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CONSEQUENCES OF EARLY UNDERNUTRITION ON RAT HYPOTHALAMIC INSULIN AND LEPTIN SIGNALING

VPLIV ZGODNJE PODHRANJENOSTI NA SIGNALIZACIJO INZULINA IN LEPTINA V HIPOTALAMUSU PODGAN

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STATEMENT

I declare that I have performed and written this Graduation Thesis solely by myself under the mentorship of Prof. Dr. Irena Mlinarič Raščan, PhD and co-mentorship of Prof. Dr. Fernando Escrivá Pons, PhD.

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ABSTRACT

Undernutrition is a worldwide problem affecting a large segment of the population and mainly prevalent in developing countries. Paradoxically, an association between early food-restriction and having a higher risk of several diseases in adult life has been evidenced, such as cardiovascular disorders, insulin resistance and obesity. Moreover, permanent alterations on the central nervous system have also been associated with this condition. In view of these findings, some hypothalamic appetite-regulating factors related to insulin and leptin pathways have been studied in the present work, since hypothalamus is a key organ in the complex system of food intake control.

The study was conducted by analyzing different developmental stages of an animal model of chronically undernourished Wistar rats. Body weight and glycemy of food restricted newborn pups did not differ from their controls. In contrast, both parameters were decreased in undernourished animals at the ages of 10 and 70 days. Specific proteins of the insulin-signaling pathway were studied: insulin receptor (IR), its phoshorylated form (p-IR) and insulin substrates (IRS1/2), which were not modified in adult food-restricted rats. However, both the leptin receptor (Ob-Rb) and Signal Transducer Activator of Transcription (STAT-3) were significantly reduced following undernutrition in these animals. In addition, we examined the hypothalamic expression of POMC and NPY (anorexigenic and orexigenic peptides, respectively) at different stages (newborn, suckling and adult). There were no differences between groups in the NPY mRNA at newborn and suckling stages, although NPY peptide was 3.7 fold higher in undernourished adult rats than in their controls. As regards POMC, this peptide was 0.5 and 0.6 fold diminished in food-restricted suckling and adult rats, respectively. The level of POMC mRNA remained unchanged in the newborns. Finally, immunohystochemical analysis of NPY indicated increased levels in the hypothalamic ARC region of both suckling and adult restricted rats.

In summary, the results provide evidence of the changes caused by early undernutrition on several factors related to insulin and leptin signals involved in the hypothalamic appetite control. Further investigation will be necessary to elucidate the consequences of these changes on the prone to obesity previously shown in early-undernourished rats.

RAZŠIRJENI POVZETEK

Podhranjenost predstavlja, prav tako kot debelost, vedno večji svetovni problem, ki zajema predvsem populacijo ljudi v nerazvitih državah. Po statističnih raziskavah Svetovne organizacije za prehrano, FAO iz leta 2011 je na svetu skoraj milijarda ljudi podhranjenih, 9 milijonov pa naj bi letno umrlo za podhranjenostjo, od tega 6 milijonov otrok, mlajših od pet let. Znano je, da je najbolj kritično obdobje prav nosečnost in prvih nekaj mesecev po rojstvu, saj podhranjenost v zgodnji fazi razvoja povzroči spremembo strukture, fiziologije in delovanja otrokovega telesa, kar poveča nagnjenost k razvoju diabetesa tipa 2, debelosti, metabolnega sindroma in srčno-žilnih obolenj v odrasli dobi. Gre za tako imenovano Barkerjevo hipotezo o zgodnjem izvoru oz. fetalnem programiranju. Večina študij o povezavi med podhranjenostjo in kasnejšimi obolenji pa izhaja iz lakote na Nizozemskem v času 2. svetovne vojne, kjer so takrat noseče ženske in njihovi potomci še vedno tarča številnih epidemioloških študij dolgoročnih posledic zgodnje podhranjenosti pri človeku.

Zmanjšan vnos hranilnih snovi v nosečnosti in v času dojenja privede do številnih zapletov, kot so npr.: znižana telesna teža ob rojstvu; spremenjena sinteza hormonov, potrebnih za rast in razvoj; slabši odziv imunskega sistema in posledično večja dovzetnost za razne infekcije; številne posledice na centralnem živčnem sistemu kot so motnje kognitivnih in motornih funkcij ter nevrološka obolenja; sledijo vaskularne spremembe, ki vodijo do pojava hipertenzije; ter dislipidemija kot rezultat porušenega ravnotežja v lipidnem sistemu. To obdobje je prav tako ključno za razvoj možganov, kjer se nahaja center za regulacijo apetita. Pri pomanjkanju hrane med nosečnostjo ali v prvih mesecih po rojstvu so možgani sposobni adaptacije in se programirajo tako, da se ob prenehanju restrikcije oz. ko hrana postane na voljo *ad libitum*, pojavi hiperfagija (povečan apetit). Sicer paradoksno, pa vendar zgodnja podhranjenost vodi v debelost v odrasli dobi, zato so mnogi znanstveniki mnenja, da bi dandanes aktualno epidemijo debelosti mogli odpravljati z zniževanjem prevalence zgodnje podhranjenosti.

Debelost povzroči vrsto zapletov, iz katerih se lahko razvijejo resna obolenja. Običajno jo spremljajo presnovne motnje, kot je leptinska rezistenca in še ne povsem dokazana neodzivnost na inzulin. Mehanizmov rezistence je več: lahko gre za oviran transport leptina čez hematoencefalno bariero ali receptorsko oz. poreceptorsko motnjo. Zaradi teh razlogov je leptin zaenkrat še neuspešen v terapiji zdravljenja debelosti.

Signalizacija teh dveh hormonov deluje po principu kaskadnih reakcij, kjer vezava inzulina na inzulinski receptor (IR) oz. leptina na leptinski receptor (Ob-Rb) sproži avtofosforilacijo tirozinskih ostankov. Le-ti povzročijo fosforilacijo ostalih signalnih molekul, kot npr. PI3K (fosfatidil inozitol 3 kinaze) in JAK (janus kinaze), kar kot končni rezultat privede do translokacije STAT-3 (signalnega transduktorja in aktivatorja transkripcije) v jedro. Ta vpliva na povišan nivo izražanja mRNA za anoreksogena POMC (pre-opiomelanokortin) in CART (cocain and amphetamin related transcript) ter zmanjšan nivo ekspresije mRNA za oreksogena NPY (nevropeptid Y) in AgRP (agouti related peptid). Inzulin in leptin vzdražita nevrone oreksogenih in anoreksogenih peptidov v arkuatnem jedru (ARC), ki nato projekcirajo v ostale regije hipotalamusa, kot so paraventrikularno jedro, lateralni hipotalamus (»center za lakoto«) in ventromedialno jedro (»center za sitost«). Končni efekt njunega delovanja je večja sitost oz. zmanjšan občutek lakote. Če nek dražljaj (npr. zmanjšan vnos hrane) zniža občutljivost teh molekul, se poveča tendenca za razvoj hiperfagije. Posledično lahko trdimo, da sta inzulin in leptin »glavna krivca« za razvoj debelosti.

V naši predhodni študiji smo želeli preučiti posledice zgodnje podhranjenosti na leptinsko in inzulinsko signalizacijo v hipotalamusu Wistar podgan in s tem povezan vpliv na mehanizme regulacije apetita. V prvem delu diplomske naloge smo primerjali splošne karakteristike podgan med kontrolno in podhranjeno populacijo, in sicer telesno težo in koncentracijo glukoze v plazmi. Kalorična restrikcija je potekala od 14 dneva nosečnosti in se nadaljevala na potomcih vse do odrasle dobe. Živali so bile hranjene s komercialno pripravljeno hrano *Sandermus S-10*; kontrolna skupina *ad libitum*, podhranjena pa s 35% celotne količine kontrolne skupine. Dokazano je, da zgodnja podhranjenost povzroči hipoglikemijo oz. znižano koncentracijo glukoze v krvi kmalu po rojstvu. Pri novorojenih podganah ni bilo razlik med populacijama, medtem ko je pri dojenih mladičih in odraslih podganah statistično značilna razlika med podhranjeno in kontrolno skupino tako v teži kot v koncentraciji glukoze. Le-to smo določili z glukozno oksidaznim testom.

S prenosom po Westernu smo želeli dokazati vpliv zgodnje podhranjenosti na vsebnost hipotalamusnega inzulinskega receptorja (IR), njegove fosforilirane oblike (p-IR), inzulinskih substratov (IRS 1/2), leptinskega receptorja (Ob-Rb) in signalnega transduktorja in aktivatorja transkripcije (STAT-3) na odraslih podganah. V naši študiji nismo dokazali razlik med vsebnostjo IR, p-IR in IRS1/2 v hipotalamusu med podhranjeno

in kontrolno skupino podgan. Nasprotno, je bila vsebnost Ob-Rb in STAT-3 značilno zmanjšana pri podhranjeni populaciji podgan. Z isto metodo smo analizirali tudi vsebnost anoreksogenega proteina POMC v hipotalamusu, in sicer smo dokazali zmanjšano vsebnost tega proteina pri podhranjenih podganah v primerjavi s kontrolo. Vsebnost NPY, ki velja za najučinkovitejšega oreksogena, nam žal ni uspelo dokazati, saj protitelesa, ki smo jih uporabili pri Western prenosu niso bila dovolj specifična zanj.

V naslednjem delu naše študije smo želeli dokazati vpliv podhranjenosti na ekspresijo genov za POMC in NPY pri različno starih populacijah z metodo RT-qPCR, in sicer smo v študijo vključili 1, 10 in 70 dni stare podgane, katere smo primerjali s kontrolnimi skupinami. Opazili smo, da ni razlik med populacijama pri novorojenih podganah. Nivo mRNA za POMC se je razlikoval pri 10 in 70 dni starih podganah, in sicer je ekspresija tega gena za 0.5 krat nižja pri podhranjenih doječih mladičih v primerjavi s kontrolo, pri odraslih podganah pa za 0.6 krat nižja kot pri kontrolni skupini. Razlika v nivoju mRNA za NPY je statistično značilna le pri odrasli populaciji (3.7 krat višja pri podhranjeni populaciji v primerjavi s kontrolo). Nazadnje smo protein NPY kvalitatitivno analizirali še z imunohistološko metodo na 10 in 70 dni starih podganah. Videti je bilo intenzivnejše obarvanje ARC in paraventrikularnega jedra pri podhranjenih podganah, ne glede na starost. Ta kontradiktorni rezultat med metodama pri 10 dni starih podganah lahko razložimo tako, da zgodnja podhranjenost sicer vpliva na večji prepis DNA v mRNA v zgodnji fazi razvoja, kar povzroči povečano sintezo proteina NPY, izrazite spremembe v nivoju mRNA pa se izrazijo šele v odrasli dobi.

V splošnem je naša predhodna študija potrdila večino hipotez, ki smo si jih zastavili pred samim pričetkom eksperimentalnega dela. Z zagotovostjo lahko trdimo, da zgodnja podhranjenost vpliva na leptinsko in inzulinsko signalizacijsko pot, in s tem povezan kompleksen mehanizem regulacije apetita, vendar so za konkretnejše in oprijemljivejše rezultate potrebne nadaljne študije, ki so že v teku.

ABBREVIATIONS

α-MSH	α-Melanocyte-Stimulating Hormone
AgRP	Agouti-Related Protein
APS	Ammonium Persulfate
ARC	Arcuate hypothalamic nuclei
cDNA	Complementary DNA
CART	Cocaine and Amphetamine – Related Transcript
CNS	Central Nervous System
DAB	3, 3' Diaminobenzidine
dH_2O	Distilled water
DNA	Deoxyribonucleic Acid
EDTA	EthyleneDiamineTetraacetic Acid
FAO	Food Agriculture Organization
IR	Insulin receptor
IRS	Insulin receptor substrate
LHA	Lateral Hypotalamic Area
mRNA	Messenger RNA
MCH	Melanin Concentrating Hormone
NPY	Neuropeptide Y
Ob-Rb	Leptin receptor
PAGE	Polyacryilamide Gel Electrophoresis
PBS	Phosphate buffered saline (pH = 7.5)
POMC	Pro-opiomelanocortin
PVN	Paraventricular Nuclei
PVDF	Polyvinyilidene Fluoride
RIPA	Radio Immuno Precipitation Assay buffer
RNA	Ribonucleic Acid
RT	Reverse Transcription
RT-qPCR	Real Time Quantitative Polymerase Chain Reaction
SE	Standard Error
SDS	Sodium Dodecyl Sulfate
STAT-3	Signal Transducer and Activator of Transcription-3

TEMED	N,N,N',N' -Tetramethyl-Ethylenediamine
TBE	Tris/Borate/EDTA buffer
TBS	Tris® buffered saline (pH = 8)
Tris	Tris (hydroxymethyl) aminomethane
TTBS	Tris® buffered saline with Tween®20
VMN	Ventromedial Nuclei
WB	Western Blotting
WHO	World Health Organization

SYMBOLS

A	absorption
µg	microgram
µm	micrometer
µL	microliter
°C	celsius
g	gram
h	hour(s)
kCal	kilo calories
min	minute(s)
mL	mililiter
M	mol/L (concentration)
rpm	revolutions per minute
s	second(s)

1. INTRODUCTION

1.1. The importance of undernutrition in the world

1.1.1 *The number and distribution of hungry people in the world*

Undernutrition is a worldwide problem affecting a large segment of the population. According to data from the Food and Agriculture Organization (FAO), almost 9 million of people, including more than 6 million children under the age of 5, die each year as a consequence of hunger. In 2010, FAO stated that 925 million people were undernourished, which means nearly 14% of the estimated world population. As the figure shows below, undernourishment is in increase from 1995-1997 to 2009-2010 (Fig. 1) and is due mainly to three factors: the neglect of agriculture relevant to less privileged by governments and international agencies, the current global economic crisis and the significant increase of food prices in the last several years (1).

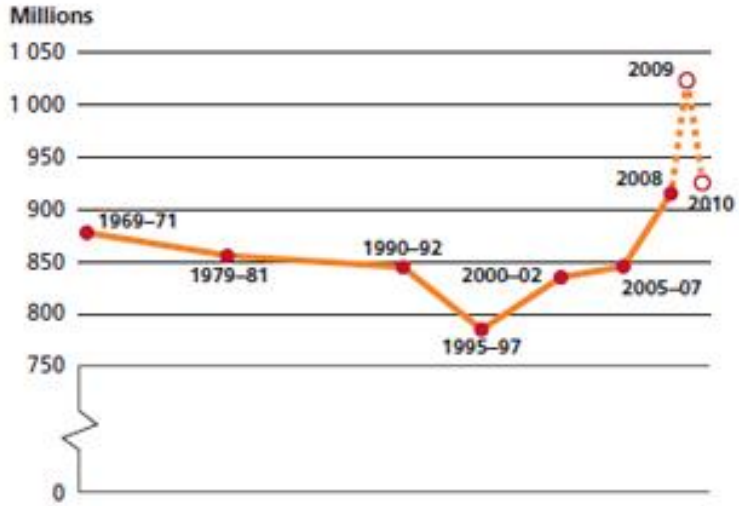

Fig. 1: Number of hungry people in the world (from 1969 to 2010) (1).

The majority of the world's undernourished population lives in developing countries, such as Asia and the Pacific regions and Sub-Saharan Africa (Fig. 2). Projections in 2010 indicated that the number of undernourished people will decline in all developing areas, although at a different pace.

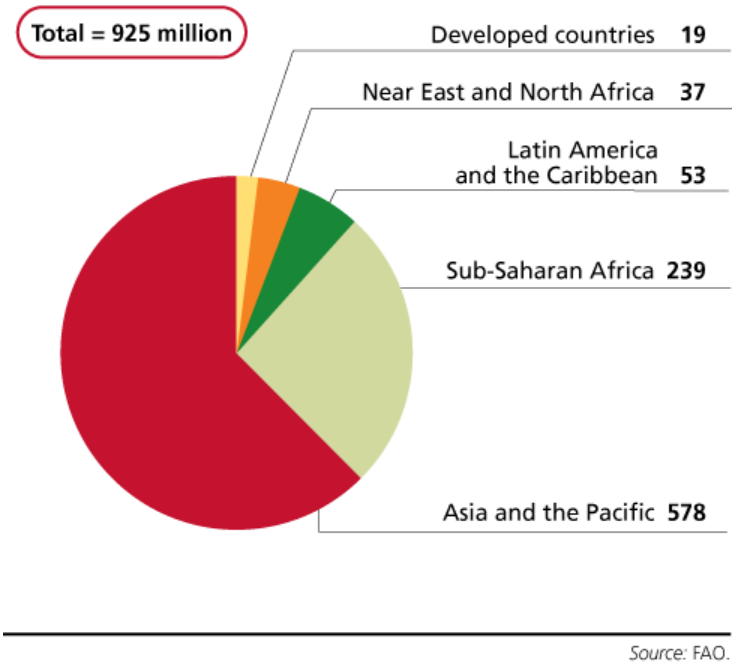

Fig. 2: Distribution of undernourished people in the world in 2010 (2).

1.1.2. *Causes of hunger*

Hunger could be mainly explained as a consequence of poverty and lack of resources within specific countries. An extremely unequal income distribution in the world and devastating conflicts must also be considered as contributing factors of hunger. Several underlying causes of poverty and hunger in the world are economic, political, and derived from social disparities. Furthermore, climate changes and natural disasters are currently worsening the situation in the most disadvantaged areas where hunger and poverty are more acute (3).

1.1.3. *Extreme scarcity of food: famines. Dutch famine*

In the past well as in the present, there have been particular situations in which the effects of hunger have been more pronounced. A sever lack of resources and extreme starvation affect both children and adults in a certain geographical area and for a long period of time. Although an outbreak of famine could be preceded by a variety of causes, the great famines in history have normally been caused by economic depressions and political conflicts. One such example is the Dutch famine of 1944-1945 (“Hunger Winter”), which

took place in the Netherlands during the Second World War. This famine is especially worth mentioning since it affected thousands of pregnant women whose descendants were the object of epidemiological studies and still provide a unique opportunity to examine long-term consequences of early undernutrition in humans (4). Although genetic background and lifestyle are the main factors that predispose an individual to suffer from metabolic diseases and type 2 diabetes, these epidemiological studies developed after the Dutch famine, proved a link between low birth weight and the subsequent higher risk of metabolic disorders in adulthood. This fact is also known as "nutritional imprinting" or "fetal programming" (5).

1.2. The impact of early undernutrition on different areas of the metabolism

Reduced intake during gestation and lactation entails negative consequences for the offspring. Early neonatal stages are crucial for the adequate operation of key processes in the development (e.g. myelination, cell proliferation, bone formation). Growth retardation and several other alterations occur as a result of such limited nutrient availability since the intrauterine stages. It is also known that poor early nutrition strongly increases the risk of suffering from several diseases in adult life, such as: diabetes mellitus, dyslipidemia, obesity, hypertension, and cardiovascular disorders. All of these pathologies are included in the metabolic syndrome (6-9). The *thrifty phenotype hypothesis* was proposed to provide a conceptual and experimentally testable basis of these relationships. The hypothesis proposes that a fetus remains adapted to a limited supply of nutrients, causing permanent alterations in its physiology and metabolism, and being unable to face different nutritional conditions. This hypothesis will be revisited later in section *4*.

1.2.1. Hormones

During fetal development, the coordinated actions of insulin, insulin-growth factors, thyroid hormones, growth hormone, gonadotropins and glucocorticoids play central roles in the control of differentiation, growth and maturation (10). Numerous studies have shown that nutrition markedly influences the synthesis and metabolism of these hormones (11).

1.2.2. The immune system

Underweight children are the most sensitive population segment for the development of diseases. They have an increased susceptibility to develop infections due mainly to

immunedeficienecies: lower levels of T lymphocytes, impairment of antibody formation, decreased complement formation, and atrophy of thymus and other lymphoid tissues. These alterations, among others, contribute to debilitating the immune system (12).

In general terms, epidemiological studies reveal that the risk of death is 2.5 times higher in children submitted to mild malnutrition compared to well-fed children. Obviously, the risk increases sharply when malnutrition worsens. The estimated proportions of deaths, in which undernutrition is an underlying cause, are roughly similar for diarrhea (61%), malaria (57%), pneumonia (52%), and measles (45%) (13, 14) (Fig. 3). Similarly, diseases can cause malnutrition and what is more, they can intensify the undernourishment state; for instance, diseases which cause diarrhea, by reducing the body's ability to convert food into usable nutrients.

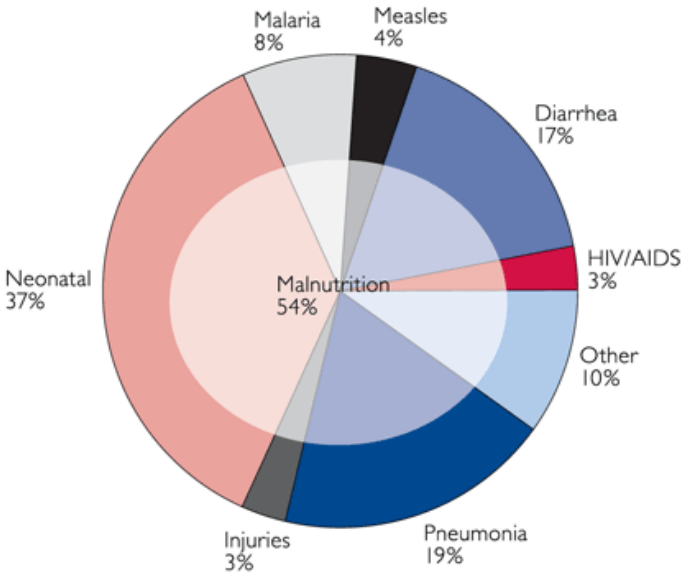

Fig. 3: The leading causes of death in children younger than 5 years in developing countries and the contributing causes of death (13).

1.2.3. Physiological disruptions

It is well known that fetal malnutrition affects both the size of the organ and its structure. For instance, the offsprings of malnourished mothers have larger livers but fewer lobules inside. Such changes must have profound implications given the important functions of the liver as the producer of acute phase proteins, now well recognised as being altered in type 2 diabetes. Birth body weights, and also the majority of the organs' weights, are significantly decreased compared to well-fed populations. However, a selective protection

of brain growth is retained (15, 16). Furthermore, adverse adaptations of food restriction affect pancreatic beta-cell insulin secretion and sensitivity to insulin (17).

1.2.4. Long lasting consequences of undernutrition: obesity and metabolic syndrome

The fetal environment seems to be a critical period for the subsequent development of obesity in adult life. This mechanism is described in section *4*.

1.3. Consequences of early undernutrition on Central Nervous System

An inadequate diet during the first period of life could adversely influence the development of the brain, which is genetically programmed to grow more quickly than the rest of the body, resulting in changes in its structure and hence functioning (18). Some of these changes are similar to the characteristics of the premature syndrom (19).

The peripheral nervous system and central nervous system (CNS) are vulnerable to protein-energy restriction. Ontogenic steps of development, such as cell proliferation and migration, neurogenesis, generation of synapsis, dendritic branching and myelination, could be negatively altered by malnutrition. Total brain and cortical size are mildly reduced as a consequence of the slowed brain growth (20). All of these CNS alterations are associated with delays in motor and cognitive functions. Attention deficit disorder, impaired school performance, decreased IQ scores, memory and learning deficiencies, as well as visuospatial functions and reduced social skills, have been associated with nutritional inadequacy (18, 21). Some pharmacological studies, based on the measurement of neurotransmitter concentration, have shown that early undernutrition may permanently alter the activity of the nervous system. However, the nature of the changes which affect brain development is not clear (22).

In animal models, it is well-known that early undernutrition has repercussions for exploratory behavior (23) and memory (24). Behavioral changes occur due to functional or morphological alterations, particularly in the neocortex and hippocampal formation (25). In humans, restricted undernutrition increases the risk of psychiatric and psychosocial disorders, such as depression (26), schizophrenia (27) and agressive behavior (28), pathologies in which specific cortical areas seem to be involved. Electroencephalographic recordings from severely undernourished infants have shown abnormalities that suggest impaired maturation of the cortex, which is the CNS region directly involved in cognitive

as well as executive functions (29). Another structure of the brain particularly affected by early undernutrition is the hippocampus, which is involved in learning processes and the storage of spatial representation of the environment in cognitive maps (30). Studies, conducted in recent decades, have shown that malnourished infants take longer to learn a task and have more difficulty in retaining the information learned (31, 32). However, undernourished children are, in general, economically unfavored, a situation that contributes to impairment of mental proficiency and masks possible specific impacts of food-restriction on learning tasks (33).

Overall, the changes produced by malnutrition are determined by the type of nutritional insult to which the organism is submitted, the severity of this insult, its duration and the period of life when it happens. Severe malnutrition during the beginning of life, during critical periods of CNS development, produces some significant and lasting behavioral changes observable in the course of a lifetime (19).

1.4. Early undernutrition and obesity in adulthood

Nowadays, obesity has become a worldwide epidemic and it represents a modern health crisis. For instance, more than 55% of adults in the United States are overweight (BMI > 25 kg/m^2) and 1 of 5 are obese (BMI > 30 kg/m^2). Obesity is considered one of the major contributing factors to the raised incidence of several related chronic-diseases, such as diabetes and hypertension (34). A fact that is crucial in relation to the work developed herein is that, paradoxically, maternal nutrient restriction imposed during early and mid-gestation or during lactation could also lead to obesity in later life (35).

1.4.1. Thrifty phenotype hypothesis

Fetal and postnatal environment may predispose an individual to develop obesity (36). Epidemiological studies have shown that adult individuals who were exposed to famine during early pregnancy had higher rates of body mass index and abdominal adipose tissue (37). Early studies linking low-birth weight and late-onset obesity laid the groundwork for the *thrifty phenotype hypothesis* (38) which proposes that a restricted nutritional environment during gestation and postnatal life may increase the risk of suffering from obesity, diabetes and other associated pathologies. The underlined hypothesis is based on the shortage of micro and macronutrients submission and the establishment of adaptations

that especially become detrimental when a high fat and dense caloric diet is supplied. At such a time, the metabolism does not face up to the new conditions and these two interrelated diseases (obesity and type 2 diabetes) could emerge (35, 38).

1.4.2. Multifactorial Pathology of Obesity

Western diets are composed predominantly of high-fat and sugar-sweetened foods and sedentary behaviours which are causing an imbalance between energetic expenditure and total amount of calories consumed. Consequently, if an obesogenic environment extends for a prolonged period of time, an individual will become overweight and obese. Obesity is a multifactorial pathology, and at the same time, a variety of metabolic signals and interactions are implicated. The control of body weight actually concerns the control of adipose tissue with the key role of the hypothalamus (39). Leptin, an adypocite hormone, and its receptor (Ob-Rb) are known to be the evident mediator of developing obesity. This hormone circulates at a concentration positively associated with body fat mass. As it has been suggested, hypothalamic "malprogramming" begins in utero, but continues in early postnatal life during the suckling period, leading to a disturbed organization and, consequently, to longlasting dysfunction in adulthood (40).

1.4.3. Leptin resistance

The hypothesis that leptin resistance can occur in association with obesity was first suggested by the finding of elevated plasma leptin levels in obese humans (41). This hypothesis suggests that some cases of human obesity may be due to reduced leptin activity in the brain and affected individuals are unlikely to respond to pharmacological treatment with leptin. Several mechanisms may contribute to leptin resistance: a decreased ability of circulating leptin to enter into the brain's interstitial fluid and impaired leptin transport across endothelial cells of the blood-brain barrier. Another potential cause of leptin resistance is reduced leptin-receptor signal transduction, among others (42).

1.4.4. Obesity and co-morbidities: metabolic syndrome

As expected, obesity brings together several associated metabolic complications (e.g. obesity, hypertriglyceridemia, low high-density lipoprotein (HDL), hypertension, and high-fasting glucose) which are collected in one term: metabolic syndrome. As previously described, there is increasing evidence that the *in utero* environment has an impact on fetal

development and alters several adult regulatory mechanisms contributing to an increase of the risk of suffering from metabolic syndrome and type 2 diabetes (43).

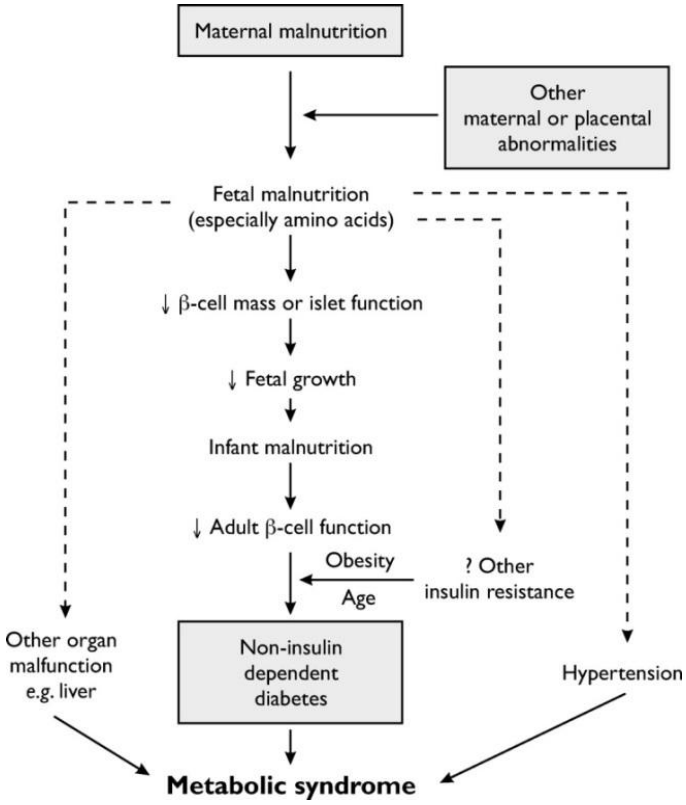

Fig. 4: The diagrammatic representation of thrifty phenotype (38).

Diabetes mellitus

Insulin resistance, obesity, ageing and physical inactivity are the most important factors in determining type 2 diabetes in adulthood (38). It has been proposed that impaired development and function of pancreatic β-cell mass (including altered vasculature and innervation of the islets of Langerhans) play an important role in relating poor early nutrition and diabetes mellitus into adulthood. For instance, the reduced capacity of insulin secretion that takes place under food-restriction could increase the risk of developing subsequent insulin resistance and associated pathologies like type 2 diabetes in adulthood.

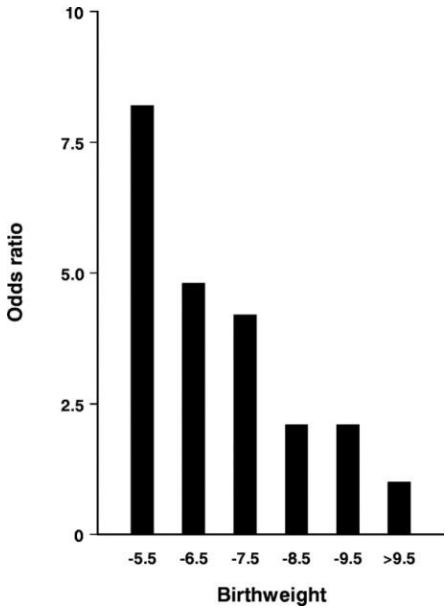

Fig. 5: Odds ratio for impaired glucose tolerance or type II diabetes according to birth weight. Study conducted with a population of 370 men, aged 64 years and born in Hetrfordshire, UK (adjusted for adult body mass index) (38).

Dyslipidemia

Lipid homeostasis is primary regulated by liver and adipose tissue. The underlying mechanisms of altered plasma trygliceride and cholesterol concentrations may include impaired hepatic expression of key enzymes; changes in bile acid synthesis, secretion, and absorption; modified hepatic growth, proliferation and morphology or altered milk composition as a result of maternal nutrient restriction. Epidemiological studies in humans show that a reduced body weight in early life is related to high levels of total and LDL-cholesterol concentrations in later life, supporting the hypothesis that early nutrition programs lipid metabolism, increasing the risk of vascular disease in adulthood (38, 44).

Hypertension

Possible mechanisms linking reduced fetal growth and raised blood pressure are persisting changes in vascular structure, including loss of elasticity in vessel walls and the effects of glucocorticoid hormones. The increased corticosterone levels found in fetuses of food restricted rats disturb the development of the hypothalamo-pituitary adrenal axis, resulting in hypertension, hyperglycemia and possibly hepatic insulin resistance (45).

1.5. Central mechanisms of appetite control

Appetite is regulated by a complex system of central and peripheral signals which interact in order to modulate the individual response to nutrient ingestion. Peripheral regulation includes adiposity and satiety signals, while central control is accomplished by several effectors, including neuropeptidergic and monoaminergic systems.

1.5.1. Hypothalamic circuitry

Hypothalamus has been identified as the area responsible for appetite-regulating pathways. The hypothalamus can be divided into a nucleus of several highly diversified neuronal populations, such as lateral hypothalamic nuclei (LHA) considered to be the "hunger" center and ventromedial nuclei (VMN), the "satiety" center (Fig. 6). In addition, paraventricular nuclei (PVN), arcuate hypothalamic nuclei (ARC) and the perifornical area (PFA) play decisive roles in the regulation of food intake and energy expenditure since hormones released from the gut and the adipose tissue converge into these areas (8, 39).

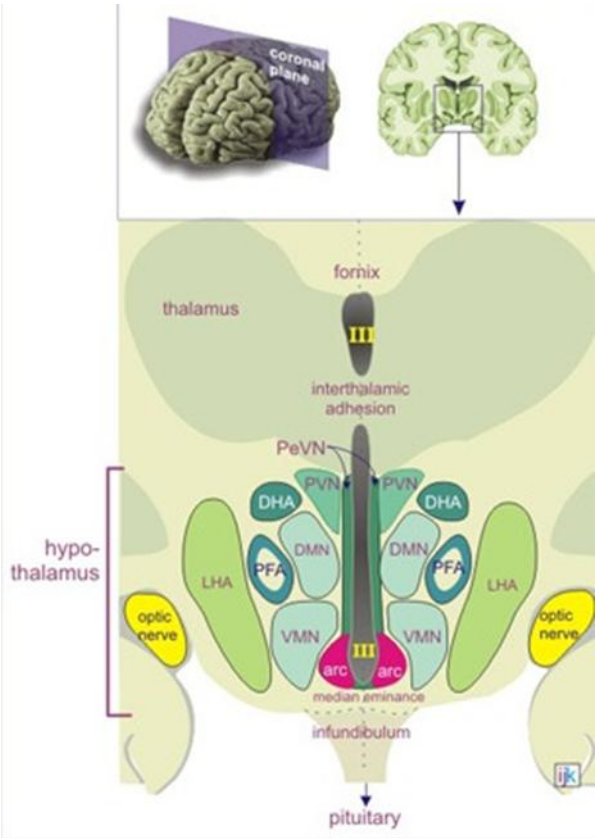

Fig. 6: The main hypothalamic regions involved in the regulation of food intake in the rat brain:

-ARC
-PVN
-PFA
-fornix (FX)
-LHA ("hunger" center)
-VMN ("satiety" center)
-dorsomedial nuclei (DMN) (46).

At the crossroads of CNS and peripheral components, there are two populations of first-order neurons within the ARC:

- anorexigenic peptide-containing neurons such as α-melanocyte-stimulating hormone (α-MSH) (a product of pro-opiomelanocortin or POMC gene) and cocaine and amphetamine-related transcript (CART),
- orexigenic peptide-containing neurons, namely neuropeptide Y (NPY) and Agouti-Related Peptide (AgRP).

Both circuits integrate circulating adiposity signals (insulin and leptin) and other blood-borne indicators of energy status (e.g. glucose, fatty acids, amino acids, gut hormones) and influence energy homeostasis. Their projections lead to other feeding centers in the hypothalamus (LHA, PVN, PFA) which are locations of second-order hypothalamic neuropeptide neurons. Corticotropin-Releasing hormone (CRH), Thyrotropin-Releasing hormone TRH, and oxytocin (OXY) are produced in PVN, while Orexins and Melanin Concentrating hormone (MCH) are secreted in PFA and LHA (35, 47).

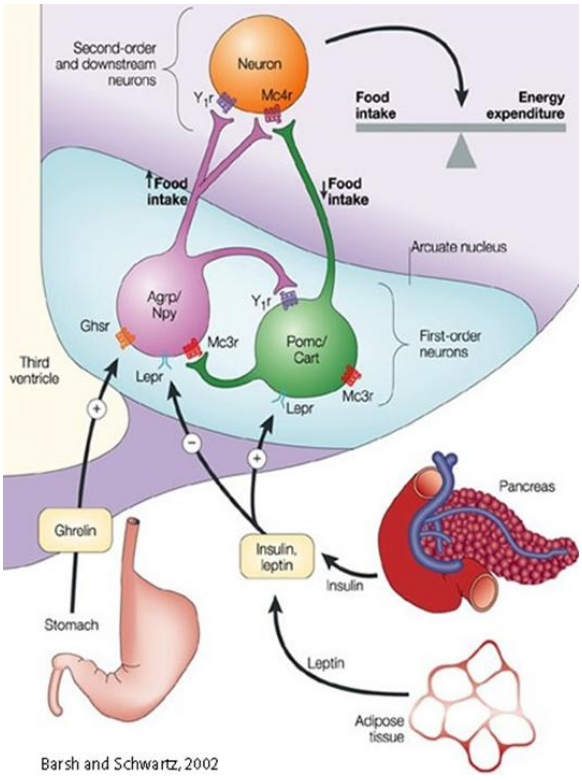

Fig. 7: Schematic diagram of ARC structure. Both insulin and leptin activate the POMC and CART neurons via their respective receptors (insulin-IR, leptin-ObRb), resulting in the release of α-MSH, which, in turn, activates downstream ”catabolic“ neurons, resulting in reduced food intake and increased energy expenditure. In contrast, the NPY/AgRP expressing neurons are stimulated by decreasing insulin and leptin levels and promote increased food intake and metabolic efficiency. The same effect promotes the gastric hormone ghrelin by activating its receptor on NPY/AgRP neurons. First-order neurons are projected to other brain areas, including PVN, zona incerta, PFA and LHA, which are locations of second-order neurons (35).

OREXIGENIC NEUROPEPTIDES

- *Neuropeptide Y*

NPY, the most potent orexigenic signal, is a 36 amino-acid peptide found throughout the peripheral and central nervous systems. At least six NPY receptor subtypes exist (Y1 to Y6), but only Y1 and Y5 are believed to play a role in appetite control. Centrally, NPY also acts in increasing the respiratory quotient and decreasing energy expenditure; while peripherally, NPY works to elevate leptin and mRNA levels of its receptor in adipose tissue (48).

- *Agouti-Related Peptide*

The AgRP is a 83-132 amino-acid protein found primarily in the ARC. It enhances appetite by antagonism of MC4R (type 4 melanocortin receptor) activation in the PVN. The expression of AgRP is elevated by fasting but supressed by leptin. Although NPY is described as the most potent orexigenic molecule, its effects are short-lived in comparison to those of the AgRP (8, 49).

- *Melanin concentrating hormone*

MCH seems to play an important role in body weight regulation because central administration of this peptide increases food intake, whereas targeted deletion of MCH or its receptor causes a weight-reduced, lean, hypermetabolic phenotype. Its expression is elevated by fasting and leptin deficiency. MCH-knockout mice have reduced food intake and are excessively lean (50, 51).

- *Orexins*

Orexins (also called hypocretins) are novel neuropeptides which were found to play a role in the stimulation of food intake and energy homeostasis. Orexin A and orexin B have been detected in the CNS, especially in LHA – the region known to be the feeding center, but also in the mucosa and neuronal gastrointestinal plexuses (GIT). Recent findings indicate that these neurons are also involved in the states of sleep and wakefulness, the coordination of emotion, reward, drug addiction, and arousal (8, 52).

ANOREXIGENIC NEUROPEPTIDES

- *Melanocortins*

Melanocortins are peptide-cleavage products from the pro-opiomelanocortin (POMC) precursor molecule. These peptides bind to the melanocortin family of receptors (MC3R and MC4R, which are located in ARC, PVN and LHA) and promote the supression of food intake. The main ligand for MC3R and MC4R is α-melanocyte-stimulating hormone (α-MSH).

There is a growing list of factors (e.g. corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH), cocaine and amphetamine-regulated transcript (CART) and interleukin-1β) which promote negative energy balance. Neuronal synthesis of these peptides is more important in response to an increased adiposity signaling in the brain (47, 53).

1.5.2. Peripheral signals and their neuronal targets

1.5.2.1. Adiposity signals: insulin and leptin

The main molecules that fulfill several criteria to be considered as adiposity signals are insulin and leptin. Their influencing consequences on food intake and body weight can be explained by identifiable signal transduction mechanisms (47).

- ***Insulin***

Insulin is a small protein composed of 51 amino acids and the major hormone secreted by pancreatic β-cells. Plasma insulin levels vary with the peripheral insulin sensitivity and the adiposity content. Insulin secretion increases rapidly after a meal. Following a receptor-mediated process, the hormone penetrates the blood-brain barrier. In the brain, insulin acts as an anorexigenic signal and food intake is inhibited. This hormone binds to its receptor (IR), present in most cells. IRs are widely distributed in the brain, with the highest concentrations found in the olfactory bulbs and the hypothalamus. Within the hypothalamus, there is a particularly high expression of insulin receptors in the ARC; they are also present in the DMH, PVN, and suprachiasmatic and periventricular regions (54, 55).

The insulin receptor exists as two splice variants: subtype A, with higher affinity for insulin and more widespread expression, and subtype B, with lower affinity and expression

in classical insulin responsive tissues such as fat, muscle and liver. There are several insulin receptor substrates (IRSs), including IRS-1 and IRS-2, both identified in neurons. Insulin and leptin exert their effects, sharing common intracellular signaling pathways via IRSs and the enzyme phosphatidil inositol 3-kinase (PI3K), resulting in downstream signal transduction (56).

- ***Leptin***

Leptin, a 16-kDa protein hormone, is produced predominantly in adipose tissue, but also at a lower level in gastric epithelium and placenta. The leptin receptor has multiple isoforms, which can be divided into three classes: long, short and secreted. The long form Ob-Rb is necessary for the effect of leptin on appetite. It acts also on afferent vagal nerves and directly on ARC neurons enhancing satiety. Leptin, like insulin, is thought to inhibit expression of orexigenic NPY/AgRP hypothalamic neurons and stimulate anorexigenic POMC/CART neuronal pathways (8, 39, 50).

- ***Insulin and leptin signaling pathway***

The binding of insulin to the extracellular α-subunit of IR results in the activation of tyrosine kinase activity, intrinsic to the intracellular domain of IR β-subunit. Following this autophosphorylation of specific tyrosine residues, IR phosphorylates a number of intracellular substrates, such as IRS-1 and IRS-2. At this time, an intracellular signaling pathway initiates. In peripheral tissues such as liver, fat and muscle, activation of the IRS-PI3K pathway is crucial for the insulin stimulation of glucose uptake and other metabolic responses. Moreover, impaired signal transduction is implicated in the pathogenesis of insulin resistance in common metabolic disorders, such as obesity and type 2 diabetes. The ARC region acts by inhibiting NPY/AgRP neurons and stimulating POMC/CART neurons, thus reducing food intake. The same effects are produced by leptin, which is transported via a saturable process across the blood-brain barrier. Its receptor is a member of the class 1 cytokine receptor superfamily. Based on this discovery, leptin receptor-mediated cell signaling was hypothesized to involve the ”JAK-STAT“ transduction cascade (Janus tyrosine Kinase-Signal Transducer and Activator of Transcription) (39, 50).

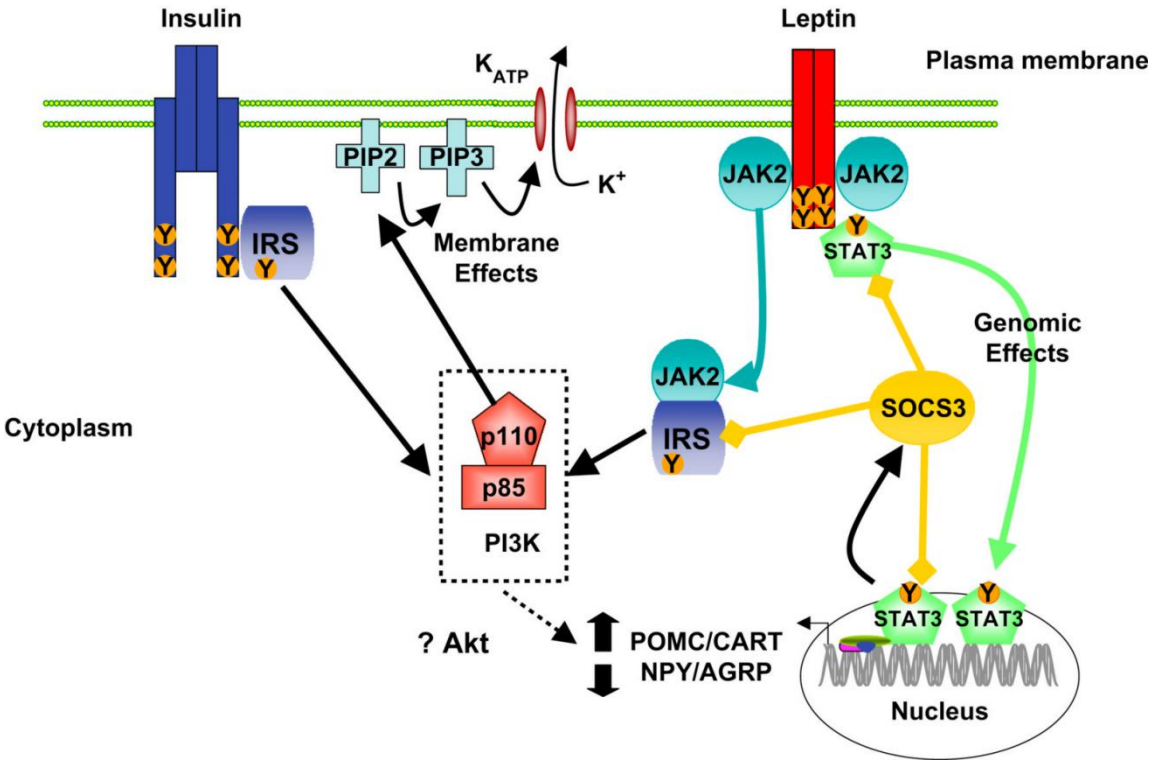

Fig. 8: Cross-talk between insulin and leptin signaling in the hypothalamus. Insulin, binding to its cell surface receptor, induces phosphorylation of the insulin receptor (IR) and several IR substrates (IRS), such as IRS-1 and IRS-2. IRS-1 tyrosine phosphorylation allows for the recruitment of PI3K, which catalyzes the formation of lipid phosphatidylinositol-3,4,5-triphosphate (PIP3) at the plasma membrane. This activates several Ser/Thr protein kinases, including Akt, which phosphorylates and prevents the translocation of the transcription factor to the nucleus. Studies also indicate a cross-talk between leptin and insulin signaling in the hypothalamus. The binding of leptin to its receptor results in the autophosphorylation of JAK2, which interacts with insulin via IRS phosphorylation of the tyrosine residues, activation and nuclear translocation of STAT3, and transcription of neuropeptides. The receptor of leptin also binds the suppressor of cytokine signaling (SOCS)3, which inhibits leptin signaling (57)
The final effect of insulin and leptin binding to their receptor is the inhibition of NPY/AgRP and the stimulation of POMC/CART, which means the reduction of food intake.

1.5.2.2. Satiety signals

During nutrient ingestion, short-term satiety signals like Cholecystokinin (CCK), Peptide YY_{3-36} (PYY_{3-36}), Glucagon-like Peptide-1 (GLP-1) are released into the circulation and promote satiety by activating neurons in hindbrain areas such as the nucleus of the solitary tract (NTS). Effects occur indirectly through activation of parasympathetic afferent neurons in the vagus nerve. This response plays a crucial role in meal termination and is, therefore, an important determinant of the amount of food consumed during individual meals (47, 58).

1.5.2.3. Other gut hormones

- ***Ghrelin***

The 28-amino acid gastric hormone ghrelin is the major gastrointestinal hormone with potent orexigenic properties, produced primarily by endocrine cells in an empty stomach. It serves as an endogenous ligand for growth hormone secretagogue receptors (GHS-R). The mechanisms of ghrelin action on appetite is suggested to be mediated through peripheral input at the ARC (where it increases the expression of NPY/AgRP neurons) and further spread to the NTS. Recent findings have shown that ghrelin negatively controls plasma release of leptin and vice-versa. This hypothesis is called "argentinian ghrelin-leptin tango". Circulating ghrelin levels also appear to reflect body weight changes over the longer term, raising the possibility that ghrelin functions as an adiposity signal (59, 60).

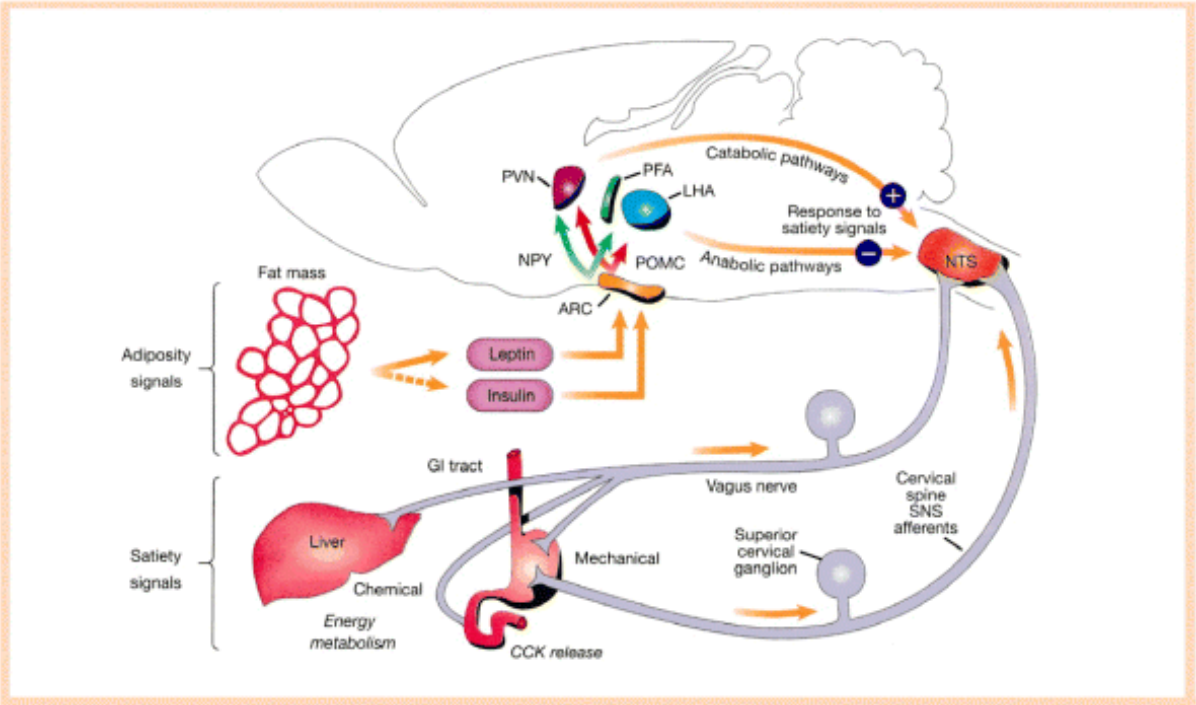

Fig. 9: Neuroanatomical model of appetite regulating pathways. Leptin and insulin are proposed to stimulate a catabolic pathway (POMC/CART neurons) and inhibit an anabolic pathway (NPY/AGRP neurons) that originates in the hypothalamic arcuate nucleus (ARC). These pathways project to the PVN and LHA/PFA, where they make connections with central autonomic pathways that project to hindbrain centres, which process satiety signals.
Afferent input related to satiety from the liver, GIT and from peptides such as CCK is transmitted through the vagus nerve and sympathetic fibres to the NTS, where they are integrated with descending hypothalamic input. Net neuronal output from hindbrain regions leads to the termination of individual meals, and is potentiated by catabolic projections from the PVN and inhibited by input from the LHA/PFA. Reduced input from adiposity signals, therefore, increases meal size by reducing brainstem responses to satiety signals (47, 61).

1.6. The impact of early undernutrition on appetite-regulating factors

Signals, factors and pathways involved in energy homeostasis have been described in the previous chapter. Alteration of one or more relevant factors during early development plays a major role in the programming of obesity and co-morbidities in adulthood.

1.6.1. Impact on central mechanisms

An altered environment during critical periods of development may impair the proper maturity of neuronal circuits that regulate food intake. Although little is known about the advance of these regulating appetite pathways and factors (such as NPY and POMC) along individual ontogeny, several studies have proven that development of these circuits occurs late in gestation and continues postnatally. The hypothalamus is a flexible organ and appears highly susceptible to changes at this part of development, for example, its neuronal circuits.

The ARC is the main regulating nuclei of food intake and energetic expenditure in the hypothalamus. As it has been previously described, ARC contains orexigenic neurons which co-express NPY and AgRP, and anorexigenic neurons which co-express POMC and CART. All of these populations of neurons present leptin receptors on their surface (62). Several studies in rodents have shown an altered organization of these hypothalamic circuits under inadequate diets submission (63-65). Density impairment of NPY neurons and an overexpression of NPY gen in fetal and neonatal tissues support the idea that NPY is a very important target in the programming of perinatal development (65, 66). Furthermore, anorexigenic pathways are also targets of nutritional programming, since caloric restriction of mothers caused diminished expression of POMC mRNA in the offspring, specifically in ARC and PVN (67, 68). Therefore, as maternal nutrient restriction can modify the peripheral factors which act on the hypothalamus of pups, this could have deleterious consequences for the hypothalamic expression of several target genes, inducing alterations in the long term in energetic balance and food intake.

1.6.2. Impact on insulin and leptin

Leptin and insulin play significant roles in the development of the appetite regulating system. In neonates, leptin has a neurotrophic effect on ARC neurons and it is probable

that leptin is in charge of promoting the projections from this structure to the PVN nucleus, DMN hypothalamus, and lateral hypothalamus (69).

While rodents are suckling, leptin is unable to alter feeding or energy expenditure (70). During the neonatal period, a surge of leptin occurs, whose origin as yet remains unknown (71). However, this neonatal hyperleptinemia is not able to affect growth, food intake or energy expenditure in mice and rats, as the neuronal circuits are still not developed (72, 73). Recently it has been suggested that this leptin surge is actually an important signal for the initiation of the development of ARC projections in the rodent (74).

Other studies have evidenced that maternal intrauterine undernutrition caused a premature surge of leptin in the offspring (68, 75). They also found impaired leptin transport to the brain and higher density of nerve terminals in the hypothalamus of these undernourished pups (75). Some studies in rodents have revealed that leptin treatment during the early postnatal period caused abnormal expression of NPY, AgRP and POMC in the ARC (73). Collectively, these findings suggest that leptin is required during critical periods of development and both deficiency and excess can have long-term detrimental effects on the hypothalamic circuitry that regulates energy homeostasis.

Insulin receptors are also highly expressed in the fetal brain of rodents and humans, however, this expression declines during the postnatal period (76). Abnormal insulin levels during a critical period of development may cause long-term defects in the regulation of energy homeostasis (77, 78). Insulin may also be an important trophic factor; however, more studies are needed to determine its role in the development of feeding circuits.

1.6.3. Impact on adipose tissue

Adipose tissue development can also be affected during the fetal and postnatal periods. The development of adipose tissue starts *in utero*, where adipocytes have the ability to develop into either brown or white adipose tissue (WAT) (79). The main function of brown adipose tissue (BAT) is to convert energy into heat (80), whereas the WAT represents an endogenous energy store that is capable of secreting a number of mediators involved in the regulation of energy metabolism, neuroendocrine function and immune function (81). Actually, an acquisition of brown-like phenotype instead of WAT normal pattern has been found in weaning rats whose mothers where submitted to a 50% food restriction (41). Over the last decade, WAT has become recognized as an important endocrine organ, able to secrete a vast number of hormones as well as expressing numerous receptors (81, 82).

2. THE OVERALL AIM OF THE STUDY

Data from clinical-epidemiological and animal studies has established that early undernutrition constitutes a risk factor for adult obesity, provided that the previously undernourished subject has energy-dense foods available in abundace later in life. To explain this, the following hypothesis has been proposed by various authors: early undernutrition could induce chronic alterations in the hypothalamic mechanisms involved in the regulation of food intake and satiety. Given these considerations, the present Graduation Thesis will focus on the impact of early undernutrition on hypothalamic appetite regulating factors by using an experimental model of early undernourished Wistar rat.

First of all, the general characteristics of undernourished animals will be compared, such as body weight and plasma glucose, with those of the controls.

Secondly, we will investigate whether the hypothalamic signaling of two peripheral anorexigenic factors, insulin and leptin, is altered by undernutrition. To this end, the insulin receptor, its phosphorylated (active) form, insulin receptor substrates (IRS1/2), leptin receptor (Ob-Rb) and transcription factor STAT-3 will be quantified in control and undernourished rats by Western blotting.

Appetite control, as a complex system, also comprises central mechanisms, being arcuate hypothalamus the main region involved. In the third part we will examine the effects of undernutrition on the hypothalamic expression of two proteins, anorexigenic NPY and orexigenic POMC, by means of real time PCR in newborn (1 day of life), suckling (10 days of life) and adult (70 days of life) rats. Also, we will determine the POMC content in the hypothalamus of both groups of rats by Western blot.

Finally, we will carry out an immunohystochemical approach to locate NPY in the hypothalamus of suckling and adult animals, in order to check for possible differences between the two populations of rats.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. *The animals and their diet*

To carry out the present study we have used Wistar rats from the animal house of the Department of Biochemistry and Molecular Biology II at the Faculty of Pharmacy, University Complutense, Madrid. The rats were bred at a constant temperature of 23 ± 1°C with artificial light/dark cycles of 12 hours. The females were caged with males, and mating was confirmed by the presence of spermatozoa in vaginal smears. Only females were selected, which were then housed individually from the 14th day of pregnancy, at which time food-restriction was established. The number of pups in each litter was evened to eight. Rats were fed a commercial standard laboratory diet (*Sandermus S-10, Sanders, Barcelona, Spain*). The composition of this diet, expressed in percentages, is as follows:

Proteins.....19%
Carbohydrates.....56%
Lipids.....3,5%
Celulose.....4,5%
Vitamins and minerals.....5%
Water.....12%

} This mix has a value of 2,54 kCal/g.

The rats were killed by decapitation without anesthesia, blood was harvested from the trunk and plasma was stored at -80°C. For the biochemical analysis, the brains were rapidly removed and placed on a chilled surface. The cerebral cortices were dissected out quickly over ice and kept at -80°C until assays were performed. All the animals were handled in accordance with the principles and procedures approved by the Committee for Animal Experimentation of the Complutense University of Madrid.

The pattern of undernutrition

On the basis of the amount of food supplied to the rats, two experimental groups were established:

- CONTROL RATS: these rats proceeded from parents always fed a standard diet *ad libitum*. After weaning, these animals were placed in separate cages and fed a standard diet *ad libitum.*

- UNDERNOURISHED RATS: the model of undernutrition is based on a restriction of the amount ingested daily by the controls, specifically 35% of the control value. It was established from the 14th day of pregnancy onwards and continued during suckling and after weaning, until adulthood.

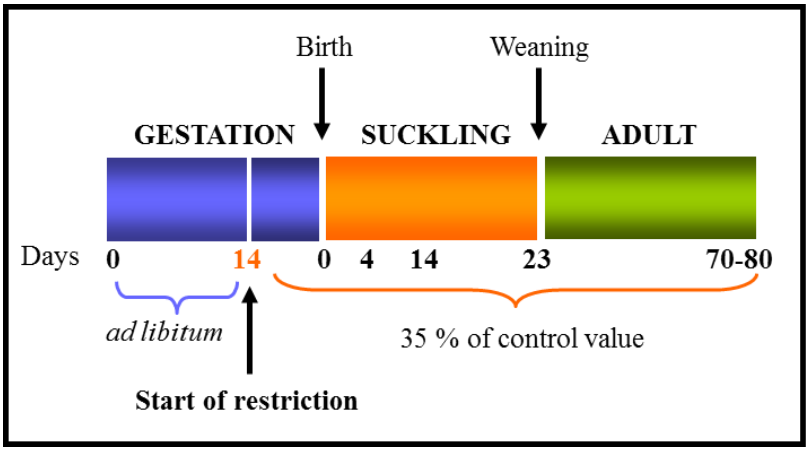

Fig. 10: Model of restriction.

Therefore, during the first two phases mentioned, the nutritional deficiency of pups was indirect, being the result of the dam's restriction. Pregnant rats received 10 g daily of the standard food until delivery. Lactating mothers received 15, 20 and 25 g daily during the 1st, 2nd and 3rd week of lactation, respectively. After weaning, undernourished rats were fed the amounts of food listed in Table I. Water was provided *ad libitum*.

Table I: Quantity of food per pup in adult undernourished rats.

Days of life	***Amount of food (g)***
25 - 29	3
30 - 34	3.75
35 - 39	5
40 - 44	5.4
45 – 49	6
50 - 54	6.2
55 - 59	6.4
60 - 64	6.75
65 - 69	6.8
70 - 74	7

3.1.2. *Chemicals and reagents*

- 2-propanol: *Panreac, Barcelona, Spain*
- Acrilamide/Bisacrilamide 40% solution 19:1, electrophoresis purity reagent: *Bio-Rad Lab., California, USA*
- Agarose: *Pronadisa, Madrid, Spain*
- Ammonium Persulfate (APS): *Sigma - Aldrich, Spain*
- Aprotinin, from bovine: *Sigma - Aldrich Co., Steinheim, Germany*
- Benzamidine: *Sigma - Aldrich Co., Steinheim, Germany*
- Bio-Rad Protein Assay (dye reagent concentrate): *Bio-Rad Lab. München, Germany*
- Boric acid: *Panreac, Barcelona, Spain*
- Bromphenol blue: *Sigma, St.Louis, MO, USA*
- β-mercaptoethanol: *E.Merck, München, Germany*
- Chloroform (trichloromethane stabilized with ethanol): *Panreac, Barcelona, Spain*
- Commercially filtered formaldehyd: *E.Merck, Darmstadt, Germany*
- Dako-Pen (liquid blocker): *Sigma Aldrich Chemie GmbH, Steinheim, Germany*
- D-limonene (xylene substitute): *DIAPATH, Martinengo (Bg), Italy*
- Deoxycholic acid (sodium salt): *Sigma Aldrich Co., Steinheim, Germany*
- Di-Sodium-hydrogen phosphate dyhidrate: *E.Merck, Darmstadt, Germany*
- ECL (Enhanced Chemiluminiscence Reagent): *Amersham Life Science, Little Chalfont, Buckinghamshire, UK*
- Ethanol absolute: *Panreac, Barcelona, Spain*
- Ethylene-diamine-tetraacetic acid – EDTA: *Sigma-Aldrich Chemic Gmbh, Steinheim, Germany*
- Ethidium Bromide solution (10 mg/mL in H_2O): *Sigma-Aldrich, St.Louis, MO, USA*
- Eukitt mounting reagent (poly(butyl methacrylate-co-methyl methacrylate)): *Panreac, Barcelona, Spain*
- Glacial acetic acid: *Panreac, Barcelona, Spain*
- Glycine: *Panreac, Barcelona, Spain*
- Hydrogen peroxide 30% aqueous solution: *Merck, Dermstadt, Germany*
- Leupeptin hemisulfate salt: *Sigma - Aldrich, St.Louis, MO, USA*
- Methanol: *Panreac, Barcelona, Spain*
- Non-fat dried milk: *Asturiana, Siero, Spain*

- NP-40 (Nonidet P40): *Roche Diagnostics, Indianapolis, USA*
- Orthovanadate: *Sigma – Aldrich, St.Louis, MO, USA*
- Paraffin pellets*: Panreac, Barcelona, Spain*
- Picric acid, aqueous solution: *Sigma - Aldrich Co., Steinheim, Germany*
- Potassium Chloride: *Sigma - Aldrich Co., Steinheim, Germany*
- Potassium dihydrogen phosphate: *E.Merck, Darmstadt, Germany*
- PMSF (Phenyl-Methyl-Sulfonyl-Fluoride): *Boehringer, Mannheim, Germany*
- Polyvinylidene difluoride membrane (PVDF): *Millipore Corporation, Bedford, MA, USA*
- Sodium Chloride: *Panreac, Barcelona, Spain*
- Sodium Dodecyl Sulfate (SDS): *Sigma - Aldrich Co., Germany*
- TEMED (N,N,N',N'-tetra-methyl-ethylenediamine): *Bio-Rad Lab., California, USA*
- Tetra-Natrium-Diphosphate-Decahydrate: *E.Merck, Darmstadt, Germany*
- Tris® (Tris Hydroxymethyl Aminomethane): *Panreac, Barcelona, Spain*
- Trizol® Reagent: *Invitrogen, California, USA*
- Tween®20: *Sigma - Aldrich Co., Steinheim, Germany*
- Xylane cyanole: *Sigma, St. Louis, MO, USA*

3.1.3. *Antibodies*

- *Western Blot*

- Anti-βactin mouse monoclonal Ref. A5316 *(Sigma-Aldrich, St. Louis, MO, USA)*
- Anti-insulin Rβ(C-19)(SC711) rabbit polyclonal IgG *(Santa Cruz Biotechnologies, Spain)*
- Anti-IRS1 rabbit polyclonal Ref. 06-248 *(Millipore, Temecula, California)*
- Anti-IRS2 rabbit polyclonal Ref. 06-506 *(Upstate, Temecula, California)*
- Anti-ObRb rabbit polyclonal SC 1834 *(Santa Cruz Biotechnologies, Spain)*
- Anti-POMC rabbit polyclonal PAB 8938 *(Abyntek, Spain)*
- Anti-STAT3 rabbit polyclonal (C-20) SC-482 *(Santa Cruz Biotechnologies, Spain)*
- Anti mouse IgG peroxidase (A4416) *(Sigma-Aldrich, St. Louis, MO, USA)*
- Anti Rabbit IgG peroxidase (A6254) *(Sigma-Aldrich, St. Louis, MO, USA)*

- *Immunochemistry*

- NPY anti-rabbit IgG (C-20)-RSC-14728-R *(Santa Cruz Biotechnology, California, USA)*
- Goat anti-rabbit IgG (L+H) affinity purified antibody, peroxidase labeled *(Gaithersburg, USA)*

3.1.4. *Laboratory equipment*

- Agitator Agimatic-S, *J.P. Selecta, Barcelona, Spain*
- Centrifuge 5415-R, *Eppendorf, Hamburg, Germany*
- Glass slides for citochemistry and immunochemistry, *Superfrost Plus, Menzel Gläser, Termo Scientific, Braunschweig, Germany*
- Hystology paraffin bath and wax dispenser, *J.P. Selecta, Barcelona, Spain*
- Laser scanning densitometry, *Molecular Dynamics, Sunnyvale, California, USA*
- Microtome, *Leica RM 2125RT, Nussloch, Germany*
- Microscope, *Leica Microsystems, Wetzlar, Germany*
- Microscope, *Nikon Eclipse 80* (associated to image program *Archimed*), *Tokyo, Japan*
- pH meter GLP 21, *Crison, Nessler, Madrid, Spain*
- Pipetman (precision microliter pipette) – P2, P10, P20, P200, P1000, P5000: *Gilson, S.A.S., France*
- Platform Rocker STR-6; *Stuart, Nessler, Madrid, Spain*
- PowerPac™ 4.5, *Bio-Rad, Hercules, California*
- Precisa Gravimetrics AG, *XB-120A, Switzerland*
- Scale Sartorius, *Gottingen, Germany*
- Termoblock, *J.P. Selecta (S.A.), Barcelona, Spain*
- ThermoSpectronic, *Biomate 3, ThermoScientific, Waltham, MA, USA*
- Ultra-turrax (T 10 basic), *IKA® WERKE GmbH & Co.KG, Staufen, Germany*
- Veriti 96 well ThermoCycler, *Applied Biosystems, Carlsbad, California, USA*
- Vortex mixer, *Heidolph (Reax top), Schwabach, Germany*
- 7900HT Fast Real-Time PCR, *Applied Biosystems, Carlsbad, California, USA*

3.1.5. *Buffers and solutions*

SDS-PAGE

- *<u>RIPA buffer</u> = lysis buffer*

Tris base.....150 mg
NaCl.....220 mg
$P_2O_7Na_4 \times 10H_2O$.....55 mg
Sodium Deoxicholate.....12 mg
dH_2O.....20 mL
SDS (10% w/v).....250 µL
NP-40 (10% w/v).....2.5 mL

pH = 7,6 (adjusted with 2M NaOH and 2M HCl)

PMSF.....100 µL
Leupeptin.....25 µL
Orthovanadate.....50 µL
Benzamidine..... 50 µL
Aprotinin.....30 µL

phosphatase and protease inhibitors (added extemporarily)

- *<u>Bradford reactive</u> (for determination of protein concentration):*

Protein Assay (1:5 diluted with water; for each sample, we need 1 mL of this dilution)

- *<u>Laemmli buffer</u> 2× (for denaturation and reduction of proteins):*

Tris-Cl 0,5M (pH = 6.8).....3 mL
SDS.....480 mg
Glicerol 20% (v/v).....2.4 mL
Bromphenol blue.....15 mg
β-mercaptoethanol.....1.2 mL
dH_2O.....5.4 mL

- *<u>Running buffer for electroforesis (10×):</u>*

Tris base.....15 g
Glycine.....71 g
SDS 10% (w/v).....50 mL
dH_2O.....till 500 mL

- *10% (w/v) Ammonium Persulfate (APS):*

APS.....0.5 g

dH_2O.....5 mL

WESTERN BLOTTING

- *Buffer for electrotransfer:*

Tris base.....3.03 g

Glycine.....14.4 g

Methanol.....10 mL

dH_2O.....990 mL

- *TBS buffer 5× (Tris-buffered saline):*

NaCl.....8.8 g
Tris base.....1.21 g
dH_2O.....till 1L
} adjust pH = 8.0

- *TTBS buffer 0,05% (TBS + Tween®20) = washing buffer*

Tween®20.....250 µL

TBS 5×.....100 mL

dH_2O.....400 mL

- *Blocking buffer (5% PBS-milk):*

Tween®20 (25%).....200 µL

Non-fat dried milk.....2.5 g

PBS.....50 mL

- *PBS buffer*

NaCl.....40 g
KCl.....1 g
KH_2PO_4.....1 g
Na_2HPO_4.....5.7 g
dH2O.....1000 mL
} adjust pH = 7.4

- *Incubation solution (with the primary antibody):*

Primary antibody.....1:500

Tween®20 (25% v/v).....20 µL

PBS.....10 mL

- *Incubation solution (with the secondary antibody):*

Secondary antibody.....1:1000

Tween®20 (25% v/v).....36 µL

PBS.....18 mL

Non-fat dried milk.....180 mg

- *Solution for the detection with ECL reagents:*

solution 1: solution 2 = 1:1; V_{final} = 0.125 mL/cm^2 of membrane

RNA ISOLATION FROM HYPOTHALAMUS

- *Agarose gel:*

Agarose.....0.3 g

TBE 1×.....30 mL

BrEt (1 mg/mL).....3 µL

- *TBE buffer 1×:*

Tris base (0,89M).....107.8 g

Boric acid (0,88M).....54.4 g

EDTA (0,01 M).....4.52 g

dH_2O.....till 1L

- *LB 6× (loading buffer):*

Glicerol (20% v/v).....3 mL

Bromphenol Blue.....25 mg

Xylene cyanol.....25 mg

dH_2O..... .till 10 mL

HISTOLOGY

- *Bouin solution:*

Satured aqueous filtered picric acid.....75 mL

Commercially filtered formaldehyd 37 % (w/v).....25 mL

Glacial acetic acid.....5 mL

- *Washing buffer = Tris/NaCl 50 mM:*

NaCl.....9 g

Tris 0.5M.....100 mL ⟶ Tris base.....60.57 g

dH_2O.....900 mL dH_2O.....till 1L

3.1.6. *Analytical kits*

GLUCOSE OXIDASE METHOD

- Glucose oxidase/peroxidase *(Biosystems S.A. Costa Brava, Barcelona, Spain):*
  - Reagent
  - Glucose/Urea/Creatinin Standard

RT-qPCR

- High capacity cDNA Reverse Transcription kit (*Applied Biosystems, CA, USA):*
  - 10× Reverse Transcript Buffer
  - 25× dNTP Mix (100 mM)
  - 10× Random Primers
  - MultiScribe Reverse Transcriptase (50 U/µL)
  - RNAse Inhibitor
  - Nuclease-free water
- FastStart Universal Probe Master (ROX) – 2x-concentrated master mix for qPCR *(Roche, Penzberg, Germany):*
  - FastStart Taq DNA Polymerase
  - Reaction Buffer
  - Nucleotides (dATP, dCTP, dGTP, dUTP)
  - Reference dye

HYSTOLOGY

- Kit from Inmunocruz Staining System (*Santa Cruz Biotechnology, CA, USA):*
  - Peroxidase block
  - Serum block (Goat serum)
- DAB substrate kit peroxidase: SK-4100 (*Vector Lab., Burlingame, CA, USA):*
  - Buffer stock solution
  - DAB stock solution
  - Hydrogen Peroxide solution

3.2. Methods

3.2.1. Determination of plasma glucose

Blood samples were treated with heparin as an anticoagulant. After centrifugation, each plasma sample was separated, and the glucose level was determined with the glucose oxidase method. By means of the coupled reactions described below, a red-coloured complex was measured using spectrophotometry. Absorption (A) of this chromogen is positively related with the native amount of glucose in the sample determined by the glucose oxidase method.

$$\text{Glucose} + \tfrac{1}{2}\,O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{Gluconate} + H_2O_2$$

$$2H_2O_2 + \text{4-aminoantipyrine} + \text{Phenol} \xrightarrow{\text{peroxidase}} \text{Quinoneimine} + 4H_2O$$

Glucose level (C_{sample}) was calculated by the general formula:

$$A_{sample}/A_{standard} \times C_{standard} = C_{sample} \qquad [1]$$

The reactifs were provided by a kit by *Biosystems.*

3.2.2. Development of the Western blotting procedure

Sample preparation

The lysis buffer RIPA was prepared as described in section *3.1.5*. We transferred the hypothalamus to an eppendorf tube with 200 µL of RIPA, previously weighed. The tissue was homogenized with ultraturrax (on speed 4) and placed on an orbital shaker in the cold room for 30 min. The supernatant was transferred to another tube and kept on ice.

Analysis of the protein concentration

The Bradford assay is a frequently-used method for protein determination. The original solution has to be diluted with water in a 1:5 proportion. 1 mL is needed for each sample. γ-globulin was used to prepare the calibration curve, which had 6 different concentrations: 5, 10, 20, 30, 40, 60 μg/μL. A dilution of Bradford was the blank value. The protein concentrations of samples were calculated from the absorbance values of the calibration curve after spectrophotometry.

SDS-PAGE

Two gels with different acrylamide/bisacrylamide concentrations were prepared, as indicated in the Table II. The concentration of running gel depends on molecular weights of proteins to be analyzed, ranging from 5% to 12%.

Table II: Preparation of gels for electrophoresis.

	RUNNING GEL (12%)	*STACKING GEL (5%)*
Acrylamide/Bisacrylamide	3.0 mL	0.625 mL
SDS 10% (w/v)	100 μL	50 μL
Tris-Cl 1,5M pH=8.8	2.5 mL	/
Tris-Cl 0,5M pH=6.8	/	1.25 mL
dH20	4.34 mL	3.04 mL
APS 10% (w/v)*	50 μL	25 μL
TEMED*	10 μL	10 μL

*These reagents were added extemporarily.

The solution for running gel was pipetted up to 4 cm from the top of the glasses. Then, a thin layer of water was deposited over it, which was removed after polymerization, about half an hour later. Each sandwich was filled with the stacking solution, the comb was inserted carefully in its place. Approximately half an hour later, the gels were ready for sample loading.

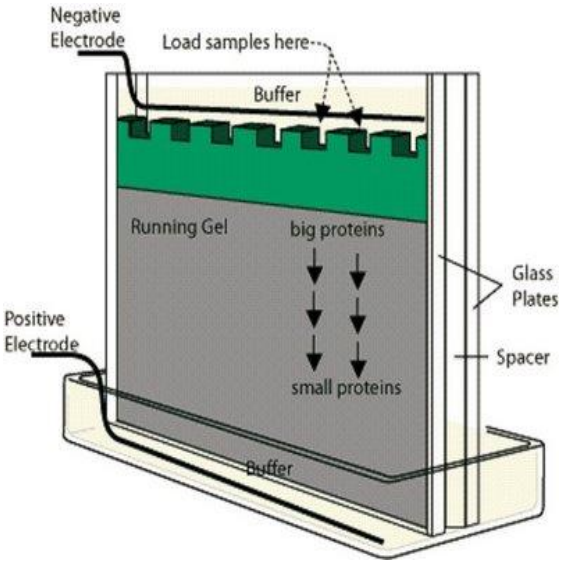

Fig. 11: View of an electrophoresis cell.

Laemmli buffer was mixed with samples in the adequate volume (each sample contained, for instance, 50 µg of proteins, in 10 µL of final volume). The mixture was heated in a Termoblock for 5 min at 95°C. The samples as well as a protein standard solution were loaded in the wells of the gel using pipette. Electrophoresis was carried out in the Bio-Rad Mini Protean Cell, following the manufacturer's instructions. The process was performed at 125 V and lasted 1-2 hrs.

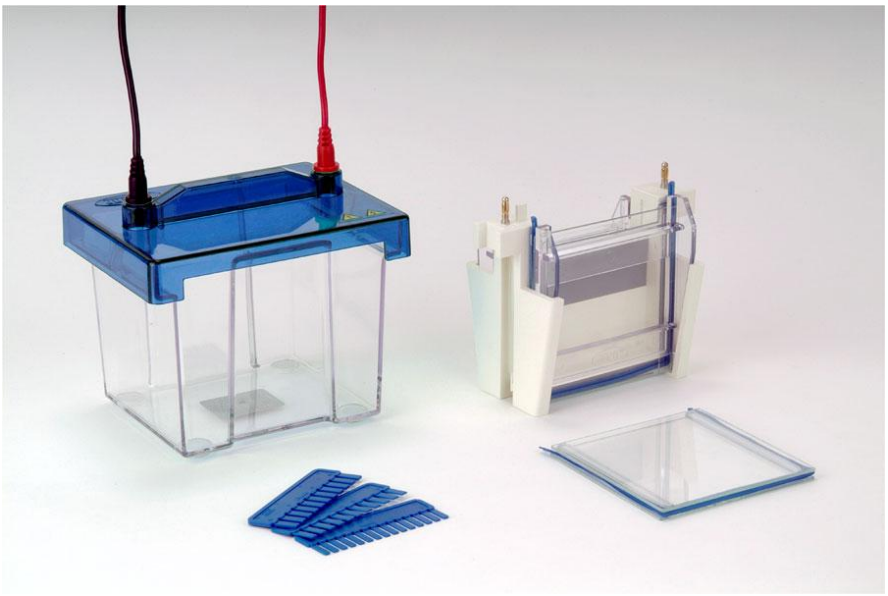

Fig. 12: Components of an electrophoresis cell: tank, plates, combs, lid (Bio-Rad Mini Protean Cell).

Electrotransfer of proteins

The proteins were electrophoretically transferred to polyvinylidene difluoride membranes (PVDF) with a cold transfer buffer. The transfer took place at 100 V for 2 h with continuous stirring. Finally, the membranes were blocked with 5% non-fat dry milk in Tris-buffered saline for 1.5 h at room temperature.

Incubations with antibodies

Following overnight incubation with the primary antibody in the cold room, the PVDF membranes were washed 4 times for 10 min each time with TTBS. Finally, the membranes were incubated with the appropriate secondary antibody, which was conjugated to horseradish peroxidase (*Sigma, St. Louis, MO, USA*) for 1 h at room temperature with continuous stirring.

Identification of proteins

At the end, the membranes were exposed to an enhanced chemiluminiscence reagent (ECL). The reaction occured where a protein formed a specific immunocomplex, leading to emission of light which was proportional to the amount of protein. The light was then detected by photographic film. Finally, the bands were quantified by laser scanning densitometry *(Image Gauge Software Ver. 4).*

Loading control

Beta actin was used as a loading control in each Western blot analysis by reprobing the blots with mouse anti-rat β-actin.

3.2.3. Development of the RT-qPCR procedure

RNA extraction from the hypothalamus

Total RNA from the rats' hypothalami was extracted by means of a protocol developed in our Department. In brief, hypothalami frozen in liquid nitrogen were grinded in a mortar and pestle and then transferred to eppendorf tubes containing 1 mL of trizol. This reactif helps to lyse the cells and maintains the integrity of the RNA (83). After vigorous vortexing for 30 s, the samples were incubated on ice for 5 min. In the next step – the separation phase - we added 200 µL of chloroform and repeated the vortexing, followed by incubation at room temperature to complete the dissociation of nucleoprotein complexes. Following centrifugation (15 min, 13000 rpm, 4°C), the mix was separated into three phases: a lower red - phenol-chloroform phase, an interphase and a colorless supernatant aqueous phase, where the RNA remained (83). The latter was carefully pipetted to a fresh tube, in which the RNA was precipitated by adding 500 µL of isopropyl alcohol. Samples

were mixed gently by inversion and kept overnight at -20°C. This last step can be shortened to 30 min at -80°C. After centrifugation (30 min, 13000 rpm, 4°C), the supernatant was aspired. The pellets were washed with 1 mL of 75% cold ethanol and centrifuged again (10 min, 13000 rpm, 4°C). At the end of the procedure, the RNA pellets were air-dried for 10 minutes and resuspended by pipetting up and down in aproximately 20 µL of RNAse-free water.

The RNA was quantified by spectrophotometry. 2 µL of the isolated RNA were diluted with 98 µL of dH_2O. RNAse-free water was used as a blank value. Absorbances at 260 and 280 nm were measured, and the ratio was used to assess the RNA purity of preparation. Pure RNA has a ratio of 2.1. Usually, 1.8 – 2 ratios indicate a high degree of purity.

The integrity of the RNA was assessed with agarose gel electrophoresis to ensure that the RNA had not degraded during the isolation procedure. Agarose gel was prepared as described in section *3.1.5*. The samples were prepared as follows: 1 µL of isolated RNA, 2 µL of buffer LSB 6× and 9 µL of dH_2O. This mixture was vortexed, spinned and deposited in the gel, running at 90 V for 30 min. As a final step, the gel was stained and visualized in a transilluminator.

Reverse transcription

Isolated and purified RNA was transformed to complementary DNA (cDNA) using a High capacity cDNA Reverse Transcription kit *(Applied Biosystems, California, USA)* according to the manufacturer's protocol with some modifications. It was demonstrated by our laboratory that the ideal concentration of cDNA needed for the qPCR was 80 ng/µL. It was calculated for a final volume of 50 µL, thus 4 µg of RNA were needed for the reaction, supposedly fully transformed into cDNA.

Previously, we had calculated the adequate volumes of RNA and nuclease-free water for each sample. To this mixture of 25 µL, 25 µL of MasterMix were added. cDNA was obtained by incubating mixtures in the thermocycler for 10 min at 25°C, followed by 2 h at 37°C.

RT-qPCR

The quantitative polymerase chain reaction is used to amplify the transcripts of cDNA previously obtained. This method is very sensitive and reliable for gene expression

analysis. TaqMan reactions for the target and housekeeping genes were prepared in separate tubes. The probe purchased was the rat primer for NpY *(TaqMan gen expression Assay, Applied Biosystems, CA, USA)* (Rn01410145_m1) and rat primer for POMC *(TaqMan gen expression Assay, Applied Biosystems, CA, USA)* (Rn00595020_m1). As the endogenous target, RpS 18 (Ribosomal protein S18) (Rn01428913_gH) was used. 4 μL of cDNA were used as template, mixed with 40 μL of nuclease-free water. From this mixture, 20 μL of each sample was replaced to another tube, while each reaction was performed in duplicate. Finally, we pippetted 19 μL of the mixture (cDNA, nuclease-free water, probe, MasterMix) into each well. The reaction was performed in the 7900HT Fast Real-Time PCR instrument (40 cycles, 95°C, 10 min each cycle).

The comparative threshold cycle (C_T) method was used to calculate the relative expression. For cuantification of gene expression, the target gene values were normalized to the expression of the endogenous reference (RpS 18). Thus, the amount of target relative to a calibrator is given by: $2^{-\Delta\Delta C}{}_T$ [$\Delta C_T = C_T$ (target gene) – C_T (RpS 18); $\Delta\Delta C_T = \Delta C_T$ for any sample – ΔC_T for the calibrator].

3.2.4. Immunohystochemical studies

Fixation and embedding of the hypothalamus

The rats' brains were extracted, removed and fixed in aqueous Bouin solution overnight at room temperature. The tissues were dehydrated through a series of graded ethanol baths at room temperature: two times per 1 h in 70%(w/v) alcohol, once per 30 min in 95% (w/v) alcohol and three times per 30 min in absolute alcohol. Then they were washed with xylene three times per 15 min and two times per 2h in parafin at 60°C. Finally, they were embedded into wax blocks.

Preparation of tissue sections for immunohistochemistry

5 μm thick tissue sections were obtained with a microtome, floated in a 37°C water bath and mounted on glass slides for cytochemistry and immunochemistry. The slides were dried overnight at room temperature (they can be stored either at room temperature or at 2-8 °C for several years).

Deparaffinization and rehydration

Before staining, the tissue sections were deparaffinized with D-limonene two times per 2 minutes and rehydrated with different alcohols: two times with absolute alcohol, once with 95%(w/v) alcohol and once with 70%(w/v) alcohol, each one per 2 min. The slides were rinsed in dH_20 and prepared for staining.

Immunohystochemical staining

Tissues were washed in a wash buffer (Tris/NaCl 0,05 M) for 2 min, dried and bordered with Dako-Pen. Then one of the following procedures was applied: the Immunocruz Staining System from *Santa Cruz* or our own protocol, which was the following. First, endogenous peroxidase was blocked with 30% hydrogen peroxide in Tris/NaCl, extemporarily prepared. After washing with Tris/NaCl two times for 2 min, the non-specific binding was blocked with 10% goat serum solution in Tris/NaCl for 10 min at room temperature, prior to the primary antibody incubation. This incubation was performed overnight at 4°C with different dilutions of a primary antibody (1/100, 1/250). The tissues were washed with Tris/NaCl two times forr 2 min and then incubated with a secondary antibody for 1 h at room temperature (dilutions of secondary antibody: 1/300, 1/500, 1/1000). Finally they were passed in dH_2O. Tris/NaCl was used as a negative control. Finally, tissues were revealed with DAB substrate as chromogen, according to manufacturer's instructions, till a brown coloration was obtained (approximately 4 min).

Dehydrating and mounting

After being washed in dH_2O, the tissues were dehydrated through a series of graded alcohols baths (70%(w/v) once, 95%(w/v) once, absolute alcohol twice) and through d-limonene (twice), each one for 2 min. Then they were mounted with eukitt, covered with coverslides and dryed overnight. After all these steps, the slides were ready to be seen under the microscope.

3.2.5. Statistical analysis

Data is reported as means ± SE. The difference between two mean values was assessed with a *t*-test. $P < 0.05$ was considered significant.

4. RESULTS

4.1. Characteristics of undernourished rats

The body weight and plasma glucose concentration of undernourished and control rats over the period studied in the present work are summarized in Table III. Newborn restricted rats weighed slightly less than controls, although the difference between the two groups did not reach statistical significance. During suckling, that is 10 days of life, undernourished rats had approximately 43% body weight deficit in relation to the controls. This deficit increased during development so that it was close to 65% in adulthood. Regarding plasma glucose concentration, maternal food restriction did not affect this parameter during pregnancy, as shown by the data corresponding to newborn animals (1 day of life). However, it was significantly below the control values during suckling and in adulthood (70 days of life), as shown in Table III.

Table III: Characteristics of newborn, suckling and adult rats in control and undernourished populations.

	NEWBORN			SUCKLING			ADULT		
	C	U	P	C	U	P	C	U	P
BW (g)	6.2 ± 0.1	5.9 ± 0.35	N.S.	21.8 ± 0,7	12.3 ± 0.2	***	211 ± 6	75 ± 5	***
PG (mg/ dl)	99 ± 9	100 ± 4	N.S.	153 ± 4	128 ± 3	***	146 ± 4	116 ± 7	**

Body weight (BW) and plasma glucose (PG) in control (C) and undernourished (U) rats at 1, 10 and 70 days of life (newborn, suckling and adult, respectively). Values are means ± SE of 6-8 independent determinations. **, $P < 0.01$; ***, $P < 0.001$.

4.2. Effects of early undernutrition on insulin and leptin hypothalamic signaling

4.2.1. *Insulin signaling*

To study the possible effects of early undernutrition on hypothalamic insulin signaling we examined some proximal steps of this pathway in both populations of rats, control and restricted; namely, the contents of the hormone receptor (β subunit) in its unphosphorylated and phosphorylated forms, as well as the contents of IRS-1 and IRS-2. These proteins were quantified by Western blotting. As shown in Figure 13, insulin

receptor abundance in the hypothalamus was not affected by undernutrition. Also, we did not find differences between undernourished rats and their controls regarding the phosphorylated form of this receptor. The same was true for the IRS-1 and IRS-2 proteins (Fig. 14).

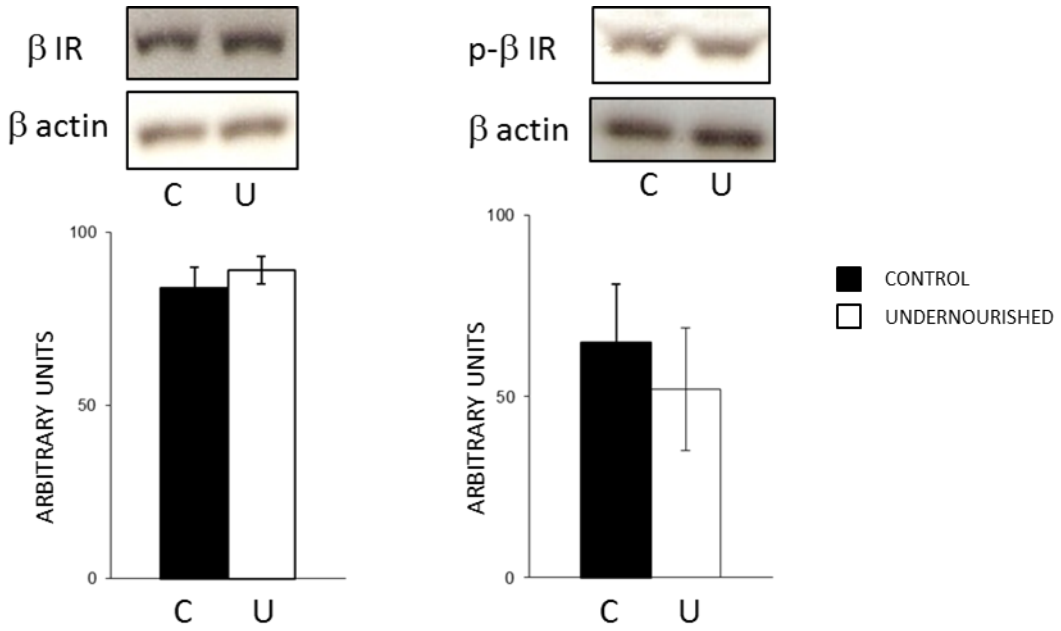

Fig. 13: Insulin receptor and its phosphorylated form in the hypothalamus of adult rats (70 days of life), control (C) and undernourished (U). Bars correspond to the mean ± SE for 6-8 independent determinations.

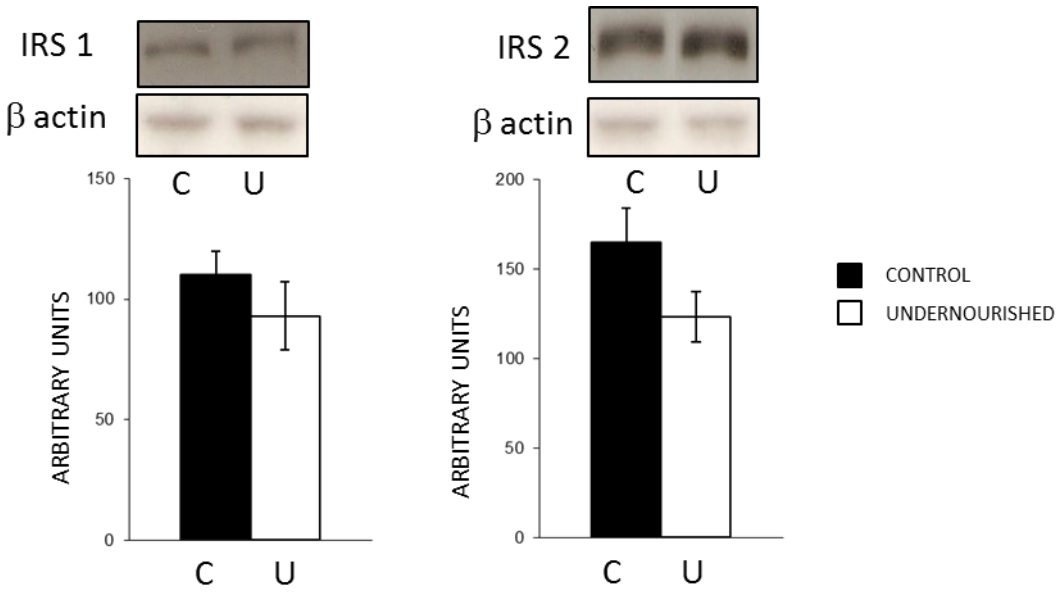

Fig. 14: IRS-1 and IRS-2 in the hypothalamus of control (C) and undernourished (U) adult rats. Bars correspond to the mean ± SE for 6-8 independent determinations.

4.2.2. *Leptin signaling*

One purpose of this study was to determine whether undernutrition alters hypothalamic responsiveness to leptin. To this end we analyzed the leptin receptor (Ob-Rb) as well as STAT-3, a protein which is phosphorylated and activated in response to this hormone. We found that the hypothalamic content of Ob-Rb was markedly decreased by food restriction (Fig. 15).

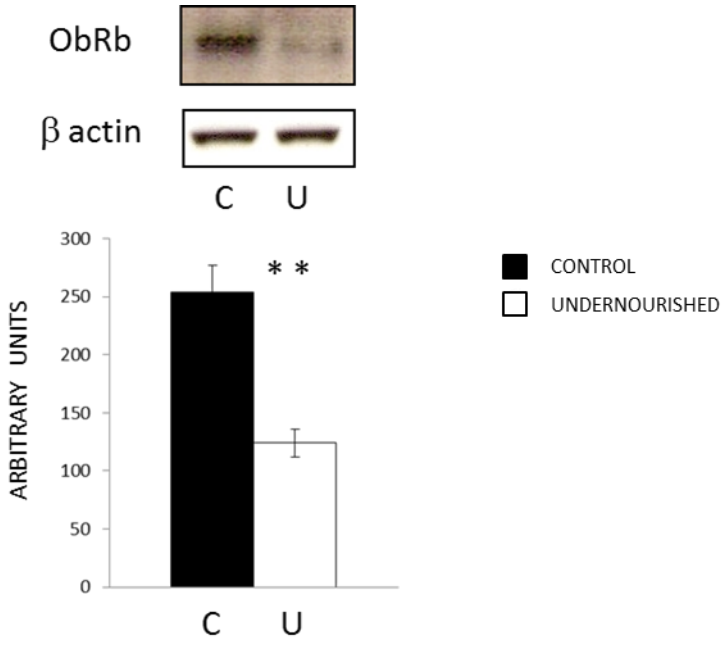

Fig. 15: Leptin receptor (Ob-Rb) in hypothalamus of control (C) and undernourished (U) 70-day-old rats. Bars correspond to the mean ± SE for 5 independent determinations. **, $P < 0.01$.

The results presented in Fig. 16 show that STAT-3 protein content in the hypothalamus of undernourished adult rats was significantly reduced as compared with their well-nourished controls.

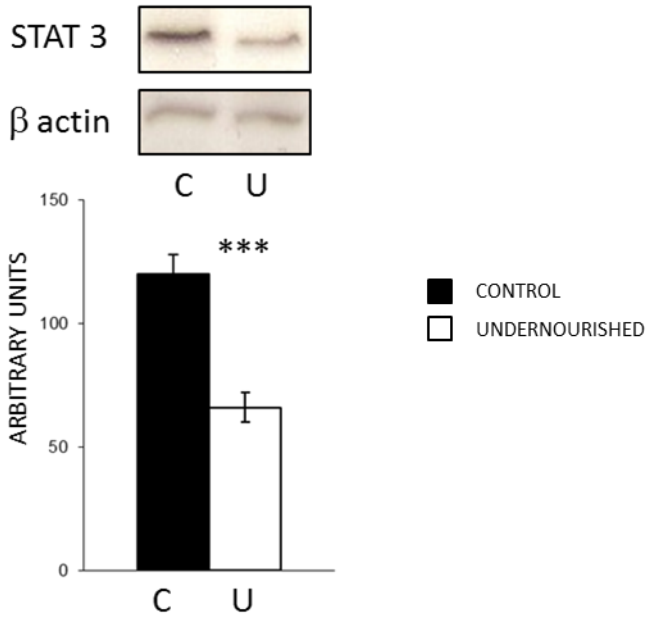

Fig. 16: STAT-3 content in the hypothalamus of control (C) and undernourished (U) adult rats. Bars correspond to the mean ± SE for 6-8 independent determinations. ***, P < 0.001.

4.3. Effects of undernutrition on hypothalamic POMC content

As shown in Fig. 17, undernutrition caused a marked decrease in the hypothalamic content of POMC, whose concentration was roughly 50% of that found in 70 day-old control rats.

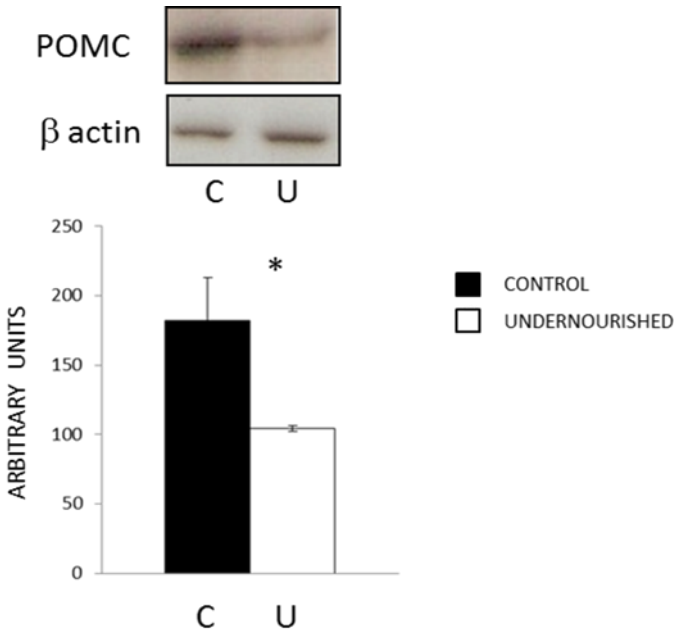

Fig. 17: Hypothalamic POMC content in control (C) and undernourished (U) 70-day-old rats. Bars are the mean ± SE for 5 independen detrminations. *, P< 0.05.

4.4. Effects of undernutrition on NPY and POMC expression in the hypothalamus

It is well known that hypothalamic neurons are implicated in the control of body weight and appetite, being the arcuate nucleus of one of the main regions involved. Consequently, we have examined the effects of undernutrition on the expression of two main proteins that participate in the orexigenic and anorexigenic mechanisms: NPY (neuropeptide Y) and POMC (pro-opiomelanocortin), respectively. Both analyses were made by real time PCR.
Undernutrition did not change the level of NPY expression in newborn nor in 10-day old rats, as seen in Fig. 18. However, the expression of this protein was markedly increased in the hypothalamus of food-restricted adult animals, reaching 370% of the control value.

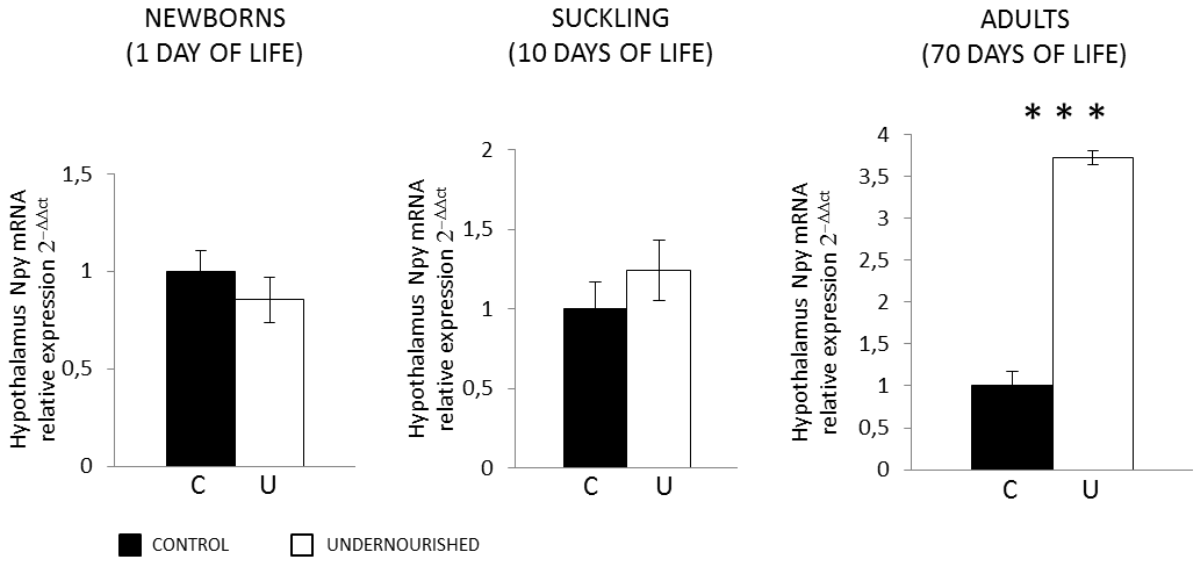

Fig. 18: NPY expression in the hypothalamus of newborns, suckling and adult rats, control (C) and undernourished (U). Values are expressed as the mean of 6-8 determinations ± SE. ***, P< 0.001.

Regarding POMC expression, we found no alterations in the hypothalamus of undernourished newborn rats. In contrast, both suckling and adult food-restricted animals experienced a significant decline: 50% and 60%, respectively, as compared to control values (Fig. 19).

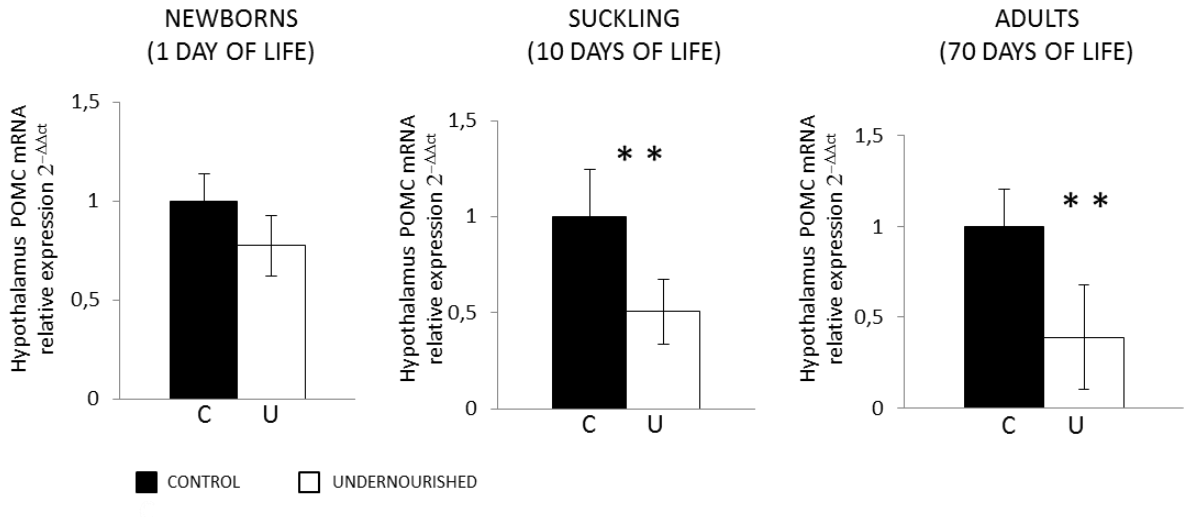

Fig. 19: POMC expression in the hypothalamus of newborns, suckling and adults rats, control (C) and undernourished (U). Values are means ± SE for 6-8 observations in each group. **, P < 0.01.

4.5. Qualitative immunohystochemical analysis of NPY

The results obtained in the immunohistochemical detection of NPY protein in several hypothalamic regions are presented in Figs. 21 and 22. Also, these regions are schematically depicted in Fig. 20.

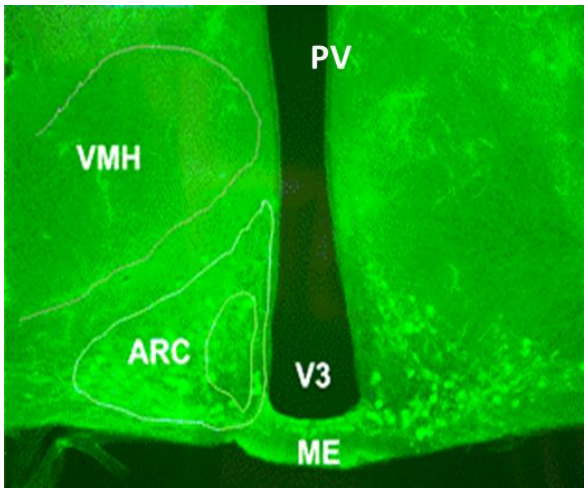

Fig. 20: A schematic cross section of an hypothalamic region surrounding the third ventricle. The locations of some representative nuclei are depicted:

V3: third ventricle,
ARC: arcuate nuclei,
ME: median eminence,
PV: paraventricular nuclei,
VMH: ventromedial nuclei.

NPY immunoreactivity was widely distributed throughout the areas studied. Small clusters of NPY neurons were observed in ARC regions of both control and suckling undernourished rats (10-day-old), being slightly more abundant in the restricted animals (Fig. 21). In the case of adults, the PV and ARC nuclei were much more immunostained in undernourished than in control rats (Fig. 22).

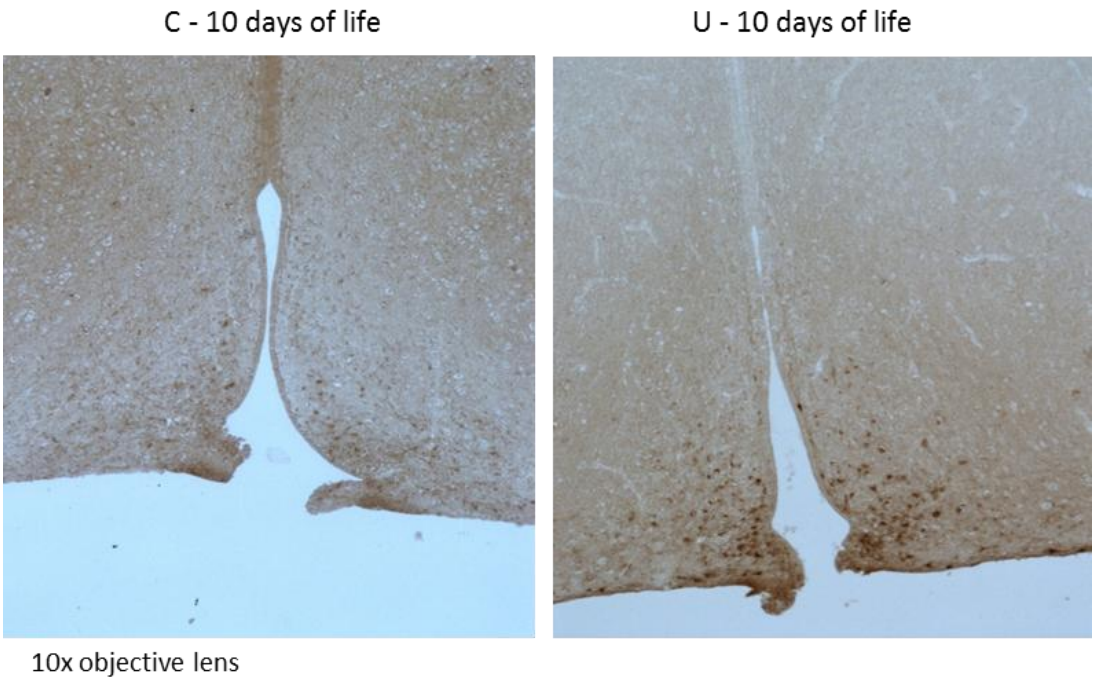

Fig. 21: Immunohistochemical staining of NPY protein present in the hypothalamus of control (C) and undernourished (U) 10-day-old rats.

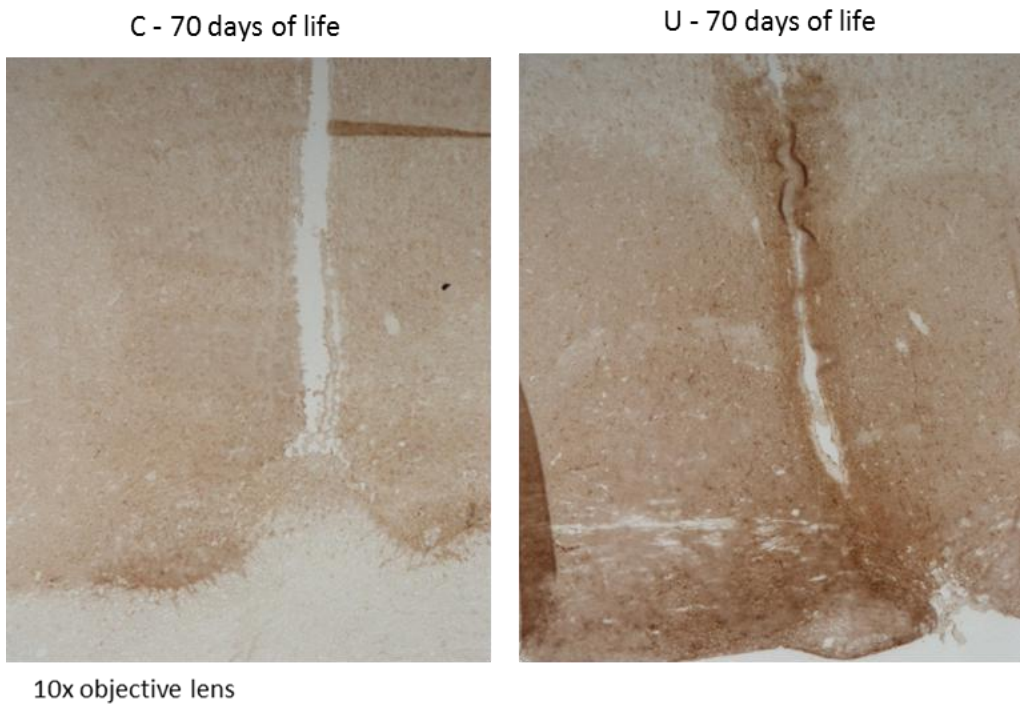

Fig. 22: Immunohistochemical staining of NPY protein present in the hypothalamus of control (C) and undernourished (U) 70-day-old rats.

5. DISCUSSION

5.1. Characteristics of undernourished rats

Food restriction during pregnancy, established in accordance with the present experimental model, induces only a slight decrease in the body weight of newborn rats which is not statistically significant, as shown by our results. Intrauterine growth is protected to some extent from adverse circumstances; this probably occurs in the case of undernutrition. We can speculate that some changes in placental characteristics may be produced as a result of adaptations to increase the delivery of substrates to the foetus, such as an improved blood flow; in that case, the deficient supply of food to the pregnant animals could be partially compensated, avoiding a more intense impairment in prenatal growth. In this context, it has been recently shown that the placenta from undernourished pregnant mice undergoes increases in the expression of glucose and aminoacid transporters, which helps to maintain the foetal growth (84). A similar change could be established in foetuses of food-restricted pregnant rats. Later, the autonomy of extra-uterine life diminished the possibility of successful adaptations, accentuating the effects of undernutrition on body weight, as shown by our data. During lactation, a large reduction in milk volume is associated with food restriction, as is well known (85); it mostly causes, undoubtedly, the significant growth retardation experienced by undernourished suckling rats. In fact, the body weight of these rats remained below the control values until adulthood. This situation mimics what actually happens in humans that are chronically undernourished from the early stages of development.

In the present work we do not provide data on brain weights. However, since the main objective of the present study is the hypothalamus, which is a part of the CNS, it seems necessary to highlight some aspects of this. It is well known that brain growth is partially preserved from deleterious effects of undernutrition, compared to other organs; so it is surprising that the brain to body weight ratio is enhanced in restricted suckling animals, a phenomena known as “brain sparing effect” (16, 86). This effect occurs despite the following: a) the fact that glucose is the main substrate for the brain; b) that undernourished rats are hypoglycaemic, as shown both by our present results and those from other authors (65). The cited research group has recently reported that the decrease in plasma glucose associated with undernutrition coincides, during suckling, with a rise in plasma ketone bodies, whose concentration remains two-fold above control values (87). It

should be noted that suckling is a stage in which the blood level of such compounds remains physiologically very high (as compared with adulthood). From the classical works of Krebs' laboratory it is known that ketone bodies are important substrates for the immature brain (88), which is capable of extracting and utilizing them at a rate many times greater than it is in adulthood. Consequently, we can speculate that the marked hyperketonemia characteristic of restricted suckling rats is one of the reasons why immature brain growth is better protected from undernutrition than other less crucial organs, because this condition allows an extra amount of such substrates to be redirected towards the brain.

It should be noted that "growth protection" does not necessarily mean that all brain functions are adequately protected and that they will not as a result be altered. In other words: the absence of major changes in the brain weight of undernourished animals (or in other macroscopic parameters) does not rule out the possibility of more subtle metabolic or microscopic abnormalities. In fact, a lot of work done on early undernourishment of both humans and animals has shown that this condition leads to further alterations in brain functions which have repercussions for behaviour, attention, cognition and other aspects. Consequently, it must be concluded that early undernutrition can induce permanent deleterious effects on the CNS, as recently reviewed (89).

5.2. Can undernutrition contribute to obesity later?

In addition to the brain functions indicated in the preceding paragraph, another basic role of this organ is the participation in appetite control. This regulatory function essentially takes place in the hypothalamus. The present work constitutes a first approach of this research group (within a larger scientific project) to study the impact of undernutrition on some hypothalamic mechanisms controlling feeding. However, before discussing the results obtained, it seems convenient to consider the aim of the present work in a broader context. One way to accomplish it is to briefly answer the following question: *how might the fact that early undernutrition influences the mechanisms controlling hunger and satiety be relevant for human beings?*

A lot of information reported over the past two decades indicates that the type of nutrition during early stages of development affects the risk of various diseases, such as type 2 diabetes mellitus. This is not surprising if one considers the fact that diabetes is the result of genetic plus environmental factors (90): the nutritional *status* is one of them. For

example: nutritional *status* can lead to obesity, which is the main risk condition for type 2 diabetes. Consequently, one might wonder whether early undernutrition can lead to obesity in later life. At first glance, the establishment of connections between undernutrition and obesity may seem paradoxical; however, it is known that both conditions coexist in populations living in economically disadvantaged countries, as well as those in which developmental prospects are improving. These countries previously had low prevalences of overweight, but the obesity rate is now increasing (90). The aetiology of this condition is multifactorial but in most cases it is associated with inadequate (excessive) food intake. The above facts suggest the following rationale: early undernutrition may modify the hypothalamic regulation of hunger and satiety, predisposing the individual to the development of obesity later in life, by moving them to over-nutrition. When would this happen? When circumstances changed and food became available in abundance. In that case, early undernutrition could amplify the propensity towards diet-induced obesity.

Therefore, we can answer the question raised above: it is of great interest to study the hypothalamic responses to hormones involved in the feeding control, by applying it in experimental models of undernutrition, in order to to check for possible alterations that could favour overweight and obesity. Consequently, we have made a preliminary approach to this problem by analysing early undernourished rats. The consistency of this approach is demonstrated by the fact that early food restriction leads to a complex disorganization of hypothalamic nuclei involved in body weight regulation, as previously shown (65, 68). So we could expect that some biochemical pathways were also modified in these CNS regions of our undernourished rats.

5.3. Effects of early undernutrition on hypothalamic insulin receptors

Insulin is the main signal arising in the periphery that influences food intake and energy expenditure. It is released by the pancreatic β-cells in response to an elevation of nutrients in the blood, mainly glucose. This hormone enters the brain by transport through the blood-brain-barrier, reaching especially the hypothalamic arcuate region, where it interacts with specific neuronal receptors (reviewed in 91). The binding of insulin to these receptors results in the auto-phosphorylation of a number of tyrosine residues; they are recognized by various adaptor proteins, such as members of the IRS family which, in turn, are phosphorylated. Next, these proteins interact with other signaling molecules working in

cascade, as described in more detail in the Introduction. The net result of insulin action on the hypothalamus is to elicit anorexigenic effects by increasing the activity of satiety signals and decreasing that of the orexigenic ones. The consequences are a reduction of food intake and the loss of body weight.

According to the results found in the present study concerning insulin receptors and IRSs, it could be concluded that early undernutrition has no effects on the hypothalamic content of these proteins. In other words: the first steps of hypothalamic insulin signaling are not apparently affected by undernutrition. That said, it should be noted that some data obtained from the analysis of other proteins located a downstream of IRSs, indicating the possibility of insulin resistance. This data remains an unpublished result, not included in the present work since it is still preliminary. The insulin resistance suggests that the anorexigenic effects of this hormone are reduced in the hypothalamus of undernourished rats, a situation that could facilitate hyperphagia and then obesity.

5.4. Effects of early undernutrition on hypothalamic leptin receptors and STAT-3

Leptin is mainly produced by white adipocytes (although the stomach and other tissues are also sources). Consequently, it is secreted in direct proportion of body fat. Leptin is transported through the blood-brain barrier and gains access to neurons in the hypothalamus, where this hormone influences feeding and energy homeostasis. It mediates phosphorylation activation (by dimerization) and nuclear translocation of STAT-3, a transcription factor which in turn modifies the level of expression of two major targets: the POMC mRNA increases (anorexigenic factor), while the NPY mRNA decreases (orexigenic factor) (92). Thus, leptin is a feeding-inhibitory signal.

Our results show that undernourished adult rats undergo a dramatic decrease in the hypothalamic content of both the leptin receptor (Ob-Rb) and STAT-3. Although we have not analysed these proteins in previous stages, it has been suggested that some defects in the hypothalamic control of feeding may begin *in utero* and continue in postnatal life (93). Thus, the possibility arises that alterations in the leptin receptor and STAT-3 observed herein were already established during the immaturity of restricted rats.

An increase of food intake can be expected when the leptin signal is reduced in the hypothalamus. Consequently, our results reinforce the hypothesis that the hypothalamus of

undernourished adult rats becomes adapted so as to induce hyperphagia, a condition that would facilitate obesity when undernutrition ceased and abundant food became available. Evidently, the insulin resistance described in the previous section would contribute to this situation. It is nevertheless true that we have not analysed the plasma level of leptin, data that would complete the picture regarding the actual leptin effects established in the hypothalamus of undernourished rats. Instead, it is well-known that plasma insulin remains dramatically low in undernourished rats (16), since food restriction decreases the number of pancreatic β-cells (17).

5.5. Effects of undernutrition on hypothalamic POMC expression and content

The hypothalamic-melanocortin system plays an important role in the regulation of food intake and body weight. This system comprises several peptides that derive from a precursor, the pro-opiomelanocortin (POMC). POMC is synthesized by neurons within the hypothalamic arcuate nucleus. Then, it is processed to produce these peptides, which act as anorexigenic factors (such as α-melanocyte stimulating hormone).

We have evaluated this precursor polypeptide in the restricted rats. The results have shown that both hypothalamic POMC mRNA expression and protein abundance are decreased in such animals. In fact, POMC mRNA expression is already reduced during suckling (at this stage, we have not analysed the protein content). Breton et al. observed no changes in hypothalamic POMC expression in adult rats following undernutrition; however, in their experimental model, the food-restriction is prolonged only until weaning and then the animals are fed *ad libitum* until they become adults (67). Thus, our results suggest that POMC deficiency seems rather linked to the chronic nature of undernutrition. In view of the anorexigenic properties of products derived of POMC, we believe that these results fit well with those described in the above paragraphs, since they show a diminution of anorexigenic factors in the hypothalamus of undernourished adult rats.

5.6. Effects of early undernutrition on hypothalamic NPY

NPY is largely synthesized by neurons whose cell bodies lie in the arcuate nucleus of the hypothalamus; they send projections to other hypothalamic regions. NPY is an important orexigenic factor; NPY administration markedly stimulates feeding and reduces thermogenesis (reviewed in 94). Since the effects of this peptide in the hypothalamus are

the opposite to those of the other factors studied herein, it seemed of interest to check whether undernutrition had any effect on it. Unfortunately, we have failed to develop a Western blot procedure to direct NPY quantification, as the antibodies available were not specific enough to unambiguously detect this peptide. Alternatively, we have evaluated the NPY mRNA expression and, to complete the view, we have set up a technique to immunohistologically detect this factor within the hypothalamus.

According to our results, early undernutrition leads to a very substantial increase in the hypothalamic expression of NPY (that is, mRNA), which is statistically significant only in the adult rats. On the other hand, increased contents of NPY immunopositive neurones were found in the arcuate and paraventricular nuclei of restricted rats. The increased staining was evident during both suckling and adulthood; however, it is notable that food-restriction produced no changes regarding mRNA during suckling, as stated above. The apparently contradictory results observed at this stage (when comparing mRNA and staining) could be explained by suggesting that undernutrition could elicit an improvement in the rate of NPY mRNA translation at this immature stage, which would result in an increased NPY peptide biosynthesis without changes in the messenger.

In general, these results agree with others showing increased NPY immunoreactivity within hypothalamic nuclei, in association with early undernutrition (95). The improvement in NPY, a potent orexigenic factor, may lead to hyperphagia, reinforcing the effects derived from the reduction in anorexigenic signals that we have described in the above sections. So it can be speculated that if food were available *ad libitum* to rats previously undernourished, these animals should consume larger amounts than the controls, favouring obesity.

6. CONCLUSIONS

The following conclusions refer to the effects of undernutrition applied to Wistar rats according to the experimental model studied in the present work.

1. Undernutrition induces a situation of hypoglycaemia which is maintained throughout suckling and reaches adulthood.
2. Undernutrition has no effect on the hypothalamic content of insulin receptors, nor that of the main insulin receptor substrates (IRS-1 and IRS-2) present in the hypothalamus.
3. Both the leptin receptor Ob-Rb and STAT-3, the transcription factor activated by this hormone, are markedly reduced in the hypothalamus of adult rats, which have been undernourished from intrauterine life.
4. Hypothalamic POMC protein content as well as its gene expression are markedly decreased in adult rats submitted to early and chronic undernutrition.
5. The gene expression of NPY is enhanced in the hypothalamus of adult rats; in accordance with the result, the immunostaining of this protein is much more intense in the regions corresponding to paraventricular and arcuate nuclei of this organ.

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