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Pre- and/or postnatal protein restriction in rats impairs learning and motivation in male offspring

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Abstract

Suboptimal developmental environments program offspring to lifelong health complications including affective and cognitive disorders. Little is known about the effects of suboptimal intra-uterine environments on associative learning and motivational behavior. We hypothesized that maternal isocaloric low protein diet during pregnancy and lactation would impair offspring associative learning and motivation as measured by operant conditioning and the progressive ratio task, respectively. Control mothers were fed 20% casein (C) and restricted mothers (R) 10% casein to provide four groups: CC, RR, CR, and RC (first letter pregnancy diet and second letter lactation diet), to evaluate effects of maternal diet on male offspring behavior. Impaired learning was observed during fixed ratio-1 operant conditioning in RC offspring that required more sessions to learn vs. the CC offspring (9.4 ± 0.8 and 3.8 ± 0.3 sessions, respectively, $p < 0.05$). Performance in fixed ratio-5 conditioning showed the RR (5.4 ± 1.1), CR (4.0 ± 0.8), and RC (5.0 ± 0.8) offspring required more sessions to reach performance criterion than CC offspring (2.5 ± 0.5 , $p < 0.05$). Furthermore, motivational effects during the progressive ratio test revealed less responding in the RR (48.1 ± 17), CR (74.7 ± 8.4), and RC (65.9 ± 11.2) for positive reinforcement vs. the CC offspring (131.5 ± 7.5 , $p < 0.05$). These findings demonstrate negative developmental programming effects due to perinatal isocaloric low protein diet on learning and motivation behavior with the nutritional challenge in the prenatal period showing more vulnerability in offspring behavior.

Keywords

developmental programming; learning; motivation; protein restriction; operant conditioning

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1. Introduction

Protein malnutrition during pregnancy increases offspring morbidity and mortality and is a major health concern (Duggan, 2003; Guilloteau et al., 2009; Stein et al., 2009). Human epidemiological and precisely controlled animal studies clearly show that the prenatal and early postnatal nutritional environments modify the development of a wide variety of systems including cardiovascular, metabolic and endocrine systems (Taylor and Poston, 2007; Nijland et al., 2008; Symonds et al., 2009; Solomons, 2009; Nuyt and Alexander, 2009; Warner and Ozanne, 2010; Bouret, 2010). Indeed there is a nutritional basis for the fetal origins of adult disease (Harding, 2001; Armitage et al., 2004). Importantly, an adequate maternal diet is vital for the development of the central nervous system in offspring (Morgane et al., 1993). Indeed poor nutrition can have profound effects on the developing brain especially neurons of the limbic system showing considerable sensitivity (Morgane et al., 2002; Bedi, 2003; Lister et al., 2005; Lister et al., 2006). Consequently, outcomes in humans demonstrate influences of perinatal diet on cognition and behavior (Lucas, 2005; Benton, 2008; Stein et al., 2009). Brain developmental impairments, e.g. modified neuron proliferation in hypothalamic and hippocampal regions, are evident in rat offspring protein restricted during gestation and/or lactation (Coupe et al., 2009). Behaviors such as anxiety, motivation and approach-avoidance/risk assessment rely heavily on intact limbic structures and associated neural networks, e.g. hippocampus, amygdala, and areas of the prefrontal cortex (Morgane et al., 2005). Therefore, studying behavioral abnormalities in animals can further our understanding of human pediatric development by suggesting areas of vulnerability.

To date a limited number of studies have focused on operant conditioning or motivation based behaviors subsequent to pre- and/or postnatal protein restriction. For instance, prenatal protein restriction (6% casein) in rats impairs the acquisition of a differential reinforcement of low rates operant task (Tonkiss et al., 1990a). However, there are conflicting results on working memory outcomes, with impairments in the radial arm maze following pre- and postnatal protein restriction (7% casein) in mice (Ranade et al., 2008) but no impairment in an operant T-maze or delayed alternation task following prenatal protein restriction (6% casein) in rats (Tonkiss and Galler, 1990). Spatial learning (Morris water maze) results show no effect (Tonkiss et al., 1994) or impairment of performance in rats prenatally protein restricted (Tonkiss et al., 1997). Additionally, a study on appetitive motivation in rats showed prenatal protein restriction increased responding to positive reinforcement (Tonkiss et al., 1990b).

Those studies establish the concept that reduced perinatal protein has effects on offspring learning and behavior when protein is reduced to 75 % of control diet (6% from 25%). Due to the limited number of studies, the effects of pre- and/or postnatal protein restriction on motivational aspects of operant conditioning remain unclear and the effects of lower amounts of protein restriction have yet to be determined. In the light of the structural and functional differences in brain and behavior resulting from maternal low protein diet during pregnancy and/or lactation, we investigated the effects of protein restriction during these two critical windows of development on learning and motivation in adult male rat offspring. Since behavioral effects have been reported following a substantial reduction of protein (in the aforementioned studies), we sought to investigate the potential effects of a more modest restriction (50%) on offspring behavior which potentially more accurately reflects the human dietary insecurities in developed countries. We hypothesized that protein restriction during pregnancy and/or lactation would affect development of the brain and be manifest in operant conditioning and motivation based behaviors.

2. EXPERIMENTAL PROCEDURES

2.1 Care and maintenance of animals

All procedures were approved by the Animal Experimentation Ethics Committee of the “Intituto Nacional de Ciencias Médicas y Nutrición, Salvador Zubirán” (INNSZ), Mexico. Details of maternal diet, breeding, and management of groups of offspring have been published in detail (Zambrano et al., 2005b). Briefly, dams were virgin female albino Wistar rats aged 21 ± 1 wk (Mean $\pm$ SEM) and weighing 240 ± 20 g, obtained from INNSZ. Dams were maintained on normal laboratory chow (Zeigler Rodent RQ 22-5, USA), under controlled lighting (lights on from 7:00 am to 7:00 pm at 22–23°C) and mated overnight with proven male breeders. The day on which spermatozoa were present in a vaginal smear was designated as conception (day 0). Only rats impregnated within 5 days of male breeder introduction were retained in the study. Pregnant rats were transferred to individual cages and allocated at random to one of two groups fed either a 20% casein (control diet—C) or 10% casein isocaloric diet (restricted diet—R, see Table 1 for diet nutrient composition) (Zambrano et al., 2005a). Food and water were available *ad libitum* with chow provided in the form of flat biscuits retained behind a tray grill through which the rats nibbled the food. Delivery occurred in the early daylight hours between 9.00 am and noon on post-conceptual day 22. Day of delivery was considered postnatal day (PND) 0. Maternal weight, litter size, and pup weight were recorded at birth. Ano-genital distance was measured with calipers. According to our published data (Zambrano et al., 2006), sex was judged according to whether the ano-genital distance was less (female) or greater than (male) 2.5 mm. To ensure homogeneity of study subjects, litters of over 14 pups, or less than 10 pups, were not included in the study. Litters were adjusted to 10 pups for each dam with no sex ratio difference observed between litters of the different diets. For the lactation period, 4 groups were established: CC, RR, CR, and RC (first letter diet received during pregnancy and second letter maternal diet during lactation) resulting in 8 animals from different litters per group (8 each of male CC, RR, CR and RC offspring). After weaning (PND 21), all pups were fed the standard chow rodent diet. At PND 120 offspring commenced operant conditioning studies, which were completed on PND 150 following the progressive ratio testing. For progressive ratio testing only 5 of the 8 animals per group were studied for unavoidable practical reasons, i.e. they could not be studied in an adequately restricted age window.

2.2 Operant Apparatus

Eight unrelated offspring from different litters per diet group were tested in two test-boxes (E10-10TC, Coulbourn-Instruments, PA, USA) enclosed in ventilated, sound-attenuating chambers (E10-20, Coulbourn-Instruments, PA, USA) and fitted with a removable response lever and a liquid dipper (E14-05, Coulbourn-Instruments, PA, USA). These boxes had two side walls of aluminum and a rear and front wall of clear Perspex. Each of the boxes contained a grid floor and during the test-sessions were illuminated with a diffuse house-light. Response lever and reward-magazine were placed on the right side wall. For exact internal measurements see (Russig et al., 2003). When a rat had performed the required number of lever presses, a light was turned on in the reward magazine and the liquid dipper transferred a drop of sucrose solution (0.01 ml, 7%) from a reservoir into the reward magazine. A nose poke into the magazine was registered by photocell receptors. A personal computer controlled, via universal environment interfaces (E91-12, Coulbourn-Instruments, PA, USA), the sessions and recorded the data.

2.3 Operant Conditioning and Progressive Ratio Testing

Two weeks prior to onset of the training sessions, the offspring were placed on water deprivation for 23h/day and one hour of free access immediately following behavioral

sessions which continued throughout conditioning and testing. Subjects were operantly assessed 7 days a week at the same time of the dark cycle for each subject ($\pm$ 30-minutes). Prior to conditioning animal subjects were given a non-contingent (free) reinforcement in the test-box. Subsequently, on the first training day the animals were conditioned to press a lever for reinforcement, according to a fixed ratio schedule (FR-1). For each trial the lever was extended until pressed, after which the subject was allowed 120 seconds to approach the reward magazine and respond with a nose poke. The registration of the nose poke into the reward magazine by the photocell receptors started the feeding for 10 seconds. Each trial was followed by an inter-trial interval of 5 seconds during which the lever was retracted. FR-1 conditioning was completed when the animal earned 20 reinforcements during a 15 minute session. After all animals reached this criterion, they were introduced to a FR-5 schedule with the identical performance criteria as for the FR-1 schedule albeit with 5 responses required per trial. Following operant conditioning sessions, the animals were placed on a progressive ratio schedule for ten days. In the progressive ratio schedule, for the first eight reinforcements each subsequent reinforcement required an additional press (progressive ratio + 1), thereafter the response increment doubled every eighth reinforcement and hence the number of lever presses required to obtain successive sucrose reinforcements was as followed: progressive ratio + 1 = 1, 2, ..., 8; progressive ratio + 2 = 10, 12, ..., 24; progressive ratio + 4 = 28, 32, ..., 56; progressive ratio + 8 = 64, 72, ..., 120; progressive ratio + 16 = 136, 152, ...etc. Each progressive ratio session lasted 30 minutes and on each trial the lever was presented for 300 seconds. The subjects were tested equally in each operant chamber.

2.4 Free Sucrose Consumption

One day after the last progressive ratio session, animals were given direct access to bottled sucrose solution (0,01 ml, 7%) for 30 minute in the familiar colony room and the amount of consumed solution was measured. For this evaluation animals were single caged and the amount of sucrose consumption was calculated by the subtraction of the bottle weight after the consummatory period from the initial weight. This procedure was performed on 3 consecutive days in order to assess appetitive and consummatory behavior.

2.5 Statistical Analyses

All data are expressed as mean $\pm$ SEM with alpha level set at 0.05. Differences in body weight and morphometric measurements at birth were calculated by t-test. Body weight at adult age was performed by one way ANOVA. Analysis for behavioral testing was by ANOVA with between-subject factor of early life manipulation (maternal diet during pregnancy and lactation). Post-hoc analyses were performed by Tukey test, with a repeated measures factor for the ten progressive ratio test days, the total number of responses and the total number of reinforcements obtained. For the free sucrose consumption test, average of the 3 days was measured per subject.

3. RESULTS

3.1 Offspring morphometrics

Table 2 shows morphometric analysis of offspring at birth. Body weight for pups of protein restricted dams was significantly reduced ($p < 0.05$) compared to pups from dams fed the control diet. Ano-genital distance relative to body weight was significantly increased ($p < 0.05$) by 27% in the prenatal protein restricted offspring. No changes were observed in other parameters at birth. In adult life both at the beginning and end of operant assessments the CC offspring weight was significantly higher ($p < 0.05$) than in RR offspring (Table 3).

3.2 Operant Conditioning

In FR-1 conditioning a significant effect of diet was determined [$F(3,31)=21.48$, $p<0.001$]. The number of FR-1 sessions required to reach performance criterion was higher in the RC offspring compared to the RR, CR and the CC offspring (Figure 1A). Additionally, during FR-5 conditioning a significant effect of diet was determined [$F(3,31)=18.59$, $p<0.001$], the RR, CR and RC offspring required more sessions to attain performance criterion compared to the CC offspring (Figure 1B).

3.3 Progressive Ratio Testing

Comparisons for total responses made revealed a significant effect of experimental diet [$F(3,19)=9.89$, $p<0.001$]. Post-hoc analysis confirmed all restricted diet groups responded less for positive reinforcement than the CC offspring vs. RR, CR, and RC offspring (Figure 2A). A comparison of the number of reinforcements earned revealed a significant effect of diet [$F(3,19)=8.19$, $p<0.005$]. Post-hoc analysis confirmed less reinforcements earned by the RR, CR, and RC vs. the CC offspring (Figure 2B).

3.4 Free Sucrose Consumption

No differences were determined (in mL) across three 30-minute sessions between the different dietary regimens in the free sucrose (7%) consumption test: CC 28.8 ± 0.7 , RR 28.0 ± 0.4 , CR 28.0 ± 0.7 , and RC 28.1 ± 0.7 mL.

4. DISCUSSION

The goal of these studies was to assess associative learning and motivational behavioral outcomes in rat male offspring exposed to protein restriction during two critical windows of embryonic/fetal and offspring development; pregnancy and lactation. Maternal protein restriction reduced the birth weight of pups, which directly reflects the importance of proper protein intake during pregnancy for fetal maturation. This morphometric outcome is consistent with models of intra-uterine growth retardation and fetal programming (Vuguin, 2007). Also, we report an increase of ano-genital distance in prenatal protein restricted offspring. An increase in the ano-genital distance of prenatal protein restricted offspring born to dams with elevated progesterone, corticosterone, oestradiol, and testosterone concentration at 19 days gestation (near term) has been reported by our group (Zambrano et al., 2005b). Maternal steroids can cross the placenta, and such exposure to transplacentally acquired androgens in fetal life can increase ano-genital distance (Hotchkiss et al., 2007). Effects of these steroids on fetal development could have a role in the current behavioral findings since human studies report impairment of spatial learning ability in males exposed to excess levels of androgens *in utero* (Puts et al., 2008).

Initial behavioral observations revealed diet specific outcomes of pre- and/or postnatal protein restriction during operant conditioning. Male RC offspring were negatively affected as demonstrated by an increase in the number of sessions to acquire operant conditioning for liquid reinforcement in FR-1 conditioning. This outcome suggests that the critical window for neural components underlying operant conditioning begin prenatally. During FR-5 schedule of reinforcement the RR, CR and RC offspring all required more sessions to perform to criterion compared to the CC offspring. So for learning assessments, prenatal protein restriction alone (RC offspring) produced impairment in FR-1 conditioning whereas during FR-5 conditioning, in which there was an increase in response requirement, all experimental offspring needed more sessions to reach performance criterion compared to the CC offspring. Also, differences in motivation were demonstrated in that the RR, RC and CR offspring responded significantly less and earned significantly fewer reinforcements than the CC offspring during progressive ratio testing. These results reveal the negative effect of

protein restriction during pregnancy and/or lactation on learning and motivation in male rat offspring with protein restriction during the prenatal period showing the most vulnerability.

These behavioral findings contribute to the current knowledge of developmental programming and postnatal cognition and motivation. The cognitive deficits we report are in agreement with global undernutrition during gestation impairing learning in adult age rat offspring (Landon et al., 2007). That study showed *in utero* diet restricted offspring perform consistently poorer than controls in a cognitive flexibility task using concurrent variable-interval schedules. Similarly adult sheep offspring prenatally undernourished display cognitive flexibility impairment as assessed by learning speed during reversal tasks in a T-maze (Erhard et al., 2004). In addition, perinatal food restriction in male rats impaired spatial learning and memory in the Morris water maze (Tonkiss et al., 1997; Zhang et al., 2010). Similar to our findings, existing literature does tell of performance impairments in offspring perinatally protein restriction in different operant tasks such as the differential reinforcement of low rates (DRL) (Tonkiss et al., 1990a) and the radial arm maze (Ranade et al., 2008). However, to our knowledge we are the first to report a learning impairment during operant conditioning in perinatal protein restricted male offspring. Although those studies do not fully reflect the dietary challenges and operant tasks we administered, a commonality exists in our experimental groups exhibiting cognitive inflexibility during operant conditioning following perinatal undernutrition of a macronutrient. The perinatal protein restricted offspring in this study performed similarly to controls during FR-1 conditioning, with the exception of the RC group which performed worse, but all protein restricted groups were subsequently impaired when response requirements increased in FR-5 operant conditioning as compared to controls.

A potential explanation for this behavioral perturbation may stem from findings by our group that show protein restriction during pregnancy increases circulating androgen and glucocorticoid levels in dams near term (Zambrano et al., 2005b) and a retrospective human study by others reporting impaired spatial task performance in males following excessive prenatal androgen exposure (Puts et al., 2008). There is also abundant evidence of excess levels of glucocorticoids *in utero* impairing brain development and later behavior in humans and animal models (Johnson et al., 1981; Uno et al., 1990; Uno et al., 1994; Szuran et al., 2000; Antonow-Schlorke et al., 2001; Matthews, 2001; Antonow-Schlorke et al., 2003; French et al., 2004; Karemaker et al., 2006; Karemaker et al., 2008; Weinstock, 2008; Seckl, 2008). Although we did not assess postnatal androgen or glucocorticoid levels in offspring, this should not preclude conclusions based on gestational exposure effecting behavioral performance. Evidence does exist of elevated androgens in male rats small for gestational age (Allvin et al., 2008).

In regards to motivation for positive reinforcement, prenatal undernourished rats show less appetitive motivation exhibiting increased preference for wheel running versus lever pressing for food which are in agreement to findings reported here (Miles et al., 2009). Continuing with our argument that maternally derived androgens and glucocorticoids due to protein restriction effect postnatal learning, prenatal exposure to androgens have also been shown to induce aversive properties to appetitive stimuli postnatally (Dominguez-Salazar et al., 2008). Our results show decreased motivation for positive reinforcement in all experimental offspring compared to CC group. In contrast, results from a previous study revealed that prenatal protein restriction increased the response for reinforcement in adult male rats (Tonkiss et al., 1990b). This may however be based on different experimental conditions as the amount of protein restriction in that study was 6% (75% reduction) casein diet while our study administered a 10% (50% reduction) casein experimental diet.

One might hypothesize that sufficient protein components required for normal neurodevelopment were lacking in our RR, RC and CR offspring resulting in an immature limbic system at the time of testing which could have effected cognition and motivation (Morgane et al., 1993; Bedi, 2003; Lister et al., 2005; Lister et al., 2006). Furthermore, we have also reported that protein restriction during pregnancy negatively impacts normal fetal brain development by changes in maternal lipid metabolism (Torres et al., 2009). Fetal tissues produce only small amounts of long chain polyunsaturated fatty acids (LC-PUFAs) due to a relative lack of desaturases and elongases and thus the fetal brain depends on the maternal source of LC-PUFAs (Hamosh and Salem, Jr., 1998). Gestational protein restriction significantly reduces maternal liver desaturase and elongase gene expression, and formation of the LC-PUFAs: arachidonic and docosahexaenoic acid (Torres et al., 2009). Thus, fetuses born to protein restricted dams exhibit low body weight as well as reduced brain fat including the content of docosahexaenoic acid in the brain (Torres et al., 2009).

From the perspective that a lack of protein during development can cause cognitive and behavior problems in humans (Galler et al., 1983; Galler et al., 1984; Galler et al., 1987; Galler and Ramsey, 1989), the need to elucidate the behaviors and underlying brain areas influenced by these nutritional challenges are imperative. Here, we show behavioral perturbations during learning and motivation based behaviors in male offspring perinatally protein restricted during the entirety of human pregnancy equivalence and thus many critical early neurogenesis stages were affected when translating between species (Clancy et al., 2007a; Clancy et al., 2007b). These outcomes are potentially due to the insufficient protein (essential amino acids) or fatty acids/lipids available during critical windows of brain development. The isocaloric diet administered not only had reduced protein but also high carbohydrate quantities which should be taken into consideration during interpretation. Hormonal response in both dam and fetus must also be considered together or separately with the protein effects as steroids greatly determine neural proliferation, differentiation, and migration (Jazin and Cahill, 2010). These differences clearly reflect impairments in cognition and motivation without affecting consummatory behavior. Further studies of discrete brain areas, especially neurons and support cells of the limbic system and the hypothalamic pituitary adrenal axis due to protein insufficiency during development are necessary to determine mechanisms.

Abbreviations

C	control diet
R	protein restricted diet
CC	control diet during pregnancy and lactation
RR	protein restricted diet during pregnancy and lactation
CR	control diet during pregnancy and restricted diet during lactation
RC	restricted diet during pregnancy and control diet during lactation
FR	fixed ratio
PND	postnatal day

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Figure 1. Operant Conditioning

A) Comparison of FR-1 conditioning shows the number of sessions to reach performance criterion. B) Comparison of FR-5 conditioning shows the number of sessions to reach performance criterion. Offspring of rats fed with control (C = 20% casein) or restricted (R = 10% casein) diet during pregnancy (first letter) and lactation (second letter). Mean $\pm$ SEM, $n = 8$ offspring from different litters. Groups not sharing a letter are statistically different, $p < 0.05$.

Figure 2. Progressive ratio

A) Average number of responses made during 10 sessions. B) Average number of reinforcements earned during 10 sessions. Offspring of rats fed with control (C = 20% casein) or restricted (R = 10% casein) diet during pregnancy (first letter) and lactation (second letter). Mean $\pm$ SEM, $n = 5$ offspring from different litters. Groups not sharing a letter are statistically different, $p < 0.05$.

Table 1

Nutritional composition of control and protein restricted diet administered during pregnancy and lactation.

	Control (%)	Restricted (%)
Casein	20	10
Vitamin mix	1	1
Mineral mix	3.5	3.5
Cystine	0.3	0.15
Choline	0.165	0.165
Fiber	5	5
Corn oil	5	5
Cornstarch	32.52	37.6
Dextrose	32.52	37.6
	3.85kcal/g	3.85kcal/g

Table 2

Pup weight and morphometric measurements at birth of rats fed the control (20% casein) or restricted (10% casein) diet during pregnancy. Mean $\pm$ SEM; n refers to pups from different litters.

	Control (n = 8)	Restricted (n = 8)
Weight (g)	5.9 $\pm$ 0.1	5.0 $\pm$ 0.2*
Length (mm)	4.7 $\pm$ 0.09	4.6 $\pm$ 0.1
Ano genital distance (mm)	3.7 $\pm$ 0.2	3.9 $\pm$ 0.08
Ano genital distance (mm/g)	0.63 $\pm$ 0.03	0.80 $\pm$ 0.03*
Head diameter (mm)	11.4 $\pm$ 0.5	11.4 $\pm$ 0.1
Abdominal diameter (mm)	12.4 $\pm$ 0.06	12.3 $\pm$ 0.05
Head:abdominal ratio	0.92 $\pm$ 0.006	0.92 $\pm$ 0.004

*p<0.05 different from control.

Table 3

Offspring weight at the beginning of the training (120 d) and at the end of the progressive ratio testing (150 d). Offspring from dams fed with control (C = 20% casein) or restricted (R = 10% casein) diet during pregnancy (first letter) and lactation (second letter). Mean $\pm$ SEM; n = 8 offspring from different litters. Groups not sharing a letter are statistically different, $p < 0.05$.

	CC	RR	CR	RC
120 d	392 $\pm$ 13 ^a	324 $\pm$ 17 ^b	352 $\pm$ 6 ^{ab}	364 $\pm$ 15 ^{ab}
150 d	420 $\pm$ 12 ^a	362 $\pm$ 19 ^b	382 $\pm$ 5 ^{ab}	403 $\pm$ 16 ^{ab}