Brain Research Bulletin 84 (2011) 31–38

Contents lists available at ScienceDirect

Brain Research Bulletin

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

Research report

Changes in neostriatal and hippocampal synaptic densities in perinatal asphyctic male and female young rats: Role of hypothermia

Elisa Cebral[a,*], César Fabián Loidl[b]

[a] Laboratorio de Reproducción y Fisiopatología Materno-Embrionaria, Instituto de Fisiología, Biología Molecular y Neurociencias (IFIBYNE-CONICET/FCEN, UBA), Departamento de Biodiversidad y Biología Experimental (DBBE), Facultad de Ciencias Exactas y Naturales (FCEyN), Universidad de Buenos Aires, Buenos Aires, Argentina
[b] Laboratorio de Neuropatología Experimental, Instituto de Biología Celular y Neurociencias "Prof. E. De Robertis", Facultad de Medicina, Universidad de Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 20 July 2010
Received in revised form
21 September 2010
Accepted 12 October 2010
Available online 20 October 2010

Keywords:
Postsynaptic density
Perinatal asphyxia
Rats
Neostriatum
Hippocampus
Hypothermia

ABSTRACT

Perinatal asphyxia (PA) may cause long-term neurological and psychiatric diseases. We evaluated, by ethanolic phosphotungstic acid (E-PTA) staining, whether PA affects postsynaptic densities (PSDs), ultrastructure of neostriatum and hippocampus of 45-day-old post-PA male and female rats. PA was induced by placing the uterine horns containing the fetuses in a 37 °C bath for 10, 15, 19 and 20 min and a 15 °C bath for 20 min (hypothermia). Striatal synaptic disorganization and PSDs thickness increase were evident after 10 and 19 min of PA in male and female rats, respectively, but striatal female PSDs thickness was lower than in males. These changes were associated with increments of the PSDs area in both sexes at 19 and 20 min PA. Thickness and PSDs area from hippocampal PA males was affected more negatively than in females. Intrahypoxic hypothermia was able to protect the brain from effects of PA. In conclusion, early PA affects neostriatal and hippocampal PSDs in a time and sex-dependent manner, while hypothermia during asphyxia is able to prevent synaptic changes by providing protection from damage.

1. Introduction

Perinatal asphyxia (PA) is an important cause of acute and chronic neurological disease [61] and involves a high risk of behavioral and neurological deficits, memory impairment, mental retardation and cerebral palsy or death [2,16,51]. The worst effects of this global hypoxic ischemic insult appear in the Central Nervous System (CNS) where the most vulnerable areas of damage are primarily located in the hippocampus, cerebral cortex and basal ganglia [1,11,41,46,58,62].

Although neurons, their axons and neuroglia, appear normal under light microscopic levels in early stages of age after a hypoxic–ischemic episode [6,42], at ultrastructural level neurons show alterations such as disaggregation of polyribosomes, abnormalities of the Golgi apparatus, edema in the oligodendrocytes, transformations of oligodendrocytes in astroglia and an age-related increase in the number of presynaptic boutons in neocortex [31]. Also, brains that have suffered PA have a long-lasting effect on the synaptic organization of the neostriatum as a direct result of damage to brain tissue [55]. However, no previous studies have examined the effect of PA on postsynaptic densities in striatum from brains of both young male and female rats.

On the other hand, although hippocampus is one of the principal brain regions involved in two different long-term outcomes, cognitive memory impairment and the psychiatric disorder schizophrenia [12], and can be damaged by PA, the morphology and structure of their synapses has been studied little. Some reports demonstrated that one-day-old mice exposed to global asphyxia produces, 60 days postinsult, slower increase rate of hippocampal synaptic junction number [54]. Also, it was shown that rat brains subjected to 20 min PA at an advanced age have significantly increased stress proteins and alterations in the excitatory system, which may lead to impaired neuronal transport and vesicle-trafficking [32]. Other studies indicated that hippocampal CA1 pyramidal cells are relatively sensitive to hypoxic–ischemic insults [50], showing some modifications of postsynaptic densities [25,43,53]. However, at present, no studies exist of long-term alterations of different PA times on the ultrastructural synapses of male and female striatum and hippocampus.

In our study, we used a model that induces global asphyxia during delivery which leads to increase striatial dopamine and decreased gamma-amino butyric acid (GABA) and aspartate levels [11,38,39,40]. Our study used conventional electron microscopy techniques together with E-PTA staining to study postsynaptic den-

* Corresponding author at: Laboratorio de Reproducción y Fisiopatología Materno-Embrionaria (IFIBYNE-CONICET/DBBE/UBA), 4to. Piso, Pab. II, Intendente Güiraldes 2160, Ciudad Universitaria (CP 1428), Buenos Aires, Argentina.
Tel.: +54 11 45763300x258; fax: +54 11 45763384.
E-mail addresses: elisacebral@yahoo.com.ar, cebral@bg.fcen.uba.ar (E. Cebral).

0361-9230/$ – see front matter © 2010 Published by Elsevier Inc.
doi:10.1016/j.brainresbull.2010.10.003

sities (PSDs) alterations in neostriatum and hippocampus 45 days post-PA insult.

Another point of consideration is that at present no effective treatment exists for asphyxia and the emphasis thus far has been on prevention methods. However, hypothermia has been demonstrated to be a simple and useful treatment to reduce or prevent cellular brain damage. The potent neuroprotective effect of hypothermia following cerebral hypoxic–ischemic injury was reported in various animal models. The pioneering work of Miller et al. [44] and several other authors [21,35,52] demonstrated that deep cooling is an effective treatment for ischemia. Our laboratory has also shown that hypothermia reduces the release of nitric oxide and free radicals and protects the brain against morphological and biochemical damage [5,6,7,40]. Since hypothermia has a dramatic effect on cell death, we also tested the effect of hypothermia on PSDs changes after perinatal asphyxia.

2. Materials and methods

2.1. Experimental animals

Pregnant Sprague–Dawley rats were obtained from the vivarium of the Universidad de Buenos Aires Medical School. They were placed in separated cages on day 14 of gestation and kept in a temperature-controlled ($21 \pm 2\,^{\circ}C$) and humidity ($65 \pm 5\%$) on a 12-h light/dark cycle (lights on at 7 a.m.). They were fed standard lab chow and tap water *ad libitum*. One group was used as surrogate mothers ($n = 15$) and another group was assigned to PA or PA + HYP procedures.

The Ethical Board of the Instituto de Medicina y Biología Experimental (IBYME), University of Buenos Aires Medical School, Buenos Aires, Argentina, approved all animal experimental procedures and care. Experiments were conducted according to principles stated in the Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996). All efforts were made to reduce the number of animals used and to minimize suffering.

2.2. Induction of perinatal asphyxia

Full-term pregnant rats (day 22 of gestation) were decapitated and immediately hysterectomized after their first pup delivered vaginally (vaginal control). One uterine horn, still containing the remaining fetuses, was placed in a 37 °C water bath for 10 (slight PA), 15 min (moderate PA), 19 min (subsevere PA) and 20 min (severe PA). The other horn was placed in a bath at 15 °C for 20 min (hypothermia during insult group, HYP 20 min). In this hypothermia procedure the temperature of the pups is expected to be higher than the water bath and also, we and others previously obtained a 100% survival rate with important protective effects using the same protocol. We chose 20 min as the maximum time of PA because 21 or more minutes of PA results in a survival rate lower than 3%. Pups obtained from the uteri without being included in the bath were the caesarian-delivered control group (0 min PA). Following asphyxia, the uterus horn was rapidly opened, the removed pups were stimulated to breathe by cleaning up the amniotic fluid and by tactile intermittent stimulation with pieces of medical wipes for a few minutes until regular breathing could be established. The umbilical cord was ligated and animals were left to recover for around 1 h before being given to surrogate mothers, which had delivered normally within the 48 h before the experiments. The different groups of pups were marked and mixed with the surrogate's normal litters. We kept litters of 8 pups with each surrogate mother. The percentage of mortality in the severe PA group was 80%. All pups in the control vaginal delivery group were breathing immediately after birth and all survived.

2.3. Post-asphyxia procedures

Male and female 45-day-old rats ($N = 4$–5 animals/group), were anaesthetized with 28% (w/v) chloral hydrate, 0.1 ml/100 g of body weight and perfused with 4% paraformaldehyde in phosphate buffer 0.1 M, pH 7.4 through the abdominal aorta. Brains were dissected and post-fixed in the same solution for 2 h, then immersed overnight in sucrose 5% in phosphate buffer 0.1 M, pH 7.4. Coronal sections from brain containing the dorsal neostriatum and ventral hippocampus (40 and 100 μm thick) were cut on an Oxford vibratome and recovered for electron microscopic studies.

2.4. Histology

Adjacent coronal sections from medial and lateral sectors of neostriatum from both male and female rats were collected and stained with cresyl violet. Different lines were drawn to define the exact area to be quantified. Medially a line was drawn from the dorsal tip of the left-brain side to the top of the corpus callosum. Dorsal and lateral boundaries were defined by the corpus callosum; a line drawn from the ventral tip of the lateral ventricle to the rhinal fissure was used as a ventral boundary. Laterally a line was drawn from the ventral tip of the lateral ventricle to the corpus callosum. Anterior and posterior boundaries for the striatum were set at bregma 1.6 mm and −0.8 mm. Bilateral counts of neurons (with well-defined nucleus, distinct cellular membrane and not shrunken) were performed by an observer blind to treatment conditions. The spatial distribution of cresyl violet staining neurons was counted using an image analysis system.

2.5. Electron microscopic studies

Coronal brain sections were cut at a thickness of 200 μm with a vibratome through the level of the dorsal neostriatum and post-fixed for 1 h with 4% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4. Then tissue sections from hypoxic and control animals were stained either by 1% E-PTA (3,4) or by the conventional osmium–uranium–lead method.

For conventional osmium–uranium–lead staining, sections were postfixed for 2 h in 1% osmium tetroxide in 0.1 M buffer cacodylate buffer, rinsed in distilled water and stained with 1% aqueous uranyl acetate overnight. Tissue sections were then dehydrated in an ascending series of ethanol to 100% followed by dry acetone and embedded in resin (Durcupan ACM, Fluka, Buchs, Switzerland) for 72 h at 60 °C. Sections were mounted on coverslips and neostriatum and hippocampus areas selected to obtain thin sections (80–100 μm), were mounted on regular grids and stained with lead citrate before EM observations. For conventional electron microscopy we took micrographs of synapses and the neuronal cell body.

For E-PTA staining, sections were dehydrated in an ascending series of ethanol to 100% and stained for 1 h with 1% PTA stain prepared by dissolving 0.1 g of PTA in 10 ml of 100% ethanol and adding four drops of 95% ethanol. Then sections were embedded in Durcupan ACM resin and treated as for conventional osmium–uranium–lead staining.

For E-PTA staining we focused on PSDs since this technique is used mainly to identify postsynaptic densities.

2.6. Quantitative analysis of sections

Tissue sections were cut at a thickness of 100 nm and examined and photographed at 80 keV at a magnification of 8300× with a Zeiss M109 electron microscope (Carl Zeiss Inc., Berlin, Germany). Observations were done on dorsal neostriatal and ventral hippocampal sections. For quantitative analyses, specimens were selected for quality of the conventional osmium–uranium–lead and E-PTA staining and degree of ultrastructural preservation. Samples analyzed were: controls: $n = 4$–5, 10 min PA: $n = 6$, 15 min PA: $n = 6$, 19 min PA: $n = 8$, 20 min PA: $n = 8$ and 20 min hypothermia (15 °C): $n = 10$. For each animal, five micrographs were taken of each dorsal neostriatal or ventral hippocampal section. Each negative image was digitalized on a PC computer. Using NIH Image 1.6, PSDs were first manually outlined and then maximal thickness, minimum thickness, length and total area of each PSD were determined. We only analyzed those synapses in which PSDs were visible. Between 45 and 70 PSDs per animal for each region was the selected criterion for analysis.

2.7. Statistical analysis

Results were expressed as means ± standard deviation (SD). Comparisons between each experimental group and the control group were done by two-tailed Dunnett's post hoc test. Differences between means and SD of control and experimental groups were considered significant when $p < 0.05$. All statistical analyses were done with SPSS 13.0 for Windows statistical package (SPSS Inc., Chicago, IL).

3. Results

3.1. Microscopic analysis of neostriatal sections

Staining of neostriatal sections with cresyl violet revealed clear nuclear condensation and a significant decrease of the number of neurons in dorsal striatum ($p < 0.01$) after 45 days in subsevere and severe brains of young male and female hypoxic rats (data not shown).

3.2. Modifications in neostriatal PSDs

In male and female rats, no obvious neostriatal alterations in the membranes of dendrites shafts, spines or neurons were observed in the neostriatal material stained with osmium–lead–citrate (Fig. 1A). Presynaptic terminals, presynaptic vesicles and PSDs ultrastructural organization also appeared intact (Fig. 1A). However, gross alterations were apparent in post asphyctic tissue stained with E-PTA (Fig. 1B). After PA, post-asphyctic PSDs were