Published in final edited form as:
Neurotoxicol Teratol. 2009 ; 31(1): 26–33. doi:10.1016/j.ntt.2008.09.001.

Abnormal Neurological Responses in Young Adult Offspring Caused by Excess Omega-3 Fatty Acid (Fish Oil) Consumption by the Mother during Pregnancy and Lactation

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Abstract

Consuming omega-3 fatty acids (ω-3 FA) during pregnancy and lactation benefits fetal and infant brain development and might reduce the severity of preterm births by prolonging pregnancy. However, diets that are relatively rich in ω-3 FA can adversely affect fetal and infant development and the auditory brainstem response (ABR), a measure of brain development and sensory function. We previously examined the offspring of female rats fed excessive, adequate or deficient amounts of ω-3 FA during pregnancy and lactation. The 24-day-old offspring in the Excess group, compared to the Control group, had postnatal growth retardation and poor hearing acuity and prolonged neural transmission times as evidenced by the ABR. The Deficient group was intermediate. The current study followed these offspring to see if these poor outcomes persisted into young adulthood. Based on prior findings, we hypothesized that the Excess and Deficient offspring would "catch-up" to the Control offspring by young adulthood. Female Wistar rats received one of the three diet conditions from day 1 of pregnancy through lactation. The three diets were the Control ω-3 FA condition (ω-3/ω-6 ratio ~ 0.14), the Excess ω-3 FA condition (ω-3/ω-6 ratio ~ 14.0) and Deficient ω-3 FA condition (ω-3/ω-6 ratio ~ 0% ratio). The Control diet contained 7 % soybean oil; whereas the Deficient and Excess ω-3 FA diets contained 7% safflower oil and 7% fish oil, respectively. One male and female offspring per litter were ABR-tested as young adults using tone pip stimuli of 2, 4, 8 and 16 kHz. The postnatal growth retardation and prolonged neural transmission times in the Excess and Deficient pups had dissipated by young adulthood. In contrast, the Excess group had elevated ABR thresholds (hearing loss) at all tone pip frequencies in comparison to the Control and Deficient groups. The Deficient group had worse ABR thresholds than the Control group in response to the 8 kHz tone pips only. The Excess group also had ABR amplitude-intensity profiles suggestive of hyperacusis. These results are consistent with the Barker hypothesis concerning the fetal and neonatal origins of adult diseases. Thus, consuming diets that are excessively rich or deficient in ω-3 FA during pregnancy and lactation seems inadvisable because of risks for long-lasting adverse effects on brain development and sensory function.

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Keywords

auditory brainstem response (ABR); Barker hypothesis; docosahexanoic acid (DHA); eicosapentanoic acid (EPA); fetal programming; fish oil; hearing loss; hyperacusis; lactation; omega-3 fatty acids (ω-3 FA); omega-6 fatty acids (ω-6 FA); over-nutrition; postnatal; pregnancy; prenatal

1. Introduction

Omega-3 fatty acids (ω-3 FA) are "essential fatty acids" because it is necessary to acquire them through food consumption. Docosahexanoic acid (DHA) and eicosapentanoic acid (EPA) are the chief ω-3 FA used as dietary supplements, usually in the form of fish oil. Maternal consumption of ω-3 FA during pregnancy and lactation can influence fetal and infant health and development. Regarding pregnancy, some studies reported that diets rich in ω-3 FA can increase birth weight and prolong pregnancy, thereby reducing the incidence and severity of preterm births and low birth weight infants [49,50,60,65]. Regarding lactation, the ω-3 FA in a mother's milk or in fortified infant formulas improve neurocognitive and visual development during the first year of life in comparison to infants who receive infant formula without ω-3 FA supplementation [2,9,33]. Thus, increasingly higher ω-3 FA doses are being recommended for pregnant women and nursing babies for advancing the health and development of preterm, low birth weight, and even normal infants [1,36,50].

Even though moderate amounts of ω-3 FAs are beneficial to the developing fetus and infant, there is new evidence that too much can be harmful. For example, several human studies found decreased gestational length and/or fetal growth retardation [25,26,48,51,58,68] and increased infant morbidity [68] from high fish oil consumption by the mother during pregnancy. Adverse effects from high ω-3 FA consumption by infants drinking formulas fortified with ω-3 FA include reduced body growth and head circumference [12,37,41], decreased blood arachidonic acid (AA) levels [8] and decreased verbal skills [40,62].

Animal studies report adverse effects as well. For example, prenatal and/or postnatal dietary supplementation with large amounts of ω-3 FA or a high ω-3/ω-6 FA ratio (due to low amounts of ω-6 FA) can result in reduced birth weight, postnatal growth impairment, increased pre- and postnatal mortality, decreased brain sizes, decreased AA levels, and/or abnormal neurobehavioral function [3,18,30,56,70] and abnormal retinal function and structure [38,74]. Studies using the auditory brainstem response (ABR), a measure of brain development and sensory function, found that high levels of dietary ω-3 FA supplementation in pregnant and lactating rats caused the offspring to have prolonged ABR wave latencies [19,30,59,67], delayed acoustic startle reflexes [30,59,67], reduced auditory acuity [18], and evidence suggesting impaired brain myelination [30] when tested as postweanling pups. Effects from pre- and postnatal diets that are rich in ω-3 FA are similar to those caused by ω-3 FA deficiency or a low ω-3/ω-6 ratio (due to ω-6 FA excess) which include impaired visual function [7,74], learning deficits, decreased brain weight and/or altered nerve FA composition [7,9,33,69,71] and ABRs indicating a faster aging brain in old age [6].

Unfortunately, the caveat that rich ω-3 FA diets can be harmful to the fetus and infant is being ignored [19]. Adverse fetal and neonatal environments can "program" the offspring for adult-onset health disorders [4,22]. Yet no one has investigated the possibility that nutritional toxicity from high levels of dietary ω-3 FA or a high ω-3/ω-6 ratio can cause fetal programming of adult-onset disorders, with the exception of a study which found altered body fat composition in adult rats born to dams receiving diets rich in ω-3 FA during pregnancy and lactation [35].

Thus, further research on the potentially harmful effects of prenatal and postnatal ω-3 FA excess and deficiency is rather important, particularly in terms of the long-term consequences.

With these issues in mind, our study's primary goal was to investigate the possibility that maternal consumption of diets that are excessively rich or deficient ω-3 FA during pregnancy and lactation cause long-term impairment of the offspring's nervous system as evidenced by the ABR. In prior studies, we found that our "ω-3 FA excess" condition caused postnatal growth retardation, hearing loss [18] and delayed neurotransmission times [19] in 24-day old rat offspring. The current study followed these offspring longitudinally to see if these poor outcomes persisted into young adulthood. A recent study on ω-3 FA deficiency during pregnancy found that the rat offspring had abnormal ABRs as pups, which became normal in young adulthood, then became abnormal again in old adulthood [6]. Others have found this same age-dependent pattern in neurological outcomes following prenatal or neonatal exposure to various brain damaging substances [5,34,43,44,47,61,73]. Consequently, we hypothesized that our ω-3 FA excess and deficient offspring would show normalization of their ABRs during young adulthood.

2. Methods

2.1. Animals and diets

Wayne State University's animal investigation committee approved the procedures for this study. Institutional and NIH guidelines were followed.

Details of our procedures are detailed elsewhere [18,19]. Briefly, female Wistar rats, 10 weeks of age, were mated individually with male Wistar rats. The presence of a sperm plug was designated as gestational day one. The females were then placed in separate polycarbonate cages (25 × 45 × 20 cm) and randomly assigned to one of the three diet conditions starting from day 1 of pregnancy through the entire period of pregnancy and lactation. The three diets were the Control ω-3 FA condition (ω-3/ω-6 ratio ~ 0.14), the Deficient ω-3 FA condition (ω-3/ω-6 ratio ~ 0% ratio) and the Excess ω-3 FA condition (ω-3/ω-6 ratio ~ 14.0). The number of pregnant dams/litters in the Control, Deficient and Excess conditions were n = 23, 31 and 22, respectively. The Deficient group had slightly more pregnant dams than the other two groups because of a better pregnancy success rate for unknown reasons. The Control diet contained 7% soybean oil. The Deficient ω-3 FA diet contained 7% safflower oil in place of soybean oil. The Excess ω-3 FA diet contained 7% menhaden oil (a type of fish oil) in place of soybean oil. Our rationale for these dose selections is detailed elsewhere [18,19]. Briefly, we considered this an excess ω-3 FA diet because it had a ω-3/ω-6 ratio that was 100 times what is adequate for pregnant and lactating rats and because our diet of 7% fish oil is consistent with other studies on "excess ω-3 FA" reporting adverse developmental effects (see Introduction). All diets were formulated according to AIN-93G standards which have determined that the ω-3/ω-6 ratio ~ 0.14 and 7% oil composition is ideal for pregnant and lactating female rats [50]. Fish oil was selected for the Excess diet because of its use in clinical studies (see Introduction). The soybean and safflower oils in the Control and Deficient diets were selected because they are commonly consumed by humans and in animal studies. We used the naturally occurring fatty acid profiles of the fish, soybean and safflower oils; nothing was artificially altered. The diets were prepared by Dyets Inc (Bethlehem, PA). All three diets contained tertiary-butylhydroquinone (TBHQ) because this preservative prevents oxidation [23,24,55]. Diets were stored at refrigeration temperatures and fresh diet was provided twice weekly to further protect against oxidation. Each diet provided 3.96 kcal/g. Detailed composition of each diet was recently published in this journal [18,19].

Dams had free access to food and water. Food consumption was assessed twice weekly when the old food was discarded and replaced by fresh diet. Dams were weighed on these days as

well. Animals were housed at ~53% relative humidity and at ~22ºC room temperature. Within 24 hours after delivery, designated as postnatal day one (PND 1), litters were counted, weighed and reduced to 8 pups per litter, consisting of 4 male and 4 female pups when possible. The remaining pups were euthanized by CO_2 exposure and decapitated to ensure death. The retained pups were weaned on PND 21 and kept on their respective experimental diets until the day of ABR testing on PND 24. After PND 24, all offspring channeled into our ABR study were switched to a standard rodent diet (5001 Rodent Diet, PMI Nutrition International).

2.2. ABR procedure

When possible, one male and one female pup/litter were randomly selected for testing in the current study. The other littermates were channeled into a study that analyzed their fatty acid tissue composition [35]. Using male/female littermate pairs allowed assessment of sex-dependent differences and controlled for within-litter effects by limiting the number of pups tested from any one litter. Of the 23 Control litters, 21 litters had both a male and female offspring that were ABR-tested whereas one litter had just one male and one litter had just one female offspring that were tested for a total of n = 44 offspring. Of the 31 Deficient litters, 30 litters had both a male and female offspring whereas one litter had just one female offspring for a total of n = 61 offspring. Of the 22 Excess litters, 21 litters had both a male and female whereas one litter had just one male offspring for a total of n = 43 offspring. Rat offspring were initially tested on PND 24 and those test results were previously reported [18,19]. These same offspring were subsequently retested as young adults and those results are reported in this article. The animals were fully mature young adults, aged 167–178 days (95% confidence interval) at the time of retesting. The rat ABR is fully mature and stable by approximately 70–100 days of age [20] and age-related hearing loss (presbycusis) usually does not occur in rats until about 17 months [15].

Our ABR procedure is detailed elsewhere [16,18]. Prior to ABR recording, each animal was given 100 mg/kg of the anesthetic ketamine (i.p.). Ketamine influences ABR latencies and amplitudes, but the effects are minor and the ABR quality is excellent [17]. Rectal temperature was monitored because temperature can influence the ABR [57] (Model 43TD, Yellow Springs Instruments Co., Yellow Springs, Ohio 45387, USA). A water-circulating heating pad was used to regulate and maintain normothermia by raising or lowering the temperature of the circulating water (Model TP500, Gaymar Industries, Orchard Park, New York 14127, USA).

The ABR was differentially recorded between two subcutaneous platinum E-2 needle electrodes. The active electrode was inserted at the vertex, the reference electrode below the left ear, and the ground electrode below the right ear. Evoked potentials were collected by a Biologic Navigator and amplified 300,000 times with a digital bandpass of 300–3000 Hz. Electrode impedances ranged from 0–9 kΩ. At least 256 responses were averaged. Recordings were made in an electrically shielded, double-walled sound attenuation chamber (Allotech, Inc., Raleigh, North Carolina 27603, USA). Binaural, ‘open field’ tone pips in the ascending order of 2000 Hz, 4000 Hz, 8000 Hz and 16000 Hz were delivered through a TDH-39P headphone positioned in front of the animal (rise/fall time = 0.5 msec, plateau = 10.0 msec, polarity = alternating, repetition rate = 19.0/sec, stimulus intensity = 15 to100 dB peSPL).

2.2.1. ABR latencies (neural transmission times)—The ABR is a series of action potentials and postsynaptic potentials. The rat ABR is composed of four components (labeled P1 to P4) occurring within 6 msec of stimulus onset [15,17,18,20]. Although the neurogenerators of the rat’s ABRs have not been determined, in the mouse they reflect neural activity chiefly from the auditory nerve (P1), the cochlear nucleus (P2), the superior olivary complex (P3), and the lateral lemniscus and/or inferior colliculus (P4) [31]. The latency of each ABR component was measured as the time from the computer’s triggering of the earphone

to a wave's positive peak, including a 0.3 msec acoustic transit time between the earphone and the animal's pinnae. Two experimenters, who were 'blind' as to each animal's treatment condition, scored the latencies of ABR waves P1, P2, P3 and P4. When scorers disagree (rarely), the scores are averaged. The primary outcome variable was the P4 latency, a measure of neural transmission time along the auditory nerve and brainstem auditory pathway inclusively, in response to the 100 dB stimuli. The secondary outcomes were the latencies of the individual ABR waves and the P1 to P4 interpeak latency (P1-P4 IPL). The P1-P4 IPL measures the brainstem portion of neural transmission by excluding the auditory nerve transmission time.

2.2.2 ABR thresholds (hearing acuity)—ABR thresholds were determined by the method of limits [16,18]. Here, serial ABRs were gathered to a range of stimulus intensities starting at 100dB, then descending to 80, 60, 50, 40, 35, 30, 25, 20, and 15 dB as the ABR threshold was reached and passed. To establish ABR threshold more precisely, 2 and 3 dB changes in stimulus intensity levels were tested around the ABR's threshold (as determined by visual detection) and multiple ABR traces (2 to 5) were collected at each near-threshold intensity level. Threshold was defined as the lowest intensity to elicit a reliably scored ABR component. An experimenter, who was 'blind' as to each animal's treatment condition, scored the ABR thresholds. A second experimenter then checked the threshold scoring for reliability purposes.

2.2.3 ABR latency-intensity profiles—An ABR latency-intensity (L-I) profile can help determine if a subject's hearing loss is a conductive hearing loss (CHL) or a sensorineural hearing loss (SNHL). A subject with a CHL would have an elevated ABR threshold and an L-I profile that is displaced upward and parallel to the normal curve. A subject with a SNHL will also have an elevated ABR threshold, however the P2 latencies will typically be normal or near normal in response to loud stimulus intensities but progressively curve upward from the normal range as the stimulus intensity decreases [16]. In rodents, one typically uses the ABR's P2 wave to derive an animal's latency-intensity (L-I) profile because the rodent's P2 wave is the largest wave and the last wave to disappear as the sound stimulus is decreased [16]. P2 latency was measured at the positive peak of this waveform at each stimulus intensity level.

2.2.4 ABR amplitude-intensity profiles—The amplitude of the P2 waveform was used as an index of the ABR's maximum amplitude because the rodent's P2 wave is the largest wave and the last wave to disappear as the sound intensity is decreased. P2 amplitude was measured from the positive peak of wave P2 to the subsequent negative trough (labeled N2). This was done at each stimulus intensity level in order to derive amplitude-intensity (A-I) profiles. Whereas the ABR amplitude is a function of neural synchrony and the number of neural units firing, the ABR amplitude can provide diagnostic information on these neural functions.

2.3. Data analysis

Analyses of variances (ANOVA) were used to assess statistical significance. Because we were interested in gender-dependent differences, male and female littermates were treated as individual units of measure. For the ABR variables, we used a three-way ANOVA to test for the effects of Diet, Sex and Tone Pip Frequency. Because Tone Pip was a within-subjects measure, the Greenhouse-Geisser adjustment was used with this variable's main effects and interactions. Because we sampled from each litter in a highly balanced manner, using only one male and one female from each litter with only rare exception (see *2.2. ABR procedure*), it was unnecessary to control for litter-dependent effects by using litter as a variable in the statistical design. If an ANOVA indicated a significant treatment (Diet) effect ($p < 0.05$), the Bonferroni test was used to make planned pair-wise comparisons between treatment groups. Two-sided tests were used to compare all groups with each other because we hypothesized that they would not differ.

Simple regression analyses were used to see if the ABR thresholds gathered when the offspring were pups (24 days of age) correlated with (predicted) their ABR thresholds gathered when they were young adults. To simplify data analyses, the ABR thresholds in response to the 2, 4, 8 and 16 kHz tone pips were averaged to derive one threshold value for each offspring when it was a pup. This procedure was repeated for each offspring's young adult threshold data. Details about the pup threshold data for the current set of offspring are published elsewhere [18].

3. Results

3.1. Maternal and birthing outcomes

Maternal and birthing data were reported previously [18,19,35]. There were no group differences in gestational length, maternal weight gain, food consumption during pregnancy or lactation, the number of pups per litter, litter or pup weights at birth, or age of teeth eruption. There was a trend for increased postnatal mortality between birth and weaning for the Excess offspring and their pinna detachment was delayed. On PND 24, we weighed the male/female littermate pairs that were channeled into the ABR study. The respective weights of these Control, Deficient and Excess male pups were 65.3 ± 1.9, 59.8 ± 2.1 and 51.0 ± 2.4 g (mean ± SEM). The respective weights of these Control, Deficient and Excess female pups were 61.3 ± 1.5, 56.1 ± 1.7 and 48.4 ± 2.1 g. The ANOVA indicated a significant effect for Diet group: $F(2, 144) = 21.20$, $p < 0.001$. Post hoc comparisons indicated that the Excess male and female pups weighed significantly less than their Control and Deficient cohorts and that the Deficient female pups weighed less than their Control cohorts. Males weighed more than females: $F(1, 144) = 4.36$, $p = 0.038$. There was no Diet-by-Sex interaction: $F(2, 144) = 0.06$, $p = 0.94$.

3.2. Adult offspring characteristics

The Control, Deficient and Excess offsprings' respective ages during the ABR recording session were 170 ± 37, 174 ± 34 and 174 ± 33 g, their body temperatures were 37.6 ± 0.4, 37.5 ± 0.4 and 37.6 ± 0.4 °C, the male weights were 653 ± 76, 641 ± 72 and 614 ± 93 g, and the female weights were 418 ± 51, 400 ± 41 and 396 ± 57 g (mean ± SEM). There were no significant Diet group differences in testing age: $F (2, 142) = 0.25$, $p = 0.78$, rectal temperature: $F (2, 142) = 0.27$, $p = 0.77$, male body weight: $F (2, 71) = 1.44$, $p = 0.24$, or female body weight: $F (2, 71) = 1.37$, $p = 0.26$. There were no significant effects for Sex or the Sex-by-Diet, except for males weighing more than females: $F (1, 142) = 437.61$, $p < 0.001$.

3.3. ABR latencies (neural transmission times)

Figure 1 shows neural transmission times (P4 latency) as functions of Diet group and Tone Pip Frequency. There were no significant differences as functions of Diet group: $F (2, 142) = 1.20$, $p = 0.30$, Sex: $F (1, 142) = 0.25$, $p = 0.62$, or the Diet-by-Sex interaction: $F (2, 142) = 0.41$, $p = 0.66$. There was a significant main effect for Tone Pip Frequency, indicating that P4 latency became gradually shorter (faster) as the tone pip frequency progressed from 2 to 16 kHz: $F (3, 426) = 261.29$, $p < 0.001$. No significant Diet or Sex effects were found for the secondary outcome variables of P1, P2 or P3 latencies or the P1-P4 IPL.

3.4. ABR thresholds (hearing acuity)

Figure 2 shows ABR thresholds as a function of Diet group and Tone Pip Frequency. The ANOVA indicated a significant effect for Diet group: $F (2, 142) = 19.40$, $p < 0.001$. Pairwise comparisons indicated that the Excess group had higher (worse) ABR thresholds than the Control and Deficient groups at all tone pip frequencies. The Deficient group had a significantly higher threshold than the Control group only during the 8 kHz tone pip condition. There were no significant effects for Sex: $F (1, 142) = 3.096$, $p = 0.08$ or the Diet-by-Sex interaction: F (2,

142) = 0.25, p = 0.78. There was a significant effect for Tone Pip Frequency, reflecting that rats have progressively better hearing acuity as the tonal frequency progresses from 2 to 16 kHz [16,18]: F (3, 426) = 891.52, p < 0.001. There was a significant interaction between Diet group and Tone Pip Frequency, indicating that Diet group differences were more dramatic in response to the 4 and 8 kHz than to the 2 and 16 kHz tone pip conditions: F (6, 42) = 3.49, p = 0.003.

Figure 3 shows serial ABRs in response to 8 kHz tone pips of descending stimulus intensity from representative young adult offspring in the Control and Excess diet groups. The Control animal (panel A) had an ABR still present at 25 dB, whereas the first Excess animal (panel B) had an ABR at 40 dB but not at 30 dB and the second Excess animal (panel C) had an ABR at 80 dB but not at 60 dB. These two Excess animals therefore had elevated ABR thresholds (technically defined as ≥ 2 standard deviations above the Control group's mean), suggesting respective hearing losses of 15 dB and ≥ 35 dB. Several animals in the Deficient group (not shown) had elevated ABR thresholds very similar to those shown in panel B of Figure 3, but only in response to the 8 kHz tone pips. The Deficient group had normal ABR thresholds at the remaining tone pip frequencies of 2, 4 and 16 kHz.

3.5. ABR latency-intensity (L-I) profiles

As mentioned in the Methods section, the L-I profile can help diagnose whether a subject's hearing loss is either CHL or SNHL. Figure 4 shows the L-I profiles in response to the 8 kHz tone pip condition for each animal in the Excess group that had a significantly elevated ABR threshold. A significant elevation in the ABR threshold was defined as being ≥ 2 standard deviations (SD) above the Control group mean [16]. The shaded region in Figure 4 is the range of normalcy derived from Control data. Figure 4 shows that the L-I profiles from all Excess animals with elevated ABR thresholds fell within the range of normalcy, indicating an absence of the typical L-I patterns that are diagnostic of CHL and SNHL. These animals did not have ABRs below 40 dB, however. Results from the 2, 4 and 16 kHz tone pip conditions (not shown) were highly similar to those illustrated in Figure 4.

3.6. ABR amplitude-intensity (A-I) profiles

The Excess group had elevated ABR thresholds without exhibiting either SNHL or CHL patterns in their L-I profiles. This suggested that the Excess group merely had small amplitude ABRs. To investigate this possibility, we measured the ABR's P2-N2 amplitude. This waveform was chosen as an index of the ABR's amplitude for reasons described in the Methods section. We expected the Excess group to have abnormally low ABR amplitudes across the complete range of stimulus intensities under all four tone pip conditions.

Figure 5 shows the A-I profiles for the P2-N2 wave in response to the 8 kHz tone pip condition for animals in the three diet groups. The ANOVA found a significant effect for Stimulus Intensity, indicating a significant decrease in ABR amplitude as the stimulus intensity decreased: F (6,852) = 2652.59, p < 0.001. There was a significant effect for Sex, reflecting that females had larger P2-N2 amplitudes than males: F (1, 142) = 45.66, p < 0.001. There was no main effect for Diet group: F (2, 142) = 0.70, p = 0.50; and there was no effect for the Diet-by-Sex interaction: F (2, 142) = 0.34, p = 0.71. There was however a significant Diet-by-Stimulus Intensity interaction: F (12, 852) = 4.75, p = 0.002. This interaction is described below.

Consistent with our expectation, univariate analyses of the 8 kHz data showed that the Excess group had lower amplitudes than the Control group for the 25 and 30 dB stimulus intensity conditions. Contrary to our expectation, the Excess group had normal P2-N2 amplitudes for the 40 through 80 dB conditions and significantly larger amplitudes than the Control group for

the 100 dB condition. The other three tone pip conditions also showed that the Excess group's P2-N2 amplitudes were smaller than the Control group's P2-N2 amplitudes at the lowest stimulus intensities of 25 and 30 dB, were within normal limits for the mid-range stimulus intensities of 40 to 80 dB, and were larger than normal at the highest stimulus intensity of 100 dB (not shown). This abnormal growth pattern in ABR amplitude is also illustrated in Fig. 3C where the Excess offspring had no ABR at 60 dB but a large amplitude ABR at 100 dB. The only exception to this pattern was that the Excess group's P2-N2 amplitudes were within normal limits at 100 dB for the 2 kHz tone pip condition. The Deficient group's amplitudes were within normal limits at all stimulus intensities under all four tone pip conditions.

Figure 6 further illustrates the Excess group's propensity for larger than normal P2-N2 amplitudes in response to loud intensity stimuli. There was a significant effect for Tone Pip Frequency, indicating that the P2-N2 amplitudes differed across the various tone pip frequencies: $F(3, 435) = 166.90$, $p < 0.001$. There were also significant effects for Diet group: $F(2, 145) = 3.35$, $p = 0.038$ and for the Diet-by-Tone Pip Frequency interaction: $F(6, 435) = 3.28$, $p = 0.006$. Subsequent univariate analyses indicated that the Excess group had larger P2-N2 amplitudes than the Control group at 4, 8 and 16 kHz, but not at 2 kHz. The P2-N2 amplitudes of the Deficient group were intermediate to the other two groups but did not differ significantly from either.

3.7. Pup ABR thresholds predicting young adult ABR thresholds

The Control, Deficient and Excess offspring had respective ABR thresholds of 28.3 ± 0.3, 29.1 ± 0.3 and 30.2 ± 0.3 dB as PND 24 pups and ABR thresholds of 29.3 ± 0.5, 30.0 ± 0.4 and 33.0 ± 0.5 dB as young adults (mean ± SEM). At both ages, the Excess offspring had significantly higher ABR thresholds than the other two groups ($p \leq 0.018$ or better) With Pearson correlation coefficients (r) of 0.088 and −0.159, the Control and Deficient offspring showed no significant associations between their pup and young adult ABR thresholds. In contrast with $r = 0.371$ ($p = 0.014$), the Excess offspring showed a significant association (see *2.3. Data analyses* for details). With regards to the 43 Excess offspring: (A) Of the17 offspring with poor thresholds as pups (defined as ≥ 2 SD above the Control group mean), six (35%) had a persistence of poor thresholds into adulthood and 11 had normalization of their thresholds by the time they reached adulthood. (B) Of the 26 offspring with normal thresholds as pups, five (19%) developed poor thresholds by adulthood and 21 retained their normal thresholds into adulthood. An Age-by-Diet ANOVA with the Greenhouse-Geisser adjustments for a within-subject measure was also performed on the ABR data. There was a significant effect for Age group (pup versus adult): $F(1, 145) = 35.59$, $p = 0.001$ and for Diet group: $F(2, 145) = 25.00$, $p = 0.001$ and for the Age-by-Diet interaction: $F(2, 145) = 4.79$, $p = 0.010$. These results indicated that the ABR thresholds were higher when the offspring were adults, that the Excess group had higher ABR thresholds than the Control and Deficient groups, and that the Excess offspring showed a greater age-related increase in their ABR thresholds than their cohorts in the other two diet groups.

4. Discussion

4.1. ABR findings

A prior study on ω-3 FA deficiency during pregnancy found that the rat offspring had abnormal ABRs as pups, which became normal in young adulthood, then became abnormal again in old adulthood [6]. Others have found the same age-dependent pattern in neurological outcomes following prenatal or neonatal exposure to various brain damaging substances [5,34,43,44, 47,61,73]. Thus, we hypothesized that our Excess and Deficient offspring would show normalization of their ABRs during young adulthood. Consistent with this hypothesis, we found that the ABR wave P4 latencies (neural transmission times) for the young adult animals in the Excess and Deficient ω-3 FA treatment groups were within normal limits. This contrasts

with the prolonged neural transmission times that were seen in these animals when they were 24-day-old pups [19]. Contrary to our hypothesis, we found that the ABR thresholds were elevated (worse hearing acuity) in the young adult Excess animals for the 2, 4, 8 and 16 kHz tone pip conditions and in the young adult Deficient animals for the 8 kHz tone pip condition when compared to the young adult Control animals. These Excess and Deficient animals had elevated ABR thresholds as 24-day-old pups [18]. Thus, they continued this abnormality into adulthood.

The Control and Deficient groups showed no correlations between their respective pup and adult ABR thresholds. In contrast, the Excess group's pup and adult ABR thresholds were significantly correlated. Of the17 Excess offspring with poor thresholds as pups, six (35%) had a persistence of poor thresholds into young adulthood, suggesting these six offspring had a permanent deficit and that the remaining 11 offspring had temporary deficits as pups that normalized with maturation. Of the 26 Excess offspring with normal thresholds as pups, five (19%) developed poor thresholds by adulthood, suggesting that these offspring experienced early adult-onset deficits and that the remaining 21 pups retained their normalcy into young adulthood.

In addition to the elevated ABR thresholds in the adult Excess offspring, we found differences in their ABR amplitudes. Specifically, the Excess offspring exhibited significantly lower amplitude ABRs in response to low stimulus intensities and significantly higher amplitude ABRs in response to high stimulus intensities compared to Controls. The Deficient offspring showed a similar but non-significant tend. ABR amplitudes reflect the number of neural units firing, their firing rates and/or their neural synchrony [21,53,66]. Thus, the elevated ABR thresholds in the Excess offspring were likely due to such mechanisms in response to low intensity stimuli. In contrast, the Excess offspring also had an over-excitation phenomenon in response to high intensity stimuli that caused the enlarged ABR amplitudes. Such patterns of decreased auditory evoked potential amplitudes at low stimulus intensities and enlarged amplitudes at high stimulus intensities are seen in hearing losses that have accompanying hyperacusis [53,63,76]. An abnormal decrease in neural inhibitory processes is the suspected mechanism underlying such hyperactivity [53,63,76].

This hyperacusis phenomenon in the Excess group was seen in response to the 4, 8 and 16 kHz tone pips, but not in response to the 2 kHz tone pips. This was likely due to differences in perceived stimulus intensity or hearing level (HL). As shown in Figure 2, the ABR thresholds for the 2 kHz condition were about 15–20 dB higher (worse) than the other three conditions. If we had compensated for this poorer hearing acuity at 2 kHz by raising the stimulus an additional 15–20 dB, we likely would have seen the hyperacusis phenomenon.

The ABR threshold and amplitude abnormalities in our young adult Excess and Deficient offspring indicate that their treatments resulted in permanent and/or early adult-onset neurological and sensory deficits. These ABR findings, as well as another study from our laboratory that found increased ω-3 FA levels and increased ω-3/ω-6 ratios in the body fatty acid composition in their male and female littermates [35], are the first studies to show abnormalities in adulthood as a result of exposure to excess amounts of ω-3 FA during pregnancy and lactation. In addition, we have observed a significant decrease in the adult life span of our Excess animals [manuscript in preparation]. Such findings are consistent with Barker's hypothesis that adverse prenatal and/or postnatal conditions can program the offspring for health disorders in adulthood [4,22,54].

4.2. Adult offspring characteristics

Although there was a tendency for the Excess and Deficient offspring as young adults to weigh less than their Control cohorts, these differences were not significant. This contrasts to the

significant postnatal growth retardation seen in these same Excess and Deficient offspring prior to weaning [18,19]. This indicates ability for catch-up growth in these offspring.

4.3. Mechanisms of ω-3 FA nutritional toxicity

There are several mechanisms by which ω-3 FA imbalances can produce adverse effects on offspring development: (A) A balanced ratio of ω-3 and ω-6 FA is required for optimal growth. A relative excess of ω-3 FA will lower AA concentrations in blood, brain and other tissues through competitive displacement. In addition, EPA and DHA are also known to inhibit the synthesis of AA [28]. Lowered concentrations of AA impair fetal and infant growth [11,13]. This is because AA promotes adipose tissue deposition and is a mediator of growth and metabolic hormones [9,29]. (B) Excess ω-3 FA can harm the developing fetus and infant by causing oxidative stress and subsequent cell apoptosis through augmented lipid peroxidation [23–25,72] and reduced antioxidant status [14]. For example, elevated peroxidation levels were found in the mammary glands, skeletal muscle, heart and carcass of female mice given a diet of 19% fish oil + 1% corn oil for four weeks [24]. (C) Various forms of over-nutrition can cause epigenetic and hormonal changes in the fetus or young child which can increase the risks for adult-onset health disorders such as diabetes, hypertension and a shortened life span [52, 54,75]. It is possible that fish oil over-nutrition can have similar effects. (D) Offspring growth retardation due to prenatal undernutrition causes reduced cell numbers in rat embryos [39] and in the adrenal glands, kidney, liver and heart of human fetuses [45]. It seems plausible that conditions causing postnatal growth retardation, such as our ω-3 FA Excess and Deficiency conditions, could similarly reduce cell numbers in the nervous system and other vital organs. (E) A diet rich in ω-3 FA can decrease the milk yield of lactating rats, resulting in postnatal growth retardation [32]. (F) Exposure of the oocyte to an environment high in ω-3 FA can result in perturbed mitochondrial distribution, metabolism, and calcium levels, adversely affecting embryo morphology and development [72].

4.4. Conclusions and health implications

In summary, a diet rich in ω-3 FA during pregnancy and lactation caused abnormal ABR thresholds and amplitudes. A diet deficient in ω-3 FA caused similar but less dramatic effects. Our study's findings have important health implications. Current United State consumption of ω-3 FA is lower than national and international recommendations [1] and large amounts of ω-3 FA are being consumed voluntarily [25,26,48,51,58,68] and being given as treatment for preterm birth [46,49,50,60,65]. Our results and those from other laboratories indicate that both excess and deficient amounts of dietary ω-3 FA during pregnancy and lactation can cause postnatal growth retardation as well as sensory and neurological abnormalities in the offspring.

Thus, consuming or administering large amounts of ω-3 FA to pregnant women or nursing infants seems inadvisable. Others have similarly stressed caution in the over-use of fish oil and other dietary sources of ω-3 FA by pregnant and lactating women [3,10,25,37,68]. In contrast to earlier studies, recent literature reviews have failed to find convincing evidence that prenatal fish oil treatments improve pregnancy outcome [42] and failed to find that postnatal ω-3 FA supplementation during nursing/bottle feeding has lasting effects on infant brain and visual function beyond 1–2 years of age [27,64]. In light of such findings, we conclude that using large amounts of ω-3 FA during pregnancy and lactation has little merit, is potentially harmful to the offspring and should not be advocated in clinical or public practice. Instead, more research is needed to determine: (A) how much perinatal ω-3 FA is too much, too little and just right for the developing human and (B) the long-term consequences in regards to the fetal programming of neurodevelopmental and sensory impairments and the adult-onset diseases of hypertension, diabetes, age-related neural degeneration and a shortened life span [4,22,39].

Acknowledgements

This project was supported by grants from the Gerber Foundation and the National Institute of General Medical Sciences (GM58905), neither of which contributed to the design, analyses or writing of this study.

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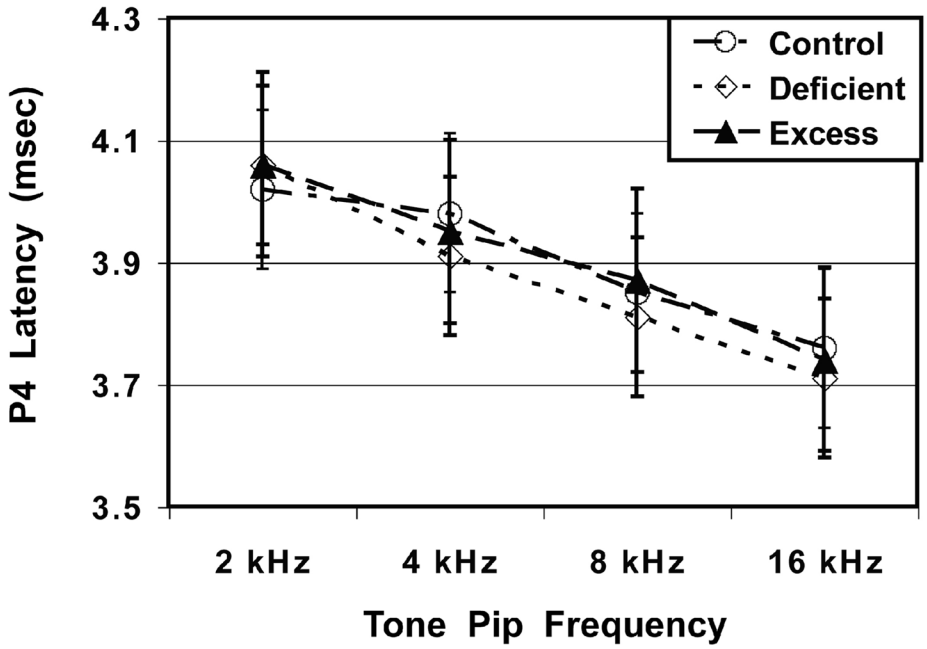

Fig. 1.

Mean (± SEM) latencies of the ABR's P4 waveform as functions of Diet group and Tone Pip Frequency. The ω-3 FA Deficiency and Excess diet conditions did not influence P4 latencies in young adult offspring.

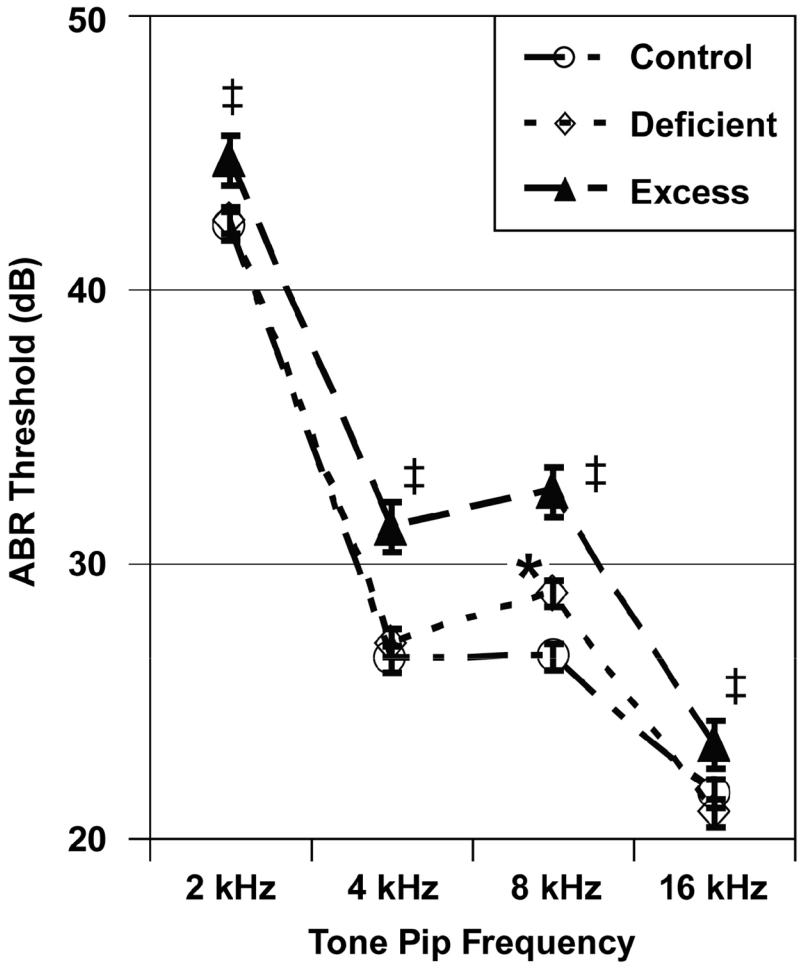

Fig. 2.

Mean (± SEM) ABR thresholds as functions of Diet group and Tone Pip Frequency. The Excess group had elevated thresholds in comparison to the Control and Deficient groups at all tone pip frequencies. The Deficient group had a significantly elevated threshold in comparison to the Control group only at 8 kHz. Footnotes: ‡ Different from Control and Deficient groups ($p < 0.05$); * Different from Control group ($p < 0.05$).

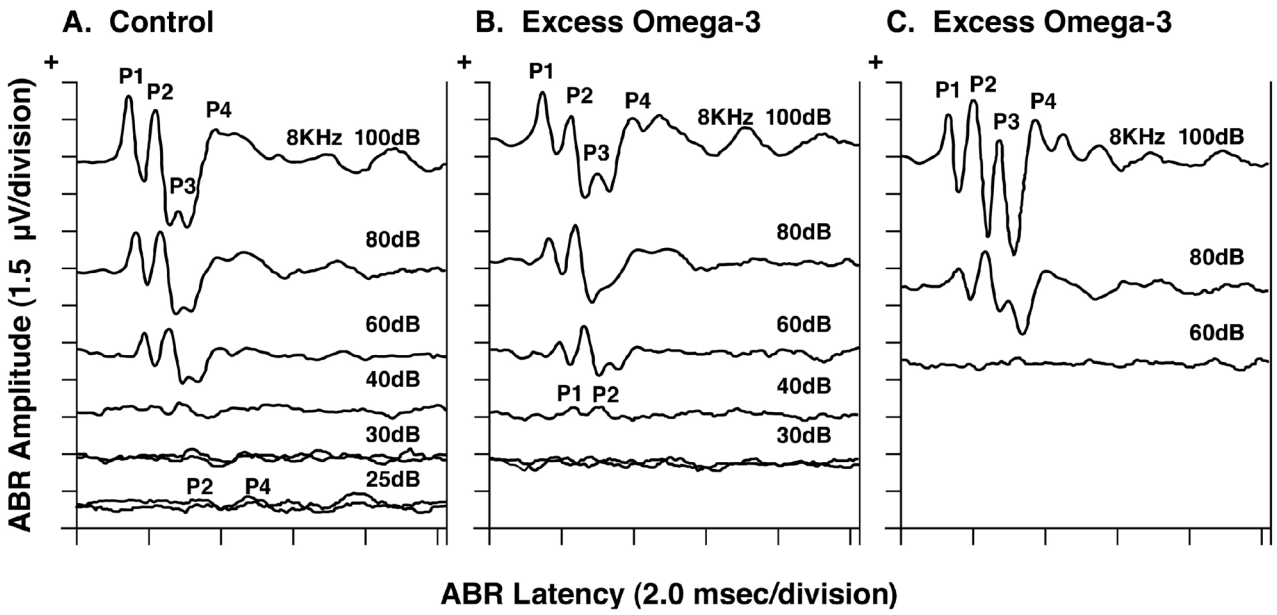

Fig. 3.

Serial ABRs in response to 8 kHz tone pips of descending stimulus intensity from representative young adult offspring in the Control and the ω-3 FA Excess diet groups. The Control animal (panel A) had an ABR still present at 25 dB, whereas the first Excess animal had an ABR at 40 dB but not at 30 dB (panel B) and the second Excess animal did not have an ABR even at 60 dB (panel C).

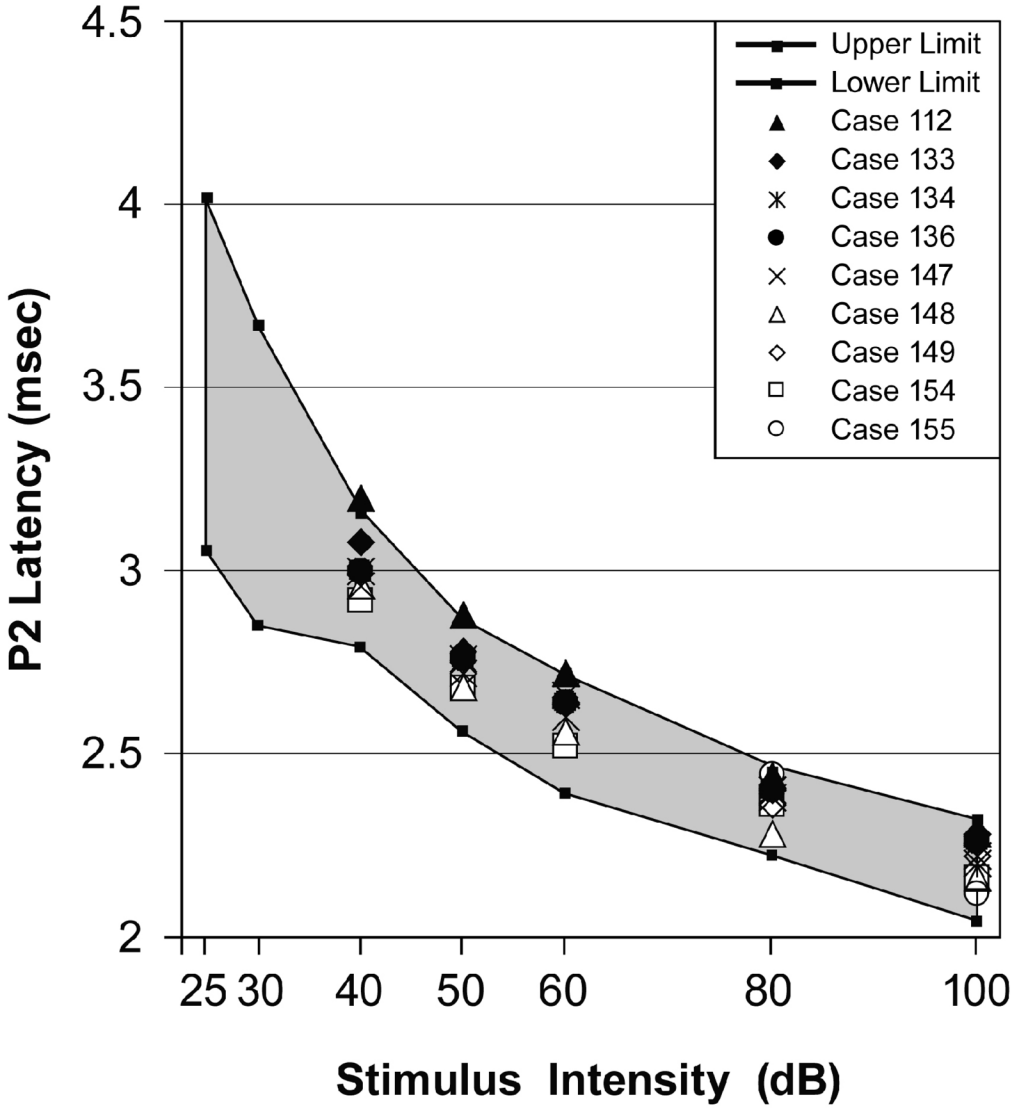

Fig. 4.

ABR latency-intensity (L-I) profiles for each Excess animal that had an elevated ABR threshold in response to the 8 kHz tone pip condition. The shaded region is the range of normalcy for the P2 wave's latency (mean ± 2 SD) as derived from the Control group data. Each of these Excess animals had L-I profiles that fell within the range of normalcy with the exception that they did not have ABRs below 40 dB.

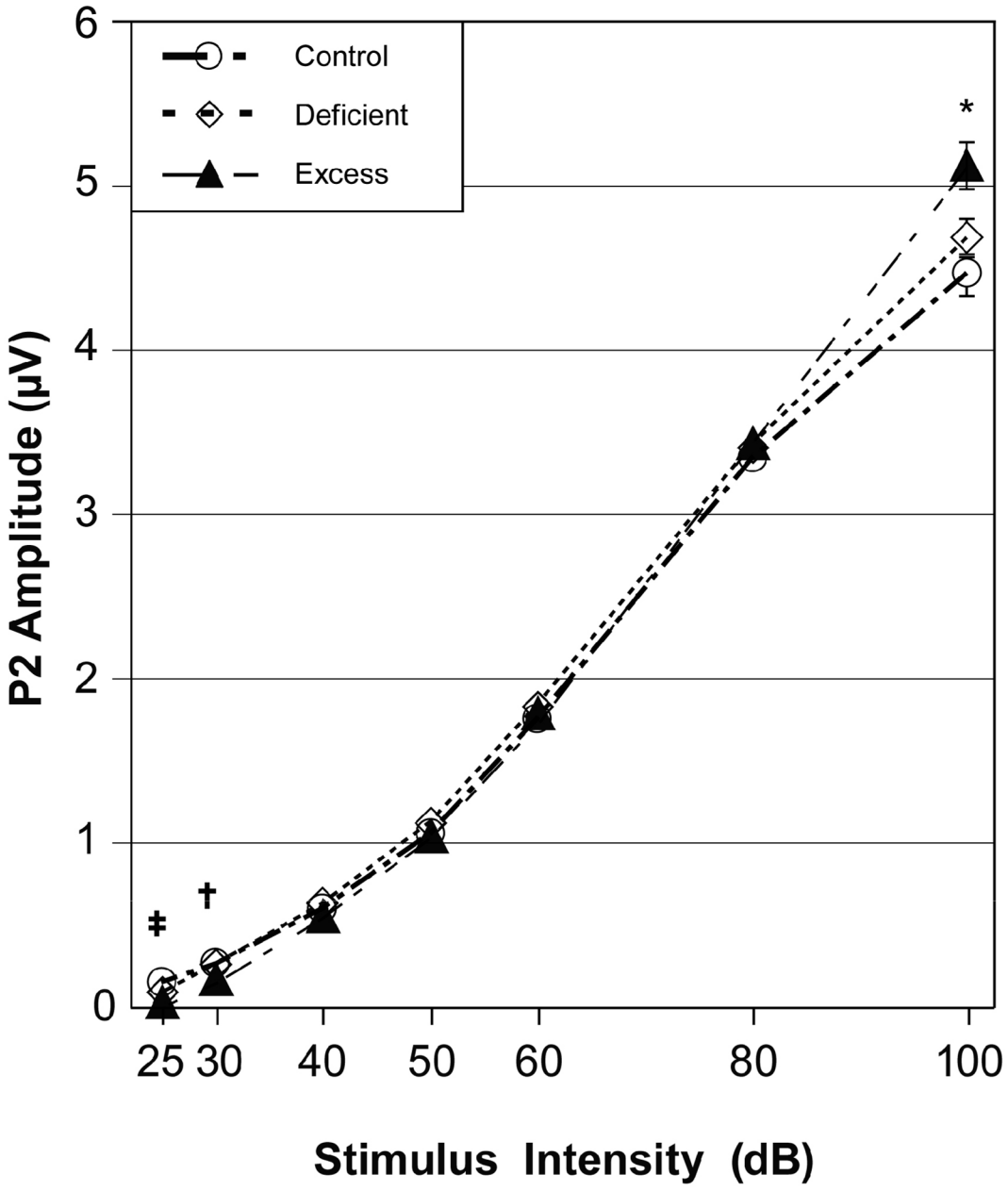

Fig. 5.

ABR amplitude-intensity (A-I) profiles in response to the 8 kHz tone pip condition for the Control, Deficient and Excess diet groups (mean ± SEM). Footnotes: *Excess greater than Control group ($p < 0.05$); † Excess smaller than Control and Deficient groups ($p < 0.05$); ‡ Excess smaller than Control group ($p < 0.05$).

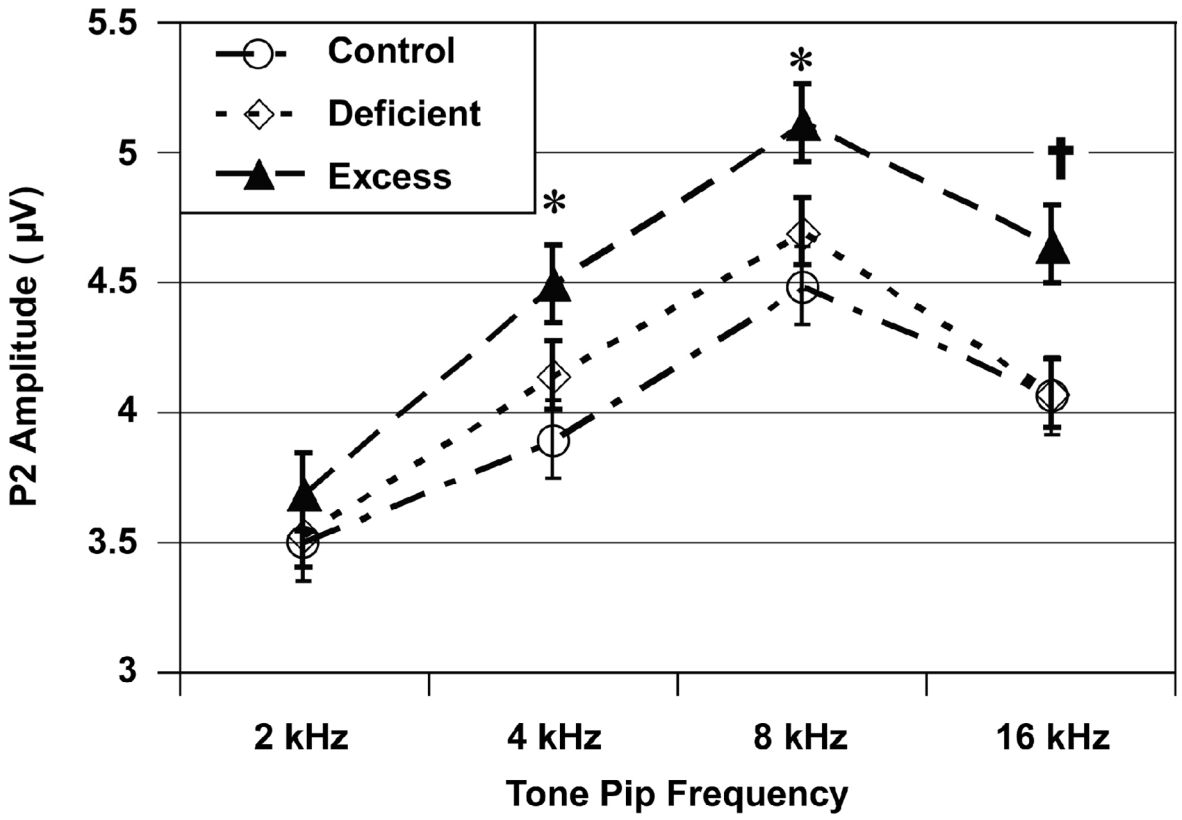

Fig. 6.

ABR's P2-N2 amplitudes as functions of Diet group and Tone Pip Frequency for the 100 dB stimulus intensity condition (mean ± SEM). Footnotes: *Excess greater than Control group ($p < 0.05$); † Excess greater than Control and Deficient groups ($p < 0.05$).