Perinatal undernourishment provokes long-lasting alterations of clusterin and fumarate hydratase expression in the rat nucleus accumbens

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Abstract

Perinatal malnutrition seems to provoke important neurochemical alterations in the brain that lead to higher vulnerability to develop neuropsychiatric disorders in the adulthood. In this work we have examined the persistence and reversibility of the changes induced by perinatal undernourishment on the expression of fumarate hydratase in the rat nucleus accumbens, bearing in mind that this expression has been previously linked with addictive disorders. Clusterin, a multifunctional protein known to be neuroprotective and possibly related to addiction in humans, was studied in parallel. Female rats under severe restriction of food during gestation, lactation and 5 months of postnatal life showed a marked upregulation of both clusterin and fumarate hydratase in the mitochondrial fraction of the nucleus accumbens, as quantified by western blot. In the case of clusterin, this upreglation was also observed in the cytosolic fraction of the nucleus accumbens. When animals were undernourished only during gestation and lactation but then switched to normal chow upon weaning, the two proteins appeared downregulated with respect to controls. The results are consistent with the idea that perinatal malnutrition provokes marked changes of brain neurochemistry that are not fully corrected by the rehabilitation of normal feeding and could be linked to behavioural disturbances in the adulthood, i.e. increased vulnerability to addiction.

Keywords: Undernutrition, nucleus accumbens, fumarate hydratase, clusterin

Introduction

Perinatal malnutrition has been shown to affect brain development and lead to behavioural disturbances in the adulthood. One of the consequences of undernourishment is a long-lasting increase in food and drug reward later in life [1-4]. This abnormal activation of reward could lead to the higher addiction vulnerability observed in animals and also in human subjects exposed to famine during gestation [5]. The neurochemical alterations underlying this condition have been examined in different animal models of perinatal malnutrition that have focused on the ventral striatum / nucleus accumbens (NAC) as a key brain area in addiction. Thus, Delta Fos B, a transcription factor closely related to drug addiction, was found upregulated in the NAC of rats undergoing protein deprivation [6]. Perinatal malnutrition affects dopaminergic and serotoninergic function in the NAC, as shown by the enhanced reactivity to 5-HT_6 receptor agonists [7] and the increased expression of the dopamine receptor D1a gene [8]. Perinatal food restriction also leads to the upregulation of fumarate hydratase (FH) in the NAC of rats, an effect that markedly affected the mitochondrial pool of the protein [9]. Bearing in mind that mitochondrial FH is involved in oxidative ATP synthesis, this finding was interpreted as a sign of metabolic activation of accumbal cells possibly linked to altered sensitivity to reward. Cytosolic FH, which is more closely related to DNA repair [10], was unaffected. Other studies have related FH expression in the NAC to both drug and food seeking in rodents [11, 12]; thus, FH density was found to correlate with the persistence of conditioned preference for environments paired to palatable food in mice [12].

In this work we have studied the persistence and reversibility of neurochemical alterations triggered by food restriction by comparing FH expression in the NAC of animals of 5 months of age that were (a) restricted during the perinatal period, (b)

restricted during both peri- and postnatal life and (c) not restricted. The prefrontal cortex (PFC) was used as a reference area since it has been observed that FH expression in this brain region is independent of reward function, unlike the NAC [13]. Besides FH, we have studied in parallel the expression of clusterin, a multifunction protein that is overexpressed and seems to play a neuroprotective role in several conditions associated to brain damage such as ischemia [14], β-amyloid deposition [15], excitotoxicity [16], trauma [17], cellular stress [18] and increased tau protein levels [19]. Since food restriction can be considered a major insult for the developing brain, it seemed relevant to study the evolution of clusterin levels in our model to find out if malnutrition also triggers clusterin upregulation in the NAC. It is important to note that clusterin has been related with loss of control over eating [20], nicotine dependence [21] and the risk of cocaine and alcohol abuse [22] in humans.

Methods

The study was conducted under an approved animal protocol (EC 280790000085) in accordance to the EC Directive 86/609/EEC. This protocol was based on a method previously described in detail [23]. Wistar rats were bred with controlled temperature and artificial dark–light cycle (light from 07:00 to 19:00) and fed ad libitum with a commercial standard laboratory diet containing by weight 19 % protein, 56 % carbohydrate (starch and sucrose) 3.5 % lipid, 4.5 % cellulose, vitamin and mineral mix, and 12 % water.

Females were caged with males for 24 h, and mating was confirmed by the presence of spermatozoa in vaginal smears. Each dam was housed individually from the 14^{th} day of pregnancy. The protocol used to undernourish animals was based on the amount of food consumed by a group of control rats (C, n = 6) which was prepared in parallel and fed

with standard diet ad libitum all along the experiment. In summary, restricted rats received 10 g of the standard food daily from 16th day of gestation until delivery, which represents 40-50 % of that ingested by controls and prevents the 20 % increase of body weight observed in these rats during this period. Next, the restricted mothers received 40 % of the food consumed by controls, that is: 15, 20 and 25 g daily of food during the 1st, 2nd and 3rd week of lactation, respectively. Upon weaning, the rats of restricted mothers were divided into two groups; those assigned to the chronic undernourished group (U, n = 9) received 35 % of the diet daily consumed by controls until the end of the experiment, while the others had free access to standard chow (U + Ad lib, n = 3). Water was given ad libitum. We selected the females of each litter for this study because previous work demonstrated a higher sensitivity of undernourished females to develop a metabolic syndrome with decreased pro-opio-melanocortin expression when undernourishment was discontinued [24, 25]. The expression of FH and clusterin in subcellular fractions of NAC and PFC was determined by western blotting. Rats were decapitated at 5 months of age, the NAC and PFC rapidly dissected as described by Heimer et al. [26], frozen in liquid N_2 and stored at −80 °C until use. For protein quantification the tissues were unfrozen, homogenated in 200 µL of lysis buffer (sucrose 25 mM; EDTA 0.5 mM; Tris 10 mM; pH = 7.4) by using a TissueLyser LT bead (Qiagen Iberia, Madrid, Spain; two cycles of 1 min at 50 oscillations/s) and centrifuged (2500 rpm, 5 min, 4 ºC). The supernatant was centrifuged again (12700 rpm, 5 min, 4 ºC) to separate the mitochondrial fraction (pellet) and the cytosolic fraction (supernatant). The efficacy of this procedure was checked by studying the presence/absence of specific proteins of each fraction (superoxide dismutase 2 for the mitochondrial fraction, AKT for the cytosolic fraction; results not shown).

The protein content of each fraction was determined by the Bradford method (Quick Start kit, Biorad, California, USA) using bovine serum albumin as the standard, and adjusted to 1 mg/ml in Laemli buffer (10% gliycerol; 2% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue in Tris 0.5 M, pH = 6.8). Samples were load into 15 % polyacrylamide/bisacrylamide gels in an electrophoresis system (Bio-Rad, Madrid, Spain). Proteins were separated by molecular weight (90 V 15 min, then 150 V 45 min) and secondly blotted (2.5 A, 25 V, 7 min) onto nitrocellulose membranes (0.2 µm pore size, Bio-Rad) by a Transblot-Turbo Transfer System (Bio-Rad). The membranes were washed for 5 min twice with washing buffer (PBS, 0.5% Tween 20, 0.1% skimmed milk), treated with blocking buffer (PBS, 0.5% Tween 20, 5% skimmed milk) for 30 min and washed again for 5 min five times. Afterwards, the membranes were incubated with primary antibodies overnight at 4 °C with agitation; we used NBP1-68308 antibody for clusterin (1:1000) and NBP1-47754 for FH (1:1000) (Novus Biologicals, Littleton, CO, USA). Once this incubation was finished, the membranes were washed for 5 min five times and then incubated for 1 h at room temperature with peroxidase-linked secondary antibodies (1:5000 anti-rabbit for clusterin and 1:5000 anti-mouse for FH; Santa Cruz Biotechnology, Dallas, TX, USA). Membranes were revealed by ECL-Supersignal™kit following manufacturer's instructions (GE Healthcare, New Jersey, USA) and the bands quantified by densitometry in a ChemiDoc XRS+ apparatus (Bio-Rad). All densitometries were expressed in arbitrary units (A.U.). In all Western blot analyses, β-actin was used as loading control; to ahieve this, the membranes were incubated with a primary antibody anti- β-actin (Santa Cruz Biotechnology; 1:5000 dilution) for 1 h at room temperature with agitation, then washed, incubated with an anti-mouse peroxidase-linked secondary antibody (sc-2005, Santa Cruz Biotechnology; 1:5000 dilution) for 30 min and revealed as previously described.

The comparison of body weights between the three experimental groups was performed by using ANOVA followed by Bonferroni's multiple comparisons tests, after checking normality of data with the Kolmogorov-Smirnov test. The same analysis was applied to compare clusterin expression in each brain area and cell fraction separately, as well as fumarate hydratase expression. Significance was considered at the 0.05 level.

Results

The dieting procedure provoked marked differences in the body weights of rats ($F(2,15) = 115.2$, $p < 0.0001$). Those animals that were undernourished during the perinatal period and postnatal life exhibited a marked reduction of body weight at 5 months of age; when restrictions were only applied during pregnancy and lactation but the rats were allowed free access to standard chow from weaning, their body weights were similar than those of control animals (Fig. 1A).

The statistical analysis also revealed a significant effect of the diet on the expression of FH and clusterin in the mitochondrial fraction of the NAC (FH: $F(2,15) = 17.79$, $p = 0.0001$; clusterin: $F(2,15) = 11.38$, $p = 0.001$). A significant effect of the diet on the cytosolic density of clusterin was also observed ($F(2,15) = 6.25$, $p < 0.02$). Chronic undernourishment upregulated both FH (Fig. 1B) and clusterin (Fig. 1C) in the mitochondrial fraction of the NAC; by contrast, when restrictions were applied only during the perinatal period, mitochondrial FH was found downregulated and clusterin expression decreased not only in the mitochondria but also in the cytosolic fraction of this brain area.

Although both FH and clusterin tended to be increased in the cytosolic fraction of the PFC of undernourished rats with respect to controls, the effect did not reach statistical

significance. ANOVA also discarded significant differences between groups concerning mitochondrial clusterin expression in the PFC.

[Figure 1 near here]

Discussion

The results obtained confirmed the region-specific upregulation of FH previously reported in the mitochondrial fraction of the NAC which was suggested to be a neurochemical correlate of increased sensitivity to reward [9]. The present experiments demonstrate that FH upregulation does not happen when the animals turn to ad libitum chow during postnatal life, however the expression of the protein is not entirely normal at 5 months of age but indeed reduced with respect to controls. It is tempting to interpret FH downregulation as a correlate of long-term decreased sensitivity to reward, but this remains to be firmly established by conducting behavioral studies. In any case, the finding that rehabilitation to normal feeding for a period as long as 5 months does not restore the normal expression of FH indicates the existence of long-lasting metabolic alterations in the reward system. This is in agreement with the broadly accepted idea that perinatal malnutrition by itself is a significant risk factor for the appearance of several neuropsychiatric disorders in the adulthood, including addiction [5]. Further experiments must address the study of the evolution of FH levels at shorter time intervals from the moment of switching the animals to chow *ad libitum*, as well as the possible parallelisms of these changes with behavioral alterations affecting food seeking and food reward.

Clusterin levels paralleled to some extent those seen with FH. To our knowledge this is the first report of diet-induced changes of clusterin in the reward system; previous work demonstrated that the central administration of clusterin provokes anorexia and weight

loss in the mouse, but this effect was related with a regulation of both leptin and ghrelin effects on hypothalamic neurons [27]. Clusterin downregulation in the NAC of animals refed *ad libitum* was even more marked than that of FH, since it also applied to the cytosolic pool of the protein. The functional consequences of clusterin changes are to be established, but they could be indicative of altered susceptibility to brain damaging stimuli if we bear in mind the neuroprotective role of the protein [28]. This neuroprotection has been partially related to an anti-apoptotic effect which involves both the cytosolic and mitochondrial pools of clusterin; thus, cytosolic clusterin seems to prevent the arrival of Bax to the mitochondria, whilst mitochondrial clusterin inhibits the formation of Bax-Bak complexes [29]. Besides, cytosolic clusterin seems to bind misfolded proteins and traffic them to the proteasome for degradation [30]. According to these mechanisms, long-term downregulation of clusterin such as that observed in the NAC could reflect a situation of higher risk of damage and reward dysfunction, which is compatible with an increased susceptibility to addiction. Obviously, this hypothesis should be addressed with specific behavioral work, on one hand, and neurotoxicity assessments, on the other. Another question that remains to be clearly established is the region specificity of the effects observed: even when statistical differences were only achieved in the NAC, some other tendencies observed in the PFC will deserve further attention.

The main limitations of the present work are the exclusive use of females and the low number of animals in the refed group. Other studies on the effects of perinatal malnutrition were only conducted in males [7,8], therefore the availability of data from both sexes will help to obtain a clearer picture of the effects of undernourishment. Concerning refeeding, the homogeneity and consistency of the results obtained in our study make it highly probable that further experimentation will confirm our findings.

In conclusion, the present experiments confirm that perinatal and postnatal food restriction provoke deep neurochemical changes in the NAC with marked upregulations of FH and clusterin. After perinatal malnutrition, the free access to a standard normal diet fails to restore normal protein levels but leads to a downregulation that could be related with the reported increase in the susceptibility to neuropsychiatric disorders, i.e. addiction.

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Declaration of interest statement

All authors state that they have no potential conflict of interest.

References

[1] Tonkiss J, Shukitt-Hale B, Formica R, Rocco J, Galler J. Prenatal protein malnutrition alters response to reward in adult rats. Physiol. Behav. 1990;48:675-80.

[2] Valdomero A, Bussolino DF, Orsingher OA, Cuadra GR. Perinatal protein malnutrition enhances rewarding cocaine properties in adult rats. Neuroscience 2006;137:221-9.

[3] Valdomero A, Velazquez EE, de Olmos S, de Olmos JS, Orsingher OA, Cuadra GR. Increased rewarding properties of morphine in perinatally protein-malnourished rats. Neuroscience 2007;150:449-58.

[4] da Silva AA, Borba TK, de Almeida Lira L, Cavalcante TC, de Freitas MF, Leandro CG et al. Perinatal undernutrition stimulates seeking food reward. Int. J. Dev. Neurosci. 2013;31:334-41.

[5] Franzek EJ, Sprangers N, Janssens AC, Van Duijn CM, Van De Wetering BJ. Prenatal exposure to the 1944-45 Dutch 'hunger winter' and addiction later in life. Addiction 2008;103:433-8.

[6] Perondi MC, Gutiérrez MC, Valdomero A, Cuadra GR. Perinatal protein deprivation facilitates morphine cross-sensitization to cocaine and enhances ΔFosB expression in adult rats. Behav. Brain Res. 2017;333:203-10.

[7] da Silva AAM, Oliveira MM, Cavalcante TCF, do Amaral Almeida LC, Cruz PLM, de Souza SL. Undernutrition during pregnancy and lactation increases the number of fos-cells in the reward system in response to a 5-HT6 receptor agonist in male adolescent rats. Int. J. Food Sci. Nutr. 2018;69:488-93.

[8] de Melo Martimiano PH, da Silva GR, Coimbra VF, Matos RJ, de Souza BF, da Silva AA et al. Perinatal malnutrition stimulates motivation through reward and enhances drd(1a) receptor expression in the ventral striatum of adult mice. Pharmacol. Biochem. Behav. 2015;134:106-14.

[9] Lizárraga-Mollinedo E, Álvarez C, Fernández-Millán E, Escrivá F, González-Martín C, Salas E et al.. Undernutrition upregulates fumarate hydratase in the rat nucleus accumbens. Metab. Brain Dis. 2013;28:111-5.

[10] Yogev O, Naamati A, Pines O. Fumarase: a paradigm of dual targeting and dual localized functions. FEBS J. 2011;278:4230-42.

[11] del Castillo C, Morales L, Alguacil, LF, Salas E, Garrido E, Alonso E, Pérez-García C. Proteomic analysis of the nucleus accumbens of rats with different vulnerability to cocaine addiction. Neuropharmacology 2009;57:41-8.

[12] Pérez-Ortiz JM, Galiana-Simal A, Salas E, González-Martín C, García-Rojo M, Alguacil LF. A high-fat diet combined with food deprivation increases food seeking and the expression of candidate biomarkers of addiction. Addiction Biology 2017;22:1002-9.

[13] Alguacil LF, Stucchi P, del Olmo N, Herradón G, Ruiz-Gayo M. Biomarkers and drug targets in addiction and obesity. Eur. J. Clin. Pharmacol. 2010;66:S85.

[14] May PC, Robison P, Fuson K, Smalstig B, Stephenson D, Clemens JA. Sulfated glycoprotein-2 expression increases in rodent brain after transient global ischemia. Brain Res. Mol. Brain Res. 1992;15:33-9.

[15] Boggs LN, Fuson KS, Baez M, Churgay L, McClure D, Becker G, May PC. Clusterin (Apo J) protects against in vitro amyloid-beta (1-40) neurotoxicity. J. Neurochem. 1996;67:1324-7.

[16] Park CH, Kim YS, Lee HK, Kim YH, Choi MY, Jung DE et al. Citicoline reduces upregulated clusterin following kainic acid injection in the rat retina. Curr. Eye Res. 2007;32:1055-63.

[17] Huang Z, Cheng C, Jiang L, Yu Z, Cao F, Zhong J et al. Intraventricular apolipoprotein ApoJ infusion acts protectively in traumatic brain injury. J. Neurochem. 2016;136:1017-25.

[18] Zinkie S, Gentil BJ, Minotti S, Durham HD. Expression of the protein chaperone, clusterin, in spinal cord cells constitutively and following cellular stress, and upregulation by treatment with Hsp90 inhibitor. Cell Stress Chaperones 2013;18:745-58.

[19] Shepherd CE, Affleck AJ, Bahar AY, Carew-Jones F, Halliday GM. Intracellular and secreted forms of clusterin are elevated early in Alzheimer's disease and associate with both Aβ and tau pathology. Neurobiol. Aging 2020;89:129-31.

[20] Rodríguez-Rivera C, Pérez-García C, Muñoz-Rodríguez JR, Vicente-Rodríguez M, Polo F, Ford RM et al. Proteomic identification of biomarkers associated with eating control and bariatric surgery outcomes in patients with morbid obesity. World J. Surg. 2019;43:744-50.

[21] Pallardo-Fernández I, Iglesias V, Rodríguez-Rivera C, González-Martín C, Alguacil LF. Salivary clusterin as a biomarker of tobacco consumption in nicotine addicts undergoing smoking cessation therapy. J. Smok. Cessat. 2020;15:171-4.

[22] Pallardo-Fernández I, Muñoz-Rodríguez JR, González-Martín C, Alguacil LF. Human clusterin gene polymorphisms associated with substance use disorders. Rev. Toxicol. 2020;37:3-5.

[23] Escrivá F, Rodriguez C, Cacho J, Álvarez C, Portha B, Pascual-Leone AM. Glucose utilization and insulin action in adult rats submitted to prolonged food restriction. Am. J. Physiol. 1992;263:E1–7.

[24] De Toro-Martín J, Fernández-Millán E, Lizárraga-Mollinedo E, López-Oliva E, Serradas P, Escrivá F et al. Predominant role of GIP in the development of a metabolic syndrome-like phenotype in female Wistar rats submitted to forced catch-up growth. Endocrinology 2014;155:3769-80.

[25] Lizárraga-Mollinedo E, Fernández-Millán E, García-San Frutos M, de Toro-Martín J, Fernández-Agulló T, Ros M et al. Early and long-term undernutrition in female rats exacerbates the metabolic risk associated with nutritional rehabilitation. J. Biol. Chem. 2015;290:19353-66.

[26] Heimer L, Zahm DS, Churchill L, Kalivas PW, Wohltmann C. Specificity in the projection patterns of accumbal core and shell in the rat. Neuroscience 1991;41:89-125.

[27] Gil SY, Youn BS, Byun K, Huang H, Namkoong C, Jang PG et al. Clusterin and LRP2 are critical components of the hypothalamic feeding regulatory pathway. Nat. Commun. 2013;4:1862.

[28] Rohne P, Prochnow H, Koch-Brandt C. The CLU-files : disentanglement of a mystery. Biomol. Concepts 2016;7:1-15.

[29] Rodríguez-Rivera C, Garcia MM, Molina-Álvarez M, González-Martín C, Goicoechea C. Clusterin: Always protecting. Synthesis, function and potential issues Biomed. Pharmacother 2021;134:111174.

[30] Satapathy S, Wilson MR. The dual roles of clusterin in extracellular and intracellular proteostasis. Trends Biochem. Sci. 2021; Online ahead of print.

FIGURE CAPTION

Figure 1. Panel A. Body weights of animals fed on chow (control, C), undernourished peri- and postnatally (U) and undernourished prenatally but fed on normal chow *ad libitum* upon weaning (U + Ad Lib). *p<0.05 vs C; +p<0.05 vs U (ANOVA + Bonferroni's posthoc tests). Panel B. Expression of fumarate hydratase (FH) in the mitochondrial (M-) and cytosolic (C-) fractions of the nucleus accumbens (NAC) and prefrontal cortex (PFC) of rats fed on chow (control, C), undernourished peri- and postnatally (U) and undernourished prenatally but fed on normal chow *ad libitum* upon weaning (U + Ad Lib). *p<0.05 vs C; +p<0.05 vs U (ANOVA + Bonferroni's posthoc tests). Panel C. Expression of clusterin (CLU) in the mitochondrial (M-) and cytosolic (C-) fractions of the nucleus accumbens (NAC) and prefrontal cortex (PFC) of rats fed on chow (control, C), undernourished peri- and postnatally (U) and undernourished prenatally but fed on normal chow *ad libitum* upon weaning (U + Ad Lib). *p<0.05 vs C; +p<0.05 vs U (ANOVA + Bonferroni's posthoc tests).