Research article

Essential fatty acid deficiency reduces cortical spreading depression propagation in rats: a two-generation study

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Cortical spreading depression (CSD) propagation was investigated in rats under dietary essential fatty acid (EFA) deficiency over two generations (F1 and F2). Wistar rat dams received diets containing 5% fat either from coconut-oil (EFA-deficient) or soybean-oil (control). F1-pups received their dams' diets until the day of CSD recording (30–40 days or 90-100 days). F2-pups were kept on their F1 dams' diet until 30–40 days. Compared to the controls, the EFA-deficient group had reduced ($P < 0.05$) body weights in both F1 and F2 conditions. This effect was more conspicuous ($P < 0.001$) in the F2-animals where brain weight was also reduced ($P < 0.05$). All EFA-deficient groups displayed lower CSD velocities ($P < 0.001$) than the corresponding controls. Within the same dietary group and generation, F1 young rats showed higher CSD velocities ($P < 0.001$) than adults. Data show that EFA deficiency reduces CSD propagation, and this effect is long lasting as it persists up to the second generation.

Keywords: successive generations' effect, cortical spreading depression, essential fatty acids, brain development, polyunsaturated fatty acids

Introduction

Long-chain polyunsaturated fatty acids (LC-PUFAs) are synthesized in mammals from their respective dietary essential fatty acid (EFA) precursors, α-linoleic acid (18:2*n*-6) and α-linolenic acid (18:3*n*-3). They can also be obtained directly from dietary sources such as vegetable oils (corn, safflower, soyabean), eggs, breast-milk and fish. In the adult brain, approximately 35% of the total lipids are in the form of LC-PUFA, mainly arachidonic acid (AA, 20:4*n*-6) and docosahexaenoic acid (DHA, 22:6*n*-3).[1] Different organs present distinct proportions of these compounds and, in the central nervous system, the amount varies depending of the brain area. In general, linoleic, linolenic and eicosapentaenoic acids are in low concentrations while DHA and AA are found in high concentrations in neuronal membranes, mainly in the pre-frontal cortex.[2]

EFAs are important constituents of structural lipids in nervous cell membranes and, as such, are involved in many brain functions.[3] Recently, a variety of functions have been demonstrated for both α-linoleic and α-linolenic acids. Both types of fatty acids are precursors of signaling molecules with opposing effects, modulating membrane fluidity and gene expression.[4,5] In addition, they are involved in the synthesis and functions of brain neurotransmitters, and in the molecules of the immune system.[3] It has also been shown that α-linolenic acid

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deficiency possibly induces an enhanced vulnerability to stress that can affect behavioural, sensory and motor performance in rodents.[6] Although the modifications cannot be precisely related to a specific neurotransmission system, there is evidence suggesting the involvement of the mono-aminergic system.[7] Under physiological conditions, EFAs have also multiple effects on the glutamatergic system; some of these effects would be expected to favor hyperexcitability,[8,9] while others could contribute to decreased synaptic glutamate transmission and increased neuroprotection.[10–12]. Vreughdenhil *et al*.[10] showed that LC-PUFAs and their metabolites may diminish neuronal excitability by modulating ion channels, *i.e*. in the presence of DHA and eicosapentaenoic acid, both sodium and calcium currents were inactivated in the CA1 hippocampal isolated neurons. Pathological conditions in the brain (such as ischemia, trauma and seizure) are accompanied by increased levels of free n-6 and n-3 LC-PUFAs, mainly AA and DHA,[13,14] which are synthesized and released from astrocytes.[15,16] A neuroprotective role has been suggested for LC-PUFAs involving the opening of K^+ channels like TREK1 and TRAAK in the neurons whose activation would be expected to hyperpolarize synaptic terminals.[11] Moreover, it has been shown that AA and DHA have various effects on different pathways of Ca^{2+} intracellular regulation in astrocytes.[12] These LC-PUFAs inhibit store-operated Ca^{2+} entry, reduce the amplitudes of Ca^{2+} responses evoked by agonists of G protein-coupled receptors and suppress intracellular Ca^{2+} concentration oscillations. Prolonged exposure of astrocytes to AA and DHA drives the cells into a new steady state with moderately elevated intracellular Ca^{2+} concentrations, where cells become virtually insensitive to external stimuli.[12] Altogether, such mechanisms have been proposed as important neuroprotective actions of LC-PUFAs, because AA and DHA released by disturbed parts of the brain protect surrounding cells from pathological overstimulation.[12] Such data are compatible with the idea that increasing dietary consumption of LC-PUFAs could prevent epileptic discharges since these compounds easily cross the blood–brain barrier and, consequently, could decrease neuronal excitability.[10–12] The relationship between EFA dietary manipulation and neuronal excitability can be experimentally studied by using the electrophysiological phenomenon known as cortical spreading depression (CSD).

CSD is characterized by a reduction of spontaneous electrical brain activity evoked by mechanical, electrical or chemical cortical stimulation. During the initial phase of CSD, a burst of neuronal electrical activity, similar to that found in epileptic EEG, can occur. Following this, the spontaneous brain electrical activity is depressed and this EEG depression spreads slowly all over the brain cortical surface.[17] This phenomenon is reversible and is characterized by particular ionic, metabolic and hemodynamic changes.[18] It is dependent of neuron–glia interactions and can be affected by several conditions including nutritional[19,20] and pharmacological[21–23] manipulations. There are several reports showing that CSD seems to be involved in various pathophysiological events including ischemia,[24] migraine,[25] and epilepsy.[26]

In this study, we used CSD as a neurophysiological parameter to investigate, in the young and in the adult rat, the long-lasting effects over two generations (F1 and F2) fed a diet deficient in both α-linolenic and α-linoleic acid. The present two-generation study is based on the evidence from others that such long-lasting treatment seems to be required to induce the brain DHA and AA decline necessary (around 50–80%) to induce more severe changes in neural function.[27] In addition, we also evaluated CSD propagation at a young and an adult age (respectively, 30–40 days and 90–100 days of life) since age is related to regional changes in fatty acid composition of brain phospholipids.[2]

Materials and methods

Animals and diets

Female Wistar rats from the colony in our department received one of the two following diets at mating: (i) the experimental diet containing 5% fat as coconut oil (Rhoster®), which is specifically deficient in both α-linolenic and α-linoleic acid (EFA-deficient); or (ii) the standard diet (control) containing 5% fat as soybean oil, which provided normal amounts of the EFA. Both diets were prepared according to Soares *et al*.[28] and were balanced in all nutrients, except for the lipid source. After weaning, all the pups were raised on their respective dams' diets. They constituted the so-called first generation group of animals. The second generation group was formed by pups born from dams of the first generation. After weaning, the pups also received the diets of their respective dams.

The animals were maintained in a room at a temperature of 23 ± 2ºC with a 12-h light:12-h dark cycle (lights on at 6:00 am) with free access to food and water. All animal procedures utilized in this study were in accordance with the *Principles of Laboratory Animal Care* (NIH, Bethesda, MD, USA) and with the norms of the Ethics Committee for Animal Research, of the Universidade Federal de Pernambuco.

CSD recordings

After birth, all the pups were weighed on days 0, 7, 14, 21 (weaning) and 30 as well as on the day of CSD recording. The CSD was recorded when the rats were

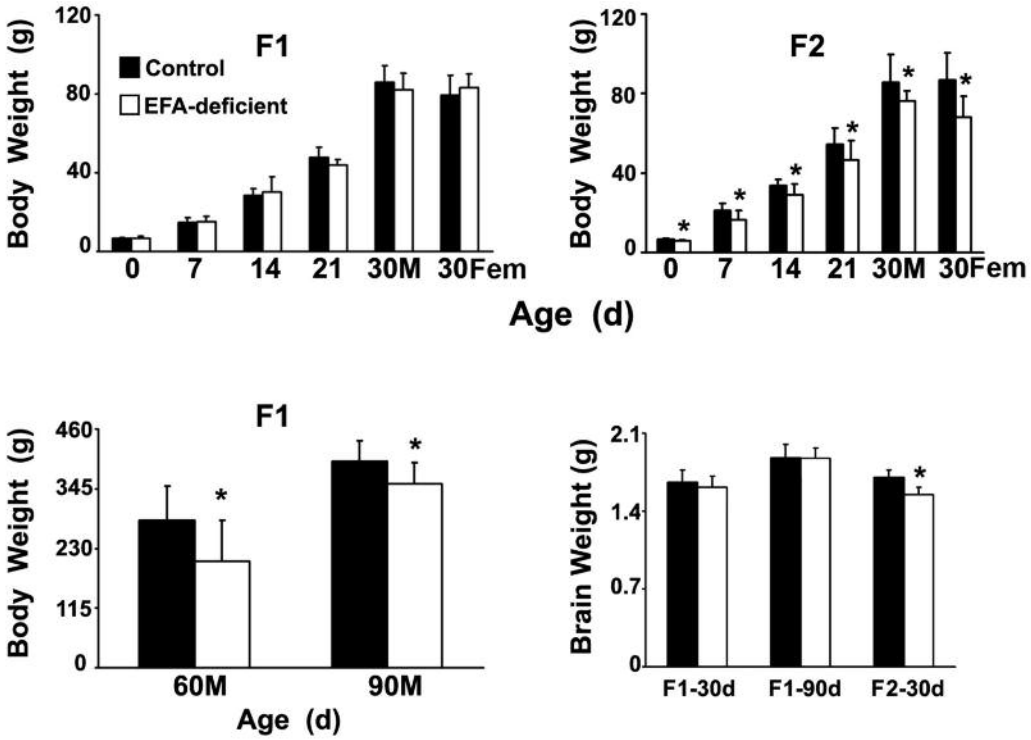

Figure 1 Body and brain weights (mean ± SD) of control and EFA-deficient rats, fed diets in which the lipid sources were, respectively, soya bean oil and coconut oil. The two upper panels compare the body weights of young animals (up to 30 days of age) in two consecutive generations (respectively, F1 and F2). The bottom-left panel shows F1 intergroup body weight differences in adult rats (60 and 90 days of age). The brain weights of young (30 days of age) F1 and F2 rats, as well as of adults (90 days) F1 animals are in the bottom-right panel. M and Fem denote, respectively, male and female animals. The asterisks indicate the EFA-deficient values that were significantly lower than the corresponding controls ($P < 0.05$; ANOVA plus Tukey test)

30–40 days-old (young rats) and at 90–100 days (adult rats) for the first generation. For the second generation, CSD was recorded only at 30–40 days of life (young rats). On the day of the electrophysiological experiment, the animals were intraperitoneally anesthetized with a mixture of 1 g/kg urethane plus 40 mg/kg chloralose. The trachea was opened and a tracheal cannula inserted, followed by the trepanation of three trephine holes on the right side of the skull. These holes were aligned in the anteroposterior direction and parallel to the midline. The first hole (2 mm diameter) was positioned on the frontal bone and was used to apply KCl to elicit CSD. The other two holes were drilled on the parietal bone (3–4 mm diameter) and were used to record the propagating CSD wave. During the recording time, rectal temperature was continuously monitored and maintained at 37 ± 1ºC by means of a heating blanket.

CSD was elicited at 20-min intervals by 1-min application of a cotton ball (1–2 mm in diameter) soaked with 2% KCl solution (approximately 0.27 M), applied to the anterior hole drilled at the frontal region. Both the slow potential change and the spontaneous cortical electrical activity (ECoG) accompanying CSD were continuously recorded for 4 h, using two Ag–AgCl agar-Ringer electrodes (one in each hole) against a common reference electrode of the same type, placed on the nasal bones. The CSD velocity of propagation was calculated from the time required for a CSD wave to pass the distance between the two cortical electrodes. After the electrophysiological recordings, the brain was immediately removed and weighed.

Statistical analysis

Data were expressed as mean values ± SD in all groups. Body and brain weights, as well as CSD propagation rates, were compared between groups by using ANOVA, followed by Tukey test, where indicated. *P*-values of less than 0.05 were considered significant.

Results

Body and brain weights

In the first generation, animals receiving the EFA-deficient diet only presented a significantly reduced ($P < 0.05$) body weight at 60 days and 90 days (205.3 ± 79.0 g and 355.1 ±

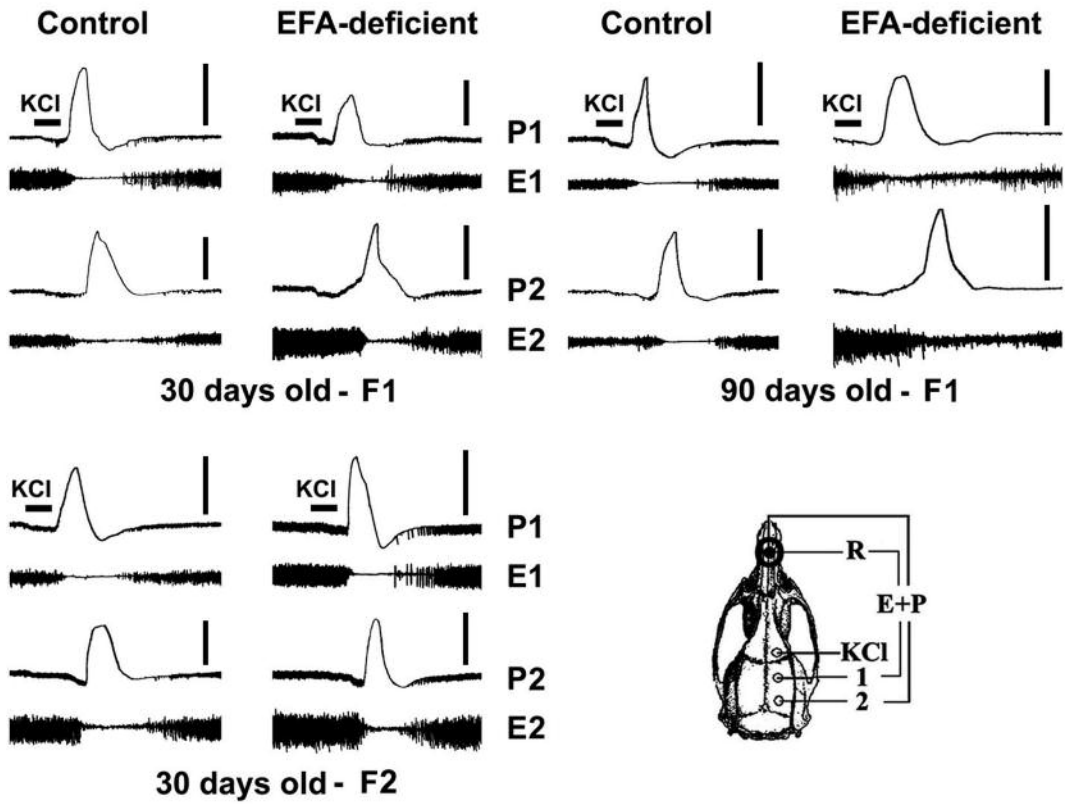

Figure 2 **Electrocorticogram (E) and slow potential change (P) recorded from two points of the right cortical parietal surface in 30-day-old (two left vertical columns) and 90-day-old (two right columns). The horizontal black bars in P1-traces indicate the period (1 min) in which stimulation with 2% KCl was applied to the frontal region to elicit CSD. The inset (bottom right) shows the recording positions 1 and 2, from which the traces marked with the same numbers were obtained. The position of the common reference electrode (R) and the application place of stimulus (KCl) are also shown. Vertical bars correspond to 10 mV in P and 1 mV in E (negative upwards)**

40.1 g, respectively), as compared with the control group (284.5 ± 65.6 g and 398.2 ± 39.5 g) as shown in Figure 1 (upper and lower left panels).

At the second generation, animals chronically fed an EFA-deficient diet showed a persistent body weight reduction ($P < 0.001$) from birth (5.69 ± 0.73 g) until 30 days of life (76.27 ± 5.01 g and 68.04 ± 10.34 g for males and females, respectively), as compared to the controls (from 6.54 ± 0.63 g at birth to 90.08 ± 10.73 g and 86.67 ± 13.61 g for 30-day-old males and females, respectively). Data are shown in Figure 1 (upper right panel).

Regarding the brain weight, rats submitted to an EFA-deficient diet for two generations presented a significant reduction at 30 days (from 1.701 ± 0.075 g to 1.548 ± 0.063 g for control and EFA-deficient, respectively). This effect was not seen at 30 days in the first generation group (1.660 ± 0.108 g and 1.616 ± 0.099 g; $P = 0.235$). Data are shown in Figure 1 (lower-right panel).

CSD velocity

Topical 2% KCl stimulation on a point in the frontal cortical surface elicited a single CSD wave. This wave propagated without interruption along the whole cortex and could be recorded (electrocorticogram and slow potential changes) by the two electrodes located at the parietal cortex. Within a few minutes after CSD had been recorded, the ECoG and the slow potential gradually returned to the pre-CSD pattern. Figure 2 presents examples of CSD recordings in control and EFA-deficient animals of both ages.

The effect of EFA-deficient diet on the CSD propagation can be seen in Figure 3, which shows the mean CSD-velocities of propagation for all groups during the 4 h of recording. F1–rats receiving the EFA-deficient diet displayed significantly lower ($P < 0.001$) CSD velocities (3.29 ± 0.10 mm/min and 3.03 ± 0.13 mm/min, for the young and adult groups, respectively) as compared to the corresponding control groups (3.73 ± 0.11 mm/min and 3.36 ± 0.09 mm/min). Furthermore, in the F1-rats, comparison of the two age-groups revealed that adult animals displayed lower CSD propagation velocities than the corresponding young animals ($P < 0.001$; two way ANOVA). The EFA-deficiency effect on CSD propagation persisted in the second generation young rats

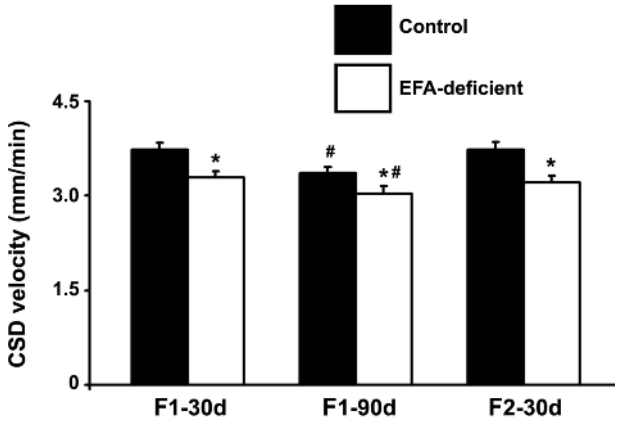

Figure 3 CSD velocities in F1 30- and 90-day-old rats, as well as in F2 30-day-old animals fed a control or an EFA-deficient diet in which the lipid sources were, respectively, soya bean oil and coconut oil. Values are mean ± SD. Asterisks indicate that all EFA-deficient rat values are significantly lower than the corresponding control values. The symbol # in the 90-day-old rats show that their velocities are significantly lower than those of the respective 30-day-old groups ($P < 0.05$; ANOVA plus Tukey test)

(control, 3.74 ± 0.12 mm/min; EFA-deficient 3.21 ± 0.10 mm/min; $P < 0.001$).

Discussion

The main finding of the present study was that chronic EFA-deficiency reduced cortical spreading depression (CSD) susceptibility, as indexed by its lower propagation velocities. This effect was seen in both rat generations investigated.

A number of experimental data obtained from our laboratory have shown that several clinically relevant conditions can interfere with CSD elicitation and/or propagation.[20] For example, improving the nutritional status lowers CSD propagation, whereas under protein deficiency CSD propagates with a faster velocity.[29–31] In our study, although both control and EFA-deficient diets were balanced in all other nutrients, the EFA-deficient diet reduced body and brain weights as well as the CSD propagation velocity, probably due to the specific absence of essential α-linolenic and α-linoleic acids. Xiao *et al.*[2] have shown that the second generation rat offspring raised on the omega-3 deficient diet have cortical phospholipid DHA contents 75% lower than those raised on the control diet. In the present experiments, we did not measure cortical phospholipid DHA and AA levels. However, considering that our dietary model for two generations included α-linolenic as well α-linoleic acid deficiency, we could speculate that similar levels of deficiency in cortical DHA and AA might be detected. This hypothesis needs to be confirmed in future experiments.

It is already documented that neurotransmitter systems, such as the serotoninergic[32] and dopaminergic[33,34] systems, can be affected by EFA-deficiency. Kodas *et al.*[32] proposed that chronic α-linolenic deficiency could increase the release of serotonin, and also decrease its re-uptake and degradation in the synaptic cleft. On the other hand, high levels in PUFAs from the omega-6 and omega-3 family can affect physiological properties of 5-HT receptors in the prefrontal cortex.[35] In agreement with these findings, our data support the possibility that serotoninergic neurotransmission could be increased in the cortex of EFA-deficient rats, since it has been shown that serotoninergic system activation exerts an antagonistic effect on the CSD phenomenon.[22,36] Therefore, an increased extracellular concentration of serotonin would explain our finding of reduced CSD propagation in the EFA-deficient animals.

In addition to the effect of serotonin on their specific receptors, an alternative mechanism that could explain the antagonistic role of serotonin on CSD would be via its action on the *N*-methyl-D-aspartate (NMDA) receptors. Experimental evidence shows that pharmacological stimulation of the serotoninergic system can structurally change NMDA receptor by affecting its pharmacological and physiological properties.[37] Furthermore, it seems that NMDA receptors play an important role in the initiation, propagation and duration of CSD since NMDA antagonists can impair those CSD features.[38–40] Electrophysiological recordings in acutely dissociated cortical cells also have indicated that, under physiological conditions, a direct excitatory effect of DHA and AA occurs on the NMDA receptor, increasing the excitability of cortical neurons.[8,9]

A number of studies in experimental animals have shown that plasma LC-PUFAs, either obtained directly from the diet or synthesized in the liver from their precursors, are the main source for the brain.[41,42] However, other studies show that both cerebral endothelium and astrocytes avidly elongate and desaturate precursors of the LC-PUFAs. AA and DHA are released from these cells supplying the neurons which are unable to carry out fatty acid desaturation.[15] Such release can be increased by activation of astroglial serotonin receptors and it has been suggested that the release of AA and DHA in response to serotonin may represent a mechanism through which astroglia provides these LC-PUFAs to neurons.[43]

It has been shown that the synthesis of DHA in the brain may be regulated by the availability of DHA or other LC–PUFAs in the brain tissue or cerebral circulation.[44,45] According to these authors, there is an inverse relationship between EFA levels in the diet and

DHA synthesis in the brain. Some pathophysiological conditions, such as ischemia and seizure where CSD seems to be involved, are accompanied by increased levels of free AA and DHA[13,14] released from astrocytes.[15,16] It has been speculated that such release could reduce the cortical excitability, especially via K^+ channel activation in neurons and suppressing intracellular Ca^{2+} concentration oscillations in astrocytes, as potential mechanisms of neuroprotection.[11,12] Although, in the present study, we did not analyze the amount of AA and DHA released in the brain, we can not discard the possibility that a fine balance in the neurochemical interactions between astrocytes and neurons, especially those involving serotonin, glutamate and ionic mobilization, could be involved in the effects induced by the EFA deficiency upon CSD propagation. Our data seem to be also consistent with potential modifications in the electrophysiological properties of plasma membranes that could be established since the growth spurt period of brain development in the young animals of F1. In accordance with this, it should be mentioned that a recent study described that relative power of fast activities in the EEG recorded from α–linolenic acid deficient rats was significantly lower than that in the rats receiving adequate DHA amounts during the lactation period of the F1 generation.[46]

Another possibility that could explain the effect of the EFA-deficient diet used in this study in reducing CSD velocity would be based on the impairment of the cerebral blood flow (CBF). The first description of CSD demonstrated dramatic changes in the tone of the cortical resistance vasculature, *i.e*. a transient dilation of pial arterioles was noticed.[47] The mechanisms responsible for regulating the cerebral circulation during CSD involve cerebral blood vessels, astroglia, neurons and perivascular nerves as functionally inter-related components of the neurovascular unit.[48] These components may, directly or secondarily, modulate cerebral blood flow through releasing neurotransmitters, neuronal and astroglial-derived factors (*e.g*. prostaglandins and thromboxane), nitric oxide, carbon monoxide, adenosine, hydrogen and potassium ions, lipoxygenase and cytochrome P-450 monooxygenase products; of note, such factors participate in the mechanisms involved in promoting and counteracting cerebral vasodilator responses consequent to CSD.[49] The role of PUFAs, particularly α-linolenic and docosahexanoic acid, in promoting vasodilation is well known[50] and this function is related to the fact that these PUFAs are potent protectors against focal and global ischemia of the brain.[11] This vasodilation seems to involve TREK-1 potassium channels, since the PUFA-mediated activation of these channels induced a robust vasodilation of the basilar arteries, where such channels are expressed.[50] Taken together, the above findings suggest that the reduction in CSD velocity could involve, at least in part, some of the effects induced by the chronic dietary PUFA deficiency in the hemodynamic changes that accompany and favor brain susceptibility to CSD.

Another important finding of the present study was the persistence of the EFA-deficiency effect on CSD propagation until the second generation offspring. The implication is that no additive mechanisms had been detected during the two-generation period where the EFA deficiency is more expressive; especially taking into account that such deficiency could induce a greater susceptibility of biological nervous membranes to stress.[51] Alternatively, if such additive effects occurred, considering that the brain weight was reduced in F2 animals, they were effective in maintaining an electrophysiological steady-state that could reduce, but did not block, the CSD propagation. On the other hand, the absence of compensatory mechanisms that could restore the CSD velocities to control levels re-inforces the importance of DHA and AA in brain development and function. The lasting CSD effect of EFA deficiency would be consistent with observations in humans showing that cognitive deficits associated with EFA deficiency occurring early-in-life persisted until high-school age.[52]

Conclusions

The data document, for the first time, an impairing effect of long-lasting EFA-deficiency on CSD propagation in the rat cortex, which persisted in the second generation EFA-deficient animals. Our data advance understanding of the mechanisms of EFA-deficiency and cerebral functional relationships. Therefore, they might be useful in shedding light on the changes in cortical excitability associated with fatty acid-dependent structural and functional neuron–glial changes involved in some neurological diseases.

Acknowledgements

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