



Auditory evoked potential in handled and non-handled iron-deficient rats

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Abstract

Iron deficiency alters metabolism, neurotransmission, glial integrity and the cortical myelin layer, besides increasing myelination time. Environmental stimulation (handling) improves morphological, biochemical, electrophysiological and behavioral aspects of both well-nourished and malnourished animals. The objective of the present study was to determine the effects of an iron-deficient diet and of handling on the brainstem auditory evoked potential (BAEP) of rats during development. Ninety-six male rats were divided since birth into Well-nourished (W, 35 mg iron/kg) and Anemic (A, 4 mg iron/kg) groups, and subdivided into Handling (H) and No Handling (NH). Body weight, hemoglobin (Hb), hematocrit (Ht), latencies of waves I, II, III IV, I-IV interpeak interval, and response threshold to auditory stimuli were evaluated at 18, 22, and 32 days. W animals presented higher Hb and Ht levels than A animals at 18, 22 and 32 days. The animals presented longer latencies of waves I, II, III and IV and I-IV interpeak interval of BAEP at 18 than at 22 and 32 days, and AH18 rats presented longer latencies of waves I and II than AH22 and AH32 rats, and longer wave I latency than WH18 animals. Iron deficiency increased the latencies of BAEP waves, suggesting damage to the myelin layer, especially during the early development, and the effects of handling were more evident along time in anemic animals. **Keywords:** iron deficiency, brainstem auditory evoked potential, handling, wave latency.

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Introduction

The idea of investigating the auditory evoked potentials of iron-deficient animals was based on an initial study that showed the effects of malnutrition (reduced amount of protein in the diet) on the brainstem auditory evoked potentials (BAEPs) of rats during development. The study was conducted in the laboratory of Nutrition and Behavior at the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, USP, under the direction of Prof. Luiz Marcellino de Oliveira (Rocinholi, De Oliveira & Colafêmia, 2001a, 2001b).

Both malnutrition and iron deficiency can produce changes in the myelin layer of different cortical brain regions and in the entire telencephalon (Felt et al., 2006; Wu et al., 2008). Although iron is detected throughout the rat brain, it is mainly located in the lateral and medial globus pallidus, in the substantia nigra, dentate nucleus, as well as in the putamen, nucleus ruber, thalamus, caudate nucleus (Morris, Candy, Oakley, Bloxham, & Edwardson, 1992), hippocampus (Wu et al., 2008) and, to a lesser extent, in the striatum (François, Nguyen-Legros & Percheron, 1981). This microelement is mainly located in oligodendrocytes (Connor & Benkovic, 1992), which are the cells responsible for myelination of the central nervous system, and may also be present in the interstitial spaces and be associated with nerve cell processes (Hill & Switzer, 1984).

Iron deficiency may alter metabolism, neurotransmission and glial integrity and may also increase the myelination time of the nervous system (Schmidt, Waldow, Grove, Salinas, & Georgieff, 2007) and reduce the magnitude of the acoustic startle response (Unger et al., 2006). However, the effects of protein- or iron-deficient diets on the myelin layer that envelops the specific neurons of the brainstem have not been reported in the literature.

Electrophysiological evaluation through BAEP is a means of investigating the effects of deficient diets on the brainstem,

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and some studies have shown that malnutrition can promote alterations in this area, increasing the latency of BAEP waves (Rocinho et al., 2001b). We assumed that this effect could be due to changes produced by malnutrition in the myelin layer, and since iron deficiency can also produce this type of alteration (Wu et al., 2008) we felt that it was reasonable to investigate the effects of an iron-deficient diet on the BAEP and on the response threshold to auditory stimuli.

On the other hand, environmental stimulation (handling and enriched environment) can improve the morphological (Lemaire, Lamarque, Le Moal, Piazza & Abrous, 2006; Lucion, Pereira, Winkelman, Sanvitto & Anselmo-Franci, 2003; Carughi, Carpenter, & Diamond, 1989), biochemical (Sullivan & Dufresne, 2006; Wiggins, Fuller & Enna, 1984), electrophysiological (Rocinho et al., 2001a, 2001b) and behavioral (Chapillon, Patin, Roy, Vincent, & Caston, 2002; Rocinho, Almeida & De Oliveira, 1997) aspects of adequately fed animals, of animals receiving protein-deficient diets and also of stressed animals (a situation also occurring in malnourished animals). In addition, it can also reduce the differences between control and experimental animals (malnourished and stressed) (Lemaire et al., 2006; Rocinho et al., 2001a, 2001b).

Thus, the objective of the present study was to investigate the effects of an iron-deficient diet and of handling on the BAEP of rats during development, a highly vulnerable period during which the myelin layer is still forming in the nervous system (Connor & Benkovic, 1992).

Methods

Subjects

On the day of birth, 33 litters consisting of 6 male neonates from the animal facility of the University of São Paulo in Ribeirão Preto, were obtained from a larger pool of pups and placed with a lactating female (n=198 pups). However, only 96 animals (n=8 per group) were used in this study, the remaining being used in another study of our laboratory. Lactating females were maintained on isocaloric diets until the end of lactation (21 days). The isocaloric diet, prepared according to AOAC - Association of Official Agricultural Chemists (Cunniff, 1995) recommendations, consisted of 20% casein, 0.15% choline, 0.27% salt mixture, 10% vitamin mixture, 20% cornstarch (Maizena®), 5% corn oil, 0.5% DL methionine and 49.38% glucose H₂O. Dams of the Well-nourished (W) group received the diet enriched with 35 mg iron/kg, while dams of the Anemic (A) group received diets containing 4 mg iron/kg (Felt & Lozof, 1996). After weaning, the pups were maintained on the same diet of the lactation period until 32 days of age.

The animals (n=96) were weighed on the 18th, 22nd and 32nd days of age and maintained on a 12L:12D cycle at 23-25° C. These conditions meet the standards for the care of laboratory animals as outlined in the

Guide for the Care and Use of Laboratory Animals of the National Research Council (National Research Council, 1996).

Apparatus

During the lactation period the animals were housed in polyethylene cages (41 x 33 x 16 cm) and after weaning they were housed in the plastic cages with different measure (30 x 19 x 11.5) with stainless steel lids, food troughs and glass drinking spouts. All animals were weighed with a Filizola® scale.

BAEPs were recorded using Bio-Logic-Traveller Unit 2 TDH-39 insertion earphones and platinum contact electrodes. The rectal temperature at 36.5-37.0° C was monitored with a digital clinical thermometer.

Procedure

All material used for animal housing and care was previously washed with a 30% (v/v) nitric acid solution and rinsed with deionized water in order to prevent contamination with iron present in the environment.

Handling

Half the pups in both diet groups were exposed to individual handling five days per week from birth to the test day (18th, 22nd or 32nd). Handling consisted of holding the animal in one hand and stroking its dorsal region for 3 minutes with the thumb of the other hand. The handling procedure during the lactation and post-weaning period has been described by Rocinho et al. (2001b). The following groups were studied (n=8 per group): Well-nourished No handling (WN), Well-nourished Handling (WH), Anemic No Handling (AN), and Anemic Handling (AH). Independent rat groups were tested on the 18th (WN18, WH18, AN18 and AH18), 22nd (WN22, WH22, AN22 and AH22) and 32nd (WN32, WH32, AN32 and AH32) days of age.

Hematology

The determinations were performed in the Laboratory of Clinical Analyses of the Faculty of Pharmaceutical Sciences of Ribeirão Preto, USP. A 1 ml blood sample was collected from each pup by cardiac puncture using a 10% aqueous solution of potassium ethylene diaminetetraacetate as anticoagulant. Hematologic determinations were performed on the same day of collection in order to assess the following parameters: hemoglobin concentration by the cyanomethemoglobin method and hematocrit by the microhematocrit method (Simmons, Ng, Harker & Hockaday, 1989).

Brainstem Auditory Evoked Potentials (BAEPs)

The animals were anesthetized intraperitoneally with 40 mg/kg body weight pentobarbital (Jewett & Romano, 1972). The positive electrode was placed on the vertex, a ground electrode on the right ear and the recording electrode on the left ear. The rectal temperature (36.5-37° C) was monitored and the animals were placed on a hot-water bottle during the recording. BAEPs were recorded

in an acoustically and electrically isolated room. BAEPs were evoked by clicks of the rarefaction polarity and intensities of 80, 70, 60, 50, 40, 30 DB n HL or until the threshold, delivered at a rate of 21.1 clicks/second with 100 μ s of pulse duration each. Stimuli were presented through earphones (TDH-39) inserted into the auditory conduit of the animal's left ear. The wave threshold was determined as the lowest intensity able to evoke this wave. Recordings with more than 200 artifacts (noise) were excluded.

The evoked signals were then amplified and filtered with a bandpass of 150-3000 Hz, and fed into the signal processor for averaging. Each BAEP recording consisted of the average response to 1000 clicks and was taken in duplicate. The different wave peaks were identified by two independent observers taking into account the morphology of each record. Data were collected for the latencies of waves I, II, III and IV, and for the I-IV interpeak interval, as described elsewhere (Rocinho et al., 2001a, 2001b).

Statistics

Body weight, hemoglobin (Hb), hematocrit (Ht) and auditory threshold were compared by three-way ANOVA for independent measures: diet (well-nourished, malnourished), stimulation (Handling, No Handling), and age (18, 22 and 32 days). The latencies of waves I, II, III and IV and the I-IV interpeak interval were log transformed and compared by four-way ANOVA: diet (well-nourished, malnourished), stimulation (Handling, No Handling), age (18, 22 and 32 days), and intensity (80, 70, 60, 50, 40, 30 dB – waves I, II, III and IV and I-IV interpeak interval), with intensity as a repeated measure. *Post hoc* analysis of all measures was conducted by the Newman-Keuls test ($p < .05$).

Table 1. Hemoglobin (Hb) and Hematocrit (Ht) of pups tested at 18, 22 and 32 days of age (Mean \pm SEM). Groups: W (Well-nourished, Handling plus No Handling, n=16) and A (Anemic, Handling plus No Handling, n=16) on the 18th, 22nd and 32nd days of age. ANOVA and the Newman-Keuls test were used. Hb and Ht: (*) W > A on the 18th, 22nd and 32nd days of age ($p < .05$) and (#) 18 and 22 < 32 d ($p < .05$); Ht: (***) A 18th and A 22nd > A 32nd ($p < .05$).

Hb	Group W	Group A
18 Days	7.73 \pm 0.25 (*)	5.47 \pm 0.15
22 Days	7.70 \pm 0.27 (*)	5.09 \pm 0.16
32 Days(#)	11.57 \pm 0.37 (*)	4.32 \pm 0.14
Ht	Group W	Group A
18 Days	25.13 \pm 0.69 (*)	18.13 \pm 0.42
22 Days	25.00 \pm 0.94 (*)	17.00 \pm 0.60
32 Days	38.05 \pm 1.06 (*)	13.68 \pm 0.37 (***)

Results

Pup Body Weight

The ANOVA revealed an effect of diet ($F(1,81) = 126.4414$), stimulation ($F(1,81) = 5.3823$), age ($F(2,81) = 468.42$), diet x stimulation ($F(1,81) = 7.27$), diet x age ($F(2,81) = 20.26$), and diet x stimulation x age ($F(2,81) = 9.13$) on pup body weight. The *post hoc* Newman-Keuls test showed that W rats weighed more than A rats, H rats weighed more than N rats, and young animals weighed less than older animals (18 < 22 < 32 days) ($p < .05$).

Regarding the diet x stimulation interaction, the *post hoc* analysis revealed that WN and WH animals weighed more than AN and AH animals, respectively. In addition, the WH group weighed more than the WN group ($p < .05$).

The diet x stimulation x age interaction showed that groups WN and WH weighed less at 18 days than at 22 and 32 days, and also less at 22 days than at 32 days. The WN group weighed less than the WH group at 18 (37.54 x 41.77 g) and 32 (108.94 x 116.5 g) days. WN (108.94 g) and WH (116.5 g) animals weighed more than AN (72 g) and AH (73.9 g) animals at 32 days of age. WH animals weighed more than AN animals at 18 (41.77 x 36.44 g) and 22 (60 x 48.4 g) days. AN animals weighed less at 18 (41.27 g) and 22 (46.5 g) days than at 32 (72 g) days. The weight of AH animals at 18 days (36.44 g) was lower than the weight of AN animals at 18 days (41.28 g) and than the weight of AH animals at 22 (48.4 g) and 32 (73.9 g) days; AH animals weighed more at 32 than at 22 days ($p < .05$).

Hemoglobin (Hb) and Hematocrit (Ht)

ANOVA revealed an effect of diet ($F(1,75) = 469.5394$), age ($F(2,75) = 6.3157$), and diet x age interaction ($F(2,75) = 56.4509$) on Hb levels in the pups. The *post hoc* analysis showed that W rats had a higher concentration of Hb than A rats ($p < .05$). The same analysis also showed that the animals had a smaller amount of Hb at 18 and 22 days than at 32 days of age. The *post hoc* analysis of the diet x age interaction showed that W animals had a greater amount of Hb than A animals at 18, 22 and 32 days of age. Regarding the percentage of Ht, the ANOVA indicated an effect of diet ($F(1,75) = 403.62$), age ($F(2,75) = 21.54$) and diet x age interaction ($F(2,75) = 70.69$). The *post hoc* analysis showed that W rats had a higher %Ht than A rats and that the %Ht was lower at 18 and 22 days than at 32 days. Also, W rats had a lower %Ht at 18 and 22 days compared to 32 days, and a higher %Ht than A rats at 18, 22 and 32 days of age. In addition, A rats had a higher %Ht at 18 and 22 days than at 32 days of age. As the statistical analyses did not show stimulation effects on Hb ($F(1,75) = 0.2488$; $p > 0.05$) and Ht ($F(1,75) = 0.9712$; $p > 0.05$), the NH and H rats were gathered in the same diet group (W or A) (Table 1).

Auditory Evaluation

ANOVA showed effects of age on waves I ($F(2,32) = 8.23$), II ($F(2,36) = 7.17$), III ($F(2,54) = 20.29$) and IV

($F(2,26) = 28.32$); and of intensity on waves I ($F(5,190) = 420.84$), II ($F(5,180) = 339.21$), III ($F(4,21) = 278.34$) and IV ($F(5,130) = 186.35$), as well as an effect of the diet x intensity interaction for waves I ($F(5,190) = 2.79$), II ($F(5,180) = 2.57$) and IV ($F(5,13) = 3.19$), and of diet x stimulation x age interaction for waves I ($F(2,38) = 3.90$) and II ($F(2,36) = 3.90$).

Post hoc comparisons showed that the animals presented longer latencies of waves I, II, III and IV of the BAEP at 18 than at 22 and 32 days. However, the latencies were longer at 22 than at 32 days only for waves I and IV ($p < .05$). Regarding the effect of intensity, the multiple comparison test revealed that all animals presented lower latencies for waves I, III and IV at higher intensities compared to lower ones ($80 < 70 < 60 < 50 < 40 < 30\text{dB}$). For wave II, the effect of intensity was demonstrated as starting only at 70 dB (80 and $70 < 60 < 50 < 40 < 30\text{dB}$) ($p < .05$) and the diet x intensity interaction indicated the same effect in both the W and A groups ($p < .05$). In this same direction, the diet x intensity interaction revealed that W and A animals had lower latencies for waves I and IV at higher intensities compared to lower ones ($W80 < W70 < W60 < W50 < W40 < W30\text{dB}$ and $A80 < A70 < A60 < A50 < A40 < A30\text{dB}$). The *post hoc* analysis for the diet x stimulation x age interaction showed that AH18 rats had longer latencies of waves I and II than AH22 and AH32 rats, as well as a longer latency of wave I than WH18 rats ($p < .05$) (Figure 1).

ANOVA indicated effects of age ($F(2,26) = 21.43$) and intensity ($F(5,130) = 30.90$) on the I-IV interpeak interval. The *post hoc* test indicated that the interpeak intervals were greater at 18 than at 22 days, and that these two groups had greater I-IV intervals than rats tested at 32 days. The *post hoc* analysis of the intensity variable showed that all groups presented a greater I-IV interpeak interval when submitted to higher intensities (80dB) compared to lower ones (30dB) ($80 < 70 < 60 < 50 < 40 < 30\text{dB}$) ($p < .05$).

ANOVA did not show effects of diet ($F(1,83) = 0.01$; $p > .05$), stimulation ($F(1,83) = 0.01$; $p > .05$) or age ($F(2,83) = 2.08$; $p > .05$) on the threshold of the responses to auditory stimuli.

Discussion

Iron is a necessary component for red blood cell formation and for cell metabolism (Schmidt et al., 2007). Its deficiency may cause a reduction in the amount of serum iron (Cunha, van Ravenzwaay, Mellert & Kaufmann, 2008) and in hematocrit and liver iron stores (Bourque, Iqbal, Reynolds, Adams & Nakatsu, 2008), components whose deficiency may impair tissue growth and consequently reduce the body weight of individuals receiving iron-poor diets (Eseh & Zimmerberg, 2005). In the present study, the iron-deficient diet imposed during the lactation period produced a lower body weight

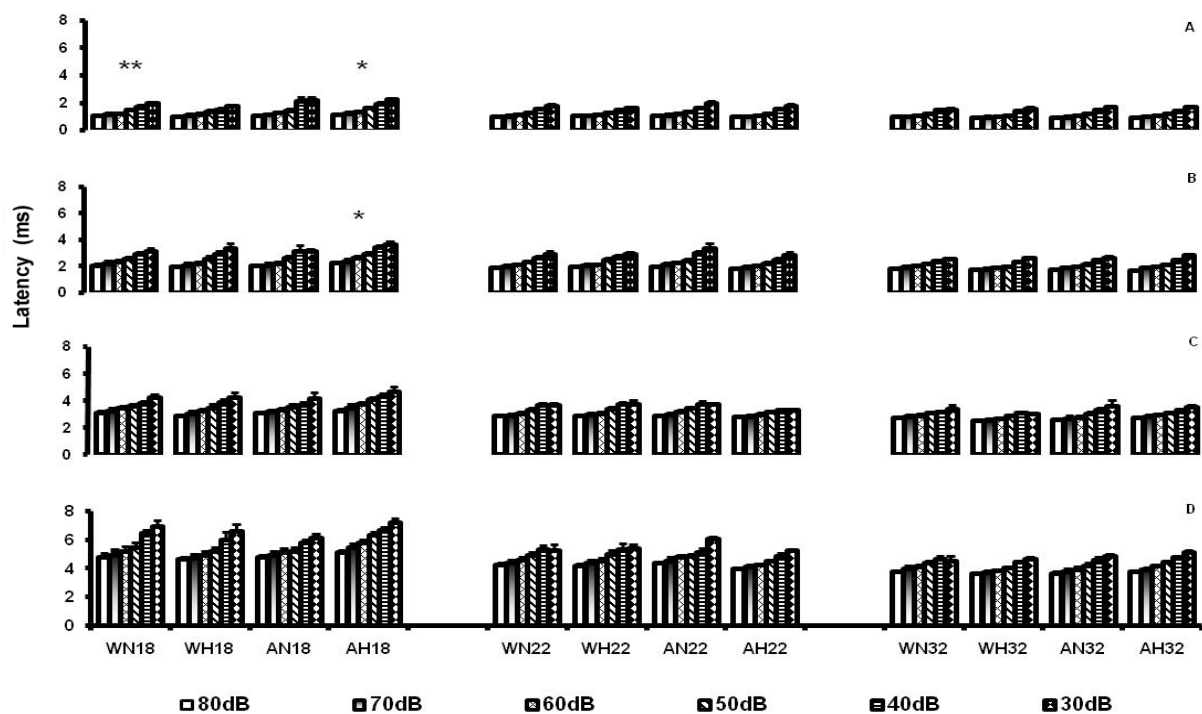


Figure 1. Latency of waves I (A), II (B), III (C) and IV (D) of the Brainstem Auditory Evoked Potential (BAEP) in response to a click intensity of 80, 70, 60, 50, 40 and 30dB. The vertical bars represent the mean \pm SEM for the groups. Groups: WN18, WN22, WN32 (Well-nourished, No Handling, $n=8$), WH18, WH22, WH32 (Well-nourished, Handling, $n=8$), AN18, AN22, AN32 (Anemic, No Handling, $n=8$) and AH18, AH22, AH32 (Anemic, Handling, $n=8$) on the 18th, 22nd and 32nd days of age. ANOVA and the Newman-Keuls test were used. Waves I and II: (*) AH18 < AH22 and AH32 (Click Intensity: 80 + 70 + 60 + 50 + 40 + 30 dB) Wave I: (**) AH18 > WH18 (Click Intensity: 80 + 70 + 60 + 50 + 40 + 30 dB).

compared to animals that received a diet with adequate amounts of iron according to the AOAC (Cunniff, 1995). These data demonstrate the importance of dietary iron for the maintenance of the quantity of cells in tissues and also of the animals' weight, in agreement with previous studies that reported a reduction of body weight in rats with iron deficiency (Eseh & Zimmerberg, 2005). In the present study, AH rats had lower body weights than AN animals at 18 days, with the effects of handling being similar to those observed in malnourished rats of the same ages (Rocinholi et al., 2001b) in which malnutrition is known to cause a reduction in the rate of cell division (Morgane, Austin-LaFrance, Bronzino, Tonkiss & Galler, 1992). However, in contrast to what was demonstrated in previous studies, WH animals had higher weights than WN animals, showing an inverse effect of handling on anemic and control animals at this age. Although the iron-deficient diet reduced the body weight of anemic rats compared to controls, revealing inadequate development, all groups presented weight gain along time.

The iron-deficient diet, in addition to reducing the body weight of group A animals, also caused lower Hb and %Ht compared to W rats of the same age (18, 22 and 32 days), demonstrating that this diet promoted a deficient nutritional condition in anemic rats, as also reported in the study by Cunha et al. (2008). Thus, it permitted the investigation of the effects of anemia on the BAEP of the rats. Although the body weight of all animals increased until the end of the experiment, only W animals showed an increase in Hb and Ht at 32 days compared to rats of 18 and 22 days of age. Anemic animals, although they gained body weight, presented lower quantities of Hb and Ht at 32 days than at 18 and 22 days of age. These results reveal that the imposition of the deficient diet up to 32 days continued to reduce the iron stores in the organism along the period evaluated, reducing Ht and Hb. However, the other components of the diet were adequate, a fact that may have provided the body weight gain, although the animals require iron for cell metabolism and growth and for the adequate development of body tissues (Schmidt et al., 2007).

BAEP recordings of the animals studied here revealed that age is an important factor that determines the maturation of the auditory pathway of the brainstem, since waves I, II, III and IV, formed by the arrival of nervous impulses to the nuclei that compose this pathway, present longer latencies in young animals (18 days) than in older ones (22 and 32 days). Since the conduction velocity of nervous impulses is directly associated with the thickness of the myelin layer and with the caliber of the axons of the neurons that compose the pathways of the nervous system, we may suggest that in the present study the younger animals were probably going through the process of myelinization, which starts at about 19 days of life and reaches the post-lactation phase (Connor & Benkovic, 1992). Thus, we may suggest that the waves have longer latencies because the nervous

impulse occurs at a lower velocity due to the fact that the myelin layer is still forming.

The analysis of the diet x stimulation x age interaction revealed that waves I and II had longer latencies in group AH at 18 days of age than at 22 and 32 days. This effect was not observed in the WH group, showing that handling reduced the effect of age in W animals and was unable to produce a similar effect in group A. Iron-deficiency can alter metabolism, neurotransmission and glial integrity and can increase the time of nervous system myelinization (Schmidt et al., 2007), while on the other hand handling can promote increased thickness of the myelin layer in the corpus callosum (Lima, 1992) and hippocampus (Iuvone, Geloso & Dell'Anna, 1996), and reduce the latency of BAEP waves in rats (Rocinholi et al., 2001b). The data suggest that the effects of handling over time became evident only in W animals since these animals have a substrate for an earlier formation of the myelin layer, a fact that does not occur in A rats due to the low amount of iron in their diet.

Since AH animals showed longer latencies of waves I and II and since these waves depend on the integrity of myelin in the system, we may assume that there was an alteration in the myelin layer of auditory nerve neurons (responsible for the formation of wave I) and of the cochlear nuclei (responsible for the formation of wave II). However, there are no reports of morphological analyses of the structures of brainstem auditory pathways of stimulated animals.

Although an increase in the latency of waves I and II was not observed in WH animals, the AH group presented greater wave I latency than the WH group at 18 days of age, showing that diet is the one factor responsible for this increase, since the myelin layer is being formed during this period of development (Connor & Benkovic, 1992), and also revealing a greater vulnerability of peripheral structures (auditory nerve) to the effects of iron deficiency, since no increase occurred in the latency of waves III and IV.

Similarly, BAEP recordings showed a shorter latency of the I-IV interpeak interval only in young animals compared to older animals, indicating the effect of age. However, no diet effect was observed, demonstrating that the brainstem was not intensely affected by the iron-deficient diet, as was the case for malnourished animals (Rocinholi et al., 2001a, 2001b).

It should also be pointed out that all BAEP waves and the I-IV interpeak interval were affected by the intensity of the auditory stimuli, whose increase produced shorter latencies in these recordings. Since auditory stimuli represent an important aspect in the expression of emotion and in species preservation by alerting an individual to dangerous environmental situations (Darwin, 1877/2000), there seems to be a biological adaptation of the auditory pathway which causes a more rapid response to intense stimuli.

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