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Omega-3 fatty acids upregulate adult neurogenesis

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Abstract

Omega-3 fatty acids play crucial roles in the development and function of the central nervous system. These components, which must be obtained from dietary sources, have been implicated in a variety of neurodevelopmental and psychiatric disorders. Furthermore, the presence of omega-6 fatty acids may interfere with omega-3 fatty acid metabolism. The present study investigated whether changes in dietary ratios of omega-3:omega-6 fatty acids influence neurogenesis in the lobster (*Homarus americanus*) brain where, as in many vertebrate species, neurogenesis persists throughout life. The factors that regulate adult neurogenesis are highly conserved among species, and the crustacean brain has been successfully utilized as a model for investigating this process. In this study, lobsters were fed one of three diets that differed in fatty acid content. These animals were subsequently incubated in 5-bromo-2′-deoxyuridine (BrdU) to detect cells in S-phase of the cell cycle. A quantitative analysis of the resulting BrdU-labeled cells in the projection neuron cluster in the brain shows that short-term augmentation of dietary omega-3 relative to omega-6 fatty acids results in significant increases in the numbers of S-phase cells, and that the circadian pattern of neurogenesis is also altered. It is proposed that the ratio of omega-3:omega-6 fatty acids may alter neurogenesis via modulatory influences on membrane proteins, cytokines and/or neurotrophins.

Keywords

adult neurogenesis; diet; circadian rhythm; LC-PUFA; α-linolenic acid

Long chain polyunsaturated fatty acids (LC-PUFAs), which make up 20% of the brain's dry weight, are critical for healthy brain development and function because of their roles in membrane structure and cytokine regulation. The large number of LC-PUFAs makes the study of nutritional impacts difficult, because not only the dietary level of specific fatty acids, but also their respective ratios, are often important in modulating the degree of impact [18]. The omega-3 fatty acids EPA (eicosapentaenoic acid, 20:5ω3) and DHA (docosahexaenoic acid, 22:6ω3) are of particular importance in the nervous system [21], and levels of ALA (α-linolenic acid, 18:3ω3), the parent omega-3 molecule, also have been implicated in various aspects of brain function [5]. These molecules must be obtained largely from dietary sources because animals cannot synthesize them *de novo*. Some fish and crustacean species can convert ALA into DHA and EPA [17], although this mechanism does not appear to contribute significantly to DHA and EPA levels in humans [1].

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Research suggests that abnormalities in fatty acid metabolism may play a part in a range of neurodevelopmental and psychiatric disorders [11,14]. For example, several studies support a connection between dietary intake of omega-3 fatty acids and the prevalence of depressive illnesses [15,19]. Recently, EPA supplementation has even emerged as a potential treatment for depression [23].

Our interest is the possible connection between depressive disorders and deficiencies in life-long neurogenesis, a link originally proposed by Jacobs et al. [16]. Several lines of evidence support this hypothesis. First, major depression is associated with a loss in volume of the hippocampus [6], one of the sites of neuronal addition throughout life. Second, treatments that have antidepressive effects in patients also influence hippocampal neurogenesis in animal studies (e.g., lithium [7]; physical exercise [29]; serotonin reuptake inhibitors [20]). Finally, factors such as stress that can lead to depression are correlated with decreased neurogenesis [10]. As recent research has established an association between omega-3 fatty acids and major depressive disorder, we were interested in whether there is also a correlation between changes in dietary intake of these molecules and the extent of neuronal proliferation in the brain.

Adult neurogenesis occurs in the brains of many vertebrate and invertebrate species. The factors that influence the birth and survival of adult-born neurons in these organisms are highly conserved [3], and therefore studies in non-vertebrate models can reveal regulatory strategies that are also important in more highly derived species. In the crustacean brain, neuronal birth persists throughout life within two populations of interneurons innervating the olfactory lobe, the functional homolog of the vertebrate olfactory bulb, and the accessory lobe, a higher-order multimodal synaptic area [12]. The somata of these interneuronal populations form two spatially distinct clusters located lateral (cluster 10) and medial (cluster 9) to the lobes. Labeling with cell-cycle markers shows that cell proliferation within each cluster occurs within restricted regions known as the proliferation zones. The crustacean brain therefore provides an accessible and sensitive system for examining the regulation of neurogenesis [3].

In the present study, we asked whether alterations in nutritional LC-PUFA content influence levels of neurogenesis in cluster 10 of the lobster brain, where cell numbers can be accurately assessed in whole mounts of juvenile brains. The fatty acid content of the lobster diet is easily manipulated, and previous studies in lobsters have demonstrated that growth and survival are highly sensitive to dietary LC-PUFA levels [27]. We also assessed the influence of LC-PUFAs on the circadian pattern of neurogenesis [9].

Materials and Methods

Animals

Lobsters were hatched from the eggs of a single female reared at the New England Aquarium (NEAq; Boston, MA), and maintained individually at 17°C in aquaria with circulating filtered seawater and a light/dark cycle of 12:12 hours (photophase @6:00-18:00 hrs). As the neuroblasts generating embryonic neurons die during late embryonic and early larval life [3, 12] only the precursors that generate neurons throughout the lobster's life are present after that time; therefore, after the larval period, this process is referred to as "adult neurogenesis". In the present study, juvenile lobsters (6–7mm carapace length) in which only the "adult" progenitors and spatial patterns of neurogenesis persist, were maintained for 25 days on one of three diets: (1) UN: unenriched brine shrimp (*Artemia franciscana*), (2) O3: omega-3 fatty acid enriched *A. franciscana*, and 3) SP: *Spirulina* enriched *A. franciscana*. The three types of *Artemia* were grown to adults in salt ponds, enriched or not, frozen by San Francisco Bay Brand, Inc. (Newark, CA), and shipped to the NEAq where they were thawed and fed on an as-needed basis. These diets were chosen because they have similar fatty acid levels (55.31, 47.72, and 49.48 mg/g dry weight respectively), vary in the ratios of omega-3:omega-6 fatty

acids (1:1, 2:1 and 2:1 respectively) as well as in EPA and ALA levels, and none of them have detectable levels of DHA (Table 1). These diets were not intended to replicate the natural diet of young lobsters in the wild, which is composed of plankton rich in EPA (12.8 mg/g dry weight) and DHA (22.3 mg/g dry weight) (Tlusty, unpublished data). Rather, these diets were designed to provide a first approximation of how controlled manipulation of LC-PUFA levels might influence the cell cycle, and specifically neurogenesis.

Fatty acids were analyzed by gas chromatography of the methyl esters (M. Arts, Environment Canada, Burlington ON). After the period of rearing on the experimental diets, the lobsters were assessed for neurogenesis during a single sample period at dusk (Experiment 1). Furthermore, the rate of neurogenesis in the lobster brain is influenced by a circadian clock [9], and hence additional experiments were conducted in which lobsters were reared on either UN or SP, and the levels of neurogenesis were assessed in six 3-hour sampling times during a 24-hour span.

In vivo bromodeoxyuridine labeling

At the end of the dietary periods, the substitute nucleoside 5-bromo-2′-deoxyuridine (BrdU) was used to label brain cells in the S phase. In the first experiment, 4 lobsters per diet were incubated in BrdU (Sigma; 2mg/ml artificial sea water) for 4 hours beginning two hours before dusk. In subsequent experiments testing the interaction between circadian changes in cell proliferation and diet, 4–6 lobsters per dietary treatment were incubated in BrdU for 3 hours at one of 6 different time periods spanning a 24-hour sampling period (a total of 30 and 31 lobsters for the SP and UN diets respectively).

Brains were dissected from the lobsters under cold lobster saline (462mM NaCl, 16mM KCl, 34mM $CaCl_2$, 17mM $MgCl_2$, 11mM α-D(+)-glucose, and 10mM HEPES buffer; pH 7.4), then fixed and processed immunocytochemically for BrdU, using fluorescent detection [see method in 26].

Confocal microscopy

Specimens were viewed using a Leica TCS SP confocal microscope. Optical sections were taken at intervals of 1μm and saved as three-dimensional stacks. BrdU-labeled cell profiles in cluster 10 in each optical section in the stacked series were traced onto a transparent sheet attached to the monitor, and then counted.

Statistical analyses

Results of Experiment 1 were analyzed with a two-way repeated measures ANOVA (SigmaStat 3.1, Systat Software Inc.). The factors were diet (UN, O3, SP), and brain side (left, right) while the repeated measure was the individual lobster. Brain side did not influence cell count ($F_{1,9} = 0.79$, $P > 0.35$). The relationship between average number of BrdU-labeled cells for the three diets and individual fatty acid constituents was assessed using a Pearson product moment correlation.

The experiments that examined the dietary influence on levels of neurogenesis over a 24-hour period first assessed how time and diet influenced the average cell proliferation per lobster using a two-way ANOVA. This analysis exhibited a significant diet versus time interaction, so the two diets were then analyzed separately. The circadian rhythm data were normalized to the average value at dusk within diet, as this was the point at which the greatest numbers of BrdU-labeled cells were produced. Normalized values were then analyzed within each diet as a two-way repeated-measures ANOVA with the factors time (the six 3-hour periods) and brain side (left, right). The repeated measure was the individual lobster. Data were assessed for normality and equal variance. The circadian cycle data failed to meet these assumptions, and

were therefore ranked prior to analysis. For all ANOVAs, significant differences between paired comparisons were conducted with the Holm-Sidak method.

Results

In the present study we compared the numbers of BrdU-labeled cells in the cluster 10 proliferation zone in the brains of lobsters fed on one of the three diets. Lobsters fed the O3 or SP diet had the greatest numbers of BrdU-labeled cells, while those fed UN had significantly fewer labeled cells ($F_{2,9} = 7.30$, $P < 0.02$; Fig. 1). The enriched diets resulted in a robust 50% increase in the numbers of cells labeled over a 4-hour period (UN vs. O3, $t = 3.15$, $P < 0.02$; UN vs. SP, $t = 3.45$, $P < 0.01$). The omega-3:omega-6 ratio and ALA levels were the two fatty acid parameters that were significantly ($P < 0.05$) correlated to average cell number ($r^2 = 0.99$ and 0.92 respectively).

We also examined the levels of neurogenesis throughout a 24-hour period in the brains of lobsters maintained on UN or SP diets. Prior studies demonstrated that neuronal proliferation in the brains of lobsters reared on the UN diet was circadian, with peak levels at dusk and lowest levels at dawn [9]. In the present study, the protocol of Goergen et al. [9] was used, and we observed a significant diet versus time interaction ($F_{5,49} = 4.74$, $P < 0.001$). A simple effects analysis demonstrated that within each time period, more neurogenesis occurred in lobsters fed the SP compared to the UN diet (for all comparisons, $t > 2.3$, $P < 0.03$). The smallest dietary influence on neurogenesis was during the dusk period (10 cells), while pre-dawn, dawn, and post-dawn all differed by > 30 cells. Examining the normalized data, the brains from the UN lobsters showed a robust circadian rhythm ($F_{5,25} = 30.38$, $P < 0.001$) with the peak of proliferative activity at dusk (Fig. 2), as previously published [9]. Cell proliferation was greater during this period than all other sampling times within this diet (for all comparisons, $t > 3.10$, $P < 0.005$). Dawn was the period of lowest cell proliferation, but was statistically similar to the period immediately preceding and following it (Fig. 2). This circadian pattern was not observed in the brains of animals that were fed on the SP diet (Fig. 2). Although the highest levels of neurogenesis in this group were at dusk, these levels were not statistically different from any of the other time periods ($F_{5,24} = 2.56$, $P > 0.05$). These data confirm an increase in the numbers of BrdU-labeled neurons with the LC- PUFA-enriched diets (Fig. 1) and show that this is primarily due to an increase in the basal levels of neurogenesis, with a relatively small, though statistically significant, increase in the maximal rate seen at dusk.

Discussion

Our experiments indicate that even a few weeks of dietary manipulation of LC-PUFA content can increase the numbers of new neurons born in the lobster brain. Specifically, the correlation between levels of neurogenesis and (1) the ratio of omega-3:omega-6 fatty acids and (2) ALA levels, suggests that one of these two dietary factors is responsible for the increase in neurogenesis. ALA, along with oleic acid (C18:1ω9c), are two of the most abundant fatty acids in the enriched diets (Table 1). Furthermore, the ratio of omega-3:omega-6 fatty acids is potentially important because undesirable ratios can inhibit the conversion of ALA to DHA and EPA [19].

It is also important to note that the fatty acid profiles of the three diets examined here differ in a number of ways (Table 1). For example, the amount of EPA, another omega-3 fatty acid critical for nervous system function, also varied among the three experimental diets. However, the changes we measured in cell proliferation due to diet (UN<O3=SP) do not correspond to the amount of EPA in the diet (O3<UN=SP). Likewise, while arachidonic acid (20:4ω6; ARA) levels increased among diet treatments (UN < O3 < SP; Table 1), cell proliferation did not show corresponding increases (UN < O3 = SP; Fig. 1). Finally, the *Spirulina* enriched

Artemia diet introduces additional components. One of the benefits of this diet is the presence of the antioxidant astaxanthin [27]. On the other hand *Artemia*, the basis for all three diets, already contains sufficient quantities of astaxanthin, as well as other carotenoids that the lobster can convert to astaxanthin [8]. Therefore, the most parsimonious explanation for our data is that increased neurogenesis is a result of a favorable omega-3:omega-6 ratio, likely caused by the increase of ALA in the enriched *Artemia* diets.

The increases in neuronal proliferation seen with LC-PUFA enrichment imply that the nervous system benefits from this nutritional enhancement. We know from our work in related species that the BrdU-labeled cells born in this cell cluster normally mature and differentiate into projection interneurons that will innervate two distinct synaptic areas in the midbrain, the olfactory and accessory lobes, and project to synaptic neuropils in the lateral protocerebrum [26]. It will be important to confirm that the additional cells born as a result of dietary manipulations survive and differentiate along these same lines.

Long-term survival studies of lobsters have shown that there is a growth, survival, and economic benefit of rearing lobsters on SP diets as opposed to UN [27]. It would be particularly instructive to assess the role that increased neurogenesis has on overall growth and survival of hatchery animals. Aquatic animals, grown in captivity for eventual release to the wild (enhancement programs), are often fed less nutritious, low-cost food. Unfortunately, feeding low-quality diets unenriched with LC-PUFAs may produce animals that are neurologically and physiologically ill-equipped to survive in the wild.

Omega-3 fatty acids have the potential to influence neurogenesis through at least two distinct mechanisms. First, omega-3 fatty acids are incorporated into neuronal membranes, where they influence the quarternary structure of membrane proteins, some of which act as transporters and receptors [4]. They also can alter membrane fluidity [31], which is important for neurotransmitter binding as well as signaling within cells. For instance, lipid fluidity modulates the binding of serotonin to neuronal membranes in the mouse brain [13]. As serotonin stimulates neurogenesis in both vertebrate [28] and invertebrate [3] species, a change in efficacy of this modulator could influence the levels of neuronal proliferation. Therefore, omega-3 fatty acids may alter the rate of neurogenesis via their contributions to the dynamic structure and function of neuronal membranes.

A second potential pathway by which these diets may influence neurogenesis is via omega-3 fatty acid modulation of cytokine levels, which in turn regulate immune function. For example, EPA inhibits the release of the proinflammatory cytokines interleukin-1 beta (IL-1β) and TNFα [24]. These and closely related molecules have been implicated in mechanisms that regulate neurogenesis and cell fate [2]. Omega-3 fatty acids also influence levels of neurotrophins, molecules that promote neuronal survival and growth. Among the neurotrophins, brain-derived neurotrophic factor (BDNF) levels are altered by dietary intake of omega-3 fatty acids [30], and BDNF is associated with alterations in neurogenesis and neuronal survival [22]. Therefore, although the specific effects of cytokines and neurotrophins on neurogenesis, cell fate and survival are still being clarified, the association between omega-3 fatty acids and the regulation these classes of molecules is no longer debatable.

These connections between the omega-3 fatty acids, cytokines, neurotrophins and neurogenesis are also intriguing from the clinical perspective. Cytokines appear to play a potentially critical role in depressive illness [25]. Stress can cause an elevation in cytokines, and selective serotonin reuptake inhibitors reduce cytokine levels. Likewise, the neurotrophin BDNF has been negatively correlated with the severity of depressive symptoms. Antidepressant medications and exercise enhance BDNF levels, while stress inhibits BDNF production. Therefore, the same molecules that are of importance in regulating neurogenesis, also are

implicated in major depressive illness. Although there are not adequate data at this time to make a conclusive statement regarding the functional relationship between neurogenesis and clinical depression, these associations are suggestive of pathways by which omega-3 fatty acids may simultaneously influence neurogenesis and depressive illnesses.

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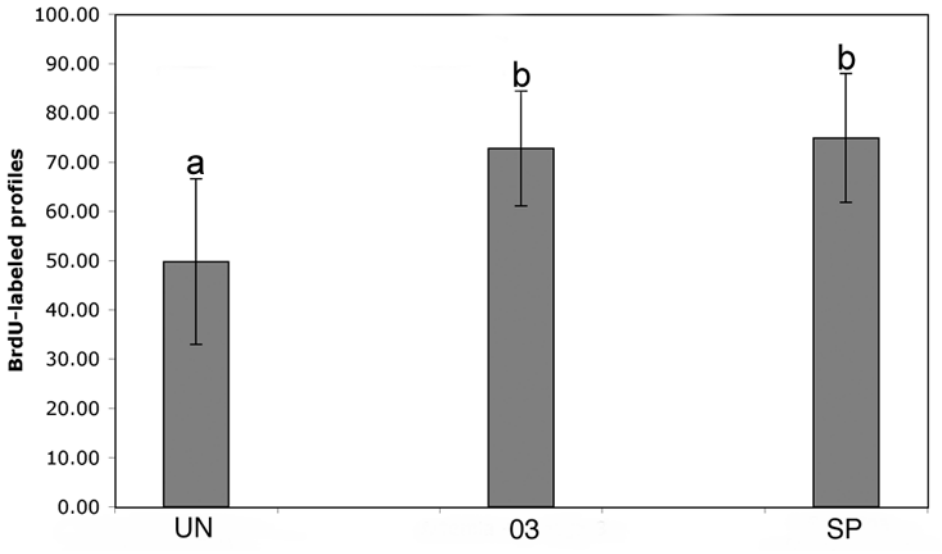

Figure 1.

The levels of neurogenesis in cluster 10 in the brains of lobsters fed UN, O3 or SP diet. Each histogram represents the mean of BrdU-labeled cell counts. Following a two-way repeated measures ANOVA ($P<0.05$), Holm-Sidak paired comparisons revealed significant differences in levels of neurogenesis between the UN vs. O3 and SP diets. Statistical similarity is indicated by the same letters (O3 and SP diets).

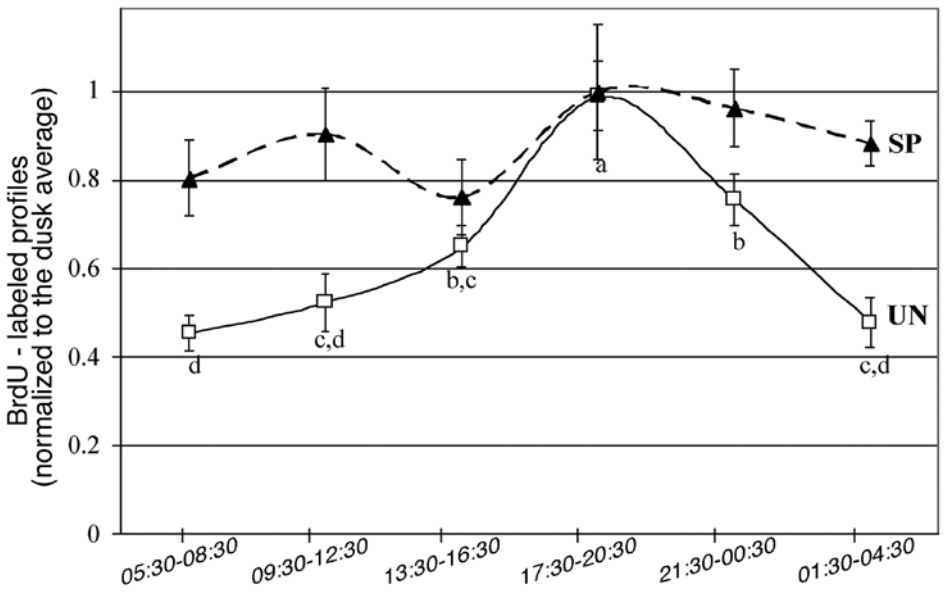

Figure 2.

Mean counts and 95% confidence intervals of BrdU-labeled cells normalized to the average value for dusk within each diet treatment, for the different sampling times over a 24-hour period. There was a significant diet versus time interaction for cell count data, and thus the normalized ranked data were analyzed separately for each diet. Statistical similarity within the UN treatment is denoted by the same letters; there were no significant differences between time periods for the SP data. When lobsters are fed unenriched *Artemia*, cell proliferation is significantly greater at dusk than at any other time of the day. Cell proliferation between 09:30 and 12:30 averages 50% that of the dusk period, and all 95% confidence intervals are < 60%. When lobsters are fed the *Spirulina* enriched *Artemia* diet, cell proliferation during the day is relatively uniform, with only the dawn (05:30–08:30) and pre-dusk (13:30–16:30) time periods averaging 80% that of the dusk period.

Table 1

The proportion (percent) of fatty acid methyl esters for each of the three experimental *Artemia* diets. Only fatty acids with > 14 carbons and detectable values are listed. The summary rows at the bottom indicate all omega-3, omega-6, saturated, mono-unsaturated, and polyunsaturated fatty acids.

		Unenriched	Omega-3 enriched	*Spirulina* enriched
C14:0	myristic acid	0.84	1.30	0.41
C15:0	pentadecanoic acid	0.52	0.00	0.38
C16:0	palmitic acid	17.67	18.36	17.19
C16:1ω7	palmitoleic acid	3.18	3.28	2.66
C17:0	heptadecanoic acid	1.12	0.68	1.12
C18:0	stearic acid	6.90	7.07	7.50
C18:1ω9t	elaidic acid	0.19	0.23	0.00
C18:1ω9c	oleic acid	24.26	27.09	25.26
C18:2ω6c	linoleic acid	11.24	11.72	8.37
C18:3ω6	γ-linolenic acid	1.26	1.37	1.22
C20:1ω9	eicosenoic acid	0.17	0.43	0.30
C18:3ω3	α-linolenic acid (ALA)	17.44	25.98	24.30
C20:2	cis-11,14-eicosadienoic acid	0.29	0.50	0.27
C22:0	behenic acid	0.24	0.00	0.00
C20:3ω6	homo-γ-linolenic acid	0.33	0.00	0.42
C20:3ω3	eicosatrienoic acid (ETA)	0.35	0.98	0.82
C20:4ω6	arachidonic acid (ARA)	9.17	0.68	4.95
C20:5ω3	eicosapentaenoic acid (EPA)	4.74	0.27	4.77
	Σω3	22.5	27.2	29.9
	Σω6	22.0	13.8	15.0
	ΣSAFA	27.3	27.4	26.6
	ΣMUFA	27.8	31.1	28.2
	ΣPUFA	44.9	41.5	45.1