International Journal of Developmental Neuroscience 75 (2019) 13–18

Contents lists available at ScienceDirect

International Journal of Developmental Neuroscience

journal homepage: www.elsevier.com/locate/ijdevneu

Undernourishment and recurrent seizures early in life impair Long-Term Potentiation and alter NMDAR and AMPAR expression in rat hippocampus

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ARTICLE INFO

Keywords:
Undernourishment
Seizures
NMDA receptors
Long-Term Potentiation

ABSTRACT

Undernourishment is a global issue, especially in developing countries, affecting newborns and children in a vulnerable period of brain development. Previous studies of undernourishment models suggested a relationship between undernourishment and epilepsy. The exposure to both undernourishment and recurrent seizures early in life appears to have detrimental effects on the developing brain. This study aims to investigate the neurobiological consequences of undernourishment and recurrent seizures exposure early in life, investigating Long-Term Potentiation (LTP) induction and gene expression of NMDA receptor subunits in the hippocampus during adulthood (P60). Animals were exposed to maternal deprivation protocol from P2 to P15 to control food intake in rat pups and Flurothyl-induced seizures from P7 to P10. Electrophysiological records of hippocampal slices were recorded and gene expression of NR1A, NR2A, NR2B, NR2C, NR2D and BDNF were investigated. Animals exposed to undernourishment or recurrent seizures failed to promote LTP after stimulation. Furthermore, seizure exposure early in life led to increased expression of hippocampal NR1A, NR2A, NR2B, NR2C and NR2D when compared to controls. Interestingly, when animals were exposed to undernourishment paradigm early in life, this upregulation of NDMA subunits was absent. In conclusion, our study showed impaired LTP after undernourishment and recurrent seizures early in life, together with differential expression of NDMA expression in the hippocampus during adulthood.

1. Introduction

Undernourishment is a global issue, especially in developing countries, that affects newborns and children throughout a vulnerable period of brain development. It has been shown that inadequate nutrition early in life leads to impairment in neurodevelopmental processes, promoting permanent cognitive deficits later in life [for review, see (Prado and Dewey, 2014)]. Besides that, child undernourishment is one of the main causes of mortality in underdeveloped countries (Rush, 2000).Coincidentally, one of the most prevalent brain disorder in underdeveloped countries is Epilepsy. The World Health Organization estimates that epilepsy affects around 50 million people worldwide and 80% of these individuals are located in underdeveloped regions. Epilepsy is characterized by recurrent seizures due to abnormal neuronal activity in the brain. Although undernourishment could not be considered a direct cause of epilepsy, previous studies using animal models of undernourishment suggested that there is a relationship between undernourishment and epilepsy (Simão et al., 2012). Understanding the neurobiological consequences of recurrent seizures episodes together with early undernourishment could provide valuable insights in pathophysiology of epilepsy.

The N-Methyl-D-Aspartate receptor (NMDAR) is a glutamate receptor found in nerve cells, with glutamate recognized as constituting the main excitatory neurotransmitter in the CNS. When glutamate binds to NMDAR, it allows positively charged ions to flow through the cell membrane (Furukawa et al., 2005). Calcium flux as a consequence of NMDAR activation is thought to play a major role in synaptic plasticity, an important cellular mechanism for learning and memory function (Li and Tsien, 2009). However, excessive glutamate signaling leads to an excitotoxic effect, destroying CNS neurons by exaggerated activation of excitatory receptors (Choi, 1992). The activation of NMDAR plays a critical role in the pathophysiology of several neurological disorders such as hypoxia-ischemia, trauma and epilepsy (Olney, 1990). Administration of NDMA receptor antagonists blocked the expression of seizures using different animal models such pilocarpine, kindling and electroshock (Sato et al., 1988). In the developing brain, NMDAR

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https://doi.org/10.1016/j.ijdevneu.2019.03.005
Received 25 January 2019; Received in revised form 19 March 2019; Accepted 28 March 2019
Available online 30 March 2019

receptor expression is age-dependent and the maximum time of NDMA expression representing the higher period of vulnerability, usually around the second and third week of postnatal life in rats (Monyer et al., 1994).

Our group previously showed that early undernourishment and seizures had detrimental effects on body and brain weight, as well as impaired spatial memory. Briefly, rats exposed early in life to recurrent seizures induced by Flurothyl spent less time in the target quadrant on the Morris Water Maze (Hemb et al., 2010). We also showed in another study from our group that this same model lead to altered plasticity in the dentate gyrus of hippocampus (Nunes et al., 2000). As part of an ongoing research line that seeks to characterize the neurobiological consequences of undernourishment and seizures episodes early in life on developing brain and also considering the clinical relevance of early undernourishment regarding seizures occurrences, this study aims to investigate whether an animal model of undernourishment and recurrent seizures could alter long-term potentiation and the expression of NMDAR and AMPAR in the hippocampus.

2. Materials and methods

2.1. Animals, undernourishment paradigm and seizures induction

The experiments were conducted under conditions approved by the Scientific and Research Ethics Committees of the Pontifícia Universidade Católica do Rio Grande do Sul. All experiments were performed in accordance with the National Institute of Health guide for the Care and use of Laboratory animals. Pregnant female Wistar rats from our breeding colony were maintained on a 12 h dark-light cycle with food and water Ad libitum. After delivery, each dam was housed individually with the litter. Each litter was culled to 10 pups and the day of birth was counted as P0. The entire litters were randomly divided in four groups and only male pups were selected for each test condition. The litters were randomly divided in 4 groups: N (nourished); NS (nourished + seizures), U (undernourished); US (undernourished + seizures);

2.2. Undernourishment paradigm

The undernourishment paradigm consisted of limiting the litter's access to nutrition by removing the dams from the cage starting at P2. The deprivation period was increased by 2 h per day for 6 consecutive days, from 2 h on P2 to 12 h on P7. From P8 to P15 the deprivation period remained at 12 h/day. After deprivation, the pups were housed with their respective dams. This method of food deprivation has been successfully used before with significant alterations in body weight of rats throughout the protocol (Hemb et al., 2010).

2.3. Flurothyl seizures

To provoke early recurrent seizures, we used flurothyl [bis(2,2,2-trifluoroethyl)ether] (99%), a volatile convulsive agent that rapidly stimulates the central nervous system, inducing generalized seizures (Liptáková et al., 2000). Pups were placed individually in an airtight chamber (9.38 L) and exposed to liquid flurothyl (20 mL/min constant flow rate) delivered through a syringe and dripped slowly onto filter paper, where the agent evaporates to provoke seizures. Animals were submitted to recurrent seizures - 5 exposures of flurothyl per day (1 h interexposure interval) from P7 to P10. Rats were then quickly removed from the chamber and allowed to recover in room air. Between each trial, the chamber was flushed with room air and cleaned.

2.4. Electrophysiology

When animal reached adulthood (P60), rats were euthanized using guillotine and brains were removed en bloc and stored briefly in ice-cold artificial cerebrospinal fluid (ACSF). Coronal hippocampal slices (400 µm thick) were cut with a vibro slicer (MA752, Campden Instruments, USA) and kept for at least 1 h in oxygenated (95% O2 plus 5% CO2) ACSF, at 23–25 °C. The composition of the ACSF was the following (in mM): 130 NaCl, 3.5 KCl, 1.3 NaH2PO4, 2 MgCl2, 2 CaCl2, 10 D-glucose, and 24 NaHCO3, pH 7.4.

For extracellular electrophysiological recordings, individual hippocampal slices from N, U, S and US groups (n = 6–10 slices/animal averaged to a final n of 4 per group) were transferred to a submersion-type recording chamber, which was continuously superfused with oxygenated (95% O2 plus 5% CO2) ACSF at a flow rate of 3.0 ml/min. Field excitatory postsynaptic potentials (fEPSP) were triggered by electrical stimulation of the Schaffer collaterals with constant-current pulses of 0.2 ms duration delivered every 20 s (0.05 Hz) using a differential alternating current stimulator (Isoflex M.P.I., Israel); the stimulation electrode consisted of a twisted bipolar pair of 75 µm platinum–iridium wires (A-M Systems, Carlsborg, WA, USA). Field EPSPs were recorded extracellularly on the pyramidal layer of the CA1 hippocampal region using an Axoclamp 2-B amplifier (Axon Instruments, Foster City, CA, USA). Field EPSPs were amplified and low-pass filtered at 600 Hz (Cyber Amp 320, Axon Instruments), digitized (Digidata 1322A, Axon Instruments) and recorded on a computer using the Axoscope 9.2 software (Axon Instruments). The amplitude of fEPSPs was measured using the Clampfit 9.2 software (Axon Instruments). Recorded values were normalized on a per recording basis and then plotted as the mean of 2-min time periods (6 fEPSPs) ± S.E.M. corresponding to two to four slices per experimental animal. At the beginning of each recording, an input–output (I/O) curve for the fEPSP amplitude relative to the stimulus intensity was recorded using 50 µA stepwise increases (ranging from 50 to 250 µA) until saturation of the fEPSP amplitude. Current intensity was then adjusted to evoke baseline fEPSP amplitude ranging from 50 to 60% of the maximal fEPSP amplitude obtained by the I/O curve. Baseline responses obtained to 0.05 Hz paired-pulse stimuli (0.2 ms) were recorded for 20 min before the induction of long-term potentiation (LTP). After reaching stable baseline fEPSP recordings, LTP was induced using a high-frequency stimulation (HFS) protocol consisting of four trains of 1 s duration delivered at 100 Hz frequency with an inter-train interval of 20 s. Field EPSPs were monitored for 60 min after tetanic stimuli.

2.5. Gene expression analysis

Mice brains were collected and whole hippocampus rapidly hand-free dissected on ice. After dissection, hippocampus was snap frozen in liquid nitrogen and maintained at −80 °C until analysis. Total RNA were isolated from tissue using RNA SV-Total (Promega Corporation) reagent following manufacturer's protocol and reconstituted in 20 u L of RNase-free water. The concentration of RNA was measured using Qubit 2.0 Fluorometer (Invitrogen) and cDNA from each sample was reverse transcribed using SuperScript VILO MasterMix (Life Technologies). In each reaction, 20 ng of cDNA were used quantitative real-time RT-PCR was performed using SYBR Select Master Mix (Applied Biosystems) on a Step One Plus Real-Time PCR System thermal cycler (Applied Biosystems). Each RT-qPCR were run in duplicates for each sample and repeated one time. The fold-change relative expression calculation using the ΔΔCt method with one naïve control group that serve as reference and the GAPDH Ct values as endogenous controls for mRNA analysis. The primers used for RT-qPCR analysis are: GAPDH: For: 5′-TGCCACTCAGAAGACTGTGGATG-3′, Rev: 5′-GCCTGCTTCACCACCTTCTGAT-3′; BDNF: 5′-AGCTGAGCGTGTGTGACAGT-3′, Rev: 5′-ACCCATGGGATTACACTTGG-3′; NR1A: For: 5′-CGGCTCTTGGAAGATACAGC-3′, Rev: 5′-GTGAAGTGGTCGTTGGGAGT-3′; NR2A: For: 5′-GGGCGTGTTCTACATGCTG-3′, Rev: 5′-AATGTGTACCCCATGGATGCA-3′; NR2B: For: 5′-GTGAGAGCTCCTTTGCCAAC-3′, Rev: 5′-GTCAGGGTAGAGCGACTTGC-3′ NR2C: For: 5′-CTTCTGGGGGATGGAGAGAC-3′, Rev: 5′-CTGAAAGCCAGCAGGAAGTC-3′; NR2D:For: 5′-TAGTGTCAGTGCGC

AGATCC-3′, Rev: 5′-ACCATGAACCAGACGTAGCC-3′; GRIA1: For: 5′-CGAGTTCTGCTACAAATCCCG-3′, Rev: 5′-TGTCCGTATGGCTTCATT GATG-3′; GRIA2: For: 5′−CCAAGGACTCGGGAAGTAAGG-3′, Rev: 5′−CCCCCGACAAGGATGTAGAA-3′.

2.6. Statistical analysis

All statistical analysis were performed using the SPSS 20.0 (IBM – New York, EUA) and the graphs were constructed using the Prism GraphPad 6.0 (La Jolla, EUA). To analyze the effects of undernourishment and seizures on body weight gain and LTP stimulation, a mixed-model ANOVA was performed using the different groups (4 levels) as between-subjects variable and fEPSP recording of each time interval as repeated measure variable. To analyze data from gene expression, a one-way ANOVA were used with Tukey's multiple comparisons. In all analysis, data are expressed as mean ± SEM and the level of statistical significance was set as 5%.

3. Results

Firstly, we looked at body weight gain between groups from P7 to P10. As expected, there is a significant group effect, were rats exposed to undernourishment and seizures, as well as both protocols combined, showed decreased body weight when compared to nourished rats [$F(1.733, 20.252) = 19.966$, $p < 0.001$, Fig. 1A]. The difference in body weight persisted on P21 (weaning), P30 and P45 [$F(4.115, 15.087) = 11.783$, $p < 0.001$, Fig. 1B]. When animals reached adulthood (P60), no differences were observed between groups regarding body weight. All animals recovered quickly following Flurothyl-induced seizures and one death was observed in the undernourished group and one in the Undernourished + Flurothyl group.

Secondly, we asked ourselves whether undernourishment or seizures episodes early in life could change LTP amplitude. Data from pretetanic (−18, −15, −12, −9, −6 and −3 min) and posttetanic (3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 42, 45, 48, 51 and 54 min) epochs were analyzed by Mixed-Model ANOVA to compare recordings from Nourished, Undernourished, Nourished + Seizures and Undernourished + Seizures groups. To look for differences in baseline synaptic transmission following the experimental manipulation, we analyze the data prior to LTP stimulation. No differences between groups were observed from 18 min to 3 min before stimulation [$F(4.737, 28.422) = 1.012$, $p = 0.427$]. Fig. 2 shows that rats of nourished group (controls) develop a fEPSP potentiation response of 181 ± 27% 3 min after electrical stimulation in Schaffer collaterals that last for more than 30 min, considered to be a Long-Term Potentiation (Bliss and Collingridge, 1993). However, animals exposed to undernourishment, as well as seizures episodes early in life failed to promote LTP after electrical stimulation in vitro [$F(1.733, 20.252) = 19.966$, $p < 0.001$, Fig. 2].

Regarding gene expression analysis, one-way ANOVA indicated no differences in BDNF mRNA expression [N: 1.51 ± 0.14, NS: 2.00 ± 0.21, U: 1.57 ± 0.22, US: 1.59 ± 0.02, $F(3,10) = 1.418$, $p = 0.29$, Fig. 3A]. NR1A mRNA level analysis showed a significant difference between groups [N: 0.96 ± 0.15, NS: 1.75 ± 0.07, U: 1.55 ± 0.10, US: 1.61 ± 0.13, $F(3,10) = 8.819$, $p = 0.004$, Fig. 3B]. Tukey's multiple comparison revealed that Nourished + Seizure, Undernourished and Undernourished + Seizure groups increased NR1A expression when compared to nourished group ($p = 0.003$, $p = 0.02$ and $p = 0.03$, respectively). When looking at NR2A expression, there is a significant difference between groups as well [N: 0.74 ± 0.08, NS: 1.33 ± 0.12, U: 0.83 ± 0.10, US: 0.73 ± 0.03, $F(3,10) = 7.004$, $p = 0.008$, Fig. 3C]. Tukey's post-hoc revealed that NS group had increased expression when compared to controls ($p = 0.01$) as well as compared to U and US groups ($p = 0.02$ and $p = 0.03$, respectively). Regarding NR2B expression, there is also a statistically significant difference between groups [N: 0.68 ± 0.10, NS: 1.36 ± 0.08, U: 0.97 ± 0.06, US: 0.88 ± 0.07, $F(3,10) = 11.100$, $p = 0.002$, Fig. 3D]. Multiple comparison analysis showed increased mRNA levels in NS group when compared to controls ($p = 0.01$) as well as compared to U and US groups ($p = 0.03$ and $p = 0.03$, respectively). NR2C mRNA levels analysis revealed differences between groups as well [N: 1.22 ± 0.30, NS: 3.71 ± 0.58, U: 1.57 ± 0.16, US: 1.73 ± 0.06, $F(3,10) = 9.036$, $p = 0.003$, Fig. 3E]. Tukey's multiple comparison analysis showed increased mRNA levels in NS group when compared to controls ($p = 0.004$) as well as compared to U and US groups ($p = 0.01$ and $p = 0.04$, respectively). Finally, NR2D mRNA analysis also presented differences between groups [N: 1.09 ± 0.15, NS: 3.10 ± 0.70, U: 0.92 ± 0.12, US: 0.55 ± 0.08, $F(3,10) = 9.835$, $p = 0.003$, Fig. 3F]. Multiple comparisons showed increased mRNA levels in NS group when compared to controls ($p = 0.01$) as well as compared to U and US groups ($p = 0.006$ and $p = 0.007$, respectively).

Additionally, we looked at AMPA receptor subunits GRIA1 and GRIA2 expression in the hippocampus. GRIA1 mRNA levels analysis revealed no differences between groups [N: 0.92 ± 0.11, NS: 1.18 ± 0.16, U: 1.82 ± 0.44, US: 1.37 ± 0.15, $F(3,10) = 1.940$, $p = 0.187$, Fig. 4A]. However, there is a significant difference between groups on GRIA2 mRNA levels in the hippocampus, [N: 1.28 ± 0.12, NS: 2.06 ± 0.23, U: 2.61 ± 0.25, US: 2.23 ± 0.10, $F(3,10) = 7.369$, $p = 0.007$, Fig. 4B]. Tukey's multiple comparison analysis showed increased mRNA levels in U group when compared to controls ($p = 0.004$).

4. Discussion

In this paper, we showed that using a model of undernourishment, as well as provoking recurrent seizures early in life lead to impaired LTP in the hippocampus during adulthood. LTP amplitude was significantly lower in the undernourished groups, as well as in the seizures groups.

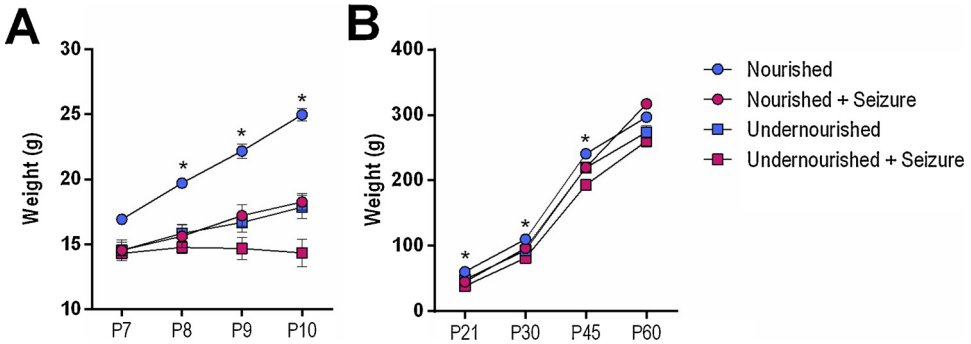

Fig. 1. Body weight changes throughout development.
A) Body weight gains from P7 to P10; B) Body weight gains from P21 to P60. Data are presented as mean ± SEM; n = 4 animals per group; * represents statistically significant difference when compared to controls ($p < 0.01$).

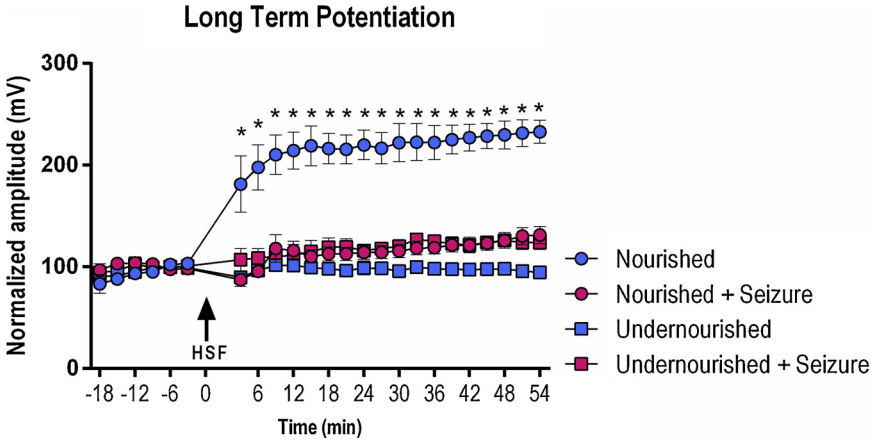

Fig. 2. Electrophysiological Records and Gene Expression Analysis.
Time-course of fEPSP recordings in hippocampal slices normalized to the pre-tetanus (HFS) amplitude after electrical stimulation in the Schaffer collaterals; Data are presented as mean ± SEM; For electrophysiological records, n = 6–10 slices/animal, 4 animals per group; * represents $p < 0.05$.

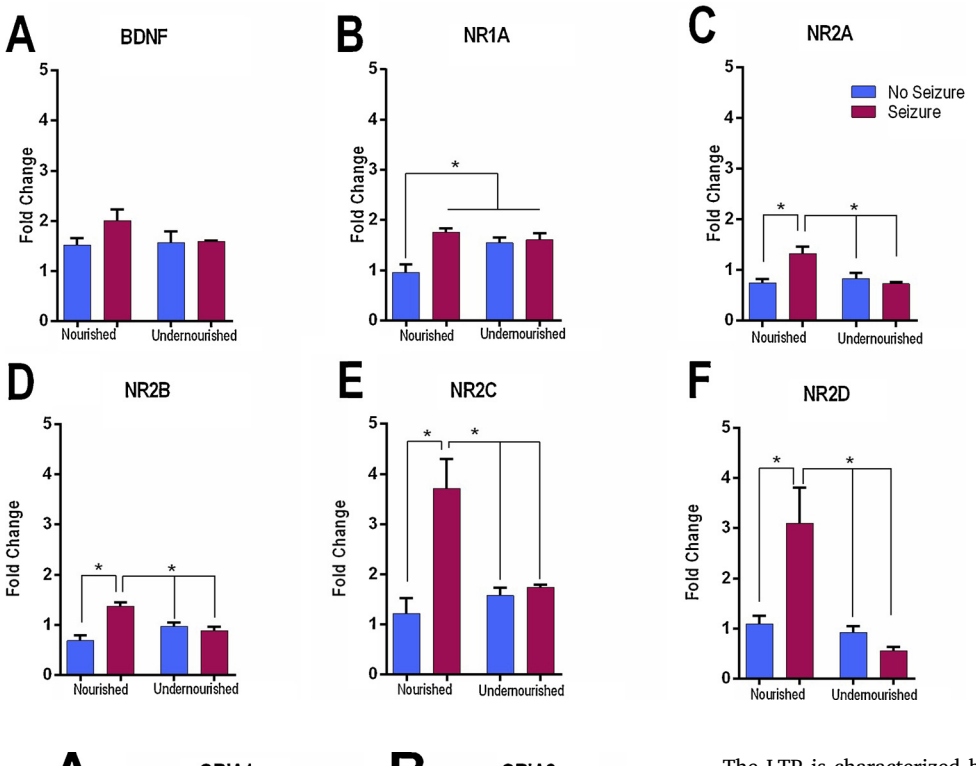

Fig. 3. Hippocampal Gene Expression of BDNF and NMDAR.
A) BDNF mRNA levels; B) NR1A mRNA levels C) NR2A mRNA levels; D) NR2B mRNA levels; E) NR2C mRNA levels and F) NR2D mRNA levels; All gene expression data were conducted in hippocampal tissue and normalized to a naïve group and using GAPDH as endogenous controls using the ΔΔcT method. Data are presented as mean ± SEM. n = 3–4 per group. * represent $p < 0.05$.

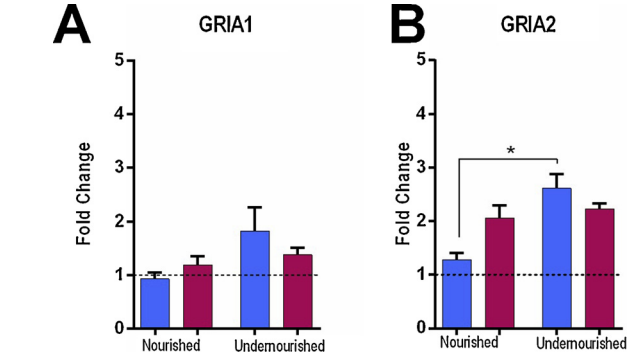

Fig. 4. Hippocampal Gene Expression of AMPAR subunits 1 and 2.
A) GRIA1 mRNA levels; B) GRIA2 mRNA levels; All gene expression data were conducted in hippocampal tissue and normalized to a naïve group and using GAPDH as endogenous controls using the ΔΔcT method. Data are presented as mean ± SEM. n = 3–4 per group. * represent $p < 0.05$.

The LTP is characterized by persistent synapsis activity between neurons and is considered one of the major cellular signaling mechanism underlying learning and memory. Our group previously showed that using the maternal deprivation model of undernourishment led to lower body weight gains between P2 to P29. In this same previously published work rats exposed to undernourishment, as well as recurrent seizures, also showed learning and memory impairments in the Morris Water Maze (Hemb et al., 2010). This set of data corroborates with previously published papers showing that rats exposed to Flurothyl from P0 to P10 or from P15 to P25 presented spatial memory deficits when compared to control rats. The effects of Flurothyl-induced seizures are apparently not specific to hippocampus. Rats exposed to Flurothyl from P0 to P10 showed alterations in pre-frontal cortex (PFC) structure, as well as behavioral flexibility, a cognitive function highly dependent of PFC functioning (Kleen et al., 2011).

Our study also showed altered gene expression of NMDA receptors in the hippocampus. We showed that seizure exposure early in life leads to increased expression of hippocampal NR1A, NR2A, NR2B, NR2C and NR2D when compared to controls. Interestingly, when animals are exposed to undernourishment paradigm early in life, this upregulation of NDMA subunits was absent. Previous studies in the literature showed

that undernourished rats are less responsive to kindling stimulation and have brain excitability affected when compared to controls rats. In addition, undernourishment also decreased several parameters of glutamatergic signaling such as decreased sensibility to quinolinic acid (an excitotoxin that act as an NMDAR agonist), decreased glutamate binding in brain synaptic membranes and decreased glutamate release (Rotta et al., 2008). It is widely known that NMDA receptors play a pivotal role in synaptic plasticity mechanisms by induction of LTP and an exaggerated activation of NMDAR promotes excitotoxic effects in neurons (Rothman and Olney, 1995). The adequate signaling of glutamate via NMDAR activation is crucial for physiological processes and the effect of undernourishment on the physiology of glutamate is still under investigated.

In addition to NMDAR, AMPAR also play an important role in both LTP and LTD. Under baseline conditions, synapses are functionally silent and lack AMPAR. During LTP, AMPAR are rapidly delivered from nonsynaptic sites to the synapse, via exocytosis-analogous mechanism of presynaptic vesicles (Isaac et al., 1995). Glutamate binding open these postsynaptic AMPAR and Na^+ flows into the cell, resulting in depolarization. Following NMDAR and AMPAR activation, one key step is the resultant activation of Ca2+/calmodulin-dependent protein kinase (CaMKII) which is an important mediator of learning and memory (Lledo et al., 1995; Yamauchi, 2005). Impaired inhibition of these previously mentioned signaling cascade and enhanced excitatory synaptic transmission (mostly due GABAa receptor activation) are among the pathomechanisms of epilepsy (Jefferys et al., 2012). Here, we used the GABAa antagonist Flurothyl to induce seizures early in life in rats exposed to undernourishment. We found no effect of Flurothyl-induced seizures and undernourishment regarding NMDAR and AMPAR gene expression. It is interesting to observe that Flurothyl blocked the increased expression of NMDAR and AMPAR (specifically GRIA2) in the hippocampus. This data could provide insights for future studies aiming to investigate Flurothyl in the perspective of finding for anesthetics, especially because GABAa receptor is the molecular target of the benzodiazepine class of tranquilizer drugs (Sigel, 2002).

It is important to mention that the effects of maternal deprivation extend beyond the amount and type of food intake. The separation of the pups from the dams on the first two weeks of postnatal life is a widely used model of early life stress, which has been shown to promote long-lasting effects in the developing brain. This model of maternal deprivation produce permanent changes in the Hypothalamic-Pituitary-Adrenal (HPA) axis, with following impacts on anxiety-like phenotype and memory (Oitzl et al., 2000; Vallee et al., 1999). Moreover, it is possible to hypothesize that glutamate signaling cascades are affected by maternal deprivation, since Roceri et al (2002) showed that this model reduced the levels of NR2A and NR2B in the hippocampus of adult rats (Roceri et al., 2002). Although the use of animal models in experimental research has provided valuable insights in neurobiological mechanisms of several pathological conditions, sometimes it may be tricky to isolate certain experimental conditions. The findings of this study should be interpreted having in mind confounds associated with maternal deprivation.

Undernourishment is a worldwide problem, especially in underdeveloped countries that present higher prevalence rates of malnutrition. The proposed relationship between seizures and undernourishment are not recent and a two-way relationship was proposed to explain this relationship, where in one hand undernourishment could contribute to the onset of epilepsy or seizures and, in the other hand, epilepsy could be responsible for malnutrition. This is, indeed, a common clinical situation where prenatal and early postnatal undernourishment (low birth weight) appears as a risk factor for the onset of seizures episode, although a clear cause-effect relationship between these two factor are not yet been stablished. However, several evidences suggest that both early undernourishment and recurrent seizures impacts the developing brain (Simão et al., 2012).

5. Conclusion

In conclusion, our study showed impaired LTP induction after undernourishment and recurrent seizures early in life, together with differential expression of NDMAR expression in the hippocampus during adulthood. It is widely known that recurrent seizures early in life can have detrimental effects on the developing brain and experimental studies aiming to investigate the neurophysiological underpinnings of seizure exposure early in life can contribute to the body of work aiming to future therapies. Although we cannot make a direct translation between laboratory animals and humans, animal models provide very interesting insights at the molecular levels that enhance our knowledge in the pathophysiology of undernourishment and recurrent seizure early in life, two important clinical factors in seizures and epilepsy.

Acknowledgements

This research was supported by the Brazilian funding agencies: Conselho Nacional de Pesquisa e Desenvolvimento (CNPq) [Grant number 306338/2017-3] and Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior – Brasil (CAPES) – Finance Code 001 and Grant 2789/2010 – Programa Nacional de Pós Doutorado (PNPD)MEC/CAPES and MCT/CNPq/FINEP.

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