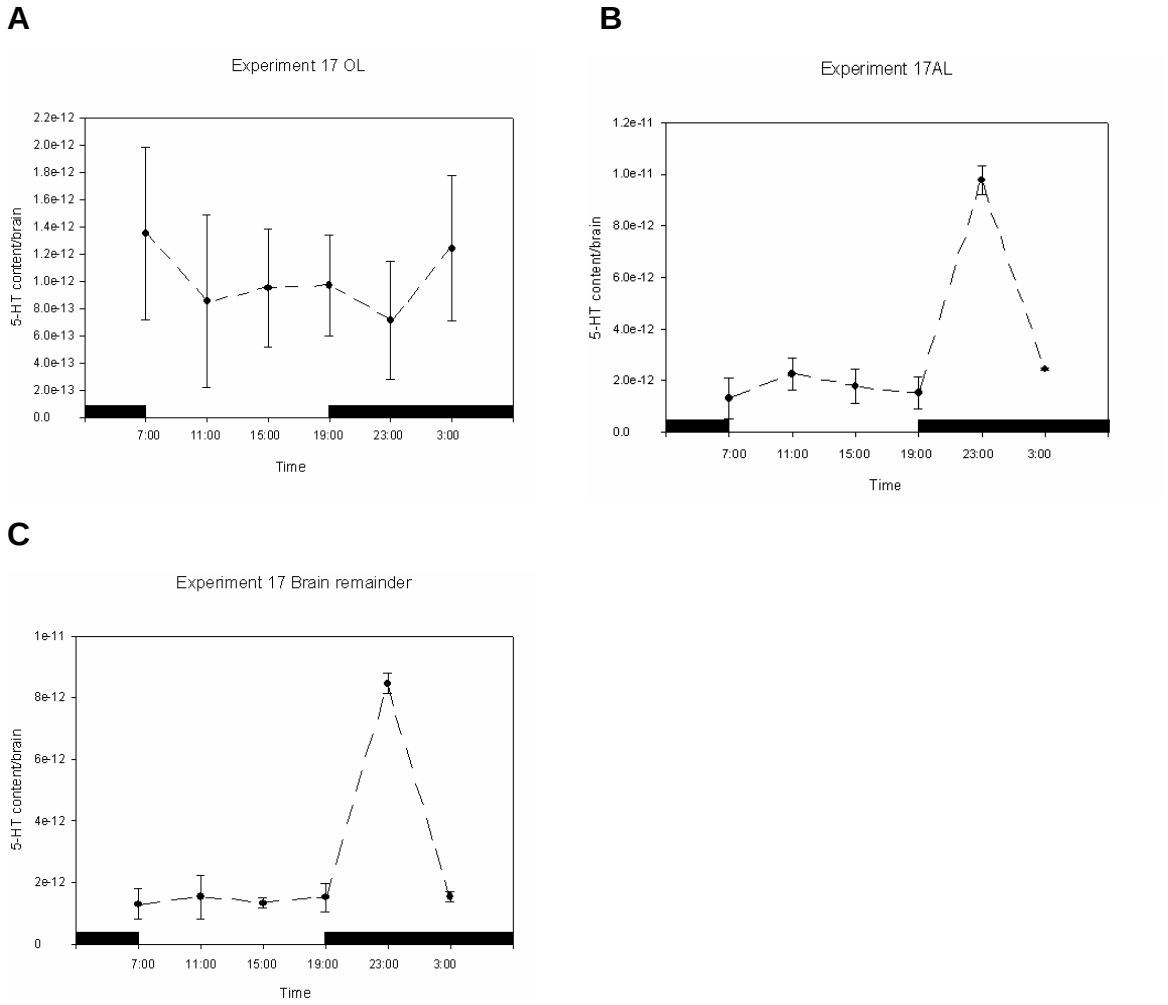

Figure 39. The contrasting circadian rhythm of serotonin within functionally distinct regions of the brain even diverges when compared to experiments performed using the same experimental protocol (compare Figure A and B). Brain serotonin levels in the A) ALs and B) brain remainder show a single peak after dusk. Because of large standard errors no significant distinction can be made for the OLs (A). n=24.

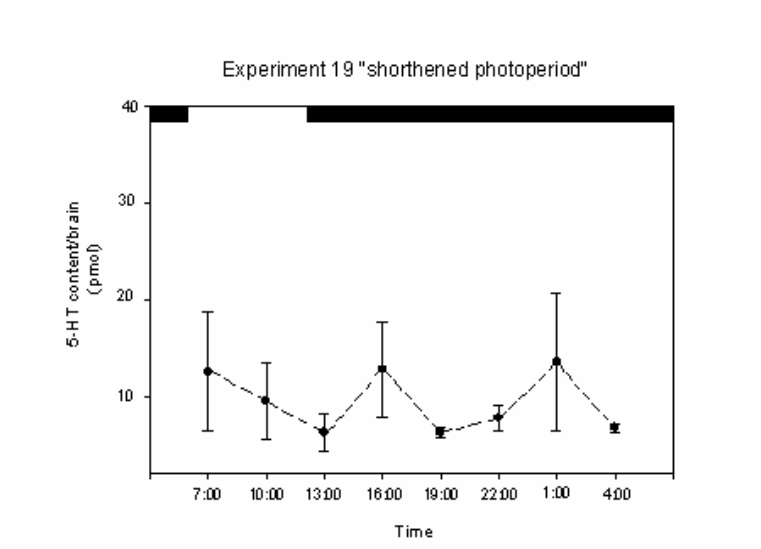

Figure 40. To test the effect of photoperiod on the circadian rhythm of brain serotonin, animals were entrained to a shortened light regime (6:18 L/D). Because the standard errors are large no characteristic pattern over 24-hours can be seen. n=32.

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- **Experiment 20 and 26 are represented in Figure 19B (see 3.2; 'fictive feeding').**
- **Experiment 24 is shown in Figure 19A-C (see 3.2; 'fictive feeding').**
- **Experiment 21 and 25 are represented in Figure 18 (see 3.2; 'surprise feeding').**
- **Experiment 22 is shown in Figure 20A (see 3.3; melatonin)**
- **Experiment 23 is shown in Figure 20B (see 3.3; melatonin)**

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7.3. List of publications and posters related to this project

2004 M.Wildt, E.M. Goergen, J.L. Benton and B.S. Beltz. Regulation of serotonin levels by multiple light-entrainable endogenous rhythms. Journal of Experimental Biology. 207, 3765-3774

2004 M. Wildt, E. M. Goergen, B.S. Beltz. The modulatory effect of feeding on serotonin levels and neurogenesis in the brain of the American lobster, *Homarus* americanus. In preparation

2004 M.Wildt, E.M. Goergen, J.L. Benton and B.S. Beltz. Feeding alters brain serotonin levels and neurogenesis. Poster at 34th Annual Meeting of the Society of Neuroscience, abstract 382.17

2004 M. Wildt, B.S. Beltz. Distribution of the serotonin transporter (SERT) at the site of life-long neurogenesis in the brain of the American lobster, *Homarus americanus*. Poster at FENS Forum 2004, abstract A145.20

2003 M. Wildt, B.S. Beltz. Serotonin Levels in Brains of Juvenile Lobsters, *Homarus americanus*, show a Diurnal Rhythm. Poster at 29th Göttingen Neurobiology Conference, abstract 935

2003 M. Wildt, B.S. Beltz. Night and Day: Serotonin levels in the brain of the American lobster, *Homarus americanus*, show a light entrainable circadian rhythm. Poster at 33nd Annual Meeting of the Society for Neuroscience, abstract 562.12

2002 M. Wildt, J.L. Benton, S.J. Kohler, B.S. Beltz. Circadian variations in brain serotonin levels in juvenile lobsters. Poster at 32nd Annual Meeting of the Society for Neuroscience, abstract 23.17

7.3.1 Talks

2004 The impact of day, night and other external synchronizers on brain serotonin levels in the American lobster *Homarus americanus*. Universität Ulm.

2004 Day, night and brain serotonin levels in lobsters. 30th Annual East Coast NerveNet Meeting, Woods Hole, MA, USA.

2004 The impact of day, night and other external synchronizers on serotonin levels in the lobster brain. Wellesley College, Wellesley, MA, USA.

2003 Tag und Nacht: Serotonin Level im Gehirn juveniler Hummer zeigen einen Circadianen Rhythmus. Neurobiologie (Abt. IV), Universität Ulm.

7.4 Curriculum vitae

Personal Details

Name:	Miriam Wildt
Date of Birth:	February 17th, 1976
Place of Birth:	Cologne, Germany
Nationality:	German

Education

Since Sept. 2001	PhD-studies (Biology) at the University of Ulm and Wellesley College, MA, USA "The impact of day, night and other external synchronizers on brain serotonin levels in the American lobster *Homarus americanus*".
April 2001	Diploma (Biology) at the University of Bielefeld "Ontogeny of the visual system of *Artemia salina* Linné, 1785 (Crustacea, Entomostraca, Branchiopoda): Proliferation of neuronal stem cells and neuropil development".
1996	Biology program at the University of Bielefeld
1994-1996	Friederich-Wilhelm Gymnasium, Köln (Abitur)
1993-1994	Gymnasium Kreuzgasse, Köln
1992-1993	Horton-District-High-School, New Minas, Nova Scotia, Canada
1986-1992	Geschwister-Scholl-Realschule, Köln
1982-1986	Pater-Delp-Grundschule, Köln

Additional activities

2002-2004	College Lecturer, The Education Cooperative (tec)/Wellesley College, MA/USA: 'Biopsychology' (a college level class for gifted high school students)
2001	Laboratory Manager- Microbiology; biotec GmbH, Gütersloh, Germany

Research collaborations

2001-2004	Wellesley College, Wellesley, MA/USA Graduate Research Assistant of Prof. B. Beltz;
Jan. 2003	Bowling Green State University, OH/USA – Prof. R. Huber;
July 2002	Whitney Laboratory in St Augustine, FL/USA – Prof. B. A. Battelle;
Aug. 2000	University of St Petersburg, Russia - S. Fleissner;
2000	University of Ulm, Germany - PD Dr. S. Harzsch;
1999	University of Ulm - PD Dr. S. Harzsch, Prof. Dr. H. Wolf and Prof. Dr. D. Walossek;
1999	State Veterinary Department Detmold, Germany – Dr. Takla

Stipends

2001-2003	Landesgraduiertenförderung Baden-Württemberg
2001-2003	Deutscher Akademischer Austauschdienst (DAAD) (2001-2003)
2003-2004	Wellesley College, Wellesley/USA

Ulm, 18.10.2004

Miriam Wildt

7.5 Abbreviations

AL	accessory lobe
AMPN	anterior median protocerebral neuropil
AN	antenna II neuropil
BrdU	Bromodeoxyuredine
CB	central body
DC	deutocerebrum
D/D	constant darkness
DGN	dorsal giant neuron
HPLC	high pressure liquid chromatography
LAN	lateral antennular neuropil
L/D	light/dark conditions
MAN	medial antennular neuropil
OES	oesophageal connectives
OGT	olfactory globular tract
OL	olfactory lobe
PCB	protocerebral bridge
PCT	protocerebral tract
PMPN	posterior median protocerebral neuropil
PB	protocerebrum
SERT	serotonin transporter
TEG	tegumentary nerves
ZT	zeitgeber time
5-HT	serotonin

Circadian rhythm of brain serotonin levels

Group 1	12:12 L/D cycle (n=24)
Group 2	12:12 L/D + 3 days in 12:12 D/D (n=24)
Group 3	4 hour phase shift of 12:12 L/D + 3 days in 12:12 D/D (n=24)

Circadian rhythm of brain serotonin levels and neurogenesis after feeding

Group 1*	'surprise feeding' (n=36)
Group 2*	'fictive feeding', No Stimulus (serotonin†: n=24; neurogenesis‡: n=36)
Group 3*	'fictive feeding', *Artemia* (serotonin†: n=24; neurogenesis‡: n=35)
Group 4*	'fictive feeding', Bead (serotonin†: n=24; neurogenesis‡: n=35)

† sampled off 3 days in D/D
‡ sampled off L/D condition

Erklärung:

Ich versichere hiermit, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich habe keine anderen als die angegebenen Hilfsmittel und Quellen benutzt, sowie wörtlich oder inhaltlich übernommene Stellen als solche kenntlich gemacht.

Ulm, 2004

Miriam Wildt