UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO
DOCTORADO EN CIENCIAS BIOMÉDICAS
FACULTAD DE MEDICINA

LA RESTRICCIÓN PROTEÍNICA MATERNA EN LA RATA INCREMENTA LA ANSIEDAD EN LA CRÍA MACHO Y GENERA DETERIORO COGNITIVO POR EFECTO DEL AUMENTO DE LOS GLUCOCORTICOIDES Y CAMBIOS ANATÓMICOS DURANTE SU DESARROLLO.

TESIS
QUE PARA OPTAR POR EL GRADO DE:
DOCTOR EN CIENCIAS

PRESENTA: LUIS ANTONIO REYES CASTRO

DIRECTOR DE TESIS:
DRA. ELENA ZAMBRANO GONZÁLEZ
Facultad de Medicina

COMITÉ TUTOR:
Dra. NORMA A. BOBADILLA SANDOVAL
Instituto de Investigaciones Biomédicas

DR. IGNACIO CAMACHO ARROYO
Facultad de Medicina

Ciudad de México, Junio 2016

El presente trabajo fue realizado en el Laboratorio 02 del Departamento de Biología de la Reproducción del Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, bajo la tutoría de la **Dra. Elena Zambrano González** y el Comité tutoral integrado por la **Dra. Norma Araceli Bobadilla Sandoval** (Instituto de Investigaciones Biomédicas) y el **Dr. Ignacio Camacho Arroyo** (Facultad de Medicina) de la UNAM.

Este Trabajo fue financiado por el Consejo Nacional de Ciencia y Tecnología **(CONACyT)** No. 48839

Durante los estudios de Doctorado, el autor de la tesis obtuvo apoyo para asistencia a congresos del Programa de Apoyo de Estudios de posgrado **(PAEP)** del Doctorado en Ciencias Biomédicas, UNAM**.**

El autor contó con beca **CONACyT** para la realización de estudios de posgrado.
No. de becario 295647/228006

AGRADECIMIENTOS:

- Antes que nada quiero agradecerle a papá Dios, por darme la paciencia y sabiduría para poder culminar mis estudios y cumplir una meta más en mi vida.

- A mi madre por estar conmigo durante los momentos difíciles, su entrega, cariño y comprensión.

- A la Dra. Elena Zambrano por su confianza, paciencia y dedicación para la realización de este proyecto.

- Al Dr. Fernando Larrea por las facilidades, dedicación y apoyo durante el proceso del Doctorado.

- A la Dra. Norma Araceli Bobadilla Sandoval y el Dr. Ignacio Camacho Arroyo por todas las aportaciones que fueron de gran utilidad para el enriquecimiento del proyecto.

- A la Dra. Gina Quirarte, Dra. Sofía Díaz y Dra. Euridice Padilla por su cariño, dedicación y confianza para enriquecer el proyecto.

- A mis amigos del laboratorio de BR: Lupita, Magaly, Rosy, Claudia Vega, Claudia, Carlitos y Lilia Vargas por su amistad, comprensión y apoyo, sin ustedes no sé que hubiese hecho, los llevo en el corazón.

- A Lourdes Boeck y Roberto Chavira por aguantar mi temperamento, por sus consejos, regaños, apoyo incondicional y colaboración, no tengo como agradecérselos.

- A la M. en C. Azucena Ruth Vázquez porque gracias a ti pude darle otro giro a mi proyecto, y por sus consejos, amistad y apoyo técnico en el Laboratorio.

- A Norma Serafín López por su gentil asistencia en el proyecto de tesis.

- A mi prima Lirio Castro, primo Jesús Almanza, las princess y Sebas por todo el cariño que me han brindado, por ayudarme en los momentos difíciles y darme los consejos precisos para continuar.

- A Gil, Ale, Vero y Andy (mi familia de elección) por estar siempre a mi lado, con toda su entrega y amor. Los quiero, recuerden que esta amistad será por siempre.

- A los chicos del laboratorio C02 del INB: Laura, Erika, Martha, Israel, Isaac, Jesús, Poncho, por compartir conmigo sus conocimientos, los ratos amenos que pasamos juntos, por su amistad y apoyo en verdad los quiero, gracias por todo. Ah creíste que te olvidaría Gargamel pues no¡¡¡¡ a ti también Zeus no tengo como agradecerte todo lo que me has aguantado.

- A Luis Alberto Estrada Flores, por su gran cariño y amistad incondicional, te adelantaste en el camino pero sé que más adelante nos volveremos a encontrar. Te mando un abrazo hasta el cielo, tqm.

- En especial a mis ratas, que fueron de gran utilidad para la obtención de resultados en el presente trabajo.

DEDICATORIA:

- A mi madre por ser una persona excepcional y digna de admiración.

- A mi abue Columba y a mí querido hermano Chuchín que han sido mi guía en cada momento, los llevo en mi mente y corazón; saben que algún día nos volveremos a ver.

- A mis compañeros y amigos por impulsarme a seguirme superando y por todos los momentos inolvidables que compartimos.

- A toda mi familia, por toda su confianza y entusiasmo que me transmitieron para seguir adelante en mi vida profesional.

No puedo presumir de mi amor por Dios porque fallo a diario, pero puedo presumir de su amor por mí ya que ÉL nunca me falla.

ÍNDICE

No. pág

1. - Pre- and/or postnatal protein restriction in rats impairs learning and motivation in male offspring. **Reyes-Castro LA**, Rodriguez JS, Rodríguez-González GL, Wimmer RD, McDonald TJ, Larrea F, Nathanielsz PW, Zambrano E. Int. J. Devl Neuroscience (2011); 29 (2): 177–182.

2. - Regulación del apetito por las hormonas esteroides. CJ Bautista, **LA Reyes Castro**, E Zambrano. Revista de la Escuela de Medicina “Dr. José Sierra Flores” (2011); 25(2): 26-37.

3. - Emergence of ageing-related changes in insulin secretion by pancreatic islets of male rat offspring of mothers fed a low-protein diet. Morimoto S, Calzada L, Sosa TC, **Reyes-Castro LA**, Rodriguez-González GL, Morales A, Nathanielsz PW, Zambrano E. British Journal of Nutrition (2012); 107(11): 1562–1565.

4. - Pre- and/or postnatal protein restriction developmentally programs affect and risk assessment behaviors in adult male rats. **Reyes-Castro LA**, Rodriguez JS, Rodríguez-González GL, Chavira R, Bautista CJ, McDonald TJ, Nathanielsz PW, Zambrano E. Behavioural Brain Research (2012); 227(2): 324–329.

5. - Maternal protein restriction in the rat during pregnancy and/or lactation alters cognitive and anxiety behaviors of female offspring. **Reyes-Castro LA**, Rodriguez JS, Charco R, Bautista CJ, Larrea F, Nathanielsz PW, Zambrano E. Int. J. Devl Neuroscience (2012); 30(1): 39–45.

6. - Maternal obesity in the rat programs male offspring exploratory, learning and motivation behavior: prevention by dietary intervention pre-gestation or in gestation. JS Rodríguez, GL Rodríguez-González, **LA Reyes-Castro,** C Ibañez, A. Ramírez, R Chavira, F Larrea, PW Nathanielsz, E Zambrano. Int. J. Devl Neuroscience; (2012) 30(2): 75–81

7. - Developmental programming of aging of isolated pancreatic islet glucose stimulated insulin secretion in female offspring of mothers fed low protein diets in pregnancy and/or lactation. Morimoto S, Sosa TC, Calzada L, **Reyes-Castro LA**,

Díaz-Díaz E, Nathanielsz PW and Zambrano E. Journal of Developmental Origins of Health and Disease (2012); 3(6): 483–488.

8. - Exercise in obese female rats has beneficial effects on maternal and male and female offspring metabolism. Vega CC, **Reyes-Castro LA**, Bautista CJ, Larrea F, Nathanielsz PW, Zambrano E. Int J Obes (2015); 39(4): 712-9.

9. - Interventions to prevent adverse fetal programming due to maternal obesity during pregnancy. Nathanielsz PW, Ford SP, Long NM, Vega CC, **Reyes-Castro LA**, Zambrano E. Nutr Rev (2013); 71(1): S78–S87.

10. - Accelerated aging of reproductive capacity in male rat offspring of protein-restricted mothers is associated with increased testicular and sperm oxidative stress. Guadalupe L. Rodríguez-González, **Luis A. Reyes-Castro**, Claudia C. Vega, Lourdes Boeck, Carlos Ibáñez, Peter W. Nathanielsz, Fernando Larrea, Elena Zambrano.AGE (2014); 36(6):9721, 1-12.

11. - Paternal line multigenerational passage of altered risk assessment behavior in female but not male rat offspring of mothers fed a low protein diet. **Reyes-Castro LA**, Rodríguez-González GL, Chavira R, Ibáñez C, Lomas-Soria C, Rodríguez JS, Nathanielsz PW, Zambrano E. Physiology & Behavior (2015); 140: 89–95.

12. - Maternal obesity and overnutrition increase oxidative stress in male rat offspring reproductive system and decrease fertility. Rodríguez-González GL, Vega CC, Boeck L, Vázquez M, Bautista C, **Reyes-Castro LA**, Saldaña O, Lovera D, Nathanielsz PW, Zambrano E. International Journal of Obesity (2015); 39(4): 549–556

13. - Ovarian Hormones and Prolactin Increase the Renal NaCl Cotransporter Phosphorylation. Rojas-Vega L, **Reyes-Castro LA**, Ramirez V, Bautista-Pérez R, Rafael C, Castañeda-Bueno M, Meade P, De Los Heros P, Arroyo-Garza I, Bernard V, Binart N, Bobadilla NA, Hadchouel J, Zambrano E, Gamba G. Am J Physiol Renal Physiol (2015); 308(8): F799–F808.

14. - Aging, glucocorticoids and developmental programming. Zambrano E, **Reyes-Castro LA**, Nathanielsz PW. AGE (2015); 37(3): 52, 9774 1-7.

15. - Resveratrol partially prevents oxidative stress and metabolic dysfunction in pregnant rats fed a low protein diet and their offspring.Vega CC, **Reyes-Castro LA**, Rodríguez-González GL, Bautista CJ, Vázquez-Martínez M, Larrea F, Chamorro-Cevallos GA, Zambrano E, Nathanielsz PW. J Physiol (2016); 594(5):1483-99.

ABREVIATURAS

Ácido araquidónico	AA
Ácido docosahexaenoico	DHA
Ácidos grasos de cadena larga	LC-PUFAS
Alfa amino 3 hidroxi 5 metilisoxazole ácido propiónico	AMPA
Amígdala basolateral	BLA
Asociación comisural	AC
Campo abierto	OF
Células granulares	CG
Células piramidales	CP
Cornu ammonis	CA
Condicionamiento operante	CO
Corteza entorrinal	CE
Corticosterona	CORT
Densidad post sináptica	PSD
Dihidrotestosterona	DHT
Distancia ano genital	DAG
Edad posnatal	PND
Eje hipotálamo hipófisis adrenal	HHA
Factor neurotrópico derivado del cerebro	BDNF
Fibras musgosas	FM
Formación hipocampal	FH
Giro dentado	GD
Glucocorticoides	GC
Glutamato	GLUT
Hormona adrenocorticotropa	ACTH
Hormona liberadora de corticotropina	CRH
Laberinto acuático de morris	LAM
Laberinto elevado en cruz	EPM
Manual Diagnóstico y Estadístico de la Asociación Psiquiátrica Americana	DSM-V
N metil D aspartato	NMDA
Núcleo para ventricular	PVN
Orígenes del desarrollo de salud y enfermedad	DOHaD
Para subiculum	PaSu
Patrón Perforante	PP
Potenciación a largo plazo	LTP

Pre subiculum	PrSu
Propiomelanocortina	POMC
Prueba de razón progresiva	PRT
Radioinmunoanálisis	RIA
Razón fija	FR
Receptor de andrógenos	AR
Receptor de glucocorticoides	GR
Receptor de mineralocorticoides	MR
Reconocimiento de objeto novedoso	RON
Sistema *Locus coeruleus*	SLC
Sistema nervioso central	SNC
Sistema nervioso simpático	SNS
Stratum lacunosum-moleculare	SLM
Stratum lucidum	SL
Stratum oriens	SO
Stratum pyramidale	SP
Stratum radiatum	SR
Subiculum	Su
Tiempo en Objeto novedoso	TON
Tiempo en Objeto familiar	TOF

RESUMEN

El desafío nutricional materno durante el desarrollo fetal y neonatal programa múltiples sistemas en la descendencia incluyendo la maduración y la función del cerebro. Poco se sabe acerca de los efectos del ambiente subóptimo en la vida intrauterina y perinatal sobre el aprendizaje, motivación, ansiedad y memoria en la vida adulta de la descendencia. La hipótesis del presente trabajo fue que la restricción proteínica materna incrementa la ansiedad y el deterioro cognitivo debido a: 1) cambios anatómicos del hipocampo en sus sitios pre (sistema de fibras musgosas) y post sinápticos (espinas dendríticas) y 2) hiperactividad del eje Hipotálamo Hipófisis Adrenal (HHA). Se emplearon ratas Wistar preñadas y alimentadas con dietas isocalóricas con contenido normal en proteína o restringido. Los grupos experimentales fueron: CC, RR, CR, y RC (la primera letra indica el tipo de dieta que recibió la madre durante la gestación y la segunda, durante la lactancia). Se analizó el efecto de la dieta materna en la conducta de las cría macho de la rata. De los 90 a 104 días de edad postnatal (PND) se evaluó la ansiedad, la conducta exploratoria y la memoria de reconocimiento visual mediante las pruebas de Laberinto elevado en cruz (EPM), campo abierto (OF) y reconocimiento de objetos novedoso (RON). A los 108 d se evaluó la memoria espacial mediante la prueba del laberinto acuático de Morris (LAM). De los 120 a 150 d se evaluó aprendizaje asociativo y motivación mediante el condicionamiento operante (CO) y la tarea progresiva (PRT). Las concentraciones de corticosterona (CORT) y ACTH se determinaron a los 110 d y posteriormente previo y después de la inmovilización a 220 d. Se midió en la región CA3 del hipocampo, el área de las fibras musgosas en el *stratum lucidum,* la densidad y tipos de espinas (cortas, largas y hongo) en las dendritas basales del *stratum oriens*. Los resultados del EPM mostraron efecto de la dieta en la disminución en el número de entradas, tiempo de permanencia y distancia recorrida en los brazos abiertos en las crías RR en comparación a CC. En la prueba del OF, las crías RR entraron menos veces a la zona central con respecto a CC, mostrando así mayor ansiedad. En la prueba de RON las crías de los grupos RR, CR y RC presentaron menor índice de discriminación con respecto al grupo CC. En la prueba de adquisición en el LAM, se demostró que el grupo CC requirió dos sesiones, el RC tres y el CR siete para aprender, pero el grupo RR no aprendió durante los ocho ensayos. En la prueba de retención evaluada 24 horas después del entrenamiento se observó que el RR, CR y RC requirieron más tiempo para localizar la plataforma y tuvieron menor número de entradas a la zona blanco; sin embargo, el RR y RC pasaron menos tiempo en la zona blanco. Durante el CO de razón fija 1, el RC requirió de mayor número de sesiones para aprender en relación al CC. En el condicionamiento de razón fija 5, los grupos RR, CR y RC requirieron más sesiones para cumplir con el criterio de desempeño. Los efectos de motivación durante el PRT revelaron que los grupos RR, CR y RC responden menos al reforzamiento positivo en comparación a CC. A los 110 d, las crías RR presentaron mayor concentración de CORT y ACTH al comparar con CC. Este mismo efecto se observó a los a los 20 y 40 min después de la inmovilización a los 220 d. En la región CA3 del hipocampo, el área de las FM, el número de las espinas totales y las espinas largas estuvieron disminuidas en RR, CR y RC. Las espinas en forma de hongo se encontraron disminuidas en RR y RC; sin embargo, las espinas cortas estuvieron aumentadas en los grupos RR, CR y RC con respecto al CC. Estos hallazgos demuestran efectos negativos de la programación debido al consumo de una dieta baja en proteína en las madres sobre la ansiedad, motivación, aprendizaje y memoria de la descendencia, teniendo mayor vulnerabilidad el período prenatal. Con base a los anterior podemos concluir que el consumo materno de una dieta baja en proteína durante la gestación y lactancia incrementa la ansiedad, retrasa el aprendizaje, genera deterioro en la retención de la memoria, debido a cambios en el sustrato anatómico del hipocampo en sitios pre y post sinápticos (sistema de las fibras musgosas y espinas dendríticas); así como a la excesiva liberación de ACTH y CORT, lo cual es indicativo de la disfunción de la actividad del eje HHA. Dichos cambios se proponen como mecanismos potenciales para explicar los déficits observados en el presente trabajo de investigación.

ABSTRACT

Maternal nutritional challenges during fetal and neonatal development results in developmental programming of multiple offspring organ system including brain maturation and function. Little is known about the effects of suboptimal intra-uterine environment during perinatal period on learning, motivation, anxiety and memory in offspring adult life. We hypothesized that maternal low protein diet increased anxiety type behavior and impaired cognitive behavior due to 1) anatomic pre and post synaptic hipocampal changes (mossy fiber system and dendrite spines) and 2) hypothalamic pituitary adrenal (HPA) activity. To perform the study pregnant Wistar rats were fed different isocaloric diets, either control or restricted mothers. The experimental groups were: CC, RR, CR, and RC (first letter indicated pregnancy and second letter lactation diet). We evaluated maternal diet effects on behaviour male offspring rat. At postnatal day (PND) 90-104 evaluated the risk assessment, anxiety, exploratory behaviors and memory of visual recognition as measured by elevated plus maze (EPM), open field (OF) and novel object recognition test (NOR). At PND 108, males were tested in the Morris water maze (MWM) to evaluated spatial learning and memory. The associative learning and motivation as measured by operant conditioning (CO) and the progressive ratio task (PRT), respectively at PND 120-150. Corticosterone (CORT) and ACTH were measured at PND 110 and before and after immobilization at PND 220. We measured in the hippocampal CA3 field, mossy fiber area in *stratum lucidum* and total density and spine types (stubby, thin and mushroom) in basal dendrites of *stratum oriens*. EPM results showed an effect of pre- and/or postnatal diet manipulation in open arm entries, time and distance ($p<0.05$) with decreases seen in the RR relative to CC offspring. In the OF, the RR offspring entered the center zone less than the CC offspring thus exhibiting increased anxiety. In the NOR task, the RR, CR and RC offspring showed a low discrimination index with comparison CC group. In the MWM acquisition test CC offspring required two sessions, RC three and CR seven sessions to learn. RR didn´t learn in eight trials. In a retention test after a further 24 h, RR, CR and RC spent more time locating the platform and performed fewer target zone entries. RR and RC offspring spent less time in the target zone. Impaired learning was observed during fixed ratio-1 operant conditioning in RC offspring that required more sessions to learn vs. the CC offspring. Performance in fixed ratio-5 conditioning showed the RR, CR and RC required more sessions to reach performance criterion than CC offspring. Furthermore, motivational effects during the PRT revealed less responding in the RR, CR and RC for positive reinforcement vs the CC offspring. At PND 110, CORT and ACTH were higher in RR in comparison with CC. The same tendencies were observed at 20 and 40 minutes after immobilization in the RR compared to CC group at PND 220. In the hippocampal CA3 field, mossy fiber area in *stratum lucidum,* total density and thin spines in basal dendrites of *stratum oriens* were lower in RR, CR and RC than CC. Mushroom spines were lower in RR and RC. Stubby spines were higher in RR, CR and RC than CC. These findings demonstrate negative developmental programming effects due to maternal isocaloric low protein diet on anxiety, motivation, learning and memory in the progeny, with more vulnerability in perinatal period. We concluded that a maternal low protein diet during pregnancy and lactation increased the anxiety type behaviour in male offspring and associative delays as well as spatial acquisition and memory retention due to alterations in hippocampal pre synaptic (mossy fiber), and postsynaptic (spines) elements and excessive released of ACTH and CORT due to HPA hyperactivity. The finding could be involved in potential mechanisms to explain observed behavior deficits showed in the present research study.

1. INTRODUCCIÓN

Estudios con animales de experimentación (Nathanielsz, 2006; Ozanne and Hales, 1999; Zambrano et al., 2006) y epidemiológicos (Ravelli et al., 1999; Strauss, 1997) han demostrado que el fenotipo de un individuo no está determinado exclusivamente por sus genes, existe además una fuerte influencia ambiental, con mayor impacto en etapas tempranas del desarrollo.

El ambiente subóptimo intrauterino y durante la lactancia modifica el crecimiento y predispone a un fenotipo de alteraciones en la vida adulta del individuo tales como: desarrollo de diabetes tipo 2 (Dallar et al., 2007), obesidad (Tian et al., 2006), dislipidemia (Davies et al., 2004), alteración en la densidad mineral ósea (Akcakus et al., 2006), secreción hormonal (Guzman et al., 2006; Zambrano et al., 2005b) y alteraciones neuroendocrinas (Gluckman and Hanson, 2004; O'Connor et al., 2005). De estas evidencias ha surgido la hipótesis de los *orígenes del desarrollo de la salud y enfermedad* (DOHaD, por sus siglas en inglés) antes conocida como "programación del desarrollo", que propone que la fisiología y metabolismo fetal y neonatal pueden ser alterados por cambios durante periodos críticos del desarrollo, como la gestación y la lactancia (Godfrey and Barker, 2000). Se ha establecido que un órgano o un tejido específico pueden ser programados en el útero por efecto de un estímulo o reto precoz en un periodo sensible del desarrollo, con consecuencias adversas en su función en la vida postnatal. El desarrollo del individuo depende entre otros factores de las condiciones nutrimentales que haya tenido en la vida intrauterina y en el periodo perinatal, ya que la dieta es elemental para la buena formación del sistema nervioso central (Morgane et al., 1993; Morgane et al., 2002). Actualmente, se sabe que la mala nutrición durante etapas tempranas puede tener efectos adversos sobre el desarrollo del cerebro, (Weinstock, 2001) siendo más susceptibles las neuronas del sistema límbico (Bedi, 2003; Lister et al., 2005; Lister et al., 2006; Morgane et al., 2005)**.**

En nuestro grupo de estudio, utilizando a la rata albina como modelo biológico, hemos reportado el impacto de la restricción proteínica durante la gestación, sobre el metabolismo de lípidos en el hígado materno y la composición

lipídica del cerebro fetal de la rata. Dado que el feto no tiene la capacidad de producir los ácidos grasos de cadena larga o LC-PUFAS (por sus siglas en inglés, long-chain polyunsatured fatty acids), la madre se encarga de proporcionarlos. En dicho estudio, comprobamos que la restricción proteínica nutricional reduce la expresión de las enzimas elongasas (sistema de alargamiento de los ácidos grasos) y desaturasas en el hígado materno encargadas de la formación de LC-PUFAS, y se asocia con la disminución en la proporción de grasa y concentración de los ácidos araquidónico (AA) y docosahexaenoico (DHA) en el cerebro fetal (Torres et al., 2010). Dado que éstos son componentes estructurales importantes del sistema nervioso central (SNC), su disminución puede ser un factor determinante en las alteraciones cognitivas en la vida adulta de los descendientes de madres sometidas a restricción proteínica durante la gestación (Ohishi et al., 2012; Ranade et al., 2008). Tales alteraciones en la cognición pueden deberse al inadecuado desarrollo de las áreas del sistema límbico, implicadas en la regulación de las emociones y la memoria como es el hipocampo y la amígdala, o debido a alteraciones en la plasticidad de sus elementos pre y post sinápticos involucrados en la regulación de estas conductas.

Estudios experimentales han sugerido que la restricción de proteína materna durante la gestación modifica la proliferación de neuronas en regiones cerebrales del hipocampo e hipotálamo (Coupe et al., 2009; Lister et al., 2005). De igual manera, se ha reportado que la restricción proteínica severa (6% proteína) previo y durante la gestación modifica el grado de respuesta de las crías (de ambos sexos) ante reforzadores de alimentos y/o soluciones de sacarosa (Tonkiss et al., 1990a)**;** incrementa la impulsividad y exploración en el laberinto elevado (son menos ansiosos) y desinhibe la conducta en el laberinto elevado en forma de T (Almeida et al., 1991; Almeida et al., 1993; Almeida et al., 1996b; Almeida et al., 1996c; Watkins et al., 2008).

Estos hallazgos ponen de manifiesto que la restricción proteínica materna durante la gestación tiene efectos negativos a niveles; morfológico, neuroquímico, neurofisiológico y funcional. Sin embargo, a la fecha existe información limitada del impacto de la restricción proteínica materna moderada (10% proteína) sobre la

conducta afectiva y cognitiva en la vida adulta de la progenie. El presente trabajo de investigación nos permitió primeramente evaluar los efectos del bajo consumo de proteína en la madre durante la gestación y/o la lactancia sobre la ansiedad, aprendizaje, motivación y memoria en las crías macho de la rata. De igual manera fue de utilidad para tratar de elucidar los posibles mecanismos relacionados con dichas alteraciones; para lo cual se estudiaron los cambios en el sustrato anatómico del hipocampo en sitios pre y post sinápticos (sistemas de fibras musgosas y espinas dendríticas, respectivamente), así como el papel de la ACTH y glucocorticoides (corticosterona) como marcadores de la actividad del eje hipotálamo hipófisis adrenal ante situaciones de estrés.

2.0 ANTECEDENTES

2.1 Programación del desarrollo

La programación del desarrollo describe el proceso mediante el cual las condiciones adversas durante la etapa intrauterina y neonatal, incrementan la susceptibilidad para presentar un fenotipo de alteraciones en la vida adulta, como sucede en la restricción global de nutrientes (macro o micro nutrientes) o la sobrealimentación, la restricción del flujo sanguíneo uterino, la exposición fetal a altos niveles inapropiados de glucocorticoides (Ashton, 2000), la diabetes materna gestacional (Capobianco et al., 2015), el tabaquismo (Ion et al., 2015) y alcoholismo (Probyn et al., 2013). La programación tiene mayor impacto durante ventanas críticas del desarrollo, en las cuales existen periodos de mayor demanda de nutrimentos como sucede en la última etapa del embarazo o al inicio de la lactancia (Gluckman, 2001; Godfrey and Barker, 2000; Good et al., 2006; Holemans et al., 2003; Sedaghat et al., 2015).

Considerando que el desarrollo fetal y neonatal son los periodos de mayor plasticidad celular (diferenciación y maduración neuronal), la etapa postnatal se ha incorporado recientemente en el campo de la programación ya que intervenciones en la edad postnatal podrían inducir efectos negativos o positivos, de acuerdo al concepto actual de la hipótesis de los Orígenes del Desarrollo de la Salud y la

Enfermedad (Barker, 2004b; McMullen and Mostyn, 2009). Así, el crecimiento durante las etapas tempranas de la vida depende del componente genético del individuo; sin embargo, el ambiente intrauterino y postnatal, influye de manera importante en el fenotipo. Durante la vida intrauterina, el feto retrasa su crecimiento en respuesta a condiciones ambientales adversas (Gluckman and Hanson, 2004), con lo que se presentan modificaciones que funcionan como respuesta adaptativa predictiva (Hales and Barker, 2001) que lo preparan para el ambiente postnatal adverso (Gluckman and Hanson, 2004; Hales and Barker, 2001). Estudios epidemiológicos y en animales de experimentación han demostrado que tales adaptaciones originan alteraciones metabólicas en la vida adulta del individuo (Phillips, 2001) tales como: obesidad, diabetes y resistencia a la insulina (Ravelli et al., 1998), dislipidemia (Davies et al., 2004), enfermedades cardiovasculares (Painter et al., 2007), alteraciones de desarrollo sexual y función reproductiva (Guzman et al., 2006; Rodriguez-Gonzalez et al., 2014; Zambrano et al., 2005b), afectivas y cognitivas (Alamy and Bengelloun, 2012). Como se mencionó anteriormente existen diferentes modelos biológicos que permiten evaluar los efectos de la programación durante estadíos críticos que afectan el desarrollo fetal y neonatal, tal es el caso de la malnutrición materna.

2.2 Malnutrición proteínica materna y programación en el cerebro de la cría

La nutrición juega un papel crucial en la maduración y funcionalidad del sistema nervioso central, por lo que los efectos de la malnutrición han sido demostrados en seres humanos de manera repetida en los últimos 40 años (Dhopeshwarkar, 1983; Dobbing, 1987; Galler et al., 1997) y en roedores (Dobbing, 1968; Morgane et al., 1992; Morgane et al., 1978; Tonkiss et al., 1993). Así, diferentes tipos de malnutrición en combinación con etapas críticas de la vida perinatal, pueden afectar la maduración del cerebro y el desarrollo de la función cognitiva, lo que provoca alteraciones del comportamiento, retraso en el aprendizaje y deterioro de la memoria.

El término "malnutrición" se refiere a las carencias, excesos o desequilibrios en la ingesta de energía, proteínas y/o otros nutrientes. La falta de micronutrientes como vitaminas o minerales pueden afectar la maduración del cerebro, la ausencia de proteínas es más crítico (Morgane et al., 2002). "La desnutrición" es una forma de malnutrición, donde todos los nutrientes requeridos están disponibles en la dieta, pero en cantidades insuficientes (Morgane et al., 2002). Estudios epidemiológicos y efectuados en animales de experimentación han demostrado que la malnutrición provoca deterioro en la progenie en cuanto a sus habilidades motoras finas, disminución en su coeficiente intelectual y trastorno de déficit de atención (Galler et al., 1987; Galler et al., 1990; Galler et al., 1997). De igual manera se han evaluado las funciones cognitivas (Bengelloun, 1990 ; Tonkiss et al., 1991; Tonkiss et al., 1993; Tonkiss et al., 1990a; Tonkiss et al., 1990b; Tonkiss et al., 1994), los efectos a nivel de neurotransmisores (Alamy et al., 2005; Almeida et al., 1996a) y en la fosforilación de proteínas y estado oxidativo en el cerebro (Bonatto et al., 2005; Feoli et al., 2006). La inadecuada nutrición es uno de los principales factores no genéticos que afectan el desarrollo del cerebro. En la actualidad es de suma importancia el estudio de la deficiencia nutricional fetal e infantil a nivel cerebral, debido a que dicho insulto conduce a déficits permanentes en el aprendizaje y el comportamiento en la descendencia (Morgane et al., 2002). La malnutrición materna induce efectos nocivos sobre estructuras cerebrales (King et al., 2004; King et al., 2002; Soto-Moyano et al., 1999), provocando la disminución del peso y disfunción cerebral, sobre todo en los periodos postnatales en la progenie (de Souza et al., 2011; de Souza et al., 2008; Joshi et al., 2003).

La malnutrición proteínica prenatal provoca cambios en la neurogénesis de las células granulares del giro dentado (Debassio et al., 1994; Debassio et al., 1996), en la morfología de las células del hipocampo (Diaz-Cintra et al., 1991); así como en el número y distribución de receptores de neurotransmisores (Almeida et al., 1996a). Estudios en roedores han mostrado que la deprivación de proteína afecta la neurotransmisión catecolaminérgica, serotoninérgica (Wiggins et al., 1984),glutamatérgica (Rotta et al., 2008) y GABAérgica (Steiger et al., 2003; Steiger et al., 2002). El consumo de una dieta baja en proteína en la madre

durante la gestación y lactancia parece afectar la neurogénesis, el ciclo celular, la migración, la diferenciación así como la mielinización y la sinaptogénesis (Debassio et al., 1994; Lukoyanov and Andrade, 2000). Asimismo, se ha demostrado que la malnutrición proteínica provoca astrogliosis en la corteza cerebral e hipocampo (Feoli et al., 2008). El consumo de una dieta baja en proteína puede conducir al aumento en el daño oxidante al disminuir la defensa antioxidante del cerebro y otros tejidos (Bonatto et al., 2005). La malnutrición a temprana edad afecta el hipocampo, estructura involucrada en funciones tales como: aprendizaje y memoria, especialmente en las de características espaciales (Morris, 1984). Por lo tanto, existe un gran número de investigaciones dedicadas a evaluar el impacto de la malnutrición en paradigmas de aprendizaje, usando pruebas tales como el laberinto radial (Jordan et al., 1981), la alternancia espacial (Goodlett et al., 1988) y el laberinto acuático de Morris (de Souza et al., 2008; Fukuda et al., 2002; Laus et al., 2011; Lukoyanov and Andrade, 2000; Tonkiss et al., 1994).

Por otro lado, se sabe que la malnutrición materna durante la etapa prenatal genera en la descendencia aumenta el número de respuestas para obtener la recompensa, lo anterior se refiere a dar un premio por realizar una actividad en un periodo de tiempo (Almeida et al., 1996b; Tonkiss et al., 1990a; Tonkiss et al., 1990b). Se ha demostrado que los descendientes de madres alimentadas con dieta baja en proteína (6%) durante la gestación presentan mayor impulsividad (menos ansiosos) cuando son sometidos a pruebas de ansiedad como el laberinto elevado en T, que implica estímulos aversivos que no son ni dolorosos ni artificiales (da Silva Hernandes et al., 2005).

2.3 Orígenes del desarrollo de la conducta de tipo ansiedad

El término **ansiedad** hace referencia al estado emocional, un síntoma o un conjunto de síntomas, que se presentan como reacción ante situaciones de peligro, estrés, conflicto, como resultado de un trauma, por consumo de drogas, o por presencia de una enfermedad; los cuales pueden deteriorar el funcionamiento normal. Se le define también como una anticipación aprehensiva de miedo futuro,

acompañada de síntomas de tensión o disforia (estado de ánimo depresivo) con respuestas relacionadas al comportamiento, dentro de las cuales se incluye la evitación de la interacción social, la vigilancia y la excitación, que se activan con la finalidad de proteger al individuo ante situaciones de peligro (Bouton et al., 2001).

En el ser humano, el estado de ansiedad es similar al temor, aunque, algunos expertos consideran necesario hacer una distinción entre estos conceptos, porque si bien ambos estados preparan al organismo para hacer frente a situaciones de peligro, en el caso del temor el peligro es reconocible, mientras que en la ansiedad el peligro no es discernible (Pritchard, 2015). En su forma patológica, la ansiedad puede interferir en la vida cotidiana del individuo, y se ha clasificado según el Manual Diagnóstico y Estadístico de la Asociación Psiquiátrica Americana (por sus siglas en ingles DSM-V) en seis trastornos: trastorno de ansiedad generalizada, fobias social y simple, trastorno de pánico, desorden de estrés postraumático y trastorno obsesivo-compulsivo, compartiendo características fisiológicas y de comportamiento común como: expectativas irrealistas, preocupación excesiva y duradera, tensión motora, inquietud, irritabilidad, dificultad para dormir, hipervigilia, episodios inesperados de terror, disnea, miedo a morir, pérdida de control, sentimiento de rechazo, y obsesivos compulsivos (Pritchard, 2015).

2.3.1 Modelos experimentales para el estudio de ansiedad

Estudios en animales de experimentación han sido de gran utilidad para elucidar los mecanismos que incrementan la conducta de tipo ansiedad en respuesta a estímulos amenazantes a lo largo de la vida del individuo, para integrar los circuitos neurobiológicos que participan en la regulación de la ansiedad y para estudiar la interacción gen-ambiente en la etiología de la ansiedad (Gross and Hen, 2004).

La evidencia experimental generada por Cannon (Cannon, 1929; Cannon, 1987), permitió proponer que el cuerpo mamilar del hipotálamo era el punto de partida de las emociones. Desde el punto de vista anatómico, Paez (Pratt, 1992) describió la

importancia del sistema límbico en la experiencia y en la expresión de la emoción. Con base en esta propuesta, se desarrollaron modelos de ansiedad basados en la estimulación de zonas discretas del SNC en felinos y roedores (Fernandez De Molina and Hunsperger, 1959). En la actualidad se sabe que el sistema límbico participa de manera integrada en la medición de respuestas emocionales (Gray, 1991). La identificación y clasificación del repertorio conductual de varias especies animales permitió el desarrollo de un grupo de paradigmas conductuales para el estudio de la ansiedad. Estos paradigmas etológicos, en contraste con las pruebas de conflicto, explotan conductas innatas, no condicionadas. Este tipo de paradigmas revela más variabilidad en los valores conductuales basales, sin embargo, tienen un nivel más alto de validez neurobiológica, no requieren entrenamiento y son menos susceptibles a la interferencia de procesos relacionados a la memoria o motivacionales (Rodgers, 1997). Algunos ejemplos incluyen, entre otros, el laberinto elevado en forma de cruz (Pellow and File, 1986), la conducta defensiva de enterramiento (Treit et al., 1981), el modelo de interacción social (File, 1992), y la prueba de campo abierto (Hall, 1934).

2.3.1.1. El **laberinto elevado en cruz**, (EPM, por sus siglas en inglés) fue propuesto por Handley and Mithani en 1984. El dispositivo se encuentra elevado desde el suelo y consta de dos brazos abiertos y dos cerrados frente a frente interconectados por una plataforma central; ha sido validado en ratas (Pellow et al., 1985) y ratones (Lister, 1987); no requiere de entrenamiento. Este modelo evalúa la exploración de la rata en un nuevo ambiente que presenta dos zonas diferentes: una potencialmente aversiva [brazos abiertos] y otra segura [brazos cerrados] (Green S and H., 1991; Rodgers, 1997). El movimiento del animal puede ser explicado en primera aproximación como el resultado de una ponderación entre la motivación de explorar y la aversión que experimenta en una determinada posición del laberinto (AE., 1993). En su estado natural la rata elige estar cerca de superficies verticales, preferiblemente rincones y lugares con poca iluminación, los campos abiertos y las alturas le causan aversión, lo que explica porque la rata permanezca más en los brazos cerrados que los brazos abiertos (Green S and H.,

1991; Rodgers, 1997). Evalúa la actividad exploratoria, y diversos parámetros tales como: número de entradas, tiempo de permanencia y distancia recorrida tanto en los brazos abiertos como cerrados, lo cual permitirá determinar el grado de ansiedad del animal.

2.3.1.2. La **prueba de campo abierto**, (OF, por sus siglas en inglés) consiste en someter a un animal (roedor), a un entorno desconocido del que intentará escapar (Walsh and Cummins, 1976). Este tipo de prueba permite registrar una serie de variables indicativas de actividad motora general, exploración y emocionalidad. Para entender los resultados de estas pruebas es necesario recordar que la rata es un animal de costumbres nocturnas que vive formando colonias en madrigueras relativamente estrechas. Estas características hacen que la rata presente una fuerte fotofobia y una marcada tendencia tigmotáxica, es decir, a desplazarse con su cuerpo en contacto físico con paredes y objetos. Teniendo en cuenta estas características, el campo abierto (particularmente cuando está fuertemente iluminado) constituye para la rata un medio adverso (nivel medio o moderado de estrés) que inducirá en el animal un aumento de la emocionalidad, aumento que suele traducirse en una pérdida de su capacidad exploratoria. Además, se debe valorar de manera distinta los desplazamientos en zonas próximas a la pared (deambulación externa) de aquellos realizados en zonas alejadas (deambulación interna), ya que la respuesta tigmotáxica sólo se inhibe cuando el nivel emocional del animal es bajo y se aventura a desplazarse hacia el centro del recinto (P., 1991).

Tanto el EPM como OF, son pruebas complementarias que permiten evaluar la ansiedad y la función motora en los roedores. Se considera que un animal es más ansioso cuando pasa menor tiempo en los brazos abiertos o en la zona central dependiendo del aparato que esté utilizando el evaluador. La función motora se determina de manera indirecta al determinar la distancia total recorrida. El equipo *per se* y su iluminación, genera estrés en los animales que puede modificar la

conducta innata a explorar los espacios desconocidos y a su vez estimular la liberación excesiva de glucocorticoides. Es por eso que dichas conductas pueden asociarse de manera indirecta con la actividad o respuesta del eje HHA. Existe evidencia experimental que demuestra que ambos equipos son sensibles para evaluar ansiedad en roedores, en los cuales utilizan modelos de estrés moderado, crónico y nutricional (Cárdenas and Navarro, 2002).

2.3.2 Ansiedad y la Amígdala

En la literatura existe información relevante, que demuestra la participación de la amígdala en la conducta de tipo ansiedad. En humanos, se relaciona el incremento de su volumen con mayor ansiedad, así como aumento en la activación de ésta estructura en pacientes con trastorno de ansiedad social (Boehme et al., 2014; Machado-de-Sousa et al., 2014; Qin et al., 2014).

Por otra parte, los ensayos de genes tempranos en roedores demuestran la activación de la amígdala después de la exposición a un ansiogénico (Butler et al., 2016; Butler et al., 2012; Silveira et al., 1993), y su inactivación farmacológica actúa como ansiolítico cuando al animal se somete a la prueba EPM (Moreira et al., 2007). Ambos estudios en humanos y roedores muestran que la amígdala es un elemento crucial en el circuito de la ansiedad (Figura 1) y las subregiones más estudiadas son: la amígdala baso lateral (BLA por sus siglas en inglés) y el núcleo central.

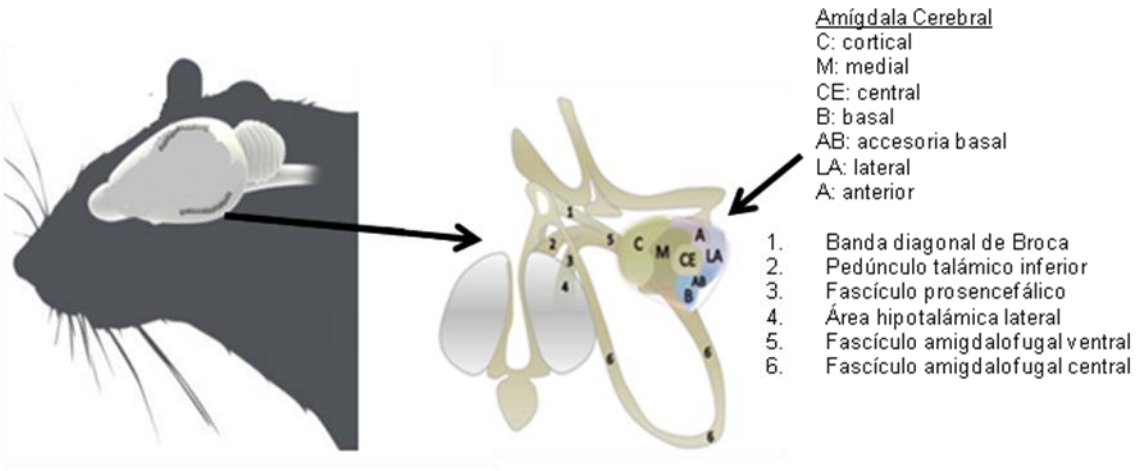

Figura 1. Componentes estructurales de la amígdala de la rata. Esta estructura participa en la señalización de los estímulos relacionados con la recompensa, ansiedad y miedo. Fuente modificada de Torras (Torras et al., 2001).

La BLA integra información altamente procesada por el medio ambiente y codifica las señales pertinentes del comportamiento (LeDoux, 2000), tiene células que responden a las señales que predicen amenazas (Amano et al., 2011) y otras que no predicen el peligro (Senn et al., 2014). Tiene proyecciones a la porción lateral y medial del núcleo central de la amígdala (Tye et al., 2011), la cual es su principal entrada excitatoria (LeDoux, 2000), cuya función es la regulación de conductas inducidas por estímulos amenazantes. Este efecto es mediado por las proyecciones del núcleo medial central al hipotálamo y del tallo cerebral (Price and Amaral, 1981); lo cuales modulan diversas características de la estado de ansiedad. La amígdala no sólo recibe aferencias corticales que procesan información de los estímulos del entorno, sino también proyecciones de la corteza pre frontal y del hipocampo ventral (Rosenkranz and Grace, 2001). La BLA regula la producción de respuestas emocionales tanto innatas como aprendidas y participa en los sistemas neurales que se relacionan con el aprendizaje asociativo (Chau and Galvez, 2012; Morgan and LeDoux, 1999) al permitir la vinculación de estímulos condicionados con respuestas somáticas previamente relacionadas con estímulos no condicionados. Estudios en animales han demostrado que la amígdala juega un papel crítico en la adquisición, consolidación y extinción de los recuerdos relacionados con el miedo (Chau and Galvez, 2012; Moustafa et al.,

2013). Además se ha mostrado que la activación de la BLA modula la consolidación de la memoria a través de proyecciones a otras regiones del cerebro como: hipocampo, núcleo caudado, núcleo basal y la corteza, implicadas en la consolidación de la memoria (McGaugh, 2002).

2.4 Neurobiología de la memoria y aprendizaje

El sistema de cognición en general se compone de procesos de funcionalidad y de aprendizaje y memoria, entre los primeros, se incluyen por una parte, procesos de "pre atención", en los que se realiza el filtrado de la información sensoriomotora para centrar la atención en los elementos más relevantes del entorno (Posner, 1995) y cuya alteración genera una sobrecarga sensorial y fragmentación cognitiva que podría contribuir a las alteraciones que se dan en distintos trastornos psicóticos (McGhie and Chapman, 1961). Por otra parte el sistema de cognición participa en los procesos de atención, como atención reflexiva, orientación visual, orientación aprendida, vigilancia, habituación y atención selectiva, mantenida o dividida. En los procesos de aprendizaje y memoria se incluyen el aprendizaje asociativo, espacial o no espacial, y la memoria a corto y largo plazo. Se puede definir el concepto de memoria como 'la retención a largo plazo de representaciones internas dependientes de la experiencia' (Dudai, 1989). El aprendizaje es el proceso de adquisición de conocimientos, habilidades o actitudes a través de la experiencia o la educación, lo que origina un cambio persistente, cuantificable y específico en el comportamiento de un individuo (Kandel ER et al., 2000). .

La memoria se puede dividir en: memoria implícita (no declarativa) y explícita (declarativa) (Baddeley and Hitch, 1993; Kandel ER et al., 2000), la primera, es la resultante de procesos de aprendizaje no consciente realizados a través de hábitos y habilidades, mediante estimulación o sensibilización previa, y en los que interviene la musculatura esquelética o bien respuestas emocionales y de aprendizaje no asociativo. La memoria explícita o declarativa engloba la retención del conocimiento de determinados acontecimientos, lugares o hechos, y en ella toma parte el lóbulo medio temporal del diencéfalo. Esta información se

retiene mediante un esfuerzo consciente y a través de asociaciones nuevas (Kandel ER et al., 2000). Estos aspectos del aprendizaje y la memoria se han estudiado a lo largo de los años gracias a los modelos animales, que han permitido conocer las estructuras cerebrales que intervienen en su funcionamiento (Figura 2). En el estudio de la memoria, se han distinguido tres etapas: adquisición, consolidación y evocación. La adquisición y subsecuente codificación se refiere a los procesos por los cuales la información nueva es procesada en un primer encuentro con determinada circunstancia o experiencia; es crítica porque establece cuán adecuadamente lo aprendido podrá luego ser recordado. La extensión de este proceso está en relación directa con el tiempo y número de sesiones necesarias para aprender una determinada tarea. La consolidación involucra aquellos procesos por los que se almacena la información adquirida, de manera reciente, transformándose en más estable y, de alguna manera, resistente a la influencia de cualquier factor de interferencia, persistiendo así por un cierto tiempo. La consolidación de la memoria a largo plazo involucra la expresión de genes y la síntesis de nuevas proteínas, dando lugar a cambios estructurales que permiten el “almacenamiento” de esa memoria de una manera estable a través del tiempo. La duración de esta etapa se establece desde el momento que termina el entrenamiento, aunque una información puede comenzar a ser consolidada mientras se sigue adquiriendo (Dudai and Eisenberg, 2004; Morris, 2006; Tse et al., 2007). La evocación es el proceso por el cual se expresa y usa la información o conocimiento adquiridos, consolidados y asociados. Involucra la manifestación simultánea de diferentes tipos de información, que pueden estar guardados en diferentes sitios y por diferentes mecanismos, por el que se puede manifestar lo aprendido. Una memoria puede ser medida por el desempeño en el momento de la evocación. En animales no humanos, la evocación de un aprendizaje se evidencia a través de cambios en el comportamiento (Iversen, 1997; Izquierdo, 1989).

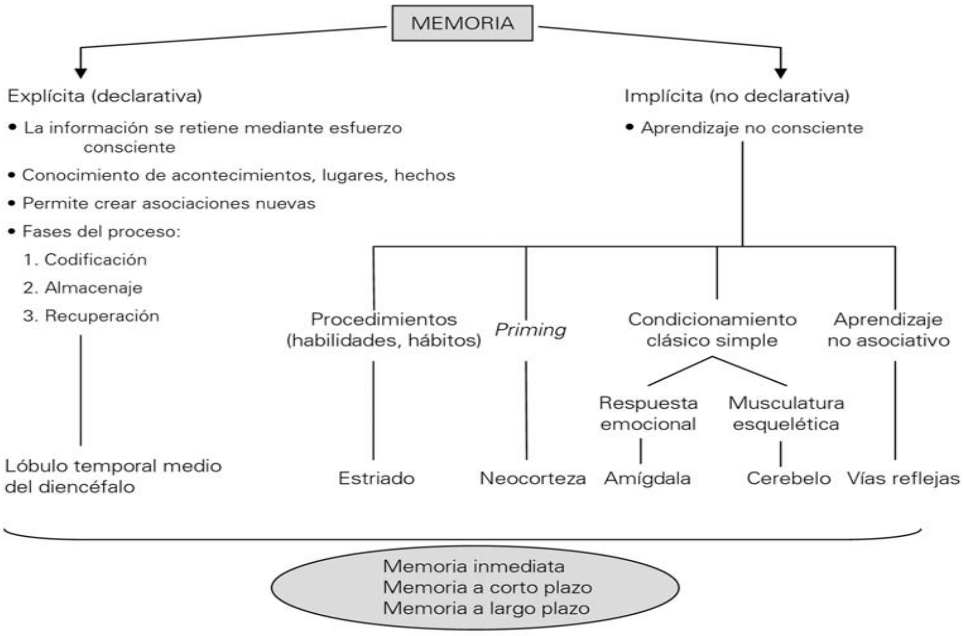

Figura 2: Tipos de memoria y estructuras cerebrales relacionadas. En función de cómo se recibe y almacena la información, cabe distinguir dos grandes tipos de memoria: la implícita, que no se requiere un aprendizaje consciente, y la explícita, producto de un esfuerzo cognitivo consciente. Fuente obtenida de Navarrete y colaboradores (Navarrete et al., 2008).

2.4.1 Modelos experimentales para el estudio de aprendizaje, motivación y memoria

2.4.1.1 Condicionamiento clásico y operante (aprendizaje asociativo)

Desde 1938, Skinner, en la publicación de su primer libro *La conducta de los organismos*, estableció la diferenciación entre dos tipos de aprendizaje mediante condicionamiento: uno regido por el *principio de sustitución* (el condicionamiento clásico-pavloviano) y otro gobernado por la *ley del efecto* (el condicionamiento instrumental), los denominó respectivamente, condicionamiento respondiente y operante. Propuso la palabra *operante* para definir la conducta que produce cambios o que opera en el medio o en el ambiente (Skinner, 1938). El condicionamiento clásico es un proceso de aprendizaje mediante el cual un estímulo que previamente no suscitaba una respuesta, acaba provocándola a consecuencia de su *asociación temporal* con otro estímulo que sí provoca la respuesta. Es el mecanismo más simple por el que los organismos aprenden a dar *respuestas nuevas* a los estímulos y aprenden *las relaciones entre los estímulos*.

Implica el aprendizaje de relaciones entre estímulos (condicionados e incondicionados). Desde 1927, Pavlov, consideró la salivación anticipatoria como una manifestación de una respuesta refleja provocada por el estímulo de la comida. Esta nueva respuesta era resultado del aprendizaje asociativo: el alimento, que ya provocaba salivación. Sugirió que tanto los animales como el hombre poseen *reflejos incondicionados* o innatos, los cuales constan de dos elementos: 1- un *estímulo incondicionado* (por ej. comida), que provoca 2- una *respuesta incondicionada* (por ej. salivación); y cuando un *estímulo ambiental neutro* aparece junto con el estimulo incondicionado, se forma un nuevo reflejo denominado *reflejo condicionado*. El estímulo neutro se convierte en el *estímulo condicionado* (por ej. un sonido o luz), y es capaz de provocar la respuesta aprendida o *respuesta condicionada* (por ej. salivar ante el sonido o luz) (Habib et al., 1994).

Los procesamientos se centran en la relación entre las apariciones o ejecuciones de una respuesta específica (instrumental) y la administración del reforzador o *refuerzo,* y se basa en la ejecución de una respuesta específica y la administración del reforzador (Habib et al., 1994). Un programa de reforzamiento es una pauta o regla que determina la forma en que se relacionan las presentaciones del reforzador con las ocurrencias de la respuesta instrumental (Domjan et al., 1986). La entrega del reforzador puede depender de varios factores, número de respuestas, paso del tiempo, etc. Los programas de reforzamiento se investigan normalmente en cajas de Skinner y permiten una observación continua de la conducta.

La motivación operacional implica la modificación de la conducta mediante diferentes operaciones que anteceden a la conducta en cuestión. Por ejemplo, el que la frecuencia de una conducta reforzada con comida aumente conforme se alarga el periodo de privación de comida, es un fenómeno denominado “motivación”. A pesar de que el condicionamiento y la motivación son procesos diferentes, inevitablemente interactúan en cualquiera de los dos tipos de experimentos. Esto es, porque la privación y la saciedad habilitan o inhabilitan la función de los estímulos como reforzadores operantes. En el caso de la comida es

claro que su función como un reforzador de alguna respuesta depende de su privación. Aún más, las conductas establecidas pueden ocurrir o dejar de ocurrir dependiendo de la privación o la saciedad del reforzador (Michael, 2007). Un excelente ejemplo de tal interacción son los estudios realizados por Skinner sobre la conducta de comer de las ratas. En este estudio, las ratas previamente entrenadas al presionar una palanca para obtener un gránulo de comida, solamente comían en una ocasión cada día, siempre a la misma hora (ejemplo, bajo una privación de 24 horas). La sesión experimental terminaba cuando dejaban de presionar la palanca por más de 30 minutos. La variable dependiente fue el curso temporal de la frecuencia de presionar la palanca para obtener la comida. La frecuencia de presiones a la palanca fue alta al iniciar la sesión experimental pero disminuyó gradualmente conforme aumentó el número de gránulos de comida obtenidas por la rata, a la manera de una curva acelerada de manera negativa (McSweeney and Murphy, 2000). Cuando se alcanzó el punto de la saciedad de comida, la comida perdió su función como reforzador de la respuesta de presionar la palanca (McSweeney and Murphy, 2000).

2.4.1.2 Aprendizaje y memoria espacial

La memoria espacial es un componente fundamental de la memoria episódica, aquella que se refiere a hechos que tuvieron lugar en un determinado tiempo y espacio y forma parte de la memoria declarativa (Kandel ER et al., 2000). El aprendizaje y la memoria espacial es la capacidad que tiene el animal de adquirir y retener asociaciones de las características del ambiente, lo que le permite desenvolverse en el espacio (Vicens et al., 2003) y consiste en múltiples mecanismos que se encargan de codificar, almacenar y recuperar información de rutas, configuraciones y localizaciones espaciales (Kessels et al., 2001). Este tipo de memoria puede ser evaluada mediante modelos experimentales con animales en los que la solución de la tarea depende de la información espacial disponible. Los roedores pueden adoptar cuatro formas principales de navegación para la resolución de tareas espaciales: orientación, guía, cartografía e integración de la ruta (Santín-Núñez et al., 2000). En el aprendizaje de orientación los animales

basan su búsqueda en movimientos aprendidos durante la ejecución de la tarea; en el aprendizaje de guía aprenden asociaciones entre los estímulos señal y la meta. Estas dos formas de navegación se explicarían mediante paradigmas asociativos de condicionamiento. El aprendizaje cartográfico, implica el uso de señales distales con las que los animales se forman una representación de su entorno mediante el que localizan la meta. Por último, la integración de la ruta consiste en un proceso de actualización de la información cuando las pistas ambientales no ofrecen la suficiente, mediante un sistema interno de referencia basado en el lugar de salida antes de iniciar la navegación, para lo que el animal podría utilizar pistas cinestésicas (movimiento) y señales vestibulares (Santín-Núñez et al., 2000). Estas estrategias de navegación espacial parecen depender de distintos sistemas de memoria. En el laberinto de agua las ratas tienden a aproximarse a la plataforma sumergida desde una dirección conocida, sugiriendo la utilización de representaciones específicas para reconocer su localización, lo que implicaría establecer las relaciones entre los distintos estímulos (Wang and Spelke, 2002).

El laberinto acuático de Morris (LAM) es uno de los modelos más empleados en el estudio de la memoria espacial en roedores. Fue diseñado en 1984 por R.G. Morris para evaluar la memoria espacial en ratas (Morris, 1984). Este paradigma resulta de especial interés, puesto que no necesita de la privación de agua o comida ni de la aplicación de una descarga eléctrica para motivar la conducta (Vicens et al., 2003). Con esta prueba es posible valorar la memoria espacial (consiste en múltiples mecanismos especializados en codificar, almacenar y recuperar información acerca de rutas, configuraciones y localizaciones espaciales). La memoria espacial es independiente de los ensayos y permite aprender el procedimiento general para la ejecución de la tarea (Morris, 1984).

La prueba de reconocimiento de objetos, fue introducida por el grupo de Ennaceur (Ennaceur et al., 1997) y su uso se ha incrementado como modelo de investigación y herramienta experimental para valorar los efectos de los fármacos sobre el aprendizaje y la memoria relacionados con los mecanismos

neurobiológicos (Baker and Kim, 2002; Okuda et al., 2004; Rampon et al., 2000; Rosa et al., 2003). Esta prueba se fundamenta en la tendencia natural de los roedores a explorar nuevos objetos y ambientes y compararlos con otros que les son familiares. La prueba se realiza en un campo abierto situado en una habitación iluminada de manera homogénea y con objetos que deben ser de similar textura, color y tamaño, pero de distinta forma. El resultado esperado es que el animal sin deterioro cognitivo explore más el objeto nuevo que el familiar. En cambio, para un animal con alguna alteración en la función cognitiva, el objeto familiar y el nuevo le parecerán igual de novedosos y no habrá diferencia entre los tiempos de exploración.

2.5 Sustrato anatomo funcional de la formación hipocampal

La formación hipocampal (FH) forma parte del sistema límbico, y está constituida por una serie de estructuras conectadas entre sí a través de un patrón intrínseco, cada una tiene una organización citoarquitectónica especial que le confiere funciones cognitivas. Las funciones complejas de la FH son la navegación espacial (Gothard et al., 1996), el aprendizaje (Bunsey and Eichenbaum, 1996; Moser et al., 1993) y la elaboración de un mapa cognitivo de la experiencia del animal en un ambiente determinado (O'Keefe, 1990). En la Figura 3, se indica la organización anatómica de la FH que la constituyen la cortezas de asociación, entorrinal (medial y lateral), peririnal y parahipocampal, así como el complejo subicular (pre y parasubiculum), el propio Cornu Ammonis (CA) con sus cuatro campos en el humano y tres en la rata (CA1-3), y el giro dentado (GD), formado por una capa de somas pequeños densamente empaquetados formando el *stratum granulare* de células granulares (CG), las cuales son de naturaleza glutamatérgica y poseen axones basales denominados fibras musgosas. En la capa más cercana a la fisura hipocámpica**,** se encuentra el *stratum moleculare* o capa molecular, formado por las prolongaciones de las dendríticas apicales de las CG, interneuronas y células gliales. La capa más profunda del giro dentado o *hilus,* se caracteriza por su naturaleza polimórfica, compuesta por una gran variedad de tipos celulares (Amaral, 1978; Amaral et al., 2007). Entre el *stratum*

granulare y el *hilus* se pueda distinguir una fina capa subgranular, compuesta por precursores neurales que poseen actividad proliferativa durante la vida adulta(Altman and Bayer, 1990; Seri et al., 2001).

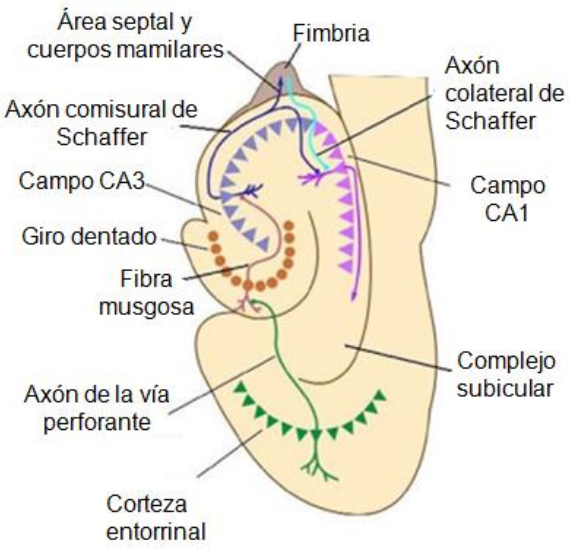

Figura 3: Corte horizontal de la formación hipocampal de la rata y sus divisiones. Se distinguen el complejo subicular (subiculum, SUB; presubiculum, PrS y parasubiculum, PaS). La corteza entorrinal (CE), el hipocampo (CA1 y CA3) y el giro dentado (GD). Esquema de Swanso, L.W. et al., 1987 (Swanson et al., 1987)

La corteza entorrinal (CE) tiene 2 subdivisiones principales: la corteza entorrinal lateral y medial, su citoarquitectura comprende seis capas bien definidas: las II y III contienen células pequeñas localizadas de manera densa que envían sus axones hacia el hipocampo; las capas V y VI están formadas por neuronas grandes que envían sus proyecciones fuera de la formación hipocampal. Las capas I y IV son acelulares (Dolorfo and Amaral, 1998).

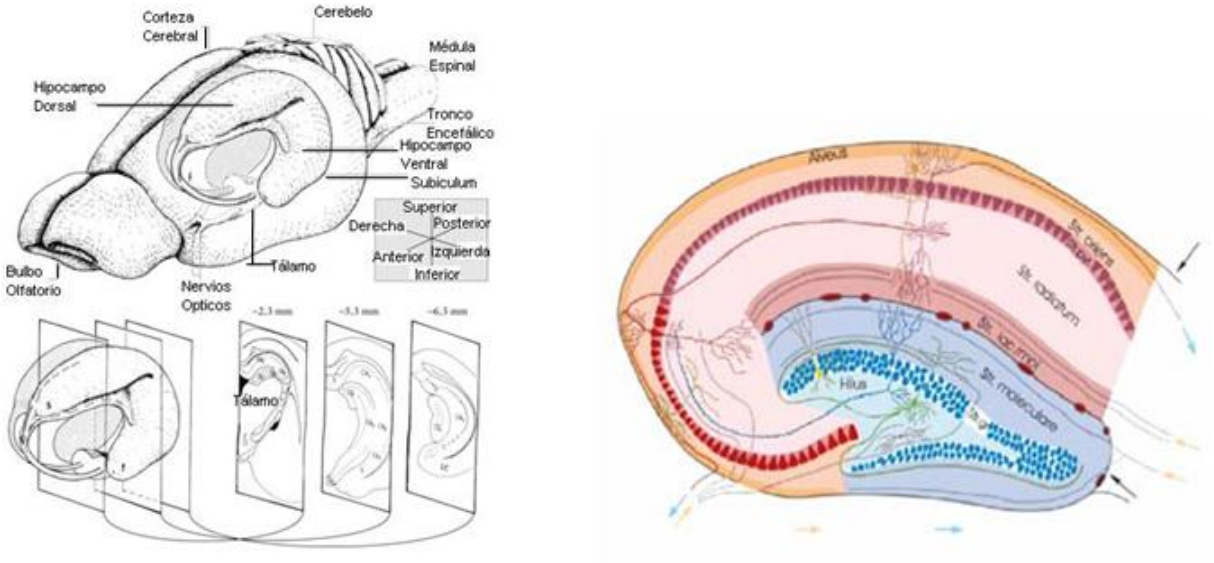

Figura 4: Hipocampo y su ubicación en el cerebro de la rata. Esquema de Amaral y Witter, 1995 (Amaral and Witter, 1995)

Es importante destacar que las células que conforman cada región de la FH, no se presentan al mismo tiempo (heterocronía) por que siguen un patrón distinto de manifestación, siendo diferente para cada especie (Tabla 1). La mayoría de éstos se inician en la etapa prenatal y se extienden más allá del nacimiento.

Tabla 1: Periodo de tiempo expresado en días, en la que se lleva a cabo la producción de células que conforman cada región de la formación hipocampal.

Animal	Tiempo de Gestación (días)	Giro Dentado	CA3	CA1	Subículum	Corteza Entorrinal
Ratón	19	G10- P15	G10- 7	G10- 17	G10- 15	G10-16
Rata	21	G14- P25	G10- 20	G14- 20	G14- 17	G13-17
Gato	63- 65	G22- P14	G22- 42	G22- 42	G22- 42	G21- 40
Mono	140-145	G39- P43	G38- 62	G38- 70	G38- 75	G36- 70

En el cerebro embrionario, muchos procesos de organización son lábiles a perturbaciones (estrés y/o desnutrición), lo cuales pueden modificar la actividad de organización durante períodos cortos de tiempo y luego cesar por completo; o bien, pueden inicialmente procesarse rápidamente y luego decaen a un nivel bajo sin cesar totalmente su actividad. Lo anterior puede generar cambios irreversibles (morfológicos, fisiológicos y bioquímicos) en el desarrollo del sistema nervioso central; es decir, si un proceso no se produce en su tiempo programado, no será un funcional (Morgane et al., 2002).

2.5.1 Hipocampo

El hipocampo se ubica en el cerebro de la rata en posición rostro-caudal desde los núcleos septales hasta el lóbulo temporal caudo-ventral (Figura 4) cuya porción dorsal y anterior recibe información derivada de las cortezas sensoriales. Las rutas de flujo de información en el hipocampo están claramente definidas por lo que esta estructura es idónea para el estudio de la función sináptica. Consta de tres regiones divididas según su conectividad: Cornu ammonis (CA)1, CA2 y CA3 (estas últimas agrupadas normalmente como región CA2/CA3).

Estas regiones tienen una estructura similar organizada en capas que se disponen, unas encima de otras, de manera paralela a la superficie del ventrículo lateral (Figura 5). Desde la zona ventricular hacia la fisura hipocámpica, las capas de las regiones CA son: el *alveus* que contiene axones aferentes y eferentes del hipocampo.

Raramente se encuentran neuronas en esta capa; estrato oriens (SO, del latín *stratum oriens*) adyacente al *alveus*, contiene las dendritas basales de las neuronas piramidales así como neuronas inhibidoras, entre ellas las que proyectan al septum medial y los axones colaterales de Schaffer, que llegan al estrato radiado (stratum radiatum del CA1), o establecen recurrencias con las piramides del CA3. Capa piramidal (SP, *stratum pyramidale*) que: contiene los somas de las neuronas piramidales (NP), de naturaleza glutamatérgica. Entremezcladas entre ellas se encuentran algunas interneuronas inhibidoras.

Estrato lúcido (SL, *stratum lucidum*): presente únicamente en la región CA3, el SL aloja las fibras musgosas del giro dentado y algunas interneuronas inhibidoras.
Estrato radiado (SR, *stratum radiatum*), adyacente al piramidal, en la región CA1, y al lúcido, en CA3, contiene las dendritas apicales de las neuronas piramidales y el estrato lacunoso molecular (SLM, *stratum lacunosum-moleculare*) formado por la parte más distal de las dendritas apicales de las neuronas piramidales del CA1 y de fibras provenientes de la corteza entorrinal (patrón perforante).

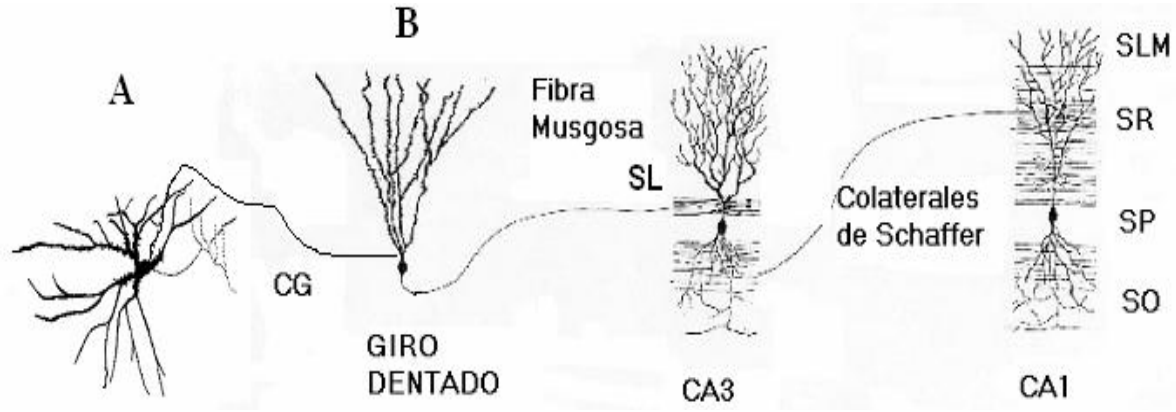

Figura 5. Células de proyección que forman parte del intracircuito del hipocampo. A, célula musgosa del hilus, B, célula granular (CG) del giro dentado de donde parte la fibra musgosa que hace sinapsis en el *stratum lucidum* (SL) de la célula piramidal del campo CA3, de esta célula parten las colaterales de Schaffer hacia el *stratum radiatum* (SR) de las células piramidales (CP) del campo CA1, en donde se localizan también los estratos *oriens* (SO) y *lacunosum moleculare* (SLM). Esquema de Ishizuka y colaboradores (Ishizuka et al., 1995).

2.5.1.1 Circuitos del hipocampo

La FH destaca por la extraordinaria complejidad de los circuitos locales (Amaral and Witter, 2004). El llamado circuito trisináptico (Figura 6) está formado básicamente por tres conexiones unidireccionales que permiten el procesamiento de información proveniente de regiones neocorticales, y el envío de información de vuelta a la neocorteza.

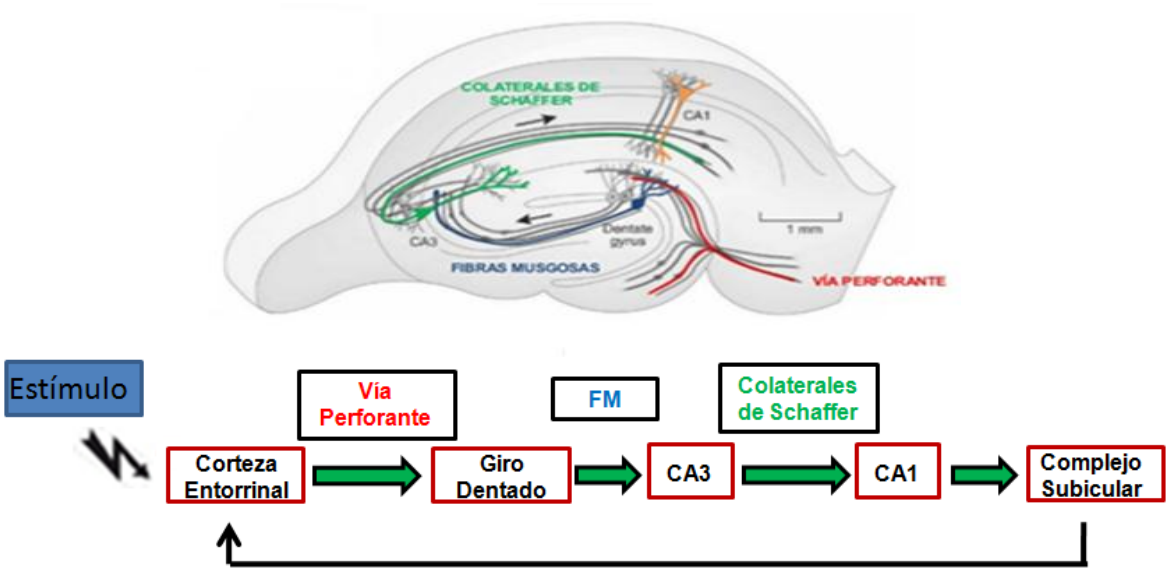

Figura 6. Circuito unidireccional del hipocampo de roedor. Se inicia con la entrada de los axones provenientes de las capas II/IV del patrón perforante de la corteza entorrinal que hacen conexiones con el giro dentado (GD) y las células piramidales del CA3, las que también reciben entradas de las fibras musgosas (FM) provenientes de las células granulares. Las células piramidales del CA3 envían axones colaterales de Schaffer al CA1, el cual también envía axones de asociación comisural (AC) y recibe axones del patrón perforante (PP) provenientes de la corteza entorrinal pero de la capas II/V. Los axones de estas células van hacia el subiculum (SU), que en turno envían axones de regreso hacia la corteza entorrinal lateral y medial. Esquema de Bortolottoy colaboradores (Bortolotto et al., 2003).

La conexión entorrino hipocámpica, o vía del patrón perforante (PP), está formada por los axones de las células piramidales de capa II de la CE que contactan con las dendritas de células granulares, situadas en la capa molecular del giro dentado, y con algunas interneuronas de esa misma capa. La vía de las fibras musgosas (FM), formada por los axones de las células granulares, no mielinizados que atraviesan el hilus para inervar el estrato lúcido de CA3, donde contactan con las dendritas de las neuronas piramidales e interneuronas, o forman recurrencias con las neuronas granulares. La vía asociativa o colateral de Schaffer está formada por axones de las neuronas piramidales del CA3 que proyectan a las dendritas de células piramidales localizadas en los estratos *oriens* y *radiatum* de la región CA1.

2.5.1.2 Hipocampo y sistema de fibras musgosas (FM)

Uno de los subsistemas del hipocampo en el que se han explorado ampliamente las modificaciones plásticas es la vía de las FM, ya que este subsistema es bastante accesible al estudio histológico, electrofisiológico y bioquímico, debido al gran tamaño relativo de sus componentes y a su estructura laminar (Amaral and Dent, 1981). Además, esta vía constituye un importante escaparate para el estudio de fenómenos plásticos, debido a su alta susceptibilidad de experimentar modificaciones de tipo funcional y anatómico (Represa et al., 1990). Las FM están conformadas por los axones no mielinizados de las células granulares del giro dentado que hacen sinapsis tanto con las células hilares como con las células piramidales del área CA3. Estas fibras viajan a través del área CA3 formando una banda gruesa llamada estrato lúcido haciendo sinapsis con la parte proximal de las dendritas apicales de las células piramidales del área CA3 (Henze et al., 2000).

A su vez, las células granulares del GD reciben aferencias masivas desde la corteza entorrinal, la cual llega a los dos tercios externos de sus dendritas y en su tercio interno reciben proyecciones del hilus ipsi y contralateral. La proyección de las FM es en la zona proximal de las dendritas apicales de las células piramidales del área CA3 en el estrato *lucidum*, a las que se les denomina conexiones suprapiramidales. Se ha observado que tras diferentes tipos de tratamiento ya sea conductuales (Dobrossy and Dunnett, 2001; Ramirez-Amaya et al., 2001), electrofisiológicos (Adams et al., 1997; Morimoto et al., 2004; Schjetnan and Escobar, 2012) o bioquímicos (Adams et al., 1997; Represa et al., 1990) se induce plasticidad de las FM. Por otro lado, las FM establecen sinapsis en las zonas proximales de las dendritas basales de las células piramidales del área CA3 en el estrato *oriens*, a las que se les denomina conexiones infrapiramidales.

Este tipo de sinapsis (FM-CA3), ha sido detectada con la técnica histoquímica de Timm y corroborada utilizando otras técnicas como la de Golgi (Ben-Ari and Represa, 1990), trazadores de vías neurales (Franck et al., 1995)o por microscopía electrónica (Amaral and Dent, 1981; Ramirez-Amaya et al., 1999). Las sinapsis FM-CA3 tienen un papel importante para el almacén y recuerdos de

la información de tipo espacial en las redes del hipocampo (Bischofberger et al., 2006; Nicoll and Malenka, 1995; Si and Treves, 2009). Las terminales axónicas de las FM están conformadas por botones sinápticos gigantes. Estos botones se encuentran invaginados por excrecencias y espinas de las células piramidales, a los que típicamente envuelven completamente, formando grandes complejos sinápticos (Henze et al., 2000; Zhao et al., 2012) (Figura 7). Cada botón contiene una amplia cantidad de vesículas sinápticas claras (aproximadamente 25,000), vesículas de núcleo denso y hasta 45 zonas activas de liberación de neurotransmisor (Rollenhagen et al., 2007).

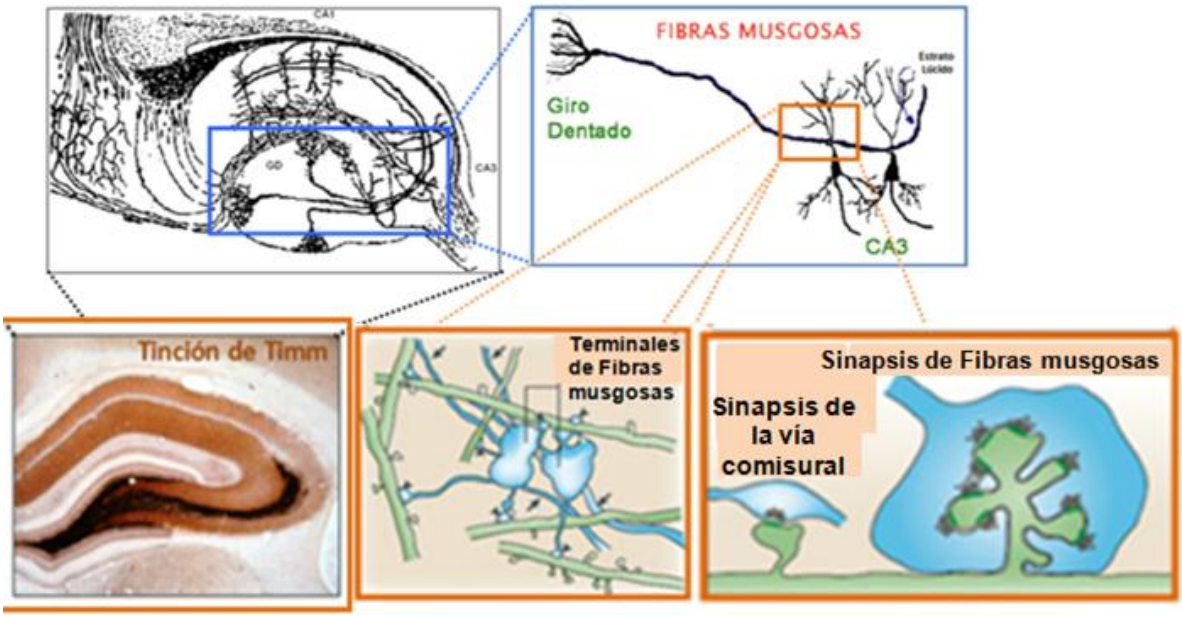

Figura 7: Las fibras musgosas del hipocampo y sus terminales axónicas. Se observa del lado izquierdo el diagrama del hipocampo y, proyectada hacia abajo, la foto de un corte coronal del hipocampo procesado con la técnica de Timm. Del lado derecho, se proyecta la vía de las fibras musgosas con sus conexiones en el estrato lucido, y abajo se observan proyectadas, representaciones de las terminales axónicas de la FM (azul), las cuales invaginan a las espinas dendríticas con las que se conectan (verde), y su comparación con las terminales de la vía comisural. Esquema modificado de Amaral y Dent, 1981; Nicoll y Schmitz, 2005 (Amaral and Dent, 1981; Nicoll and Schmitz, 2005)

La neurotransmisión excitatoria de las FM, depende de la liberación de glutamato y de la activación de receptores post sinápticos a glutamato – el principal transmisor excitatorio en mamíferos – el cual despolariza las neuronas hipocampales, más aún, la estimulación eléctrica provoca su liberación. El glutamato activa tres tipos de receptores que regulan la transmisión excitatoria ionotrópica: Kainato, α-amino-3-hidroxi-5metil-isoxazole-ácido propiónico (AMPA) y N-metil-D-aspartato (NMDA). El blanco celular dicta no sólo el tipo de receptor presináptico sino también el tipo de plasticidad que esta expresada en una sinapsis particular de la FM. Es importante mencionar que también hay varias formas de plasticidad sináptica a largo plazo (Henze et al., 2000). La potenciación a largo plazo (LTP por sus siglas en ingles) es una intensificación duradera en la transmisión de señales entre dos neuronas que resulta de la estimulación sincrónica de ambas (Cooke and Bliss, 2006). Dado que se piensa que los recuerdos están codificados por modificaciones de la fuerza sináptica (Bliss and Collingridge, 1993), se considera al LTP como uno de los mecanismos celulares principales que puede encontrarse de manera prominente en estructuras (amígdala y el hipocampo) que involucran al aprendizaje y la memoria (Bliss and Collingridge, 1993; Cooke and Bliss, 2006). La LTP ha sido estudiada en dos tipos de sinapsis excitatorias que dependen de receptores a NMDA en células piramidales (CP) del CA3 en roedores: 1) sinapsis formadas por los axones de fibras comisurales/asociación y requiere de la activación del receptor de NMDA y 2) sinapsis formada entre las colaterales de Schaffer y neuronas piramidales de CA1. Además, se ha demostrado que en la sinapsis de las FM y las CP del CA3, la LTP es independiente de la activación de receptores a NMDA (Nicoll and Roche, 2013; Zalutsky and Nicoll, 1990) y dependiente de la activación de receptores a opioides Mu, Delta y péptidos opioides como las encefalinas y dimorfinas que juegan un papel importante en la inducción de la LTP en terminales de las FM (Derrick and Martinez, 1994; Derrick et al., 1992; Escobar et al., 1997).

Por otro lado, se sabe que otro de los elementos que se encuentra modulando la plasticidad sináptica de la vía excitatoria glutamatérgica en las terminaciones axónicas de las FM es el zinc, un catión divalente que se encuentra

dentro de las vesículas de los botones sinápticos gigantes. La tinción de Timm, que revela al zinc, puede ser usada para el estudio de la inervación de las FM. Existen múltiples mecanismos del zinc extracelular que puede estar modulando receptores y transportadores a glutamato (Legendre et al., 1993). Hay evidencia de que el zinc puede ser liberado de las FM durante la estimulación sináptica y además es co-liberado con L-Glutamato. La LTP requiere de la entrada de zinc desde el espacio extracelular a la neurona postsináptica usando vías permeables de Ca^{+2} como receptores AMPA o Kainato o a través de canales de iones dependientes de voltaje (Li et al., 2001). Trabajos realizados por (Frotscher et al., 2006) indican una mayor complejidad funcional de las FM con las interneuronas GABAérgicas tanto del hilus como del CA3 que modulan el circuito inhibitorio del hipocampo. Las alteraciones en el zinc y en la homeostasis lo han sugerido como un factor clave en el desarrollo de varios trastornos neuropsiquiátricos. Se ha demostrado que a carencia del zinc reduce la neurogénesis y provoca apoptosis neural, lo cual conlleva a un déficit de aprendizaje y deterioro cognitivo. Los circuitos neuronales son definidos por las estructuras de axones y dendritas, y las sinapsis conectadas por estos.

2.5.2 Espinas dendríticas

La capacidad de las neuronas de funcionar dentro de los circuitos neuronales es mediada a través sitios de contacto denominados sinapsis. Las sinapsis químicas regulan la comunicación eléctrica dentro de las redes neuronales y transmiten información desde la terminal axónica pre sinápticas a regiones dendríticas post-sinápticas. La construcción de los circuitos neuronales durante el desarrollo del cerebro requiere del control preciso para que la actividad de la red neuronal funcione de manera adecuada. La mayoría de las sinapsis de tipo excitador en el cerebro de mamífero se establecen sobre pequeñas protuberancias dendríticas, llamadas espinas dendríticas (Bourne and Harris, 2008). Se ha mostrado que la eficacia sináptica puede inducir cambios morfológicos en el número de espinas dendríticas (Kasai et al., 2003). Además, se sabe que el almacén de información en el cerebro está dado por el fortalecimiento o el debilitamiento de las sinapsis

existentes, así como por la aparición o eliminación de las espinas dendríticas. Estos cambios funcionales y estructurales en las espinas y las sinapsis se cree son la base del aprendizaje y la memoria (Kasai et al., 2010). Las espinas dendríticas son minúsculas protuberancias protoplasmáticas que revisten la superficie de muchas neuronas (Koch et al., 1992) y representan el sitio de contacto sináptico excitador en neuronas del hipocampo, la neocorteza y otras regiones cerebrales. Fueron descritas inicialmente por Ramón y Cajal (1888), quien propuso que las neuronas se conectan por medio de axones y dendritas, y que son elementos estructurales del sistema nervioso (S., 1888). Por otro lado, diversos estudios han demostrado que las proteínas de unión al citoesqueleto de actina son fundamentales en la formación, eliminación, motilidad, estabilidad, tamaño y forma de las espinas dendríticas (Renner et al., 2008; Schubert and Dotti, 2007; Tada and Sheng, 2006). Además, en las sinapsis, el citoesqueleto de actina no sólo contribuye a la estructura morfológica de la neurona, sino también participa en la organización de las proteínas presentes en la densidad post-sináptica, así como en el anclaje de diferentes tipos de receptores post-sinápticos necesarios para la recepción del mensaje y la transmisión de la información sináptica (Renner et al., 2008)

2.5.2.1 Estructura y función

Las espinas dendríticas representan el sitio de contacto post sináptico de tipo excitador por excelencia, su densidad es de 1 a 10 por micrómetro (μm) a lo largo de la longitud de la dendrita (Sorra and Harris, 2000). La estructura de las dendritas consta de tres elementos básicos: a) la base en el cruce con el eje dendrítico, b) el cuello, y c) la cabeza que puede hacer contacto con el axón. Su forma y tamaño es variable, su longitud va de 0,2 a 2μm, con un volumen de 0,001 a 1μm^3. Con base en su morfología se clasifican en: espinas delgadas, cortas sin cuello y con cabeza ancha en forma de hongo (Bourne and Harris, 2008) (Figura 8). La dendritas no son estáticas, y su morfología cambia continuamente, incluso a lo largo de la edad adulta, lo que refleja la naturaleza de la plasticidad de las conexiones sinápticas (Grutzendler et al., 2002). La plasticidad puede ser

modificada por la actividad neuronal *in vitro* e in vivo por la experiencia (Roberts et al., 2010). Los patrones de actividad que inducen la LTP, son uno de los principales mecanismos celulares que subyacen al aprendizaje y la memoria e inducen el alargamiento de las espinas por lo que se ha sugerido que estos cambios son fundamentales en la formación de los trazos de memoria (Kasai et al., 2003).

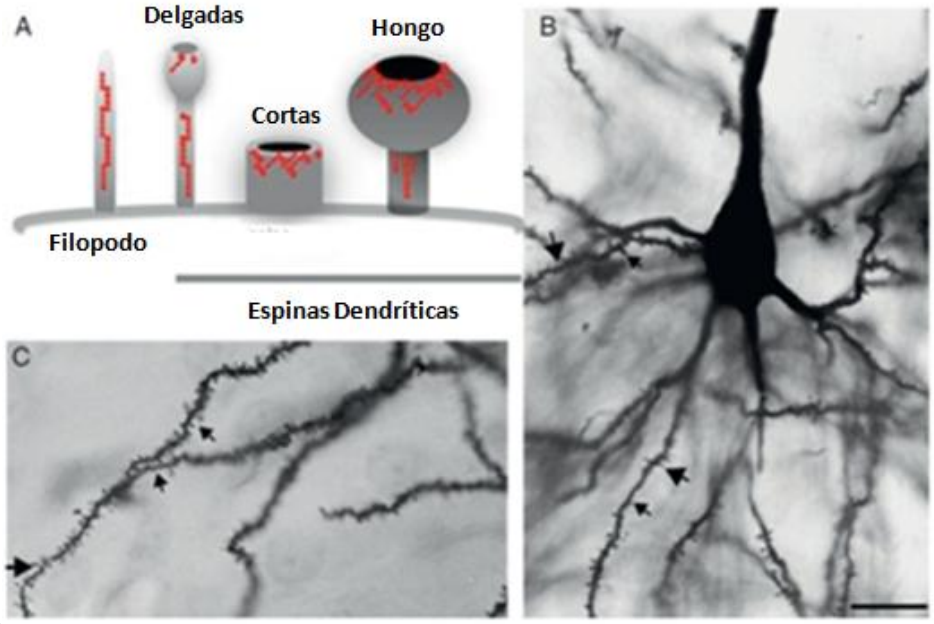

Figura 8. A) Representación esquemática de los filopodios y las espinas dendríticas: delgada, corta sin cuello y hongo. B y C) Microfotografía de una neurona piramidal de la rata. En la cual se muestra las diferentes formas de espinas dendríticas a lo largo de la dendrita basal. Técnica de Golgi modificado. Barra de 25 µm. Esquema de Sekino et al, 2007 (Sekino et al., 2007).

Las espinas dendríticas contienen la maquinaria pos sináptica necesaria para la trasmisión del impulso nervioso; incluyen receptores a glutamato, proteínas de densidad post-sináptica (PDS) y el citoesqueleto de actina, así como una amplia variedad de organelos del sistema de endomembranas (Figura 9): el retículo endoplasmático liso, las mitocondrias y los endosomas (Sheng and Hoogenraad, 2007). La zona de la PDS se encuentra en la parte posterior de la cabeza de la espina dendrítica y frontal a la zona activa pre sináptica. La PDS funciona como una estructura organizadora de diferentes grupos de receptores,

moléculas de adhesión y canales iónicos. Una gran variedad de moléculas de señalización en la membrana post sináptica (Kennedy et al., 2005). La mayoría de las vías de señalización intracelular parecen controlar la forma de las espinas y además convergen de manera directa en el citoesqueleto de actina.

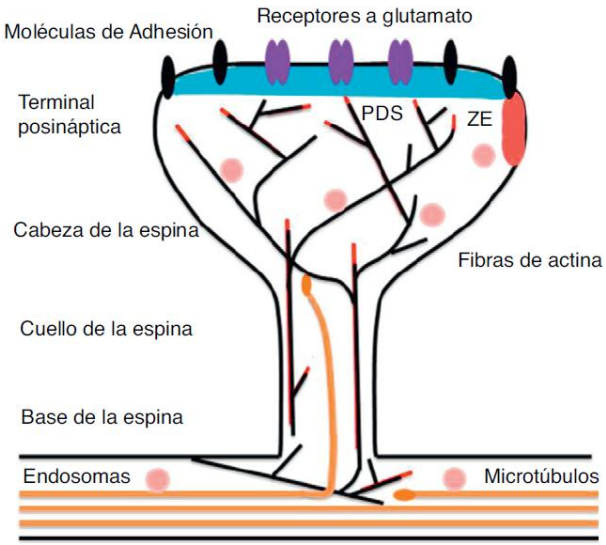

Figura 9: Representación esquemática de una espina en forma de hongo en la que se muestra la maquinaria post-sináptica necesaria para la trasmisión del impulso nervios. Esquema modificado de Hotulainen y Hoogenraad (Hotulainen and Hoogenraad, 2010)

Las modificaciones dendríticas de las neuronas del hipocampo pueden ser producidas bajo una variedad de condiciones de estrés y pueden ser revertidas después de un período de recuperación libre de estrés (Magarinos and McEwen, 1995), así como exposición a diversas tareas de aprendizaje (Carasatorre et al., 2013). El mecanismo propuesto es la liberación de los glucocorticoides, los cuales se liberan por la activación del eje Hipotálamo-Hipófisis-Adrenal (Ulrich-Lai and Herman, 2009).

2.5.3 Estrés, eje hipotálamo-hipófisis-adrenal y glucocorticoides

El estrés se ha definido como un estado de homeostasis alterada, en la que el organismo responde activando una serie de respuestas adaptativas centrales y periféricas (Charmandari et al., 2005; Chrousos and Kino, 2007; Johnson et al., 1992). Actualmente se considera que el estrés es una condición del organismo en la que las demandas ambientales y situaciones que son impredecibles e incontrolables, exceden la capacidad reguladora del organismo. Fisiológicamente, el estrés se caracteriza por la ausencia de una respuesta anticipada (impredecible) así como por una baja recuperación después de la reacción neuroendocrina (Koolhaas et al., 2011). La respuesta de estrés es dirigida por estructuras del sistema nervioso central (SNC) y por órganos periféricos. Los dos componentes principales del sistema de estrés son: los sistemas *Locus Coeruleus* (sistema-LC), nervioso simpático (SNS)-médula adrenal, y el eje Hipotálamo-Hipófisis-Adrenal. Ambos componentes interaccionan entre sí (Charmandari et al., 2005; Habib et al., 2001). El sistema de estrés recibe e integra diversas señales cognitivas, emocionales, neurosensoriales y periféricas que llegan a través de diferentes vías (Charmandari et al., 2005).

Estudios realizados en ratas han demostrado que la exposición materna al estrés durante el embarazo, o la separación materna durante la vida postnatal temprana se asocia con incremento de la ansiedad en la descendencia (Brunton and Russell, 2010; Fan et al., 2009; Fride and Weinstock, 1988; Vallee et al., 1997), desregulación del eje HHA (Brunton and Russell, 2010; Fan et al., 2009; Koenig et al., 2005; McCormick et al., 1995; Takahashi and Kalin, 1991; Weinstock, 2001), deterioro del desarrollo neuronal (Lemaire et al., 2000), déficits cognitivos (Lemaire et al., 2000; Paris et al., 2011; Paris and Frye, 2011) y comportamientos sociales aberrantes (Frye and Orecki, 2002; Holson et al., 1995; Lee et al., 2007; Patin et al., 2005) en la vida adulta de los descendientes.

En humanos, el estrés y la ansiedad materna durante el embarazo también se asocia con alteración del neuro-desarrollo infantil, incluyendo retraso en el desarrollo motor, deterioro cognitivo, problemas emocionales, el temperamento

negativo y síntomas de trastorno de déficit de atención (Blair et al., 2011; Glover, 2015; King et al., 2012; Schuurmans and Kurrasch, 2013).

2.5.3.1 Eje hipotálamo hipófisis adrenal (HHA)

El eje HHA es un sistema que consta de tres estructuras: el hipotálamo, la hipófisis y las glándulas suprarrenales (también conocida como adrenales en roedores), mismas que a su vez liberan diferentes hormonas: la liberadora de corticotropina (CRH), la adenocorticotropa (ACTH) y los glucocorticoides (GC), respectivamente. Durante la respuesta de estrés, la activación del eje HHA causa la liberación de CRH, un péptido de 41 aminoácidos, sintetizado y liberado por las neuronas del núcleo paraventricular (PVN) del hipotálamo (Bloom et al., 1982). La CRH es liberada al sistema porta-hipofisiario, a través del cual es transportada hacia la hipófisis anterior. La CRH estimula en los corticotropos adenohipofisiarios la síntesis y secreción de ACTH, a través de la unión a receptores específicos en esas células (Aguilera et al., 2008; Rivier and Vale, 1983). La ACTH liberada al torrente sanguíneo se une a receptores de alta afinidad, localizados en la membrana de las células de la corteza suprarrenal, donde estimula la síntesis y liberación de GC (Ulrich-Lai and Herman, 2009).

La retroalimentación del cortisol modula la actividad del HHA a través de la activación de los receptores de glucocorticoides (GR) y mineralocorticoides (MR) en el hipocampo, y el del GR en el PVN y pituitaria anterior (Moisiadis and Matthews, 2014). El incremento y respuesta prolongada del eje HHA al estrés es asociado con la reducción de la expresión de GR, MR o ambos en el hipocampo (Brunton and Russell, 2010; Henry et al., 1994; Weinstock, 2001), indicando un deterioro de la retroalimentación negativa de GC (Figura 10). El estrés (pre o postnatal) está asociado con la desregulación del eje HHA, así como el incremento en la ansiedad y deterioro de la función cognitiva (Brunton, 2015)

2.5.3.2 Glucocorticoides

Los GC (cortisol en humanos y corticosterona en la rata) son hormonas de naturaleza esteroidea sintetizadas en la zona *fasciculata* y *reticularis* de la corteza suprarrenal (Ganong et al., 1974; Nicolaides et al., 2015), a partir del colesterol y son liberados a la circulación en general. La corticosterona (CORT) en el cerebro de la rata actúa con otros componentes del sistema de la respuesta de estrés, de esta forma muestra dos modos de operación. El primero, donde la CORT mantienen la actividad basal del eje HHA y controla la sensibilidad del sistema de respuesta de estrés. Esta hormona coordina eventos de carácter circadiano, tales como el ciclo sueño-vigilia, la ingesta de alimento y está implicada en procesos que refuerzan la atención selectiva, la integración de la información sensorial y la elección de la respuesta conductual del organismo (Nicolaides et al., 2015).

En segundo modo, el sistema de retroalimentación de la CORT ayuda a terminar con la activación del eje HHA inducido por el estrés (De Kloet et al., 1998). Los efectos agudos de la CORT son euforogénicos, mientras que la elevación de forma crónica de los mismos produce depresión en un gran número de sujetos (Bohus et al., 1983). A nivel periférico, los GC actúan en el hígado incrementando la síntesis de las enzimas que favorecen la gluconeogénesis (síntesis de glucosa a partir de sustratos no glucosídicos, como los aminoácidos, el lactato y el glicerol). Los GC también estimulan la movilización de ácidos grasos desde los adipocitos. Estos efectos metabólicos de los GC aumentan la disponibilidad de energía, tanto para el cerebro como para los tejidos periféricos (Yates et al., 1980).

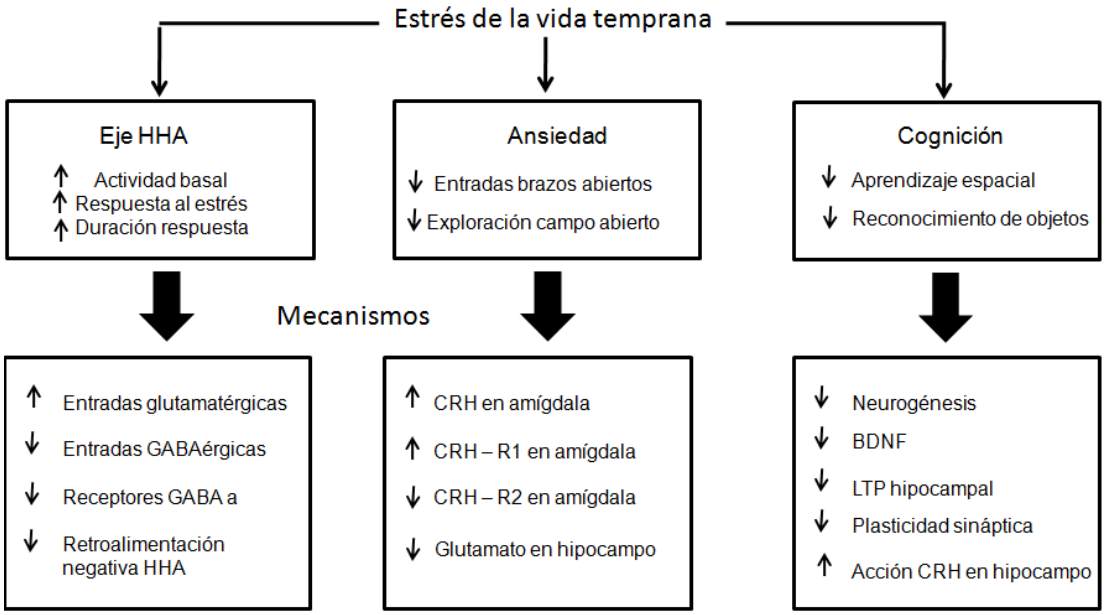

Figura 10. Resumen de las consecuencias del estrés (en animales de experimentación) durante la vida temprana (pre o posnatales) y los posibles mecanismos centrales involucrados. El estrés (pre o postnatal) está asociado con la desregulación del eje HHA, así como el incremento en la ansiedad y deterioro de la función cognitiva. Esquema tomado de Brunton (Brunton, 2015)

2.5.3.3 Receptores de Glucocorticoides (GC)

Los receptores de GC se encuentran en todo el cerebro, y su acción sobre el SNC está mediada por dos tipos de receptores: tipo I y tipo II (De Kloet et al., 1998; Reul and de Kloet, 1985). Los tipo I se encuentran en las neuronas del hipocampo y del septum, pertenecientes al sistema límbico (McEwen et al., 1968), participan en la modulación de la respuesta a los estímulos ambientales y emocionales (miedo, ansiedad, ingesta de alimento, etc), y tienen una alta afinidad por la corticosterona (Reul and de Kloet, 1985; Sapolsky et al., 1986). En el sistema límbico, tienen alta especificidad por los agonistas de la corticosterona, mientras que los mineralocorticoides, como la aldosterona, parecen ser antagonistas competitivos (De Kloet et al., 1998). Los receptores tipo I que se encuentran en los órganos circunventriculares y funcionan como receptores a mineralocorticoides que responden a la aldosterona, y participan en la regulación homeostática del

sodio, el control cardiovascular, además de controlar el apetito por la sal (Sapolsky et al., 1986).

Los receptores a GC tipo II están presentes en altas concentraciones en el hipotálamo, en las neuronas CRH y en áreas del cerebro que contienen proopiomelanocortina (POMC), como es el hipocampo, el septum lateral, la amígdala y el núcleo del tracto solitario (McEwen et al., 1968). Los GC ejercen retroalimentación negativa en respuesta al estrés, suprimiendo en la hipófisis la secreción de ACTH y en el hipotálamo, la secreción de CRH. Tienen efectos a largo plazo sobre el comportamiento adaptativo, por la vía de los receptores tipo II. (Keller-Wood and Dallman, 1984; Sapolsky et al., 1986). Los receptores de GC se localizan en el citosol y el núcleo de las neuronas y de los corticotropos, aunque también se localizan en la membrana plasmática de estas células (De Kloet et al., 1998). Estos mecanismos permiten al organismo mantener estables los niveles circulantes de GC. Durante el estrés, la ocupación de los receptores tipo I cambia poco, mientras que la ocupación de los receptores tipo II aumenta (Reul and de Kloet, 1985), lo que permite al eje HHA disminuir su actividad en respuesta al estrés.

3.0 **JUSTIFICACIÓN**

Los efectos adversos de la malnutrición materna durante el embarazo tanto en madres como en sus descendientes constituyen un gran problema de salud pública a nivel mundial, especialmente en los países en vías de desarrollo (Rojas and Salazar, 2009). Según la última Encuesta Nacional de Salud y Nutrición (ENSANUT 2012), en nuestro país el 12% de mujeres en edad reproductiva y el 18% de las embarazadas, son clasificadas como anémicas, y se ubican con mayor prevalencia en áreas rurales (Nutrición, 2012). Por lo que la morbilidad fetal y de la descendencia asociada con la baja nutrición materna durante el desarrollo es un problema alarmante de salud a nivel global ya que predispone a la descendencia a

desarrollar enfermedades como: la diabetes, hipertensión y obesidad en su vida adulta.

La inadecuada nutrición fetal e infantil sigue siendo uno de los principales factores no genéticos que afectan el desarrollo del cerebro. En la literatura existe evidencia experimental que demuestra que los efectos del reto nutricional en el cerebro en desarrollo son de larga duración y conducen a déficits permanentes en el aprendizaje y el comportamiento en la descendencia (Morganeet al., 2002).

Durante el período perinatal hay mayor vulnerabilidad para la programación adecuada del cerebro, ya que en esta etapa se llevan a cabo procesos importantes como la neurogénesis, sinaptogénesis, arborización axonal y dendríticas, la muerte celular programada y la mielinización por mencionar algunos (Maccari et al., 2014).

En los últimos años, se han estudiado en animales de experimentación los efectos que provoca el consumo de una dieta baja en proteína (malnutrición severa) previa y durante el embarazo y sus efectos en los descendientes a nivel neurofisiológico, morfológicos, neuroquímicos y plasticidad sináptica que correlacionan con las alteraciones cognitivas del individuo en la vida adulta.

Sin embargo, a la fecha existe información limitada acerca de los efectos de la malnutrición moderada (10% proteína) durante la gestación y/o la lactancia sobre las alteraciones afectivas y cognitivas en las crías macho de la rata. El presente trabajo de investigación nos permitió tratar de elucidar los posibles mecanismos relacionados con dichas alteraciones para lo cual se estudiaron los cambios en el sustrato anatómico del hipocampo en sitios pre y post sinápticos (sistemas de fibras musgosas y espinas dendríticas, respectivamente), así como el papel de los glucocorticoides como marcadores de la actividad del eje hipotálamo hipófisis adrenal ante situaciones de estrés.

4.0 PREGUNTAS DE INVESTIGACIÓN

El bajo consumo de proteína materna durante la gestación, la lactancia o en ambos periodos, repercutirá en el aprendizaje, ansiedad y memoria en la vida adulta de las crías macho de la rata.

En caso de que existan alteraciones a nivel afectivo y cognitivo en las crías macho, se determinará si existen cambios en la plasticidad sináptica del hipocampo y en la liberación de glucocorticoides (para evaluar la actividad del eje Hipotálamo Hipófisis Adrenal), como posibles mecanismos implicados en la programación negativa del cerebro durante etapas tempranas del desarrollo del individuo.

5.0 HIPÓTESIS

La restricción proteínica materna durante la gestación y/o la lactancia:

-Incrementará la ansiedad en la cría macho de la rata, por aumento en la liberación los glucocorticoides (hiperactividad del eje Hipotálamo Hipófisis Adrenal).

- Retrasará la adquisición, la memoria y la motivación en la cría macho de la rata, debido a cambios anatómicos en los elementos presinápticos (fibras musgosas) y post sinápticos (espinas) en el hipocampo.

6.0 OBJETIVOS

6.1 OBJETIVOS GENERALES

Determinar el impacto de la restricción proteínica de la rata gestante y/o lactante:

---- sobre la ansiedad, aprendizaje, motivación y memoria en las crías macho de la rata.

---- sobre la liberación de los glucocorticoides, así como cambios en el sustrato anatómico pre (fibras musgosas) y post sinápticos (espinas) del hipocampo en las crías macho de la rata.

6.2 OBJETIVOS PARTICULARES

1. Evaluar la tendencia innata a explorar espacios desconocidos (conducta de tipo ansiedad) en la vida adulta de las crías macho proveniente de madres restringidas en proteína.
2. Determinar el índice de discriminación y de reconocimiento en crías provenientes de madres malnutridas.
3. Explorar el aprendizaje y memoria de tipo espacial en las crías macho en el Laberinto acuático de Morris.
4. Evaluar la motivación por el trabajo en las crías macho cuyas madres fueron expuestas a restricción proteínica.
5. Cuantificar la concentración de corticosterona en heces después de evaluar la ansiedad en las crías macho de madres malnutridas.
6. Determinar la concentración de corticosterona y ACTH en los diferentes grupos experimentales.
7. Cuantificar la concentración de corticosterona y ACTH previo y después de la prueba de estrés (inmovilización) en la cría macho.
8. Medir el área de las fibras musgosas en *stratum lucidum* de la región CA3 del hipocampo de las crías macho.
9. Determinar el número total de espinas y tipo de espinas en la dendritra basal del *stratum oriens* de la región CA3 del hipocampo de las crías macho.

7.0 MATERIAL Y MÉTODOS

7.1 Manejo de animales

Se emplearon ratas hembras de la cepa Wistar de 20–22 semanas de edad y con un peso 220 a 260 g, crecidas y mantenidas en el Bioterio del Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMSZ), bajo condiciones controladas de temperatura (22 ± 2 ºC), así como de humedad (75%) y ciclo de luz /oscuridad (12 h de luz y 12 h de oscuridad). El alimento y agua fueron proporcionados *ad libitum*. Los procedimientos involucrados con los animales fueron aprobados por el Comité de Investigación en Animales (CINVA) del INCMNSZ y están acordes con la guía para el uso y cuidado de los animales de laboratorio del National Research Council (EUA).

Las hembras se alojaron en cajas de acrílico con un macho sin ningún tipo de tratamiento para el apareamiento durante varios días, revisándose diariamente en la mañana para determinar mediante un frotis vaginal, la presencia de espermatozoides, y designado como día cero de la gestación. El día del parto (día 22), fue asignado como inicio de la lactancia (día cero). Al día dos de nacimiento las camadas fueron ajustadas a 10 crías, en una proporción 1:1 (hembra: macho) en medida de lo posible. Las madres con menos de 10 o más de 13 crías fueron excluidas del estudio, para evaluar los efectos ocasionados por la restricción nutricional materna y no debido al número de crías por camada.

7.2 Alimentación

Las ratas hembras fueron transferidas a cajas individuales y divididas de manera aleatoria en dos grupos que fueron alimentados con dietas isocalóricas con diferente contenido de proteína: dieta control (C), con el 20 % de caseína, y dieta restringida (R), con el 10 % de caseína (Tabla 2). El uso de dietas a base de caseína ha sido reportado por nuestro grupo de trabajo (Zambrano et al., 2005b) y acorde con la dieta AIN 93G (Reeves et al., 1993), preparado en forma de galletas sólidas, con el fin de que las ratas pudieran roer el alimento. Este tipo de dieta es ideal para el crecimiento y mantenimiento de ratas durante la gestación y lactancia

(Reeves et al., 1993). En la dieta R, se incrementó el contenido de carbohidratos para proporcionar el mismo aporte energético en ambas dietas.

Tabla 2. Composición de las dietas isocalóricas experimentales: C (dieta control caseína-20%) y R (restringida caseína-10%). Todos los componentes fueron de grado alimenticio.

	Dieta Control 20% (g/100g)	Dieta Restringida 20% (g/100g)
Caseína libre de vitaminas	20	10
L-Cistina	0.3	0.15
Clorhidrato de colina	0.165	0.165
Mezcla de vitaminas	1	1
Mezcla de minerales	3.5	3.5
α-Celulosa	5	5
Aceite de maíz	5	5
Almidón de maíz	32.5	37.5
Dextrosa Anhidra	32.5	37.5
Contenido energético	3.85 kcal/g	3.85 kcal/g

La mezcla de vitaminas (Teklad AIN-93VX TD94047) contiene: ácido nicotínico, pantotenato de calcio, piridoxina, tiamina, riboflavina, ácido fólico, D biotina, vitamina B 12, acetato de tocoferol, vitamina A, vitamina D3, vitamina K y sacarosa.La mezcla de minerales (Teklad AIN-93 G-MX TD94046) contiene: calcio, potasio, sodio, magnesio, hierro, zinc, manganeso, molibdeno, yodo, fósforo, cloro, azufre, cobre, niquel, vanadio, silicio. La caseína libre de vitaminas y colina fue obtenida de Indianápolis, Indiana. E.U.A NP 160040. El aceite de maíz (marca La Gloria, México D.F) fue adquirido en supermecados. Almidón y dextrosa (HarlanTeklad, E.U.A.) y Celulosa y Cistina de Sigma Aldrich St. Louis MO. E.U. A 63103.

7.3 Grupos experimentales

El diseño experimental consistió en diferentes esquemas de alimentación durante la gestación, la lactancia o ambos periodos (Figura 11). La designación de los grupos fue al azar. La primera letra indica el tipo de dieta que recibió la madre durante la gestación y la segunda, durante la lactancia, formando así los

siguientes grupos experimentales: **CC,** madres alimentadas con dieta C durante el embarazo y la lactancia; **RR,** madres alimentadas con la dieta R durante el embarazo y la lactancia; **CR,** madres alimentadas con dieta C durante el embarazo y con la dieta R durante la lactancia; **RC,** madres alimentadas con dieta R durante el embarazo y con la dieta C durante la lactancia. Al destete, sólo se utilizaron las crías macho, las cuales fueron alimentadas con dieta control hasta el final del estudio.

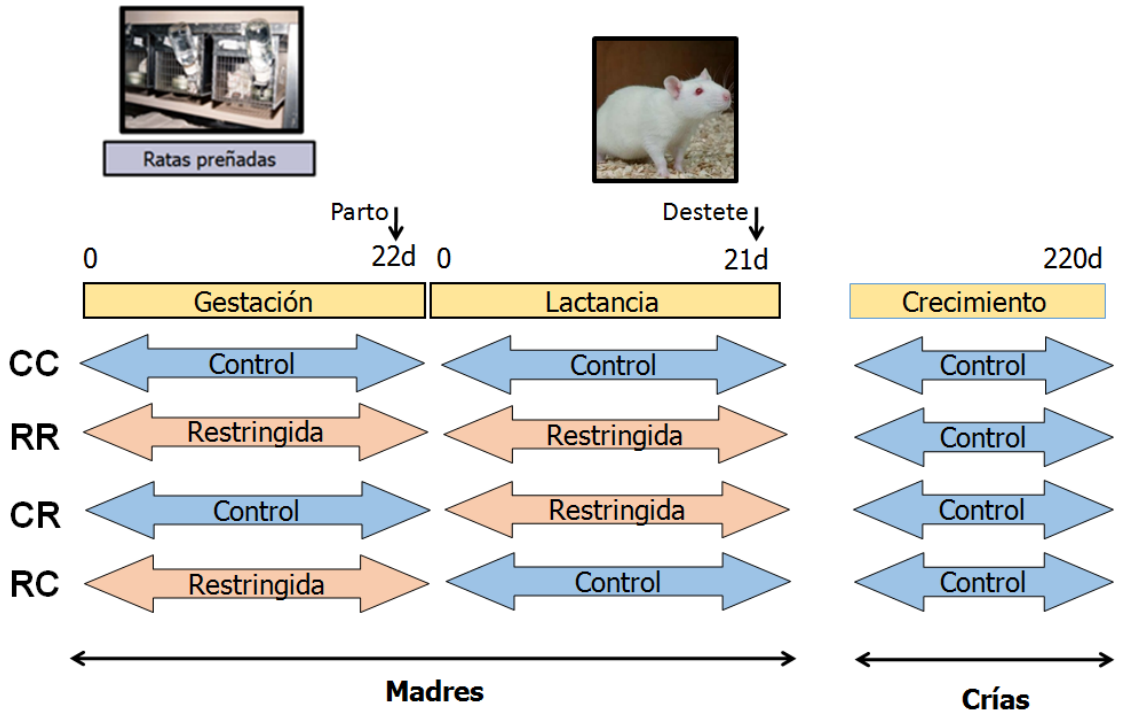

Figura 11: Representación esquemática de los 4 grupos experimentales: Las madres fueron alimentadas con dieta C (caseína 20%) o R (caseína 10%). La primera letra indica el tipo de dieta que recibió la madre en gestación y la segunda durante la lactancia.

Experimento A: Se aparearon las ratas y éstas fueron asignadas a un grupo experimental como se describió, realizando el ajuste de camadas y excluyendo a las madres que no cumplieran con el criterio de inclusión del estudio, quedando 8 madres por grupo (n= 8) madres para cada grupo. Elegimos al azar una cría de cada camada (n=8) y de cada grupo experimental diferente para la evaluación de las siguientes pruebas conductuales: Laberinto elevado en cruz, prueba del campo

abierto, condicionamiento operante, motivación y curvas de respuesta al estrés en la descendencia.

Experimento B: Se realizó el mismo procedimiento que el experimento A, para la obtención de las crías pero con 10 animales por grupo experimental (n=10). Las crías de este grupo fueron utilizadas para evaluar: la prueba de reconocimiento de objetos, el laberinto acuático de Morris, las concentraciones de los GC (corticosterona y ACTH) en suero, así como el análisis histológico de las fibras musgosas y espinas en el hipocampo.
Dado que surgió la inquietud de una nueva pregunta de investigación durante la primera fase del experimento, se decidió llevar a cabo un segundo experimento que nos permitiera replicar los hallazgos encontrados en el experimento A y a su vez contestar la nueva pregunta de investigación planteada.

7.4 Manipulación de las madres

La ganancia de peso en las ratas se registró diariamente durante el periodo de gestación y lactancia, de los cuatro grupos experimentales (CC, RR, CR y RC). Los datos fueron de utilidad para determinar el porcentaje (%) de peso ganado por la madre durante la gestación y la lactancia por cada intervención.

7.5 Análisis de la ingesta alimenticia en la madre

La cantidad de alimento consumido fue determinado por diferencia de peso entre los gramos de alimento inicial en el comedor y los gramos de alimento en el comedero después de 24 horas. Se colocaron diariamente en los comederos alrededor de 50 g por rata con la finalidad de que los animales siempre tuvieran disponible buena cantidad de alimento.

7.6 Manejo de las crías

Al nacimiento, todas las crías fueron pesadas y clasificadas por sexo, se registraron el número de crías por camada, el peso total de la camada y las medidas morfométricas para evaluar el efecto de la restricción proteínica materna.

De igual manera se determinó sólo en la cría macho, el peso corporal diario desde el nacimiento hasta el destete, así como en las diferentes edades en que se evaluaron las pruebas conductuales (Figura 12).

Después del destete todas las crías fueron alimentadas con dieta Chow (dieta comercial de Harlan Teklan Purina Chow, fórmula No. 5001) basada en dieta AIN93G (Reeves et al., 1993) para crecimiento y conservación de roedores. Para el presente estudio sólo se evaluó el efecto de la malnutrición en las crías macho de la rata.

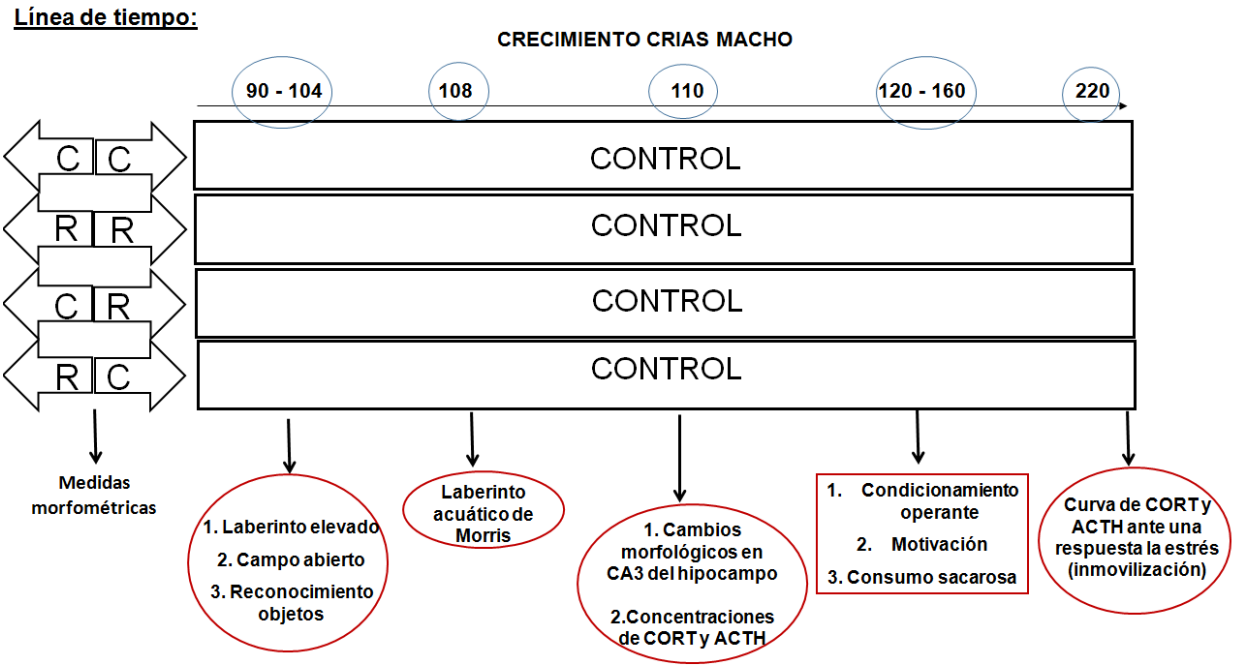

Figura 12. Representación de la línea de tiempo, que muestras los parámetros evaluados en los diferentes grupos experimentales. Las madres fueron alimentadas con dieta C (caseína 20%) o R (caseína 10%) durante la gestación-primera letra y/o durante la lactancia-segunda letra. Después del destete las crías fueron alimentadas con dieta control.

7.7 Parámetros morfométricos:

Mediante el uso de un vernier se determinó la talla de las crías al nacimiento, se midió desde la punta de la nariz a la base de la cola. El diámetro cefálico fue medido tomando como base la altura de las orejas. También se determinó el diámetro abdominal midiendo en la base de las costillas y la región ano genital midiendo la distancia entre el ano y el poro genital. La DAG en muchas especies proporciona un marcador externo de diferenciación sexual al nacer (Figura 13).

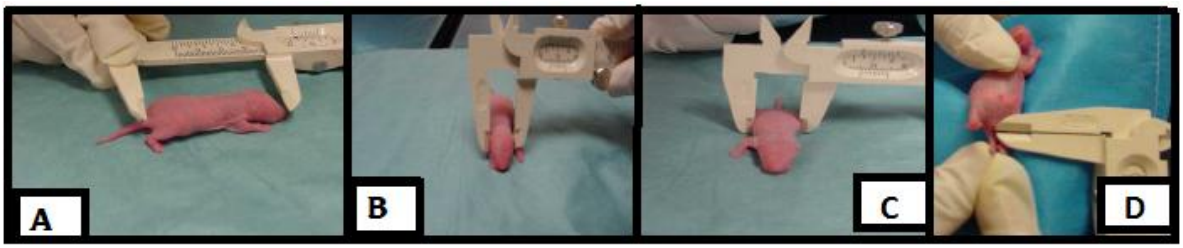

Figura 13 Parámetros morfométricos al nacimiento: (A) talla, (B) diámetro cefálico, (C) diámetro abdominal y (D) distancia ano genital. Todas las mediciones fueron determinadas mediante el uso del vernier.

7.8 Pruebas conductuales

Todas las pruebas conductuales se realizaron en la fase de obscuridad para lo cual dos semanas antes del inicio de la pruebas se hizo el cambio del ciclo de luz/oscuridad de los cuartos de alojamiento de las ratas (a las 17:00 pm se prendía la luz y 5:00 am se apagaba) ya que en la oscuridad estos animales presentan mayor actividad. Al dirigirse las ratas al cuarto de procedimiento para realizar sus pruebas conductuales fueron cubiertas con una tela oscura para evitar el contacto con la luz. De los 90 a 95 días de edad, se realizaron las pruebas conductuales para evaluar la ansiedad.

Laberinto elevado en cruz (EPM, por sus siglas en Inglés), es una estructura de conductos en forma de cruz que se encuentra a 64 cm de altura del suelo teniendo dos conductos o brazos abiertos y dos cerrados (43 X 10 cm). La rata se colocó en el centro del laberinto (de cara a los brazos abiertos) y se evaluó el tiempo de exploración de la rata en un nuevo ambiente potencialmente aversivo (conductos abiertos). La duración de la prueba fue de 5 minutos. Se registró el número de entradas, tiempo de permanencia y distancia recorrida en cada zona. Esta prueba permitió evaluar el grado de ansiedad del animal (Cannizzaro et al., 2006). El nivel de luz fue de 30 lux en los brazos abiertos y 6 lux en los brazos cerrados. La posición de la rata en el laberinto se monitoreó con ayuda de una cámara de video, la cual fue colocada en el techo centrada al laberinto y se conectó al sistema de video de seguimiento de análisis de movimiento, que se ejecuta en una computadora personal. Una vez finalizada la exploración en el EPM (Figura 14), la rata se regresó a su jaula y el equipo se limpió con etanol al

70% para eliminar olores; este procedimiento se realizó cada vez que un animal concluía su exploración.

Figura 14: Laberinto elevado en cruz: equipo utilizado para evaluar la conducta de tipo ansiedad en las crías macho de la rata.

La prueba de Campo Abierto (OF por sus siglas en Inglés**)**

Una vez finalizadas las pruebas en el EPM, de los 93 a los 95 días de edad (a los mismos animales) se les evaluó la prueba del OF. El campo abierto (Figura 15) es un aparato de forma cuadrada, abierto de la parte superior y está delimitado por bordes de acrílico, con dimensiones de 101 x 101 x 34 cm, el cual se colocó en un cuarto de experimentación iluminado por una luz baja (12 lux). Una cámara de video fue colocada en el techo centrada al campo conectado a un monitor y un sistema de video de análisis de movimiento para registrar la actividad. Cada rata fue colocada individualmente en el centro del campo abierto en el equipo durante 60 min. Después de cada periodo de sesión entre animal, la zona se limpió con etanol al 70%. Este equipo nos permitió evaluar el número entradas, tiempo de permanencia y distancia recorrida en ambas zonas (central y periférica).

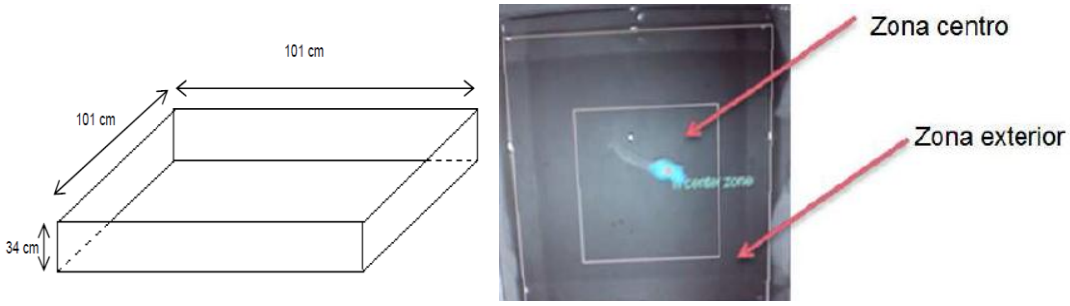

Figura 15: Campo abierto: equipo utilizado para evaluar la conducta de tipo ansiedad y actividad motora en las crías macho de la rata

La prueba de reconocimiento de objeto novedoso

En la rata macho de los 96 a los 104 días de edad, se realizó la prueba de reconocimiento de objetos para analizar la memoria (episódica) de reconocimiento visual. Este paradigma fue de utilidad para evaluarla preferencia innata a investigar un objeto novedoso por sobre un objeto familiar. Este tipo de prueba conductual no es aversivo (no estresante). En esta prueba los animales fueron colocados 10 minutos (Figura 16) en una caja negra de plástico (50x50x60cm) sin tapa durante tres días consecutivos. Al cuarto día, los animales se introdujeron en la misma caja pero en esta ocasión dentro de ella fueron colocados dos objetos iguales (situados a la misma distancia) y mediante una cámara de video se registró el tiempo de exploración a cada uno de los objetos (habituación). Al día siguiente, se intercambió uno de los objetos por uno nuevo y se registró la permanencia en ambos objetos. Entre cada animal la caja se limpió con etanol al 70%. La exploración se definió como el tiempo que el animal se acerca a oler o tocar el objeto con la nariz y/o lo toca con las patas delanteras. Cuando el animal que quedó sentado en el objeto no se consideró como exploración. Una vez finalizadas las pruebas se analizaron los videos con la finalidad de determinar el tiempo de permanencia en el objeto novedoso (TON) y familiar (TOF). Con base a éstos resultados se determinó el índice de discriminación y el índice de reconocimiento mediante el uso de las siguientes formulas:

$$\text{Índice de discriminación} = \frac{(TON - TOF)}{(TON + TOF)}$$

$$\text{Índice de reconocimiento} = \frac{TON}{(TON + TOF)}$$

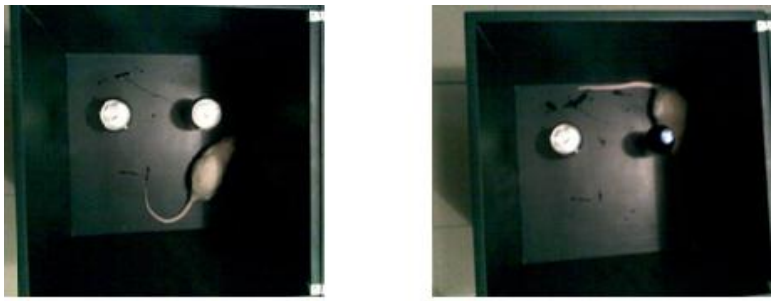

Figura 16: Prueba de reconocimiento de objetos: equipo utilizado para evaluar memoria de reconocimiento visual de objetos en las crías macho de la rata.

Laberinto acuático de Morris (LAM)

En las rata macho de los 108 a los 110 días se evaluó el aprendizaje y memoria espacial en el LAM (Figura 17). Para esta tarea se utilizó un tanque circular de color negro de plástico con diámetro de 154 cm, con una altura de 60 cm y una base de metal de 58 cm, basado en el modelo propuesto por Morris (Morris, 1984). El tanque se llenó con agua hasta una profundidad de 26 cm con una temperatura constante (25 ± 1 °C). Se establecieron cuatro posiciones de partida (norte, sur, este y oeste) dividiendo la superficie del laberinto en cuatro cuadrantes imaginarios. Se utilizó una plataforma cuadrada de acrílico negra (11.7 x 11.7cm), que se colocó dentro de uno de los cuadrantes del laberinto y quedó sumergida a 1 cm de profundidad bajo el nivel del agua. El cuarto en el que se encuentra el laberinto acuático tiene una dimensión de 236 x 225 x 242 cm, es sonoamortiguado y está pintado de blanco. En tres de sus paredes se colocaron carteles como señales. El tanque y las señales fueron iluminados por tres lámparas equidistantes. Durante el experimento las imágenes fueron capturadas con una cámara de video que se localizaba en el techo del cuarto. Las imágenes fueron analizadas mediante el sistema computacional SMART 2.5 (San Diego instruments, CA).

Adquisición. El entrenamiento consistió en una sesión de 8 ensayos. En el primer ensayo, cada animal se introdujo en el tanque con dirección hacia las paredes del mismo, se dejó nadar durante un máximo de 60 seg o hasta que se subió a la plataforma, donde se le permitió permanecer durante 20 seg. Si los animales no la

encontraban fueron dirigidas manualmente y colocados en ella por un periodo de 20 seg para que asociarán las señales espaciales en la sala con la posición de la plataforma de escape. Una vez transcurrido ese tiempo, se extrajo el animal, se envolvió en una toalla seca y se colocó en una caja de acrílico que se encontraba debajo de una lámpara de calentamiento en el cuarto adjunto, se dejó ahí por 30 seg y posteriormente se introdujo de nuevo en el tanque para iniciar un ensayo nuevo y así sucesivamente hasta completar los 8 ensayos de adquisición. Los puntos de partida fueron alternados al azar por cada ensayo, pero la plataforma sumergida se mantuvo en la misma posición; el intervalo entre cada ensayo fue de 30 seg. El tiempo en segundos que tardó la rata en encontrar la plataforma oculta recibe el nombre de latencia de escape.

Memoria espacial. Al día siguiente, la plataforma fue retirada y las ratas fueron colocadas en el cuadrante opuesto al sitio de localización de la plataforma durante la prueba de adquisición. La duración de la prueba fue de 120 seg. Si la rata aprendió el día anterior, ésta regresaba al cuadrante donde ella recordaba que se encontraba la plataforma. La única variación era que la plataforma se eliminó, y el parámetro medido fue la latencia de escape (s) para llegar al lugar donde estaba la plataforma, el número de entradas y tiempo de permanencia en el cuadrante blanco (donde se encontraba la plataforma).

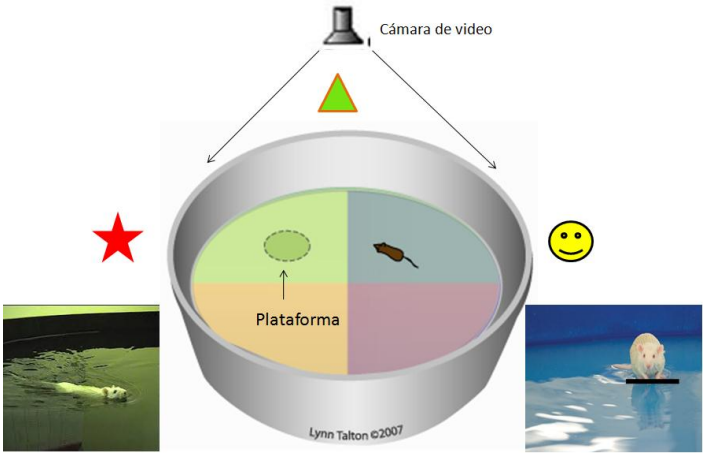

Figura 17: Prueba del Laberinto acuático de Morris: equipo utilizado para evaluar aprendizaje y memoria de tipo espacial en las crías macho de la rata.

Condicionamiento operante (CO)

Dos semanas previas al inicio del entrenamiento operante los animales fueron colocados en privación de agua durante 23 horas del día, con una hora de acceso libre al consumo de agua; esta condición se conservó durante los días de las pruebas con la hora de acceso libre al agua inmediatamente después de las sesiones de conducta. Las pruebas del CO, se iniciaron a los 120 días de edad y fueron realizadas los 7 días de la semana entre las 8 a.m. y 2 p.m. Esta prueba fue de utilidad para evaluar en una cámara operante (figura 18) el aprendizaje de tipo asociativo, para lo cual se requirió de un reforzador para fortalecer o incrementar una respuesta. Las cámaras operantes (E10-10TC, Coulbourn-Instruments, PA, E.U.A.) cuentan con ventilación, paredes de atenuación del sonido (E10-20, Coulbourn-Instruments, PA, E.U.A.), una palanca de respuesta retráctil y un recipiente para contener y dispensar líquido (E14-05, Coulbourn-Instruments, PA, E.U.A). Estas cajas tienen dos paredes laterales de aluminio y una pared posterior y frontal de plexiglás claro. También cuentan con un suelo de rejilla y durante las sesiones de pruebas se iluminaron con luz difusa. La palanca de respuesta y el compartimiento de la recompensa (solución de sacarosa 7%) se encuentran en la pared lateral derecha. En este equipo, el animal se condicionó para asociar el encendido de la luz dentro de la cámara con la retracción de la palanca y obtención de la recompensa

Figura 18: Prueba de Condicionamiento operante: equipo utilizado para evaluar aprendizaje de tipo asociativo en las crías macho de la rata.

Las pruebas de CO y de razón fija y progresiva (Progressive Ratio Test, PRT por sus siglas en inglés) se llevaron a cabo en el siguiente orden:

1. Reforzamiento libre,
2. Adquisición y reforzamiento, mediante la Prueba de Razón Fija 1, FR-1,
3. Retención, Prueba FR-5,
4. Evento Progresivo, razón variable, PR y
5. Consumo libre de sacarosa.

Reforzamiento libre. Antes del entrenamiento los animales se colocaron por primera vez en la cámara operante, por un tiempo de 10 minutos, en esta etapa los animales tuvieron acceso libre a la solución de sacarosa al 7% (reforzador positivo, primario). Esta prueba permitió que el animal reconociera y se adaptará al área y condiciones de trabajo.

Adquisición y reforzamiento. Subsecuente al primer día del entrenamiento, se les enseñó a los animales a asociar la presión de la palanca retráctil (estímulo condicionado) con la obtención de la recompensa (reforzador) bajo un esquema de Razón Fija 1 (FR-1), en esta prueba fue necesario que los animales presionaran la palanca una vez para recibir la recompensa. El registro del aprovechamiento se llevó a cabo gracias a los fotorreceptores de la cámara operante, y se inició una vez que el animal consumiera su recompensa. El entrenamiento FR-1 finalizó cuando el animal obtuvo al menos 20 respuestas durante la sesión de 15 min. Esta prueba fue de utilidad para determinar el tiempo que tardó el animal en adquirir el aprendizaje.

Retención. Después de haber completado la prueba FR-1, los animales fueron introducidos a la prueba de FR-5 Reforzamiento de Razón Fija 5, con criterios de funcionamiento idénticos a los empleados en la prueba FR-1, sólo que en esta fase el animal tenía que presionar 5 veces la palanca retráctil para obtener la recompensa. Las sesiones fueron de 15 minutos y finalizaron cuando el animal obtuvo al menos 20 respuestas en una sesión.

Evento Progresivo. Después de las sesiones de entrenamiento los animales fueron colocados en la Prueba Progresiva o de razón variable (PRT) por diez días consecutivos. En esta fase, para poder obtener una recompensa, el número de veces que se debe oprimir la palanca retráctil incrementa al doble cada 8reforzamientos, es por ello que el número de presiones sucesivas de la palanca para obtener la recompensa fue de la siguiente manera PR + 1 = 1, 2,…, 8; PR + 2= 10, 12, …, 24; PR + 4 = 28, 32, ..., 56; PR + 8 = 64, 72, …, 120; PR + 16 = 136,152, ….etc. Cada sesión de PR duró 30 minutos, esta prueba permitió evaluar la motivación por el trabajo para la obtención de la recompensa en las crías macho.

Consumo libre de sacarosa: un día después de la última sesión de la PRT, y durante tres días consecutivos, los animales tuvieron acceso directo a la solución de sacarosa 7% en una botella de 100 mL durante 30 minutos. Para esta evaluación se colocaron los animales en cajas individuales tamaño jumbo y la cantidad de consumo de sacarosa se calculó mediante la sustracción del volumen de la botella después del periodo de consumo del volumen inicial. Este procedimiento se realizó con la finalidad de investigar que la conducta observada en la prueba progresiva no se deba a la poca afinidad al consumo de sacarosa.

7.9 Recolección de muestras biológicas

Treinta minutos después de haber finalizado la prueba de retención en el LAM (a los 110 d), los animales fueron anestesiados i.p. con una combinación de Ketamina / Xilacina (65 / 7.5 mg/Kg); para obtener mediante una punción cardiaca la muestra sanguínea. La sangre se transfirió inmediatamente a un tubo sin anticoagulante y otro con anticoagulante (EDTA) y se mantuvieron en frío. Posteriormente las muestras se centrifugaron a 4°C durante 15 min a 3500 rpm y el suero y plasma obtenido se almacenaron a -20°C hasta su procesamiento. Se determinaron en suero y plasma las concentraciones de corticosterona y ACTH respectivamente.

7.9.1 Colecta de materia fecal y almacenamiento de las muestras

Una vez finalizadas las pruebas del EPM, los machos fueron regresados a cajas individuales y se dejaron transcurrir 90 min después de haber finalizado las pruebas conductuales, para colectar la materia fecal excretada durante este tiempo. Es importante aclarar que dado que los mismos animales se continuaron evaluando en otras pruebas conductuales, no fue conveniente extraer muestra sanguínea, razón por la cual sólo se recolectaron las heces fecales para la determinación de la corticosterona como marcador de estrés.

Las muestras fueron almacenadas en congelación a 4 °C hasta el día de extracción y análisis de la materia fecal. Este procedimiento fue realizado para la determinación de corticosterona en heces. El criterio de selección de muestras consistió en tomar heces firmes de la cama, basado en que para la preparación del homogenizado de muestra la eliminación digestiva se considera un acumulado real de la hormona durante el día. El proceso de extracción se inició colocando 0.6 g de materia fecal cuya consistencia fue similar. A cada tubo se añadieron 4 mL de metanol absoluto y se agitó a 1000 rpm por un periodo de 12 horas. Trascurrido ese tiempo, las muestras fueron centrifugadas a 3000 rpm durante 15 min. El sobrenadante fue depositado en tubos de 13x100 mm y se evaporó a sequedad. A continuación, la muestra fue re-suspendida en 3 mL de solución amortiguadora de albúmina sérica bovina al 0.04% y se realizó el radioinmunoanálisis. Además, se utilizaron 50 µL de muestra re-suspendida en amortiguador de albúmina sérica bovina, por duplicado para la cuantificación de la corticosterona por radioinmunoanálisis (RIA), utilizando un estuche comercial para corticosterona en rata.

Cálculos

El ensayo radioinmunológico produce resultados con unidades de ng/ml; como en este caso se analiza materia fecal, es necesario hacer la conversión correspondiente a unidades de ng/g. Para ello primero se obtiene el factor de dilución de la muestra, por medio de la siguiente fórmula:

$$\text{Factor de dilución} = \frac{\text{mL de amortiguador de albúmina sérica bovina}}{\text{(mL de metanol absoluto/ 0.05 mL)}} = 15.0 \text{ mL}$$

Dado que se utilizaron 3.0 mL de amortiguador de albúmina sérica bovina y 4.0 mL de metanol. Los siguientes 0.05 mL corresponden a los 50 µL de muestra utilizada para el RIA. Así el factor de dilución correspondiente es 15 mL. A continuación, los ng/mL obtenidos en el ensayo, se dividen entre los gramos de muestra iniciales y son multiplicados por el factor de dilución:

$$\text{(ng/mL) (1/g de muestras) (15 mL)} = \text{ng/g}$$

De esta manera los datos obtenidos son transformados de ng/mL a ng/g de muestra. Es importante señalar que esta conversión se realiza únicamente en muestras fecales.

7.9.2 Inmovilización y obtención de sangre

Al finalizar las pruebas del evento progresivo (alrededor de 220d de edad), una muestra de sangre fue tomada de la cola de la rata para determinar la concentración basal de corticosterona y ACTH (t=0). Los animales fueron físicamente restringidos del movimiento en un inmovilizador de acrílico durante 20 min; después de la inmovilización se obtuvieron muestras de sangre inmediatamente después a los 20, 40, 80 y 120min. Las muestras de sangre se centrifugaron a 4°C durante 15 min a 3500 rpm. El suero se almacenó a -20°C hasta el análisis de las muestras para la cuantificación de corticosterona y ACTH.

7.10 Determinación de Corticosterona y ACTH

La corticosterona se determinó mediante radioinmunoensayo utilizando un estuche comercial para ratas, DPC Coat-a-count (TKRC1) (Los Angeles, CA, EUA). La variabilidad intra e inter-ensayo fueron <6% y <7%respectivamente. La cantidad de suero utilizada para la determinación fue de 50µL.

La ACTH se determinó mediante el ensayo de quimioluminiscencia utilizando un estuche comercial (Siemens 06601153 IMMULITE® ACTH Kit). La variabilidad intra e inter ensayo fue de <8.4 y 9.2% respectivamente. El kit se utilizó de acuerdo con las instrucciones del fabricante y las muestras se midieron por duplicado. La cantidad de plasma utilizada para la determinación fue de 75µL. En ambas determinaciones las muestras se procesaron por duplicado.

7.11 Recolección de tejido para la Técnica de Timm

Un total de 20 animales (cinco por grupo: CC, RR, CR y RC) fueron anestesiados con una combinación de Ketamina / Xilacina y perfundidos con una solución amortiguadora de sulfuro de sodio (5,85 g de Na_2S, 5,95 g de NaH_2PO_4 H_2O en 500 ml de agua destilada) seguida del fijador Karnovsky (1% de paraformaldehído y 1.25% de glutaraldehído en solución de fosfatos 0.1 M, pH 7,4) durante 10 minutos, descrito por West y colaboradores (West et al., 1988). Los cerebros fueron preparados con técnica de Timm la cual se basa en la conversión del zinc a sulfuro de zinc de los botones gigantes de las terminales nerviosas de las FM. En estas fibras se deposita el nitrato de plata cuando los tejidos son puestos en una solución que contiene un estabilizador (goma arábiga), un agente reductor que precipita la plata (hidroquinona) (Danscher, 1981). Se estimóel área de FM en el haz suprapiramidal (West et al., 1988). Los cerebros así procesados, fueron extraídos y colocados para su crioprotección, en frascos con una solución de Karnovsky con sacarosa al 30% en refrigeración hasta que el tejido bajó al fondo del frasco. Los cerebros crioprotegido fueron cortados con un microtomo, localizando al hipocampo desde el primer corte; todas las secciones (de 40µm cada una) fueron colectadas en estricta seriación y procesadas usando una modificación de la técnica de Timm sulfuro de plata (Cintra et al., 1997). Las secciones que contenían al hipocampo, fueron montadas en dos series (una contrateñida con Violeta de cresilo-Nissl para facilitar la correlación citoarquitectural del área objeto de estudio), y se diferenciaron en la oscuridad a temperatura ambiente en una mezcla 12:6:2 de goma árabiga (20%), hidroquinona (5.6%), solución amortiguadora de citrato y 1 ml de una solución de nitrato de plata

al 17% (Padilla-Gomez et al., 2012). Para el análisis de morfometría, cada portaobjetos recibió un número aleatorio para asegurar que evitar sesgos (Figura 19). La morfometría, se efectuó con la identificación del haz de las FM que corresponde al área del s*tratum lucidum,* midiendo sólo el área en el hipocampo izquierdo dorsal, cada quinta sección; la primera sección se obtuvo a partir de una posición inicial aleatoria dentro de los primeros cinco cortes de la serie montada (Amaral and Dent, 1981; Gaarskjaer, 1985). La captura de las imágenes se realizó con una cámara digital acoplada a un microscopio fotónico a través de un objetivo 4x y analizadas con el programa Image "J", de cada 5ª sección, dando una separación de 160 µm entre los cortes siguientes hasta capturar seis imágenes por animal. Para este propósito, una sección coronal del hipocampo se obtuvo inmediatamente después de la perfusión usando un microtomo de congelación

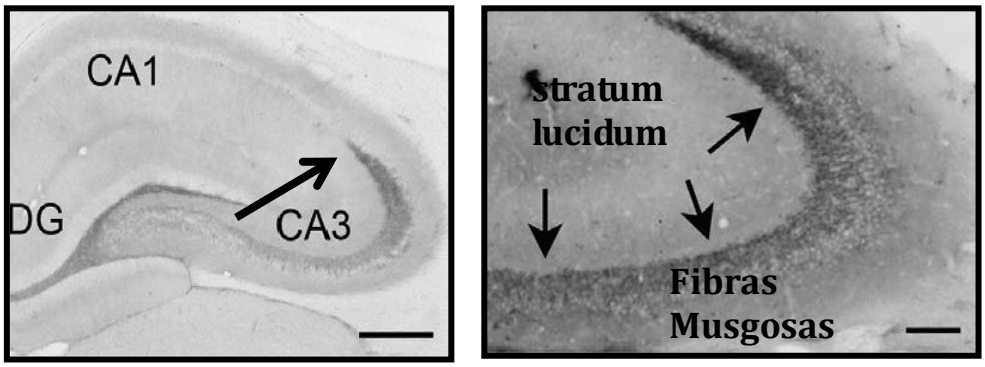

Figura 19: Representación de la regiones del hipocampo donde se localiza el área de las fibras musgosas.

7.12 Impregnación argéntica de Golgi rápido

El segundo grupo de ratas (cinco por grupo) previamente anestesiadas, fueron perfundidas por vía intracardiaca con una solución de formalina al 10% en solución amortiguadora de PBS (300 ml/rata). Una vez que el fijador penetró en el encéfalo, se seccionó la cabeza y se dejó durante 24 horas envuelta en papel aluminio, se extrajo el cerebro y se obtuvieron bloques coronales homogéneos de 4 mm de ancho abarcando el área del hipocampo dorsal (Bregma - 2.8 a - 4.2; Paxinos y Watson, 1998). Los cortes se colocaron en una solución de dicromato de potasio al 4.5% con ácido ósmico al 1 % en una proporción de 8:1. Se dejaron

en esta solución por un lapso de 10 a 13 días, en continua agitación, luego los bloques se diferenciaron en una solución de nitrato de plata al 0.75% durante 24 horas. A continuación se procedió a la deshidratación del tejido en alcoholes graduales a partir de alcohol al 50 % hasta alcohol etílico absoluto y éter, permaneciendo 30 min en cada uno de los cambios. Para poder cortarlos en el microtomo, primero se embebieron en nitrocelulosa de baja viscosidad la cual se preparó a diferentes concentraciones, empezando con 5%, aumentando 10%, 15%, 20% y 30% cada 24 horas. Finalmente los bloques se incluyeron en nitrocelulosa al 30% en moldes de plástico de 8 mm x 8 mm durante 12 horas en un desecador que contenía vapores de cloroformo, el cual se conectó a vacío. Ya endurecidos los bloques se fijaron en una platina de metal, la que a su vez se colocó en un micrótomo de deslizamiento (Leitz Wetlar 47160) y se obtuvieron cortes frontales de 120 µm de grosor, las cuales se deshidrataron en alcoholes graduales y se aclararon con terpineol y xileno, para ser montadas en portaobjetos usando entellan para colocar el cubreobjetos (técnica empleada por Díaz-Cintra, y colaboradores (Diaz-Cintra et al., 1994). El análisis morfométrico fue realizado para obtener la cantidad y forma de las espinas dendríticas en el segmento medial de la dendrita basal del *stratum oriens* en la región CA3, por medio de microscopía fotónica.

Análisis morfométrico

Un total de 240 neuronas completas y bien impregnadas fueron analizadas (12 neuronas por animal) usando un microscopio Nikon. Para el análisis de utilizó: A) un objetivo de 20X (0.5 NA) con una retícula calibrada ópticamente y B) un objetivo 100X (1.3 NA) con óptica plana cromática. Una imagen de la neurona piramidal seleccionada fue capturada con el objetivo 20x, después de una cuidadosa selección del plano óptico en el que las dendritas basales eran visibles (Figura 20A)

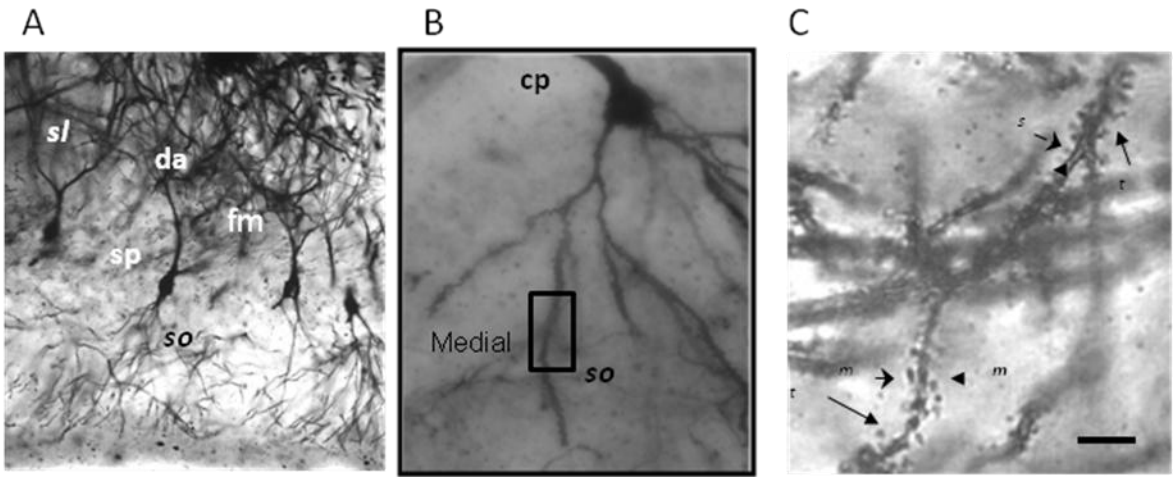

Figura 20: Fotografía representativa: A) de la región CA3: stratum lucidum (sl), oriens (so) y piramidal (sp) con su dendrita apical (da) y las fibras musgosas (FM), B) acercamiento de la región media y C) del so con sus diferentes tipos de espinas dendríticas: cortas (s), largas (t) y hongo (m). Barra de calibración = 100 μm.

Las neuronas piramidales del CA3 bien impregnadas fueron clasificadas y analizadas para determinar el número total y tipo de espinas dendríticas. Sólo las neuronas cuyas dendritas alcanzaron el *stratum oriens* fueron seleccionados para el análisis. Un segmento de 10 micras de la parte medial de la dendrita basal secundaria bien definida y completa fue seleccionada; (Figura 20B) para las mediciones con el objetivo de inmersión (100x) del número total y tipos de espinas (cortas, largas y hongo) (Figura 20C). Los criterios para la clasificación de las espinas fueron los mismos que utilizan varios investigadores (Beltran-Campos et al., 2011; Bourne and Harris, 2008; Sorra and Harris, 2000; Tashiro and Yuste, 2003). Las espinas delgadas fueron clasificadas como aquellas cuyo cuello era más largo que el bulbo de su cabeza; las espinas de hongos presentan una cabeza más grande sin cuello y, finalmente, las espinas cortas son aquellas sin cabeza o cuello, mostrando sólo una protuberancia de la dendrita.

7.13 ANÁLISIS ESTADÍSTICO

Todos los datos se expresaron como media ± error estándar. Al nacimiento, dado que eran dos grupos experimentales (Control y Restringido) se empleó la prueba de t de student. Después del nacimiento, las comparaciones entre grupos se realizaron utilizando análisis de variancia (ANOVA).

El peso e ingesta materno, el peso de crías a diferentes edades, la cantidad del alimento, las concentraciones de corticosterona y ACTH, fueron analizadas con ANOVA de una vía seguida de la prueba de post hoc Tukey. De igual manera se utilizó esta prueba estadística para analizar los resultados de las pruebas conductuales en EPM, OF, reconocimiento de objetos, prueba de retención en LAM, condicionamiento operante, motivación, así como para analizar el área de fibras musgosas y el número de espinas.

Por otro lado, los resultados de las concentraciones de la corticosterona y ACTH (inmovilización por 20 min), así como los resultados de la adquisición en el LAM se analizaron entre diferentes tiempos y grupos experimentales, con un ANOVA de dos vías de medidas repetidas seguida de una prueba post hoc de Bonferroni. El análisis estadístico se realizó utilizando la versión 2.0de Sigma Stat. Se consideró significativo con una $p<0.05$.

8.0 RESULTADOS

8.1 Efecto de la restricción proteínica en las madres durante la gestación y la lactancia

8.1.1 Peso materno durante gestación y lactancia.

Al inicio de la gestación, el peso corporal de las madres no fue diferente entre los grupos experimentales. Este mismo efecto se observó al final de la gestación e inicio de la lactancia; sin embargo, a partir del día 10 y 15 de lactancia el grupo CC presentó mayor ganancia de peso con respecto a CR y RR respectivamente, tal como se muestra en la Figura 21.

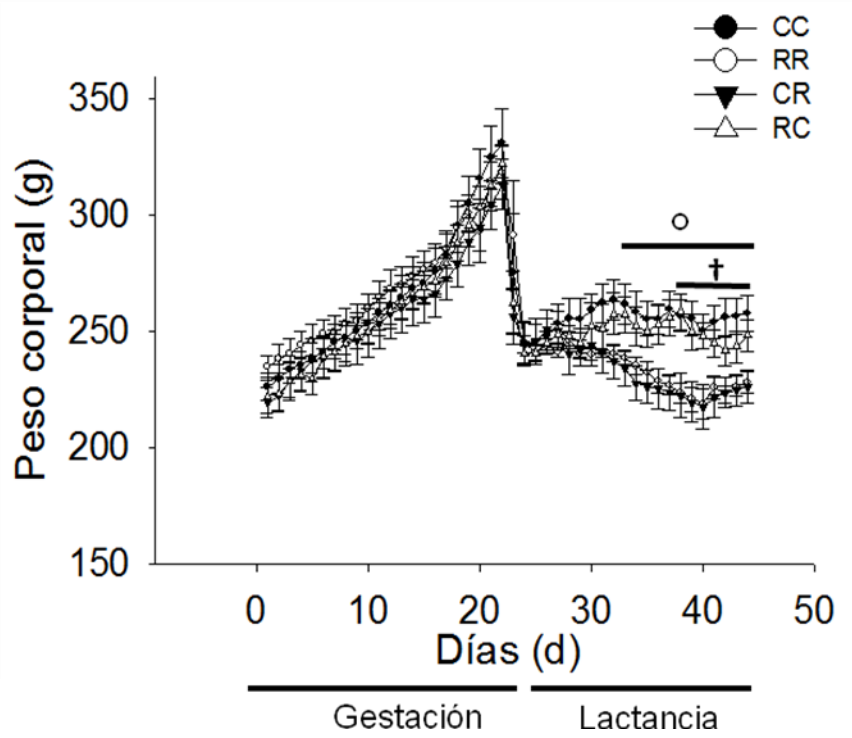

Figura 21. Ganancia de peso materno durante el periodo de gestación y lactancia. Las madres fueron alimentadas con dieta control (C=20% caseína) o restringida (R=10% caseína) durante la gestación (primera letra) y lactancia (segunda letra). Media ± EE; n=8 ANOVA, post hoc tukey; $p \leq 0.05$, † CC vs RR y ° CC vs CR.

Al final de la lactancia, las madres que fueron alimentadas con dieta restringida durante la lactancia (RR y CR) presentaron menor peso en comparación con el grupo CC, no encontrándose diferencias entre CC, RR y CR con el RC. La ganancia de peso durante el periodo de gestación y lactancia fue significativamente menor en las madres RR con respecto a CC. Este mismo comportamiento se observó al expresarlos resultados en porcentaje. Por otro lado, las madres CR tuvieron menor % de peso ganado respecto al grupo RC y CC, no encontrándose diferencias con RR (ver Tabla 3).

Tabla 3. Peso materno durante la gestación y lactancia.

Periodo	CC	RR	CR	RC
Peso (g) al inicio de la gestación	229 ± 6	238 ± 15	227 ± 6	223 ± 6
Peso (g) al final de la gestación	331 ± 14	318 ± 11	320 ± 9	322 ± 7
Peso (g) al inicio de la lactancia	245 ± 9	244 ± 4	238 ± 10	240 ± 4
Peso (g) al final de la lactancia	258 ± 8 **a**	228 ± 4 **b**	225 ± 8 **b**	248 ± 6 **ab**
Ganancia de peso (g) durante la gestación	102 ± 11	80 ± 6	93 ± 6	99 ± 4.1
Ganancia de peso durante la lactancia	13 ± 4	16 ± 4	13 ± 5	16 ± 4
Ganancia de peso durante la gestación y lactancia	28 ± 2 **a**	17± 4 **b**	18 ± 5 **ab**	25 ± 3 **ab**
% Ganancia de peso durante la gestación	44 ± 4	33 ± 2.0	41 ± 3	44 ± 2
% Ganancia de peso durante la lactancia	7 ± 2	8 ± 2	8.3 ± 2	6.5 ± 2
% Ganancia de peso durante la gestación y lactancia	12 ± 0.5 **a**	7 ± 2 **bc**	5 ± 2 **b**	11 ± 0.9 **ac**

Las madres fueron alimentadas con dieta control (C=20% caseína) o restringida (R=10% caseína) durante el embarazo (primera letra) y lactancia (segunda letra). Datos expresados en Media ± EE; n=8 madres. Grupos que no comparten la misma letra son estadísticamente diferentes, ANOVA, post hoc tukey, $p<0.05$

8.1.2 Ingesta alimenticia materna durante gestación y lactancia

La ingesta de alimento no fue diferente entre las madres de los diferentes grupos experimentales durante la gestación y los primeros diez días de lactancia (Figura 22); sin embargo, para la segunda mitad de la lactancia las madres que fueron restringidas durante la lactancia (RR y CR) presentaron menor ingesta con respecto a CC y RC. Es importante aclarar que a partir de la semana y media de lactancia (día 11), las crías ya comenzaron a comer directamente del comedero, razón por la cual se observó mayor consumo de alimento.

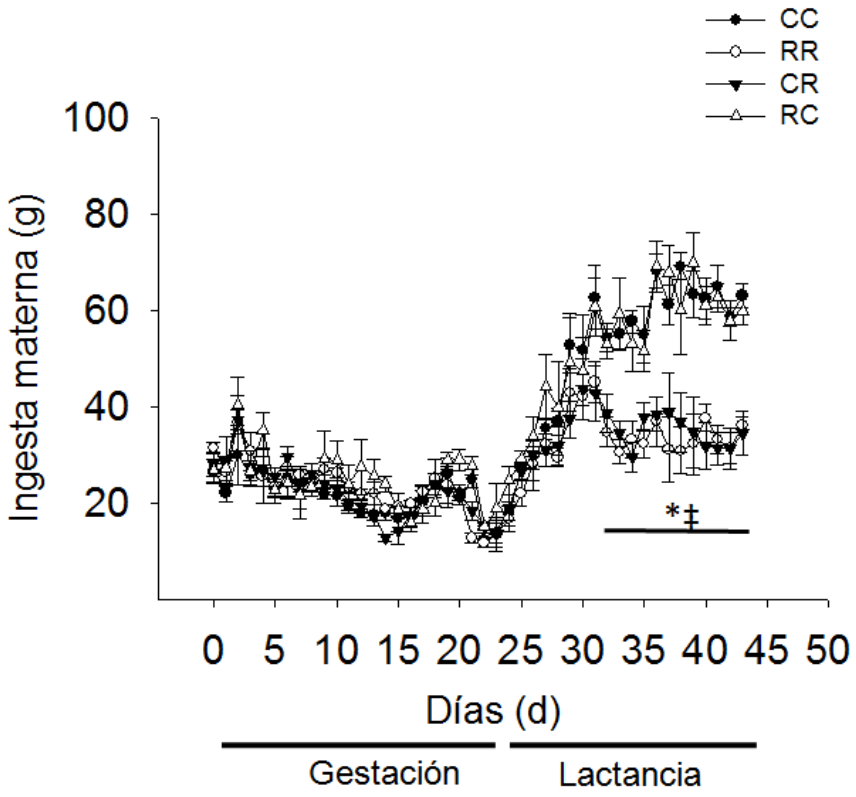

Figura 22. Ingesta de alimento materna durante el periodo de gestación y lactancia. Las madres fueron alimentadas con dieta control (C=20% caseína) o restringida (R=10% caseína) durante el embarazo (primera letra) y lactancia (segunda letra). Media ± EE; n=8 madres, ANOVA, post hoc tukey; $p \leq 0.05$, *‡ RR y CR vs CC y RC.

8.2 Efecto de la restricción proteínica materna en la descendencia

8.2.1 Parámetros morfológicos en las crías macho al nacimiento

Al nacimiento, el peso total de la camada y el peso corporal de la cría macho de madres alimentadas con dieta restringida durante la gestación fue menor comparado con el de las crías de madres CC (Tabla 4). Otro parámetro que se encontró modificado fue la distancia ano-genital al expresarse de manera relativa al peso, la cual estuvo incrementada un 7 % en el grupo R con respecto a C. Por otro lado, no se encontraron diferencias entre grupos en talla, diámetro cefálico y abdominal.

Tabla 4: Medidas morfométricas al nacimiento

Crías recién nacidas	Control (n=8)	Restringida (n=8)
No crías por camada	10.1 ± 0.2	10.0 ± 0.1
Peso (g)	5.8 ± 0.03	5.2 ± 0.01 *****
Peso (g) total de la camada	57 ± 1.2	51 ± 1.3 *****
Talla (cm)	4.6 ± 0.03	4.5 ± 0.05
Distancia anogenital (mm)	3.4 ± 0.01	3.2 ± 0.02
Distancia anogenital (mm/g)	0.58 ± 0.01	0.62 ± 0.01*****
Diámetro cefálico (mm)	11.4 ± 0.02	11.4 ± 0.02
Diámetro abdominal (mm)	12.2 ± 0.03	12.2 ± 0.03
Radio cefálico:abdominal	0.93 ± 0.01	0.93 ± 0.01

Crías machos provenientes de madres alimentadas con dieta control (C=20% caseína) o restringida (R=10% caseína) durante el embarazo. Datos expresados en Media ± EE; * vs Control; $p<0.05$.n=8, el número de camadas de los cuales se obtuvieron las crías macho.

8.2.2 Tasa de crecimiento:

8.2.2.1 Tasa de crecimiento a los 21d de vida postnatal.

Durante el periodo de lactancia, se evaluó la ganancia de peso de las crías macho desde el nacimiento hasta el destete (día 21 de lactancia), encontrándose que las crías provenientes de madres restringidas durante la lactancia (RR y CR) presentaron menor ganancia de peso con respecto a CC y RC (Figura 23).

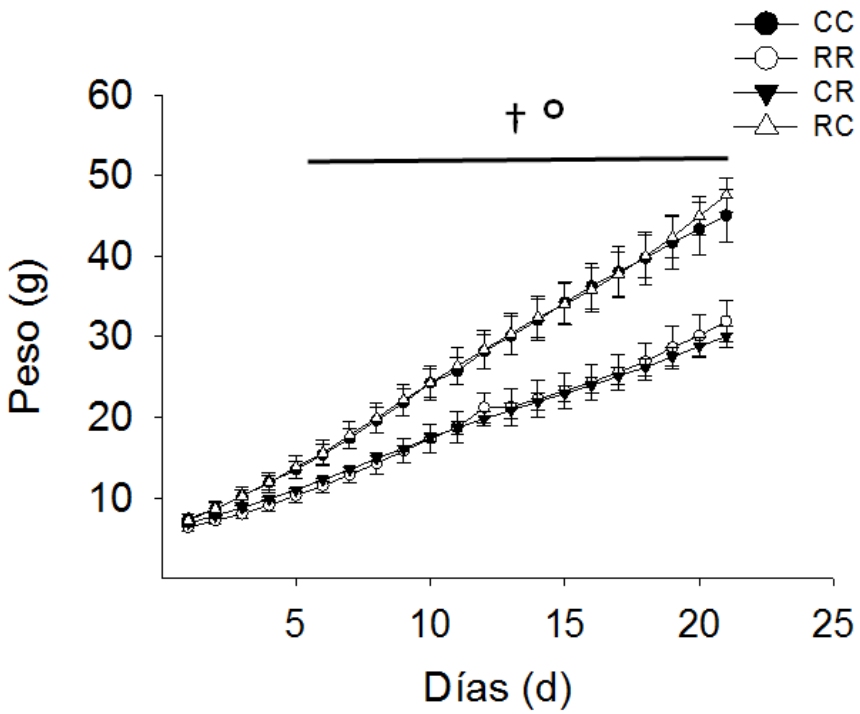

Figura 23. Ganancia de peso durante la lactancia de las crías macho. Crías de madres alimentadas con dieta control (C=20% caseína) o restringida (R=10% caseína) durante el embarazo (primera letra) y lactancia (segunda letra). Media ± EE; n=8 crías de diferente camada, ANOVA, post hoc tukey, $p \leq 0.05$ † ° CC y RC vs RR y CR.

8.2.2.2 Tasa de crecimiento de los machos en diferentes etapas de la adultez

A los 90 días de edad, cuando se comenzaron a evaluar las pruebas conductuales, el grupo CR presentó menor peso corporal con respecto a las crías CC y RC, mientras las crías RR no fueron diferentes de los demás grupos experimentales. A los 110 días, las crías RR fueron menos pesadas con respecto a las crías CC. Este mismo efecto se observó a los 150 y 220 días de vida postnatal (Tabla 5).

Tabla 5: Peso de las crías a las diferentes edades cuando se evaluaron las pruebas conductuales.

EDAD	CC	RR	CR	RC
90d	330 ± 13 **a**	298 ± 13 **ab**	281 ± 6 **b**	340 ± 7 **a**
110d	370 ± 5 **a**	329 ± 5 **b**	354 ± 14 **a**	368 ± 7 **a**
150d	420 ± 12 **a**	362 ± 19 **b**	382 ± 5 **ab**	403 ± 16 **ab**
220d	444 ± 19 **a**	381 ± 12 **b**	409 ± 4.1 **ab**	454 ± 14 **a**

Crías macho de madres alimentadas con dieta control (C=20% caseína) o restringida (R=10% caseína) durante el embarazo (primera letra) y lactancia (segunda letra). Datos expresados en Media ± EE; n=8-10 machos de diferente camada. Grupos que no comparten la misma letra son estadísticamente diferentes, ANOVA post hoc tukey, $p<0.05$.

8.2.3 Pruebas conductuales

8.2.3.1 Conducta de tipo ansiedad

Laberinto Elevado en cruz

Al comparar el número de entradas a los brazos abiertos, pudimos observar que las crías macho proveniente de madres restringidas en ambos periodos (RR) presentaron menor número de entradas con respecto al CC, no encontrándose diferencias con el CR y RC (Figura 24-A). Este mismo comportamiento se observó en el tiempo de permanencia y distancia recorrida en los brazos abiertos (Figura 24-B y C). La distancia total recorrida en los brazos abiertos como cerrados no fue diferente entre los grupos experimentales en la (Figura 24-D).

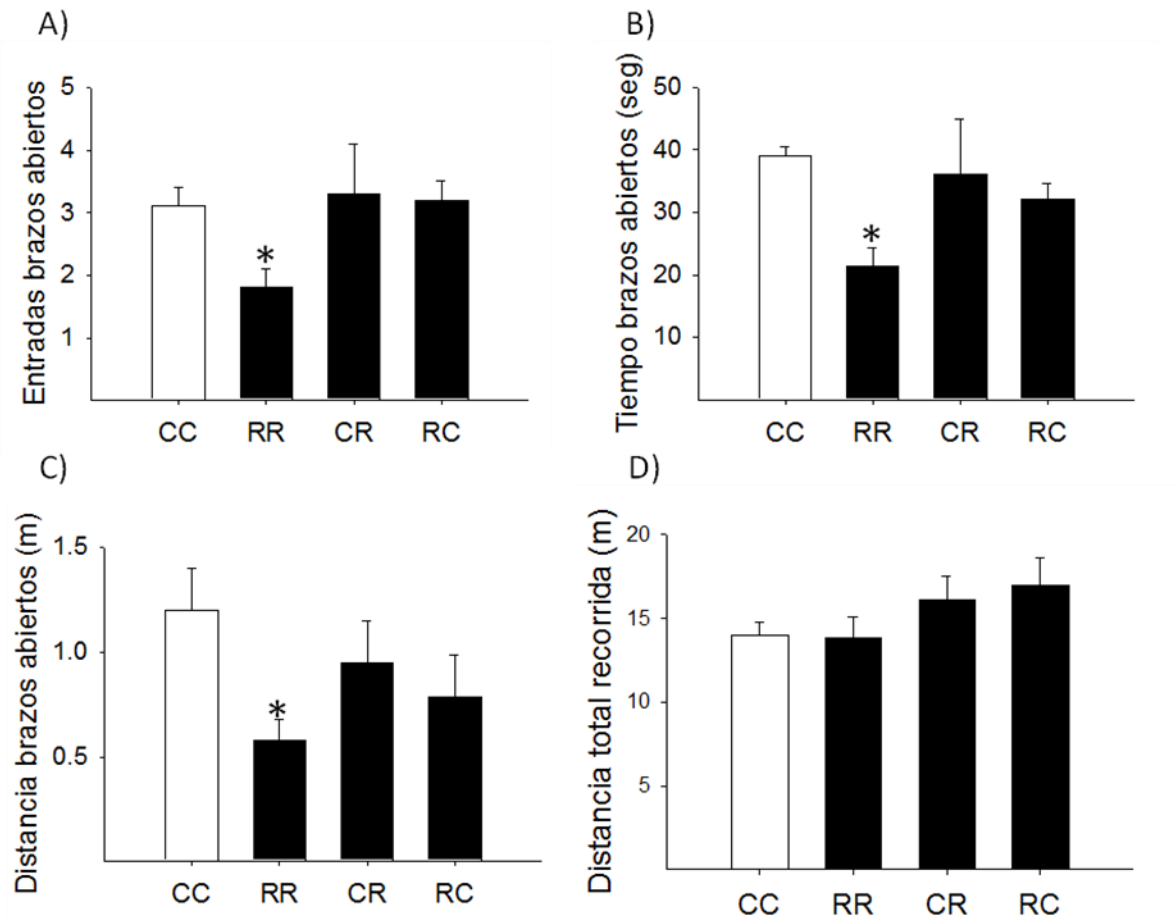

Figura 24. Prueba del Laberinto elevado en cruz: A) entradas, B) tiempo de permanencia, C) distancia recorrida en brazos abiertos y D) distancia total. Crías de madres alimentadas con dieta control (C=20% caseína) o restringida (R=10% caseína) durante el embarazo (primera letra) y lactancia (segunda letra). Media ± EE; n=8 crías de diferente camada, ANOVA post hoc tukey, $p \leq 0.05$ * vs CC

Campo abierto

La tabla 6 muestra los resultados de la conducta explorativa durante 60 minutos de los machos en la prueba del campo abierto. En esta prueba pudimos apreciar que el grupo restringido en ambos periodos (RR) presentó menor número de entradas en la zona central con respecto al CC; sin embargo las crías CR y RC no fueron diferentes de CC y RR. Por otro lado, no se encontraron diferencias estadísticas entre los grupos experimentales en el tiempo de permanencia y distancia recorrida en la zona central y periférica así como en la distancia total recorrida en ambas zonas.

Tabla 6. Evaluación del comportamiento de las crías macho en la prueba del campo abierto durante 60 min.

	Número de entradas en centro	Tiempo en centro (seg)	Tiempo en la periferia (seg)	Distancia recorrida en el centro (m)	Distancia recorrida en la periferia (m)	Distancia Total
CC	35.8 ± 6.6	124.6 ± 25.2	3475.4 ± 25.1	14.1 ± 2.4	114.5 ± 10.3	128.7 ± 9
RR	17.6 ± 4.2 *	116.7 ± 33	3483.3 ± 33.0	9.4 ± 2.4	72.5 ± 12.3	82.0 ± 11
CR	34.4 ± 5.1	127.7 ± 17	3472.3 ± 17.0	13.4 ± 2.4	74.6 ± 10.8	88.1 ± 11.2
RC	19.2 ± 3.6	141.0 ± 59.2	3459.0 ± 59.2	7.2 ± 1.7	76.2 ± 16.0	83.5 ± 15.4

Crías macho de madres alimentadas con dieta control (C=20% caseína) o restringida (R=10% caseína) durante el embarazo (primera letra) y lactancia (segunda letra). Media ± EE; n=8 crías de diferente camada. ANOVA post hoc tukey, $p<0.05$ * vs CC

8.2.3.2 Aprendizaje, memoria y motivación

Reconocimiento de objeto novedoso

Con base a los resultados obtenidos en la prueba de reconocimiento de objetos, pudimos apreciar que durante la etapa de habituación no se encontraron diferencias entre los grupos experimentales en el tiempo de exploración en ambos objetos. Durante la etapa de prueba, pudimos observar que las crías provenientes de madres restringidas durante la gestación, lactancia o ambas condiciones presentaron menor tiempo de permanencia en el objeto novedoso con respecto al control (CC: 124 ± 3**a**, RR: 36 ± 4**b**, CR: 58± 8**b** y RC 27 ± 4**b** segundos). Por otro lado, al expresar los resultados como índices de discriminación y reconocimiento (Figura 25-A y B), pudimos observar que el índice de discriminación fue significativamente menor en el grupo RR con respecto al CC; sin mostarse cambios al comparar con CR y RC. El índice de reconocimiento fue menor en los tres grupos experimentales al comparar con CC.

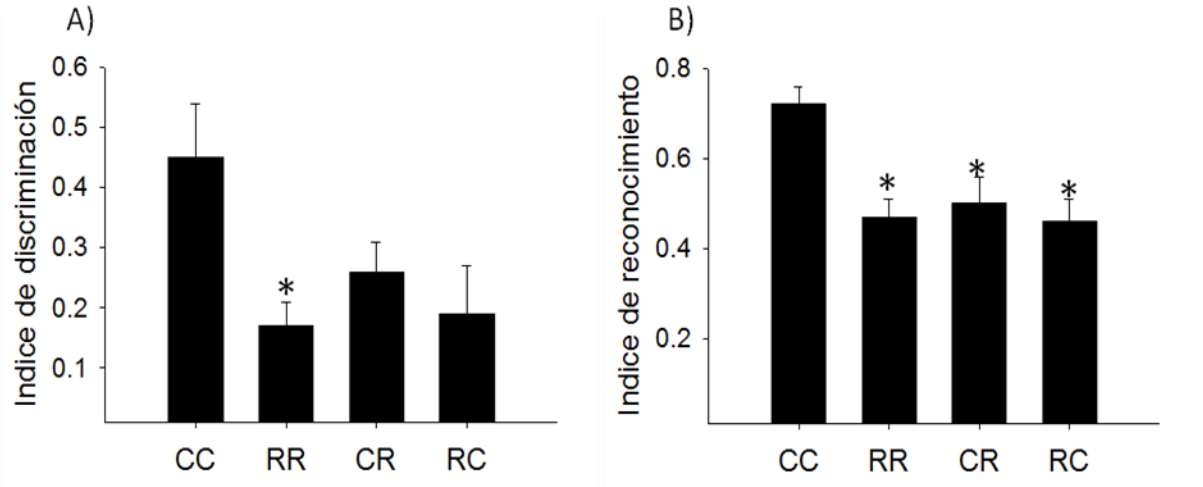

Figura 25. Entrenamiento en la prueba de reconocimiento de objetos: A) índice de discriminación, B) índice de reconocimiento. Crías de madres alimentadas con dieta control (C=20% caseína) o restringida (R=10% caseína) durante el embarazo (primera letra) y lactancia (segunda letra). Media ± EE, n=8 crías de diferente camada, ANOVA post hoc tukey, $p<0.05$ * vs CC

Laberinto acuático de Morris

Durante la prueba de adquisición, al analizar los datos por ensayo entre grupos experimentales pudimos observar que en el ensayo 1 y 3 no se observaron diferencias significativas. En el ensayo 2, las crías RC y CR presentaron una latencia de escape mayor con respecto al control. Desde el ensayo 4 al 8 las crías RR requirieron de mayor tiempo para llegar a la plataforma en comparación al grupo CC. En los ensayos 5 y 6 las crías CR mostraron una latencia incrementada al comparar con las crías CC (Figura 26). Por otro lado, al comparar los 8 ensayos entre misma dieta experimental (primero versus otros), pudimos apreciar que las crías del grupo CC aprendieron en el segundo ensayo, las crías CR en el séptimo ensayo y las crías RC en el tercero; sin embargo, las crías RR no aprendieron a localizar la plataforma, a pesar de haber completado los 8 ensayos.

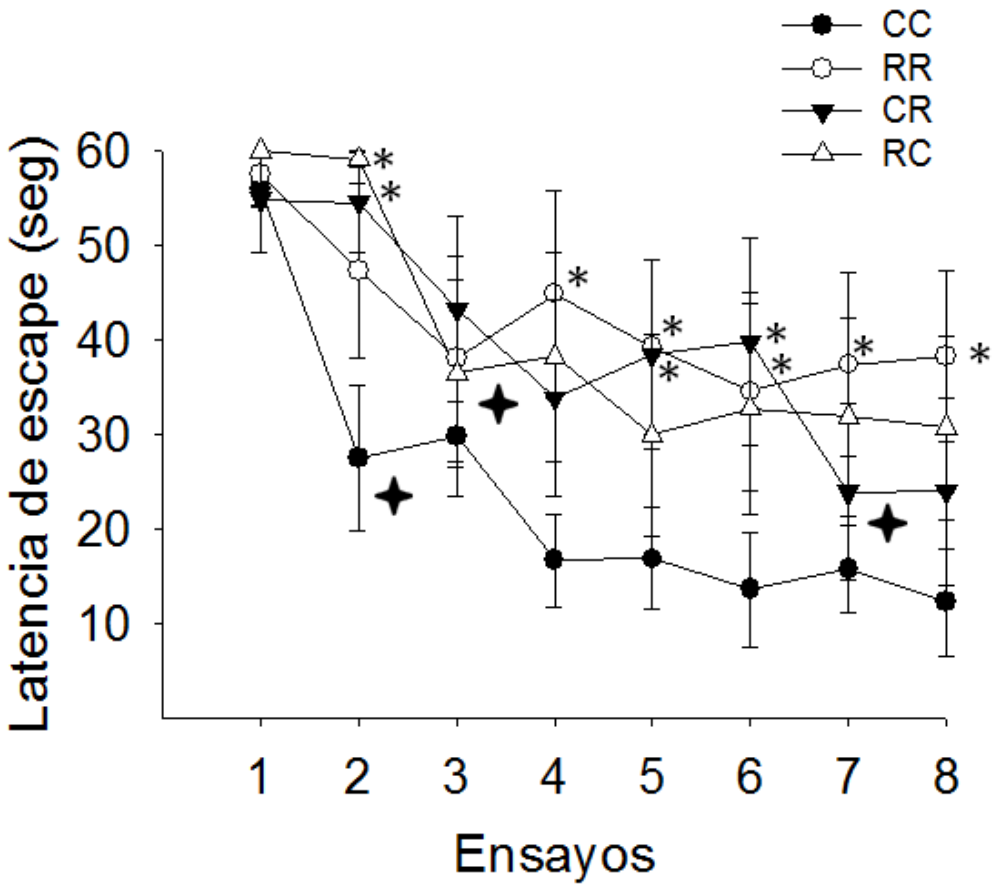

Figura 26: La adquisición en la tarea del Laberinto acuático de Morris está representado como la media de la latencia de escape (seg) en los 8 ensayos del entrenamiento.Crías de madres alimentadas con dieta control (C=20% caseína) o restringida (R=10% caseína) durante el embarazo (primera letra) y lactancia (segunda letra). Media ± EE, n=10 de diferente camada, ANOVA post hoc Bonferroni; ✦ $p<0.05$ vs primer ensayo mismo grupo experimental. *vs CC mismo ensayo.

Al analizar el área bajo la curva de la latencia de escape para llegar a la plataforma durante la prueba de adquisición pudimos observar que fue significativamente mayor en los grupos RR, CR y RC versus el control (CC: 126 ± 15 **a,** RR 294 ± 26**b**, CR 237 ± 21**b**, RC 250 ± 24**b** segundos, $p<0.05$).

En la prueba de retención (24 horas después al entrenamiento), se observó que las crías RR, CR y RC requirieron de más tiempo para localizar el área donde se encontraba la plataforma (Figura 27).

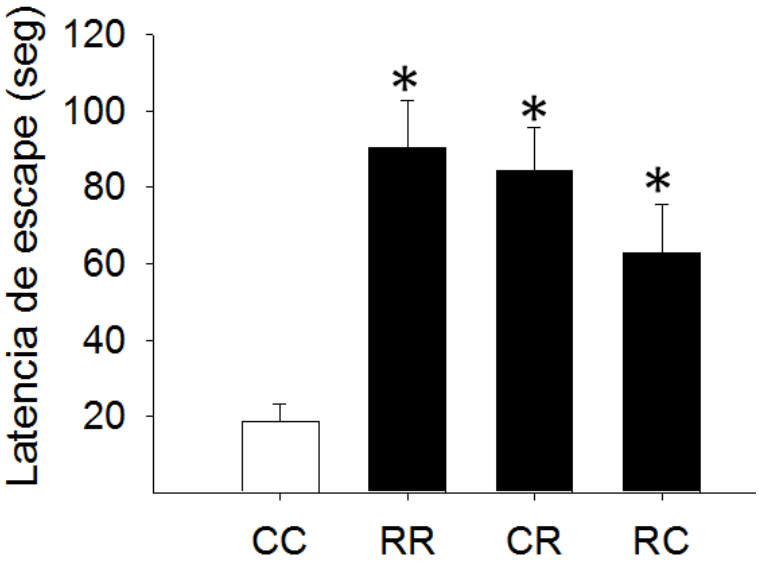

Figura 27. Latencia de escape (seg) para localizar el area de la plataforma en la prueba de retención (memoria espacial) en el Laberinto acuático de Morris. Crías de madres alimentadas con dieta control (C=20% caseína) o restringida (R=10% caseína) durante el embarazo (primera letra) y lactancia (segunda letra). Media ± EE, n=10 de diferente camada, ANOVA post hoc tukey, $p<0.05$ * vs CC.

Por otro lado, las crías RR, CR y RC mostraron menor número de entradas en la zona blanco con respecto al control. Los grupos RR y RC tuvieron menor tiempo de permanencia en la zona blanco al comparar con CC. El mismo efecto se observó tanto en el número de entradas y tiempo de permanencia en la zona opuesta (Figura 28-A y B). En la sección C de la figura 28, podemos apreciar una representación del patrón de nado (recorrido) de los cuatro grupos experimentales dentro la piscina durante la prueba de retención en el Laberinto acuático de Morris.

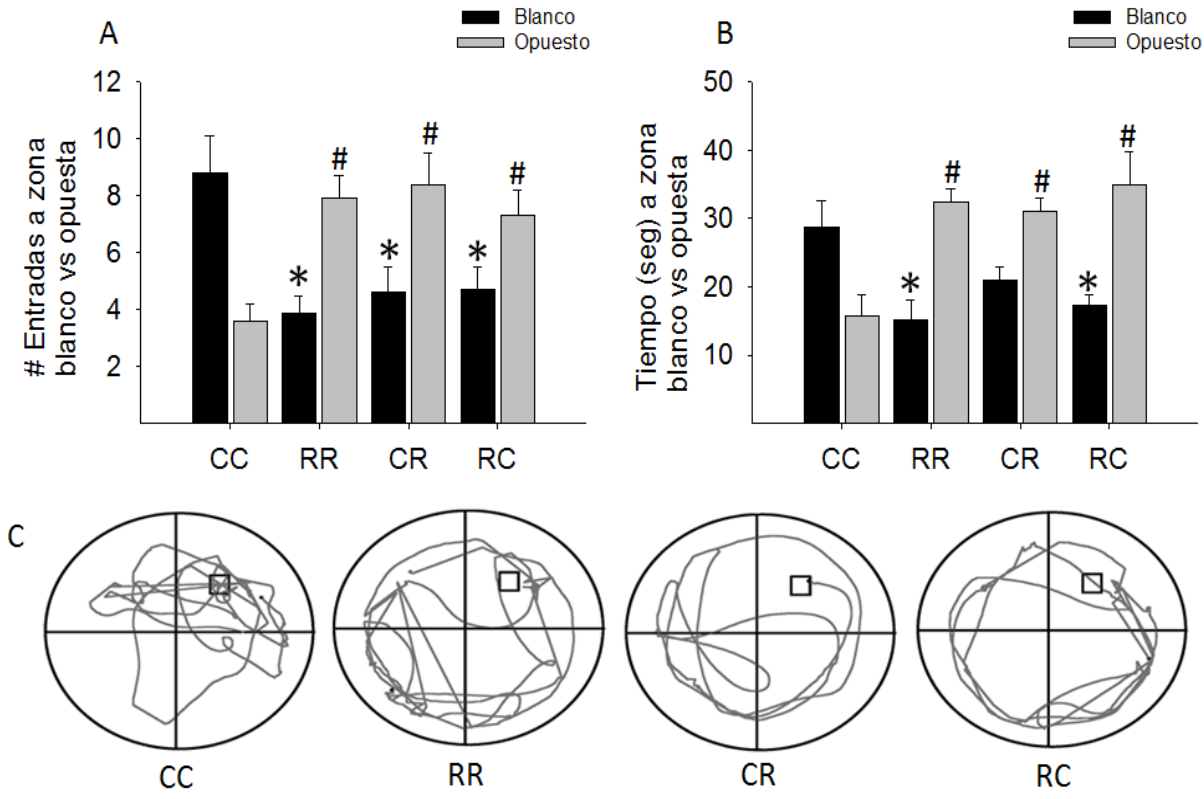

Figura 28: Laberinto acuático de Morris: A) Número de entradas, B) tiempo de permanencia en la zona blanco vs opuesta, C) representación del patrón de nado (recorrido) dentro de la piscina durante la prueba de retención; Crías de madres alimentadas con dieta control (C=20% caseína) o restringida (R=10% caseína) durante el embarazo (primera letra) y lactancia (segunda letra). Media ± EE, n=10 de diferente camada, ANOVA post hoc tukey, $p<0.05$ * vs CC en la zona blanco; # vs CC en la zona opuesta.

Condicionamiento operante

Adquisición

Al analizar los datos del condicionamiento para evaluar al aprendizaje asociativo, pudimos observar que las crías RR, CR y RC requirieron de mayor número de sesiones para presionar por primera vez la palanca y obtener la recompensa con respecto al CC. Además, las crías RC fueron significativamente diferentes de RR y CR (Figura 29).

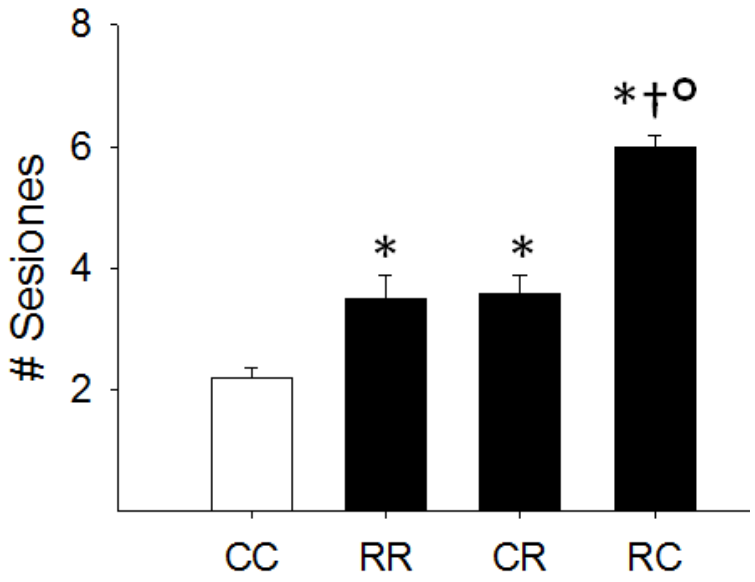

Figura 29: Condicionamiento operante: Número de sesiones necesarias para presionar por primera vez la palanca (adquisición). Crías de madres alimentadas con dieta control (C=20% caseína) o restringida (R=10% caseína) durante el embarazo (primera letra) y lactancia (segunda letra). Media ± EE; n=8 crías de diferente camada. ANOVA post hoc tukey, p≤ 0.05; * vs CC, * † ° vs CC, RR y CR.

Reforzamiento y retención (RF-1 y RF-5)

Para el condicionamiento RF-1, el número de sesiones requeridas en las crías fue mayor en el grupo RC, comparado con el resto de los grupos (Figura 30A). Adicionalmente, para el condicionamiento RF-5, los animales de los grupos RR, CR y RC requirieron de más sesiones para alcanzar el criterio de desempeño en comparación con el grupo CC. Además, entre los grupos RR y CR se encontraron diferenciasen cuanto el número de sesiones necesarias para cumplir con los criterios del condicionamiento RF-5 (Figura 30B).

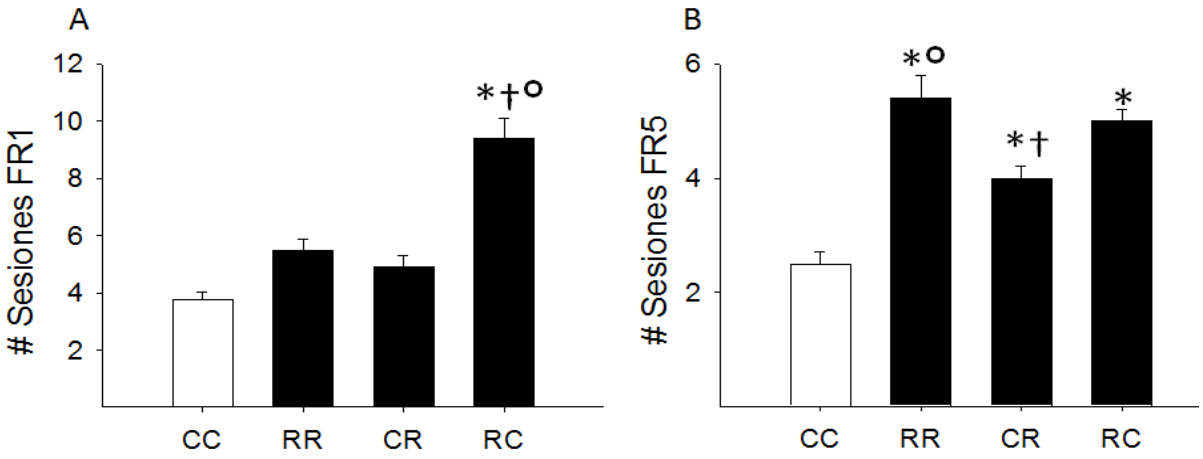

Figura 30: Condicionamiento operante: Número de sesiones necesarias para cumplir con el criterio de reforzamiento A) RF-1 y B) RF-5. Crías de madres alimentadas con dieta control (C=20% caseína) o restringida (R=10% caseína) durante el embarazo (primera letra) y lactancia (segunda letra). Media ± EE; n=8 crías de diferente camada, ANOVA post hoc tukey, p ≤ 0.05; * vs CC, ***†**° vs CC, RR y CR. ***†** vs CC y RR, *° vs CC y CR.

Evento progresivo (motivación)

En cuanto al número de respuesta obtenidas (presión de la palanca) por sesión en la prueba progresiva del condicionamiento operante, observamos que fue significativamente menor en las crías de madres restringidas de proteína en algún periodo – RR, CR y RC – y por consiguiente menor número de recompensas con respecto al grupo CC (Figura 31 –A y B).

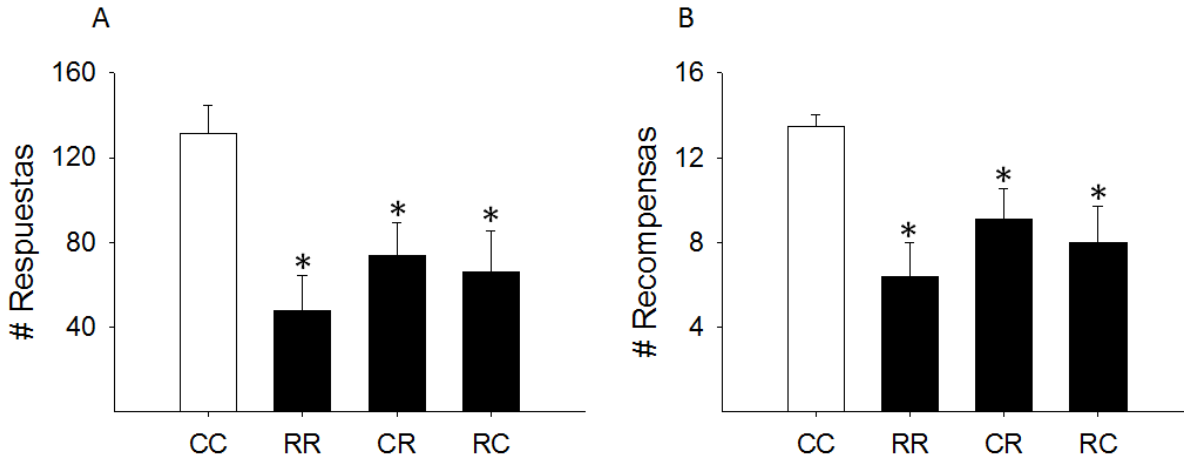

Figura 31: Prueba del evento progresivo A) Número de respuestas, B) Número de recompensas durante 10 días consecutivos. Crías de madres alimentadas con dieta control (C=20% caseína) o restringida (R=10% caseína) durante el embarazo (primera letra) y lactancia (segunda letra). Media ± EE; n=8 crías de diferente camada, ANOVA post hoc tukey, p ≤ 0.05 * vs CC

Consumo libre de sacarosa

No se encontraron diferencias al promediar los tres días consecutivos evaluados (30 minutos cada día) en el consumo libre de la solución de sacarosa al 7% entre los grupos (CC: 28.8 ± 0.7, RR 28 ± 0.4, CR 28 ± 0.7 y RC 28.1 ± 0.7 mL)

8.2.4 Evaluación de los glucocorticoides

8.2.4.1 Concentración de corticosterona entre los 90 – 92 días

Una vez finalizadas las pruebas del laberinto elevado en cruz se recolectaron las heces fecales de los diferentes grupos experimentales, encontrándose que las crías provenientes de madres restringidas durante la gestación – RR y RC – presentaron incremento en la concentración de corticosterona en comparación con el grupo control; además, el grupo RC fue significativamente mayor al CR, y sin cambio con respecto a RR (Figura 32).

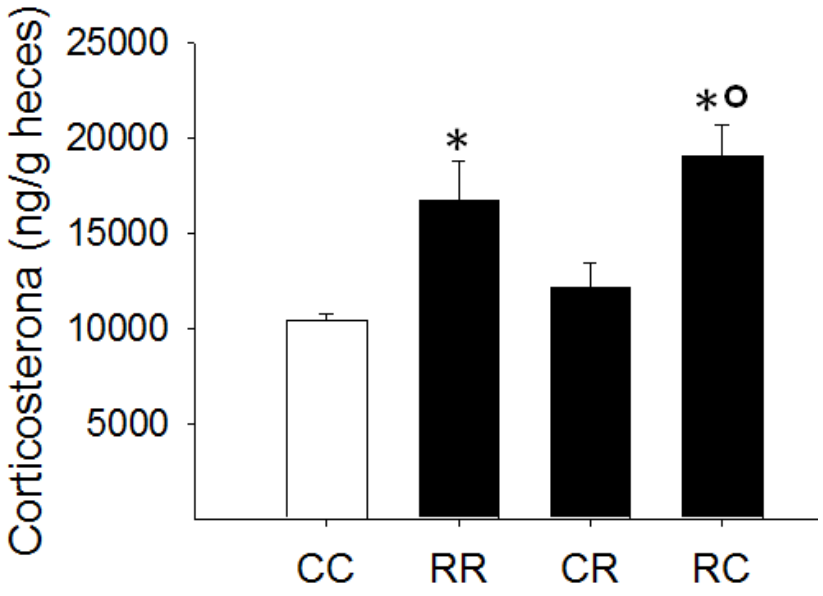

Figura 32: Corticosterona (ng/g) en heces fecales de las crías macho después de finalizar la prueba del EPM. Crías de madres alimentadas con dieta control (C=20% caseína) o restringida (R=10% caseína) durante el embarazo (primera letra) y lactancia (segunda letra). Media ± EE; n=8 crías de diferente camada, ANOVA post hoc tukey, $p \leq 0.05$ * vs CC, *° vs CC y CR.

8.2.4.2 Concentración de corticosterona a los 110 días

La concentración de corticosterona en suero fue mayor en las crías RR, CR y RC en comparación al control; a su vez, la concentración del esteroide en los grupos CR y RC fue significativamente mayor de RR (Figura 33-A). Con respecto a las concentraciones de ACTH, las crías provenientes de madres restringidas en ambos periodos (RR) mostraron incremento en sus concentraciones con respecto a CC. No encontrándose diferencias en CR y RC con respecto a CC (Figura 33-B).

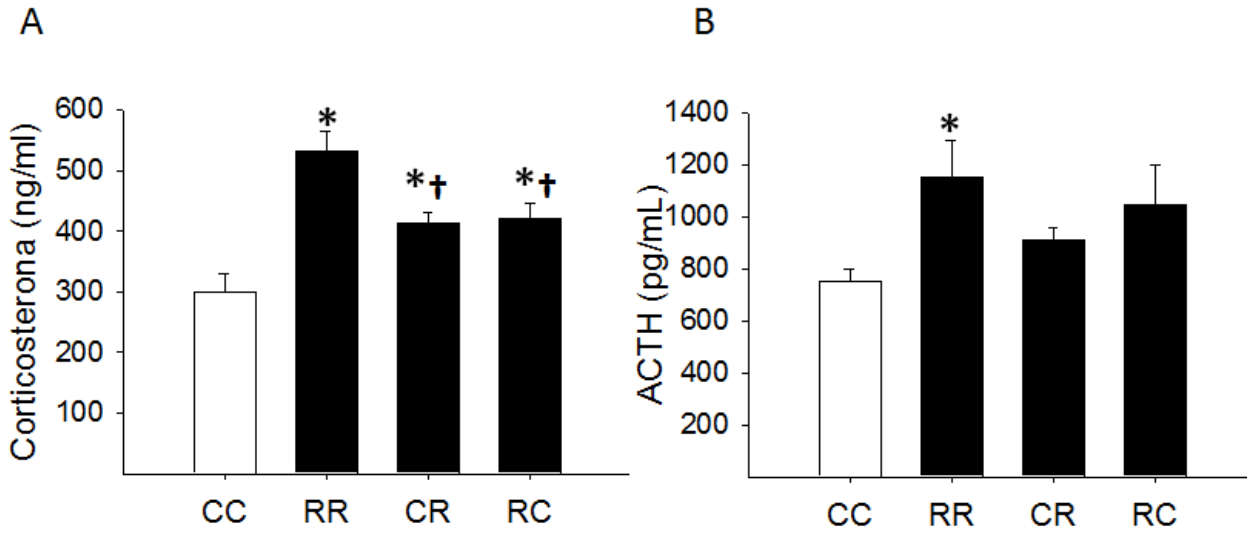

Figura 33: Concentraciones en suero de A) Corticosterona y B) ACTH a 110 días. Crías de madres alimentadas con dieta control (C=20% caseína) o restringida (R=10% caseína) durante el embarazo (primera letra) y lactancia (segunda letra). Media ± EE, n=10 diferente camada. ANOVA post hoc tukey, $p<0.05$, * vs CC, *† vs CC y RR.

8.2.4.3 Concentración de corticosterona a los 220 días.

Al analizar las concentraciones de corticosterona después del condicionamiento operante, pudimos apreciar que las crías RR presentaron mayor concentración con respecto al CC; sin embargo, los grupos CR y RC tuvieron valores intermedios entre CC y RR, sin mostrar diferencias entre ellos. Este mismo comportamiento se observó a los 20 y 40 minutos posteriores a la prueba de restricción de movimiento (reto con inmovilización). No se encontraron cambios a los 80 y 120 minutos entre los diferentes grupos experimentales.

Por otro lado, al comparar la concentración previo a inmovilización con los demás tiempos evaluados entre la misma dieta experimental, pudimos apreciar que ésta fue significativamente mayor en las crías CC, RR y RC a los 20 min en todos los grupos a los 40 min; en CC, CR y RC a los 80 min; por último en CC y CR a los 120 min (Figura 34). El área bajo la curva de las concentraciones de corticosterona fue significativamente mayor en el grupo RR con respecto a CC. No encontrándose diferencia en CR y RC con respecto a CC y RR (CC: 52 ± 1.5**a**, RR: 60± 2.1**b**, CR: 53.3 ± 2.1**ab**, RC: 55 ± 2.3**ab**)

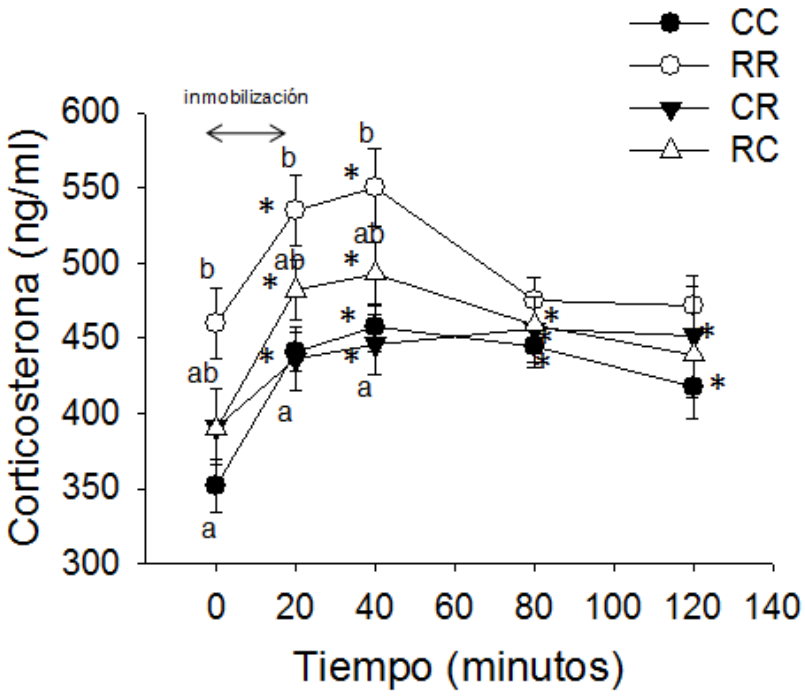

Figura 34: Concentraciones de corticosterona previo y posterior a la inmovilización tiempo=20, 40, 80 y 120 min. Crías de madres alimentadas con dieta control (C=20% caseína) o restringida (R=10% caseína) durante el embarazo (primera letra) y lactancia (segunda letra). Media ± EE; n=8 crías de diferente camada. ANOVA post hoc Bonferroni, p< 0.05, * vs basal mismo grupo experimental. Datos que no comparten la misma letra son estadísticamente diferentes.

Al analizar las concentraciones de ACTH, se pudo apreciar que las crías RR y RC presentaron mayor concentración con respecto al CC y CR. Este mismo comportamiento se observó a los 20 minutos posteriores a la prueba de restricción de movimiento (inmovilización). A los 40 minutos, la concentración fue menor en CR en comparación a RC y RR. A los 80 y 120 minutos no se observaron

diferencias entre los diferentes grupos experimentales. Al comparar la concentración (previo a la inmovilización) del mismo grupo experimental a los diferentes tiempos evaluados, pudimos apreciar esta fue significativamente mayor a los 20 min en los diferentes grupos; a los 40 min me mantuvo incrementada en CC, RR y CR; a los 80 min en CC y CR; por último a los 120 min, no se encontraron diferencias significativas (Figura 35).

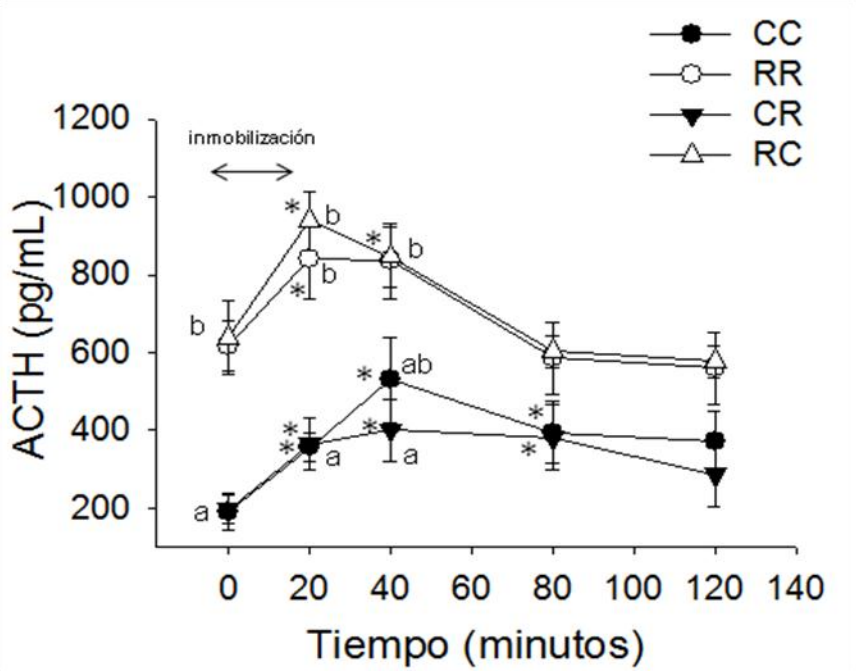

Figura 35: Concentraciones de ACTH previo y posterior a la inmovilización tiempo=20, 40, 80 y 120 min. Crías de madres alimentadas con dieta control (C=20% caseína) o restringida (R=10% caseína) durante el embarazo (primera letra) y lactancia (segunda letra). Media ± EE; n=8 crías de diferente camada. ANOVA post hoc Bonferroni, $p< 0.05$, * vs basal mismo grupo experimental. Datos que no comparten la misma letra son estadísticamente diferentes.

8.2.5 Cambios morfológicos en la región CA3 del hipocampo

8.2.5.1 Peso del cerebro

A los 110 d no se encontraron diferencias significativas entre los grupos experimentales en el peso del cerebro (CC: 2.04 ± 0.02, RR: 2.02 ± 0.03, CR: 2.09 ± 0.01 y RC: 2.05 ± 0.03); sin embargo al determinar el peso relativo con respecto al peso corporal, se observó un incremento del 11.3% en las crías provenientes de madres restringidas durante la gestación y lactancia (RR) con respecto al control, sin cambios en CR y RC.

8.2.5.2 Área de las fibras musgosas (FM):

El área total de las fibras musgosas en *stratum lucidum* fue significativamente menor en las crías RR (-21.9%), CR y RC (-11%) con respecto al grupo control. Además, el área en los grupos CR y RC fue significativamente mayor (+13.9%) que RR (Figura 36). En la Figura 36-C se puede apreciar la sección representativa del área en cada grupo experimental.

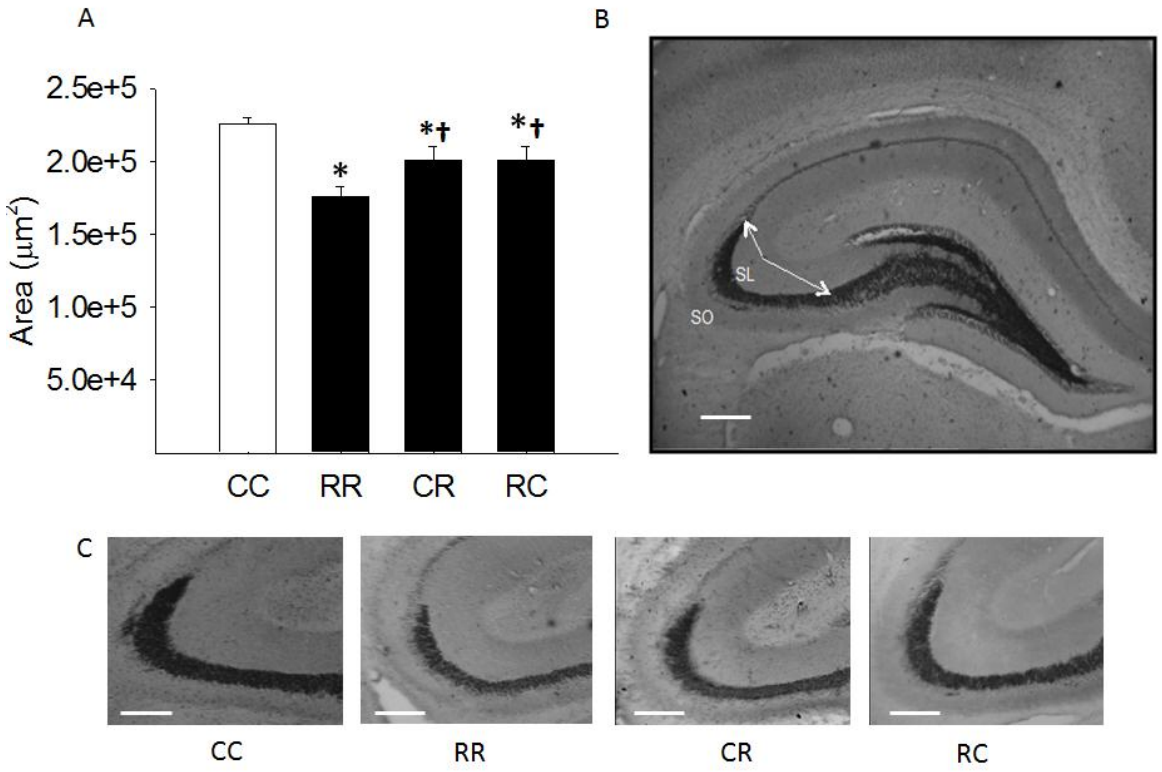

Figura 36: Área de las fibras musgosas (FM) in *stratum lucidum* (SL), B) representación de la sección coronal del hipocampo usado para estimar el área de las fibras musgosas en SL indicado por los límites de la flecha. Barra = 100 μm, C) sección representativa del área medida en cada grupo.Crías de madres alimentadas con dieta control (C=20% caseína) o restringida (R=10% caseína) durante el embarazo (primera letra) y lactancia (segunda letra). Media ± EE; n=5 crías de diferente camada. Datos que no comparten la misma letra son estadísticamente diferentes, $p< 0.05$ * vs CC, *† vs CC y RR.

8.2.5.3 Espinas totales y tipos de espinas

Al analizar la densidad de las espinas en el segmento medial de las dendritas basales del *stratum oriens*, pudimos observar que las crías provenientes de madres restringidas de proteína durante la gestación, lactancia o ambos periodos presentaron menor número de espinas totales [RR (-14.4%), CR (-12.2%) y RC (-7.7%)] con respecto al grupo control; además que el grupo RC fue significativamente mayor (7%) que RR. Las espinas largas se encontraron disminuidas en RR (-21.2%), CR (-25.2%) y RC (-13.4%) en comparación a CC. RC fue significativamente diferente a CR (+15.7%). Las espinas en forma de hongo estuvieron disminuidas en RR (-28%) y RC (32%), sin cambios en CR con los demás grupos experimentales. Las espinas cortas se encontraron aumentadas en RR (19.2%), CR (54.6%) y RC (38.4%) con respecto a CC (Figura 37).

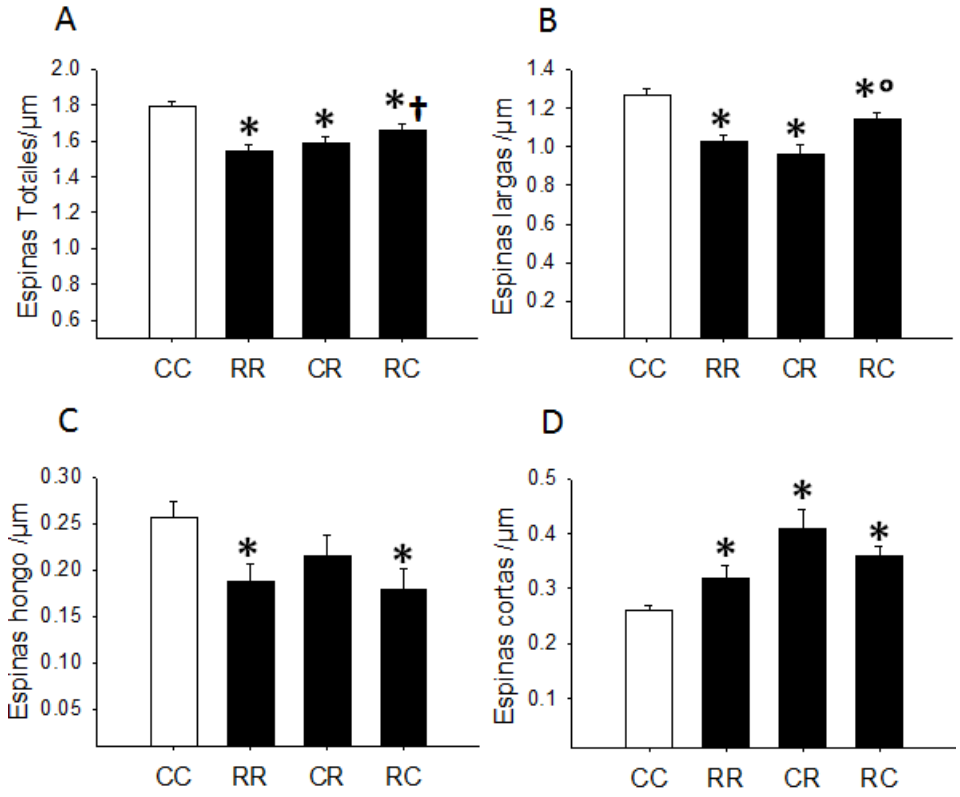

Figura 37: Densidad de las espinas en el segmento medial de las dendritas basales del *stratum oriens* (SO): A) totales, B) largas, C) hongo y D) cortas. Crías de madres alimentadas con dieta control (C=20% caseína) o restringida (R=10% caseína) durante el embarazo (primera letra) y lactancia (segunda letra). Media ± EE; n=5 crías de diferente camada. $p \leq 0.05$ * vs CC, *† vs CC y RR, *° vs CC y CR.

8.0 DISCUSIÓN

El objetivo del presente trabajo fue estudiar los efectos del bajo consumo de proteína materno durante la gestación, la lactancia o durante ambos periodos sobre la ansiedad, aprendizaje, y memoria en la vida adulta de las crías macho. De igual manera se estudiaron los cambios en el sustrato anatómico del hipocampo en sitios pre y post sinápticos y papel de los glucocorticoides, como posibles mecanismos implicados en la programación negativa del cerebro durante etapas tempranas del desarrollo del individuo. Los resultados obtenidos indican que la restricción proteínica materna provoca alteraciones a nivel afectivo y cognitivo debido a alteraciones en la plasticidad sináptica e incremento de la actividad de los glucocorticoides.

Diversos estudios en el área de la programación ponen en evidencia la susceptibilidad de los ejes neuroendócrinos, incluido el eje adrenal a condiciones adversas en las etapas tempranas de la vida durante el desarrollo fetal y neonatal (Guzman et al., 2006; Guzman et al., 2014; Padmanabhan et al., 2006; Rodriguez-Gonzalez et al., 2014; Seckl, 2004; Spencer, 2013). En la rata, gran parte de los eventos cruciales en el desarrollo del eje hipotálamo-hipófisis-adrenal se presentan durante la gestación (Matthews, 2002), por lo que esta etapa representa un periodo crítico y susceptible para la programación de alteraciones a nivel afectivo y cognitivo (Yam et al., 2015).

El uso de dietas isocalóricas durante la gestación y/o la lactancia permitieron identificar los efectos ocasionados únicamente por la restricción de la proteína, sin modificar el aporte energético de la dieta (Guzman et al., 2014; Zambrano et al., 2005b). La ganancia de peso durante el embarazo depende de la calidad de la nutrición materna (Langley-Evans et al., 1999; MacLaughlin et al., 2005; Nathanielsz, 1999; Rush, 2001). La alimentación materna durante la lactancia juega un papel importante (Zambrano et al., 2005a; Zambrano et al., 2005b), debido a que en esta etapa algunos órganos permanecen sensibles a las influencias ambientales y nutricionales (Barraclough, 1961; Barraclough and Gorski, 1961). En el presente estudio, la restricción proteínica modificó la

ganancia de peso materna durante la segunda mitad de la lactancia, encontrándose una disminución en las madres alimentadas con dieta baja en proteína durante la lactancia (RR y CR). Este mismo comportamiento se observó en la ingesta de alimento en las madres. Los efectos de la restricción proteica materna durante la gestación (reducción del 50% de proteína) sobre las medidas morfométricas fueron determinadas, encontrándose menor peso corporal e incrementó en la distancia ano-genital (DAG) en las crías macho. Estudios en animales de experimentación han demostrado que la restricción proteínica produce diferentes efectos, ya sea disminución (Guzman et al., 2006; Holemans et al., 1999; Langley-Evans, 2000; Langley-Evans et al., 1999; Vickers et al., 2001), aumento (Langley-Evans et al., 1996) o sin cambio alguno (Langley et al., 1994; Zambrano et al., 2005b) en el peso corporal al nacimiento, lo cual pueda atribuirse al nivel de restricción proteínica y/o a la compensación de carbohidratos en una dieta isocalórica. Nuestros resultados del peso al nacimiento cotejan con los resultados reportados en modelos de restricción intrauterina retardada y la programación fetal (Vuguin, 2007). De igual manera, el incremento en la DAG en la descendencia de madres restringidas en proteína replica nuestros estudios publicados (Reyes-Castro et al., 2011). La DAG proporciona un marcador externo de diferenciación sexual al nacer. La exposición a andrógenos y esteroides por vía tras placentaria durante la vida fetal impacta en la ontogenia de órganos clave, tales como: el cerebro, útero y gónadas, no sólo en el número y morfología, sino también en la regulación de los receptores esteroides. En nuestro grupo de estudio, hemos asociado el incremento de la DAG (hiperandrogenización) con elevadas concentraciones en la madre de las hormonas esteroideas tales como: progesterona, estradiol y testosterona al día 19 de gestación [cerca del término del embarazo] (Zambrano et al., 2005b). La exposición prenatal a testosterona es asociada con alteraciones en los marcadores de desarrollo sexual (retraso en el descenso de testículo y retracción del prepucio) en etapas tempranas del desarrollo (Rodriguez-Gonzalez et al., 2014).

El peso al nacimiento ha sido considerado como un buen indicador predictivo de la salud en la etapa adulta (Barker, 2004a). Se propone que la discrepancia entre el ambiente intrauterino y el pos natal ocasionan fenotipos mal adaptados, debido a respuestas adaptativas predictivas, generadas durante el desarrollo fetal, y que no coinciden con el ambiente postnatal esperado (Wells, 2007). En nuestro estudio, como era de esperarse cada grupo presentó alteraciones fenotípicas y funcionales diferentes, debido a que cada uno recibió un estímulo específico. En el caso del grupo CR, las crías no nacieron preparadas para la escases, por lo que el cambio de las madres a un ambiente posnatal con bajo contenido de proteína, originó que las crías tuvieran que realizar los ajustes necesarios que les ayudaran a sobrevivir, comprometiendo así el desarrollo de sus órganos. Por el contrario, el grupo RC se preparó para un medio adverso, adquirió adaptaciones en la vida fetal para ese medio, y creció en un medio con disponibilidad de nutrimentos mayor a la esperada. Las respuestas adaptativas predictivas que desarrolló no fueron adecuadas y mostró mala adaptación siendo un grupo obeso y con diversas alteraciones metabólicas. Se ha demostrado que estas fallas metabólicas generan un desempeño reproductivo alterado en la vida adulta: menor concentración de testosterona en suero, disminución de la cuenta espermática y motilidad que provoca disminución de la tasa de fertilidad en la vida adulta (Guzman et al., 2006; Rodriguez-Gonzalez et al., 2014; Zambrano et al., 2005a; Zambrano et al., 2005b) y cognitivo (Belluscio et al., 2014; Martinez et al., 2009; Reyes-Castro et al., 2012a; Reyes-Castro et al., 2012b; Reyes-Castro et al., 2011). Entre los grupos experimentales el que tendría un mejor pronóstico en este aspecto, es el grupo RR pues se “adaptó” a un medio restringido en proteína y creció en el medio para el cual se preparó. Por lo que sus respuestas predictivas corresponden al medio en que se desarrolló. Sin embargo, hemos publicado que las crías de este grupo también presentan alteraciones metabólicas tales como resistencia a la insulina y leptina en la vida adulta (Zambrano et al., 2006). En estudios previos hemos observado que una de las diferencias fenotípicas más características de las crías en la vida adulta es el peso corporal, pues los grupos RR y CR son los que mostraron menor ganancia de peso a lo largo del desarrollo, por el contrario las

crías RC son las que tienden a ganar mayor peso corporal (Rodriguez-Gonzalez et al., 2014; Zambrano et al., 2006).

Con respecto a los resultados de las pruebas conductuales, las observaciones en el laberinto elevado en cruz revelaron diferencias en conductas de acercamiento/ evitación al medio adverso (brazos abiertos) en las crías RR, las cuales presentaron menor número de entradas, tiempo de permanencia y distancia recorrida en los brazos abiertos en comparación con el control. La restricción proteínica materna durante la etapa pre y postnatal disminuyó en la crías macho la tendencia innata a explorar nuevos ambientes, lo cual podría ser resultado de un aumento en los niveles de precaución y/o a una menor impulsividad (más ansiosos) para explorar espacios desconocidos. Estudios en animales de experimentación han demostrado que la malnutrición severa (consumo dieta baja en proteína al 6%) previo, durante el embarazo y la lactancia, incrementa la conducta de evitación de riesgo, dichos reportes avalan los resultados obtenidos en el presente trabajo de investigación (Almeida et al., 1991; Almeida et al., 1993; Almeida et al., 1996b; Belluscio et al., 2014; da Silva Hernandes et al., 2005; Jaiswal et al., 1996; Santucci et al., 1994).

En la prueba de campo abierto sólo las crías machos provenientes de madres restringidas de proteínas durante la vida pre y posnatal (RR) mostraron disminución en el número de entradas a la zona central (más ansiosos), lo cual confirma los resultados obtenidos en la prueba del Laberinto elevado en cruz. Este comportamiento se asoció al aumento en las concentraciones de corticosterona determinado en la descendencia una vez finalizadas las pruebas de ansiedad. La elevación de la corticosterona puede ser una respuesta fisiológica en las crías RR, que correlaciona con los resultados obtenidos en el laberinto elevado y el campo abierto. En la literatura existen grupos de investigación que han reportado resultados similares a los obtenidos en este estudio (Belluscio et al., 2014; Gallo, 1981; Levay et al., 2008; Reyes-Castro et al., 2012a; Trzctnska et al., 1999; Watkins et al., 2008). Por otro lado, al darle continuidad a este estudio pudimos demostrar que las alteraciones emocionales pueden ser trasmitidas por la vía

paterna multigeneracionalmente a los descendientes, encontrándose afectada la conducta de tipo ansiedad en las crías hembras F_2, sin cambios en los machos F_2 (Reyes-Castro et al., 2015). Dichos efectos son mediados por la hiperactividad del eje HHA (Bertram et al., 2008; Lesage et al., 2006) y mecanismos epigenéticos (metilación DNA y modificación de las histonas) que causan cambios en la expresión de genes en la línea germinal, los cuales pueden ser heredables (Franklin et al., 2010; Jablonka and Raz, 2009).

En la actualidad, se sabe que la tarea de reconocimiento de objetos novedoso (Ennaceur and Delacour, 1988) es un procedimiento en el que los roedores muestran su capacidad para discriminar un objeto novedoso de un objeto familiar, con base en su tendencia natural para explorar nuevas características en su entorno (Dere et al., 2007; Mumby, 2001; Mumby et al., 2007). En el presente trabajo se pudo apreciar que las crías RR presentaron un índice de discriminación y de reconocimiento disminuido con respecto al control, lo cual es indicativo de deterioro de la memoria. Estos resultados cotejan con los resultados previamente publicados por Valadares y colaboradores (Valadares et al., 2010). En la literatura existe poca evidencia de los efectos de la malnutrición sobre la memoria relacionada con eventos o acontecimientos (episódica); sin embargo, algunos experimentos que implican la prueba de RON han demostrado que tanto el estrés prenatal, la administración de glucocorticoides y la elevación de los glucocorticoides por exposición a patógenos, afecta la preferencia por el objeto novedoso durante la prueba de ensayo (Kawashima and Kusnecov, 2002; Marco et al., 2013; Salomon et al., 2011; Vargas-Lopez et al., 2015)**.** Dichos cambios han sido asociados con el retraso del aprendizaje y/o a la alteración en la evocación de la memoria (Baker and Kim, 2002; Howland and Cazakoff, 2010; Li et al., 2012b; Okuda et al., 2004). Sin embargo, todavía no está claro si estos efectos pueden atribuirse al deterioro de la memoria exclusivamente (Cazakoff et al., 2010), a cambios inducidos por el alteración de memoria emocional (Okuda et al., 2004; Rosellini and Widman, 1989; Urani et al., 2011), o a perturbaciones en procesos de búsqueda (Eagle et al., 2013).

La memoria espacial es la capacidad que se tiene para adquirir y retener asociaciones del ambiente, lo que permite desenvolverse en el espacio (Vicens et al., 2003). El laberinto acuático es el modelo más utilizado para el estudio de este tipo de memoria en roedores. En este trabajo pudimos observar que la malnutrición materna moderada (10% proteína) durante la gestación, lactancia o ambos periodos, provoca retraso en el aprendizaje y deterioro de la memoria de tipo espacial. Estudios realizados en roedores, han demostrado los efectos de la restricción proteínica (6%), y calórica (50%) sobre procesos de aprendizaje y memoria espacial; dichos reportes son muy similares a los que se obtuvieron en el presente estudio (de Souza et al., 2008; Fukuda et al., 2007; Gilbert et al., 2010; Tonkiss et al., 1997; Valadares et al., 2010; Wang and Xu, 2007; Zhang et al., 2010).

En la prueba del condicionamiento operante, pudimos observar que las crías provenientes de madres restringidas durante la gestación (RC) fueron afectadas de manera negativa al incrementarse el número de sesiones tanto en el condicionamiento de FR-1 como FR-5. Estos resultados sugieren que la ventana crítica para programar los componentes neurales implicados en la adquisición y retención del aprendizaje de tipo asociativo comienza en la etapa prenatal, lo anterior se demostró al observar que las crías macho RC requerían de mayor número de sesiones para cumplir el criterio de desempeño. Durante la tarea progresiva, se encontraron diferencias en las crías RR, CR y RC; ya que respondieron menos para la obtención de la recompensas con respecto a CC. Esto sugiere que las crías se encontraban menos motivadas para presionar la palanca y obtener la recompensa de sacarosa. Estos resultados ponen de manifiesto el efecto negativo de la restricción de proteínica durante el embarazo y/o lactancia en el aprendizaje asociativo y motivación de las crías macho de la rata.

Dichos hallazgos conductuales contribuyen al conocimiento actual de la programación del desarrollo de la cognición y la motivación postnatal. Los déficits cognitivos que reportamos aquí son consistentes con las alteraciones cognitivas encontradas en las crías de madres desnutridas (30% reducción del consumo ad

libitum) durante la gestación (Landon et al., 2007). Dicho estudio mostró que las crías provenientes de madres alimentadas con dieta restringida *in utero* presentaban menor sensibilidad al reforzamiento con respecto al control en una tarea de flexibilidad cognitiva utilizando un esquema concurrente de intervalos variables. Del mismo modo, crías ovejas adultas provenientes de madres prenatalmente desnutridas (reducción 50%) mostraron deterioro cognitivo determinado por medio de un retardo en la velocidad de aprendizaje durante la tarea del laberinto en T (Erhard et al., 2004). Similar a nuestros hallazgos, en la literatura existente información que revela que en los descendientes restringidos de proteína prenatalmente hay deterioro del rendimiento en tareas operantes tales como esquema de reforzamiento diferencial de baja velocidad (DRL, por sus siglas en inglés, differential reinforcement of low rates) (Finger et al., 1986; Tonkiss et al., 1990a)y el laberinto radial (Ranade et al., 2008). Un estudio en ratas demostró que las crías de madres malnutridas (reducción 30%) incrementaron la preferencia por subirse a la rueda para correr versus presionar la palanca para obtener la recompensa, disminuyendo la motivación por el trabajo (Miles et al., 2009). En un estudio realizado en primates no humanos, demostraron que la restricción nutricional durante el embarazo y lactancia disminuye en los descendientes el número de respuestas y recompensas en una tarea progresiva tal y como se demostró en nuestro estudio (Keenan et al., 2013).

Una posible explicación de esta perturbación conductual puede derivarse de los hallazgos de nuestro grupo que demuestran que la restricción de proteínas durante embarazo aumenta las concentraciones circulantes de andrógenos y glucocorticoides al día 19 de gestación en la madre (Zambrano et al., 2005b). Los esteroides androgénicos en el desarrollo fetal podrían tener implicaciones en la conducta. En un estudio realizado en humanos, se ha comprobado que la exposición prenatal excesiva a andrógenos tiene repercusiones en el desempeño de las tareas espaciales (Puts et al., 2008). De igual manera existe evidencia experimental y epidemiológica que demuestra que la exposición *in útero* a elevadas concentraciones de glucocorticoides programa negativamente el desarrollo cerebral, lo cual repercute a nivel cognitivo en la vida adulta de los

descendientes (Antonow-Schlorke et al., 2001; Antonow-Schlorke et al., 2003; French et al., 2004; Johnson et al., 1981; Karemaker et al., 2006; Karemaker et al., 2008; Matthews, 2001; Seckl, 2008; Szuran et al., 2000; Uno et al., 1994; Uno et al., 1990; Weinstock, 2008). Existe evidencia que demuestra que los GC actúan tanto como neuroprotectores o como neurodegenerativos dependiendo de la concentración de los mismos. Concentraciones normales aumentan la plasticidad sináptica y tienen efectos benéficos sobre el aprendizaje y la memoria, mientras que la exposición excesiva tiene efectos opuestos (Martinez Sanchis, 2006).

En este estudio, las concentraciones de corticosterona se encontraron elevadas a la edad de 110 días, en las crías RR, CR y RC; y la ACTH solamente en RR, indicando que el bajo consumo de proteína durante la gestación y la lactancia tiene efectos a largo plazo en la programación del eje Hipotálamo Hipófisis Adrenal como se ha publicado previamente por otros investigadores en modelos de restricción proteínica y calórica (Chisari et al., 2001; Kapoor et al., 2008; Lesage et al., 2006; Schaffer et al., 2009; Zambrano et al., 2005b).

En roedores, se ha demostrado que la desnutrición afecta el eje HHA del feto, induciendo pequeños cambios en la etapa de adulto joven que se exacerban en la madurez. Lo anteriormente mencionado, se asocia con frecuencia a la hiperactividad crónica del eje neuroendocrino (Leonhardt et al., 2002; Lesage et al., 2006; Sebaai et al., 2004; Sebaai et al., 2002; Vieau et al., 2007). Dichos estudios indican que la malnutrición fetal y/o neonatal genera cambios persistentes en la retroalimentación del eje (Chisari et al., 2001). El estrés es unos de los principales moduladores negativos de la neurogénesis en el hipocampo. La estimulación crónica que conlleva a la hipersecreción de GC, lo cual ha sido implicado en la patología de algunos trastornos sistémicos, neurodegenerativos y afectivos (Martinez Sanchis, 2006). Se ha reportado que la actividad del eje HHA puede ser modificado por situaciones de estrés y además existe dimorfismo sexual, en donde las hembras liberan más GC en comparación con los machos, lo cual ha sido asociado a cambios hormonales durante el ciclo estral (Quinn et al., 2014).

En el presente estudio, pudimos apreciar que las crías RR presentaron mayor concentración de corticosterona y ACTH basal con respecto al CC a los 220 días; este mismo comportamiento se observó a los 20 y 40 minutos posteriores a la prueba de restricción de movimiento (inmovilización). Estos resultados correlacionan con estudios publicados en donde se observó incremento en las concentraciones de corticosterona y ACTH después de un periodo de deprivación de comida o de agua (Leonhardt et al., 2002; Sebaai et al., 2004; Sebaai et al., 2002). Evidencia experimental, sugiere que los glucocorticoides participan en la modificación de genes implicados en la conducta, tal es el caso del factor neurotrópico derivado del cerebro (BDNF) el cual ha sido relacionado a desórdenes de estrés. Por ejemplo, se demostró que la inmovilización leve provoca cambios en la expresión de este gen a nivel de hipocampo, lo cual se asocia a cambios epigenéticos, como la acetilación de las histonas (Fuchikami et al., 2009). Efectos similares fueron demostrados no sólo debido a la exposición a estrés moderado, sino también por la administración de corticosterona en ratas (Hunter et al., 2009). Por otro lado, se ha reportado, que la restricción calórica materna (50%) durante el periodo perinatal, produce disminución de la expresión del gen del receptor de glucocorticoides (GR) en hipocampo con respecto al grupo control (Vieau et al., 2007). Dichas alteraciones se han asociado con la hiperactividad del eje HHA y efectos adversos a nivel cognitivo.

Las perturbaciones a nivel afectivo y cognitivo (ansiedad, exploración, aprendizaje, memoria y motivación) observadas en la descendencia pueden asociarse potencialmente a la deficiencia de proteína (aminoácidos esenciales) o ácidos grasos/lípidos disponibles durante las ventanas críticas del desarrollo del cerebro. Actualmente, se sabe que el inadecuado consumo de proteína lleva a disfunciones persistentes del sistema límbico y corteza en los descendientes al tiempo de la prueba lo cual puede afectar la cognición y motivación (Bedi, 2003; Lister et al., 2005; Lister et al., 2006; Morgane et al., 1993). Por otro lado, hemos demostrado que la restricción proteínica materna durante el embarazo afecta negativamente el desarrollo adecuado del cerebro debido a cambios en el metabolismo lipídico materno (Torres et al., 2010). Los tejidos fetales sólo

producen pequeñas cantidades de LC-PUFAS debido a la falta relativa de las enzimas desaturasas y elongasas; es por ello, que el cerebro depende de la fuente materna durante etapas fetales (Hamosh and Salem, 1998). La restricción proteínica gestacional disminuye en el hígado y glándula materna, la expresión génica de desaturasas y elongasas encargadas de la formación de LC-PUFAS: AA y DHA (Bautista et al., 2013; Torres et al., 2010). Encontrándose que los fetos de madres restringidas pesaban menos, su cerebro tenía disminuida la cantidad de grasa total y específicamente el contenido de DHA, lo cual es un posible mecanismo implicado en las alteraciones cognitivas. Estudios realizados en animales experimentales indican que la restricción nutricional durante el desarrollo fetal conduce a cambios permanentes en la neuroquímica del cerebro (Mokler et al., 2003; Mokler et al., 2007; Rotta et al., 2008), y los cambios en la plasticidad sináptica (Austin et al., 1986; Hernandez et al., 2008; Holtmaat et al., 2005; Soto-Moyano et al., 2005; Wang et al., 2006).

En este proyecto se estudió al hipocampo como estructura implicada en las conductas evaluadas y además por ser una estructura que es altamente preservada durante la evolución. De las diversas subregiones del hipocampo, el área CA3 juega un papel importante en la integración de la información multimodal recibida desde de áreas como la corteza entorrinal (Vinogradova, 2001), se considera un controlador principal de la memoria asociativa y al compararlo con la región CA1 (Andrade et al., 1991; Beltran-Campos et al., 2011; Cintra et al., 1997; Garin-Aguilar et al., 2012; Zhang et al., 2013) poco se sabe acerca de los efectos de la malnutrición materna y la respuesta presináptica (fibras musgosas)y post sináptica (espinas) en los *strata lucidum* y *oriens,* respectivamente. A esta región llegan las proyecciones de las fibras musgosas provenientes de las células granulares del giro dentado y terminan en el *stratum lucidum*, en la dendrita apical, en una región dendrítica que forma una estructura post sináptica denominada excrecencias espinosas, sobre todo al primer tercio de las neuronas piramidales (Gonzales et al., 2001). Diversos estudios han demostrado correlaciones entre la extensión de las fibras musgosas del hipocampo y la capacidad de aprendizaje de tipo espacial (Bernasconi-Guastalla et al., 1994; Crusio et al., 1993). Son

altamente plásticas y estructuralmente sensibles a experiencias como el aprendizaje (Ramirez-Amaya et al., 2001; Ramirez-Amaya et al., 1999). Por otro lado, se ha demostrado que la inactivación o lesión en la conectividad que establecen estas fibras musgosas entre el giro dentado y CA3 pueden afectar la adquisición de las tareas espaciales (Lassalle et al., 2000; Steffenach et al., 2002). De igual manera, lesiones en la amígdala (estructura límbica implicada en las conductas de estrés y modulación de la memoria) genera alteraciones en la potenciación a largo plazo y memoria de tipo espacial (Kim et al., 2005).

En el presente trabajo experimental, con respecto a los elementos pre sinápticos (Fibras musgosas), se observó que su área total en el *stratum lucidum* fue menor en las crías RR (-21.9%), CR y RC (-11%) con respecto al grupo control. Se ha reportado disminución en el número de conexiones sinápticas entre los axones de las células granulares y las dendritas de células piramidales de la región CA3 en animales adultos después del consumo de largos períodos de dieta baja en proteínas (Andrade et al., 1991). De igual manera, en ratas, se encontraron disminuidos tanto el sistema de fibras musgosas como el volumen total en crías provenientes de madres restringidas de proteína (6 %) durante la gestación (Cintra et al., 1997; Granados-Rojas et al., 2002). Dichos estudios corroboran los hallazgos obtenidos por nuestro grupo de investigación. Estos hallazgos podrían ser en relación con las sinapsis FM-CA3 y las recurrencias colaterales las cuales son también sensibles a los efectos de Zn^{+2} (Vogt and Regehr, 2001) y está regulado por la inducción de la plasticidad sináptica, y sinapsis excitatorias en las espinas dendríticas (Mellone et al., 2015). La disminución del área de las fibras musgosas provocado por el bajo consumo de proteína materno, parece ser diferente dependiendo de la ventana de exposición (ya sea durante gestación y/o lactancia). Ambos paradigmas dietarios resaltan la vulnerabilidad de los componentes clave del circuito trisináptico del hipocampo, esencial para la memoria y el aprendizaje (Lister et al., 2005; Lister et al., 2006; Morgane et al., 1993; Morgane et al., 2002). Además, las vesículas sinápticas contenidas en el elemento presináptico de los axones de las fibras musgosas de las células granulares del giro dentado contienen glutamato (GLUT), neuropéptidos, zinc

(Zn^{+2}), ATP / adenosina, y el GABA (Blaabjerg and Zimmer, 2007). El Zn^{+2}modula la expresión de la plasticidad sináptica (Gundelfinger and Fejtova, 2012). La asociación selectiva de zinc con las vesículas sinápticas que contienen glutamato sugiere que podría ser un co-transmisor en algunas sinapsis excitatorias (Paoletti et al., 2009). El Zn^{+2} y GLUT se co-localizan por excitación de las fibras musgosas del hipocampo (Takeda et al., 2009). En la literatura, existe un estudio que demuestra, que la deficiencia de zinc durante el embarazo y la lactancia genera alteraciones a nivel de aprendizaje y memoria de tipo espacial, lo cual se debe a cambios en la ultraestructura de las neuronas hipocampales. Se han descrito los efectos del BDNF, Zn^{+2}, 17 β estradiol y los andrógenos en la sinapsis hipocampal y su plasticidad. Las células granulares sintetizan BDNF en cerebro de ratas normales (Scharfman and MacLusky, 2014) y la transportan a sus axones (las FM) donde éste es empacado en vesículas centrales densas presinápticas (Dissen et al., 2009). Existe evidencia que documenta que el BDNF y los andrógenos modulan por la vía de las FM la plasticidad sináptica en el hipocampo (Scharfman and MacLusky, 2014). Estudios realizados en ratas de experimentación, han demostrado que la restricción calórica (50%) y proteínica (6%) materna produce cambios en el contenido de BDNF en hipocampo (Coupe et al., 2009; Wang and Xu, 2007). Dichos hallazgos repercuten en el crecimiento y función neural, debido a que pueden verse implicado en los cambios anatómicos encontrados en el área de las fibras musgosas, lo cual repercute a nivel conductual.

Por otro lado, las espinas dendríticas representan los compartimentos post sinápticos para la mayoría de las sinapsis excitatorias (glutamatérgicas). Un reporte indica que el aprendizaje y la memoria de respuestas condicionadas están acompañados por la génesis de las espinas dendríticas en el hipocampo y en consecuencia la poda dendrítica está relacionada con el procedimiento de extinción (Garin-Aguilar et al., 2012). Si de manera natural, el hipocampo bajo condiciones de estrés, puede alterar o modular sus funciones y su citoarquitectura. La desnutrición prenatal puede ser considerada como un factor predisponente al estrés; tal condición puede ser asociada dado a los cambios anatómicos [disminución área de FM y reducción del número total de espinas (poda dendrítica)

de las dendritas basales] observados en el presente estudio; en el grupo RR con una disminución del 14 %, seguido de un 12% en CR y 8% en RC. Las espinas dendríticas son especializadas para recibir entradas sinápticas, y un cambio en la morfología se correlaciona con la fuerza y la madurez de cada sinapsis. Tanto la estructura como el funcionamiento sirven como componentes bioquímicos y eléctricos dentro de las neuronas (Grienberger et al., 2015); especialmente el calcio, el cual proporciona el aislamiento bioquímico necesario para la plasticidad sináptica específica. Además, la compartimentación esta medida por la resistencia de lo alto del cuello de la espina. Debido a caracterizaciones anatómicas, se ha propuesto que la forma de una espina varía con su maduración (Holtmaat and Svoboda, 2009). Por lo tanto, las espinas cortas, que son pequeñas protuberancias dendríticas sin cuello y cabeza bien definida, se consideran inmaduras (Bourne and Harris, 2007) y más dinámicas. Las espinas largas, tienen densidades postsinápticas más pequeñas que contienen receptores de NMDA y pocos receptores AMPA. Esto permite que cambien su eficiencia mediante la modificación del número de receptores de AMPA (Matsuzaki et al., 2001). Esta etapa morfológica transitoria mantiene la flexibilidad estructural, lo que conduce al alargamiento o contracción. Por lo tanto, las espinas largas son consideradas espinas de aprendizaje (Bourne and Harris, 2007). Las espinas en hongo, presentes en las PSD largas, pueden regular el Ca^{2+} a nivel local y tienen más probabilidades de contener retículo endoplásmico liso y polirribosomas para la síntesis de proteínas (Spacek and Harris, 1997) con las subunidades del receptor AMPA GluR1 después de condicionamiento al miedo, haciendo hincapié en la importancia de las espinas en hongo para el almacenamiento de información (Bourne and Harris, 2007), y es el tipo de espina más estable (Holtmaat and Svoboda, 2009).

En este estudio, al analizar los tipos de espinas dendríticas en la región CA3 del hipocampo entre los diferentes grupos experimentales, encontramos resultados interesantes, en donde las espinas cortas (inmaduras) se incrementaron significativamente en los tres grupos experimentales; lo cual puede ser indicativo de cambios plásticos adaptativos y compensatorios. Tanto las

espinas largas (asociadas con aprendizaje) como en hongo (asociadas a la memoria) se encontraron reducidas en RR y RC; lo cual es congruente con los resultados obtenidos en las pruebas conductuales evaluadas (aprendizaje y memoria) en los descendientes de madres malnutridas. Por otro lado, se sabe que la densidad de las espinas dendríticas en las neuronas piramidales CA1 del hipocampo se incrementa después del entrenamiento en el Laberinto acuático de Morris (Moser et al., 1994; Ramirez-Amaya et al., 2001; Ramirez-Amaya et al., 1999; Rusakov et al., 1997). La actividad gonadal también se ha asociado con la regulación de la densidad de las espinas dendríticas y los niveles anormalmente bajos de estrógenos y andrógenos circulantes se asocia con déficits en tareas hipocampo-dependiente (MacLusky et al., 2006; Scharfman and MacLusky, 2014).

La testosterona ejerce muchos de sus efectos a través del receptor de andrógenos (AR). Dicho receptor está distribuido en el tejido nervioso y en particular en las neuronas piramidales del hipocampo, que desempeñan un papel fundamental en las tareas de memoria espacial. Estudios en animales de experimentación han demostrado disminución en la densidad de las espinas (región CA1 del hipocampo) en ratas adulto joven con gonadectomía. Dichas alteraciones se revierten al suplementar con dihidrotestosterona (DHT) y testosterona (Leranth et al., 2004; Leranth et al., 2003). Beltrán y colaboradores, previamente han demostrado poda dendrítica en la región CA1 del hipocampo, debido a la reducción de estrógenos y se observó recuperación en un solo tipo de espina (en forma de hongo) con una dosis baja de 17 β estradiol (Beltran-Campos et al., 2011). En un estudio realizado en ratones transgénicos Thy1 GFP (por sus siglas en inglés proteína verde fluorescente) donde analizaron la morfología de las espinas dendríticas en el área de CA1 del hipocampo; demostraron que la gonadectomía disminuyó la densidad de las espinas en hongo (alrededor de un 50%) con respecto al control, pero incrementó las espinas cortas y largas en el *stratum radiatum* proximal, este mismo comportamiento se observó en la región distal. No encontrándose cambios en el *stratum lacunoso moleculare*. Dichos cambios fueron revertidos con la administración de testosterona. Los cambios en la maduración de las espinas dendríticas inducidos por la gonadectomía están en

función de la expresión de BDNF y PSD-95, el cual es una molécula clave en la regulación de la estructura de las espinas dendríticas (Li et al., 2012a). Estos resultados revelan el papel de la testosterona en la respuesta diferencial de maduración en las subcapas de las espinas dendríticas en el área CA1 través de las acciones del BDNF y PSD-95. En un estudio realizado en roedores, se demostraron cambios en la densidad y morfología de las espinas dendríticas en las crías provenientes de madres expuestas a testosterona prenatalmente. En nuestro grupo de estudio, se ha demostrado que la disminución del consumo de proteína durante el embarazo provoca en las crías macho incremento del daño oxidativo en testículo y espermatozoide, y disminución las concentraciones en suero de testosterona (Rodriguez-Gonzalez et al., 2014), DHT y androstendiona [datos no publicados]; así como disminución de la expresión AR en el testículo (Rodriguez-Gonzalez et al., 2012), lo cual provoca un envejecimiento prematuro de la función reproductiva. Dichos cambios en las concentraciones de las hormonas esteroides puede ser otro factor modulador de los cambios a nivel pre y post sinápticos observados en el presente estudio.

10.0 CONCLUSIÓN

Estos hallazgos demuestran efectos negativos de la programación debido al consumo de una dieta baja en proteína en la madre sobre la ansiedad, motivación, aprendizaje y memoria en la descendencia, teniendo mayor vulnerabilidad el período prenatal.

Con base a lo anterior podemos concluir que el consumo materno de una dieta baja en proteína durante la gestación y lactancia incrementa en la cría macho de la rata la ansiedad, retrasa el aprendizaje y genera deterioro en la retención de la memoria, debido a cambios en el sustrato anatómico del hipocampo en sitios pre y post sinápticos(sistema de fibras musgosas y espinas dendríticas), así como a la excesiva liberación de ACTH y corticosterona, lo cual es indicativo de la disfunción de la actividad del eje hipotálamo hipófisis adrenal. Dichos cambios se proponen como mecanismos potenciales para explicar los déficits observados en el presente trabajo de investigación.

11.0 REFERENCIAS

Adams B, Lee M, Fahnestock M, Racine RJ. 1997. Long-term potentiation trains induce mossy fiber sprouting. Brain Res 775(1-2):193-7.

AE. K. 1993. Locomotor activity and exploration. En: Sahgal A, editor. Behvioural neuroscience: a practical approach. London: Oxford University Press.

Aguilera G, Subburaju S, Young S, Chen J. 2008. The parvocellular vasopressinergic system and responsiveness of the hypothalamic pituitary adrenal axis during chronic stress. Prog Brain Res 170:29-39.

Akcakus M, Koklu E, Kurtoglu S, Kula M, Koklu SS. 2006. The relationship among intrauterine growth, insulinlike growth factor I (IGF-I), IGF-binding protein-3, and bone mineral status in newborn infants. Am J Perinatol 23(8):473-80.

Alamy M, Bengelloun WA. 2012. Malnutrition and brain development: an analysis of the effects of inadequate diet during different stages of life in rat. Neurosci Biobehav Rev 36(6):1463-80.

Alamy M, Errami M, Taghzouti K, Saddiki-Traki F, Bengelloun WA. 2005. Effects of postweaning undernutrition on exploratory behavior, memory and sensory reactivity in rats: implication of the dopaminergic system. Physiol Behav 86(1-2):195-202.

Almeida SS, de Oliveira LM, Graeff FG. 1991. Early life protein malnutrition changes exploration of the elevated plus-maze and reactivity to anxiolytics. Psychopharmacology (Berl) 103(4):513-8.

Almeida SS, Garcia RA, de Oliveira LM. 1993. Effects of early protein malnutrition and repeated testing upon locomotor and exploratory behaviors in the elevated plus-maze. Physiol Behav 54(4):749-52.

Almeida SS, Tonkiss J, Galler JR. 1996a. Malnutrition and reactivity to drugs acting in the central nervous system. Neurosci Biobehav Rev 20(3):389-402.

Almeida SS, Tonkiss J, Galler JR. 1996b. Prenatal protein malnutrition affects avoidance but not escape behavior in the elevated T-maze test. Physiol Behav 60(1):191-5.

Almeida SS, Tonkiss J, Galler JR. 1996c. Prenatal protein malnutrition affects exploratory behavior of female rats in the elevated plus-maze test. Physiol Behav 60(2):675-80.

Altman J, Bayer SA. 1990. Prolonged sojourn of developing pyramidal cells in the intermediate zone of the hippocampus and their settling in the stratum pyramidale. J Comp Neurol 301(3):343-64.

Amano T, Duvarci S, Popa D, Pare D. 2011. The fear circuit revisited: contributions of the basal amygdala nuclei to conditioned fear. J Neurosci 31(43):15481-9.

Amaral DG. 1978. A Golgi study of cell types in the hilar region of the hippocampus in the rat. J Comp Neurol 182(4 Pt 2):851-914.

Amaral DG, Dent JA. 1981. Development of the mossy fibers of the dentate gyrus: I. A light and electron microscopic study of the mossy fibers and their expansions. J Comp Neurol 195(1):51-86.

Amaral DG, Scharfman HE, Lavenex P. 2007. The dentate gyrus: fundamental neuroanatomical organization (dentate gyrus for dummies). Prog Brain Res 163:3-22.

Amaral DG, Witter MP. 1995. Hippocampal formation. en G. Paxinos (Ed.), The rat nervous system. (pp. 443-493). San Diego: Academic Press.

Amaral DG, Witter MP. 2004. "Hippocampal formation". in The rat nervous system.G. Paxinos. Amsterdam ; Boston, Elsevier Academic Press: xvii, 1309 p.

Andrade JP, Cadete-Leite A, Madeira MD, Paula-Barbosa MM. 1991. Long-term low-protein diet reduces the number of hippocampal mossy fiber synapses. Exp Neurol 112(1):119-24.

Antonow-Schlorke I, Kuhn B, Muller T, Schubert H, Sliwka U, Nathanielsz PW, Schwab M. 2001. Antenatal betamethasone treatment reduces synaptophysin immunoreactivity in presynaptic terminals in the fetal sheep brain. Neurosci Lett 297(3):147-50.

Antonow-Schlorke I, Schwab M, Li C, Nathanielsz PW. 2003. Glucocorticoid exposure at the dose used clinically alters cytoskeletal proteins and presynaptic terminals in the fetal baboon brain. J Physiol 547(Pt 1):117-23.

Ashton N. 2000. Perinatal development and adult blood pressure. Braz J Med Biol Res 33(7):731-40.

Austin KB, Bronzino J, Morgane PJ. 1986. Prenatal protein malnutrition affects synaptic potentiation in the dentate gyrus of rats in adulthood. Brain Res 394(2):267-73.

Baddeley AD, Hitch G. 1993. The recency effect: implicit learning with explicit retrieval? Mem Cognit 21(2):146-55.

Baker KB, Kim JJ. 2002. Effects of stress and hippocampal NMDA receptor antagonism on recognition memory in rats. Learn Mem 9(2):58-65.

Barker DJ. 2004a. The developmental origins of adult disease. J Am Coll Nutr 23(6 Suppl):588S-595S.

Barker DJ. 2004b. Developmental origins of adult health and disease. J Epidemiol Community Health 58(2):114-5.

Barraclough CA. 1961. Production of anovulatory, sterile rats by single injections of testosterone propionate. Endocrinology 68:62-7.

Barraclough CA, Gorski RA. 1961. Evidence that the hypothalamus is responsible for androgen-induced sterility in the female rat. Endocrinology 68:68-79.

Bautista CJ, Rodriguez-Gonzalez GL, Torres N, Hernandez-Pando R, Ramirez V, Rodriguez-Cruz M, Nathanielsz PW, Zambrano E. 2013. Protein restriction in the rat negatively impacts long-chain polyunsaturated fatty acid composition and mammary gland development at the end of gestation. Arch Med Res 44(6):429-36.

Bedi KS. 2003. Nutritional effects on neuron numbers. Nutr Neurosci 6(3):141-52.

Beltran-Campos V, Prado-Alcala RA, Leon-Jacinto U, Aguilar-Vazquez A, Quirarte GL, Ramirez-Amaya V, Diaz-Cintra S. 2011. Increase of mushroom spine density in CA1 apical dendrites produced by water maze training is prevented by ovariectomy. Brain Res 1369:119-30.

Belluscio LM, Berardino BG, Ferroni NM, Ceruti JM, Canepa ET. 2014. Early protein malnutrition negatively impacts physical growth and neurological reflexes and evokes anxiety and depressive-like behaviors. Physiol Behav 129:237-54.

Ben-Ari Y, Represa A. 1990. Brief seizure episodes induce long-term potentiation and mossy fibre sprouting in the hippocampus. Trends Neurosci 13(8):312-8.

Bengelloun WA. 1990 Kwashiorkor: behavioural indices of neurological sequelae. In: (Mal) Nutrition and the Infant Brain. Wiley-Liss, Inc, pp. 65–81.

Bernasconi-Guastalla S, Wolfer DP, Lipp HP. 1994. Hippocampal mossy fibers and swimming navigation in mice: correlations with size and left-right asymmetries. Hippocampus 4(1):53-63.

Bertram C, Khan O, Ohri S, Phillips DI, Matthews SG, Hanson MA. 2008. Transgenerational effects of prenatal nutrient restriction on cardiovascular and hypothalamic-pituitary-adrenal function. J Physiol 586(8):2217-29.

Bischofberger J, Engel D, Frotscher M, Jonas P. 2006. Timing and efficacy of transmitter release at mossy fiber synapses in the hippocampal network. Pflugers Arch 453(3):361-72.

Blaabjerg M, Zimmer J. 2007. The dentate mossy fibers: structural organization, development and plasticity. Prog Brain Res 163:85-107.

Blair MM, Glynn LM, Sandman CA, Davis EP. 2011. Prenatal maternal anxiety and early childhood temperament. Stress 14(6):644-51.

Bliss TV, Collingridge GL. 1993. A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361(6407):31-9.

Bloom FE, Battenberg EL, Rivier J, Vale W. 1982. Corticotropin releasing factor (CRF): immunoreactive neurones and fibers in rat hypothalamus. Regul Pept 4(1):43-8.

Boehme S, Ritter V, Tefikow S, Stangier U, Strauss B, Miltner WH, Straube T. 2014. Brain activation during anticipatory anxiety in social anxiety disorder. Soc Cogn Affect Neurosci 9(9):1413-8.

Bohus B, De Kloet E, Veldhuis H. 1983. Adrenal steroids and behavioral adaptation: Relationships to brain corticoid receptors. In. Ganten, R.; Pfaff, D., eds. Progress in Neuroendocrinology (2). 1: Berlin: Springer-Verlag .

Bonatto F, Polydoro M, Andrades ME, da Frota Junior ML, Dal-Pizzol F, Rotta LN, Souza DO, Perry ML, Moreira JC. 2005. Effect of protein malnutrition on redox state of the hippocampus of rat. Brain Res 1042(1):17-22.

Bortolotto ZA, Lauri S, Isaac JT, Collingridge GL. 2003. Kainate receptors and the induction of mossy fibre long-term potentiation. Philos Trans R Soc Lond B Biol Sci 358(1432):657-66.

Bourne J, Harris KM. 2007. Do thin spines learn to be mushroom spines that remember? Curr Opin Neurobiol 17(3):381-6.

Bourne JN, Harris KM. 2008. Balancing structure and function at hippocampal dendritic spines. Annu Rev Neurosci 31:47-67.

Bouton ME, Mineka S, Barlow DH. 2001. A modern learning theory perspective on the etiology of panic disorder. Psychol Rev 108(1):4-32.

Brunton PJ. 2015. Programming the brain and behaviour by early-life stress: a focus on neuroactive steroids. J Neuroendocrinol 27(6):468-80.

Brunton PJ, Russell JA. 2010. Prenatal social stress in the rat programmes neuroendocrine and behavioural responses to stress in the adult offspring: sex-specific effects. J Neuroendocrinol 22(4):258-71.

Bunsey M, Eichenbaum H. 1996. Conservation of hippocampal memory function in rats and humans. Nature 379(6562):255-7.

Butler RK, Oliver EM, Sharko AC, Parilla-Carrero J, Kaigler KF, Fadel JR, Wilson MA. 2016. Activation of corticotropin releasing factor-containing neurons in the rat central amygdala and bed nucleus of the stria terminalis following exposure to two different anxiogenic stressors. Behav Brain Res 304:92-101.

Butler RK, White LC, Frederick-Duus D, Kaigler KF, Fadel JR, Wilson MA. 2012. Comparison of the activation of somatostatin- and neuropeptide Y-containing neuronal populations of the rat amygdala following two different anxiogenic stressors. Exp Neurol 238(1):52-63.

Cannizzaro C, Plescia F, Martire M, Gagliano M, Cannizzaro G, Mantia G, Cannizzaro E. 2006. Single, intense prenatal stress decreases emotionality and enhances learning performance in the adolescent rat offspring: interaction with a brief, daily maternal separation. Behav Brain Res 169(1):128-36.

Cannon WB. 1929. Bodily changes in pain, hunger, fear and rage. Appleton D (ed.) Nueva York and London.

Cannon WB. 1987. The James-Lange theory of emotions: a critical examination and an alternative theory. By Walter B. Cannon, 1927. Am J Psychol 100(3-4):567-86.

Capobianco E, Fornes D, Linenberg I, Powell TL, Jansson T, Jawerbaum A. 2015. A novel rat model of gestational diabetes induced by intrauterine programming is associated with alterations in placental signaling and fetal overgrowth. Mol Cell Endocrinol.

Carasatorre M, Ramirez-Amaya V, Diaz Cintra S. 2013. Structural synaptic plasticity in the hippocampus induced by spatial experience and its implications in information processing. Neurologia.

Cárdenas J, Navarro JF. 2002. Modelos animales de ansiedad incondicionada (I). Psiquiatría Biológica 09(01):18-32.

Cazakoff BN, Johnson KJ, Howland JG. 2010. Converging effects of acute stress on spatial and recognition memory in rodents: a review of recent behavioural and pharmacological findings. Prog Neuropsychopharmacol Biol Psychiatry 34(5):733-41.

Cintra L, Granados L, Aguilar A, Kemper T, DeBassio W, Galler J, Morgane P, Duran P, Diaz-Cintra S. 1997. Effects of prenatal protein malnutrition on mossy fibers of the hippocampal formation in rats of four age groups. Hippocampus 7(2):184-91.

Cooke SF, Bliss TV. 2006. Plasticity in the human central nervous system. Brain 129(Pt 7):1659-73.

Coupe B, Dutriez-Casteloot I, Breton C, Lefevre F, Mairesse J, Dickes-Coopman A, Silhol M, Tapia-Arancibia L, Lesage J, Vieau D. 2009. Perinatal undernutrition modifies cell proliferation and brain-derived neurotrophic factor levels during critical time-windows for hypothalamic and hippocampal development in the male rat. J Neuroendocrinol 21(1):40-8.

Crusio WE, Schwegler H, Brust I. 1993. Covariations between hippocampal mossy fibres and working and reference memory in spatial and non-spatial radial maze tasks in mice. Eur J Neurosci 5(10):1413-20.

Charmandari E, Tsigos C, Chrousos G. 2005. Endocrinology of the stress response. Annu Rev Physiol 67:259-84.

Chau LS, Galvez R. 2012. Amygdala's involvement in facilitating associative learning-induced plasticity: a promiscuous role for the amygdala in memory acquisition. Front Integr Neurosci 6:92.

Chisari AN, Giovambattista A, Perello M, Spinedi E. 2001. Impact of maternal undernutrition on hypothalamo-pituitary-adrenal axis and adipocyte functions in male rat offspring. Endocrine 14(3):375-82.

Chrousos GP, Kino T. 2007. Glucocorticoid action networks and complex psychiatric and/or somatic disorders. Stress 10(2):213-9.

da Silva Hernandes A, Francolin-Silva AL, Valadares CT, Fukuda MT, Almeida SS. 2005. Effects of different malnutrition techniques on the behavior of rats tested in the elevated T-maze. Behav Brain Res 162(2):240-5.

Dallar Y, Dilli D, Bostanci I, Ogus E, Dogankoc S, Tug E. 2007. Insulin sensitivity obtained from the oral glucose tolerance test and its relationship with birthweight. Ann Saudi Med 27(1):13-7.

Danscher G. 1981. Histochemical demonstration of heavy metals. A revised version of the sulphide silver method suitable for both light and electronmicroscopy. Histochemistry 71(1):1-16.

Davies AA, Smith GD, Ben-Shlomo Y, Litchfield P. 2004. Low birth weight is associated with higher adult total cholesterol concentration in men: findings from an occupational cohort of 25,843 employees. Circulation 110(10):1258-62.

De Kloet ER, Vreugdenhil E, Oitzl MS, Joels M. 1998. Brain corticosteroid receptor balance in health and disease. Endocr Rev 19(3):269-301.

de Souza AS, Fernandes FS, do Carmo M. 2011. Effects of maternal malnutrition and postnatal nutritional rehabilitation on brain fatty acids, learning, and memory. Nutr Rev 69(3):132-44.

de Souza AS, Pacheco Lda C, Castro Pda S, Hokoc JN, Rocha MS, do Carmo M. 2008. Brain fatty acid profiles and spatial learning in malnourished rats: effects of nutritional intervention. Nutr Neurosci 11(3):119-27.

Debassio WA, Kemper TL, Galler JR, Tonkiss J. 1994. Prenatal malnutrition effect on pyramidal and granule cell generation in the hippocampal formation. Brain Res Bull 35(1):57-61.

Debassio WA, Kemper TL, Tonkiss J, Galler JR. 1996. Effect of prenatal protein deprivation on postnatal granule cell generation in the hippocampal dentate gyrus. Brain Res Bull 41(6):379-83.

Dere E, Huston JP, De Souza Silva MA. 2007. The pharmacology, neuroanatomy and neurogenetics of one-trial object recognition in rodents. Neurosci Biobehav Rev 31(5):673-704.

Derrick BE, Martinez JL, Jr. 1994. Opioid receptor activation is one factor underlying the frequency dependence of mossy fiber LTP induction. J Neurosci 14(7):4359-67.

Derrick BE, Rodriguez SB, Lieberman DN, Martinez JL, Jr. 1992. Mu opioid receptors are associated with the induction of hippocampal mossy fiber long-term potentiation. J Pharmacol Exp Ther 263(2):725-33.

Dhopeshwarkar GA. 1983. Nutrition and Brain Development. Plenum Press, New York.

Diaz-Cintra S, Cintra L, Galvan A, Aguilar A, Kemper T, Morgane PJ. 1991. Effects of prenatal protein deprivation on postnatal development of granule cells in the fascia dentata. J Comp Neurol 310(3):356-64.

Diaz-Cintra S, Garcia-Ruiz M, Corkidi G, Cintra L. 1994. Effects of prenatal malnutrition and postnatal nutritional rehabilitation on CA3 hippocampal pyramidal cells in rats of four ages. Brain Res 662(1-2):117-26.

Dissen GA, Garcia-Rudaz C, Ojeda SR. 2009. Role of neurotrophic factors in early ovarian development. Semin Reprod Med 27(1):24-31.

Dobbing J. 1968. Effects of experimental undernutrition on development of the nervous system. In: Scrimshaw, N.S., Gordon, J.E. (Eds.), Malnutrition, Learning and Behaviour. M.I.T. Press, Boston, pp. 181–202

Dobbing J. 1987. Early Nutrition and Later Achievement. Academic Press, London.

Dobrossy MD, Dunnett SB. 2001. The influence of environment and experience on neural grafts. Nat Rev Neurosci 2(12):871-9.

Dolorfo CL, Amaral DG. 1998. Entorhinal cortex of the rat: topographic organization of the cells of origin of the perforant path projection to the dentate gyrus. J Comp Neurol 398(1):25-48.

Domjan M, Lyons R, North NC, Bruell J. 1986. Sexual Pavlovian conditioned approach behavior in male Japanese quail (Coturnix coturnix japonica). J Comp Psychol 100(4):413-21.

Dudai Y. 1989. The neurobiology of memory: concepts, findings, trends. New York: Oxford University Press.

Dudai Y, Eisenberg M. 2004. Rites of passage of the engram: reconsolidation and the lingering consolidation hypothesis. Neuron 44(1):93-100.

Eagle AL, Fitzpatrick CJ, Perrine SA. 2013. Single prolonged stress impairs social and object novelty recognition in rats. Behav Brain Res 256:591-7.

Ennaceur A, Delacour J. 1988. A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. Behav Brain Res 31(1):47-59.

Ennaceur A, Neave N, Aggleton JP. 1997. Spontaneous object recognition and object location memory in rats: the effects of lesions in the cingulate cortices, the medial prefrontal cortex, the cingulum bundle and the fornix. Exp Brain Res 113(3):509-19.

Erhard HW, Boissy A, Rae MT, Rhind SM. 2004. Effects of prenatal undernutrition on emotional reactivity and cognitive flexibility in adult sheep. Behav Brain Res 151(1-2):25-35.

Escobar ML, Barea-Rodriguez EJ, Derrick BE, Reyes JA, Martinez JL, Jr. 1997. Opioid receptor modulation of mossy fiber synaptogenesis: independence from long-term potentiation. Brain Res 751(2):330-5.

Fan JM, Chen XQ, Jin H, Du JZ. 2009. Gestational hypoxia alone or combined with restraint sensitizes the hypothalamic-pituitary-adrenal axis and induces anxiety-like behavior in adult male rat offspring. Neuroscience 159(4):1363-73.

Feoli AM, Leite MC, Tramontina AC, Tramontina F, Posser T, Rodrigues L, Swarowsky A, Quincozes-Santos A, Leal RB, Gottfried C and others. 2008. Developmental changes in content of glial marker proteins in rats exposed to protein malnutrition. Brain Res 1187:33-41.

Feoli AM, Siqueira IR, Almeida L, Tramontina AC, Vanzella C, Sbaraini S, Schweigert ID, Netto CA, Perry ML, Goncalves CA. 2006. Effects of protein malnutrition on oxidative status in rat brain. Nutrition 22(2):160-5.

Fernandez De Molina A, Hunsperger RW. 1959. Central representation of affective reactions in forebrain and brain stem: electrical stimulation of amygdala, stria terminalis, and adjacent structures. J Physiol 145(2):251-65.

File SE. 1992. Usefulness of animal models with newer anxiolytics. Clin Neuropharmacol 15 Suppl 1 Pt A:525A-526A.

Finger S, Almli CR, Green L, Wolf C, Morgane PJ. 1986. Severe early malnutrition and DRL performance in the rat. Physiol Behav 38(5):731-4.

Franck JE, Pokorny J, Kunkel DD, Schwartzkroin PA. 1995. Physiologic and morphologic characteristics of granule cell circuitry in human epileptic hippocampus. Epilepsia 36(6):543-58.

Franklin TB, Russig H, Weiss IC, Graff J, Linder N, Michalon A, Vizi S, Mansuy IM. 2010. Epigenetic transmission of the impact of early stress across generations. Biol Psychiatry 68(5):408-15.

French NP, Hagan R, Evans SF, Mullan A, Newnham JP. 2004. Repeated antenatal corticosteroids: effects on cerebral palsy and childhood behavior. Am J Obstet Gynecol 190(3):588-95.

Fride E, Weinstock M. 1988. Prenatal stress increases anxiety related behavior and alters cerebral lateralization of dopamine activity. Life Sci 42(10):1059-65.

Frotscher M, Jonas P, Sloviter RS. 2006. Synapses formed by normal and abnormal hippocampal mossy fibers. Cell Tissue Res 326(2):361-7.

Frye CA, Orecki ZA. 2002. Prenatal stress alters reproductive responses of rats in behavioral estrus and paced mating of hormone-primed rats. Horm Behav 42(4):472-83.

Fuchikami M, Morinobu S, Kurata A, Yamamoto S, Yamawaki S. 2009. Single immobilization stress differentially alters the expression profile of transcripts of the brain-derived neurotrophic factor (BDNF) gene and histone acetylation at its promoters in the rat hippocampus. Int J Neuropsychopharmacol 12(1):73-82.

Fukuda MT, Francolin-Silva AL, Almeida SS. 2002. Early postnatal protein malnutrition affects learning and memory in the distal but not in the proximal cue version of the Morris water maze. Behav Brain Res 133(2):271-7.

Fukuda MT, Francolin-Silva AL, Hernandes AS, Valadares CT, Almeida SS. 2007. Effects of early protein malnutrition and scopolamine on learning and memory in the Morris water maze. Nutr Neurosci 10(5-6):251-9.

Gaarskjaer FB. 1985. The development of the dentate area and the hippocampal mossy fiber projection of the rat. J Comp Neurol 241(2):154-70.

Galler JR, Ramsey FC, Forde V, Salt P, Archer E, . 1987. Long-term effects of early kwashiorkor compared with marasmus. II. Intellectual performance. Journal of Pediatric Gastroenterology and Nutrition 6, 847–854.

Galler JR, Ramsey FC, Morley DS, Archer E, Salt P, . 1990. The long-term effects of early kwashiorkor compared with marasmus. IV. Performance on the national high school entrance examination. Pediatric Research 28, 235–239.

Galler RJ, Shumsky JS, Morgane PJ. 1997. Malnutrition and brain development. In: Walker, W.A., Decker, B.C. (Eds.), Nutrition in Pediatrics. Part II. Hamilton, London, pp. 196–212.

Gallo PV. 1981. Physiological and behavioral consequences of maternal and post-weaning protein restriction. Physiol Behav 26(1):77-84.

Ganong WF, Alpert LC, Lee TC. 1974. ACTH and the regulation of adrenocortical secretion. N Engl J Med 290(18):1006-11.

Garin-Aguilar ME, Diaz-Cintra S, Quirarte GL, Aguilar-Vazquez A, Medina AC, Prado-Alcala RA. 2012. Extinction procedure induces pruning of dendritic spines in CA1 hippocampal field depending on strength of training in rats. Front Behav Neurosci 6:12.

Gilbert ME, MacPhail R, Baldwin J, Moser VC, Chernoff N. 2010. Moderate developmental undernutrition: Impact on growth and cognitive function in youth and old age. Neurotoxicol Teratol 32(3):362-72.

Glover V. 2015. Prenatal stress and its effects on the fetus and the child: possible underlying biological mechanisms. Adv Neurobiol 10:269-83.

Gluckman PD. 2001. Editorial: nutrition, glucocorticoids, birth size, and adult disease. Endocrinology 142(5):1689-91.

Gluckman PD, Hanson MA. 2004. Living with the past: evolution, development, and patterns of disease. Science 305(5691):1733-6.

Godfrey KM, Barker DJ. 2000. Fetal nutrition and adult disease. Am J Clin Nutr 71(5 Suppl):1344S-52S.

Gonzales RB, DeLeon Galvan CJ, Rangel YM, Claiborne BJ. 2001. Distribution of thorny excrescences on CA3 pyramidal neurons in the rat hippocampus. J Comp Neurol 430(3):357-68.

Good CH, Bay KD, Buchanan RA, McKeon KA, Skinner RD, Garcia-Rill E. 2006. Prenatal exposure to cigarette smoke affects the physiology of pedunculopontine nucleus (PPN) neurons in development. Neurotoxicol Teratol 28(2):210-9.

Goodlett CR, Nonneman AJ, Valentino ML, West JR. 1988. Constraints on water maze spatial learning in rats: implications for behavioral studies of brain damage and recovery of function. Behav Brain Res 28(3):275-86.

Gothard KM, Skaggs WE, McNaughton BL. 1996. Dynamics of mismatch correction in the hippocampal ensemble code for space: interaction between path integration and environmental cues. J Neurosci 16(24):8027-40.

Granados-Rojas L, Larriva-Sahd J, Cintra L, Gutierrez-Ospina G, Rondan A, Diaz-Cintra S. 2002. Prenatal protein malnutrition decreases mossy fibers-CA3 thorny excrescences asymmetrical synapses in adult rats. Brain Res 933(2):164-71.

Gray TS. 1991. Limbic pathways and neurotrasmitter as mediator of autonomic and neuroendocrine responses to stress.The amygdala. En: stress Neurobiology and Neuroendocrinology. Brown M.R., Koob, G.F., Rivier, C (eds). Marcel Dekker, Inc, Nueva York. (

).

Green S, H. H. 1991. Animal models of anxiety. En: Willner P, editor. Behavioral models in psychopharmacology. London: Cambridge University Press, 1991;21-49. 3.

.

Grienberger C, Chen X, Konnerth A. 2015. Dendritic function in vivo. Trends Neurosci 38(1):45-54.

Gross C, Hen R. 2004. The developmental origins of anxiety. Nat Rev Neurosci 5(7):545-52.

Grutzendler J, Kasthuri N, Gan WB. 2002. Long-term dendritic spine stability in the adult cortex. Nature 420(6917):812-6.

Gundelfinger ED, Fejtova A. 2012. Molecular organization and plasticity of the cytomatrix at the active zone. Curr Opin Neurobiol 22(3):423-30.

Guzman C, Cabrera R, Cardenas M, Larrea F, Nathanielsz PW, Zambrano E. 2006. Protein restriction during fetal and neonatal development in the rat alters reproductive function and accelerates reproductive ageing in female progeny. J Physiol 572(Pt 1):97-108.

Guzman C, Garcia-Becerra R, Aguilar-Medina MA, Mendez I, Merchant-Larios H, Zambrano E. 2014. Maternal protein restriction during pregnancy and/or lactation negatively affects follicular ovarian development and steroidogenesis in the prepubertal rat offspring. Arch Med Res 45(4):294-300.

Habib KE, Gold PW, Chrousos GP. 2001. Neuroendocrinology of stress. Endocrinol Metab Clin North Am 30(3):695-728; vii-viii.

Habib M, Landou JF, Ansidéi D, Massot BL. 1994. Bases neurológicas de las conductas: Masson.

Hales CN, Barker DJ. 2001. The thrifty phenotype hypothesis. Br Med Bull 60:5-20.

Hall CS. 1934. Emotional behavior in the rat. I. Defecation and urination as measures of individual differences in emotionality. Journal of Comparative Psychology 18(3): 385-403.

Hamosh M, Salem N, Jr. 1998. Long-chain polyunsaturated fatty acids. Biol Neonate 74(2):106-20.

Henry C, Kabbaj M, Simon H, Le Moal M, Maccari S. 1994. Prenatal stress increases the hypothalamo-pituitary-adrenal axis response in young and adult rats. J Neuroendocrinol 6(3):341-5.

Henze DA, Urban NN, Barrionuevo G. 2000. The multifarious hippocampal mossy fiber pathway: a review. Neuroscience 98(3):407-27.

Hernandez A, Burgos H, Mondaca M, Barra R, Nunez H, Perez H, Soto-Moyano R, Sierralta W, Fernandez V, Olivares R and others. 2008. Effect of prenatal protein malnutrition on long-term potentiation and BDNF protein expression in the rat entorhinal cortex after neocortical and hippocampal tetanization. Neural Plast 2008:646919.

Holemans K, Aerts L, Van Assche FA. 2003. Fetal growth restriction and consequences for the offspring in animal models. J Soc Gynecol Investig 10(7):392-9.

Holemans K, Gerber R, Meurrens K, De Clerck F, Poston L, Van Assche FA. 1999. Maternal food restriction in the second half of pregnancy affects vascular function but not blood pressure of rat female offspring. Br J Nutr 81(1):73-9.

Holson RR, Gough B, Sullivan P, Badger T, Sheehan DM. 1995. Prenatal dexamethasone or stress but not ACTH or corticosterone alter sexual behavior in male rats. Neurotoxicol Teratol 17(4):393-401.

Holtmaat A, Svoboda K. 2009. Experience-dependent structural synaptic plasticity in the mammalian brain. Nat Rev Neurosci 10(9):647-58.

Holtmaat AJ, Trachtenberg JT, Wilbrecht L, Shepherd GM, Zhang X, Knott GW, Svoboda K. 2005. Transient and persistent dendritic spines in the neocortex in vivo. Neuron 45(2):279-91.

Hotulainen P, Hoogenraad CC. 2010. Actin in dendritic spines: connecting dynamics to function. J Cell Biol 189(4):619-29.

Howland JG, Cazakoff BN. 2010. Effects of acute stress and GluN2B-containing NMDA receptor antagonism on object and object-place recognition memory. Neurobiol Learn Mem 93(2):261-7.

Hunter RG, McCarthy KJ, Milne TA, Pfaff DW, McEwen BS. 2009. Regulation of hippocampal H3 histone methylation by acute and chronic stress. Proc Natl Acad Sci U S A 106(49):20912-7.

Ion RC, Wills AK, Bernal AL. 2015. Environmental Tobacco Smoke Exposure in Pregnancy is Associated With Earlier Delivery and Reduced Birth Weight. Reprod Sci 22(12):1603-11.

Ishizuka N, Cowan WM, Amaral DG. 1995. A quantitative analysis of the dendritic organization of pyramidal cells in the rat hippocampus. J Comp Neurol 362(1):17-45.

Iversen SD. 1997. Behavioural evaluation of cholinergic drugs. Life Sci 60(13-14):1145-52.

Izquierdo I. 1989. Different forms of post-training memory processing. Behav Neural Biol 51(2):171-202.

Jablonka E, Raz G. 2009. Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution. Q Rev Biol 84(2):131-76.

Jaiswal AK, Upadhyay SN, Satyan KS, Bhattacharya SK. 1996. Behavioural effects of prenatal and postnatal undernutrition in rats. Indian J Exp Biol 34(12):1216-9.

Johnson EO, Kamilaris TC, Chrousos GP, Gold PW. 1992. Mechanisms of stress: a dynamic overview of hormonal and behavioral homeostasis. Neurosci Biobehav Rev 16(2):115-30.

Johnson JW, Mitzner W, Beck JC, London WT, Sly DL, Lee PA, Khouzami VA, Cavalieri RL. 1981. Long-term effects of betamethasone on fetal development. Am J Obstet Gynecol 141(8):1053-64.

Jordan TC, Cane SE, Howells KF. 1981. Deficits in spatial memory performance induced by early undernutrition. Dev Psychobiol 14(4):317-25.

Joshi S, Garole V, Daware M, Girigosavi S, Rao S. 2003. Maternal protein restriction before pregnancy affects vital organs of offspring in Wistar rats. Metabolism 52(1):13-8.

Kandel ER, Kupfermann I, Iversen S. 2000. Learning and memory. Principles of neural science. New York: McGraw-Hill.

Kapoor A, Petropoulos S, Matthews SG. 2008. Fetal programming of hypothalamic-pituitary-adrenal (HPA) axis function and behavior by synthetic glucocorticoids. Brain Res Rev 57(2):586-95.

Karemaker R, Heijnen CJ, Veen S, Baerts W, Samsom J, Visser GH, Kavelaars A, van Doornen LJ, van Bel F. 2006. Differences in behavioral outcome and motor development at school age after neonatal treatment for chronic lung disease with dexamethasone versus hydrocortisone. Pediatr Res 60(6):745-50.

Karemaker R, Kavelaars A, ter Wolbeek M, Tersteeg-Kamperman M, Baerts W, Veen S, Samsom JF, Visser GH, van Bel F, Heijnen CJ. 2008. Neonatal dexamethasone treatment for chronic lung disease of prematurity alters the hypothalamus-pituitary-adrenal axis and immune system activity at school age. Pediatrics 121(4):e870-8.

Kasai H, Fukuda M, Watanabe S, Hayashi-Takagi A, Noguchi J. 2010. Structural dynamics of dendritic spines in memory and cognition. Trends Neurosci 33(3):121-9.

Kasai H, Matsuzaki M, Noguchi J, Yasumatsu N, Nakahara H. 2003. Structure-stability-function relationships of dendritic spines. Trends Neurosci 26(7):360-8.

Kawashima N, Kusnecov AW. 2002. Effects of staphylococcal enterotoxin A on pituitary-adrenal activation and neophobic behavior in the C57BL/6 mouse. J Neuroimmunol 123(1-2):41-9.

Keenan K, Bartlett TQ, Nijland M, Rodriguez JS, Nathanielsz PW, Zurcher NR. 2013. Poor nutrition during pregnancy and lactation negatively affects neurodevelopment of the offspring: evidence from a translational primate model. Am J Clin Nutr 98(2):396-402.

Keller-Wood ME, Dallman MF. 1984. Corticosteroid inhibition of ACTH secretion. Endocrine Review. 5(1):1-24.

Kennedy MB, Beale HC, Carlisle HJ, Washburn LR. 2005. Integration of biochemical signalling in spines. Nat Rev Neurosci 6(6):423-34.

Kessels RP, de Haan EH, Kappelle LJ, Postma A. 2001. Varieties of human spatial memory: a meta-analysis on the effects of hippocampal lesions. Brain Res Brain Res Rev 35(3):295-303.

Kim JJ, Koo JW, Lee HJ, Han JS. 2005. Amygdalar inactivation blocks stress-induced impairments in hippocampal long-term potentiation and spatial memory. J Neurosci 25(6):1532-9.

King RS, DeBassio WA, Kemper TL, Rosene DL, Tonkiss J, Galler JR, Blatt GJ. 2004. Effects of prenatal protein malnutrition and acute postnatal stress on granule cell genesis in the fascia dentata of neonatal and juvenile rats. Brain Res Dev Brain Res 150(1):9-15.

King RS, Kemper TL, DeBassio WA, Ramzan M, Blatt GJ, Rosene DL, Galler JR. 2002. Birthdates and number of neurons in the serotonergic raphe nuclei in the rat with prenatal protein malnutrition. Nutr Neurosci 5(6):391-7.

King S, Dancause K, Turcotte-Tremblay AM, Veru F, Laplante DP. 2012. Using natural disasters to study the effects of prenatal maternal stress on child health and development. Birth Defects Res C Embryo Today 96(4):273-88.

Koch C, Zador A, Brown TH. 1992. Dendritic spines: convergence of theory and experiment. Science 256(5059):973-4.

Koenig JI, Elmer GI, Shepard PD, Lee PR, Mayo C, Joy B, Hercher E, Brady DL. 2005. Prenatal exposure to a repeated variable stress paradigm elicits behavioral and neuroendocrinological changes in the adult offspring: potential relevance to schizophrenia. Behav Brain Res 156(2):251-61.

Koolhaas JM, Bartolomucci A, Buwalda B, de Boer SF, Flugge G, Korte SM, Meerlo P, Murison R, Olivier B, Palanza P and others. 2011. Stress revisited: a critical evaluation of the stress concept. Neurosci Biobehav Rev 35(5):1291-301.

Landon J, Davison M, Krageloh CU, Thompson NM, Miles JL, Vickers MH, Fraser M, Breier BH. 2007. Global undernutrition during gestation influences learning during adult life. Learn Behav 35(2):79-86.

Langley-Evans SC. 2000. Critical differences between two low protein diet protocols in the programming of hypertension in the rat. Int J Food Sci Nutr 51(1):11-7.

Langley-Evans SC, Gardner DS, Jackson AA. 1996. Association of disproportionate growth of fetal rats in late gestation with raised systolic blood pressure in later life. J Reprod Fertil 106(2):307-12.

Langley-Evans SC, Welham SJ, Jackson AA. 1999. Fetal exposure to a maternal low protein diet impairs nephrogenesis and promotes hypertension in the rat. Life Sci 64(11):965-74.

Langley SC, Browne RF, Jackson AA. 1994. Altered glucose tolerance in rats exposed to maternal low protein diets in utero. Comp Biochem Physiol Physiol 109(2):223-9.

Lassalle JM, Bataille T, Halley H. 2000. Reversible inactivation of the hippocampal mossy fiber synapses in mice impairs spatial learning, but neither consolidation nor memory retrieval, in the Morris navigation task. Neurobiol Learn Mem 73(3):243-57.

Laus MF, Vales LDMF, Costa TMB, Almeida SS. 2011. Early postnatal proteincalorie malnutrition and cognition: a review of human and animal studies. International Journal of Environmental Research and Public Health 8, 590–612.

LeDoux JE. 2000. Emotion circuits in the brain. Annu Rev Neurosci 23:155-84.

Lee PR, Brady DL, Shapiro RA, Dorsa DM, Koenig JI. 2007. Prenatal stress generates deficits in rat social behavior: Reversal by oxytocin. Brain Res 1156:152-67.

Legendre P, Rosenmund C, Westbrook GL. 1993. Inactivation of NMDA channels in cultured hippocampal neurons by intracellular calcium. J Neurosci 13(2):674-84.

Lemaire V, Koehl M, Le Moal M, Abrous DN. 2000. Prenatal stress produces learning deficits associated with an inhibition of neurogenesis in the hippocampus. Proc Natl Acad Sci U S A 97(20):11032-7.

Leonhardt M, Lesage J, Dufourny L, Dickes-Coopman A, Montel V, Dupouy JP. 2002. Perinatal maternal food restriction induces alterations in hypothalamo-pituitary-adrenal axis activity and in plasma corticosterone-binding globulin capacity of weaning rat pups. Neuroendocrinology 75(1):45-54.

Leranth C, Hajszan T, MacLusky NJ. 2004. Androgens increase spine synapse density in the CA1 hippocampal subfield of ovariectomized female rats. J Neurosci 24(2):495-9.

Leranth C, Petnehazy O, MacLusky NJ. 2003. Gonadal hormones affect spine synaptic density in the CA1 hippocampal subfield of male rats. J Neurosci 23(5):1588-92.

Lesage J, Sebaai N, Leonhardt M, Dutriez-Casteloot I, Breton C, Deloof S, Vieau D. 2006. Perinatal maternal undernutrition programs the offspring hypothalamo-pituitary-adrenal (HPA) axis. Stress 9(4):183-98.

Levay EA, Paolini AG, Govic A, Hazi A, Penman J, Kent S. 2008. Anxiety-like behaviour in adult rats perinatally exposed to maternal calorie restriction. Behav Brain Res 191(2):164-72.

Li M, Masugi-Tokita M, Takanami K, Yamada S, Kawata M. 2012a. Testosterone has sublayer-specific effects on dendritic spine maturation mediated by BDNF and PSD-95 in pyramidal neurons in the hippocampus CA1 area. Brain Res 1484:76-84.

Li S, Fan YX, Wang W, Tang YY. 2012b. Effects of acute restraint stress on different components of memory as assessed by object-recognition and object-location tasks in mice. Behav Brain Res 227(1):199-207.

Li Y, Hough CJ, Suh SW, Sarvey JM, Frederickson CJ. 2001. Rapid translocation of Zn(2+) from presynaptic terminals into postsynaptic hippocampal neurons after physiological stimulation. J Neurophysiol 86(5):2597-604.

Lister JP, Blatt GJ, DeBassio WA, Kemper TL, Tonkiss J, Galler JR, Rosene DL. 2005. Effect of prenatal protein malnutrition on numbers of neurons in the principal cell layers of the adult rat hippocampal formation. Hippocampus 15(3):393-403.

Lister JP, Tonkiss J, Blatt GJ, Kemper TL, DeBassio WA, Galler JR, Rosene DL. 2006. Asymmetry of neuron numbers in the hippocampal formation of prenatally malnourished and normally nourished rats: a stereological investigation. Hippocampus 16(11):946-58.

Lister RG. 1987. The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology (Berl) 92(2):180-5.

Lukoyanov NV, Andrade JP. 2000. Behavioral effects of protein deprivation and rehabilitation in adult rats: relevance to morphological alterations in the hippocampal formation. Behav Brain Res 112(1-2):85-97.

Maccari S, Krugers HJ, Morley-Fletcher S, Szyf M, Brunton PJ. 2014. The consequences of early-life adversity: neurobiological, behavioural and epigenetic adaptations. J Neuroendocrinol 26(10):707-23.

MacLaughlin SM, Walker SK, Roberts CT, Kleemann DO, McMillen IC. 2005. Periconceptional nutrition and the relationship between maternal body weight changes in the periconceptional period and feto-placental growth in the sheep. J Physiol 565(Pt 1):111-24.

MacLusky NJ, Hajszan T, Prange-Kiel J, Leranth C. 2006. Androgen modulation of hippocampal synaptic plasticity. Neuroscience 138(3):957-65.

Machado-de-Sousa JP, Osorio Fde L, Jackowski AP, Bressan RA, Chagas MH, Torro-Alves N, Depaula AL, Crippa JA, Hallak JE. 2014. Increased amygdalar and hippocampal volumes in young adults with social anxiety. PLoS One 9(2):e88523.

Magarinos AM, McEwen BS. 1995. Stress-induced atrophy of apical dendrites of hippocampal CA3c neurons: comparison of stressors. Neuroscience 69(1):83-8.

Marco EM, Valero M, de la Serna O, Aisa B, Borcel E, Ramirez MJ, Viveros MP. 2013. Maternal deprivation effects on brain plasticity and recognition memory in adolescent male and female rats. Neuropharmacology 68:223-31.

Martinez Sanchis S. 2006. Hormonas, estado de ánimo y función cognitiva. Delta publicaciones.

Martinez Y, Diaz-Cintra S, Leon-Jacinto U, Aguilar-Vazquez A, Medina AC, Quirarte GL, Prado-Alcala RA. 2009. Effects of postnatal malnutrition and senescence on learning, long-term memory, and extinction in the rat. Behav Brain Res 203(1):48-53.

Matsuzaki M, Ellis-Davies GC, Nemoto T, Miyashita Y, Iino M, Kasai H. 2001. Dendritic spine geometry is critical for AMPA receptor expression in hippocampal CA1 pyramidal neurons. Nat Neurosci 4(11):1086-92.

Matthews SG. 2001. Antenatal glucocorticoids and the developing brain: mechanisms of action. Semin Neonatol 6(4):309-17.

Matthews SG. 2002. Early programming of the hypothalamo-pituitary-adrenal axis. Trends Endocrinol Metab 13(9):373-80.

McCormick CM, Smythe JW, Sharma S, Meaney MJ. 1995. Sex-specific effects of prenatal stress on hypothalamic-pituitary-adrenal responses to stress and brain glucocorticoid receptor density in adult rats. Brain Res Dev Brain Res 84(1):55-61.

McEwen BS, Weiss JM, Schwartz LS. 1968. Selective retention of corticosterone by limbic structures in rat brain. Nature 220(5170):911-2.

McGaugh JL. 2002. Memory consolidation and the amygdala: a systems perspective. Trends Neurosci 25(9):456.

McGhie A, Chapman J. 1961. Disorders of attention and perception in early schizophrenia. Br J Med Psychol 34:103-16.

McMullen S, Mostyn A. 2009. Animal models for the study of the developmental origins of health and disease. Proc Nutr Soc 68(3):306-20.

McSweeney FK, Murphy ES. 2000. Criticisms of the satiety hypothesis as an explanation for within-session decreases in responding. J Exp Anal Behav 74(3):347-61.

Mellone M, Pelucchi S, Alberti L, Genazzani AA, Di Luca M, Gardoni F. 2015. Zinc transporter-1: a novel NMDA receptor-binding protein at the postsynaptic density. J Neurochem 132(2):159-68.

Michael J. 2007. Motivating operations. In J. O. Cooper, T. E. Heron, & W. L. Heward (Eds.), Applied behavior analysis, 2nd edition (pp. 374-391). Upper Saddle River, NJ: Pearson.

Miles JL, Landon J, Davison M, Krageloh CU, Thompson NM, Triggs CM, Breier BH. 2009. Prenatally undernourished rats show increased preference for wheel running v. lever pressing for food in a choice task. Br J Nutr 101(6):902-8.

Moisiadis VG, Matthews SG. 2014. Glucocorticoids and fetal programming part 1: outcomes. Nat Rev Endocrinol 10(7):391-402.

Mokler DJ, Galler JR, Morgane PJ. 2003. Modulation of 5-HT release in the hippocampus of 30-day-old rats exposed in utero to protein malnutrition. Brain Res Dev Brain Res 142(2):203-8.

Mokler DJ, Torres OI, Galler JR, Morgane PJ. 2007. Stress-induced changes in extracellular dopamine and serotonin in the medial prefrontal cortex and dorsal hippocampus of prenatally malnourished rats. Brain Res 1148:226-33.

Moreira CM, Masson S, Carvalho MC, Brandao ML. 2007. Exploratory behaviour of rats in the elevated plus-maze is differentially sensitive to inactivation of the basolateral and central amygdaloid nuclei. Brain Res Bull 71(5):466-74.

Morgan MA, LeDoux JE. 1999. Contribution of ventrolateral prefrontal cortex to the acquisition and extinction of conditioned fear in rats. Neurobiol Learn Mem 72(3):244-51.

Morgane PJ, Austin-LaFrance R, Bronzino J, Tonkiss J, Diaz-Cintra S, Cintra L, Kemper T, Galler JR. 1993. Prenatal malnutrition and development of the brain. Neurosci Biobehav Rev 17(1):91-128.

Morgane PJ, Austin-LaFrance RJ, Bronzino JD, Tonkiss J, Diaz-Cintra S, Cintra L, Kemper T, Galler JR. 1992. Malnutrition and the developing central nervous system. In: Issacson, R.L., Jensen, K.F. (Eds.), The Vulnerable Brain: Nutrition and Toxins. Plenum Press, New York, pp. 3–44.

Morgane PJ, Galler JR, Mokler DJ. 2005. A review of systems and networks of the limbic forebrain/limbic midbrain. Prog Neurobiol 75(2):143-60.

Morgane PJ, Miller M, Kemper T, Stern WC, Forbes W, Hall R, Bronzino JD, Caisson J, Hawrylewicz E, Resnick O. 1978. The effects of protein malnutrition on the developing central nervous system of the rat. Neuroscience & Biobehavioral Reviews 2, 137–230.

Morgane PJ, Mokler DJ, Galler JR. 2002. Effects of prenatal protein malnutrition on the hippocampal formation. Neurosci Biobehav Rev 26(4):471-83.

Morimoto K, Fahnestock M, Racine RJ. 2004. Kindling and status epilepticus models of epilepsy: rewiring the brain. Prog Neurobiol 73(1):1-60.

Morris R. 1984. Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 11(1):47-60.

Morris RG. 2006. Elements of a neurobiological theory of hippocampal function: the role of synaptic plasticity, synaptic tagging and schemas. Eur J Neurosci 23(11):2829-46.

Moser E, Moser MB, Andersen P. 1993. Spatial learning impairment parallels the magnitude of dorsal hippocampal lesions, but is hardly present following ventral lesions. J Neurosci 13(9):3916-25.

Moser MB, Trommald M, Andersen P. 1994. An increase in dendritic spine density on hippocampal CA1 pyramidal cells following spatial learning in adult rats suggests the formation of new synapses. Proc Natl Acad Sci U S A 91(26):12673-5.

Moustafa AA, Gilbertson MW, Orr SP, Herzallah MM, Servatius RJ, Myers CE. 2013. A model of amygdala-hippocampal-prefrontal interaction in fear conditioning and extinction in animals. Brain Cogn 81(1):29-43.

Mumby DG. 2001. Perspectives on object-recognition memory following hippocampal damage: lessons from studies in rats. Behav Brain Res 127(1-2):159-81.

Mumby DG, Piterkin P, Lecluse V, Lehmann H. 2007. Perirhinal cortex damage and anterograde object-recognition in rats after long retention intervals. Behav Brain Res 185(2):82-7.

Nathanielsz PW. 1999. Life in the womb. The origin of health and desease. Ithaca, N.Y.: Promethean press. 1-363.

Nathanielsz PW. 2006. Animal models that elucidate basic principles of the developmental origins of adult diseases. ILAR J 47(1):73-82.

Navarrete F, Pérez-Ortiz J, Femenía T, García-Gutiérrez M, García-Payá M, Leiva-Santana C, Manzanares J. 2008. Métodos de evaluación de trastornos cognitivos en modelos animales. Revista de neurología 47(3):137-145.

Nicolaides NC, Kyratzi E, Lamprokostopoulou A, Chrousos GP, Charmandari E. 2015. Stress, the stress system and the role of glucocorticoids. Neuroimmunomodulation 22(1-2):6-19.

Nicoll RA, Malenka RC. 1995. Contrasting properties of two forms of long-term potentiation in the hippocampus. Nature 377(6545):115-8.

Nicoll RA, Roche KW. 2013. Long-term potentiation: peeling the onion. Neuropharmacology 74:18-22.

Nicoll RA, Schmitz D. 2005. Synaptic plasticity at hippocampal mossy fibre synapses. Nat Rev Neurosci 6(11):863-76.

Nutrición ENd. 2012. México, D.F., Secretaría de Salud Pública.

O'Connor TG, Ben-Shlomo Y, Heron J, Golding J, Adams D, Glover V. 2005. Prenatal anxiety predicts individual differences in cortisol in pre-adolescent children. Biol Psychiatry 58(3):211-7.

O'Keefe J. 1990. A computational theory of the hippocampal cognitive map. Prog Brain Res 83:301-12.

Ohishi T, Wang L, Akane H, Shiraki A, Sato A, Uematsu M, Suzuki K, Mitsumori K, Shibutani M. 2012. Adolescent hyperactivity of offspring after maternal protein restriction during the second half of gestation and lactation periods in rats. J Toxicol Sci 37(2):345-52.

Okuda S, Roozendaal B, McGaugh JL. 2004. Glucocorticoid effects on object recognition memory require training-associated emotional arousal. Proc Natl Acad Sci U S A 101(3):853-8.

Ozanne SE, Hales CN. 1999. The long-term consequences of intra-uterine protein malnutrition for glucose metabolism. Proc Nutr Soc 58(3):615-9.

P. W. 1991. Behavioural models in psychopharmacology. London: Cambridge University Press.

Padilla-Gomez E, Beltran-Campos V, Montes S, Diaz-Ruiz A, Quirarte GL, Rios C, Diaz-Cintra S. 2012. Effect of 17ss-estradiol on zinc content of hippocampal mossy fibers in ovariectomized adult rats. Biometals 25(6):1129-39.

Padmanabhan V, Manikkam M, Recabarren S, Foster D. 2006. Prenatal testosterone excess programs reproductive and metabolic dysfunction in the female. Mol Cell Endocrinol 246(1-2):165-74.

Painter RC, de Rooij SR, Bossuyt PM, de Groot E, Stok WJ, Osmond C, Barker DJ, Bleker OP, Roseboom TJ. 2007. Maternal nutrition during gestation and carotid arterial compliance in the adult offspring: the Dutch famine birth cohort. J Hypertens 25(3):533-40.

Paoletti P, Vergnano AM, Barbour B, Casado M. 2009. Zinc at glutamatergic synapses. Neuroscience 158(1):126-36.

Paris JJ, Brunton PJ, Russell JA, Frye CA. 2011. Immune stress in late pregnant rats decreases length of gestation and fecundity, and alters later cognitive and affective behaviour of surviving pre-adolescent offspring. Stress 14(6):652-64.

Paris JJ, Frye CA. 2011. Juvenile offspring of rats exposed to restraint stress in late gestation have impaired cognitive performance and dysregulated progestogen formation. Stress 14(1):23-32.

Patin V, Lordi B, Vincent A, Caston J. 2005. Effects of prenatal stress on anxiety and social interactions in adult rats. Brain Res Dev Brain Res 160(2):265-74.

Pellow S, Chopin P, File SE, Briley M. 1985. Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods 14(3):149-67.

Pellow S, File SE. 1986. Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: a novel test of anxiety in the rat. Pharmacol Biochem Behav 24(3):525-9.

Phillips DI. 2001. Fetal growth and programming of the hypothalamic-pituitary-adrenal axis. Clin Exp Pharmacol Physiol 28(11):967-70.

Posner MI. 1995. Attention in cognitive neuroscience. Cambridge:MIT Press;.

Pratt JA. 1992. The neuroanatomical basis of anxiety. Pharmacol Ther 55(2):149-81.

Price JL, Amaral DG. 1981. An autoradiographic study of the projections of the central nucleus of the monkey amygdala. J Neurosci 1(11):1242-59.

Pritchard D. 2015. Classification in psychiatry: from a symptom based to a cause based model? Psychiatr Danub 27 Suppl 1:7-20.

Probyn ME, Lock EK, Anderson ST, Walton S, Bertram JF, Wlodek ME, Moritz KM. 2013. The effect of low-to-moderate-dose ethanol consumption on rat mammary gland structure and function and early postnatal growth of offspring. Am J Physiol Regul Integr Comp Physiol 304(10):R791-8.

Puts DA, McDaniel MA, Jordan CL, Breedlove SM. 2008. Spatial ability and prenatal androgens: meta-analyses of congenital adrenal hyperplasia and digit ratio (2D:4D) studies. Arch Sex Behav 37(1):100-11.

Qin S, Young CB, Duan X, Chen T, Supekar K, Menon V. 2014. Amygdala subregional structure and intrinsic functional connectivity predicts individual differences in anxiety during early childhood. Biol Psychiatry 75(11):892-900.

Quinn M, Ramamoorthy S, Cidlowski JA. 2014. Sexually dimorphic actions of glucocorticoids: beyond chromosomes and sex hormones. Ann N Y Acad Sci 1317:1-6.

Ramirez-Amaya V, Balderas I, Sandoval J, Escobar ML, Bermudez-Rattoni F. 2001. Spatial long-term memory is related to mossy fiber synaptogenesis. J Neurosci 21(18):7340-8.

Ramirez-Amaya V, Escobar ML, Chao V, Bermudez-Rattoni F. 1999. Synaptogenesis of mossy fibers induced by spatial water maze overtraining. Hippocampus 9(6):631-6.

Rampon C, Tang YP, Goodhouse J, Shimizu E, Kyin M, Tsien JZ. 2000. Enrichment induces structural changes and recovery from nonspatial memory deficits in CA1 NMDAR1-knockout mice. Nat Neurosci 3(3):238-44.

Ranade SC, Rose A, Rao M, Gallego J, Gressens P, Mani S. 2008. Different types of nutritional deficiencies affect different domains of spatial memory function checked in a radial arm maze. Neuroscience 152(4):859-66.

Ravelli AC, van der Meulen JH, Michels RP, Osmond C, Barker DJ, Hales CN, Bleker OP. 1998. Glucose tolerance in adults after prenatal exposure to famine. Lancet 351(9097):173-7.

Ravelli AC, van Der Meulen JH, Osmond C, Barker DJ, Bleker OP. 1999. Obesity at the age of 50 y in men and women exposed to famine prenatally. Am J Clin Nutr 70(5):811-6.

Reeves PG, Nielsen FH, Fahey GC, Jr. 1993. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 123(11):1939-51.

Renner M, Specht CG, Triller A. 2008. Molecular dynamics of postsynaptic receptors and scaffold proteins. Curr Opin Neurobiol 18(5):532-40.

Represa A, Tremblay E, Ben-Ari Y. 1990. Sprouting of mossy fibers in the hippocampus of epileptic human and rat. Adv Exp Med Biol 268:419-24.

Reul JM, de Kloet ER. 1985. Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology 117(6):2505-11.

Reyes-Castro LA, Rodriguez-Gonzalez GL, Chavira R, Ibanez C, Lomas-Soria C, Rodriguez JS, Nathanielsz PW, Zambrano E. 2015. Paternal line multigenerational passage of altered risk assessment behavior in female but not male rat offspring of mothers fed a low protein diet. Physiol Behav 140:89-95.

Reyes-Castro LA, Rodriguez JS, Charco R, Bautista CJ, Larrea F, Nathanielsz PW, Zambrano E. 2012a. Maternal protein restriction in the rat during pregnancy and/or lactation alters cognitive and anxiety behaviors of female offspring. Int J Dev Neurosci 30(1):39-45.

Reyes-Castro LA, Rodriguez JS, Rodriguez-Gonzalez GL, Chavira R, Bautista CJ, McDonald TJ, Nathanielsz PW, Zambrano E. 2012b. Pre- and/or postnatal protein restriction developmentally programs affect and risk assessment behaviors in adult male rats. Behav Brain Res 227(2):324-9.

Reyes-Castro LA, Rodriguez JS, Rodriguez-Gonzalez GL, Wimmer RD, McDonald TJ, Larrea F, Nathanielsz PW, Zambrano E. 2011. Pre- and/or postnatal protein restriction in rats impairs learning and motivation in male offspring. Int J Dev Neurosci 29(2):177-82.

Rivier C, Vale W. 1983. Modulation of stress-induced ACTH release by corticotropin-releasing factor, catecholamines and vasopressin. Nature 305(5932):325-7.

Roberts TF, Tschida KA, Klein ME, Mooney R. 2010. Rapid spine stabilization and synaptic enhancement at the onset of behavioural learning. Nature 463(7283):948-52.

Rodgers RJ. 1997. Animal models of 'anxiety': where next? Behav Pharmacol 8(6-7):477-96; discussion 497-504.

Rodriguez-Gonzalez GL, Reyes-Castro LA, Vega CC, Boeck L, Ibanez C, Nathanielsz PW, Larrea F, Zambrano E. 2014. Accelerated aging of reproductive capacity in male rat offspring of protein-restricted mothers is associated with increased testicular and sperm oxidative stress. Age (Dordr) 36(6):9721.

Rodriguez-Gonzalez GL, Vigueras-Villasenor RM, Millan S, Moran N, Trejo R, Nathanielsz PW, Larrea F, Zambrano E. 2012. Maternal protein restriction in pregnancy and/or lactation affects seminiferous tubule organization in male rat offspring. J Dev Orig Health Dis 3(5):321-6.

Rojas D, Salazar L. 2009. Efecto de la restriccióncalórico-proteico sobre la adipogénesis. Biológicas; (11): p22-8.

Rollenhagen A, Satzler K, Rodriguez EP, Jonas P, Frotscher M, Lubke JH. 2007. Structural determinants of transmission at large hippocampal mossy fiber synapses. J Neurosci 27(39):10434-44.

Rosa RM, Flores DG, Appelt HR, Braga AL, Henriques JA, Roesler R. 2003. Facilitation of long-term object recognition memory by pretraining administration of diphenyl diselenide in mice. Neurosci Lett 341(3):217-20.

Rosellini RA, Widman DR. 1989. Prior exposure to stress reduces the diversity of exploratory behavior of novel objects in the rat (Rattus norvegicus). J Comp Psychol 103(4):339-46.

Rosenkranz JA, Grace AA. 2001. Dopamine attenuates prefrontal cortical suppression of sensory inputs to the basolateral amygdala of rats. J Neurosci 21(11):4090-103.

Rotta LN, Leszczinski DN, Brusque AM, Pereira P, Brum LF, Nogueira CW, Frizzo ME, Perry ML, Souza DO. 2008. Effects of undernutrition on glutamatergic parameters in the cerebral cortex of young rats. Physiol Behav 94(4):580-5.

Rusakov DA, Davies HA, Harrison E, Diana G, Richter-Levin G, Bliss TV, Stewart MG. 1997. Ultrastructural synaptic correlates of spatial learning in rat hippocampus. Neuroscience 80(1):69-77.

Rush D. 2001. Maternal nutrition and perinatal survival. Nutr Rev 59(10):315-26.

S. RyC. 1888. Estructura de los centros nerviosos de las aves. Rev Trim Hist Norm Pat.;1:1—10.

Salomon S, Bejar C, Schorer-Apelbaum D, Weinstock M. 2011. Corticosterone mediates some but not other behavioural changes induced by prenatal stress in rats. J Neuroendocrinol 23(2):118-28.

Santín-Núñez LJ, Rubio S, Begega A, Miranda-García R, Arias JL. 2000. Aprendizaje espacial e hipocampo. RevNeurol 31(05):0455-462.

Santucci LB, Daud MM, Almeida SS, de Oliveira LM. 1994. Effects of early protein malnutrition and environmental stimulation upon the reactivity to diazepam in two animal models of anxiety. Pharmacol Biochem Behav 49(2):393-8.

Sapolsky RM, Krey LC, McEwen BS. 1986. The neuroendocrinology of stress and aging: the glucocorticoid cascade hypothesis. Endocr Rev 7(3):284-301.

Schaffer L, Muller-Vizentini D, Burkhardt T, Rauh M, Ehlert U, Beinder E. 2009. Blunted stress response in small for gestational age neonates. Pediatr Res 65(2):231-5.

Scharfman HE, MacLusky NJ. 2014. Differential regulation of BDNF, synaptic plasticity and sprouting in the hippocampal mossy fiber pathway of male and female rats. Neuropharmacology 76 Pt C:696-708.

Schjetnan AG, Escobar ML. 2012. In vivo BDNF modulation of hippocampal mossy fiber plasticity induced by high frequency stimulation. Hippocampus 22(1):1-8.

Schubert V, Dotti CG. 2007. Transmitting on actin: synaptic control of dendritic architecture. J Cell Sci 120(Pt 2):205-12.

Schuurmans C, Kurrasch DM. 2013. Neurodevelopmental consequences of maternal distress: what do we really know? Clin Genet 83(2):108-17.

Sebaai N, Lesage J, Breton C, Vieau D, Deloof S. 2004. Perinatal food deprivation induces marked alterations of the hypothalamo-pituitary-adrenal axis in 8-month-old male rats both under basal conditions and after a dehydration period. Neuroendocrinology 79(4):163-73.

Sebaai N, Lesage J, Vieau D, Alaoui A, Dupouy JP, Deloof S. 2002. Altered control of the hypothalamo-pituitary-adrenal axis in adult male rats exposed perinatally to food deprivation and/or dehydration. Neuroendocrinology 76(4):243-53.

Seckl JR. 2004. Prenatal glucocorticoids and long-term programming. Eur J Endocrinol 151 Suppl 3:U49-62.

Seckl JR. 2008. Glucocorticoids, developmental 'programming' and the risk of affective dysfunction. Prog Brain Res 167:17-34.

Sedaghat K, Zahediasl S, Ghasemi A. 2015. Intrauterine programming. Iran J Basic Med Sci 18(3):212-20.

Sekino Y, Kojima N, Shirao T. 2007. Role of actin cytoskeleton in dendritic spine morphogenesis. Neurochem Int 51(2-4):92-104.

Senn V, Wolff SB, Herry C, Grenier F, Ehrlich I, Grundemann J, Fadok JP, Muller C, Letzkus JJ, Luthi A. 2014. Long-range connectivity defines behavioral specificity of amygdala neurons. Neuron 81(2):428-37.

Seri B, Garcia-Verdugo JM, McEwen BS, Alvarez-Buylla A. 2001. Astrocytes give rise to new neurons in the adult mammalian hippocampus. J Neurosci 21(18):7153-60.

Sheng M, Hoogenraad CC. 2007. The postsynaptic architecture of excitatory synapses: a more quantitative view. Annu Rev Biochem 76:823-47.

Si B, Treves A. 2009. The role of competitive learning in the generation of DG fields from EC inputs. Cogn Neurodyn 3(2):177-87.

Silveira MC, Sandner G, Graeff FG. 1993. Induction of Fos immunoreactivity in the brain by exposure to the elevated plus-maze. Behav Brain Res 56(1):115-8.

Skinner BF. 1938. The behavior of organisms: An experimental analysis.

Sorra KE, Harris KM. 2000. Overview on the structure, composition, function, development, and plasticity of hippocampal dendritic spines. Hippocampus 10(5):501-11.

Soto-Moyano R, Fernandez V, Sanhueza M, Belmar J, Kusch C, Perez H, Ruiz S, Hernandez A. 1999. Effects of mild protein prenatal malnutrition and subsequent postnatal nutritional rehabilitation on noradrenaline release and neuronal density in the rat occipital cortex. Brain Res Dev Brain Res 116(1):51-8.

Soto-Moyano R, Valladares L, Sierralta W, Perez H, Mondaca M, Fernandez V, Burgos H, Hernandez A. 2005. Mild prenatal protein malnutrition increases alpha2C-adrenoceptor density in the cerebral cortex during postnatal life and impairs neocortical long-term potentiation and visuo-spatial performance in rats. J Neurochem 93(5):1099-109.

Spacek J, Harris KM. 1997. Three-dimensional organization of smooth endoplasmic reticulum in hippocampal CA1 dendrites and dendritic spines of the immature and mature rat. J Neurosci 17(1):190-203.

Spencer SJ. 2013. Perinatal programming of neuroendocrine mechanisms connecting feeding behavior and stress. Front Neurosci 7:109.

Steffenach HA, Sloviter RS, Moser EI, Moser MB. 2002. Impaired retention of spatial memory after transection of longitudinally oriented axons of hippocampal CA3 pyramidal cells. Proc Natl Acad Sci U S A 99(5):3194-8.

Steiger JL, Alexander MJ, Galler JR, Farb DH, Russek SJ. 2003. Effects of prenatal malnutrition on GABAA receptor alpha1, alpha3 and beta2 mRNA levels. Neuroreport 14(13):1731-5.

Steiger JL, Galler JR, Farb DH, Russek SJ. 2002. Prenatal protein malnutrition reduces beta(2), beta(3) and gamma(2L) GABA(A) receptor subunit mRNAs in the adult septum. Eur J Pharmacol 446(1-3):201-2.

Strauss RS. 1997. Effects of the intrauterine environment on childhood growth. Br Med Bull 53(1):81-95.

Swanson LW, Köhler C, Björklund A. 1987. Handbook of Chemical Neuroanatomy. Vol. 5 Integrated systems of the SNC, part 1. Amsterdam: Elseiver Science publishers.

Szuran TF, Pliska V, Pokorny J, Welzl H. 2000. Prenatal stress in rats: effects on plasma corticosterone, hippocampal glucocorticoid receptors, and maze performance. Physiol Behav 71(3-4):353-62.

Tada T, Sheng M. 2006. Molecular mechanisms of dendritic spine morphogenesis. Curr Opin Neurobiol 16(1):95-101.

Takahashi LK, Kalin NH. 1991. Early developmental and temporal characteristics of stress-induced secretion of pituitary-adrenal hormones in prenatally stressed rat pups. Brain Res 558(1):75-8.

Takeda A, Ando M, Kanno S, Oku N. 2009. Unique response of zinc in the hippocampus to behavioral stress and attenuation of subsequent mossy fiber long-term potentiation. Neurotoxicology 30(4):712-7.

Tashiro A, Yuste R. 2003. Structure and molecular organization of dendritic spines. Histol Histopathol 18(2):617-34.

Tian JY, Cheng Q, Song XM, Li G, Jiang GX, Gu YY, Luo M. 2006. Birth weight and risk of type 2 diabetes, abdominal obesity and hypertension among Chinese adults. Eur J Endocrinol 155(4):601-7.

Tonkiss J, Foster GA, Galler JR. 1991. Prenatal protein malnutrition and hippocampal function: partial reinforcement extinction effect. Brain Res Bull 27(6):809-13.

Tonkiss J, Galler J, Morgane PJ, Bronzino JD, Austin-LaFrance RJ. 1993. Prenatal protein malnutrition and postnatal brain function. Ann N Y Acad Sci 678:215-27.

Tonkiss J, Galler JR, Formica RN, Shukitt-Hale B, Timm RR. 1990a. Fetal protein malnutrition impairs acquisition of a DRL task in adult rats. Physiol Behav 48(1):73-7.

Tonkiss J, Shukitt-Hale B, Formica RN, Rocco FJ, Galler JR. 1990b. Prenatal protein malnutrition alters response to reward in adult rats. Physiol Behav 48(5):675-80.

Tonkiss J, Shultz P, Galler JR. 1994. An analysis of spatial navigation in prenatally protein malnourished rats. Physiol Behav 55(2):217-24.

Tonkiss J, Shultz PL, Shumsky JS, Galler JR. 1997. Development of spatial navigation following prenatal cocaine and malnutrition in rats: lack of additive effects. Neurotoxicol Teratol 19(5):363-72.

Torras M, Portell I, Morgado I. 2001. La amígdala: implicaciones funcionales. RevNeurol 33(05):0471-476.

Torres N, Bautista CJ, Tovar AR, Ordaz G, Rodriguez-Cruz M, Ortiz V, Granados O, Nathanielsz PW, Larrea F, Zambrano E. 2010. Protein restriction during pregnancy affects maternal liver lipid metabolism and fetal brain lipid composition in the rat. Am J Physiol Endocrinol Metab 298(2):E270-7.

Treit D, Pinel JP, Fibiger HC. 1981. Conditioned defensive burying: a new paradigm for the study of anxiolytic agents. Pharmacol Biochem Behav 15(4):619-26.

Trzctnska M, Tonkiss J, Galler JR. 1999. Influence of prenatal protein malnutrition on behavioral reactivity to stress in adult rats. Stress 3(1):71-83.

Tse D, Langston RF, Kakeyama M, Bethus I, Spooner PA, Wood ER, Witter MP, Morris RG. 2007. Schemas and memory consolidation. Science 316(5821):76-82.

Tye KM, Prakash R, Kim SY, Fenno LE, Grosenick L, Zarabi H, Thompson KR, Gradinaru V, Ramakrishnan C, Deisseroth K. 2011. Amygdala circuitry mediating reversible and bidirectional control of anxiety. Nature 471(7338):358-62.

Ulrich-Lai YM, Herman JP. 2009. Neural regulation of endocrine and autonomic stress responses. Nat Rev Neurosci 10(6):397-409.

Uno H, Eisele S, Sakai A, Shelton S, Baker E, DeJesus O, Holden J. 1994. Neurotoxicity of glucocorticoids in the primate brain. Horm Behav 28(4):336-48.

Uno H, Lohmiller L, Thieme C, Kemnitz JW, Engle MJ, Roecker EB, Farrell PM. 1990. Brain damage induced by prenatal exposure to dexamethasone in fetal rhesus macaques. I. Hippocampus. Brain Res Dev Brain Res 53(2):157-67.

Urani A, Philbert J, Cohen C, Griebel G. 2011. The corticotropin-releasing factor 1 receptor antagonist, SSR125543, and the vasopressin 1b receptor antagonist, SSR149415, prevent stress-induced cognitive impairment in mice. Pharmacol Biochem Behav 98(3):425-31.

Valadares CT, Fukuda MT, Francolin-Silva AL, Hernandes AS, Almeida SS. 2010. Effects of postnatal protein malnutrition on learning and memory procedures. Nutr Neurosci 13(6):274-82.

Vallee M, Mayo W, Dellu F, Le Moal M, Simon H, Maccari S. 1997. Prenatal stress induces high anxiety and postnatal handling induces low anxiety in adult offspring: correlation with stress-induced corticosterone secretion. J Neurosci 17(7):2626-36.

Vargas-Lopez V, Torres-Berrio A, Gonzalez-Martinez L, Munera A, Lamprea MR. 2015. Acute restraint stress and corticosterone transiently disrupts novelty preference in an object recognition task. Behav Brain Res 291:60-6.

Vicens P, Redolat R, Carrasco MDC. 2003. Aprendizaje espacial y laberinto de agua: metodología y aplicaciones. Psicothema 15(4):539-544.

Vickers MH, Reddy S, Ikenasio BA, Breier BH. 2001. Dysregulation of the adipoinsular axis -- a mechanism for the pathogenesis of hyperleptinemia and adipogenic diabetes induced by fetal programming. J Endocrinol 170(2):323-32.

Vieau D, Sebaai N, Leonhardt M, Dutriez-Casteloot I, Molendi-Coste O, Laborie C, Breton C, Deloof S, Lesage J. 2007. HPA axis programming by maternal undernutrition in the male rat offspring. Psychoneuroendocrinology 32 Suppl 1:S16-20.

Vinogradova OS. 2001. Hippocampus as comparator: role of the two input and two output systems of the hippocampus in selection and registration of information. Hippocampus 11(5):578-98.

Vogt KE, Regehr WG. 2001. Cholinergic modulation of excitatory synaptic transmission in the CA3 area of the hippocampus. J Neurosci 21(1):75-83.

Vuguin PM. 2007. Animal models for small for gestational age and fetal programming of adult disease. Horm Res 68(3):113-23.

Walsh RN, Cummins RA. 1976. The Open-Field Test: a critical review. Psychol Bull 83(3):482-504.

Wang H, Hu Y, Tsien JZ. 2006. Molecular and systems mechanisms of memory consolidation and storage. Prog Neurobiol 79(3):123-35.

Wang L, Xu RJ. 2007. The effects of perinatal protein malnutrition on spatial learning and memory behaviour and brain-derived neurotrophic factor concentration in the brain tissue in young rats. Asia Pac J Clin Nutr 16 Suppl 1:467-72.

Wang R, Spelke E. 2002. Human spatial representation: insights from animals. Trends Cogn Sci 6(9):376.

Watkins AJ, Wilkins A, Cunningham C, Perry VH, Seet MJ, Osmond C, Eckert JJ, Torrens C, Cagampang FR, Cleal J and others. 2008. Low protein diet fed exclusively during mouse oocyte maturation leads to behavioural and cardiovascular abnormalities in offspring. J Physiol 586(8):2231-44.

Weinstock M. 2001. Effects of maternal stress on development and behaviour in rat offspring. Stress 4(3):157-67.

Weinstock M. 2008. The long-term behavioural consequences of prenatal stress. Neurosci Biobehav Rev 32(6):1073-86.

Wells JC. 2007. The thrifty phenotype as an adaptive maternal effect. Biol Rev Camb Philos Soc 82(1):143-72.

West MJ, Coleman PD, Flood DG. 1988. Estimating the number of granule cells in the dentate gyrus with the disector. Brain Res 448(1):167-72.

Wiggins RC, Fuller G, Enna SJ. 1984. Undernutrition and the development of brain neurotransmitter systems. Life Sci 35(21):2085-94.

Yam KY, Naninck EF, Schmidt MV, Lucassen PJ, Korosi A. 2015. Early-life adversity programs emotional functions and the neuroendocrine stress system: the contribution of nutrition, metabolic hormones and epigenetic mechanisms. Stress 18(3):328-42.

Yates CM, Simpson J, Russell D, Gordon A. 1980. Cholinergic enzymes in neurofibrillary degeneration produced by aluminium. Brain Res 197(1):269-74.

Zalutsky RA, Nicoll RA. 1990. Comparison of two forms of long-term potentiation in single hippocampal neurons. Science 248(4963):1619-24.

Zambrano E, Bautista CJ, Deas M, Martinez-Samayoa PM, Gonzalez-Zamorano M, Ledesma H, Morales J, Larrea F, Nathanielsz PW. 2006. A low maternal protein diet during pregnancy and lactation has sex- and window of exposure-specific effects on offspring growth and food intake, glucose metabolism and serum leptin in the rat. J Physiol 571(Pt 1):221-30.

Zambrano E, Martinez-Samayoa PM, Bautista CJ, Deas M, Guillen L, Rodriguez-Gonzalez GL, Guzman C, Larrea F, Nathanielsz PW. 2005a. Sex differences in transgenerational alterations of growth and metabolism in progeny (F2) of female offspring (F1) of rats fed a low protein diet during pregnancy and lactation. J Physiol 566(Pt 1):225-36.

Zambrano E, Rodriguez-Gonzalez GL, Guzman C, Garcia-Becerra R, Boeck L, Diaz L, Menjivar M, Larrea F, Nathanielsz PW. 2005b. A maternal low protein diet during pregnancy and lactation in the rat impairs male reproductive development. J Physiol 563(Pt 1):275-84.

Zhang Y, Li N, Yang Z. 2010. Perinatal food restriction impaired spatial learning and memory behavior and decreased the density of nitric oxide synthase neurons in the hippocampus of adult male rat offspring. Toxicol Lett 193(2):167-72.

Zhang Y, Wei J, Yang Z. 2013. Perinatal undernutrition attenuates field excitatory postsynaptic potentials and influences dendritic spine density and morphology in hippocampus of male rat offspring. Neuroscience 244:31-41.

Zhao S, Studer D, Chai X, Graber W, Brose N, Nestel S, Young C, Rodriguez EP, Saetzler K, Frotscher M. 2012. Structural plasticity of spines at giant mossy fiber synapses. Front Neural Circuits 6:103.

Int. J. Devl Neuroscience 29 (2011) 177–182

Contents lists available at ScienceDirect

International Journal of Developmental Neuroscience

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

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

L.A. Reyes-Castro[a], J.S. Rodriguez[b], G.L. Rodríguez-González[a], R.D. Wimmer[b], T.J. McDonald[b], F. Larrea[a], P.W. Nathanielsz[b], E. Zambrano[a,*]

[a] Department of Reproductive Biology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, 14000, Mexico
[b] Center for Pregnancy and Newborn Research, Department of Obstetrics, University of Texas Health Science Center San Antonio, San Antonio, TX, 78229, USA

ARTICLE INFO

Article history:
Received 4 August 2010
Received in revised form 22 October 2010
Accepted 9 November 2010

Keywords:
Developmental programming
Learning
Motivation
Protein restriction
Operant conditioning

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.

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). A 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.,

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.

* Corresponding author. Tel.: +52 55 5487 0900x2417; fax: +52 55 5655 9859.
E-mail address: zamgon@servidor.unam.mx (E. Zambrano).

0736-5748/$36.00 © 2010 ISDN. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.ijdevneu.2010.11.002

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
	3.85 kcal/g	3.85 kcal/g

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 "Instituto 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 ± 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, 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 23 h/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 (±30-min). 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 s 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 s. Each trial was followed by an inter-trial interval of 5 s during which the lever was retracted. FR – 1 conditioning was completed when the animal earned 20 reinforcements during a 15 min 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 10 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 min and on each trial the lever was presented for 300 s. 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 min 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 ± 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.

Table 2
Pup weight and morphometric measurements at birth of rats fed the control (20% casein) or restricted (10% casein) diet during pregnancy. Mean ± SEM; n refers to litters. *$p < 0.05$ different from control.

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

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 ± 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 ± 13^{a}	324 ± 17^{b}	352 ± 6^{ab}	364 ± 15^{ab}
150 d	420 ± 12^{a}	362 ± 19^{b}	382 ± 5^{ab}	403 ± 16^{ab}

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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 2B).

3.4. Free sucrose consumption

No differences were determined (in mL) across three 30-min 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

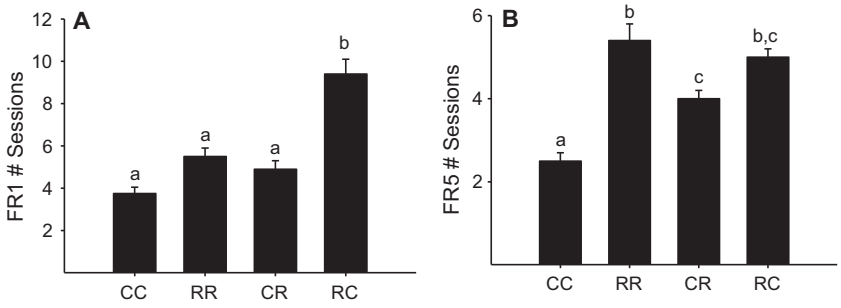

Fig. 1. Operant conditioning. (A) Comparison of FR – 1 conditioning shows the number of sessions to reach performance criterion. (B) Comparison of FR – 5 conditioning shows the number of sessions to reach performance criterion. Offspring of rats fed with control (C = 20% casein) or restricted (R = 10%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

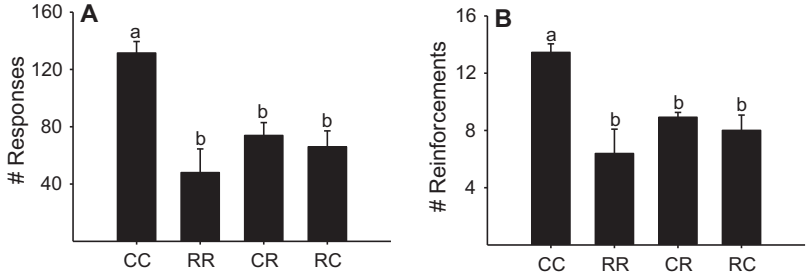

Fig. 2. Progressive ratio. (A) Average number of responses made during 10 sessions. (B) Average number of reinforcements earned during 10 sessions. Offspring of rats fed with control (C = 20% casein) or restricted (R = 10% casein) diet during pregnancy (first letter) and lactation (second letter). Mean ± SEM, $n = 5$ offspring from different litters. Groups not sharing a letter are statistically different, $p < 0.05$.

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 vs. 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, 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,b). 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.

Acknowledgements

L Reyes-Castro and GL Rodriguez-Gonzalez are graduate students from Doctorado en Ciencias Biomédicas, Facultad de Medicina, Universidad Nacional Autónoma de México and are recipients of CONACyT fellowship. This work was partially supported by Consejo Nacional de Ciencia y Tecnología (CONACyT - 48839) México and the NIH HD21350.

References

Allvin, K., Ankarberg-Lindgren, C., Fors, H., Dahlgren, J., 2008. Elevated serum levels of estradiol, dihydrotestosterone, and inhibin B in adult males born small for gestational age. J. Clin. Endocrinol. Metab. 93, 1464–1469.

Antonow-Schlorke, I., Kuhn, B., Muller, T., Schubert, H., Sliwka, U., Nathanielsz, P.W., Schwab, M., 2001. Antenatal betamethasone treatment reduces synaptophysin immunoreactivity in presynaptic terminals in the fetal sheep brain. Neurosci. Lett. 297, 147–150.

Antonow-Schlorke, I., Schwab, M., Li, C., Nathanielsz, P.W., 2003. Glucocorticoid exposure at the dose used clinically alters cytoskeletal proteins and presynaptic terminals in the fetal baboon brain. J. Physiol. 547, 117–123.

Armitage, J.A., Khan, I.Y., Taylor, P.D., Nathanielsz, P.W., Poston, L., 2004. Developmental programming of the metabolic syndrome by maternal nutritional imbalance: how strong is the evidence from experimental models in mammals? J. Physiol. 561, 355–377.

Bedi, K.S., 2003. Nutritional effects on neuron numbers. Nutr. Neurosci. 6, 141–152.

Benton, D., 2008. The influence of children's diet on their cognition and behavior. Eur. J. Nutr. 47 (Suppl. 3), 25–37.

Bouret, S.G., 2010. Development of hypothalamic neural networks controlling appetite. Forum Nutr. 63, 84–93.

Clancy, B., Finlay, B.L., Darlington, R.B., Anand, K.J., 2007a. Extrapolating brain development from experimental species to humans. Neurotoxicology 28, 931–937.

Clancy, B., Kersh, B., Hyde, J., Darlington, R.B., Anand, K.J., Finlay, B.L., 2007b. Web-based method for translating neurodevelopment from laboratory species to humans. Neuroinformatics 5, 79–94.

Coupe, B., Dutriez-Casteloot, I., Breton, C., Lefevre, F., Mairesse, J., ckes-Coopman, A., Silhol, M., Tapia-Arancibia, L., Lesage, J., Vieau, D., 2009. Perinatal undernutrition modifies cell proliferation and brain-derived neurotrophic factor levels during critical time-windows for hypothalamic and hippocampal development in the male rat. J. Neuroendocrinol. 21, 40–48.

Dominguez-Salazar, E., Camacho, F.J., Paredes, R.G., 2008. Perinatal inhibition of aromatization enhances the reward value of sex. Behav. Neurosci. 122, 855–860.

Duggan, M.B., 2003. Nutritional update: relevance to maternal and child health in East Africa. Afr. Health Sci. 3, 136–143.

Erhard, H.W., Boissy, A., Rae, M.T., Rhind, S.M., 2004. Effects of prenatal undernutrition on emotional reactivity and cognitive flexibility in adult sheep. Behav. Brain Res. 151, 25–35.

French, N.P., Hagan, R., Evans, S.F., Mullan, A., Newnham, J.P., 2004. Repeated antenatal corticosteroids: effects on cerebral palsy and childhood behavior. Am. J. Obstet. Gynecol. 190, 588–595.

Galler, J.R., Ramsey, F., 1989. A follow-up study of the influence of early malnutrition on development: behavior at home and at school. J. Am. Acad. Child Adolesc. Psychiatry 28, 254–261.

Galler, J.R., Ramsey, F., Solimano, G., 1984. The influence of early malnutrition on subsequent behavioral development III. Learning disabilities as a sequel to malnutrition. Pediatr. Res. 18, 309–313.

Galler, J.R., Ramsey, F., Solimano, G., Lowell, W.E., Mason, E., 1983. The influence of early malnutrition on subsequent behavioral development. I. Degree of impairment in intellectual performance. J. Am. Acad. Child Psychiatry 22, 8–15.

Galler, J.R., Ramsey, F.C., Forde, V., Salt, P., Archer, E., 1987. Long-term effects of early kwashiorkor compared with marasmus. II. Intellectual performance. J. Pediatr. Gastroenterol. Nutr. 6, 847–854.

Guilloteau, P., Zabielski, R., Hammon, H.M., Metges, C.C., 2009. Adverse effects of nutritional programming during prenatal and early postnatal life, some aspects of regulation and potential prevention and treatments. J. Physiol. Pharmacol. 60 (Suppl 3), 17–35.

Hamosh, M., Salem Jr., N., 1998. Long-chain polyunsaturated fatty acids. Biol. Neonate 74, 106–120.

Harding, J.E., 2001. The nutritional basis of the fetal origins of adult disease. Int. J. Epidemiol. 30, 15–23.

Hotchkiss, A.K., Lambright, C.S., Ostby, J.S., Parks-Saldutti, L., Vandenbergh, J.G., Gray Jr., L.E., 2007. Prenatal testosterone exposure permanently masculinizes anogenital distance, nipple development, and reproductive tract morphology in female Sprague-Dawley rats. Toxicol. Sci. 96, 335–345.

Jazin, E., Cahill, L., 2010. Sex differences in molecular neuroscience: from fruit flies to humans. Nat. Rev. Neurosci. 11, 9–17.

Johnson, J.W., Mitzner, W., Beck, J.C., London, W.T., Sly, D.L., Lee, P.A., Khouzami, V.A., Cavalieri, R.L., 1981. Long-term effects of betamethasone on fetal development. Am. J. Obstet. Gynecol. 141, 1053–1064.

Karemaker, R., Heijnen, C.J., Veen, S., Baerts, W., Samsom, J., Visser, G.H., Kavelaars, A., van Doornen, L.J., van, B.F., 2006. Differences in behavioral outcome and motor development at school age after neonatal treatment for chronic lung disease with dexamethasone versus hydrocortisone. Pediatr. Res. 60, 745–750.

Karemaker, R., Kavelaars, A., ter, W.M., Tersteeg-Kamperman, M., Baerts, W., Veen, S., Samsom, J.F., Visser, G.H., van, B.F., Heijnen, C.J., 2008. Neonatal dexamethasone treatment for chronic lung disease of prematurity alters the hypothalamus-pituitary-adrenal axis and immune system activity at school age. Pediatrics 121, e870–e878.

Landon, J., Davison, M., Krageloh, C.U., Thompson, N.M., Miles, J.L., Vickers, M.H., Fraser, M., Breier, B.H., 2007. Global undernutrition during gestation influences learning during adult life. Learn. Behav. 35, 79–86.

Lister, J.P., Blatt, G.J., DeBassio, W.A., Kemper, T.L., Tonkiss, J., Galler, J.R., Rosene, D.L., 2005. Effect of prenatal protein malnutrition on numbers of neurons in the principal cell layers of the adult rat hippocampal formation. Hippocampus 15, 393–403.

Lister, J.P., Tonkiss, J., Blatt, G.J., Kemper, T.L., DeBassio, W.A., Galler, J.R., Rosene, D.L., 2006. Asymmetry of neuron numbers in the hippocampal formation of prenatally malnourished and normally nourished rats: a stereological investigation. Hippocampus 16, 946–958.

Lucas, A., 2005. Long-term programming effects of early nutrition—implications for the preterm infant. J. Perinatol. 25 (Suppl 2), S2–S6.

Matthews, S.G., 2001. Antenatal glucocorticoids and the developing brain: mechanisms of action. Semin. Neonatol. 6, 309–317.

Miles, J.L., Landon, J., Davison, M., Krageloh, C.U., Thompson, N.M., Triggs, C.M., Breier, B.H., 2009. Prenatally undernourished rats show increased preference for wheel running v. lever pressing for food in a choice task. Br. J. Nutr. 101, 902–908.

Morgane, P.J., Austin-LaFrance, R., Bronzino, J., Tonkiss, J., Diaz-Cintra, S., Cintra, L., Kemper, T., Galler, J.R., 1993. Prenatal malnutrition and development of the brain. Neurosci. Biobehav. Rev. 17, 91–128.

Morgane, P.J., Galler, J.R., Mokler, D.J., 2005. A review of systems and networks of the limbic forebrain/limbic midbrain. Prog. Neurobiol. 75, 143–160.

Morgane, P.J., Mokler, D.J., Galler, J.R., 2002. Effects of prenatal protein malnutrition on the hippocampal formation. Neurosci. Biobehav. Rev. 26, 471–483.

Nijland, M.J., Ford, S.P., Nathanielsz, P.W., 2008. Prenatal origins of adult disease. Curr. Opin. Obstet. Gynecol. 20, 132–138.

Nuyt, A.M., Alexander, B.T., 2009. Developmental programming and hypertension. Curr. Opin. Nephrol. Hypertens. 18, 144–152.

Puts, D.A., McDaniel, M.A., Jordan, C.L., Breedlove, S.M., 2008. Spatial ability and prenatal androgens: meta-analyses of congenital adrenal hyperplasia and digit ratio (2D:4D) studies. Arch. Sex Behav. 37, 100–111.

Ranade, S.C., Rose, A., Rao, M., Gallego, J., Gressens, P., Mani, S., 2008. Different types of nutritional deficiencies affect different domains of spatial memory function checked in a radial arm maze. Neuroscience 152, 859–866.

Russig, H., Pezze, M.A., Nanz-Bahr, N.I., Pryce, C.R., Feldon, J., Murphy, C.A., 2003. Amphetamine withdrawal does not produce a depressive-like state in rats as measured by three behavioral tests. Behav. Pharmacol. 14, 1–18.

Seckl, J.R., 2008. Glucocorticoids, developmental 'programming' and the risk of affective dysfunction. Prog. Brain Res. 167, 17–34.

Solomons, N.W., 2009. Developmental origins of health and disease: concepts, caveats, and consequences for public health nutrition. Nutr. Rev. 67 (Suppl. 1), S12–S16.

Stein, A.D., Pierik, F.H., Verrips, G.H., Susser, E.S., Lumey, L.H., 2009. Maternal exposure to the Dutch famine before conception and during pregnancy: quality of life and depressive symptoms in adult offspring. Epidemiology 20, 909–915.

Symonds, M.E., Sebert, S.P., Hyatt, M.A., Budge, H., 2009. Nutritional programming of the metabolic syndrome. Nat. Rev. Endocrinol. 5, 604–610.

Szuran, T.F., Pliska, V., Pokorny, J., Welzl, H., 2000. Prenatal stress in rats: effects on plasma corticosterone, hippocampal glucocorticoid receptors, and maze performance. Physiol. Behav. 71, 353–362.

Taylor, P.D., Poston, L., 2007. Developmental programming of obesity in mammals. Exp. Physiol. 92, 287–298.

Tonkiss, J., Galler, J.R., 1990. Prenatal protein malnutrition and working memory performance in adult rats. Behav. Brain Res. 40, 95–107.

Tonkiss, J., Galler, J.R., Formica, R.N., Shukitt-Hale, B., Timm, R.R., 1990a. Fetal protein malnutrition impairs acquisition of a DRL task in adult rats. Physiol. Behav. 48, 73–77.

Tonkiss, J., Shukitt-Hale, B., Formica, R.N., Rocco, F.J., Galler, J.R., 1990b. Prenatal protein malnutrition alters response to reward in adult rats. Physiol. Behav. 48, 675–680.

Tonkiss, J., Shultz, P., Galler, J.R., 1994. An analysis of spatial navigation in prenatally protein malnourished rats. Physiol. Behav. 55, 217–224.

Tonkiss, J., Shultz, P.L., Shumsky, J.S., Galler, J.R., 1997. Development of spatial navigation following prenatal cocaine and malnutrition in rats: lack of additive effects. Neurotoxicol. Teratol. 19, 363–372.

Torres, N., Bautista, C.J., Tovar, A.R., Ordaz, G., Rodriguez-Cruz, M., Ortiz, V., Granados, O., Nathanielsz, P.W., Larrea, F., Zambrano, E., 2009. Protein restriction during pregnancy affects maternal liver lipid metabolism and fetal brain lipid composition in the rat. Am. J. Physiol Endocrinol. Metab..

Uno, H., Eisele, S., Sakai, A., Shelton, S., Baker, E., DeJesus, O., Holden, J., 1994. Neurotoxicity of glucocorticoids in the primate brain. Horm. Behav. 28, 336–348.

Uno, H., Lohmiller, L., Thieme, C., Kemnitz, J.W., Engle, M.J., Roecker, E.B., Farrell, P.M., 1990. Brain damage induced by prenatal exposure to dexamethasone in fetal rhesus macaques. I. Hippocampus. Brain Res. Dev. Brain Res. 53, 157–167.

Vuguin, P.M., 2007. Animal models for small for gestational age and fetal programming of adult disease. Horm. Res. 68, 113–123.

Warner, M.J., Ozanne, S.E., 2010. Mechanisms involved in the developmental programming of adulthood disease. Biochem. J. 427, 333–347.

Weinstock, M., 2008. The long-term behavioural consequences of prenatal stress. Neurosci. Biobehav. Rev. 32, 1073–1086.

Zambrano, E., Bautista, C.J., Deas, M., Martinez-Samayoa, P.M., Gonzalez-Zamorano, M., Ledesma, H., Morales, J., Larrea, F., Nathanielsz, P.W., 2006. A low maternal protein diet during pregnancy and lactation has sex- and window of exposure-specific effects on offspring growth and food intake, glucose metabolism and serum leptin in the rat. J. Physiol. 571, 221–230.

Zambrano, E., Martinez-Samayoa, P.M., Bautista, C.J., Deas, M., Guillen, L., Rodriguez-Gonzalez, G.L., Guzman, C., Larrea, F., Nathanielsz, P.W., 2005a. Sex differences in transgenerational alterations of growth and metabolism in progeny (F2) of female offspring (F1) of rats fed a low protein diet during pregnancy and lactation. J. Physiol. 566, 225–236.

Zambrano, E., Rodriguez-Gonzalez, G.L., Guzman, C., Garcia-Becerra, R., Boeck, L., Diaz, L., Menjivar, M., Larrea, F., Nathanielsz, P.W., 2005b. A maternal low protein diet during pregnancy and lactation in the rat impairs male reproductive development. J. Physiol. 563, 275–284.

Zhang, Y., Li, N., Yang, Z., 2010. Perinatal food restriction impaired spatial learning and memory behavior and decreased the density of nitric oxide synthase neurons in the hippocampus of adult male rat offspring. Toxicol. Lett. 193, 167–172.

Behavioural Brain Research 227 (2012) 324–329

Contents lists available at ScienceDirect

Behavioural Brain Research

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

Research report

Pre- and/or postnatal protein restriction developmentally programs affect and risk assessment behaviors in adult male rats

L.A. Reyes-Castro[a,1], J.S. Rodriguez[b,1], G.L. Rodríguez-González[a], R. Chavira[a], C.J. Bautista[a], T.J. McDonald[b], P.W. Nathanielsz[b], E. Zambrano[a,*]

[a] Department of Reproductive Biology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, 14000, Mexico
[b] Center for Pregnancy and Newborn Research, Department of Obstetrics, University of Texas Health Science Center San Antonio, San Antonio, TX, 78229, USA

ARTICLE INFO

Article history:
Received 29 October 2010
Received in revised form 26 May 2011
Accepted 9 June 2011
Available online 17 June 2011

Keywords:
Anxiety
Stress
Locomotion
Protein restriction
Emotional reactivity

ABSTRACT

Developmental programming resulting from a suboptimal intrauterine environment can predispose offspring to a wide-range of lifelong health complications. Little is known about the effects maternal protein restriction during pregnancy and/or lactation has on offspring neurodevelopment. We hypothesized that maternal isocaloric low protein diet during pregnancy and/or lactation would negatively influence male offspring affect and risk assessment behaviors as measured by elevated plus maze and open field tests. Control mothers received 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 offspring risk assessment, anxiety and exploratory behaviors. Elevated plus maze results showed an effect of pre- and/or postnatal diet manipulation in open arm time ($p < 0.05$) with increases seen in the RR (157 ± 22.7 s), CR (137 ± 23.2 s) and RC (146.8 ± 10.8 s) offspring relative to CC (52 ± 8.6 s) offspring. This behavior indicates decreased avoidance (less anxiety) and increased exploration by experimental groups. However, in the open field test the RR (17 ± 4.2 entries) offspring entered the center zone less than the CC (35 ± 6.6 entries) offspring thus exhibiting increased anxiety with no other groups showing effects. Elevated levels of corticosterone were measured before, during and after immobilization in the RR compared to CC offspring. These findings show protein restriction during critical periods of development negatively program offspring behavior. The underlying anatomical structures affected remain to be elucidated.

1. Introduction

Fetal and offspring morbidity associated with poor maternal nutrition during development is a global health concern and results in developmental programming of adult disease in offspring [1]. In particular, a well-balanced maternal diet is vital for the development of the fetal and neonatal central nervous system [2]. For example, cognition and behaviors such as learning and memory, anxiety, and risk assessment depend on the proper development of the limbic system and associated neural areas [3]. Impaired development of limbic system neurons, which play an important role in normal affect and cognition, occurs when maternal protein intake is reduced with resulting brain abnormalities [4–8]. Epidemiological studies also show that insufficient protein availability during development has detrimental effects on the brain and subsequent behavior, cognition and emotional reactivity [9–16]. These critical findings indicate the need for precisely controlled animal models to study perinatal nutritional challenges and their effects on offspring development and behavior.

Several studies report effects of poor maternal nutrition on the development of brain and behavioral outcomes in offspring. For example, protein restriction during gestation and/or lactation modifies neuronal proliferation in the hypothalamic and hippocampal regions in rat offspring [17]. Behavioral outcomes following prenatal protein restriction (6–9% casein) or other types of malnutrition in rodent offspring reveal high impulsiveness and exploration (less anxiety) in the elevated plus maze (EPM) and short inhibitory avoidance (disinhibition) latencies in the elevated T-maze [18–22]. However, several outcomes have been described in the open field test – an anxious phenotype, decreased locomotion as well as no effects [22–27]. Although equivocal and sometimes

Abbreviations: C, control diet; R, protein restriction diet; CC, control diet during pregnancy and lactation; RR, protein restricted diet during pregnancy and lactation; CR, control diet during pregnancy and protein restriction during lactation; RC, protein restriction during pregnancy and control diet during lactation; PND, postnatal day; HPA, hypothalamo-pituitary–adrenal axis; EPM, elevated plus maze.

* Corresponding author. Tel.: +52 55 5487 0900x2417, fax: +52 55 5655 9859.
E-mail address: zamgon@servidor.unam.mx (E. Zambrano).

[1] These authors equally contributed as first author.

0166-4328/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbr.2011.06.008

contradictory, these findings on emotional reactivity are indicative of potential behavioral modifications due to neurodevelopmental modulation subsequent to insufficient protein in early life and establish the concept that reduced perinatal protein has effects on emotional reactivity when protein is reduced by 75% of control diet (25% protein diet reduced to 6%) [18–27]. However, the effects of smaller decreases in perinatal protein have yet to be determined. Since behavioral effects have been reported following extreme reduction in protein, we sought to investigate the potential effects on offspring behavior of a more modest protein restriction (a 50% decrease) which potentially more accurately reflects the human dietary insecurities in developed countries (for review see Ref. [28]). We assessed anxiety and exploratory behaviors, using the EPM and open field testing, in adult male rat offspring protein restricted during pregnancy and/or lactation. Control mothers received 20% casein (C) while restricted mothers (R) received 10% casein to provide four groups: CC, RR, CR, and RC (first letter pregnancy diet and second letter lactation diet). These dietary manipulation combinations have not previously been administered in the context of anxiety and exploratory assessments. To more directly match human conditions, offspring were not cross-fostered but kept with their biological dams during development. We hypothesized that perinatal protein restriction would affect development of anxiety and exploratory-based behaviors.

2. Materials and methods

2.1. Care and maintenance of animals

All procedures were approved by the Animal Experimentation Ethics Committee of the "Instituto Nacional de Ciencias Médicas y Nutrición, Salvador Zubirán" (INNSZ), Mexico. Details of maternal diet, breeding, and management of the four offspring groups have been published in detail [29]. Briefly, 40 virgin female albino Wistar rats 11 to 13 weeks of age and weighing 212 ± 6 g (mean ± SEM) were obtained from INNSZ. Female rats with regular estrous cycles were fed normal laboratory chow (control diet, Zeigler Rodent RQ 22-5, USA) under controlled lighting (lights on from 7:00 a.m. to 7:00 p.m. at 22–23 °C) and mated overnight with proven male breeders. The day spermatozoa were present in the vaginal smear was designated as day of conception (day 0). Only rats impregnated within 5 days following introduction of the fertile male were retained in the study. Pregnant rats were transferred to individual acrylic cages and allocated randomly to one of two groups: fed either 20% casein ($n = 20$, control diet—C) or 10% casein isocaloric diet ($n = 20$, restricted diet—R) [30]. Food and water were available *ad libitum* and chow provided in the form of flat biscuits retained behind a tray grill through which the rats nibbled the food.

Offspring delivery occurred in the early daylight hours between 9:00 a.m. and noon on post-conception day 22, which was designated postnatal day (PND) 0. Pup weight and morphometric parameters were recorded at birth. Ano-genital distance was measured with calipers and expressed as ano-genital distance/body weight as previously reported [29–34]. Using our previously published data [31], sex was judged according to whether the ano-genital distance was <(female) or >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 while maintaining as close to a 1:1 sex ratio as possible. Morphometric measurements were performed on the litters prior to culling them to 10. 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 C diet and housed 4 offspring in the same treatment group per cage. Two weeks prior to behavioral testing (PND 75) and during the behavioral experiments, subjects were maintained on a reverse 12 h light/dark cycle (lights off at 7 a.m. and on at 7 p.m.). Behavioral assessments occurred during the dark phase on PNDs 90–95. The EPM was administered first followed by the open field test on the next day.

2.2. Elevated plus maze

Eight unrelated naïve subjects per treatment group were tested. The EPM was constructed of dark grey plastic situated 64 cm above the floor. It consisted of two unprotected arms (open arms) facing each other, and two arms protected by high grey walls (closed arms). The four arms (45 cm × 10 cm each) extended from a common central platform (10 cm × 10 cm). The light level was 30 lux in the open arms and 6 lux in the closed arms. The rat's position on the maze was recorded via a video camera mounted on the ceiling above the center of the maze. The camera was connected to a video tracking motion analysis system (Ethovision, Noldus Information Technology by Wageningen, The Netherlands) running on a personal computer. To start the session, each rat was placed individually into the center of the maze facing an open arm. After 10 min of EPM exploration, the rat was returned to its home cage and the EPM was cleaned with 70% ethanol. An experimenter naïve to treatment group of the subject being tested manually scored the number of entries into the predefined zones of the open and closed arms, while the Ethovision system monitored the distance moved and the time spent in the different zones. An arm entry was scored only if the rat's center of gravity entered into the arm. The animals were tested in a randomized sequence based on early life dietary manipulation. We excluded animals that fell off the EPM during testing (2 from the RC group and 1 from the RR) and substituted that animal with another male from the same litter.

2.3. Open field

The same animals studied in the EPM were evaluated the next day in a 60-min open field test. The open field, made of dark grey Plexiglas, consisted of a four square arena (101 cm × 101 cm and 34 cm high) which was located in an experimental room illuminated by low light (12 lux). A video camera was mounted above the arena connected to a monitor and a video tracking motion analysis system (Ethovision, Noldus Information Technology by, Wageningen, The Netherlands) to record the locomotor activity of the rats in the entire arena (total distance measured in meters). A virtual square center was defined in the open field arena and the locomotor activity of the rats inside this zone was measured. Each rat was placed individually into the center of the open field arena and subjects of each group were tested equally in all open field apparatuses. Following each session all zones were cleaned with 70% ethanol.

2.4. Immobilization and corticosterone measurement

Following the open field test but prior to immobilization/corticosterone assessment, progressive ratio sessions commenced on PND 96 to assess motivation (findings to be reported elsewhere) during which the subjects were water restricted. Subjects were returned to *ad libitum* water following that task. Starting at 12:00 h on PND 220, a blood sample was taken from the tail vein to determine time 0 corticosterone serum levels. Animals were then immobilized for 20 min and blood samples obtained at 20, 40, 80, and 120 min. Blood samples were centrifuged at 4 °C for 15 min at 3500 rpm to remove red blood cells and serum stored at −20 °C until all samples were analyzed. Corticosterone serum levels were determinate by radioimmunoassay using commercial rat kits, DPC Coat-a-count (TKRC1) from Diagnostic Products (Los Angeles, CA, USA). Intra- and inter-assay variability were <6 and <7%. The kit was used in accordance with manufacturer's instructions and samples were measured in duplicate.

2.5. Statistical analyses

All data are presented as mean ± SEM, alpha level was 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. Behavioral endpoints and corticosterone area under the curve were analyzed by one-way ANOVA with post hoc analyses using Tukey's (SigmaStat 3.5). Corticosterone levels across time points and between treatment groups were compared by using 2-way repeated measures ANOVA followed by Bonferroni post hoc tests (GraphPad Prism 5.0).

3. Results

3.1. Offspring morphometrics

Of the forty dams utilized in this study, 4 dams were eliminated from each of the C and R groups for various factors. Additionally, we have previously reported differences in the amount of food intake in dams at 10 days lactation [29] and no differences in preweaning offspring [35], so food and water intake were not measured in this study. Table 1 shows morphometric analysis of offspring at birth. Body weight of pups born to R dams was lower than that of pups from dams fed the C diet. Ano-genital distance relative to body weight was increased by 7% in the R offspring. No changes were observed in other parameters at birth. As adults when operant assessments began (PND 90), the CR offspring body weight was lower than that of CC and RC offspring while RR offspring did not differ from any of the other groups (Table 1).

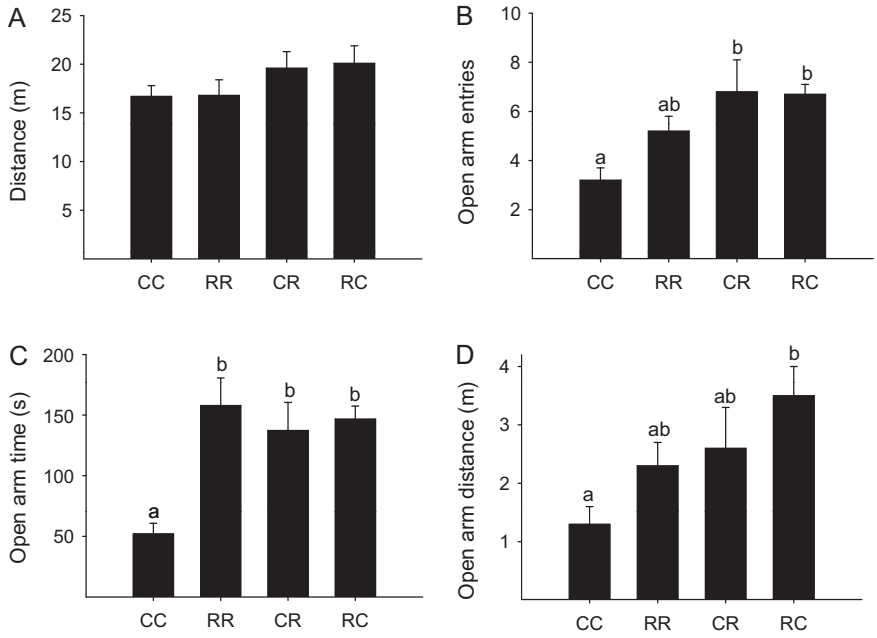

Fig. 1. Elevated plus maze comparisons. (A) Distance traveled shows no difference between groups. (B) Open arm entries reveal the CR and RC made more entries than the CC group. (C) Open arm time shows the RR, CR and RC offspring spent more time in the open arm as compared to the CC offspring. (D) Open arm distance shows the RC group traveled more in the open arm as compared to the CC group with no differences in the RR and CR offspring. Groups not sharing a letter are statistically different, $p < 0.05$. Mean ± SEM, $n = 8$ offspring from different litters.

Table 1
Pup weight and birth morphometrics from offspring born to dams fed the control (20% casein) or restricted (10% casein) diet during pregnancy and adult weights (PND 90) from CC, RR, CR and RC offspring are shown. * denotes different from control; groups not sharing a letter are statistically different, $p < 0.05$. Mean ± SEM; $n = 8$, the number of litters from which subjects were obtained.

Newborn offspring	Control ($n = 8$)	Restricted ($n = 8$)
Weight (g)	5.8 ± 0.07	5.2 ± 0.01*
Length (mm)	4.6 ± 0.03	4.5 ± 0.05
Ano-genital distance (mm)	3.4 ± 0.10	3.2 ± 0.02
Ano-genital distance (mm/g)	0.58 ± 0.01	0.62 ± 0.01*
Head diameter (mm)	11.4 ± 0.02	11.4 ± 0.02
Abdominal diameter (mm)	12.2 ± 0.03	12.2 ± 0.03
Head:abdominal ratio	0.93 ± 0.01	0.93 ± 0.01

Adult Offspring	CC	RR	CR	RC
Body weight (g)	330 ± 13a	298 ± 13ab	281 ± 6b	340 ± 7a

3.2. Elevated plus maze

Elevated plus maze comparisons for total distance traveled revealed no differences between groups [$F_{(3,31)} = 1.17$, $p = 0.34$, Fig. 1A]. Comparisons for open arm entries showed the CR and RC offspring made more open arm entries compared to the CC offspring but not RR [$F_{(3,35)} = 4.54$, $p < 0.05$, Fig. 1B]. RR, CR and RC offspring spent an increased amount of time in the open arms compared with the CC offspring [$F_{(3,35)} = 7.45$, $p < 0.05$, Fig. 1C]. RC offspring showed increased open arm distance traveled compared with CC offspring but not RR and CR [$F_{(3,35)} = 3.08$, $p < 0.05$, Fig. 1D].

3.3. Open field

Table 2 shows the comparisons in the open field behavioral endpoints. An overall effect of pre- and/or postnatal diet in offspring was determined for number of entries into the center zone [$F_{(3,35)} = 3.69$, $p < 0.05$], with a post hoc borderline effect ($p = 0.07$) in the RR offspring making less entries than the CC with no differences in the CR and RC groups. Comparisons for time spent in the center or border zones, distance traversed in center zone or border zone and total distance showed no differences between groups.

3.4. Immobilization and corticosterone levels

There was an overall effect of group ($F_{(3,20)} = 4.08$, $p < 0.05$) and time ($F_{(4,20)} = 20.93$, $p < 0.0001$) on serum corticosterone levels but no interaction ($F_{(12,80} = 1.79$, $p = 0.06$) between variables (Fig. 2A). Fig. 2A represents average corticosterone serum levels at 0, 20, 40, 80 and 120 min time points during physical restraint (immobilization) in the CC, RR, CR and RC offspring. Increases in corticosterone were observed in the CC, RR and RC groups at 20 min; in all groups at 40 min; in the CC, CR and RC groups at 80 min; and in the CC and CR offspring at 120 min. Fig. 2B shows corticosterone area under the curve (AUC) for the entire duration of behavioral assessment. RR offspring displayed an overall increased corticosterone

Table 2
Open field test comparisons. An overall effect of perinatal diet was determined for number of center zone entries ($p < 0.05$), post hoc comparisons showed the RR offspring entered the center zone marginally less than the CC offspring (†, $p = 0.07$). Mean ± SEM; $n = 8$ offspring from different litters.

	Number entries center zone	Time center zone (s)	Time border zone (s)	Distance center zone (m)	Distance border zone (m)	Total Distance (m)
CC	35.8 ± 6.6	124.6 ± 25.2	3475.4 ± 25.1	14.1 ± 2.4	114.5 ± 10.3	128.7 ± 9
RR	17.6 ± 4.2†	116.7 ± 33.0	3483.3 ± 33.0	9.4 ± 2.4	72.5 ± 12.3	82.0 ± 11
CR	34.4 ± 5.1	127.7 ± 17.0	3472.3 ± 17.0	13.4 ± 2.4	74.6 ± 10.8	88.1 ± 11.2
RC	19.2 ± 3.6	141.0 ± 59.2	3459.0 ± 59.2	7.2 ± 1.7	76.2 ± 16.0	83.5 ± 15.4

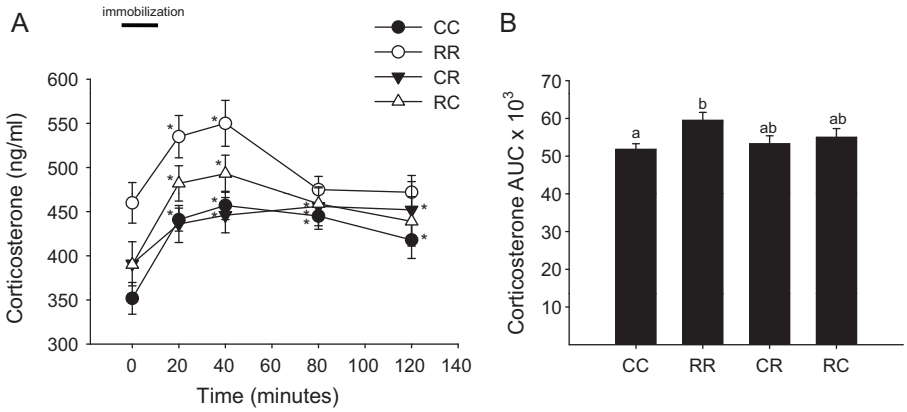

Fig. 2. Immobilization and corticosterone measurement at PND 220. (A) An overall effect of perinatal diet on corticosterone levels was determined between groups during immobilization and differences within groups are shown across time; * indicates within group differences from time 0, $p < 0.05$. (B) Area under the curve (AUC) analysis indicates that RR offspring had increased corticosterone production compared to CC offspring during the immobilization test. Groups not sharing a letter are statistically different, $p < 0.05$. Mean ± SEM, $n = 8$ offspring from different litters.

AUC compared with CC offspring ($p < 0.05$), while the CR and RC offspring had intermediate values that did not reach significance.

4. Discussion

The goal of these studies was to assess anxiety/stress and exploratory/locomotor behavior in male offspring delivered by dams fed a reduced protein diet in two specific windows of offspring development; pregnancy and lactation. Morphometric effects due to maternal protein restriction (50% reduced) were determined in offspring, such as reduced birth weights and increased ano-genital distance, demonstrating the negative effects of decrease in protein intake during pregnancy on fetal maturation. Many studies have reported different effects of maternal protein restriction on birth weight including a decrease [33,36–40], no change [29,41] or increase [42] which may be attributed to the level of protein restriction and/or carbohydrate compensation in an isocaloric diet. Our birth weight results are consistent with outcomes reported in models of intra-uterine growth restriction and fetal programming [43]. In addition, increases in the ano-genital distance of offspring born to protein restricted dams replicates our previous studies [34]. We have also reported a concomitant increase of the ano-genital distance in protein restricted offspring and elevated maternal androgen levels at term [29]. Maternal steroids can cross the placenta, and such exposure to transplacentally acquired androgens in fetal life can increase the ano-genital distance [44]. Effects of androgenic steroids on fetal development could have a role in the observed behavioral findings since human studies report impairment of spatial learning ability and emotional reactivity abnormalities in offspring exposed to excess levels of androgens *in utero* [45,46].

Behavioral observations in the EPM revealed differences in approach/avoidance behavior: the CR and RC offspring made more open arm entries and the RR, CR, and RC offspring spent more time in the open arms than the CC offspring. However, only the RC offspring traveled more in the open arms. Taken together, these pre- and/or postnatal protein restricted offspring show increased approach and less avoidance behavior (less anxiety) and thus exhibited disinhibition of avoidance as compared to controls. The results demonstrate the negative effect of reduced protein during pregnancy and/or lactation in male rats during risk assessment behaviors in the EPM and are supported by previous findings in rats of the same age subsequent to exposure to a greater degree of protein restriction during development [18–21]. The increased exploratory behavior in the RR, CR and RC groups in the EPM is unlikely due to increased food seeking since no differences were observed during a free sucrose consumption test [34].

In the open field test the pre- and postnatal protein restricted offspring (RR) showed a marginal decrease in the number of center zone entries; less than half the amount of CC center zone entries although it did not reach significant. This behavior is potentially linked to the increase in corticosterone measured in the RR offspring during physical restraint in the immobilization test. The elevated corticosterone levels could be a general physiological response in the RR offspring and thus a similar response could be predicted in the increased anxiety state displayed in the open field apparatus, although no attempts to detect corticosterone levels were implemented during evaluation of that behavior. The lack of effect in the CR and RC groups is in agreement with results obtained with similar developmental programming paradigms [25,26]. Similarly our findings of increased anxiety in the RR offspring are supported by others [22–24,27]. These inconsistent behavioral responses of the experimental groups in the two anxiety/stress based tests were not unexpected. Studies have shown stress-based tests that rely on unconditioned responses, such as in the two tests used here, assess different aspects of affect and that emotional reactivity is multidimensional [47–50]. A recent review states that the EPM, open field apparatus and the light/dark box tests should be administered concomitantly to more adequately assess anxiety/stress since time and/or sequence of test administration and other factors can influence outcomes [47]. One potential effect of testing sequentially across days is that this leads to reduction in observed effects with each subsequent test, in this case the open field. This appears to be a likely explanation for the CR and RC offspring behavior. When rats were administered the open field test they were no longer test naive, since they had previously been exposed to the EPM apparatus [49]. Alternatively, inconsistent results obtained across tests may be due to construct differences between tests or to uncontrolled, intra-individual fluctuations in behavior [47].

Mechanistically one might hypothesize that adequate availability of protein components required during normal neurodevelopment resulted in persistent dysfunction of the limbic system at the time of testing [2,4,7,8]. The effects of perinatal protein restriction on brain development have been reported in rats and swine. In most cases protein was restricted by roughly two-thirds of control diet during gestation and lactation [51,52] or only during pregnancy [53] or lactation [54,55]. Those studies in general show brain sparing in terms of weight. However, another study demonstrates reduced brain weight and volume in rat offspring delivered from dams administered an 8% casein diet during pregnancy or lactation

[54]. As adults those offspring displayed partial recovery of neural cell density with the RC offspring showing permanent residual deficiency versus the CR offspring implicating the prenatal period as a more critical neurodevelopmental time window. Recently, we have shown that protein restriction during gestation significantly decreases delivery to the fetus of essential fatty acids and lipids required for normal brain development in rat [56]. This deficiency in brain fatty acid content could affect important brain regions involved in approach/risk assessment behavior in the experimental offspring resulting in disinhibited behavior specific to the EPM. However, the potentially divergent mechanisms that are responsible for the different outcomes in the two behavioral tests remain to be established.

Human and animal studies show that early exposure to global under nutrition during the perinatal period is often associated with altered hypothalamic pituitary adrenal (HPA) axis function in later life [57,58]. In rodents, maternal under nutrition blunts HPA axis function in fetuses, induces small changes in young adults and is frequently associated with chronic hyperactivity of the neuroendocrine axis in older adults [57,59–61]. However, increased basal corticosterone concentrations have been measured in pre- and postnatal food restricted weaned and adult rats [59,62], which is in agreement with our current findings. Those studies indicate that developmental programming of the HPA axis by fetal and early postnatal under nutrition can result in persistent impairment of feedback [63]. In this report we measured an increase in postnatal corticosterone during immobilization in RR offspring with intermediate corticosterone levels in the RC and CR offspring relative to CC. These results suggest pre- and postnatal protein restriction in combination can have a negative effect on HPA axis function during physical restraint. This observation is in agreement with reports showing increased corticosterone or adrenocorticotropic hormone levels following dehydration stress in pre- and postnatal nutrient restricted offspring [59,62]. Developmental programming of the HPA axis seems a likely contributor to the behavioral and physiological observations reported here since dysregulation of the HPA axis occurs in response to perinatal protein restriction. At 2 days postnatal age offspring corticosterone levels are decreased, potentially due to the inhibition by high levels of steroid passing from the mother in the milk [33]. After weaning this maternal influence is removed and by 220 days HPA activity was increased in the present study.

In conclusion, human studies have shown negative effects on numerous developmental parameters such as affect, learning, intellect, neuromotor and behavioral development in offspring following perinatal malnutrition [9–16,64]. Our results show that protein restriction during the pre- and postnatal periods (RR), postnatal period alone (CR) or prenatal period alone (RC) disinhibits risk assessment in male offspring in the EPM. However, in the open field apparatus only the RR offspring showed increased avoidance, which could be related to the increased corticosterone levels observed during restraint testing. Additionally, during immobilization the CR and RC offspring had intermediate corticosterone levels demonstrating that protein insufficiency during either pre- or postnatal periods can have unwanted effects on post natal HPA axis function. The behavioral outcomes indicate the complexity of emotional reactivity assessment and the multidimensional nature of emotionality as evident from the differing results in the EPM and open field apparatus. The reduced protein in the RR offspring exacerbated anxiety in the open field, but facilitated less avoidance in the EPM, whereas the CR and RC offspring only showed less avoidance in the EPM and no effects in the open field. The behavioral outcomes observed in male offspring in anxiety and exploratory based behavior show diet specific effects potentially due to the insufficient protein (essential amino acids) or fatty acids/lipids available during critical windows of brain development and effects on HPA axis activity. Hormonal responses in both dams and fetus must also be considered as regulatory factors together and separately with the protein effects. These differences clearly reflect impairments in affect with only the RR offspring exhibiting obvious concomitant HPA axis abnormality. Further studies of discrete brain areas, especially neurons and support cells of the limbic system, and HPA axis are necessary to determine the mechanisms of insufficiency protein during development.

Role of funding source

This work was partially supported by Consejo Nacional de Ciencia y Tecnología (CONACyT – 48839) México and the NIH HD21350. Funding sources had no role in the study design, collection, analyses, interpretation of data, or the preparation and submission of this report.

Disclosure

This work is original findings that have not been submitted nor are being considered for publication in any other journal.

Conflict of interest

There is no conflict of interest to declare for all authors.

Acknowledgements

L.A. Reyes-Castro and G.L. Rodríguez-González are graduate students from Doctorado en Ciencias Biomédicas, Facultad de Medicina, Universidad Nacional Autónoma de México and are recipients of CONACyT fellowship.

References

[1] Warner MJ, Ozanne SE. Mechanisms involved in the developmental programming of adulthood disease. Biochem J 2010;427(3):333–47.
[2] Morgane PJ, Austin-LaFrance R, Bronzino J, Tonkiss J, Díaz-Cintra S, Cintra L, et al. Prenatal malnutrition and development of the brain. Neurosci Biobehav Rev 1993;17(1):91–128.
[3] Morgane P, Galler J, Mokler D. A review of systems and networks of the limbic forebrain/limbic midbrain. Prog Neurobiol 2005;75(2):143–60.
[4] Lister J, Blatt G, DeBassio W, Kemper T, Tonkiss J, Galler J, et al. Effect of prenatal protein malnutrition on numbers of neurons in the principal cell layers of the adult rat hippocampal formation. Hippocampus 2005;15(3):393–403.
[5] Morgane P, Mokler D, Galler J. Effects of prenatal protein malnutrition on the hippocampal formation. Neurosci Biobehav Rev 2002;26(4):471–83.
[6] Rosene D, Lister J, Schwagerl A, Tonkiss J, McCormick C, Galler J. Prenatal protein malnutrition in rats alters the c-Fos response of neurons in the anterior cingulate and medial prefrontal region to behavioral stress. Nutr Neurosci 2004;7(5–6):281–9.
[7] Lister J, Tonkiss J, Blatt G, Kemper T, DeBassio W, Galler J, et al. Asymmetry of neuron numbers in the hippocampal formation of prenatally malnourished and normally nourished rats: a stereological investigation. Hippocampus 2006;16(11):946–58.
[8] Bedi K. Nutritional effects on neuron numbers. Nutr Neurosci 2003;6(3):141–52.
[9] Galler JR, Ramsey F, Solimano G, Lowell WE, Mason E. The influence of early malnutrition on subsequent behavioral development. I. Degree of impairment in intellectual performance. J Am Acad Child Psychiatry 1983;22(1):8–15.
[10] Galler JR, Ramsey F, Solimano G. The influence of early malnutrition on subsequent behavioral development III. Learning disabilities as a sequel to malnutrition. Pediatr Res 1984;18(4):309–13.
[11] Galler JR, Ramsey F, Solimano G, Kucharski LT, Harrison R. The influence of early malnutrition on subsequent behavioral development. IV. Soft neurologic signs. Pediatr Res 1984;18(9):826–32.
[12] Galler JR, Ramsey F, Solimano G. A follow-up study of the effects of early malnutrition on subsequent development. II. Fine motor skills in adolescence. Pediatr Res 1985;19(6):524–7.
[13] Galler JR, Ramsey FC, Forde V, Salt P, Archer E. Long-term effects of early kwashiorkor compared with marasmus. II. Intellectual performance. J Pediatr Gastroenterol Nutr 1987;6(6):847–54.

[14] Galler JR, Ramsey FC, Salt P, Archer E. Long-term effects of early kwashiorkor compared with marasmus. III. Fine motor skills. J Pediatr Gastroenterol Nutr 1987;6(6):855–9.
[15] Galler JR, Ramsey FC, Morley DS, Archer E, Salt P. The long-term effects of early kwashiorkor compared with marasmus. IV. Performance on the national high school entrance examination. Pediatr Res 1990;28(3):235–9.
[16] Stein AD, Pierik FH, Verrips GHW, Susser ES, Lumey LH. Maternal exposure to the Dutch famine before conception and during pregnancy: quality of life and depressive symptoms in adult offspring. Epidemiology 2009;20(6):909–15.
[17] Coupe B, Dutriez-Casteloot I, Breton C, Lefevre F, Mairesse J, Vickes-Coopman A, et al. Perinatal undernutrition modifies cell proliferation and brain-derived neurotrophic factor levels during critical time-windows for hypothalamic and hippocampal development in the male rat. J Neuroendocrinol 2009;21(1): 40–8.
[18] da Silva Hernandes A, Françolin-Silva AL, Valadares CT, Fukuda MTH, Almeida SS. Effects of different malnutrition techniques on the behavior of rats tested in the elevated T-maze. Behav Brain Res 2005;162(2):240–5.
[19] Almeida SS, de Oliveira LM, Graeff FG. Early life protein malnutrition changes exploration of the elevated plus-maze and reactivity to anxiolytics. Psychopharmacology (Berl) 1991;103(4):513–8.
[20] Almeida SS, Garcia RA, de Oliveira LM. Effects of early protein malnutrition and repeated testing upon locomotor and exploratory behaviors in the elevated plus-maze. Physiol Behav 1993;54(4):749–52.
[21] Almeida S, Tonkiss J, Galler J. Prenatal protein malnutrition affects exploratory behavior of female rats in the elevated plus-maze test. Physiol Behav 1996;60(2):675–80.
[22] Watkins A, Wilkins A, Cunningham C, Perry V, Seet M, Osmond C, et al. Low protein diet fed exclusively during mouse oocyte maturation leads to behavioural and cardiovascular abnormalities in offspring. J Physiol 2008;586(8): 2231–44.
[23] Trzctnska M, Tonkiss J, Galler J. Influence of prenatal protein malnutrition on behavioral reactivity to stress in adult rats. Stress 1999;3(1):71–83.
[24] Gallo PV. Physiological and behavioral consequences of maternal and post-weaning protein restriction. Physiol Behav 1981;26(1): 77–84.
[25] Wiener SG, Robinson L, Levine S. Influence of perinatal malnutrition on adult physiological and behavioral reactivity in rats. Physiol Behav 1983;30(1):41–50.
[26] Nolan LA, Hart EJ, Windle RJ, Wood SA, Hu XW, Levi AJ, et al. Lack of effect of protein deprivation-induced intrauterine growth retardation on behavior and corticosterone and growth hormone secretion in adult male rats: a long-term follow-up study. Endocrinology 2001;142(7):2996–3005.
[27] Levay E, Paolini A, Govic A, Hazi A, Penman J, Kent S. Anxiety-like behaviour in adult rats perinatally exposed to maternal calorie restriction. Behav Brain Res 2008;191(2):164–72.
[28] Kursmark M, Weitzman M. Recent findings concerning childhood food insecurity. Curr Opin Clin Nutr Metab Care 2009;12(3):310–6.
[29] Zambrano E, Rodriguez-Gonzalez G, Guzman C, Garcia-Becerra R, Boeck L, Diaz L, et al. A maternal low protein diet during pregnancy and lactation in the rat impairs male reproductive development. J Physiol 2005;563(Pt 1):275–84.
[30] Zambrano E, Martinez-Samayoa P, Bautista C, Deas M, Guillen L, Rodriguez-Gonzalez G, et al. Sex differences in transgenerational alterations of growth and metabolism in progeny (F2) of female offspring (F1) of rats fed a low protein diet during pregnancy and lactation. J Physiol 2005;566(Pt 1):225–36.
[31] Zambrano E, Bautista C, Deas M, Martinez-Samayoa P, Gonzalez-Zamorano M, Ledesma H, et al. A low maternal protein diet during pregnancy and lactation has sex- and window of exposure-specific effects on offspring growth and food intake, glucose metabolism and serum leptin in the rat. J Physiol 2006;571(Pt 1):221–30.
[32] Graham S, Gandelman R. The expression of ano-genital distance data in the mouse. Physiol Behav 1986;36(1):103–4.
[33] Guzman C, Cabrera R, Cardenas M, Larrea F, Nathanielsz P, Zambrano E. Protein restriction during fetal and neonatal development in the rat alters reproductive function and accelerates reproductive ageing in female progeny. J Physiol 2006;572(Pt 1):97–108.
[34] Reyes-Castro LA, Rodriguez JS, Rodríguez-González GL, Wimmer RD, McDonald TJ, Larrea F, et al. Pre- and/or postnatal protein restriction in rats impairs learning and motivation in male offspring. Int J Dev Neurosci 2010;29(2): 177–82.
[35] Bautista C, Boeck L, Larrea F, Nathanielsz P, Zambrano E. Effects of a maternal low protein isocaloric diet on milk leptin and progeny serum leptin concentration and appetitive behavior in the first 21 days of neonatal life in the rat. Pediatr Res 2008;63(4):358–63.
[36] Langley-Evans S. Critical differences between two low protein diet protocols in the programming of hypertension in the rat. Int J Food Sci Nutr 2000;51(1):11–7.
[37] Langley SC, Jackson AA. Increased systolic blood pressure in adult rats induced by fetal exposure to maternal low protein diets. Clin Sci 1994;86(2):217–22 [discussion 121].
[38] Vickers MH, Reddy S, Ikenasio BA, Breier BH. Dysregulation of the adipoinsular axis—a mechanism for the pathogenesis of hyperleptinemia and adipogenic diabetes induced by fetal programming. J Endocrinol 2001;170(2):323–32.
[39] Holemans K, Gerber R, Meurrens K, De CF, Poston L, Van Assche F. Maternal food restriction in the second half of pregnancy affects vascular function but not blood pressure of rat female offspring. Br J Nutr 1999;81(1):73–9.
[40] Galler J, Tonkiss J. Prenatal protein malnutrition and maternal behavior in Sprague-Dawley rats. J Nutr 1991;121(5):762–9.
[41] Langley SC, Browne RF, Jackson AA. Altered glucose tolerance in rats exposed to maternal low protein diets in utero. Comp Biochem Physiol Physiol 1994;109(2):223–9.
[42] Langley-Evans S, Gardner D, Jackson A. Association of disproportionate growth of fetal rats in late gestation with raised systolic blood pressure in later life. J Reprod Fertil 1996;106(2):307–12.
[43] Vuguin PM. Animal models for small for gestational age and fetal programming of adult disease. Horm Res 2007;68(3):113–23.
[44] Hotchkiss AK, Lambright CS, Ostby JS, Parks-Saldutti L, Vandenbergh JG, Gray LE. Prenatal testosterone exposure permanently masculinizes anogenital distance, nipple development, and reproductive tract morphology in female Sprague-Dawley rats. Toxicol Sci 2007;96(2):335–45.
[45] Meyer-Bahlburg HFL. Brain development and cognitive, psychosocial, and psychiatric functioning in classical 21-hydroxylase deficiency. Endocr Dev 2011;20:88–95.
[46] Puts DA, McDaniel MA, Jordan CL, Breedlove SM. Spatial ability and prenatal androgens: meta-analyses of congenital adrenal hyperplasia and digit ratio (2D:4D) studies. Arch Sex Behav 2008;37(1):100–11.
[47] Ramos A. Animal models of anxiety: do I need multiple tests? Trends Pharmacol Sci 2008;29(10):493–8.
[48] Ramos A, Mormède P. Stress and emotionality: a multidimensional and genetic approach. Neurosci Biobehav Rev 1998;22(1):33–57.
[49] Trullas R, Skolnick P. Differences in fear motivated behaviors among inbred mouse strains. Psychopharmacology (Berl) 1993;111(3):323–31.
[50] Vendruscolo LF, Takahashi RN, Brüske GR, Ramos A. Evaluation of the anxiolytic-like effect of NKP608, a NK1-receptor antagonist, in two rat strains that differ in anxiety-related behaviors. Psychopharmacology (Berl) 2003;170(3):287–93.
[51] Atallah M, Barbeau I, Pellett P. Metabolic and development changes in growing rats born to dams restricted in protein and/or energy intake. J Nutr 1977;107(4):650–5.
[52] Forbes W, Tracy C, Resnick O, Morgane P. Effects of maternal dietary protein restriction on growth of the brain and body in the rat. Brain Res Bull 1977;2(2):131–5.
[53] Pond W, Yen J, Yen L. Body weight deficit in the absence of reduction in cerebrum weight and nucleic acid content in progeny of swine restricted in protein intake during pregnancy. Proc Soc Exp Biol Med 1988;188(2): 117–21.
[54] Siassi F, Siassi B. Differential effects of protein-calorie restriction and subsequent repletion on neuronal and nonneuronal components of cerebral cortex in newborn rats. J Nutr 1973;103(11):1625–33.
[55] Desai M, Crowther N, Lucas A, Hales C. Organ-selective growth in the offspring of protein-restricted mothers. Br J Nutr 1996;76(4):591–603.
[56] Torres N, Bautista CJ, Tovar AR, Ordaz G, Rodriguez-Cruz M, Ortiz V, et al. Protein restriction during pregnancy affects maternal liver lipid metabolism and fetal brain lipid composition in the rat. Am J Physiol Endocrinol Metab 2009;298(2):E270–277.
[57] Lesage J, Sebaai N, Leonhardt M, Dutriez-Casteloot I, Breton C, Deloof S, et al. Perinatal maternal undernutrition programs the offspring hypothalamo-pituitary–adrenal (HPA) axis. Stress 2006;9(4):183–98.
[58] Schäffer L, Müller-Vizentini D, Burkhardt T, Rauh M, Ehlert U, Beinder E. Blunted stress response in small for gestational age neonates. Pediatr Res 2009;65(2):231–5.
[59] Sebaai N, Lesage J, Breton C, Vieau D, Deloof S. Perinatal food deprivation induces marked alterations of the hypothalamo-pituitary–adrenal axis in 8-month-old male rats both under basal conditions and after a dehydration period. Neuroendocrinology 2004;79(4):163–73.
[60] Vieau D, Sebaai N, Leonhardt M, Dutriez-Casteloot I, Molendi-Coste O, Laborie C, et al. HPA axis programming by maternal undernutrition in the male rat offspring. Psychoneuroendocrinology 2007;32(Suppl. (1)):S16–20.
[61] Sebaai N, Lesage J, Vieau D, Alaoui A, Dupouy J, Deloof S. Altered control of the hypothalamo-pituitary–adrenal axis in adult male rats exposed perinatally to food deprivation and/or dehydration. Neuroendocrinology 2002;76(4):243–53.
[62] Leonhardt M, Lesage J, Dufourny L, Dickes-Coopman A, Montel V, Dupouy J. Perinatal maternal food restriction induces alterations in hypothalamo-pituitary–adrenal axis activity and in plasma corticosterone-binding globulin capacity of weaning rat pups. Neuroendocrinology 2002;75(1):45–54.
[63] Chisari A, Giovambattista A, Perello M, Spinedi E. Impact of maternal undernutrition on hypothalamo-pituitary–adrenal axis and adipocyte functions in male rat offspring. Endocrine 2001;14(3):375–82.
[64] Galler JR, Bryce CP, Waber D, Hock RS, Exner N, Eaglesfield D, et al. Early childhood malnutrition predicts depressive symptoms at ages 11–17. J Child Psychol Psychiatry 2010;51(7):789–98.

Int. J. Devl Neuroscience 30 (2012) 39–45

Contents lists available at SciVerse ScienceDirect

International Journal of Developmental Neuroscience

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

Maternal protein restriction in the rat during pregnancy and/or lactation alters cognitive and anxiety behaviors of female offspring

L.A. Reyes-Castro[a], J.S. Rodriguez[b], R. Charco[a], C.J. Bautista[a], F. Larrea[a], P.W. Nathanielsz[b], E. Zambrano[a,*]

[a] Department of Reproductive Biology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City 14000, Mexico
[b] Center for Pregnancy and Newborn Research, Department of Obstetrics, University of Texas, Health Science Center San Antonio, San Antonio, TX 78229, USA

ARTICLE INFO

Article history:
Received 14 July 2011
Received in revised form 11 October 2011
Accepted 12 October 2011

Keywords:
Developmental programming
Risk assessment
Learning
Motivation
Operant conditioning

ABSTRACT

Maternal protein deficiencies can developmentally program offspring to lifelong dysfunction of many physiological systems. We hypothesized that maternal isocaloric low protein diet during fetal and early postnatal development would negatively affect female offspring anxiety, exploration, associative learning and motivation as measured by the elevated plus maze (EPM), open field test (OFT), operant conditioning and the progressive ratio task, respectively. Control mothers (C) received a 20% casein diet and restricted mothers (R) a 10% casein diet to provide four groups: CC, RR, CR, and RC (first letter pregnancy diet and second lactation diet) to enable evaluation of offspring effects influenced by maternal diet during pregnancy and lactation. Maternal protein restriction decreased open arm time and distance in RR and RC offspring, increased anxiety behavior, in the EPM. In the OFT, the RR and RC offspring displayed decreased exploration (increased stress) as indexed by decreased distance in the center zone. These behaviors in the EPM and OFT was associated with increased corticosterone levels during an immobilization test in the RR offspring with intermediary effects in the RC offspring. Learning impairment was observed in the RR, CR and RC offspring during fixed ratio 5 schedule of reinforcement. Motivational effects were measured in RR offspring responding less, decreased motivation, and CR offspring making more responses, increased motivation, than CC offspring. These findings reveal the negative effects of developmental protein restriction on female offspring behavior. The underlying basis for these negative outcomes remains to be elucidated.

1. Introduction

In many societies nutritional insecurity remains a serious problem (Jaron and Galal, 2009; Tanumihardjo et al., 2007). Reduced maternal nutrition certainly affects fetal development with the brain particularly susceptible to the intrauterine environment in both altricial and precocial species (Morgane et al., 1993; Rice and Barone Jr, 2000). We have recently shown in baboon that an adequate maternal diet is vital for the development of the central nervous system (Antonow-Schlorke et al., 2011). Poor nutrition during development profoundly affects the fetal brain especially neurons of the frontal cortex and limbic system (Antonow-Schlorke et al., 2011; Bedi, 2003; Lister et al., 2005, 2006; Morgane et al., 2002). Behaviors such as learning and memory, emotional regulation, motivation and risk assessment rely on intact cortical and limbic structures and associated neural networks (Morgane et al., 2005). In humans, poor maternal nutrition is a major cause of intrauterine growth restriction which is associated with an increased risk of perinatal mortality and long-term morbidity (McIntire et al., 1999). In addition, intrauterine growth restriction is associated with neurodevelopmental delays (Taylor and Howie, 1989) and alterations of brain structure and neurochemistry (Almeida et al., 1996a). Epidemiological studies in human offspring demonstrate negative influences of insufficient perinatal nutrients on cognition and behavior (Benton, 2008; Galler et al., 1990, 2005; Lucas, 2005; Stein et al., 2009).

Various studies have reported effects of poor maternal nutrition on brain development and risk assessment behavioral outcomes in offspring with reduction in fetal brain fat (Torres et al., 2010) modification to neuronal proliferation in the hypothalamic and hippocampal regions in rat offspring (Coupe et al., 2009; Jahnke and Bedi, 2007). Behavioral outcomes following prenatal protein restriction (6–9% casein) in rodent offspring reveal high

Abbreviations: C, control diet; CC, control diet during pregnancy and lactation; FR, fixed ratio; CR, control diet during pregnancy and protein restriction during lactation; EPM, elevated plus maze; R, protein restriction; RR, protein restriction during pregnancy and lactation; RC, protein restriction during pregnancy and control diet during lactation; PND, postnatal day.

* Corresponding author. Tel.: +52 55 5487 0900x2417; fax: +52 55 5655 9859.
E-mail address: zamgon@servidor.unam.mx (E. Zambrano).

0736-5748/$36.00 © 2011 ISDN. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.ijdevneu.2011.10.002

impulsiveness and exploration (less anxiety) in the elevated plus maze (EPM) and short inhibitory avoidance (disinhibition) latencies in the elevated T-maze (Almeida et al., 1991, 1996b; da Silva et al., 2005; Watkins et al., 2008). Increases in anxiety have also been reported in the EPM in offspring protein restricted during lactation (Almeida et al., 1993). We have recently reported decreased avoidance behavior in the EPM and no effects in the open field (OFT) in male rats pre and/or postnatal protein restricted (Reyes-Castro et al., 2011a).

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 adult male rats impairs the acquisition of a differential reinforcement of low rates operant task (Tonkiss et al., 1990a) and impairments in the radial arm maze following pre- and postnatal protein restriction (7% casein) in mice (Ranade et al., 2008). Spatial learning (Morris water maze) results show impairment of performance in rats prenatally protein restricted (Tonkiss et al., 1997; Zhang et al., 2010). We have previously reported impaired learning in pre and/or postnatal protein restricted male rats (Reyes-Castro et al., 2011b). In regards to motivation for positive reinforcement, prenatally undernourished rats show less appetitive motivation (Miles et al., 2009). We also have reported less motivation in male rats following pre and/or postnatal protein restriction (10% casein) (Reyes-Castro et al., 2011b).

These findings on anxiety, exploration, learning and motivation are indicative of the negative behavioral effects in offspring subsequent to insufficient protein in early life. Previous studies of insufficient perinatal protein during development have been associated with a two-thirds reduction of protein from the control diet (25% protein diet reduced to 6%). However, the effects of smaller decreases in perinatal protein have only been determined in male offspring (Reyes-Castro et al., 2011a,b). Therefore we sought to investigate the negative effects of a 50% reduction in perinatal protein on female offspring behavior. This level of protein reduction more accurately reflects the human dietary insecurities in developed countries (for review see Ref. McIntire et al., 1999). For this study we assessed adult female rat offspring born to protein restricted mothers in anxiety, exploration, learning and motivation behaviors, using the EPM, OFT, operant conditioning and progressive ratio, respectively. Control mothers (C) received 20% casein while restricted mothers (R) received 10% casein to provide four groups: CC, RR, CR, and RC (first letter pregnancy diet and second letter lactation diet). These dietary manipulation combinations have not previously been administered in the context of female development and behavioral effects following maternal protein restriction. We hypothesized that maternal protein restriction during pregnancy and/or lactation would negatively affect neurodevelopment and subsequent behavior in female offspring.

2. Experimental procedures

2.1. Care and maintenance of animals

All procedures were approved by the Animal Experimentation Ethics Committee of the "InstitutoNacional de CienciasMédicas y Nutrición, Salvador Zubirán" (INNSZ), Mexico. Details of maternal diet, breeding, and management of the four groups of offspring have been published in detail (Zambrano et al., 2005). Briefly, mothers (obtained from INNSZ) were virgin female albino Wistar rats 122 ± 9 days of age (mean ± SEM) and weighed 221.8 ± 3.8 g (mean ± SEM). Female rats with regular estrous cycles were fed normal laboratory chow (control diet, Zeigler Rodent RQ 22-5, USA) under controlled lighting (lights on from 7:00 a.m. to 7:00 p.m. at 22–23 °C) and mated overnight with proven male breeders. The day spermatozoa were present in the vaginal smear was designated as day of conception (day 0). Only rats impregnated within 5 days following introduction of the fertile male were retained in the study. Pregnant rats were transferred to individual acrylic cages and allocated randomly to one of two groups: fed either 20% casein ($n = 20$, control diet-C) or 10% casein isocaloric diet ($n = 20$, restricted diet-R) (Morgane et al., 1993). Food and water were available *ad libitum* and chow provided in the form of flat biscuits.

Offspring delivery occurred in the early daylight hours between 9.00 a.m. and 12 p.m. on post-conception day 22 which was designated postnatal day (PND) 0. Pup weight and morphometric parameters were recorded at birth. Ano-genital distance was measured with calipers and expressed as ano-genital distance/body weight as previously reported (Miles et al., 2009; Morgane et al., 1993, 2002, 2005; Ranade et al., 2008; Reyes-Castro et al., 2011a). Using our previously published data (Morgane et al., 2005), sex was judged according to whether the ano-genital distance was < (female) or > 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 mother while maintaining as close to a 1:1 sex ratio as possible. Morphometric measurements were performed on the litters prior to culling them to 10. 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 female CC, RR, CR and RC offspring). After weaning (PND 21), all pups were fed the C diet and housed 4 offspring in the same treatment group per cage. Two weeks prior to behavioral testing and during the behavioral experiments, subjects were maintained on a reverse 12 h light/dark cycle (lights off at 7 a.m. and on at 7 p.m.). Behavioral assessments occurred during the dark phase on PNDs 90–220 regardless of the stage of the estrous cycle. Order of task administration was: (1) EPM (PNDs 90–92), (2) open field (PNDs 91–93), (3) operant condition (PNDs 110–135), (4) progressive ratio (PNDs 135–146), (5) sucrose consumption (PNDs 147–150) and (6) immobilization (PND 220).

2.2. Elevated plus maze

Eight unrelated naïve subjects per treatment group were tested. The EPM was constructed of dark grey plastic situated 64 cm above the floor. It consisted of two unprotected open arms 180 degrees apart, and two closed arms protected by high grey walls 90 degrees from the open arms. The four arms (45 cm × 10 cm each) extended from a common central platform (10 cm × 10 cm). The light level was 30 lux in the open arms and 6 lux in the closed arms. The rat's position on the maze was recorded via a video camera mounted on the ceiling above the center of the maze. The camera was connected to a video tracking motion analysis system (Ethovision, Noldus Information Technology by Wageningen, The Netherlands) running on a personal computer. To start the session, each rat was placed individually into the center of the maze facing an open arm. Subjects were assessed for 5 min, the rat was returned to its home cage and the EPM was cleaned with 70% ethanol. An experimenter naïve to treatment group manually scored the number of entries into the predefined zones of the open and closed arms, while the Ethovision system monitored the distance moved and the time spent in the different zones. An arm entry was scored only if the rat's center of gravity entered into the arm. The animals were tested in a randomized sequence based on early life dietary manipulation.

2.3. Open field

The same animals studied in the EPM were evaluated the next day in a 60-min OFT. The open field, made of dark grey Plexiglas, consisted of a square arena (101 cm × 101 cm and 34 cm high) which was located in an experimental room illuminated by low light (12 lux). A video camera mounted above the arena connected to a monitor and served as the video tracking motion analysis system (Ethovision, Noldus Information Technology, Wageningen, The Netherlands). This system recorded subject locomotion in the entire arena (total distance measured in meters). A virtual square center was defined in the open field arena and the locomotor activity of the rats inside this zone was measured. Each rat was placed individually into the center of the open field arena and tested equally in all open field apparatuses. Following each session all zones were cleaned with 70% ethanol.

2.4. Operant apparatus

Eight unrelated offspring from different litters per diet group were tested in 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 box contained a grid floor and was illuminated with a diffuse house-light during testing. Response lever and reward-magazine were placed on the right sidewall. When a rat had performed the required number of lever presses, the light was turned on in the reward magazine and the liquid dipper transferred a drop of sucrose solution (7%) from a reservoir into the reward magazine. A nose poke into the magazine was registered by photocell receptors. A personal computer controlled task parameters via universal environment interfaces (E91-12, Coulbourn-Instruments, PA, USA).

2.5. Operant conditioning and progressive ratio procedure

Prior to testing offspring were placed on water deprivation for 23 h/day. Subjects were given one hour of free access immediately following behavioral sessions which continued throughout training and testing. To begin training subjects were given a non-contingent reinforcement in the test-box. Subsequently, on the first

Table 1
Pup weight/morphometric measurements at birth from mothers fed the control (20% casein) or restricted (10% casein) diet during pregnancy. Mean ± SEM, *n* refers to litters.

	Control (*n* = 16)	Restricted (*n* = 16)
Litter size (pups/litter)	11.2 ± 0.3	10.9 ± 0.2
Weight (g)	5.6 ± 0.05	5.1 ± 0.04*
Length (mm)	4.5 ± 0.06	4.4 ± 0.06
Ano genital distance (mm)	2.4 ± 0.1	2.5 ± 0.07
Ano genital distance (mm/g)	0.42 ± 0.03	0.51 ± 0.02*
Head diameter (mm)	11.3 ± 0.02	11.2 ± 0.03
Abdominal diameter (mm)	12.4 ± 0.07	12.2 ± 0.02
Head:abdominal ratio	0.91 ± 0.01	0.92 ± 0.01

* $p < 0.05$ different from control.

training day subjects were conditioned to press a lever for reinforcement, according to a fixed ratio schedule (FR-1). On each trial the lever was presented until the subject pressed it. After which the subject was allowed 120 s to approach there ward magazine and respond with a nose poke. The registration of the nose poke into their ward magazine by the photocell receptors started the feeding which lasted approximately 10 s. Each trial was followed by an inter-trial interval of 5 s during which the lever was retracted. FR-1 training was completed when subjects made more than 20 responses during a 15-min session. After all subjects reached this criterion, they were introduced to a FR-5 schedule with the identical performance criteria as in the FR-1 schedule albeit with 5 responses required per trial. After the training sessions, subjects were placed on a progressive ratio schedule for ten days. In the progressive ratio schedule, the response increment doubled every eighth reinforcement and hence the number of lever presses required to obtain successive sucrose reinforcements was as follows: 1, 2, . . ., 8; 10, 12, . . ., 24; 28, 32, . . ., 56; 64, 72, . . ., 120;136, 152, . . ., etc. Each progressive ratio session lasted 30-min. The subjects of the different groups were tested in equal numbers across test boxes.

2.6. Free sucrose consumption

The day following the last progressive ratio session the subjects were given direct access to bottled sucrose solution for 30-min in the familiar colony room and the amount of consumed solution was measured. For this evaluation subjects were single caged and the amount of sucrose consumption was calculated by the subtraction of the bottle weight before and after the consummatory period. This procedure was performed in 3 consecutive days and administered in order to assess for differences in sucrose consummatory behavior.

2.7. Immobilization and corticosterone measurement

Following all behavioral testing, subjects were physically restrained for 20 min and corticosterone levels were measured. Starting at 12:00 hours on PND 220, a blood sample was taken from the tail vein to determine time 0 corticosterone serum levels. Subjects were then immobilized for 20 min and blood samples were obtained at 20, 40, 80, and 120 min. Blood samples were centrifuged at 4 °C for 15 min at 3500 rpm to remove the red blood cells. The serum was stored at −20 °C until all samples were analyzed. Corticosterone serum levels were determined by radioimmunoassay using commercial rat kits, DPC Coat-a-count (TKRC1) from Diagnostic Products (Los Angeles, CA, USA). Intra- and inter-assay variability was <6 and <7%. The kit was used in accordance with manufacturer's instructions and samples were measured in duplicate.

2.8. Statistical analyses

All data are presented as mean ± SEM. Differences in body weight and morphometric measurements at birth were calculated by *t*-test. Body weight at adult age and behavioral endpoints were analyzed by one-ANOVA with between-subject factors of early life manipulation (maternal diet during pregnancy and lactation). Post-hoc analyses were performed by Tukey's test (Sigma Stat 3.5). Corticosterone measurements were analyzed by two-way ANOVA with Bonferroni's post hoc test (GraphPad Prism 4.0).

3. Results

3.1. Offspring morphometrics

Table 1 shows morphometric analysis of offspring at birth. Body weight for pups of protein restricted mothers was reduced compared to pups from mothers fed the control diet ($p < 0.05$). Ano-genital distance relative to body weight was increased 19% in the protein restricted female offspring ($p < 0.05$). No changes were observed in other parameters at birth. Offspring weights were measured on PND 21, 90, 120, 150 and 220 (Table 2, top panel). The RR offspring weighed less that CC offspring on PND 21 and 220 while the CR offspring weighed less than CC on PND 21, 120, 150 and 220.

Table 2
Offspring weight as adults at the beginning of the training (PND 120) and before immobilization (PND 220) (Top table). Free sucrose consumption test – average intake of 30 min during 3 days (Bottom table). Offspring from mothers fed with control (C = 20% casein) or restricted (R = 10% casein) diet during pregnancy (first letter) and lactation (second letter). Mean ± SEM; *n* = 8 pups from different litters. Groups not sharing a letter are statistically different, $p < 0.05$.

Offspring weights (g)	CC	RR	CR	RC
PND 21	39 ± 0.8a	24 ± 0.7b	25 ± 1.5b	42 ± 1.1a
PND 90	209 ± 4	199 ± 10	190 ± 6	200 ± 7
PND 120	226 ± 3a	206 ± 7ab	198 ± 4b	219 ± 5ab
PND 150	236 ± 5a	217 ± 7ab	206 ± 5b	226 ± 5ab
PND 220	256 ± 8a	223 ± 11bc	222 ± 9b	247 ± 6ac

7% Sucrose	CC	RR	CR	RC
mL	28.5 ± 0.5	29.1 ± 0.7	28.7 ± 0.8	28.8 ± 0.8

3.2. Elevated plus maze

Differences were measured in the total distance with a decrease in the RR offspring compared to CC and no differences in the CR and RC (Fig. 1A, $p < 0.05$). Differences were also measured in open arm time and open arm distance with decreases in the RR and RC offspring compared to CC and no difference in the CR (Fig. 1C and D, $p < 0.05$). No differences were revealed in open arm entries (Fig. 1B).

3.3. Open field

Differences in the number of center zone and border zone entries showed the RC offspring had decreased entries in both zones versus CC offspring with intermediary effects in RR and CR (Fig. 2A and B, $p < 0.05$). Decrease in center zone distance was observed in the RR and RC offspring with intermediary effects in the CR (Fig. 2E, $p < 0.05$). No differences were determined for center or border zones time, border zone distance or total distance (Fig. 2).

3.4. Operant conditioning

For FR-1 training a significant effect of diet was determined ($p < 0.001$), the number of FR-1 training sessions required to reach performance criterion in both the RR and CR groups was increased compared to CC offspring (Fig. 3A, $p < 0.05$). Additionally, for FR-5 training a significant effect of diet was determined ($p < 0.001$) RR, CR and RC offspring required more sessions to attain performance criterion compared to CC offspring (Fig. 3B, $p < 0.05$).

3.5. Progressive ratio

Analysis of number of responses made per session show a significant effect of diet ($p < 0.001$). Number of responses were decreased in the RR offspring while the CR offspring responded more than the CC group with no difference found in the RC group (Fig. 4A). Comparisons for number of rewards earned per session show a significant effect of diet ($p < 0.001$). Post-hoc analysis confirmed the CR group earned more rewards than the CC group with no difference found in the RR and RC groups (Fig. 4B).

3.6. Free sucrose consumption

No differences were determined between experimental offspring in the free sucrose consumption test (Table 2, bottom panel).

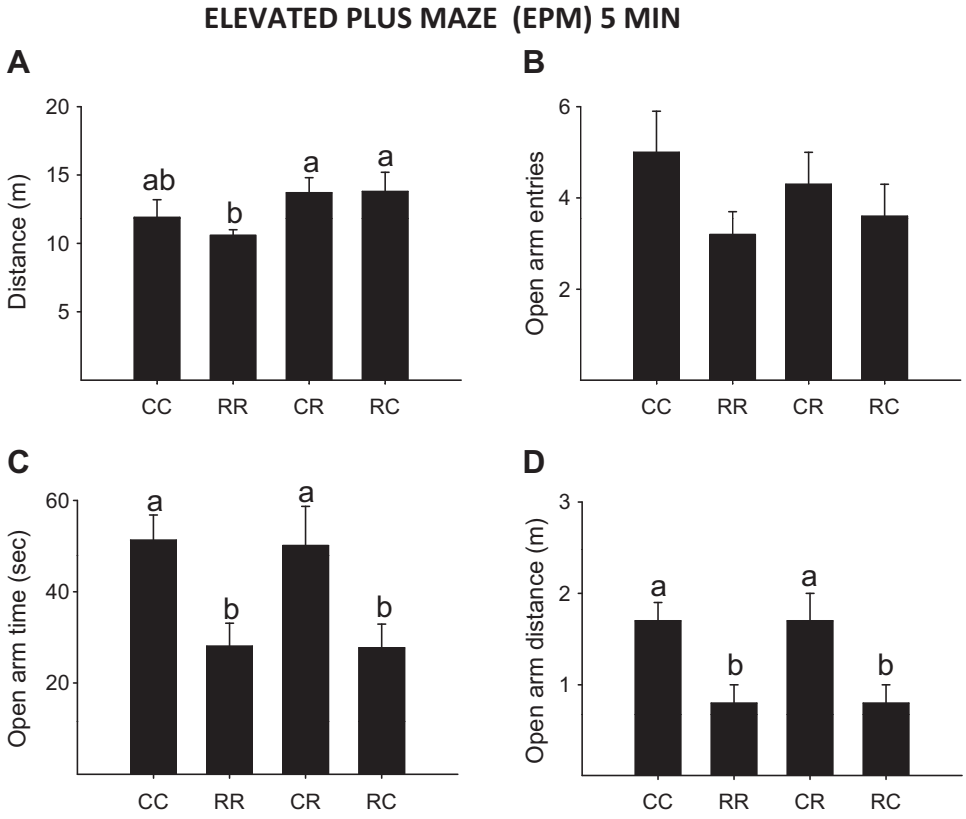

Fig. 1. Elevated plus maze comparisons. (A) Total distance, (B) open arm entries, (C) open arm time and (D) open arm distance. Offspring of rats fed with control (C = 20% casein) or restricted (R = 10% casein) diet during pregnancy (first letter) and lactation (second letter). Mean ± SEM, $n = 8$ offspring from different litters.

3.7. Immobilization and corticosterone measurements

Analysis of corticosterone levels at 0, 20, 40, 80 and 120 min show the RR offspring had increased levels at the 0 min time point versus CC and CR offspring with the RC having intermediary levels (Fig. 5A; letter symbols, $p < 0.05$). Corticosterone increase (Δ) after immobilization (between 0 and 20) was higher in CC in comparison with RR and RC, with no difference in the CR (CC 350 ± 53a, RR 154 ± 50b, CR 287 ± 53ab, RC 184 ± 30b, ng/ml; $p < 0.05$). No group differences were observed from 20–120 min. Within group differences were observed for all time points subsequent to the 0 min time point measurement (Fig. 5A; * and # symbols, $p < 0.05$). Corticosterone levels remained elevated from 20–120 min time points in the CC, CR and RC offspring. In the RR offspring corticosterone

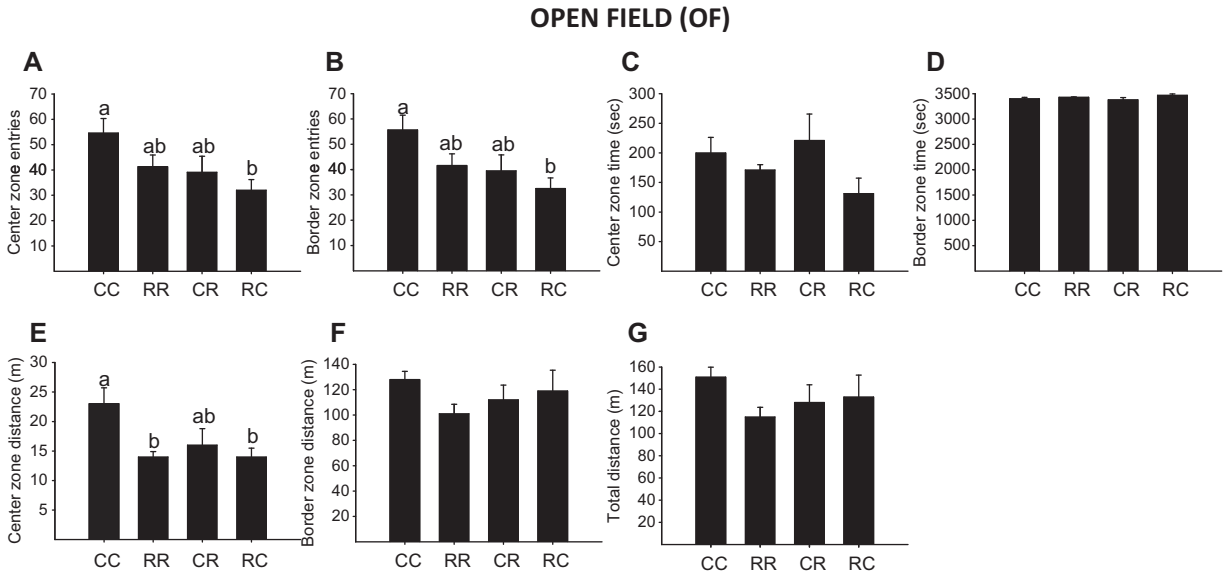

Fig. 2. Open field test. (A) Center zone entries, (B) border zone entries, (C) center zone time, (D) border zone time, (E) center zone distance, (F) border zone distance and (G) total distance. Offspring of rats fed with control (C = 20% casein) or restricted (R = 10% casein) diet during pregnancy (first letter) and lactation (second letter). Mean ± SEM, $n = 8$ pups from different litters. Groups not sharing a letter are statistically different, $p < 0.05$.

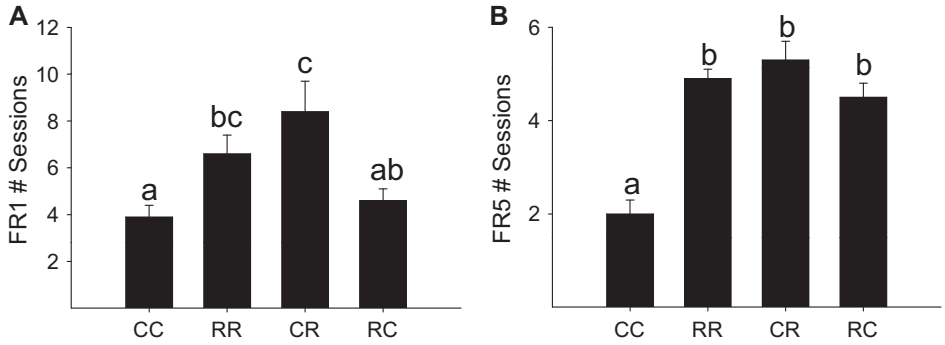

Fig. 3. Operant conditioning. (A) Number of FR1 sessions and (B) FR5sessions to reach performance criterion. Offspring of rats fed with control (C = 20% casein) or restricted (R = 10% casein) diet during pregnancy (first letter) and lactation (second letter). Mean ± SEM, $n = 8$ pups from different litters. Groups not sharing a letter are statistically different, $p < 0.05$.

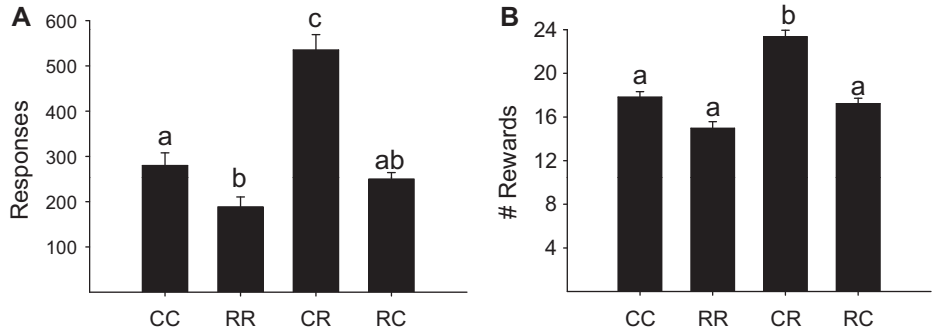

Fig. 4. Progressive ratio test. (A) Average number of responses and (B) reinforcements earned during 10 sessions. Offspring of rats fed with control (C = 20% casein) or restricted (R = 10% casein) diet during pregnancy (first letter) and lactation (second letter). Mean ± SEM, $n = 8$ pups from different litters. Groups not sharing a letter are statistically different, $p < 0.05$.

levels subsided to 0 time point levels by 120 min. There were no differences among groups for overall corticosterone levels – area under the curve (Fig. 5B).

4. Discussion

The goal of these studies was to assess anxiety, exploration, associative learning and motivation behaviors in female offspring born to mothers administered a 50% reduction in protein during pregnancy and lactation. In addition to behavioral outcomes, morphometric and corticosterone measurements were determined in offspring. Maternal protein restriction reduced pup birth weights and increased the ano-genital distance which in many species provides an external marker of sexual differentiation at birth (Manno III, 2008). An increase in the ano-genital distance indicates prenatal exposure to maternally derived steroids (Hotchkiss et al., 2007). We have also reported increases in ano-genital distance in offspring born to prenatally protein restricted mothers, following the identical diets used here, resulting in elevated maternal progesterone, corticosterone, estradiol, and testosterone concentration at 19 days gestation (Guzman et al., 2006; Zambrano et al., 2005). Although speculative, effects of these steroids on fetal development could have a role in the current behavioral findings since we previously reported behavioral impairments in male offspring administered the identical protein restriction diet used in this study (Reyes-Castro et al., 2011a,b).

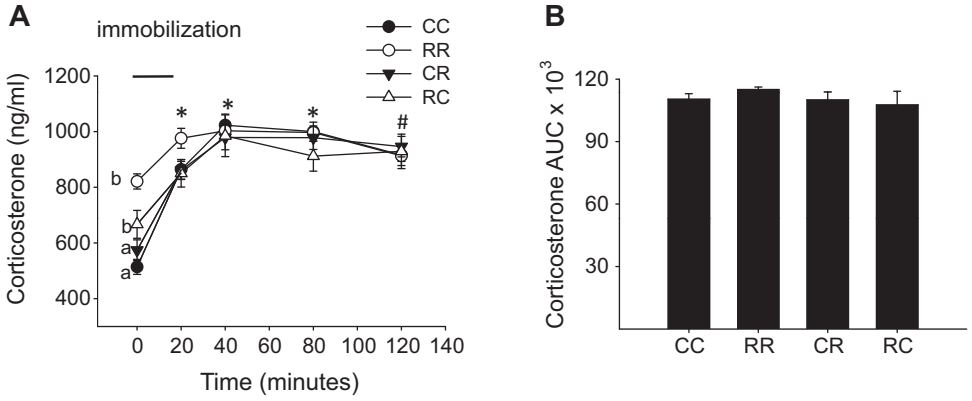

Fig. 5. (A) Immobilization and corticosterone measurement at PND 220. Groups not sharing a letter are statistically different; * indicates within group differences from 0 time point; # indicates difference from 0 time point for all groups except the RR offspring, $p < 0.05$. (B) Area under the curve (AUC) for overall corticosterone levels. Mean ± SEM, $n = 8$ offspring from different litters.

Results from the EPM did show perinatal diet differences – decreases in open arm time and distance in RR and RC offspring. These behavioral changes are consistent to previous studies in similar rodent models and indicative of increased anxiety phenotype (Almeida et al., 1993). In males we have shown the CR and RC offspring make more open arm entries and the RR, CR, and RC offspring spend more time in the open arms than the CC offspring (Reyes-Castro et al., 2011a). Taken together, the pre and/or postnatal protein restricted male offspring show increased approach and less avoidance behavior (less anxiety) and thus exhibited disinhibition of avoidance as compared to controls (Reyes-Castro et al., 2011a). However, the current observations in female offspring submitted to the same protein restrictions during development did not duplicate the findings in males and hence is an example of sex specific differences in our perinatal protein restriction paradigm.

In the open field, differences were detected in the RR and RC offspring traveling less distance in the center zone with only the RC offspring showing decreases in center and border zone entries. This increase in avoidance/risk assessment is indicative of decreased exploration and possibly increased anxiety or stress in both the RR and RC offspring which is supported by other studies showing a similar behavioral phenotype (Gallo, 1981; Levay et al., 2008; Trzctnska et al., 1999; Watkins et al., 2008). In males, pre and postnatal protein restricted offspring (RR) showed a marginal decrease in the number of center zone entries, thus suggesting an increased anxiety state (Reyes-Castro et al., 2011a), similar to the female RC offspring in this study.

Behavioral observations during operant conditioning revealed the RR and CR offspring required more sessions to attain performance criterion during acquisition of FR-1 schedule and the RR, CR and RC offspring all required more sessions to perform to criterion during FR-5 schedule of reinforcement compared to CC offspring. So, during FR-1 training pre and postnatal protein restriction in combination (RR offspring) or postnatal protein restriction alone (CR offspring) caused learning impairments. However, all experimental offspring required more sessions to reach performance criterion during FR-5 sessions. The increased number of sessions to attain performance criterion during operant conditioning implies impairment in procedural and/or associative learning in the experimental groups. We have recently reported learning impairment during FR-1 conditioning in male RC offspring and in male RR, CR and RC offspring during FR-5 conditioning (Reyes-Castro et al., 2011b). So in this perinatal protein restriction paradigm, prenatal protein restriction alone (RC offspring) in males impaired FR-1 performance while in females postnatal protein restriction (RR and CR) impaired FR-1 performance. These results suggest that the critical window for the development of the brain areas underlying task performance is earlier in males than females.

The cognitive deficits we report in these experimental offspring are consistent with impaired learning in adult rat offspring globally undernourished during gestation (30% reduction from *ad libitum* consumption) using concurrent variable-interval schedules (Landon et al., 2007). Similarly adult sheep offspring prenatally undernourished (a 50% reduction in diet) display impaired cognitive flexibility as assessed by learning speed during reversal tasks in a T-maze (Erhard et al., 2004). Perinatal diet restriction or protein restriction impairs spatial learning and memory in the Morris water maze (Tonkiss et al., 1997; Zhang et al., 2010), in the differential reinforcement of low rates operant task (Tonkiss et al., 1990a) and the radial arm maze (Ranade et al., 2008) in male rat offspring. Although those studies do not precisely reflect the dietary challenges and operant tasks we administered, a commonality exists in our offspring exhibiting learning impairment during operant conditioning. Additionally, we have recently reported learning impairment in male offspring administered the identical protein restriction protocol (Reyes-Castro et al., 2011b). It should be noted that our paradigm is only a 50% reduction of protein in an isocaloric diet (10% casein) which is a more modest protein restriction versus those studies mentioned above which administered an approximately 70% protein reduction (6% casein).

In this study differences in motivation during progressive ratio testing were observed – the RR group responded less and the CR group responded more than CC offspring and no differences were observed between groups during the free sucrose consumption implying similar appetitive/consummatory behaviors. These results indicate that pre and postnatal protein restriction in combination (RR offspring) reduces motivation, similarly to what we have reported in male protein restricted offspring (Reyes-Castro et al., 2011b). On the other hand, protein restriction during the postnatal period alone (CR offspring) increases motivation. As a basis for decreased motivation in the RR offspring, prenatal exposure to androgens have been shown to induce aversive properties to appetitive stimuli postnatally (Dominguez-Salazar et al., 2008). We have previously reported increases in maternal steroids near term in an identical protein restriction model (Guzman et al., 2006; Zambrano et al., 2005). The increased motivation displayed by the CR offspring is consistent with a previous study that revealed prenatal protein restriction increases the response for reward in adult male rats (Tonkiss et al., 1990b). In males we have reported decreased motivation in all experimental groups, i.e. RR, RC and CR offspring (Reyes-Castro et al., 2011b). Our studies in pre and/or postnatal protein restricted males and females show sex specific outcomes during progressive ratio testing.

Corticosterone levels measured during immobilization show that the RR and RC offspring had elevated corticosterone levels at the 0 min time point of immobilization and no difference in the CR offspring. This increased basal corticosterone could be a general physiological change in these offspring consistent with what has been previously reported – increased basal corticosterone concentrations have been measured in pre and postnatal food restricted weaned and adult rats (Leonhardt et al., 2002; Sebaai et al., 2004). Those studies suggest that developmental programming of the HPA axis by fetal and early postnatal malnutrition can result in persistent impairment of feedback (Chisari et al., 2001). In this report we measured an increase in basal corticosterone prior to immobilization in RR and RC offspring suggesting pre and postnatal protein restriction in combination or prenatal protein restriction alone can have a negative effect on HPA axis function during physical restraint. However as reported, in males there was an overall difference in corticosterone levels (area under the curve) in the RR offspring (Reyes-Castro et al., 2011a) but in this study there was no cumulative difference.

In conclusion, the behavioral perturbations observed in female offspring in anxiety, exploration, learning and motivation behavior are potentially due to the insufficient protein (essential amino acids) or fatty acids/lipids available during critical windows of brain development. Differential hormonal responses to the dietary challenge in both mother and fetus must also be considered as potential programming mechanisms as steroids in particular greatly determine neural proliferation, differentiation, and migration (Jazin and Cahill, 2010). Observed differences clearly reflect impairments in anxiety, exploration, cognition and motivation without affecting consummatory behavior, as determined by the free sucrose consumption test. Mechanistically one might hypothesize that the lack of sufficient protein components required for normal neurodevelopment resulted in persistent dysfunction of the limbic system and cortex (Bedi, 2003; Lister et al., 2005, 2006; Morgane et al., 1993) at the time of testing. We have also shown that protein restriction during pregnancy negatively impacts normal fetal brain development by changes in maternal lipid metabolism (Torres et al., 2010). Prenatal protein restriction significantly reduces maternal liver desaturase and elongase gene expression, and formation

of the LC-PUFAs: arachidonic and docosahexaenoic acid (Torres et al., 2010). Thus, fetuses born to protein restricted mothers exhibit low body weight as well as reduced brain fat including the content of docosahexaenoic acid in the brain (Torres et al., 2010). This study shows that a modest reduction in protein during the perinatal period can have profound effects on offspring. These findings are similar to what we have previously reported in male offspring protein restricted during the same critical developmental windows (Reyes-Castro et al., 2011a,b). Further studies of discrete brain areas, especially neurons and support cells of the limbic system and the hypothalamic pituitary adrenal axis are necessary to determine mechanisms.

Acknowledgements

LRC is a graduate student from Doctorado en Ciencias Biomédicas, Facultad de Medicina, Universidad Nacional Autónoma de México and a recipient of CONACyT fellowship.

References

Almeida, S.S., de Oliveira, L.M., Graeff, F.G., 1991. Early life protein malnutrition changes exploration of the elevated plus-maze and reactivity to anxiolytics. Psychopharmacology (Berl.) 103, 513–518.
Almeida, S.S., Garcia, R.A., de Oliveira, L.M., 1993. Effects of early protein malnutrition and repeated testing upon locomotor and exploratory behaviors in the elevated plus-maze. Physiol. Behav. 54, 749–752.
Almeida, S.S., Tonkiss, J., Galler, J.R., 1996a. Malnutrition and reactivity to drugs acting in the central nervous system. Neurosci. Biobehav. Rev. 20, 389–402.
Almeida, S.S., Tonkiss, J., Galler, J.R., 1996b. Prenatal protein malnutrition affects exploratory behavior of female rats in the elevated plus-maze test. Physiol. Behav. 60, 675–680.
Antonow-Schlorke, I., Schwab, M., Cox, L.A., Li, C., Stuchlik, K., Witte, O.W., Nathanielsz, P.W., McDonald, T.J., 2011. Vulnerability of the fetal primate brain to moderate reduction in maternal global nutrient availability. Proc. Natl. Acad. Sci. U.S.A. 108, 3011–3016.
Bedi, K.S., 2003. Nutritional effects on neuron numbers. Nutr. Neurosci. 6, 141–152.
Benton, D., 2008. The influence of children's diet on their cognition and behavior. Eur. J. Nutr. 47 (Suppl. 3), 25–37.
Chisari, A.N., Giovambattista, A., Perello, M., Spinedi, E., 2001. Impact of maternal undernutrition on hypothalamo-pituitary-adrenal axis and adipocyte functions in male rat offspring. Endocrine 14, 375–382.
Coupe, B., Dutriez-Casteloot, I., Breton, C., Lefevre, F., Mairesse, J., Dickes-Coopman, A., Silhol, M., Tapia-Arancibia, L., Lesage, J., Vieau, D., 2009. Perinatal undernutrition modifies cell proliferation and brain-derived neurotrophic factor levels during critical time-windows for hypothalamic and hippocampal development in the male rat. J. Neuroendocrinol. 21, 40–48.
da Silva, H.A., Francolin-Silva, A.L., Valadares, C.T., Fukuda, M.T., Almeida, S.S., 2005. Effects of different malnutrition techniques on the behavior of rats tested in the elevated T-maze. Behav. Brain Res. 162, 240–245.
Dominguez-Salazar, E., Camacho, F.J., Paredes, R.G., 2008. Perinatal inhibition of aromatization enhances the reward value of sex. Behav. Neurosci. 122, 855–860.
Erhard, H.W., Boissy, A., Rae, M.T., Rhind, S.M., 2004. Effects of prenatal undernutrition on emotional reactivity and cognitive flexibility in adult sheep. Behav. Brain Res. 151, 25–35.
Galler, J.R., Ramsey, F.C., Morley, D.S., Archer, E., Salt, P., 1990. The long-term effects of early kwashiorkor compared with marasmus. IV. Performance on the national high school entrance examination. Pediatr. Res. 28, 235–239.
Galler, J.R., Waber, D., Harrison, R., Ramsey, F., 2005. Behavioral effects of childhood malnutrition. Am. J. Psychiatry 162, 1760–1761.
Gallo, P.V., 1981. Physiological and behavioral consequences of maternal and post-weaning protein restriction. Physiol. Behav. 26, 77–84.
Guzman, C., Cabrera, R., Cardenas, M., Larrea, F., Nathanielsz, P.W., Zambrano, E., 2006. Protein restriction during fetal and neonatal development in the rat alters reproductive function and accelerates reproductive ageing in female progeny. J. Physiol. 572, 97–108.
Hotchkiss, A.K., Lambright, C.S., Ostby, J.S., Parks-Saldutti, L., Vandenbergh, J.G., Gray Jr., L.E., 2007. Prenatal testosterone exposure permanently masculinizes anogenital distance, nipple development, and reproductive tract morphology in female Sprague-Dawley rats. Toxicol. Sci. 96, 335–345.
Jahnke, S., Bedi, K.S., 2007. Undernutrition during early life increases the level of apoptosis in the dentate gyrus but not in the CA2 + CA3 region of the hippocampal formation. Brain Res. 1143, 60–69.
Jaron, D., Galal, O., 2009. Food security and population health and well being. Asia Pac. J. Clin. Nutr. 18, 684–687.
Jazin, E., Cahill, L., 2010. Sex differences in molecular neuroscience: from fruit flies to humans. Nat. Rev. Neurosci. 11, 9–17.
Landon, J., Davison, M., Krageloh, C.U., Thompson, N.M., Miles, J.L., Vickers, M.H., Fraser, M., Breier, B.H., 2007. Global undernutrition during gestation influences learning during adult life. Learn. Behav. 35, 79–86.
Leonhardt, M., Lesage, J., Dufourny, L., ckes-Coopman, A., Montel, V., Dupouy, J.P., 2002. Perinatal maternal food restriction induces alterations in hypothalamo-pituitary-adrenal axis activity and in plasma corticosterone-binding globulin capacity of weaning rat pups. Neuroendocrinology 75, 45–54.
Levay, E.A., Paolini, A.G., Govic, A., Hazi, A., Penman, J., Kent, S., 2008. Anxiety-like behaviour in adult rats perinatally exposed to maternal calorie restriction. Behav. Brain Res. 191, 164–172.
Lister, J.P., Blatt, G.J., DeBassio, W.A., Kemper, T.L., Tonkiss, J., Galler, J.R., Rosene, D.L., 2005. Effect of prenatal protein malnutrition on numbers of neurons in the principal cell layers of the adult rat hippocampal formation. Hippocampus 15, 393–403.
Lister, J.P., Tonkiss, J., Blatt, G.J., Kemper, T.L., DeBassio, W.A., Galler, J.R., Rosene, D.L., 2006. Asymmetry of neuron numbers in the hippocampal formation of prenatally malnourished and normally nourished rats: a stereological investigation. Hippocampus 16, 946–958.
Lucas, A., 2005. Long-term programming effects of early nutrition – implications for the preterm infant. J. Perinatol. 25 (Suppl. 2), S2–S6.
Manno III, F.A., 2008. Measurement of the digit lengths and the anogenital distance in mice. Physiol. Behav. 93, 364–368.
McIntire, D.D., Bloom, S.L., Casey, B.M., Leveno, K.J., 1999. Birth weight in relation to morbidity and mortality among newborn infants. N. Engl. J. Med. 340, 1234–1238.
Miles, J.L., Landon, J., Davison, M., Krageloh, C.U., Thompson, N.M., Triggs, C.M., Breier, B.H., 2009. Prenatally undernourished rats show increased preference for wheel running. V. Lever pressing for food in a choice task. Br. J. Nutr. 101, 902–908.
Morgane, P.J., Austin-LaFrance, R., Bronzino, J., Tonkiss, J., Diaz-Cintra, S., Cintra, L., Kemper, T., Galler, J.R., 1993. Prenatal malnutrition and development of the brain. Neurosci. Biobehav. Rev. 17, 91–128.
Morgane, P.J., Galler, J.R., Mokler, D.J., 2005. A review of systems and networks of the limbic forebrain/limbic midbrain. Prog. Neurobiol. 75, 143–160.
Morgane, P.J., Mokler, D.J., Galler, J.R., 2002. Effects of prenatal protein malnutrition on the hippocampal formation. Neurosci. Biobehav. Rev. 26, 471–483.
Ranade, S.C., Rose, A., Rao, M., Gallego, J., Gressens, P., Mani, S., 2008. Different types of nutritional deficiencies affect different domains of spatial memory function checked in a radial arm maze. Neuroscience 152, 859–866.
Reyes-Castro, L.A., Rodriguez, J.S., Rodriguez-Gonzalez, G.L., Chavira, R., Bautista, C.J., McDonald, T.J., Nathanielsz, P.W., Zambrano, E., 2011a. Pre and/or postnatal protein restriction developmentally programs affect and risk assessment behaviors in adult male rats. Behav. Brain Res..
Reyes-Castro, L.A., Rodriguez, J.S., Rodriguez-Gonzalez, G.L., Wimmer, R.D., McDonald, T.J., Larrea, F., Nathanielsz, P.W., Zambrano, E., 2011b. Pre- and/or postnatal protein restriction in rats impairs learning and motivation in male offspring. Int. J. Dev. Neurosci. 29, 177–182.
Rice, D., Barone Jr., S., 2000. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ. Health Perspect. 108 (Suppl. 3), 511–533.
Sebaai, N., Lesage, J., Breton, C., Vieau, D., Deloof, S., 2004. Perinatal food deprivation induces marked alterations of the hypothalamo–pituitary–adrenal axis in 8-month-old male rats both under basal conditions and after a dehydration period. Neuroendocrinology 79, 163–173.
Stein, A.D., Pierik, F.H., Verrips, G.H., Susser, E.S., Lumey, L.H., 2009. Maternal exposure to the Dutch famine before conception and during pregnancy: quality of life and depressive symptoms in adult offspring. Epidemiology 20, 909–915.
Tanumihardjo, S.A., Anderson, C., Kaufer-Horwitz, M., Bode, L., Emenaker, N.J., Haqq, A.M., Satia, J.A., Silver, H.J., Stadler, D.D., 2007. Poverty, obesity, and malnutrition: an international perspective recognizing the paradox. J. Am. Diet. Assoc. 107, 1966–1972.
Taylor, D.J., Howie, P.W., 1989. Fetal growth achievement and neurodevelopmental disability. Br. J. Obstet. Gynaecol. 96, 789–794.
Tonkiss, J., Galler, J.R., Formica, R.N., Shukitt-Hale, B., Timm, R.R., 1990a. Fetal protein malnutrition impairs acquisition of a DRL task in adult rats. Physiol. Behav. 48, 73–77.
Tonkiss, J., Shukitt-Hale, B., Formica, R.N., Rocco, F.J., Galler, J.R., 1990b. Prenatal protein malnutrition alters response to reward in adult rats. Physiol. Behav. 48, 675–680.
Tonkiss, J., Shultz, P.L., Shumsky, J.S., Galler, J.R., 1997. Development of spatial navigation following prenatal cocaine and malnutrition in rats: lack of additive effects. Neurotoxicol. Teratol. 19, 363–372.
Torres, N., Bautista, C.J., Tovar, A.R., Ordaz, G., Rodriguez-Cruz, M., Ortiz, V., Granados, O., Nathanielsz, P.W., Larrea, F., Zambrano, E., 2010. Protein restriction during pregnancy affects maternal liver lipid metabolism and fetal brain lipid composition in the rat. Am. J. Physiol. Endocrinol. Metab. 298 (2), E270–E277.
Trzctnska, M., Tonkiss, J., Galler, J.R., 1999. Influence of prenatal protein malnutrition on behavioral reactivity to stress in adult rats. Stress 3, 71–83.
Watkins, A.J., Wilkins, A., Cunningham, C., Perry, V.H., Seet, M.J., Osmond, C., Eckert, J.J., Torrens, C., Cagampang, F.R., Cleal, J., Gray, W.P., Hanson, M.A., Fleming, T.P., 2008. Low protein diet fed exclusively during mouse oocyte maturation leads to behavioural and cardiovascular abnormalities in offspring. J. Physiol. 586, 2231–2244.
Zambrano, E., Rodriguez-Gonzalez, G.L., Guzman, C., Garcia-Becerra, R., Boeck, L., Diaz, L., Menjivar, M., Larrea, F., Nathanielsz, P.W., 2005. A maternal low protein diet during pregnancy and lactation in the rat impairs male reproductive development. J. Physiol. 563, 275–284.
Zhang, Y., Li, N., Yang, Z., 2010. Perinatal food restriction impaired spatial learning and memory behavior and decreased the density of nitric oxide synthase neurons in the hippocampus of adult male rat offspring. Toxicol. Lett. 193, 167–172.

Int. J. Devl Neuroscience 30 (2012) 75–81

Contents lists available at SciVerse ScienceDirect

International Journal of Developmental Neuroscience

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

Maternal obesity in the rat programs male offspring exploratory, learning and motivation behavior: prevention by dietary intervention pre-gestation or in gestation

J.S. Rodriguez [b], G.L. Rodríguez-González [a], L.A. Reyes-Castro [a], C. Ibáñez [a], A. Ramírez [a], R. Chavira [a], F. Larrea [a], P.W. Nathanielsz [b], E. Zambrano [a,*]

[a] Department of Reproductive Biology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, México City 14000, Mexico
[b] Center for Pregnancy and Newborn Research, Department of Obstetrics and Gynecology, University of Texas Health Sciences Center, San Antonio, TX 78229, USA

ARTICLE INFO

Article history:
Received 15 August 2011
Received in revised form
16 December 2011
Accepted 28 December 2011

Keywords:
Developmental programming
Cognition
Stress response
Consummatory behavior
Corticosterone

ABSTRACT

We studied the effects of maternal high fat diet (HFD, 25% calories from fat administered before and during pregnancy and lactation) and dietary intervention (switching dams from HFD to control diet) at different periconceptional periods on male offspring anxiety related behavior, exploration, learning, and motivation. From weaning at postnatal day (PND) 21, female subjects produced to be the mothers in the study received either control diet (CTR – 5% calories from fat), HFD through pregnancy and lactation (MO), HFD during PNDs 21–90 followed by CTR diet (pre-gestation (PG) intervention) or HFD from PND 21 to 120 followed by CTR diet (gestation and lactation (G) intervention) and bred at PND 120. At 19 days of gestation maternal serum corticosterone was increased in MO and the PG and G dams showed partial recovery with intermediate levels. In offspring, no effects were found in the elevated plus maze test. In the open field test, MO and G offspring showed increase zone entries, displaying less thigmotaxis; PG offspring showed partial recuperation of this behavior. During initial operant conditioning MO, PG and G offspring displayed decreased approach behavior with subsequent learning impairment during the acquisition of FR-1 and FR-5 operant conditioning for sucrose reinforcement. Motivation during the progressive ratio test increased in MO offspring; PG and G intervention recuperated this behavior. We conclude that dietary intervention can reverse negative effects of maternal HFD and offspring outcomes are potentially due to elevated maternal corticosterone.

1. Introduction

Maternal obesity negatively influences maternal, fetal and offspring life-time phenotype including unwanted effects on offspring brain development, behavior, affect and cognition [for review see (Sullivan et al., 2011; Tsoi et al., 2010)]. One prospective clinical study of maternal obesity outcomes reported high inattention scores and a two-fold increase in risk of difficulties with emotional regulation in 5-year-old children (Rodriguez, 2010). In animal models, maternal obesity causes brain developmental abnormalities in offspring hypothalamic and hippocampal areas, and in the serotonergic, dopaminergic and opioid systems which result in increased anxiety, impairment in spatial learning and memory and desensitization of the reward system (Bilbo and Tsang, 2010; Bouret, 2010b; Naef et al., 2008, 2011; Naef and Woodside, 2007; Sullivan et al., 2010; Tozuka et al., 2010; Vucetic et al., 2010; Walker et al., 2008; Wright et al., 2011). Since controlled, experimental dietary manipulation combined with the required intensive behavioral testing of offspring is not possible in humans, it is necessary to use animal models to examine the effects of specific models of maternal over nutrition with and without dietary intervention on offspring development and behavior.

Human epidemiological (Dabelea, 2007; Solomons, 2009; Wadhwa et al., 2009) and animal studies (Bautista et al., 2008; Bouret, 2010a; Han et al., 2004; Nijland et al., 2008; Nuyt and Alexander, 2009; Symonds et al., 2009; Taylor and Poston, 2007; Warner and Ozanne, 2010) demonstrate that the periconceptional, fetal and early post-natal nutritional environments modify the development of offspring physiological systems including cardiovascular, metabolic and endocrine function. These observations have led to the concept of a nutritional basis for the developmental origins of adult disease (Armitage et al., 2004; Warner and Ozanne, 2010). Developmental programming of offspring resulting in metabolic disorders or obesity can occur following either maternal under-nutrition (da Silva et al., 2011; Desai et al., 2007; Hyatt

* Corresponding author. Tel.: +52 55 5487 0900x2417; fax: +52 55 5655 9859.
E-mail address: zamgon@unam.mx (E. Zambrano).

0736-5748/$36.00 © 2012 ISDN. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.ijdevneu.2011.12.012

et al., 2011; Sebert et al., 2010; Zambrano et al., 2006) or overnutrition (Bayol et al., 2010; Wright et al., 2011; Zambrano et al., 2010). We have recently reported developmental programming effects of pre and/or postnatal protein restriction in rat offspring showing reduced motivation, impaired learning and decreased thigmotaxis at adult age (Reyes-Castro et al., 2011a,b; Torres et al., 2010). However, there a few data on the developmental programming effects of maternal obesity and accompanying excess nutrient intake which are becoming major concerns since more than 60% of childbearing age women in developed countries are overweight (King, 2006).

We recently reported on the potential of dietary intervention to modify offspring metabolic outcomes resulting from maternal obesity and HFD prior to pregnancy (Zambrano et al., 2010). In the present study we wished to determine if dietary intervention, *i.e.* returning the dam from a HFD to a normal diet, at different periconceptional periods would influence offspring behavioral effects. We hypothesized that in male offspring (1) maternal HFD would negatively impact aspects of anxiety related behavior, exploration, learning and motivation behaviors and (2) dietary intervention would ameliorate some of these negative outcomes in a manner dependent on the timing of the dietary recuperation. Four groups of weanling female rats were administered either control diet (CTR – 5% calories from fat), a high fat diet (HFD – 25% calories from fat) from postnatal day (PND) 21 through pregnancy and lactation (MO group), the HFD during PND's 21–90 followed by CTR diet during pregnancy and lactation (pre-gestation (PG) dietary intervention group) and the HFD from PND 21 to 120 followed by CTR diet during pregnancy and lactation (gestation (G) dietary intervention group). Male offspring behavior was assessed to determine HFD effects on anxiety, exploration, learning, and motivation and offspring improvement by maternal dietary intervention.

2. Methods

2.1. Animal care and use

2.1.1. Subjects used to produce the dams for the pregnancies studied

Female albino Wistar rats were born and maintained in the colony of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INNSZ), Mexico City, Mexico: an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International accredited facility. All procedures were approved by the Animal Experimentation Ethics Committee of the INNSZ, Mexico City, Mexico. Subjects were housed in a light-controlled environment (lights on from 07:00 to 19:00 h at 22–23 °C) and fed normal laboratory chow (Zeigler Rodent RQ 22-5, USA) containing 22.0% protein, 5.0% fat, 31.0% polysaccharide, 31.0% simple sugars, 4.0% fiber, 6.0% minerals and 1.0% vitamins (w/w), energy 4.0 kcal g^{-1} (0.2 kcal g^{-1} from fat). Between 14 and 16 weeks of age (average weight 220 ± 20 g), females were bred with a non-litter mate proven male breeder. Dams delivered naturally at term and on postnatal day 2 litters were culled to 10 pups maintaining as near a 1:1 ratio of males and females.

2.2. Experimental dams

At weaning (PND 21), the prospective mothers were randomly assigned to either a control (CTR; $n = 12$) diet that received laboratory chow or a maternal obesity diet (MO; $n = 36$) – a high fat diet containing 23.5% protein, 20.0% animal lard, 5.0% fat, 20.2% polysaccharide, 20.2% simple sugars, 5.0% fiber, 5.0% mineral mix, 1.0% vitamin mix (w/w), energy 4.9 kcal g^{-1} (1.23 kcal g^{-1} from animal lard and fat). Only one female from any one litter was assigned to a group. At PND 90, 1 month before breeding, 12 MO females were assigned at random to the dietary intervention (DINT) pre-gestation group and placed back on CTR diet for the rest of the study, *i.e.* before and during pregnancy and lactation. At PND 120 all groups were bred and the day spermatozoa were present in a vaginal smear designated as day of conception. At this time 12 MO females were assigned at random to the DINT group during gestation (G) and switched to CTR diet for the rest of the study, *i.e.* pregnancy and lactation. The other 3 groups were fed their pre-pregnancy diet throughout pregnancy and lactation (see Table 1 for groups). At 19 days of gestation 6 dams from each of the 4 groups were euthanized to collect serum for corticosterone measurements. All remaining dams (6 per group) delivered spontaneously. The day of delivery was considered as PND 0. Food and water were available *ad libitum*. Pregnant and lactating rats were weighed every day through pregnancy and until pups were removed at weaning.

2.3. Maintenance of offspring

Litter size and pup weight were recorded at birth. Ano-genital distance, anterior–posterior abdominal distance and head diameter were measured with calipers. Our published data indicate that ano-genital distance is 1.67 ± 0.13 mm ($n = 291$ pups from 43 litters; mean ± SEM) in female pups and 3.26 ± 0.22 mm ($n = 252$ pups from 43 litters) in males at birth (Zambrano et al., 2005a). Since a value of 2.5 mm is more than 2 SDs from the mean of either group, sex was judged according to whether the ano-genital distance was >2.5 mm for males. To ensure homogeneity of offspring evaluated, all litters studied were adjusted to 10 pups per dam. The sex ratio was maintained as close to 1:1 as possible. Pups continued to be weighed every week.

2.4. Elevated plus maze (EPM)/open field

Two weeks prior to all behavioral testing, a reverse light cycle was implemented (lights off at 7 a.m. and on at 7 p.m.) with testing occurring during the dark phase. Subjects were assessed 7 days a week at the same time of the dark cycle for each subject between 8 a.m. and 4 p.m. At PND 75 six male unrelated naïve subjects per treatment group were tested. The specifications of data collection, the EPM and the open field apparatus have been described in detail (Reyes-Castro et al., 2011a).

2.5. Operant conditioning

On PND 80 six unrelated male offspring from different litters per diet group were tested in operant chambers (E10-10TC, Coulbourn-Instruments, PA, USA) as previously described (Reyes-Castro et al., 2011b). Two weeks prior to onset of operant training offspring were placed on water deprivation for 23 h/day with 1 h of free access. This continued throughout training and testing with the 1 h of free access immediately following behavioral sessions. For each trial the lever was extended until pressed, after which the subject was allowed 120 s 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 s. Each trial was followed by an inter-trial interval of 5 s during which the lever was retracted. FR-1 conditioning was complete when subjects earned 20 reinforcements during a 15-min session. After all subjects 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.

2.6. Progressive ratio testing

Following operant conditioning, each subject commenced progressive ratio testing for 10 days. In the progressive ratio schedule, an additional lever press is required for all subsequent reinforcements for the first eight reinforcements (progressive ratio + 1). For instance, one press for the first reinforcement and four presses for the fourth reinforcement. Following every eighth reinforcement, the response increment doubles 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. Progressive ratio sessions were 30 min in length.

2.7. Free sucrose consumption

To assess sucrose consumption behavior, subjects were given direct access to bottled sucrose solution (7%) for 30 min in the familiar colony room 1 day after the last progressive ratio session. For this evaluation subjects were single caged and sucrose consumption was calculated by subtraction of the bottle weight at the end of the session from the initial weight. This procedure was performed on 3 consecutive days.

2.8. Corticosterone measurements

In dams at 19 days of gestation and in the male offspring on PND 110, subjects were sacrificed by decapitation and blood samples taken from the neck to determine corticosterone serum levels. Blood samples were centrifuged at 4 °C for 15 min at 3500 rpm to remove red blood cells and serum stored at −20 °C until all samples were analyzed. Corticosterone serum levels were determinate by radioimmunoassay using a commercial rat kit, DPC Coat-a-count (TKRC1) from Diagnostic Products (Los Angeles, CA, USA). Intra- and inter-assay variability was <6% and <7%. The kit was used in accordance with manufacturer's instructions and samples were measured in duplicate.

2.9. Statistical analyses

All data are presented as Mean ± SEM, alpha level was set at 0.05. Behavioral endpoints and corticosterone levels were analyzed by ANOVA with between-subject factor of early life manipulation (maternal diet during the different periods). Post hoc analyses were performed by Tukey test using Sigma Stat 3.5.

Table 1
Experimental groups.

Groups	Maternal diet				Offspring diet
	PND 21–90	PND 90–120	Pregnancy	Lactation	
Control (CTR)	Control	Control	Control	Control	Control
Maternal obesity (MO)	High fat	High fat	High fat	High fat	Control
Pre-gestational dietary intervention (PG)	High fat	Control	Control	Control	Control
Gestational dietary intervention (G)	High fat	High fat	Control	Control	Control

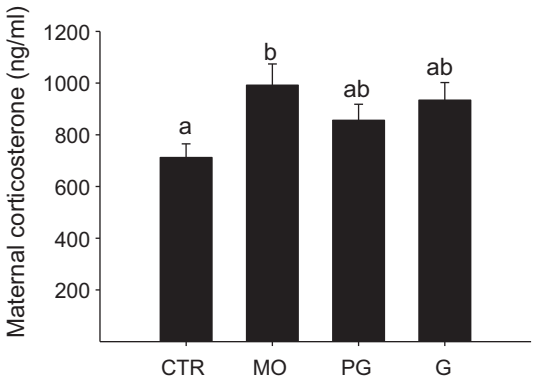

Fig. 1. Maternal serum corticosterone levels at 19 days gestation. Mean ± SEM, $n = 6$ dams. Data not sharing a letter are statistically different, $p < 0.05$.

3. Results

3.1. Maternal corticosterone

Maternal corticosterone was higher at 19 days gestation in MO than CTR dams (Fig. 1, $p < 0.05$) with intermediate levels in the PG and G which did not reach significance.

3.2. Elevated plus maze

On PND 75 male offspring were administered the EPM to measure anxiety related behaviors. Offspring displayed no differences in the number of entries, time spent or distance traveled in the open arms, or total distance traveled (Fig. 2A–D).

3.3. Open field

Analyses of behavioral endpoints in the open field test revealed differences in experimental offspring. The MO and G offspring had increased border zone entries compared to CTR offspring (Table 2, $p < 0.05$). The G offspring had increased center zone entries *versus* CTR and increased center zone distance traveled compared to CTR and PG offspring (Table 2, $p < 0.05$). No differences were found for total distance, border zone time, border zone distance, and center zone time (Table 2).

3.4. Operant conditioning and progressive ratio

In offspring initial exposure to the operant chamber revealed differences in the number of sessions before approach and response to the reinforcement contingent lever. The MO, PG and G offspring required more sessions to respond *versus* the CTR offspring (Fig. 3A, $p < 0.05$). For fixed ratio 1 schedule of reinforcement (FR-1), group differences were determined for the number of sessions before

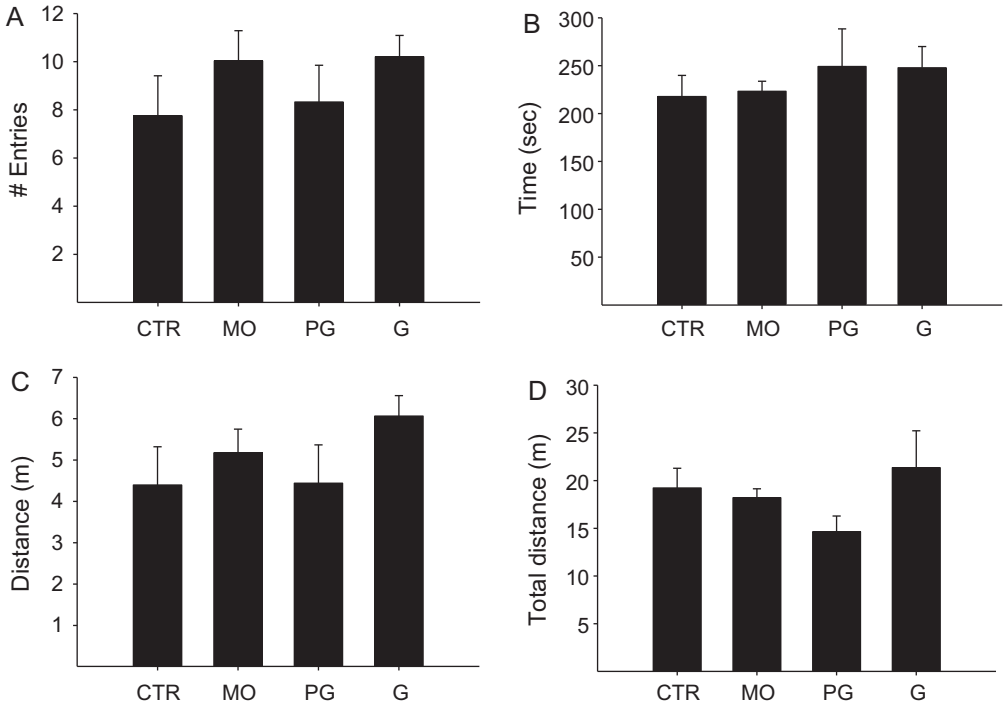

Fig. 2. Elevated plus maze endpoints. (A) Open arm entries, (B) open arm time (s), (C) open arm distance traveled (m), (D) total distance traveled (m). Mean ± SEM, $n = 6$ from different litter. Data not sharing a letter are statistically different, $p < 0.05$.

Table 2
Open field.

Group	Total distance	Border zone entries	Border zone time	Border zone distance	Center zone entries	Center zone time	Center zone distance
CTR	44.3 ± 4.8	12.3 ± 1.7[a]	519 ± 45.7	39.1 ± 4.2	11.6 ± 1.9[a]	81 ± 45.7	5.2 ± 0.9[a]
MO	56.6 ± 10.8	22.3 ± 2.9[b]	539 ± 6.0	48.7 ± 10.2	21.3 ± 2.9[ab]	61 ± 5.9	7.9 ± 0.9[ab]
PG	50.5 ± 5.0	16.3 ± 2.4[ab]	547 ± 9.3	43.8 ± 4.1	15.8 ± 2.5[ab]	53 ± 9.3	6.6 ± 1.2[a]
G	48.5 ± 4.5	25 ± 6.2[b]	461 ± 17.4	36.5 ± 3.2	25.3 ± 2.6[b]	139 ± 17.4	12.0 ± 1.6[b]

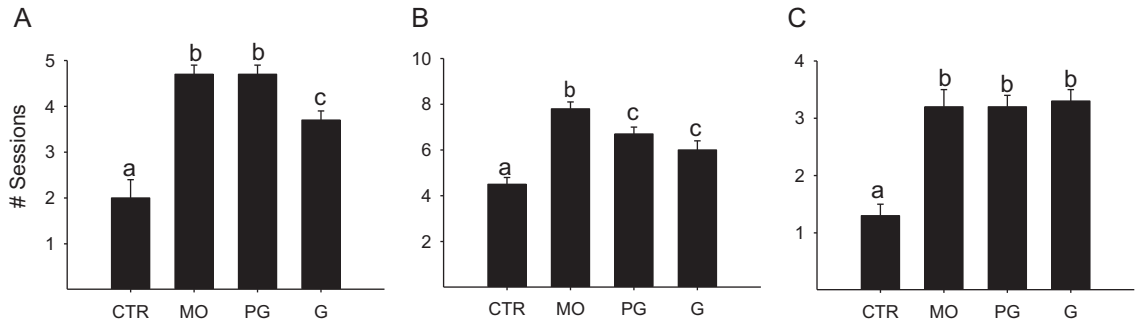

Fig. 3. (A) Number of sessions required for offspring to press the operant lever for initial positive reinforcement. (B) Number of sessions required for offspring to attain FR-1 performance criterion. (C) Number of sessions required for offspring to attain FR-5 performance criterion. Mean ± SEM, $n = 6$ pups. Data not sharing a letter are statistically different, $p < 0.05$.

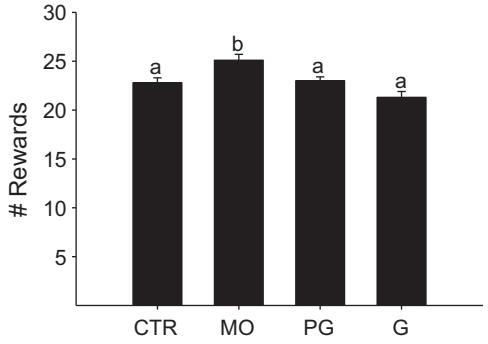

Fig. 4. Number of rewards received during progressive ratio sessions. Mean ± SEM, $n = 6$ pups. Data not sharing a letter are statistically different, $p < 0.05$.

offspring attained criterion. The MO, PG and G offspring all required more sessions to attain performance criterion with the MO group requiring the most sessions (Fig. 3B, $p < 0.05$). Fixed ratio 5 schedule of reinforcement (FR-5) shows MO, PG and G offspring all required more session to reach performance criterion *versus* CTR offspring (Fig. 3C, $p < 0.05$). Effects on motivation as assessed by progressive ratio tasks show increased responding in MO offspring compared to CTR, PG and G groups (Fig. 4, $p < 0.05$).

3.5. Free sucrose consumption

No overall treatment effect was determined for free access to 7% sucrose during three 30 min sessions (Table 3).

3.6. Offspring corticosterone

Corticosterone male serum levels were measured on PND 110. The MO offspring show decreased corticosterone levels compared to the PG and G offspring but not *versus* CTR offspring (Fig. 5, $p < 0.05$).

Table 3
7% sucrose consumption during 30 min (average of 3 days).

	CTR	MO	PG	G	ANOVA
(ml)	18.4 ± 0.3	17.3 ± 0.3	18.5 ± 0.3	18.0 ± 0.3	$P = 0.051$

4. Discussion

Developmental exposure to environmental challenges in offspring can influence various aspects of behavior. In a model of maternal obesity in the rat (Zambrano et al., 2010), we sought to determine the behavioral effects in male offspring born to dams administered a HFD from PND 21 through pregnancy and lactation (maternal obesity, MO group), HFD from PND 21 to 90 and switched to control diet 1 month before mating and during pregnancy and lactation (pre-gestation dietary intervention, PG group) and from PND 21 to 120 but not during pregnancy and lactation (gestational dietary intervention, G group). The different windows of HFD regimens administrated produced physiological differences in the mothers (Zambrano et al., 2010), of particular importance, maternal corticosterone serum levels at 19 days of gestation were increased in the MO group but to a lesser degree in the PG or G groups. Increased maternal corticosterone has been demonstrated in many situations that result in developmental programming of offspring by altered maternal nutrition and may constitute a common feature that explains some of the similarities in outcomes from

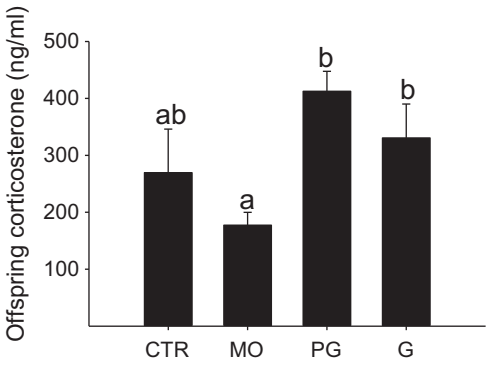

Fig. 5. Offspring corticosterone serum levels. Mean ± SEM, $n = 6$ from different litter. Data not sharing a letter are statistically different, $p < 0.05$.

different challenges (Cottrell and Seckl, 2009; Langley-Evans, 2009; Zambrano et al., 2005b; Guzman et al., 2006).

4.1. Anxiety related behavior and exploratory effects

Consistent with our findings here, anxiolytic effects in offspring following early postnatal overfeeding in male and female offspring (Spencer and Tilbrook, 2009) and following maternal HFD during lactation in male offspring have previously been reported (Wright et al., 2011). Those studies demonstrated that raising rats in small litters (Spencer and Tilbrook, 2009) or dams fed a cafeteria diet during pre-gestation, gestation and/or lactation (Wright et al., 2011) induces obesity in offspring and reduces anxiety in the EPM and the open field. Additionally, chronic consumption of a HFD during pregnancy causes perturbations in the serotonergic system, such as increased expression of tryptophan hydroxylase 2 and serotonin 1 A receptor in the rostral raphe nucleus, and increases anxiety-like behavior in rhesus monkey offspring (Sullivan et al., 2010).

In this study the anxiolytic effects were not apparent in the experimental offspring during EPM but were observed during the open field test. These inconsistent behavioral responses in the EPM and open field tests are not surprising since studies indicate that behavioral tests that rely on unconditioned responses assess different aspects of affect and that emotional reactivity is multidimensional (Ramos, 2008; Ramos et al., 2008; Trullas and Skolnick, 1993; Vendruscolo et al., 2003). A current review contends that the EPM, open field apparatus and the light/dark box tests should be administered concomitantly to more adequately assess affect since time and/or sequence of test administration can influence outcomes (Ramos, 2008). One potential effect of testing sequentially across days is that effects are reduced as testing progresses (Ramos, 2008). However, here we report negative results in the first task administered (EPM) and positive results in the subsequent task (open field) which makes the preceding argument less likely. Alternatively, inconsistent results obtained across tests may be due to construct differences between tests or to uncontrolled, intra-individual fluctuations in behavior (Ramos, 2008).

4.2. Learning effects

During FR-1 and FR-5 operant conditioning MO, PG and G offspring displayed impaired learning. So in experimental offspring, maternal dietary intervention did not prevent these particular cognitive deficiencies. In support of our findings in the MO group, previous studies report hippocampal brain derived neurotropic factor is decreased along with hippocampal neurogenesis and impairment in spatial learning in mice offspring exposed to a HFD during pregnancy and lactation (Tozuka et al., 2009, 2010). It should be noted that maternal dietary intervention at either period, 1 month before pregnancy or at the beginning of pregnancy, did not prevent learning impairment suggesting that maternal diet long-term prior to conception is critical for male development.

We have previously measured elevated corticosterone, estradiol, testosterone (Zambrano et al., 2005b) and progesterone (Guzman et al., 2006), concentrations near term (19 days gestation) in prenatal protein restricted rat dams with subsequent cognitive impairment in offspring (Reyes-Castro et al., 2011b, 2012). In the present study maternal corticosterone levels were increased in MO dams and marginally, though not significantly, increased in the PG and G dams. Maternal steroids can cross the placenta, and such exposure to transplacentally acquired androgens and glucocorticoids in fetal life can result in developmental perturbations which 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* (Meyer-Bahlburg, 2011; 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 (Antonow-Schlorke et al., 2001, 2003; French et al., 2004; Johnson et al., 1981; Karemaker et al., 2006, 2008; Matthews, 2001; Rodriguez et al., 2011; Seckl, 2008; Szuran et al., 2000; Uno et al., 1990, 1994; Weinstock, 2008) which could explain the cognitive deficits demonstrated in this study.

4.3. Motivation effects

During motivation assessment, MO offspring display increased motivation. In this context, dietary intervention normalized motivation in male PG and G offspring. The increased motivation displayed by the MO offspring is consistent with models showing increased appetitive and/or consummatory drive following maternal or early life over nutrition (Chang et al., 2008; Desai et al., 2007; Sebert et al., 2009). Similar to the MO offspring outcomes in this study, rats born to dams fed a junk food diet during gestation and lactation develop hyperphagia and a preference for fatty, sugary and salty foods over protein-rich foods compared to offspring fed a balanced chow diet prior to weaning or during lactation alone (Bayol et al., 2008). In the present study, effects of maternal HFD on offspring sucrose consumption behavior were measured there was no overall effect of perinatal diet, so the influence of consummatory behavior on progressive ratio (motivation) behavior is not applicable.

5. Conclusions

Maternal HFD administration produces an altered behavioral phenotype in male offspring. Dietary intervention recuperated or ameliorated certain indices in dams and offspring. In dams, corticosterone levels were reduced in the PG and G groups to between CTR and MO levels. However effects on offspring learning could still have been affected by the increased levels of maternal prenatal corticosterone as all experimental offspring displayed learning impairment. Prenatal exposure to increased levels of glucocorticoids changes hypothalamic pituitary adrenal axis function as well as associated receptors expression levels [for review see (Kapoor et al., 2008)]. Normal levels of motivation were restored by PG and G intervention. Additionally during exploratory behaviors, PG intervention prevented the increased exploration behavior displayed by the MO and G offspring in the open field. These findings show the importance of optimizing maternal diet and avoiding the complication of obesity. It also holds out the hope that recuperation of the diet prior to pregnancy can have beneficial long-term effects.

Acknowledgements

L.A. Reyes-Castro and G.L. Rodríguez-González are graduate students from Doctorado en Ciencias Biomédicas, Facultad de Medicina, Universidad Nacional Autónoma de México and are recipients of CONACyT fellowship. This work was supported by Consejo Nacional de Ciencias y Tecnología (CONACyT 155166), México and NIH HD 21350.

References

Antonow-Schlorke, I., Kuhn, B., Muller, T., Schubert, H., Sliwka, U., Nathanielsz, P.W., Schwab, M., 2001. Antenatal betamethasone treatment reduces synaptophysin immunoreactivity in presynaptic terminals in the fetal sheep brain. Neurosci. Lett. 297, 147–150.

Antonow-Schlorke, I., Schwab, M., Li, C., Nathanielsz, P.W., 2003. Glucocorticoid exposure at the dose used clinically alters cytoskeletal proteins and presynaptic terminals in the fetal baboon brain. J. Physiol. 547, 117–123.

Armitage, J.A., Khan, I.Y., Taylor, P.D., Nathanielsz, P.W., Poston, L., 2004. Developmental programming of the metabolic syndrome by maternal nutritional

imbalance: how strong is the evidence from experimental models in mammals? J. Physiol. 561, 355–377.
Bautista, C.J., Boeck, L., Larrea, F., Nathanielsz, P.W., Zambrano, E., 2008. Effects of a maternal low protein isocaloric diet on milk leptin and progeny serum leptin concentration and appetitive behavior in the first 21 days of neonatal life in the rat. Pediatr. Res. 63, 358–363.
Bayol, S.A., Simbi, B.H., Bertrand, J.A., Stickland, N.C., 2008. Offspring from mothers fed a 'junk food' diet in pregnancy and lactation exhibit exacerbated adiposity that is more pronounced in females. J. Physiol. 586, 3219–3230.
Bayol, S.A., Simbi, B.H., Fowkes, R.C., Stickland, N.C., 2010. A maternal junk food diet in pregnancy and lactation promotes nonalcoholic Fatty liver disease in rat offspring. Endocrinology 151, 1451–1461.
Bilbo, S.D., Tsang, V., 2010. Enduring consequences of maternal obesity for brain inflammation and behavior of offspring. FASEB J. 24, 2104–2115.
Bouret, S.G., 2010a. Development of hypothalamic neural networks controlling appetite. Forum Nutr. 63, 84–93.
Bouret, S.G., 2010b. Role of early hormonal and nutritional experiences in shaping feeding behavior and hypothalamic development. J. Nutr. 140, 653–657.
Chang, G.Q., Gaysinskaya, V., Karatayev, O., Leibowitz, S.F., 2008. Maternal high-fat diet and fetal programming: increased proliferation of hypothalamic peptide-producing neurons that increase risk for overeating and obesity. J. Neurosci. 28, 12107–12119.
Cottrell, E.C., Seckl, J.R., 2009. Prenatal stress, glucocorticoids and the programming of adult disease. Front Behav. Neurosci. 3, 19.
da Silva, L.N., Gaspar de, M.E., Cottini Fonseca, P.M., Firmino Nogueira, N.J., Martha, R.A., de, O.E., Cristina, L.P., 2011. Early weaning causes undernutrition for a short period and programmes some metabolic syndrome components and leptin resistance in adult rat offspring. Br. J. Nutr. 105, 1405–1413.
Dabelea, D., 2007. The predisposition to obesity and diabetes in offspring of diabetic mothers. Diabetes Care 30 (Suppl. 2), S169–S174.
Desai, M., Babu, J., Ross, M.G., 2007. Programmed metabolic syndrome: prenatal undernutrition and postweaning overnutrition. Am. J. Physiol. Regul. Integr. Comp. Physiol. 293, R2306–R2314.
French, N.P., Hagan, R., Evans, S.F., Mullan, A., Newnham, J.P., 2004. Repeated antenatal corticosteroids: effects on cerebral palsy and childhood behavior. Am. J. Obstet. Gynecol. 190, 588–595.
Guzman, C., Cabrera, R., Cardenas, M., Larrea, F., Nathanielsz, P.W., Zambrano, E., 2006. Protein restriction during fetal and neonatal development in the rat alters reproductive function and accelerates reproductive ageing in female progeny. J. Physiol. 572, 97–108.
Han, H.C., Austin, K.J., Nathanielsz, P.W., Ford, S.P., Nijland, M.J., Hansen, T.R., 2004. Maternal nutrient restriction alters gene expression in the ovine fetal heart. J. Physiol. 558, 111–121.
Hyatt, M.A., Gardner, D.S., Sebert, S., Wilson, V., Davidson, N., Nigmatullina, Y., Chan, L.L., Budge, H., Symonds, M.E., 2011. Suboptimal maternal nutrition, during early fetal liver development, promotes lipid accumulation in the liver of obese offspring. Reproduction 141, 119–126.
Johnson, J.W., Mitzner, W., Beck, J.C., London, W.T., Sly, D.L., Lee, P.A., Khouzami, V.A., Cavalieri, R.L., 1981. Long-term effects of betamethasone on fetal development. Am. J. Obstet. Gynecol. 141, 1053–1064.
Kapoor, A., Petropoulos, S., Matthews, S.G., 2008. Fetal programming of hypothalamic-pituitary-adrenal (HPA) axis function and behavior by synthetic glucocorticoids. Brain Res. Rev. 57, 586–595.
Karemaker, R., Heijnen, C.J., Veen, S., Baerts, W., Samsom, J., Visser, G.H., Kavelaars, A., van Doornen, L.J., van, B.F., 2006. Differences in behavioral outcome and motor development at school age after neonatal treatment for chronic lung disease with dexamethasone versus hydrocortisone. Pediatr. Res. 60, 745–750.
Karemaker, R., Kavelaars, A., ter, W.M., Tersteeg-Kamperman, M., Baerts, W., Veen, S., Samsom, J.F., Visser, G.H., van, B.F., Heijnen, C.J., 2008. Neonatal dexamethasone treatment for chronic lung disease of prematurity alters the hypothalamus-pituitary-adrenal axis and immune system activity at school age. Pediatrics 121, e870–e878.
King, J.C., 2006. Maternal obesity, metabolism, and pregnancy outcomes. Annu. Rev. Nutr. 26, 271–291.
Langley-Evans, S.C., 2009. Nutritional programming of disease: unravelling the mechanism. J. Anat. 215, 36–51.
Matthews, S.G., 2001. Antenatal glucocorticoids and the developing brain: mechanisms of action. Semin. Neonatol. 6, 309–317.
Meyer-Bahlburg, H.F., 2011. Brain development and cognitive, psychosocial, and psychiatric functioning in classical 21-hydroxylase deficiency. Endocr. Dev. 20, 88–95.
Naef, L., Moquin, L., Dal, B.G., Giros, B., Gratton, A., Walker, C.D., 2011. Maternal high-fat intake alters presynaptic regulation of dopamine in the nucleus accumbens and increases motivation for fat rewards in the offspring. Neuroscience 176, 225–236.
Naef, L., Srivastava, L., Gratton, A., Hendrickson, H., Owens, S.M., Walker, C.D., 2008. Maternal high fat diet during the perinatal period alters mesocorticolimbic dopamine in the adult rat offspring: reduction in the behavioral responses to repeated amphetamine administration. Psychopharmacology (Berl) 197, 83–94.
Naef, L., Woodside, B., 2007. Prolactin/Leptin interactions in the control of food intake in rats. Endocrinology 148, 5977–5983.
Nijland, M.J., Ford, S.P., Nathanielsz, P.W., 2008. Prenatal origins of adult disease. Curr. Opin. Obstet. Gynecol. 20, 132–138.
Nuyt, A.M., Alexander, B.T., 2009. Developmental programming and hypertension. Curr. Opin. Nephrol. Hypertens. 18, 144–152.
Puts, D.A., McDaniel, M.A., Jordan, C.L., Breedlove, S.M., 2008. Spatial ability and prenatal androgens: meta-analyses of congenital adrenal hyperplasia and digit ratio (2D:4D) studies. Arch. Sex Behav. 37, 100–111.
Ramos, A., 2008. Animal models of anxiety: do I need multiple tests? Trends Pharmacol. Sci. 29, 493–498.
Ramos, A., Pereira, E., Martins, G.C., Wehrmeister, T.D., Izidio, G.S., 2008. Integrating the open field, elevated plus maze and light/dark box to assess different types of emotional behaviors in one single trial. Behav. Brain Res. 193, 277–288.
Reyes-Castro, L.A., Rodriguez, J.S., Rodriguez-Gonzalez, G.L., Chavira, R., Bautista, C.J., McDonald, T.J., Nathanielsz, P.W., Zambrano, E., 2011a. Pre and/or postnatal protein restriction developmentally programs affect and risk assessment behaviors in adult male rats. Behav. Brain Res., doi:10.1016/j.bbr.2011.06.008.
Reyes-Castro, L.A., Rodriguez, J.S., Rodriguez-Gonzalez, G.L., Wimmer, R.D., McDonald, T.J., Larrea, F., Nathanielsz, P.W., Zambrano, E., 2011b. Pre- and/or postnatal protein restriction in rats impairs learning and motivation in male offspring. Int. J. Dev. Neurosci. 29, 177–182.
Reyes-Castro, L.A., Rodriguez, J.S., Charco, R., Bautista, C.J., Larrea, F., Nathanielsz, P.W., Zambrano, E., 2012. Maternal protein restriction in the rat during pregnancy and/or lactation alters cognitive and anxiety behaviors of female offspring. Int. J. Dev. Neurosci. 30, 39–45.
Rodriguez, A., 2010. Maternal pre-pregnancy obesity and risk for inattention and negative emotionality in children. J. Child Psychol. Psychiatry 51, 134–143.
Rodriguez, J.S., Zurcher, N.R., Keenan, K.E., Bartlett, T.Q., Nathanielsz, P.W., Nijland, M.J., 2011. Prenatal betamethasone exposure has sex specific effects in reversal learning and attention in juvenile baboons. Am. J. Obstet. Gynecol., doi:10.1016/j.ajog.2011.01.063.
Sebert, S.P., Hyatt, M.A., Chan, L.L., Patel, N., Bell, R.C., Keisler, D., Stephenson, T., Budge, H., Symonds, M.E., Gardner, D.S., 2009. Maternal nutrient restriction between early and midgestation and its impact upon appetite regulation after juvenile obesity. Endocrinology 150, 634–641.
Sebert, S.P., Hyatt, M.A., Chan, L.L., Yiallourides, M., Fainberg, H.P., Patel, N., Sharkey, D., Stephenson, T., Rhind, S.M., Bell, R.C., Budge, H., Gardner, D.S., Symonds, M.E., 2010. Influence of prenatal nutrition and obesity on tissue specific fat mass and obesity-associated (FTO) gene expression. Reproduction 139, 265–274.
Seckl, J.R., 2008. Glucocorticoids, developmental 'programming' and the risk of affective dysfunction. Prog. Brain Res. 167, 17–34.
Solomons, N.W., 2009. Developmental origins of health and disease: concepts, caveats, and consequences for public health nutrition. Nutr. Rev. 67 (Suppl. 1), S12–S16.
Spencer, S.J., Tilbrook, A., 2009. Neonatal overfeeding alters adult anxiety and stress responsiveness. Psychoneuroendocrinology 34, 1133–1143.
Sullivan, E.L., Grayson, B., Takahashi, D., Robertson, N., Maier, A., Bethea, C.L., Smith, M.S., Coleman, K., Grove, K.L., 2010. Chronic consumption of a high-fat diet during pregnancy causes perturbations in the serotonergic system and increased anxiety-like behavior in nonhuman primate offspring. J. Neurosci. 30, 3826–3830.
Sullivan, E.L., Smith, M.S., Grove, K.L., 2011. Perinatal exposure to high-fat diet programs energy balance, metabolism and behavior in adulthood. Neuroendocrinology 93, 1–8.
Symonds, M.E., Sebert, S.P., Hyatt, M.A., Budge, H., 2009. Nutritional programming of the metabolic syndrome. Nat. Rev. Endocrinol. 5, 604–610.
Szuran, T.F., Pliska, V., Pokorny, J., Welzl, H., 2000. Prenatal stress in rats: effects on plasma corticosterone, hippocampal glucocorticoid receptors, and maze performance. Physiol. Behav. 71, 353–362.
Taylor, P.D., Poston, L., 2007. Developmental programming of obesity in mammals. Exp. Physiol. 92, 287–298.
Torres, N., Bautista, C.J., Tovar, A.R., Ordáz, G., Rodríguez-Cruz, M., Ortiz, V., Granados, O., Nathanielsz, P.W., Larrea, F., Zambrano, E., 2010. Protein restriction during pregnancy affects maternal liver lipid metabolism and fetal brain lipid composition in the rat. Am. J. Physiol. Endocrinol. Metab. 298, E270–E277.
Tozuka, Y., Kumon, M., Wada, E., Onodera, M., Mochizuki, H., Wada, K., 2010. Maternal obesity impairs hippocampal BDNF production and spatial learning performance in young mouse offspring. Neurochem. Int. 57, 235–247.
Tozuka, Y., Wada, E., Wada, K., 2009. Diet-induced obesity in female mice leads to peroxidized lipid accumulations and impairment of hippocampal neurogenesis during the early life of their offspring. FASEB J. 23, 1920–1934.
Trullas, R., Skolnick, P., 1993. Differences in fear motivated behaviors among inbred mouse strains. Psychopharmacology (Berl) 111, 323–331.
Tsoi, E., Shaikh, H., Robinson, S., Teoh, T.G., 2010. Obesity in pregnancy: a major healthcare issue. Postgrad. Med. J. 86, 617–623.
Uno, H., Eisele, S., Sakai, A., Shelton, S., Baker, E., DeJesus, O., Holden, J., 1994. Neurotoxicity of glucocorticoids in the primate brain. Horm. Behav. 28, 336–348.
Uno, H., Lohmiller, L., Thieme, C., Kemnitz, J.W., Engle, M.J., Roecker, E.B., Farrell, P.M., 1990. Brain damage induced by prenatal exposure to dexamethasone in fetal rhesus macaques. I. Hippocampus. Brain Res. Dev. Brain Res. 53, 157–167.
Vendruscolo, L.F., Takahashi, R.N., Bruske, G.R., Ramos, A., 2003. Evaluation of the anxiolytic-like effect of NKP608, a NK1-receptor antagonist, in two rat strains that differ in anxiety-related behaviors. Psychopharmacology (Berl) 170, 287–293.
Vucetic, Z., Kimmel, J., Totoki, K., Hollenbeck, E., Reyes, T.M., 2010. Maternal high-fat diet alters methylation and gene expression of dopamine and opioid-related genes. Endocrinology 151, 4756–4764.
Wadhwa, P.D., Buss, C., Entringer, S., Swanson, J.M., 2009. Developmental origins of health and disease: brief history of the approach and current focus on epigenetic mechanisms. Semin. Reprod. Med. 27, 358–368.

Walker, C.D., Naef, L., d'Asti, E., Long, H., Xu, Z., Moreau, A., Azeddine, B., 2008. Perinatal maternal fat intake affects metabolism and hippocampal function in the offspring: a potential role for leptin. Ann. N.Y. Acad. Sci. 1144, 189–202.

Warner, M.J., Ozanne, S.E., 2010. Mechanisms involved in the developmental programming of adulthood disease. Biochem. J. 427, 333–347.

Weinstock, M., 2008. The long-term behavioural consequences of prenatal stress. Neurosci. Biobehav. Rev. 32, 1073–1086.

Wright, T., Langley-Evans, S.C., Voigt, J.P., 2011. The impact of maternal cafeteria diet on anxiety-related behaviour and exploration in the offspring. Physiol. Behav. 103, 164–172.

Zambrano, E., Bautista, C.J., Deas, M., Martinez-Samayoa, P.M., Gonzalez-Zamorano, M., Ledesma, H., Morales, J., Larrea, F., Nathanielsz, P.W., 2006. A low maternal protein diet during pregnancy and lactation has sex- and window of exposure-specific effects on offspring growth and food intake, glucose metabolism and serum leptin in the rat. J. Physiol. 571, 221–230.

Zambrano, E., Martinez-Samayoa, P.M., Bautista, C.J., Deas, M., Guillen, L., Rodriguez-Gonzalez, G.L., Guzman, C., Larrea, F., Nathanielsz, P.W., 2005a. Sex differences in transgenerational alterations of growth and metabolism in progeny (F2) of female offspring (F1) of rats fed a low protein diet during pregnancy and lactation. J. Physiol. 566, 225–236.

Zambrano, E., Martinez-Samayoa, P.M., Rodriguez-Gonzalez, G.L., Nathanielsz, P.W., 2010. Dietary intervention prior to pregnancy reverses metabolic programming in male offspring of obese rats. J. Physiol. 588, 1791–1799.

Zambrano, E., Rodriguez-Gonzalez, G.L., Guzman, C., Garcia-Becerra, R., Boeck, L., Diaz, L., Menjivar, M., Larrea, F., Nathanielsz, P.W., 2005b. A maternal low protein diet during pregnancy and lactation in the rat impairs male reproductive development. J. Physiol. 563, 275–284.

Physiology & Behavior 140 (2015) 89–95

Contents lists available at ScienceDirect

Physiology & Behavior

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

Paternal line multigenerational passage of altered risk assessment behavior in female but not male rat offspring of mothers fed a low protein diet

L.A. Reyes-Castro [a], G.L. Rodríguez-González [a], R. Chavira [a], C. Ibáñez [a], C. Lomas-Soria [a,b], J.S. Rodriguez [b], P.W. Nathanielsz [b], E. Zambrano [a,*]

[a] Department of Reproductive Biology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, Mexico
[b] Center for Pregnancy and Newborn Research, Department of Obstetrics and Gynecology, University of Texas Health Science Center at San Antonio, TX 78229, USA

HIGHLIGHTS

- Multigenerational behavior effects by paternal line
- Maternal protein restriction causes multigenerational effects on corticosterone.
- Multigenerational sex behavioral difference by paternal line

ARTICLE INFO

Article history:
Received 6 August 2014
Received in revised form 4 December 2014
Accepted 8 December 2014
Available online 10 December 2014

Keywords:
Developmental programming
Protein restriction
Multigenerational
Paternal lineage
Behavior

ABSTRACT

Maternal low protein (MLP) diets in pregnancy and lactation impair offspring brain development and modify offspring behavior. We hypothesized multigenerational passage of altered behavioral outcomes as has been demonstrated following other developmental programming challenges. We investigated potential multigenerational effects of MLP in rat pregnancy and/or lactation on offspring risk assessment behavior. Founder generation mothers (F_0) ate 20% casein (C) or restricted (R) 10% casein diet, providing four groups: CC, RR, CR, and RC (first letter pregnancy, second letter lactation diet) to evaluate offspring (F_1) effects influenced by MLP in F_0. On postnatal day (PND 250), F_1 males were mated to non-colony siblings producing F_2. On PND 90, F_2 females (in diestrous) and F_2 males were tested in the elevated plus maze (EPM) and open field. Corticosterone was measured at PND 110. Female but not male CR and RC F_2 made more entries and spent more time in EPM open arms than CC females. Overall activity was unchanged as observed in male F_1 fathers. There were no open field differences in F_2 of either sex, indicating that multigenerational MLP effects are due to altered risk assessment, not locomotion. MLP in pregnancy reduced F_1 male and F_2 female corticosterone. We conclude that MLP in pregnancy and/or lactation increases the innate tendency to explore novel environments in F_2 females via the paternal linage, suggesting lower levels of caution and/or higher impulsiveness to explore unknown spaces. Further studies will be necessary to identify the epigenetic modifications in the germ line through the paternal linage.

1. Introduction

Developmental programming of offspring as a result of a suboptimal intra-uterine environment predisposes to adverse health outcomes in offspring throughout life [1–4]. Epigenetic factors may also play a role in the long-term effects on progeny through multiple generations [4–7]. Recently studies show that germ line epigenetic changes can influence offspring brain and behavior development [8]. The impetus to such multigenerational effects can result from chemical environmental exposure or sub-optimal maternal diet.

Female F_2 rats conceived by F_1 intra-uterine growth restricted mothers and then transferred as embryos to control rats show hyperglycemia, hyperinsulinemia, increased hepatic weight and unsuppressed hepatic glucose production in F_2 female offspring [9] while perinatal protein restriction results in increased body mass in F_2 male rats [10]. Glucose metabolism is also adversely affected in the F_3 generation of F_0 female rats fed with a low protein diet during early development [11].

F_0 maternal undernutrition during pregnancy programs reduced birth weight, glucose intolerance and obesity in both the F_1 and F_2.

* Corresponding author at: Department of Reproductive Biology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City 14000, Mexico.
E-mail address: zamgon@unam.mx (E. Zambrano).

http://dx.doi.org/10.1016/j.physbeh.2014.12.017
0031-9384/

Sex-specific transmission of phenotypes could implicate several mechanisms including alterations in metabolic environment (transmaternal inheritance of obesity), gene expression, mediated by developmental and epigenetic pathways (transpaternal inheritance of low body weight), or both (impaired glucose tolerance) [12]. In addition multigenerational effects are often offspring sex dependent. For example, male and female F_3 progeny exhibit opposite anxiety-like behavior following F_0 exposure to vinclozolin, a fungicide with antiandrogenic activity [13].

We have shown that the timing of protein restriction during development mediates sex-dependent inheritance in the F_2, as male rats developed insulin resistance in response to post-natal protein restriction while females developed sensitivity following prenatal protein restriction [14]. In addition to multigenerational effects of restricted maternal diet, maternal high fat diet during pregnancy and lactation produces increased body length, reduced insulin sensitivity, and reduced leptin levels over two generations [15]. Only females displayed the increased body size phenotype in the F_3 generation and this was via the paternal lineage [16].

To date no study to our knowledge has investigated multigenerational behavioral effects following perinatal protein restriction by paternal line. We hypothesized that perinatal protein restriction in F_0 dams results in altered behavioral outcomes in F_2 progeny via the paternal linage.

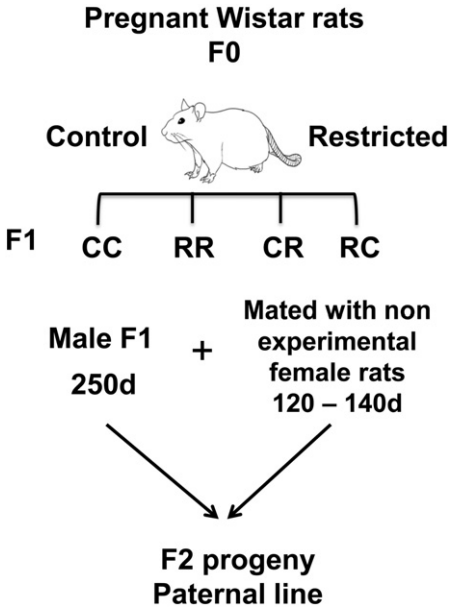

Fig. 1. Schematic of the study design: first letter defines the diet during pregnancy and second letter means diet during lactation. Control (C) and restricted (R).

2. Methods

2.1. Care and maintenance of animals

2.1.1. Breeding and maintenance of F_0 female rats

All procedures were approved by the animal Experimentation Ethics of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City. Fifty virgin female albino Wistar rats aged 16–18 weeks, weighing 220 ± 20 g were obtained from the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (Mexico City, Mexico). Rats were maintained under controlled lighting (lights from 07.00 to 19.00 h) at 22–23 °C. Prior to breeding male and female subjects were maintained on Zeigler, RQ 22-5 rodent diet. Female rats were mated overnight with male breeders and the day on which spermatozoa were present in a vaginal smear was designated as day of conception (day 0). Only rats that were pregnant within 5 days were retained in the study. Pregnant rats were transferred to individual cages and allocated at random to one of two groups to be fed either 20% casein (control diet) or 10% casein isocaloric diet (restricted). Food and water were available ad libitum for all animals.

All F_0 rats delivered the F_1 offspring by spontaneous vaginal delivery. Timing of delivery of the F_1 and F_2 pups and morphometric measurements was recorded at birth. Ano-genital distance was measured with calipers to determine offspring sex [17]. To ensure homogeneity of study subjects, litters of over 14, or less than 10, pups were excluded from the study. Litters were adjusted to 10 pups for each dam while maintaining as close to a 1:1 sex ratio as possible.

Four groups were established. C represents control and R the restricted diet. The first letter defines the diet mothers received during pregnancy and the second the diet in lactation: CC control during pregnancy and lactation, RR restricted during pregnancy and lactation, CR restricted only during lactation and RC restricted only during pregnancy. After weaning all pups were maintained on Zeigler RQ 22-5 rodent diet.

2.2. Breeding of F_1 males

To produce the F_2, F_1 males aged 250 days were bred with non-experimental female Wistar rats aged 16–18 weeks with regular estrous cycles. During pregnancy and for the rest of the study, all rats were fed with control diet. All F_1 females underwent spontaneous vaginal delivery (Fig. 1).

2.3. Elevated plus maze (EPM)

At PND 75, two weeks prior to all behavioral testing, a reverse light cycle was implemented (lights off at 07.00 h, and on at 19.00 h) so that testing could be conducted in the animals' dark phase corresponding to the investigators' day time. For females behavioral assessments were performed during the diestrous cycle. Body weight was recorded at the beginning of the behavioral test. Subjects were assessed at the same time of the dark cycle between 08.00 h and 14.00 h. The EPM was constructed of dark gray plastic and was situated 64 cm above the floor. It consisted of two unprotected arms (open arms, 45 cm × 10 cm each) facing each other and two arms protected by high gray walls at 90° from the open arms (closed arms, 45 cm × 10 cm each), all extended from a common central platform (10 cm × 10 cm). The light level was 30 lx in the open arms and 6 lx in the closed arms. The rat's position on the maze was recorded via a video camera mounted on the ceiling above the center of the maze. The camera was connected to a video tracking motion analysis system (Ethovision, Noldus Information Technology by Wageningen, The Netherlands) running on a personal computer. To start the session each rat was placed individually at the center of the maze facing an open arm. After 10 min of EPM exploration, the rat was returned to its home cage and the EPM was cleaned with 70% ethanol. An experimenter blind to the subject's treatment group manually scored the number of entries into the predefined zones of the open and closed arms, while the Ethovision system measured the distance traveled and the time spent in the different areas. An arm entry was scored only if the rat's center of gravity entered into the arm. All subjects were tested in a randomized sequence.

2.4. Open field

The day following EPM testing, the same subjects were evaluated in a 60-minute open field test. The open field, made of dark gray Plexiglas, consisted of a square arena (101 cm × 101 cm and 34 cm high) which was located in an experimental room illuminated by low light (12 lx).

A video camera, mounted above the arena, was connected to a monitor and served as the video tracking motion analysis system (Ethovision, Noldus Information Technology by, Wageningen, The Netherlands). This system recorded subject locomotion in the entire arena (total distance measured in meters). A virtual square center was defined in the open field arena and the distance, number of entries and time spent inside this center zone were measured. To begin a session each rat was placed individually into the center of the open field arena. Following each session all zones were cleaned with 70% ethanol.

2.5. Corticosterone measurement

At PND 110 blood samples were taken from the tail to determine serum corticosterone levels in both F_1 and F_2 subjects. Blood samples were centrifuged at 4 °C for 15 min at 3500 rpm to remove red blood cells and serum stored at −20 °C until all samples were analyzed. Serum corticosterone levels were determined by radioimmunoassay using the DPC Coat-a-count (TKRC1) rat kit from Diagnostic Products (Los Angeles, CA, USA). Intra-assay and inter-assay variability were <6 and <7% respectively. The kit was used in accordance with manufacturer's instructions and samples were measured in duplicate.

2.6. Statistical analyses

All data are presented as mean ± SEM, alpha level was set at 0.05. Behavioral end points and corticosterone levels were analyzed by one way-ANOVA. Post-hoc analyses were performed by Tukey's test using SigmaStat 3.5. Analyses were not set up between female and male because of the known mark behavior variation between the sexes. Data from F_1 males have been published [18] and are included in the figures where they are needed to show the relationship to male and female F_2 through the paternal line.

Table 1
F_2 female and male data from F_1 paternal linage: morphometric measurements at birth and adult weight at beginning of training (PND 90) from F_1 male born to dams fed control (20% casein) or restricted (10% casein) diet during pregnancy. Mean ± SEM; n = 8 pups from 8 different litters.

	CC	RR	CR	RC
Female F_2				
Weight (g)	5.4 ± 0.2	5.8 ± 0.1	5.7 ± 0.1	5.8 ± 0.06
Length (cm)	5.0 ± 0.02	5.0 ± 0.02	5.0 ± 0.01	4.9 ± 0.03
Ano-genital distance (mm)	1.2 ± 0.07	1.5 ± 0.1	1.3 ± 0.08	1.2 ± 0.04
Ano-genital distance (mm/g)	0.22 ± 0.01	0.25 ± 0.03	0.23 ± 0.02	0.21 ± 0.005
Head diameter (mm)	10.3 ± 0.09	10.5 ± 0.06	10.4 ± 0.04	10.4 ± 0.1
Abdominal diameter (mm)	11.2 ± 0.1	11.5 ± 0.1	11.2 ± 0.05	11.3 ± 0.1
Head:abdominal ratio	0.92 ± 0.004	0.91 ± 0.005	0.92 ± 0.003	0.92 ± 0.005
Male F_2				
Weight (g)	5.6 ± 0.2	6.2 ± 0.2	5.7 ± 0.07	6.2 ± 0.1
Length (cm)	5.0 ± 0.04	5.0 ± 0.09	5.0 ± 0.01	5.1 ± 0.04
Ano-genital distance (mm)	3.6 ± 0.06	3.7 ± 0.1	3.6 ± 0.07	3.4 ± 0.1
Ano-genital distance (mm/g)	0.65 ± 0.02	0.61 ± 0.02	0.63 ± 0.01	0.56 ± 0.01
Head diameter (mm)	10.4 ± 0.1	10.5 ± 0.1	10.5 ± 0.1	10.6 ± 0.1
Abdominal diameter (mm)	11.4 ± 0.1	11.5 ± 0.1	11.3 ± 0.05	11.3 ± 0.2
Head:abdominal ratio	0.91 ± 0.02	0.61 ± 0.02	0.63 ± 0.01	0.56 ± 0.01
Adult offspring PND 90				
Weight (g) female F_2	257 ± 8.3	244 ± 4.8	259 ± 2.5	258 ± 5.0
Weight (g) male F_2	365 ± 8.0	351 ± 18	334 ± 8.5	332 ± 3.8

3. Results

3.1. Offspring parameters at birth

Table 1 shows no differences in morphometric analysis measures at birth and body weight at beginning of the training (PND 90) in F_2 females and males from the F_1 paternal linage.

3.1.1. Elevated plus maze (EPM)

F_2 female RR, CR and RC offspring showed increased open arm entries compared with F_2 CC (Fig. 2B). There were no differences between the male F_2 groups (Fig. 2C). The number of EPM open arm entries increased in F_1, CR and RC males compared to F_1 CC offspring (Fig. 2A — previously published data for comparison). F_2 female RR, CR and RC offspring increased the time spent in the open arms compared with F_2 CC (Fig. 3B). In contrast, F_2 males showed no differences between groups (Fig. 3C). Time spent in the EPM open arms was increased in male F_1 RR, CR and RC compared to F_1 CC (Fig. 3A). F_2 female RR, CR and RC traversed greater distances than CC (Fig. 4B). Results from F_2 show no effect in male experimental groups (Fig. 4C). The distance in the EPM open arm increased in F_1 RC compared to CC offspring with intermediate distance in the RR and CR groups (Fig. 4 A). No differences were observed in total distance traversed in female (CC: 20.4 ± 1.3, RR: 20.3 ± 1.0, CR: 18.5 ± 1.1, RC 22.1 ± 1.3 m) and male F_2 (CC: 15.2 ± 1.4, RR: 14.9 ± 0.9, CR 20.4 ± 1.8, RC 16.2 ± 1.2 m).

3.1.2. Open field

Table 2 shows the comparisons in the open field behavioral endpoints. F_1 male RR offspring made numerically fewer entries into the center zone than CC (post-hoc borderline effect, $p = 0.07$) with no differences in the CR and RC groups. Results from F_2 show no differences in center zone entries in female and male. Comparison for time spent in the center zone, distance traversed in center zone and total distance showed no differences between male F_1 and female and male F_2 groups.

3.1.3. Corticosterone measurement

Female F_2 show decreased corticosterone levels in RC offspring compared to CC, RR and CR groups (Fig. 5B) while in male F_2, the RR group had lower corticosterone levels (Fig. 5C) compared to CC, CR and RC groups. Corticosterone levels in F_1 offspring decreased in male RC compared to CC offspring (Fig. 5A).

4. Discussion

Transgenerational passage of acquired characteristics can be defined as the transmission of an acquired physiological phenotype to subsequent generations that are not directly exposed to the original environmental challenge that caused the phenotype change [19,20]. In contrast multigenerational transmission between generations involves direct exposure [20] to the challenge at each generation such as when both F_1 and F_2 germ lines, embryos or fetuses are directly exposed to the challenge presented in the F_0 pregnant mothers e.g. gestational diabetes that recurs in the daughters of mothers with gestational diabetes. Studies of multigenerational effects of prenatal maternal manipulation on behavior in the F_2 generation and beyond are limited [21,22]. Experimental animal studies have explored more exhaustively multigenerational programmed effects by maternal linage on birth weight and cardiovascular disease [23], as well as metabolism [9]. Therefore, the present study explored multigenerational effects on behavior in F_2 of grandmothers (F_0) fed a low protein diet by paternal linage (F_1).

We previously reported that prenatal protein restriction in the rat reduces fetal (F_1) brain docosahexaenoic acid content [24] and gender specific outcomes in both EPM and open field test in adult F_1 of F_0 mothers on a maternal low protein diet during pregnancy and/or lactation [18,25]. F_1 male showed increased approach and less avoidance behavior (less anxiety) and thus exhibited disinhibition of avoidance as

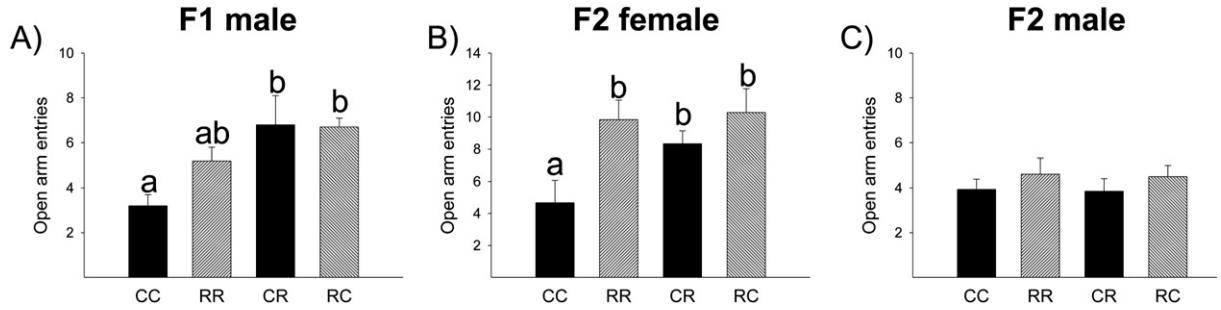

Fig. 2. EPM test at PND 90. Open arm entries. CC (control–control), RR (restricted–restricted), CR (control–restricted), and RC (restricted–control). Mean ± SEM. Data not sharing a letter are statistically different, $p < 0.05$, $n = 8$.

compared to controls. The results demonstrate the negative effect of low protein diet during pregnancy and/or lactation in the risk assessment behaviors in the EPM. In the open field test the F_1 RR group showed a marginal decrease in the number of center zone entries in comparison to controls although it did not reach significance. This behavior can be linked to the increase in corticosterone [18]. The same alterations in the EPM were observed in F_2 female but not male F_2. There were no gender specific differences in the open field, indicating that the transgenerational maternal protein restriction effect was due to changes in anxiety behavior and not in locomotion problems. As we previously found for the F_1 behavioral responses [18], there were similar inconsistent F_2 behavioral responses of the experimental groups in the two anxiety/stress based tests. Some studies have shown stress-based tests that rely on unconditioned responses, such as in the two tests used here, assess different aspects of affect and that emotional reactivity is multidimensional [26–29]. These studies provided the rational to determine altered behavioral outcomes in the F_2.

Supplementation of maternal diet with methyl donors generates a multigenerational phenotype with sex dependent transmission characteristics. Folate, betaine and choline supplementations during pregnancy induces hypermethylation of the Agouti viable yellow (Avy) locus and results in a darker coat color phenotype in offspring [30–33]. However, this darker coat color persists into the second generation only when transmitted through the female lineage [34]. Clearly, the transmission of transgenerational outcomes can be specific to the maternal or paternal lineage. Not surprisingly, the inherence of epigenetic traits can also occur in an offspring sex-dependent manner. We have shown sex specific effects in 2nd generation rats, as post-natal protein restriction resulted in insulin resistance in male rats while females developed insulin sensitivity [14]. The transmission of obesity and glucose intolerance following maternal caloric restriction is sex dependent as male and female 2nd generation mice offspring exhibit reduced birth weight via the paternal lineage while obesity is transmitted through the maternal lineage [12].

Environmental experiences have enduring effects on neurobiology and behavior, but there is little evidence for the impact of maternal manipulation and subsequent behavioral effects across multiple generations. Multigenerational effects of stress are potentially mediated via modulation of the hypothalamic–pituitary–adrenal axis (HPA) as well as epigenetic mechanisms causing heritable changes in gene expression [35–37].

In the present study, multigenerational effects on the HPA axis were demonstrated with male F_1 RC offspring showing blunted corticosterone levels that transferred to female F_2 RC offspring who also displayed reduced corticosterone but not F_2 males. These results suggest that epigenetic modifications through the paternal lineage are manifested more robustly in female F_2 behavior which demonstrates germ line transfer. A great deal of research has focused on developmental programming and more recently on epigenetic modifications [5]. In contrast with the female F_2 by paternal linage behavior, female F_1 from our maternal protein restricted model showed decreased open arm time and distance in both groups restricted during pregnancy, which means increased anxiety behavior in the EPM, associated with higher corticosterone serum levels [25]. Embryo and fetal development conditions in female F_1 from restricted mothers and female F_2 from paternal protein restricted linage were different, since F_0 restricted mothers presented higher corticosterone serum levels in comparison with control mothers while fathers (F_1) from restricted mothers had lower corticosterone serum levels. Corticosterone is one of the key hormones in programming and that might be one of the explanations of different effects in behavior programming between female F_1 from protein restricted mothers (F_0) and female F_2 by paternal F_1 linage protein restricted during gestation.

Evidence suggests that glucocorticoids are involved in histone modification of important behavioral genes for example, brief immobilizations of rats caused a decrease in the expression of the brain-derived neurotrophic factor (BDNF), and this was due to altered histone acetylation in promoters of this gene [38]. Similar effects have been found as a result not only of restraint stress, but also of administration of corticosterone to rats [39].

An early behavioral study showed that stress during pregnancy in rats results in increased activity in an open field environment in F_2 offspring [40]. In mice, chronic and unpredictable maternal separation

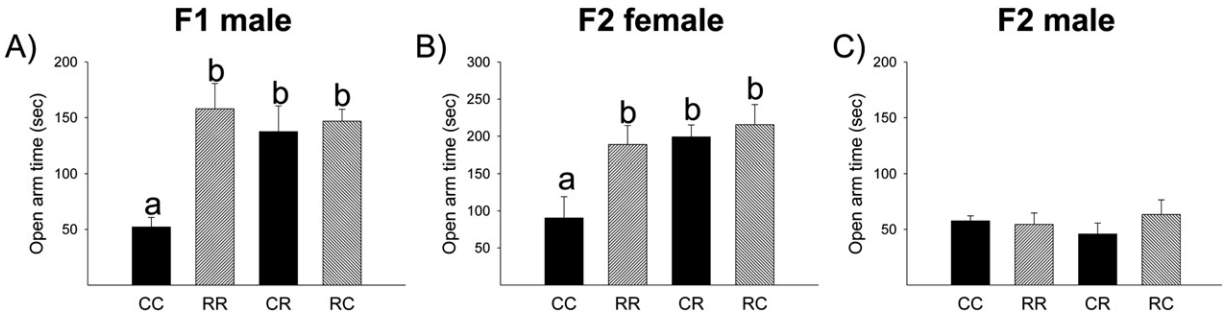

Fig. 3. EPM test at PND 90. Open arm time (sec). CC (control–control), RR (restricted–restricted), CR (control–restricted), and RC (restricted–control). Mean ± SEM. Data not sharing a letter are statistically different, $p < 0.05$, $n = 8$.

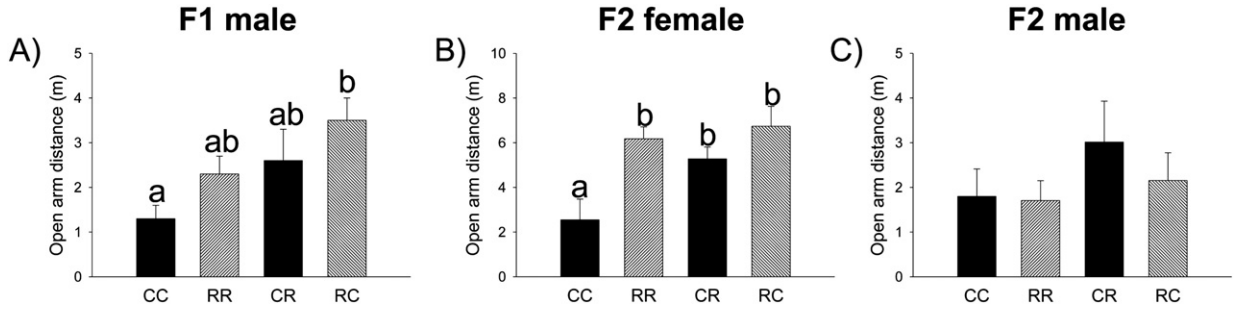

Fig. 4. EPM test at PND 90. Open arm distance (m). CC (control–control), RR (restricted–restricted), CR (control–restricted), and RC (restricted–control). Mean ± SEM, data not sharing a letter are statistically different. $p < 0.05$, $n = 8$.

from postnatal day 1 to 14 induces depressive-like behaviors when adult, in addition, transient embryonic exposure to the endocrine disrupter vinclozolin, led to a decrease in anxiety-like behavior in F_3 generation males, but an increase in anxiety-like behavior in F_3 generation females [13]. Multigenerational effects to stress response have been reported in various experimental models [41]. In a related issue to behavioral assessments, maternal undernutrition modified both basal and activated HPA functions for two generations in the guinea pig through maternal transmission — increased basal cortisol and increased ACTH responsiveness to challenge in F_1 and F_2 offspring [42]. Taken together, these studies clearly show that pregnancy and lactation manipulation can lead to robust transgenerational influences on behavior in offspring and that changes are dependent on parental lineage and offspring sex. However, the underlying mechanisms are not clear so far.

In rats and mice, studies based on paternal lines have shown that such transmission associated with epigenetic modification of the genome can often be traced through several generations [43] and changes in DNA methylation of several genes have been observed in the sperm of both F_2 and F_3 males [31,44–46]. Demonstration of epigenetic changes in transmission to subsequent generation is required to show that the phenotype is indeed transgenerational germ line-dependent, and is not a direct effect of the treatment itself [20]. Growing interest in the field also provided new experimental evidence that alteration in DNA methylation in germ cells may underlie epigenetic inheritance [47]. A wide range of environmental factors are known to be associated with changes in DNA methylation in both humans and rodents; it is now clear that epigenetic inheritance may be occurring on a much broader scale than previously thought. Further to DNA methylation, other epigenetic mechanisms such as RNA interference, histone post-translational modifications, and DNA repair may also contribute to epigenetic inheritance [48]. Reactive oxygen species are known to induce DNA damage and alter the methylation pattern in the sperm. Data from our group shows that maternal protein restriction during pregnancy increases male F_1 sperm oxidative stress [49].

In a study conducted in mice it was shown that F_0 sperm with high concentrations of ROS programs female F_1 for metabolic syndrome and obesity; interestingly male offspring was less affected, demonstrating sex-specific impacts as was shown in the present study [50].

In this study we can only speculate that the multigenerational behavioral changes in male F_1 and female F_2 progeny are based on epigenetic modifications through the paternal germline [47]. It is important to point out that multigenerational behavioral F_2 effects through the maternal line were not explored in the present study, therefore it is possible that similar effects may or may not occur via F_1 female.

Positive multigenerational cognitive and neurophysiological effects have been reported with enhancement of learning following F_0 exposure to an enriched environment in mice [51–52]. Exposure of pregnant rats to an enriched environment enhanced not only their performance ability in a maze but also the ability of their future offspring [53]. Enhanced learning ability across generations has also been reported in offspring when the dam had been exposed to environmental enrichment before pregnancy [54]. Indeed evidence of transgenerational effects and specifically cognitive and affective characteristics in progeny are growing as more research elucidates these non-genomic inheritable traits.

Table 2
Open field test comparisons from F_1 male born to dams fed control (20% casein) or restricted (10% casein) diet during pregnancy, and F_2 female and male from paternal linage. Mean ± SEM; $n = 8$ pups from 8 different litters.

	CC	RR	CR	RC
Number entries center zone				
Male F_1	35.8 ± 6.6	17.6 ± 4.2*	34.4 ± 5.1	19.2 ± 3.6
Female F_2	99.5 ± 9.2	96.0 ± 18.1	101 ± 24.1	115 ± 12.8
Male F_2	47.3 ± 7.8	35.0 ± 5.7	40.3 ± 9.4	26.1 ± 1.5
Time center zone (s)				
Male F_1	124. 6 ± 25.2	116.7 ± 33	127.7 ± 17	141 ± 59.2
Female F_2	459.4 ± 86.7	472.8 ± 85.2	369.9 ± 56.5	480.4 ± 48.9
Male F_2	106.7 ± 23.6	93.3 ± 19.9	89.9 ± 9.8	65.4 ± 17.3
Distance center zone (m)				
Male F_1	14.1 ± 2.4	9.4 ± 2.4	13.4 ± 2.3	7.2 ± 1.7
Female F_2	45.8 ± 5.4	43.7 ± 7.0	36.2 ± 5.6	49.2 ± 3.5
Male F_2	11.3 ± 1.1	10.0 ± 1.8	10.3 ± 2.1	7.5 ± 1.1
Total distance (m)				
Male F_1	128.7 ± 9.0	82.0 ± 11	88.1 ± 11.2	83.5 ± 15.4
Female F_2	189 ± 8.9	180.1 ± 18.3	182.5 ± 21.2	184.7 ± 5.8
Male F_2	134.6 ± 7.9	141.8 ± 25.3	120.2 ± 10.4	128.8 ± 12.9

* $p < 0.07$ vs C.

5. Conclusion

In the present study we found multigenerational effects in F_2 following perinatal protein restriction during development of the F_1 paternal lineage. Interestingly, the behavioral differences – disinhibited EPM behavior – displayed in male F_1 RR, CR and RC offspring transferred to female F_2 experimental progeny more than to male F_2.

Role of the funding source

This work was partially supported by the Consejo Nacional de Ciencia y Tecnología (CONACyT-155166) México. Funding sources had no role in the study design, collection, analyses, interpretation of data, or the preparation and submission of this report.

Disclosure statement

There is no conflict of interest to declare for all authors.

Submission declaration

This work is an original finding that has not been submitted nor is being considered for publication in any other journal.

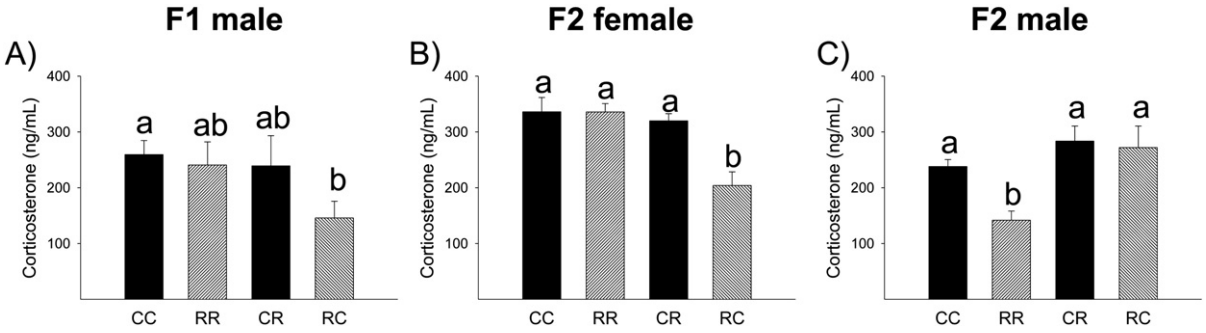

Fig. 5. Corticosterone serum levels at PND 110. CC (control–control), RR (restricted–restricted), CR (control–restricted), and RC (restricted–control). Mean ± SEM, data not sharing a letter are statistically different. $p < 0.05$, $n = 8$.

Author contributions

Reyes-Castro LA and Rodríguez-González GL: researched data and behavioral assessments for first and second generation; Chavira R, Ibáñez C and Lomas-Soria C: care and maintenance of animals; Rodríguez JS: contributed to discussion; and Nathanielsz PW and Zambrano E: study design and manuscript writing.

Acknowledgments

L.A. Reyes-Castro and G.L. Rodríguez-González are graduate students from Doctorado en Ciencias Biomédicas, Facultad de Medicina, Universidad Nacional Autónoma de México and are recipients of CONACyT fellowship.

References

[1] S.C. Langley-Evans, S. McMullen, Developmental origins of adult disease, Med. Princ. Pract. 19 (2010) 87–98.
[2] M.J. Nijland, S.P. Ford, P.W. Nathanielsz, Prenatal origins of adult disease, Curr. Opin. Obstet. Gynecol. 20 (2008) 132–138.
[3] L. Poston, Developmental programming and diabetes — the human experience and insight from animal models, Best Pract. Res. Clin. Endocrinol. Metab. 24 (2010) 541–552.
[4] P.D. Wadhwa, C. Buss, S. Entringer, J.M. Swanson, Developmental origins of health and disease: brief history of the approach and current focus on epigenetic mechanisms, Semin. Reprod. Med. 27 (2009) 358–368.
[5] D. Crews, Epigenetic modifications of brain and behavior: theory and practice, Horm. Behav. 59 (2011) 393–398.
[6] M.K. Skinner, Role of epigenetics in developmental biology and transgenerational inheritance, Birth Defects Res. C Embryo Today 93 (2011) 51–55.
[7] M.K. Skinner, Environmental epigenomics and disease susceptibility, EMBO Rep. 12 (2011) 620–622.
[8] D. Crews, R. Gillette, S.V. Scarpino, M. Manikkam, M.I. Savenkova, M.K. Skinner, Epigenetic transgenerational inheritance of altered stress responses, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 9143–9148.
[9] M. Thamotharan, M. Garg, S. Oak, L.M. Rogers, G. Pan, F. Sangiorgi, et al., Transgenerational inheritance of the insulin-resistant phenotype in embryo-transferred intrauterine growth-restricted adult female rat offspring, Am. J. Physiol. Endocrinol. Metab. 292 (2007) E1270–E1279.
[10] A.R. Pinheiro, I.D. Salvucci, M.B. Aguila, C.A. Mandarim-de-Lacerda, Protein restriction during gestation and/or lactation causes adverse transgenerational effects on biometry and glucose metabolism in F1 and F2 progenies of rats, Clin. Sci. (Lond.) 114 (2008) 381–392.
[11] D.C. Benyshek, C.S. Johnston, J.F. Martin, Glucose metabolism is altered in the adequately-nourished grand-offspring (F3 generation) of rats malnourished during gestation and perinatal life, Diabetologia 49 (2006) 1117–1119.
[12] J.C. Jimenez-Chillaron, E. Isganaitis, M. Charalambous, S. Gesta, T. Pentinat-Pelegrin, R.R. Faucette, et al., Intergenerational transmission of glucose intolerance and obesity by in utero undernutrition in mice, Diabetes 58 (2009) 460–468.
[13] M.K. Skinner, M.D. Anway, M.I. Savenkova, A.C. Gore, D. Crews, Transgenerational epigenetic programming of the brain transcriptome and anxiety behavior, PLoS One 3 (2008) e3745.
[14] E. Zambrano, P.M. Martinez-Samayoa, C.J. Bautista, M. Deas, L. Guillen, G.L. Rodriguez-Gonzalez, et al., Sex differences in transgenerational alterations of growth and metabolism in progeny (F2) of female offspring (F1) of rats fed a low protein diet during pregnancy and lactation, J. Physiol. 566 (2005) 225–236.
[15] G.A. Dunn, T.L. Bale, Maternal high-fat diet promotes body length increases and insulin insensitivity in second-generation mice, Endocrinology 150 (2009) 4999–5009.
[16] G.A. Dunn, T.L. Bale, Maternal high-fat diet effects on third-generation female body size via the paternal lineage, Endocrinology 152 (2011) 2228–2236.
[17] E. Zambrano, G.L. Rodriguez-Gonzalez, C. Guzman, R. Garcia-Becerra, L. Boeck, L. Diaz, et al., A maternal low protein diet during pregnancy and lactation in the rat impairs male reproductive development, J. Physiol. 563 (2005) 275–284.
[18] L.A. Reyes-Castro, J.S. Rodriguez, G.L. Rodriguez-Gonzalez, R. Chavira, C.J. Bautista, T.J. McDonald, et al., Pre- and/or postnatal protein restriction developmentally programs affect and risk assessment behaviors in adult male rats, Behav. Brain Res. 227 (2012) 324–329.
[19] M.K. Skinner, Endocrine disruptors and epigenetic transgenerational disease etiology, Pediatr. Res. 61 (2007) 48R–50R.
[20] M.K. Skinner, What is an epigenetic transgenerational phenotype? F3 or F2, Reprod. Toxicol. 25 (2008) 2–6.
[21] S.G. Matthews, D.I. Phillips, Transgenerational inheritance of stress pathology, Exp. Neurol. 233 (2012) 95–101.
[22] M.M. McCarthy, A.P. Auger, T.L. Bale, G.J. De Vries, G.A. Dunn, N.G. Forger, et al., The epigenetics of sex differences in the brain, J. Neurosci. 29 (2009) 12815–12823.
[23] A.J. Drake, B.R. Walker, The intergenerational effects of fetal programming: non-genomic mechanisms for the inheritance of low birth weight and cardiovascular risk, J. Endocrinol. 180 (2004) 1–16.
[24] N. Torres, C.J. Bautista, A.R. Tovar, G. Ordaz, M. Rodriguez-Cruz, V. Ortiz, et al., Protein restriction during pregnancy affects maternal liver lipid metabolism and fetal brain lipid composition in the rat, Am. J. Physiol. Endocrinol. Metab. 298 (2009) E270–E277.
[25] L.A. Reyes-Castro, J.S. Rodriguez, R. Charco, C.J. Bautista, F. Larrea, P.W. Nathanielsz, et al., Maternal protein restriction in the rat during pregnancy and/or lactation alters cognitive and anxiety behaviors of female offspring, Int. J. Dev. Neurosci. 30 (2012) 39–45.
[26] A. Ramos, Animal models of anxiety: do I need multiple tests? Trends Pharmacol. Sci. 29 (2008) 493–498.
[27] A. Ramos, P. Mormede, Stress and emotionality: a multidimensional and genetic approach, Neurosci. Biobehav. Rev. 22 (1998) 33–57.
[28] R. Trullas, P. Skolnick, Differences in fear motivated behaviors among inbred mouse strains, Psychopharmacology (Berl) 111 (1993) 323–331.
[29] L.F. Vendruscolo, R.N. Takahashi, G.R. Bruske, A. Ramos, Evaluation of the anxiolytic-like effect of NKP608, a NK1-receptor antagonist, in two rat strains that differ in anxiety-related behaviors, Psychopharmacology (Berl) 170 (2003) 287–293.
[30] C.A. Cooney, A.A. Dave, G.L. Wolff, Maternal methyl supplements in mice affect epigenetic variation and DNA methylation of offspring, J. Nutr. 132 (2002) 2393S–2400S.
[31] R.L. Jirtle, M.K. Skinner, Environmental epigenomics and disease susceptibility, Nat. Rev. Genet. 8 (2007) 253–262.
[32] R.A. Waterland, R.L. Jirtle, Transposable elements: targets for early nutritional effects on epigenetic gene regulation, Mol. Cell. Biol. 23 (2003) 5293–5300.
[33] G.L. Wolff, R.L. Kodell, S.R. Moore, C.A. Cooney, Maternal epigenetics and methyl supplements affect agouti gene expression in Avy/a mice, FASEB J. 12 (1998) 949–957.
[34] J.E. Cropley, C.M. Suter, K.B. Beckman, D.I. Martin, Germ-line epigenetic modification of the murine A^{vy} allele by nutritional supplementation, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 17308–17312.
[35] V.C. Goerlich, D. Natt, M. Elfwing, B. Macdonald, P. Jensen, Transgenerational effects of early experience on behavioral, hormonal and gene expression responses to acute stress in the precocial chicken, Horm. Behav. 61 (2012) 711–718.
[36] J. Lesage, N. Sebaai, M. Leonhardt, I. Dutriez-Casteloot, C. Breton, S. Deloof, et al., Perinatal maternal undernutrition programs the offspring hypothalamo-pituitary-adrenal (HPA) axis, Stress 9 (2006) 183–198.
[37] L. Schaffer, D. Muller-Vizentini, T. Burkhardt, M. Rauh, U. Ehlert, E. Beinder, Blunted stress response in small for gestational age neonates, Pediatr. Res. 65 (2009) 231–235.
[38] M. Fuchikami, S. Morinobu, A. Kurata, S. Yamamoto, S. Yamawaki, Single immobilization stress differentially alters the expression profile of transcripts of the brain-derived neurotrophic factor (BDNF) gene and histone acetylation at its promoters in the rat hippocampus, Int. J. Neuropsychopharmacol. 12 (2009) 73–82.
[39] R.G. Hunter, K.J. McCarthy, T.A. Milne, D.W. Pfaff, B.S. McEwen, Regulation of hippocampal H3 histone methylation by acute and chronic stress, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 20912–20917.

[40] F. Wehmer, R.H. Porter, B. Scales, Pre-mating and pregnancy stress in rats affects behaviour of grandpups, Nature 227 (1970) 622.
[41] S.G. Matthews, D.I. Phillips, Transgenerational inheritance of stress pathology, Exp. Neurol. 233 (2011) 95–101.
[42] C. Bertram, O. Khan, S. Ohri, D.I. Phillips, S.G. Matthews, M.A. Hanson, Transgenerational effects of prenatal nutrient restriction on cardiovascular and hypothalamic–pituitary–adrenal function, J. Physiol. 586 (2008) 2217–2229.
[43] T.B. Franklin, I.M. Mansuy, Epigenetic inheritance in mammals: evidence for the impact of adverse environmental effects, Neurobiol. Dis. 39 (2010) 61–65.
[44] M.D. Anway, A.S. Cupp, M. Uzumcu, M.K. Skinner, Epigenetic transgenerational actions of endocrine disruptors and male fertility, Science 308 (2005) 1466–1469.
[45] T.B. Franklin, H. Russig, I.C. Weiss, J. Graff, N. Linder, A. Michalon, et al., Epigenetic transmission of the impact of early stress across generations, Biol. Psychiatry 68 (2010) 408–415.
[46] E. Jablonka, G. Raz, Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution, Q. Rev. Biol. 84 (2009) 131–176.
[47] K. Gapp, A. Jawaid, P. Sarkies, J. Bohacek, P. Pelczar, J. Prados, et al., Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice, Nat. Neurosci. 17 (2014) 667–669.
[48] M. Godmann, R. Lambrot, S. Kimmins, The dynamic epigenetic program in male germ cells: its role in spermatogenesis, testis cancer, and its response to the environment, Microsc. Res. Tech. 72 (2009) 603–619.
[49] G.L. Rodríguez-González, L.A. Reyes-Castro, C.C. Vega, L. Boeck, C. Ibáñez, P.W. Nathanielsz, et al., Accelerated aging of reproductive capacity in male rat offspring of protein-restricted mothers is associated with increased testicular and sperm oxidative stress, Age (Dordr) 36 (2014) 9721.
[50] M. Lane, N.O. McPherson, T. Fullston, M. Spillane, L. Sandeman, W.X. Kang, et al., Oxidative stress in mouse sperm impairs embryo development, fetal growth and alters adiposity and glucose regulation in female offspring, PLoS One 9 (2014) e100832.
[51] J.A. Arai, L.A. Feig, Long-lasting and transgenerational effects of an environmental enrichment on memory formation, Brain Res. Bull. 85 (2011) 30–35.
[52] J.A. Arai, S. Li, D.M. Hartley, L.A. Feig, Transgenerational rescue of a genetic defect in long-term potentiation and memory formation by juvenile enrichment, J. Neurosci. 29 (2009) 1496–1502.
[53] S. Kiyono, M.L. Seo, M. Shibagaki, M. Inouye, Facilitative effects of maternal environmental enrichment on maze learning in rat offspring, Physiol. Behav. 34 (1985) 431–435.
[54] P.A. Dell, F.D. Rose, Transfer of effects from environmentally enriched and impoverished female rats to future offspring, Physiol. Behav. 39 (1987) 187–190.

AGE (2015) 37: 52
DOI 10.1007/s11357-015-9774-0

Aging, glucocorticoids and developmental programming

E. Zambrano · L. A. Reyes-Castro · P. W. Nathanielsz

Received: 18 December 2014 / Accepted: 2 April 2015 / Published online: 9 May 2015

Abstract Glucocorticoids are pleiotropic regulators of multiple cell types with critical roles in physiological systems that change across the life-course. Although glucocorticoids have been associated with aging, available data on the aging trajectory in basal circulating glucocorticoids are conflicting. A literature search reveals sparse life-course data. We evaluated (1) the profile of basal circulating corticosterone across the life-course from weaning (postnatal day—PND 21), young adult PND 110, adult PND 450, mature adult PND 650 to aged phase PND 850 in a well-characterized homogeneous rat colony to determine existence of significant changes in trajectory in the second half of life; (2) sex differences; and (3) whether developmental programming of offspring by exposure to maternal obesity during development alters the later-life circulating corticosterone trajectory. We identified (1) a fall in corticosterone between PND 450 and 650 in both males and females ($p<0.05$) and (2) higher female than male concentrations ($p<0.05$). (3) Using our five life-course time-point data set, corticosterone fell at a similar age but from higher levels in male and female offspring of obese mothers. In all four groups studied, there was a second half of life fall in corticosterone. Higher corticosterone levels in offspring of obese mothers may play a role in their shorter life-span, but the age-associated fall occurs at a similar time to control offspring. Although even more life-course time-points would be useful, a five life-course time-point analysis provides important new information on normative and programmed aging of circulating corticosterone.

Keywords Aging · Glucocorticoids · Developmental programming · Maternal obesity

Abbreviations

BMI	Body mass index
C	Control
dG	Days gestation
PND	Postnatal day

Introduction

Glucocorticoids are pleiotropic regulators of multiple cell types with critical regulatory roles in many physiological systems that change across the life-course. Exposure to both high (Cushing's disease) and low glucocorticoid concentrations (Addison's disease) produces weakness and frailty. Data in the literature are conflicting as to whether aging is accompanied by an increase or decrease in basal circulating glucocorticoid concentrations. Our search of the literature indicated that there is a lack of measurements across the complete life-

E. Zambrano · L. A. Reyes-Castro
Reproductive Biology Department, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City 14000, Mexico

P. W. Nathanielsz (✉)
Department of Animal Science, University of Wyoming, Laramie, WY 82071-3684, USA
e-mail: peter.nathanielsz@uwyo.edu

course in any dataset available. In the light of the key roles that glucocorticoids play at different stages of development (Zambrano et al. 2014), we propose that the first step in understanding glucocorticoids' role in aging is to establish the timing of life-course changes in basal circulating glucocorticoids with normal aging. To set a baseline, there is a need for data from as many life-course stages as practically possible. Measurements are needed even before a clear aged phenotype emerges. There is also a need to determine any differences between males and females.

Differences in conclusions about timing and extent of aging related basal glucocorticoid function are likely due to several factors. Importantly, heterogeneity in prior life-course history of subjects significantly affects the aging trajectory. This is especially true for developmental programming by early life factors whose outcomes may lay dormant to emerge later. Existing reviews discuss glucocorticoids' as well as adrenal dehydroepiandrosterone sulfate's role in programming lifetime health and suggest a potential key role in aging (Langie et al. 2012; Maestripieri et al. 2009) life-course glucocorticoid profiles.

Normative basal values are required to determine the critical time windows at which to seek mechanisms involved in normal, premature, or augmented aging. One problem in establishing the normative aging trajectory is the life-course time-range of available data. Plasma glucocorticoid concentrations are often only obtained at one or two life-course time-points without the baseline data needed at early ages, making the timing of progression through the aging process difficult to analyze and interpret (Bowman et al. 2006; Ferrari et al. 2001; Peeters et al. 2007). While measures from one or two stages of the life-course are valuable, obviously, the greater the number of time-points determined, the firmer the conclusions that can be drawn. We propose that studies assessing life-course changes in aging should preferably cover at least five life-course time-points (Fig. 1).

Human (Kral et al. 2006; Villamor and Cnattingius 2006) and experimental animal (Nivoit et al. 2009; Samuelsson et al. 2008) studies have shown that offspring obesity is one of the adverse outcomes of maternal obesity. Many different mechanisms are involved in this predisposition. Glucocorticoids play a critical role during gestation in maturing a variety of fetal organs by stimulating differentiation and inhibiting growth and proliferation. We have previously published (Nathanielsz et al. 2013; Rodriguez et al. 2012; Vega et al. 2015; Zambrano and Nathanielsz 2013) that

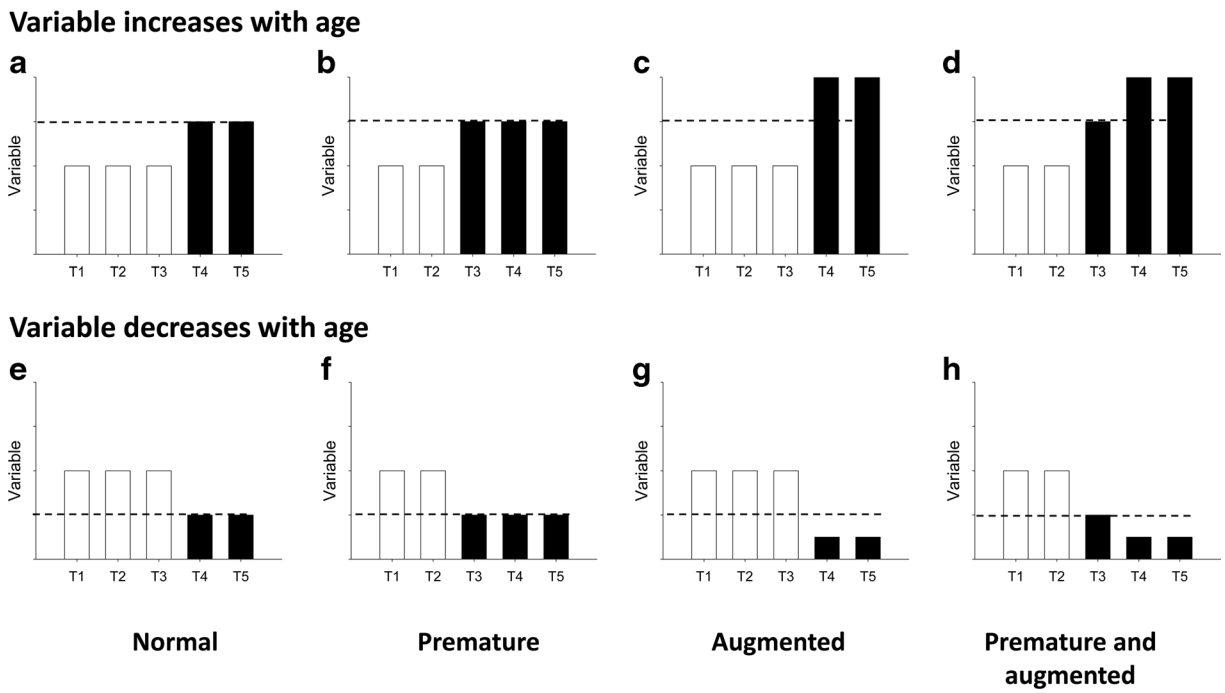

Fig. 1 Proposed strategy to determine at five life-course time-points. The timing of an age-related change represented by *dash line* in a variable that increases (**a**–**d**) or decreases (**e**–**h**) with age. **a**, **e** Represent a normal; **b**, **f** a premature; **c**, **g** an augmented; and **d**, **h** a premature and augmented aging process

maternal serum corticosterone concentrations in maternal obesity (MO) were higher than controls (C) at the time of breeding, end of gestation, 19 days gestation (dG), and end of lactation, postnatal day (PND) 21. Prenatal exposure to increased levels of glucocorticoids changes hypothalamic pituitary adrenal axis function (Braun et al. 2013).

We determined (1) the profile of basal circulating corticosterone in the rat across the complete life-course from weaning (PND 21) to old age (PND 850) in a well-characterized homogeneous colony to provide evidence for the existence of any significant change in trajectory in the second half of life; (2) differences between absolute corticosterone concentrations in males and females; and (3) whether developmental programming of offspring by exposure to maternal obesity during development alters the later-life circulating corticosterone trajectory.

Materials and methods

Care and maintenance of animals

All procedures were approved by the Animal Experimentation Ethics Committee of the Instituto Nacional de Ciencias Médicas y Nutrición, Salvador Zubirán (INNSZ), Mexico, and in accordance with the guidelines of Mexican law on animal protection (NOM-062-ZOO-1999). General procedures relating to maternal diet, breeding, and management of control and obese mothers have been previously described in detail (Zambrano et al. 2010). Briefly, at 4 months of age, 28 female Wistar rats weighing between 220 and 260 g were obtained from the INNSZ animal colony and maintained on normal laboratory chow (Zeigler Rodent RQ 22–5, USA) containing 22.0 % protein, 5.0 % fat, 31.0 % polysaccharide, 31.0 % simple sugars, 4.0 % fiber, 6.0 % minerals and 1.0 % vitamins (w/w), energy 4.0 kcal g^{-1}, under controlled lighting (lights-on from 7:00 a.m. to 7:00 p.m. at 22–23 °C). Female rats were mated overnight with proven male breeders. The day on which spermatozoa were detected in a vaginal smear was designated as conception day 0. Only rats that were pregnant within 5 days of mating with males were studied. All rats were delivered vaginally. To ensure homogeneity, only litters between 12 and 15 pups were studied. Litters were adjusted to 12 pups for each mother while maintaining as close to a 1:1 sex ratio as possible. After birth, all mother were fed with C diet. The females in these litters constituted the founder generation (F_0) mothers. At weaning, one F_0 female from each litter was placed on chow diet (controls C) (n=14) and one on the high fat high energy obesogenic diet containing 23.5 % protein, 20.0 % animal lard, 5.0 % fat, 20.2 % polysaccharide, 20.2 % simple sugars, 5.0 % fiber, 5.0 % mineral mix, 1.0 % vitamin mix (*w*/*w*), energy 4.9 kcal g^{-1} diet from weaning until they were bred at PND 120 (Zambrano et al. 2010) when obese F_0 mothers were 17 % heavier than control F_0 mothers. High-fat diet continued to be fed to the obese F_0 mothers in pregnancy and lactation. F_0 control mothers ate normal laboratory chow throughout. The high-fat diet increased obese F_0 insulin, glucose, HOMA, leptin, triglycerides, and retroperitoneal fat before breeding (Vega et al. 2015). Control and obese F_0 mothers were sisters and thus F_1 (the offspring of F_0) were cousins, helping to homogenize genetic factors. F_1 of both control and obese mothers ate standard laboratory diet after weaning. Food and water were available ad libitum.

Postnatal maintenance

After weaning (PND 21), both F_1 male and female pups were separated into groups of three to four per cage and fed standard rodent chow diet ad libitum throughout the study. At PND 21 (weaning), 110 (young adult), 450 (adult), 650 (mature adult), and 850 (aged phase), after 6 h of fasting, rats were euthanized between 12:00 p.m. and 2:00 p.m. by decapitation using a rodent guillotine (Thomas Scientific, NJ) by trained and experienced personnel. For each age group, trunk blood was collected and serum separated. F_1 evaluated at each of the five ages were siblings as far as possible.

Corticosterone measurements

For each age group, blood was collected, centrifuged, and serum frozen until assayed for corticosterone. Corticosterone was measured in fasting serum by radioimmunoassay (Rodriguez et al. 2012).

Statistical analysis

Data are expressed as means±SEM. Data were in-transformed. Male and female data for each point were analyzed by *t* test and were different, therefore sexes were analyzed separately. For the timing of the corticosterone fall in each group, we used the approach shown

in Fig. 1 to analyze data from five points across the life-course to set baselines and determine the time at which changes occur related to aging. The strategy uses the unpaired *t* test to compare data from the oldest age available (T5) to determine a significant difference from the preceding time-point (T4). If there is no difference, T4 and T3 are then compared, repeating the analysis to determine all points at which the later of the two points compared differs from the one before. For C vs. obese F_1 at the three ages in each sex, two-way ANOVA and Sidak's multiple comparison tests were used.

Results

Normative fall in corticosterone with aging

Corticosterone concentrations in the control animals were higher in females than in males (Fig. 2). Using the criteria established in Fig. 1, corticosterone fell between PND 450 and 650 in both male and female from control and F_1 of obese mothers (Fig. 2a, b).

Having established the timing of the normative corticosterone fall in both males and females, we examined the same stage of life in the developmental programming paradigm of F_1 offspring of obese F_0 mothers. F_1 offspring of obese F_0 mothers themselves become obese even on the same diet eaten by control F_1 (Nathanielsz et al. 2013). Corticosterone in male and female F_1 offspring of obese mothers fell between PND 450 and 650, a similar time window as control F_1 (Fig. 2).

Discussion

One major confound in the interpretation of available human aging data on life-course profiles of glucocorticoids is the inclusion of data from subjects with chronic diseases such as Alzheimer's, hypertension, or diabetes (Huang et al. 2009), which themselves can alter glucocorticoid production. In one study that included subjects with high blood pressure and heart disease, young subjects were all men to avoid ovarian cycle changes, while the older population was mixed male and female subjects (Zhao et al. 2003). Our data above show clearly the need to separate male and female data. One comprehensive human study on eight young (18–35 years) and eight elderly men (60–72 years) demonstrated a higher circadian cortisol rhythm mesor in aged males (Bergendahl et al. 2000). However, this study only included data at two time-points in the life-course. In one study in rhesus monkeys, values in older monkeys were higher than younger animals (Downs et al. 2008). In another study, plasma cortisol concentration was measured on day 2 after capture and transfer of 53 rhesus macaque mothers from a free-ranging situation to single cages (Maestripieri et al. 2009). Ages ranged between 15 and 25 years, and subjects had been pregnant and delivered at variable times before sampling.

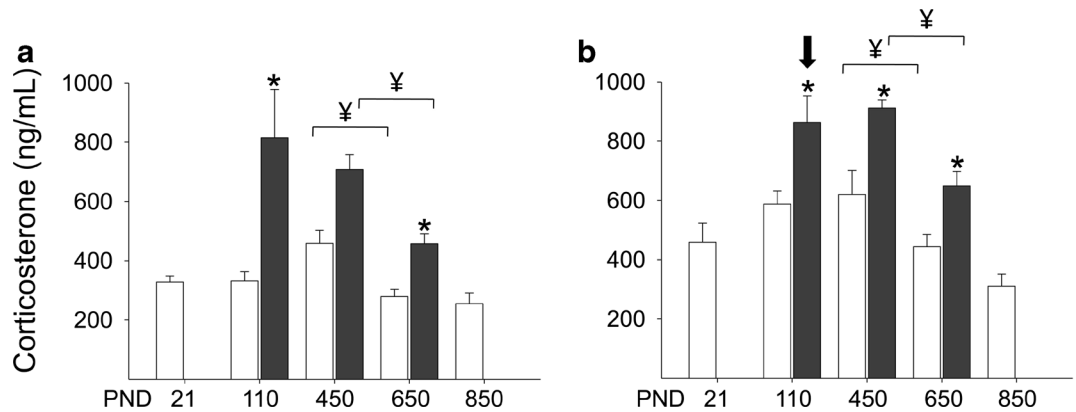

Fig. 2 Plasma corticosterone in **a** male and **b** female control rats (open histograms) at five life-course stages (PND postnatal day). The *yen sign* indicates the first significant change when comparing each age with the one prior starting with the oldest age as described for the analysis used in Fig. 1. Data are also presented (solid histogram) for F_1 of obese mothers **a** male and **b** female. Data are mean±SEM; group n=5–14. Data were in-transformed, and sexes were analyzed separately. Female corticosterone values were higher than male in all groups at all ages except in offspring of obese group at PND 110 (*downward-pointing arrow*). For the timing of the corticosterone fall in each group analysis was as described in relation to Fig. 1. For C vs. obese at the three ages in each sex, two-way ANOVA with Sidak's multiple comparison tests. $p<0.05$ (*asterisk*) vs. C

There was a negative relationship between cortisol and age that did not quite reach significance ($p=0.09$). These conflicting results show the need for data from homogeneous well-controlled subjects that fulfill the criteria given in Fig. 1.

One might consider that this question is easier to resolve in rodents than other species for many practical reasons. However, even in rodents, there is a lack of data from longitudinal studies of glucocorticoid concentrations across the life-span such as we present here (Fig. 2). Beginning studies in early life is important because early events set a baseline as well as themselves potentially influencing future function. We standardized the study subjects to remove disease-related confounds of the aging process. All animals were from our well-characterized colony of healthy male and female rats that has been maintained for several generations in standard conditions (Rodriguez et al. 2012; Vega et al. 2015; Zambrano et al. 2010). Background patrilineal and matrilineal data were available from all subjects to ensure homogeneity and lack of siblings among F_0 mothers in different studies. It is important to note that all study animals and their mothers are reared under very constant conditions with detailed observations on growth and general health throughout their life. Thus, study animals are not exposed to the variety of environmental confounds that inevitably occur in human studies.

The question needs to be posed "Is it possible to determine normal aging or should the aging process always be defined in the context of the life-course history of the individual." In addition to clear evidence of a strong genetic component, the final trajectory of aging will be determined by gene-environment interactions (nature and nurture) that occur even before conception. For example, maternal obesity affects gametes and alters offspring phenotype throughout life in ways that potentially influencing the rate of aging (Igosheva et al. 2010). The aging trajectory is clearly modified by age-related diseases. However, it is necessary to reconcile two opposing points of view. One holds that aging produces age-related diseases, and the other, that age-related diseases such as diabetes are themselves mechanisms that modify the aging trajectory.

There is support for the view that glucocorticoids accelerate aging processes (Anderson et al. 2014) as well as for development of both glucocorticoid resistance and glucocorticoid enhanced aging in different metabolic pathways (Chen et al. 2013). We propose that circulating glucocorticoids follow an age-related trajectory, which can be affected by both the external environment and internal physiological events. Importantly, life-span effects are influenced by developmental programming by challenges such as altered F_1 nutrition during their development. The mechanisms are likely multiple and include both programmed changes within the hypothalamo-pituitary adrenal axis (Braun et al. 2013; Zambrano et al. 2014) and altered peripheral production of glucocorticoids in adipose tissue that are influenced by maternal nutrition (Guo et al. 2013). The influence of developmental programming is clearly shown by the difference in the corticosterone concentrations in the F_1 from control and obese mothers. This part of the study was terminated at PND 650 because F_1 offspring of obese mothers begin to die around PND 650; thus, no data are available in this group at PND 850, which is equivalent to 75 years in human life (Quinn 2005), although making age comparisons between humans and rat life-span is not directly linear. Maximal rat life-span is dependent on the strain and—as this study indicates programming effects in the history of the animals under study—the probable maximum is around 3 years (Quinn 2005), but the differences in development must be taken into consideration when age is a crucial factor for comparison with human life.

The relationship of shorter life-span and high body mass index (BMI) in human populations is complicated by a wide variety of confounders and is not a linear relationship. However, human BMI levels in the obese range are correlated with shortened life-span (National Research Council 2011).

In conclusion, our goal was to address three precise and limited objectives for which no data exist in the literature. We conclude that, in rats, if samples cover a sufficient period of the life-course, corticosterone levels fall with aging in both normal controls and F_1 of obese F_0 mothers. The timing of the decrease in corticosterone concentrations appears similar in F_1 females and males of control and obese F_0 mothers, but the fall occurs from higher levels in F_1 from obese F_0 mothers. Corticosterone levels were higher in control and obese females than in males at all ages except PND 110 F_1 of obese mothers. Further studies are needed to determine whether this fall from a higher level and plays a role in the earlier death of F_1 from obese mothers that we have observed. Further, our observations are compatible with the view that the elevated corticosterone levels, of themselves, contribute to this aging process. Future studies

need to evaluate a wider range of glucocorticoid endpoints such as the parameters of the cosinor analysis of circadian rhythms.

Acknowledgments LARC is a graduate student from Doctorado en Ciencias Biomédicas, Facultad de Medicina, Universidad Nacional Autónoma de México and is a recipient of the CONACyT fellowship; this work was supported by CONACyT 155166 México. We are grateful to Guadalupe L. Rodríguez-González for assistance with the manuscript.

Conflict of interest The authors have nothing to disclose and have no conflict of interest.

References

Anderson RM, Birnie AK, Koblesky NK, Romig-Martin SA, Radley JJ (2014) Adrenocortical status predicts the degree of age-related deficits in prefrontal structural plasticity and working memory. J Neurosci 34:8387–8397. doi:10.1523/JNEUROSCI.1385-14.2014

Bergendahl M, Iranmanesh A, Mulligan T, Veldhuis JD (2000) Impact of age on cortisol secretory dynamics basally and as driven by nutrient-withdrawal stress. J Clin Endocrinol Metab 85:2203–2214. doi:10.1210/jcem.85.6.6628

Bowman RE, Maclusky NJ, Diaz SE, Zrull MC, Luine VN (2006) Aged rats: sex differences and responses to chronic stress. Brain Res 1126:156–166. doi:10.1016/j.brainres.2006.07.047

Braun T, Challis JR, Newnham JP, Sloboda DM (2013) Early-life glucocorticoid exposure: the hypothalamic-pituitary-adrenal axis, placental function, and long-term disease risk. Endocr Rev 34:885–916. doi:10.1210/er.2013-1012

Chen KC et al (2013) Glucocorticoid-dependent hippocampal transcriptome in male rats: pathway-specific alterations with aging. Endocrinology 154:2807–2820. doi:10.1210/en.2013-1139

Downs JL, Mattison JA, Ingram DK, Urbanski HF (2008) Effect of age and caloric restriction on circadian adrenal steroid rhythms in rhesus macaques. Neurobiol Aging 29:1412–1422. doi:10.1016/j.neurobiolaging.2007.03.011

Ferrari E et al (2001) Age-related changes of the hypothalamic-pituitary-adrenal axis: pathophysiological correlates. Eur J Endocrinol 144:319–329

Guo C, Li C, Myatt L, Nathanielsz PW, Sun K (2013) Sexually dimorphic effects of maternal nutrient reduction on expression of genes regulating cortisol metabolism in fetal baboon adipose and liver tissues. Diabetes 62:1175–1185. doi:10.2337/db12-0561

Huang CW, Lui CC, Chang WN, Lu CH, Wang YL, Chang CC (2009) Elevated basal cortisol level predicts lower hippocampal volume and cognitive decline in Alzheimer's disease. J Clin Neurosci 16:1283–1286. doi:10.1016/j.jocn.2008.12.026

Igosheva N, Abramov AY, Poston L, Eckert JJ, Fleming TP, Duchen MR, McConnell J (2010) Maternal diet-induced obesity alters mitochondrial activity and redox status in mouse oocytes and zygotes. PLoS One 5, e10074. doi:10.1371/journal.pone.0010074

Kral JG, Biron S, Simard S, Hould FS, Lebel S, Marceau S, Marceau P (2006) Large maternal weight loss from obesity surgery prevents transmission of obesity to children who were followed for 2 to 18 years. Pediatrics 118:e1644–e1649. doi:10.1542/peds.2006-1379

Langie SA, Lara J, Mathers JC (2012) Early determinants of the ageing trajectory. Best Pract Res Clin Endocrinol Metab 26:613–626. doi:10.1016/j.beem.2012.03.004

Maestripieri D, Hoffman CL, Anderson GM, Carter CS, Higley JD (2009) Mother-infant interactions in free-ranging rhesus macaques: relationships between physiological and behavioral variables. Physiol Behav 96:613–619. doi:10.1016/j.physbeh.2008.12.016

Nathanielsz PW, Ford SP, Long NM, Vega CC, Reyes-Castro LA, Zambrano E (2013) Interventions to prevent adverse fetal programming due to maternal obesity during pregnancy. Nutr Rev 71(Suppl 1):S78–S87. doi:10.1111/nure.12062

National Research Council (2011) Explaining divergent levels of longevity in high-income countries. In: Crimmins EM, Preston SH, Cohen B (eds) Panel on understanding divergent trends in longevity in high-income countries. Committee on population, division of behavioral and social sciences and education. The National Academies Press, Washington DC

Nivoit P, Morens C, Van Assche FA, Jansen E, Poston L, Remacle C, Reusens B (2009) Established diet-induced obesity in female rats leads to offspring hyperphagia, adiposity and insulin resistance. Diabetologia 52:1133–1142. doi:10.1007/s00125-009-1316-9

Peeters GM, van Schoor NM, Visser M, Knol DL, Eekhoff EM, de Ronde W, Lips P (2007) Relationship between cortisol and physical performance in older persons. Clin Endocrinol 67:398–406. doi:10.1111/j.1365-2265.2007.02900.x

Quinn R (2005) Comparing rat's to human's age: how old is my rat in people years? Nutrition 21:775–777. doi:10.1016/j.nut.2005.04.002

Rodriguez JS et al (2012) Maternal obesity in the rat programs male offspring exploratory, learning and motivation behavior: prevention by dietary intervention pre-gestation or in gestation. Int J Dev Neurosci 30:75–81. doi:10.1016/j.ijdevneu.2011.12.012

Samuelsson AM et al (2008) Diet-induced obesity in female mice leads to offspring hyperphagia, adiposity, hypertension, and insulin resistance: a novel murine model of developmental programming. Hypertension 51:383–392. doi:10.1161/HYPERTENSIONAHA.107.101477

Vega CC, Reyes-Castro LA, Bautista CJ, Larrea F, Nathanielsz PW, Zambrano E (2015) Exercise in obese female rats has beneficial effects on maternal and male and female offspring metabolism. Int J Obes (Lond) 39(4):712–719. doi:10.1038/ijo.2013.150

Villamor E, Cnattingius S (2006) Interpregnancy weight change and risk of adverse pregnancy outcomes: a population-based study. Lancet 368:1164–1170. doi:10.1016/S0140-6736(06)69473-7

Zambrano E, Nathanielsz PW (2013) Mechanisms by which maternal obesity programs offspring for obesity: evidence from

animal studies. Nutr Rev 71(Suppl 1):S42–S54. doi:10.1111/nure.12068

Zambrano E, Martinez-Samayoa PM, Rodriguez-Gonzalez GL, Nathanielsz PW (2010) Dietary intervention prior to pregnancy reverses metabolic programming in male offspring of obese rats. J Physiol 588:1791–1799. doi:10.1113/jphysiol.2010.190033

Zambrano E et al (2014) Increased central and peripheral glucocorticoid synthesis act as an orchestrator of developmental programming. In: stress and developmental programming of health and disease: beyond phenomenology nova Science Publishers, Inc

Zhao ZY, Lu FH, Xie Y, Fu YR, Bogdan A, Touitou Y (2003) Cortisol secretion in the elderly. Influence of age, sex and cardiovascular disease in a Chinese population. Steroids 68: 551–555