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Artículo original

Effect of chronic undernutrition on postnatal expression of short-dystrophin in rat brain

Froylán M. Vargas[1,2], José Carlos Guadarrama-Olmos[1], Silvia Mariscal, Bertha Segura-Alegría[3], Ismael Jiménez-Estrada[1]

ABSTRACT

Maternal undernutrition results in impaired fetal and postnatal growth of the newborn and it is associated with increased risks of developing cardiovascular and metabolic syndrome type diseases in adulthood. Undernourished offsprings show altered electrophysiological and neurochemical properties producing behavioral, learning and memory deficits. Dystrophin protein plays an important role as part of a scaffold-signaling associated protein complex (DAPC), known to contribute to Na^+, K^+, and Ca^{2+} ion channels modulation, in muscle and brain cells. Short dystrophin of 71 kDal (Dp71) is the main form identified in neuronal and glial cells which covers most of the scaffolding functions as shown in muscle cells. The goal of this study was to analyze the postnatal expression of this Dp71 as a first approach to comprehend the many functional imbalances occurring during postnatal development in the brain from rats subjected to chronic undernutrition (CU) treatment. We found that Dp71 is present at postnatal day 7 (P7) and attains maximal level of expression at 16 days of age (P16) in the brain of pup rats born from mothers fed a control diet. In addition, Dp71 protein expression at P7 and P16 was significantly lower in brain samples of pup rats born from dams under a CU feeding conditions as compared to the values of control groups or 58±13% *vs* 24±7% and 98±2% *vs* 57±24%, respectively, and practically no significant differences were observed at P26 and P45. Our data shows that CU treatment delays expression of Dp71 in the rat brain during the first three wks postnatally. Since the DAPC contribute to the ionic homeostasis in the cell it is plausible to suggest that Dp71 expression delay causes a rise in $[Ca^{2+}]i$ that disturbs brain cells maturation, thus contributing to the physiological and neurochemical deficits observed in these CU treated rats.

Key words: dystrophin, undernourishment, brain development, rats.

Efecto crónico sobre la expresión posnatal de distrofina en el cerebro de rata

RESUMEN

La desnutrición maternal impone la restricción del crecimiento fetal y del recién nacido además de incrementar significativamente los riesgos de desarrollar enfermedades cardiovasculares y síndrome metabólico en general. Los recién nacidos de mamás desnutridas muestran propiedades electrofisiológicas y neuroquímicas anormales conduciendo a un comportamiento anómalo, y a una reducción en la capacidad de aprendizaje y de memoria. La distrofina es una proteína estructural que forma parte del complejo proteico de señalización denominado complejo de proteínas asociadas a la distrofina (DAPC, por sus siglas en inglés), que tiene un papel importante en la modulación de los canales iónicos del Na^+, K^+, y Ca^{2+} en la células musculares y del tejido cerebral. La distrofina corta de peso molecular de 71 kDal (Dp71) es la forma molecular más abundante en el cerebro de mamíferos. Es por estos motivos que decidimos analizar la expresión de la Dp71 en el cerebro de rata durante el desarrollo posnatal, tanto de ratas con alimento *ad libitum* (controles) como de ratas sometidas a una dieta restringida durante la gestación y posnatal (CU, desnutrición crónica). Observamos que Dp71 esta presente desde el día 7 posnatal (P7) y alcanza su nivel máximo de expresión a los 16 días de edad (P16) en el cerebro de ratitas nacidas de ratas controles. El análisis de la expresión de la Dp71 a los 7 y 16 días de edad en muestras cerebrales de ratitas desnutridas reveló una disminución significativa con respecto a las muestras controles de 58±13 *vs* 24±7% y de 98±2 *vs* 57±24%, respectivamente. A los 26 y 45 días de edad no se observó ninguna diferencia significativa en la expresión de Dp71 entre las muestras CU y las controles con respecto a la Dp71 observada a los 60 días de edad, considerada como el control adulto. Estos datos muestran que la desnutrición crónica retarda la expresión de la Dp71 en el cerebro de rata durante las 3 primeras semanas posnatales. Dado que la Dp71 es la proteína principal de anclaje del DAPC, es posible plantear la hipótesis de que el retardo en la expresión de la Dp71 disminuye la formación del DAPC, afectando negativamente la maduración del tejido cerebral debido quizás a un aumento en el [Ca^{2+}]i, lo cual conduce a un desarrollo fisiológico y neuroquímico deficiente en las ratas sometidas a desnutrición crónica.

Palabras clave: distrofina, rata, cerebro, desnutrición, ratas.

Maternal undernutrition results in impaired fetal and postnatal growth of the newborn and mposes increased risks of developing diseases in adult life such as behavioral, learning and memory deficits, hypertension, cardiovascular diseases, neurodegenerative disorders, and non-insulin dependent diabetes, or the metabolic syndrome type[1-4]; as shown in Duchenne muscular dystrophy (DMD) patients[5-7]. Indeed, malnourished brain preparations from rats subjected to a CU diet showed abnormal neurotransmitters and receptors levels[8-11], lower antioxidant defenses, and develop hipertension early in life[12,13]; as a result of increased levels of corticosterone during fetal and postnatal life[4]. In addition, prenatal undernourished rats showed a decrease in the strenght of LTP in the hippocampal formation throughout its life[3], which was suggested as a putative mechanism for the deficits in memory and learning in animals exposed to prenatal undernutrition[3,8]. Thus, it has been proposed that sustained imbalance in the regulation of [Ca^{2+}]i may be an important contributing factor of learning and memory deficits, altered behavior, and neuronal degeneration in patients attained by undernutrition or DMD disease[4,7,14], as evidenced, also, in Alzheimer´s disease model mice and aged rats[15,16].

Dystrophin protein is the main product of the DMD gene in mammalian cells[7]. Brain dystrophin is expressed in CNS areas involved in cognitive and motor functions, such as the hippocampus, neocortex, stiatum, and cerebellum[6,7,17]. It has been detected in neurons and glial cells and it is mostly localized in postsynaptic densities[18,19], and found to correspond with the C-terminus of the dystrophin protein of 71 kDa of size (Dp71)[20]. Dp71 is part of an elaborate associated protein complex (DAPC) in muscle and nerve cells that bridges the inner cystoskeleton (F-actin) and the extracellular matrix, through the transmembrane beta-dystroglycan glycoprotein, functioning as a major scaffold-signaling system in peripheral organs[7,21], and in the brain regulating water intake by astrocytes associated to aquaporin-4[5,22]. DPAC appears to play an important role on the molecular cascade that regulate [Ca^{2+}]i in muscle cells[21], and possibly in neuronal and glial cells during postnatal development and mature brain[19,22]. Accordingly, DAPC comprises five classes of proteins: dystroglycans, syntrophins, dystrobrevins, sarcoglycans and sarcospan, composed each by several members or isoforms which are assembled around dystrophin or its autosomal homologue utrophin[7,21]. In brain, alfa-syntrophins link the DAPC with Na^+, K^+, and Ca^{2+} ion channels[5,7,21], and possibly with the Ca^{2+}-ATPase as shown in muscle cells[23]. Nitric oxide synthase (NOS) a key modulator of the [Ca^{2+}]i homeostasis is also associated with the DAPC through the alpha-syntrophin PDZ binding domain[21,23].

Moreover, cerebellar granule cells from a dystrophin-deficient *mdx* mouse, a murine model of DMD, showed higher [Ca^{2+}]i levels as compared to normals[24], and enhanced sensitivity of hippocampal pyramidal neurons from *mdx* mice to hypoxic-induced loss of synaptic transmission[14], and, Purkinje cells of the *mdx* mouse showed altered inhibitory input due to a reduction of GABA-A receptor clusters[3,17]. Thus, the DAPC may, also, be a key component of the [Ca^{2+}]i homeostasis modulation in brain cells, such as its proposed model for muscle cells[21] since all its components and interacting signaling proteins are found in the brain[5,6,19].

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[1]Departamento Fisiología, Biofísica y Neurociencias, CINVESTAV, IPN; [2]Visiting Profesor; [3]FES-Iztacala, UNAM. Corresponding: Froylán M. Vargas Ph.D. Depto. Fisiología, Biofísica y Neurociencias. Centro de Investigación y Estudios Avanzados, IPN. Av. IPN 2508, Col. San Pedro Zacatenco. 07360 México, D. F. E-Mail: fmvargas@fisio.cinvestav.mx

Therefore, since dystrophin *mdx* mice showed higher [Ca^{2+}]i levels in cerebellar neurons as compared to wild type[7,24], and malnourished brain preparations showed altered electrophysiological properties, such as reduced hippocampal LTP, decreased ability of callosal-cortical synapses to perform temporal summation, and alterations in the excitability of the cortico-spinal tract and the spinal cord[3,6,25] due, likely, to an altered ion channels modulation, we decided to analyze and compare the expression of Dp71, in brain tissue, during postnatal development of the rat fed a normal and undernourished diets. Previous developmental and regional studies of dystrophin in CNS have shown the presence of DMD gene protein products of larger molecular weight, mainly the 400-427 and 110-140 kDa[5,7,26], without providing a clear evidence of Dp71 presence in rat brain tissue during early postnatal life[27]. Thus, since Dp71 is the main dystrophin protein form in adult rat brain it is necessary to clarify its presence during postnatal development[5,6], given its putative role in the ionic environment homeostasis in brain cells, for optimal synaptic functions[6,7,17]. This report shows a clear evidence of Dp71 protein expression in early postnatal development in rat brain, and the effect of CU on Dp71 postnatal expression in newborn rats, as judged by western blotting analysis

MATERIAL AND METHODS

Antibodies used in this study were: Polyclonal antibody (pAb) H4 against C-dystrophin which has been produced and characterized in detail[28,29]. This antibody was selected on the basis of its large and strong reactivity pattern in several tissues, from different species; and Mandra 1, an antibody that recognizes an epitope located on the 128 amino acids at the end of the C-terminal domain of the human dystrophin molecule, amino acid residues 3558 - 3684 (Sigma Chemical Co., San Louis Mo.). Actin antibody was also from Sigma Chemicals Co.

Animals: Two groups of female Wistar rats were subjected to the following feeding conditions: (a) control group (Ctrl) consisting of females rats and their offspring allowed free access of food (Lab Diet, Formulab 5008); and (b) chronic undernourished group (CU) fed with half the total amount of the food intake eaten by control animals starting three weeks before mating, during pregnancy and lactation periods[25,30]. After weaning (P21) offsprings were also fed following their corresponding dams as to have Ctrl and CU rat groups. All animals had free access to water and were housed under identical conditions of temperature (22-24 ºC) and standard 12h light (6.00 - 18.00) and 12h dark (18.00 - 6.00) cycles. After parturition, the sex and number of pups was determined and adjusted to 8 males per litter and their body weight was measured every day. All procedures were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and reviewed and approved by the CINVESTAV *Animal Care Committee* (SAGARPA NUM-062-ZOO-1999, México).

Brain tissue samples preparation: Newborn rats aged 7-45 days old were killed by upper spinal cord dislocation. Ice-cold saline solution was perfused through the third ventricle (5-10 mL) after the cave vein was opened. Brain tissue was carefully removed mid and upper brain without cerebellum, nor spinal cord. Dissected fresh whole brain tissue from three rats, either control (Ctrl) or CU male pups at postnatal 7 (P7), P16, P26 and P45 days of age, was immediatedly homogeneized in 5 volumes of cold phosphate buffered-isotonic saline solution (NaCl 0.9% plus potassium phosphate buffer 10 mM, pH 7.0, at 4ºC), or 1 gram of fresh tissue in 5 mL, using a Potter-Elvehjem with a teflon pestle (0.1 mm clearance), giving eight up and down strokes. Homogenates were mixed 1:1 vol/vol with Laemmli SDS-solubilization solution (2X-concentrated) containing: Tris-Cl 125 mM, SDS 2%, Glycerol 10 % and bromophenol blue (BioRad, 4 mg per 100 mL) at pH 6.9[31,32]. 2-Mercaptoethanol was added to the mixed samples, 50 microL per 1 mL of solubilized (4-6ºC) mixture; agitated and stand for 10 min. Solubilized samples were boiled for 5 min at 98ºC (Reactive-Therm, Pierce). Boiled samples were left 15 min at 4-6ºC, then stored at -20ºC. All procedures were performed in a cold room. Protein content was determined by Bradford´s method as described by Bio-Rad notice, using bovine serum albumin as standard.

SDS-PAGE and Western Blot Analysis: Proteins of boiled samples were defrozen and adjusted to 4.0 – 5.0 mg of protein per mL with SDS-solubilization buffer (1X -concentrated) containing 50 µL of 2-mercaptoethanol per mL. Samples were mixed and centrifuged for 1 min at 1000 rpm in an Eppendorf table centrifuge at room temperature (RT). A tiny pellet was discarded. Transparent solubilized sample was used for electrophoresis; eigthy µg of protein from original homogenate was applied per well. Sonication was used for the elimination of lightly-elastic type material whenever needed. Samples were separated on the SDS-PAGE according to Laemmli, as described previously[31,32], by using 7-5 % or 10% separating stacking gel. Acrylamide/bis-acrylamide ratio was maintained at 30/0.6 (w/w) to allow the migration of 427 kDa dystrophin and the optimal separation of Dp71 isoforms. High molecular weight standards were used for calibration (Precision Plus Protein standards, Bio-Rad); then, separated proteins were transferred to nitrocellulose membrane (BA83. Schleicher and Schuell Inc., Keene NH, USA), using a Genie blotter (Idea Scientific Co., U.S.A.), in a buffer containing Tris 12.5 mM, glycine 60 mM, SDS 0.1%, and methanol 15% (v/v). Then, the nitrocellulose membrane transfers were stained with Ponceau´s solution (Sigma Chemical). Non-specific binding sites were blocked by shaking the blot in TBS containing Tween-20, 0.05% (v/v), (TBS-T) and dry skim milk 5%, for two hours at RT; followed by 4 washes in TBS-T 10 mL for 5 min each wash at RT. Dystrophin positive proteins to specific Dp71 antibodies were immunodetected with Mandra 1 and pAb-H4 (diluted 1:1000 and 1:3000 fold, respectively, in TBS-T). These C-terminal Dp71 antibodies have been characterized previously [28]. Blots were incubated overnight with the antibody at 4ºC (or 1 hr at RT). The membranes were then washed as described above and incubated in TBS-T. Proteins were revealed by incubating the membranes with a peroxidase coupled goat anti-mouse IgG from Bio-Rad, diluted 1:5000 for monoclonal antibodies and 1:10,000 for polyclonal antibodies. Incubated nitrocellulose membranes were washed as before. The signal was revealed by enhanced chemiluminiscence (ECL reagents, Amersham, Life Sciences). Emitted light was detected by AX-Ray Film (Konica Minolta) in an X-ray exposure cassette. Immunodetection was done also, when indicated, with actin antibody, as a control for protein content in the western blot analysis procedure. The staining intensity of protein bands was determined using Kodak Digital Science 1D Image Analysis Software. In order to quantify and compare the density of the different bands for the developmental expression time course, the Dp71 band intensity for adult levels were obtained from sixty days old rats fed ad libitum, representing 100 % of Dp71 band intensity or maximum protein expression.

Data were analyzed by one-way ANOVA with Tukey tests. All data are expressed as mean values with their standard errors (four independent experiments with several immunobloting assays each).Statistical significance was achieved with P d»0.01.

RESULTS

Body weight. Postnatal body weight of pups born to dams fed with 50% of the food intake eaten by the Ctrl group was significantly lower than pups born to dams fed normal diet, containing 23.4% of protein (figure 1 A). CU rats grew at a very low rate as reported

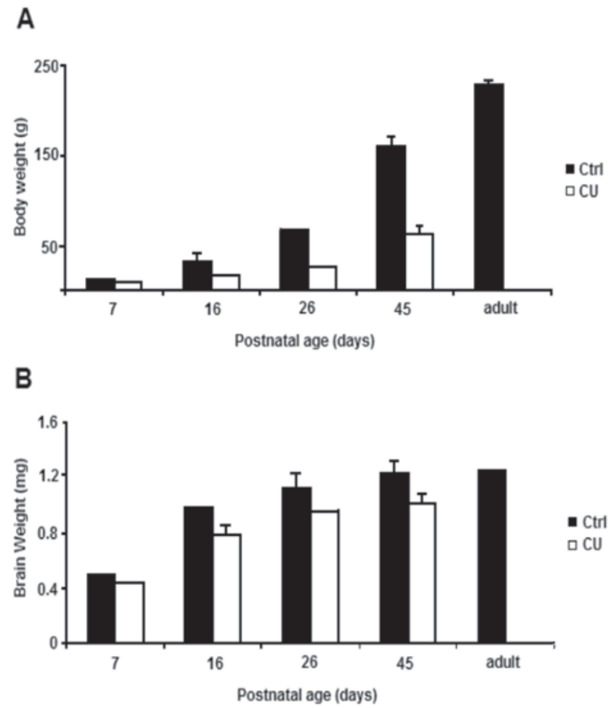

Figure 1. Body (A) and brain (B) weights of normal (Ctrl) and chronic undernourished (CU) rats of 7, 16, 26, 45 and 60 days of age. Values are means ± S.E.M., (A) p dd0.005, (B) dd0.01 p, n= 4 (n= independent experiments).

previously[25,30]. Brain weight was also significantly lower in CU animals (figure 1 B). Ctrl and CU male pups were used at random in groups of three at every chosen postnatal age: P7, P16, P26, and P45 days old. Individual differences between pups of the same group were diminished by pooling the six halves brains and preparing the homogenate accordingly. Typical immunoblotting pattern of the newborn rat brain samples is shown in figure 2A, using Mandra 1, the molecular mass of the main and single band was about 71 kDa corresponding to Dp71. Dp71 band density was maximum in adult brain samples obtained at 45 and 60 days of age. Figure 2B shows mean ± S.E.D. values of Dp71 bands at four different postnatal days from two different littermates, pooling the results obtained with Mandra1 and H4 antibodies. CU produced a significant reduction in dystrophin protein expression at 7 and 16 days of age as compared to control groups (58±13 *vs* 24±7% and 98±2% *vs* 57±24%, respectively) and this effect was minor thereafter and without significative difference at 45 days of age. Actin band intensities remained constant in all samples examined either Ctrl or CU (figure 2A). There was none significant change in actin band intensities.

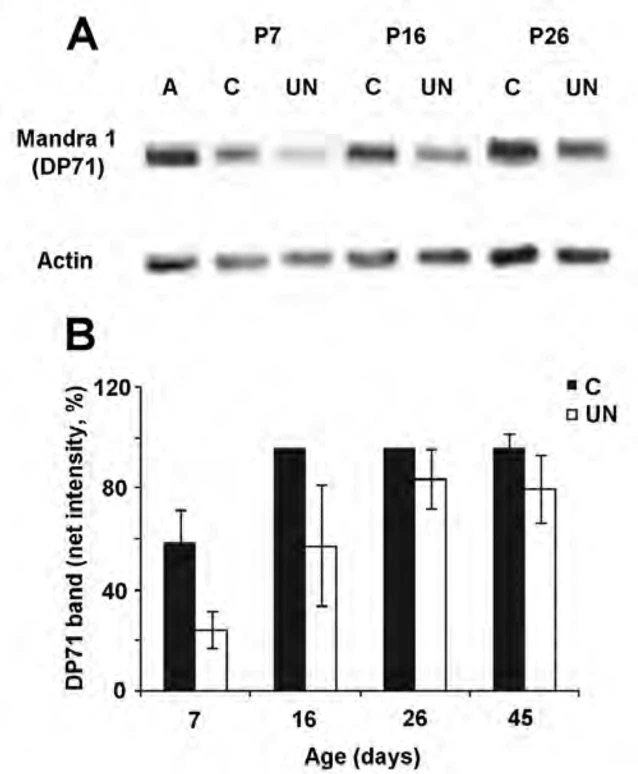

Figure 2. Immunoblotting analysis of short dystrophin, Dp71, in extracts from newborn rat brains. Rats were fed either ad libitum (control rats, Ctrl), or chronically undernourished (CU), and sacrificed at different postnatal age. **(A)** Brain samples were prepared from at P7, P16, P26, P45, and 60 days old, from newborn rats, either Ctrl or CU. Ctrl samples are lanes 2, 4, 6; CU samples are lanes 3, 5, 7. Samples of 80 ug of protein homogenate per gel-lane were developed on a 10% gel. Protein bands were detected by ECL with antibodies recognizing Dp71 (Mandra1 or H4). Dp71 band intensity for adult levels were obtained from sixty days old rats fed ad libitum, representing 100% Dp71-band density or adult Dp71 levels, line A**. (B)** Comparative postnatal semi-quantitative estimation of Dp71 expression from Ctrl and CU rat brain. Bars show the average values and standard deviation of Dp71 bands densities from Ctrl (filled bars) and CU (open bars) from five different western blot analysis using both Mandra1 and H4 antibodies, representative of 4 independent experiments, $p \leq 0.01$.

DISCUSSION

During development neural activity regulate a wide range of brain growth proceses, including glial maturation, neuronal differentiation, axon myelination, and circadian rhythms establishment[3,11,33,34]. Mature astrocytes support neuronal migration, outgrowth of axons, and production and secretion of factors that contribute to brain cytoarchitecture maturation, modulating neuronal differentiation[4,22,35], where calcium-dependent cell signaling mechanisms plays a major role in postnatal organs maturation[6,36,37].

Little is known, however, of the DAPC putative role in these developmental processes. This study presents a clear evidence of Dp71 expression during postnatal development in rat brain, and shows the effect of CU on Dp71 protein expression in newborn rat brain. Dp71 expression follows the normal brain growth pattern rather than a synaptogenesis developmental pattern as shown for other pre- and postsynaptic proteins[17,31]. CU delays the appearance of Dp71 in rat brain tissue, this effect was higher during the lactation periods, e.g. the postnatal first 3 wk, attaining young adult levels, closely, at P26 as the control rat brain tissue samples.

Nonetheless Dp71 levels are recovered in adult malnourished rat brains similar to the Dp71 expression values observed in control animals, its reduced levels during postnatal development, before weaning, may contribute to the alterations of the electrophysiological properties, abruptly diminishing synaptic plasticity, neuronal connectivity and maturation[4,8,14,22], given its role as a modulator of the ionic environment in brain cells, as main component of the DAPC in neurons and glial cells[5,21,22]. Noteworthy, mice deficient for Dp71 showed reduced levels of DAPC proteins in the brain and altered inhibitory input to Purkinje cells[38,39]. Therefore, delayed Dp71 expression during postnatal development, in the brains of CU rats, contribute to the neurochemical imbalance causing an impairment of learning and memory functions observed in neonate malnourished rodents[1,6,12,13,40,41].

Dystrophin enrichment in brain nuclei involved in cognitive and motor functions, its neuronal localization mostly in the postsynapsis[6,17,20] and in isolated postsynaptic densities[19], suggest its involvement in synaptic plasticity[6,17]. In addition, cytoskeletal disruption appears to alter Ca^{2+} channel kinetics by decreasing the ability of the DAPC to modulate DHPR and TRPC calcium channels leading to an increase of cytosolic calcium in muscle cells[21,40,42], and likely, in brain cells which would alter synaptic function[6,29,43], and water homeostasis by glial cells[22].

Undernutrition induce changes in these neuron-glia interactions diminishing neurotrophic and glial factors such as IGF-1, IGF-II[44], glial fibrillary acidic protein (GFAP), vasoactive intestinal peptide and vasopre-ssin[4,34,43], highly necessary for differentiation of astrocytes, neuronal maturation and synaptogenesis during early postnatal life in the rat[3-5,35]. Interestingly, Dp71 protein postnatal expression pattern showed in this study resembles the developmental expression of the astrocyte GFAP, which protein expression is also delayed in cortical and hypothalamic structures by undernutrition[43]. In addition, Dp71 expression favors PC12 cells differentiation induced by NGF, since Dp71 antisenseRNA blocked NGF effect, while senseRNA produced an overexpression of Dp71 promoting an increased outgrowth of neurites branches in the presence of NGF[28]. Therefore, postnatal Dp71 expression represents an important DAPC component for pursuing future studies on this scaffold-signaling protein complex involvement in ion transport modulation and brain nuclei maturation under CU and prenatal undernutrition[3,4,6,8].

In summary, CU treatment reduced Dp71 expression in rat brain during early postnatal life (P7 - P16) delaying the appearance of this important component of the DAPC in developing rat brain, contributing to the overall alterations observed in the malnourished offspring.

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