UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA

João Paulo de Arruda Amorim

"CARACTERIZAÇÃO DO COMPORTAMENTO MATERNO E SUAS IMPLICAÇÕES NO DESENVOLVIMENTO FÍSICO, NA FUNÇÃO REPRODUTIVA E NO PERFIL HORMONAL DA PROLE FEMININA DE RATAS UCHA E UCHB (CONSUMIDORAS VOLUNTÁRIAS DE ETANOL A 10%)"

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) *João Paulo de Arruda Amorim*

e aprovada pela Comissão Julgadora.

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Celular e Estrutural, na área de Anatomia.

Orientador: Prof. Dr. Francisco Eduardo Martinez

Co-Orientador: Prof. Dr. Wilson De Mello Junior

Campinas, 2012

FICHA CATALOGRÁFICA ELABORADA POR
ROBERTA CRISTINA DAL' EVEDOVE TARTAROTTI – CRB8/7430
BIBLIOTECA DO INSTITUTO DE BIOLOGIA - UNICAMP

Am68c

Amorim, João Paulo de Arruda, 1981-
Caracterização do comportamento materno e suas implicações no desenvolvimento físico, na função reprodutiva e no perfil hormonal da prole feminina de ratas UChA e UChB (consumidoras voluntárias de etanol a 10%) / João Paulo de Arruda Amorim. – Campinas, SP: [s.n.], 2012.

Orientador: Francisco Eduardo Martinez.
Coorientador: Wilson de Mello Junior.
Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Comportamento materno. 2. Rato - Desenvolvimento. 3. Reprodução. 4. Hormônios. 5. Rato UChA. 6. Rato UChB. I. Martinez, Francisco Eduardo. II. Mello Junior, Wilson de. III. Universidade Estadual de Campinas. Instituto de Biologia. IV. Título.

Informações para Biblioteca Digital

Título em Inglês: Maternal care evaluation and effects on the physical development, reproductive functions and hormonal profile of UChA and UChB rats female offspring (10% v/v ethanol voluntary intake)
Palavras-chave em Inglês:
Maternal behavior
Rats - Development
Reproduction
Hormones
UChA rat
UChB rat
Área de concentração: Anatomia
Titulação: Doutor em Biologia Celular e Estrutural
Banca examinadora:
Francisco Eduardo Martinez [Orientador]
Tânia Mara Segatelli
Fernanda Cristina Alcântara dos Santos
Wagner José Fávaro
Wellerson Rodrigo Scarano
Data da defesa: 27-01-2012
Programa de Pós Graduação: Biologia Celular e Estrutural

Campinas, 27 de janeiro de 2012.

BANCA EXAMINADORA

Prof. Dr. Francisco Eduardo Martinez (Orientador) _____
Assinatura

Profa. Dra. Tânia Mara Segatelli _____
Assinatura

Profa. Dra. Fernanda Cristina Alcântara dos Santos _____
Assinatura

Prof. Dr. Wagner José Fávaro _____
Assinatura

Prof. Dr. Wellerson Rodrigo Scarano _____
Assinatura

Prof. Dr. Robson Francisco Carvalho _____
Assinatura

Prof. Dr. Luís Fernando Barbisan _____
Assinatura

Profa. Dra. Flávia Karina Delella _____
Assinatura

Há um tempo em que é preciso abandonar as roupas usadas, que já tem a forma do nosso corpo, e esquecer os nossos caminhos, que nos levam sempre aos mesmos lugares. É o tempo da travessia: e, se não ousarmos fazê-la, teremos ficado, para sempre, à margem de nós mesmos."

Fernando Pessoa

Agir, eis a inteligência verdadeira. Serei o que quiser. Mas tenho que querer o que for. O êxito está em ter êxito, e não em ter condições de êxito. Condições de palácio tem qualquer terra larga, mas onde estará o palácio se não o fizerem ali?

Fernando Pessoa

Dedicatória

"Ainda que eu falasse as línguas dos homens e dos anjos,
se eu não tivesse amor, seria como bronze que soa
ou como címbalo que retine.
Ainda que eu tivesse o dom da profecia e
conhecesse todos os mistérios e toda a ciência;
Ainda que eu tivesse tamanha fé,
a ponto de transportar montanhas,
se não tivesse amor eu nada seria."
(Paulo, 1 Coríntios 13, 1- 2)

Dedico este trabalho a minha esposa, Elaine
e a minha ingênua e amada filha, Bianca.
Minhas decisões serão sempre para vocês.

Agradecimentos

Ao Prof. Dr. Francisco Eduardo Martinez, pela orientação, amizade, respeito e ensinamentos dedicados durante todos esses anos de convivência.

Ao Prof. Dr. Wilson de Mello Junior, pela co-orientação e ensinamentos.

Aos docentes do Departamento de Anatomia Humana (Wagner, Patrícia, Anchieta, Jair, Raquel, Sérgio) pela amizade e constante disponibilidade.

Aos Profs. Drs. Luís Fernando Tirapelli, Maíra Aparecida Stefanini e Isabel Cristina Cherici Camargo pelas correções e sugestões realizadas na análise prévia deste trabalho.

Ao programa de Pós-graduação em Biologia Celular e Estrutural pela oportunidade de realizar o doutorado.

A melhor secretária que eu conheço, Lilian Alves Senne Panágio, excelente pessoa e profissional extremamente dedicada ao programa e aos alunos. Muito obrigado.

A fundação de Amparo a Pesquisa do Estado de São Paulo – FAPESP – (Procs. 07/59355-1 e 08/56229-8) e a CAPES/PROEX e CAPES/PROAP pelo apoio financeiro, sem o qual eu não poderia realizar esse trabalho.

Aos funcionários do Departamento de Anatomia.

Aos grandes amigos que a anatomia me proporcionou: Gustavo, Leonardo, Giovana, Beatriz e Olegário, pela amizade, profissionalismo e dedicação nas intermináveis horas de observação do comportamento materno.

A Elaine, por decidir viver ao meu lado, sempre acreditando em mim e em meus ideais, muito obrigado por fazer parte da minha vida.

Aos meus pais, Valdivino Luiz de Amorim (*in memorian*) e Maria da Graças de Arruda, por sempre incentivarem os meus estudos, pela educação recebida e pela forma de amar.

E principalmente a Deus, por me conceder o dom da vida.

Sumário

Resumo

Estudos realizados com mães dependentes de etanol demonstraram que elas apresentam maior dificuldade em cuidar de suas crianças, quando comparadas às mães não dependentes, evidenciando um distúrbio no comportamento materno durante o período pós-natal, que corresponde ao período onde as primeiras ligações sociais do animal são formadas e o organismo está muito sensível aos efeitos de estímulos ambientais. Vários estudos têm documentado as conseqüências do uso de etanol durante a gestação para a saúde do infante, porém pouca atenção tem sido dada à relação materno-infantil em mulheres alcoólicas durante o período pós-natal e as consequências dessa relação para prole feminina na vida adulta. O presente trabalho teve o objetivo de caracterizar o comportamento materno das ratas da variedade consumidora de etanol (UChA e UChB) e verificar as influências da variação do comportamento materno no desenvolvimento físico, na função reprodutiva e no status hormonal da prole feminina. O comportamento foi avaliado observando os seguintes parâmetros: carregar, lamber, amamentar com o dorso arcado e lamber, amamentar com o dorso arcado, amamentar passivamente e não contato com a prole. A avaliação do desenvolvimento físico da prole feminina considerou o dia do nascimento dos pêlos, da abertura dos olhos e do descolamento de orelhas. Para avaliar o desenvolvimento sexual inicial foram analisados os dias da abertura vaginal e idade do primeiro e segundo estro. A função reprodutiva foi avaliada pela regularidade de ciclo estral, pela expressão dos receptores AR, ER-α e ER-β no ovário e pelo perfil hormonal da prole feminina (níveis plasmáticos de FSH, LH, 17β-estradiol, progesterona e corticosterona). As fêmeas UChA apresentaram maiores frequências dos comportamentos

de carregar, de lamber/limpar e de amamentar os filhotes. Mães muito cuidadosas apresentaram concentrações elevadas de corticosterona e 17β-estradiol. A prole UChA apresentou maior ganho de peso corporal, aceleração da abertura dos olhos, da abertura vaginal, da instalação da puberdade e sincronização do ciclo estral. A prole feminina que recebeu baixo cuidado materno (UChB) revelou maior duração do ciclo estral, aumento das concentrações de corticosterona e 17β-estradiol e de seus receptores ovarianos (ER-α e ER-β), maior peso dos ovários, maior número de folículos primordiais, antrais e maduros e mais imunomarcações positivas do Ki67 nos folículos ovarianos. Concluímos que a variedade de ratas UChB, apresenta acentuada variação do comportamento materno, sendo classificada como mãe pouco cuidadosa e essa variação do cuidado materno afeta diretamente o desenvolvimento físico, a instalação da puberdade, os níveis hormonais, desregula o ciclo estral e a foliculogênese e regula diferencialmente a expressão dos receptores ER-α e ER-β nos ovários de ratas adultas.

Palavras chave: Cuidado materno, desenvolvimento, função reprodutiva, hormônios, ovários e ratos UCh.

Abstract

Studies focused on drug-dependent mothers (mainly ethanol-dependent mothers) have demonstrated that there is an enormous difference in the care of their children compared to non-dependent mothers, showing an disorder in maternal behavior during the postnatal period, which corresponds to the period where the first social bonds are formed and the animal's organism is very sensible to the effects of environmental stimuli. Various studies have documented the consequences of ethanol use during pregnancy for the health of the infant, but little attention has been given to the mother-child relationship in alcoholic female during the postnatal period and the consequences of this relationship to female offspring in adulthood. The aim of the present work is to evaluate maternal care in ethanol-preferring rats (UChA and UChB) and its effects on physical development, in sexual function and in status hormones in female offspring. The behavior was evaluated by observing the following parameters: carry, licking/grooming, arched-back nursing and licking/grooming, arched-back nursing, passive nursing, contact and not with the pups. The evaluation of the physical development of the female offspring considered the day of birth of hair, eye opening and detached ears. To evaluate the early sexual development were analyzed days of vaginal opening and age of first and second estrous. The reproductive function was evaluated by the regularity of the estrous cycle, the expression of receptors AR, ER-α and ER-β in the ovary and the hormonal status of female offspring (plasma levels of FSH, LH, 17β-estradiol, progesterone and corticosterone). UChA mothers showed higher frequencies of carrying, licking/grooming and nursing the pups. Mothers high care evidencing the highest plasma corticosterone levels and 17β-estradiol. The UChA offspring

showed greater body weight gain, accelerated eye opening, vaginal opening, the installation and synchronization of estrous cycle. The female offspring who received low maternal care (UChB) showed an increase of the estrous cycle, concentrations of corticosterone and 17β-estradiol and ovarian receptors (ER-α and ER-β, higher ovarian weight and increased number of primordial, antral and mature follicles and higher Immunoreactivity for Ki-67 in the ovarian follicles. We conclude that UChB rats show marked variations in maternal care, being classified as low maternal care and the variation of maternal care directly affects the physical, the installation of puberty, hormone levels, deregulate the estrous cycle and folliculogenesis and differentially regulates the expression of receptors ER-α and ER-β in the ovaries of adult rats.

Key Words: Maternal care, developmental, reproductive function, hormones, ovary and UCh rats.

Introdução geral

Comportamento materno e a relação mãe-filhote

O comportamento materno em ratas consiste de vários elementos integrados relacionados com a nutrição e com o cuidado da prole, podendo envolver diretamente os filhotes (amamentar, lamber/limpar, aquecer e busca de filhotes) ou não (construção de ninhos e agressão materna). Perto do parto, a mãe inicia a seqüência de mudanças comportamentais que visam receber adequadamente os filhotes. Ela muda seus padrões de limpeza corporal, levando mais tempo na região das mamas e alguns dias antes do parto constroem o ninho com o substrato disponível (Todeschini, 2002).

Depois do nascimento, alguns hormônios facilitam a resposta materna, entre eles os estrógenos, ocitocina e prolactina (Nelson, 1995; Pedersen & Boccia, 2003; Afonso *et al*., 2009). Além das mudanças hormonais, as lactantes têm a produção crônica de calor neste período, o que auxilia no aquecimento dos filhotes (Kitrell & Satinoff, 1988). As mudanças hormonais e comportamentais induzem e habilitam a fêmea a proteger e orientar a ninhada (Stern & Johnson, 1990, Michel & Tyler, 2007). Entretanto, uma vez estabelecida à interação mãe-filhote, a resposta materna passa a ser induzida por estímulos dos filhotes. O principal estímulo é a presença dos filhotes que atraem a atenção da mãe com vocalizações, movimentos do corpo e pelo olfato (Moore, 1985; Polan & Hofer, 1999; Coutellier *et al*., 2008).

A rata lactante fica a maior parte do tempo sobre os filhotes para mantê-los aquecidos (Grota & Ader, 1969). Além disso, as mães mantêm-se por cima dos filhotes com uma postura arqueada fazendo com que as mamas fiquem mais expostas e facilitando a

ejeção do leite pelo estímulo de sucção dos filhotes ou fazendo a limpeza corporal e lambendo o filhote (Stern & Johnson, 1990; Albert & Walsh, 1995).

Nos ratos, o comportamento materno ocorre com maior freqüência nos primeiros dez dias pós-parto, principalmente nas primeiras horas do dia durante a fase clara (Champagne *et al*., 2003a). Depois de duas semanas do nascimento há um declínio gradual dos cuidados maternos, até que ocorra a rejeição dos filhotes pela mãe no desmame (Reisbick *et al*., 1975), que ocorre aproximadamente aos 28 dias de vida.

O comportamento de lamber os filhotes é um importante estímulo táctil que pode influenciar no desenvolvimento social de machos e fêmeas adultos (Birke & Sadler, 1987; Moore & Power, 1992). Tal procedimento influencia o comportamento materno futuro, proporcionando fêmeas mais cuidadosas com seus filhotes (Francis et al., 1999b). Este "ambiente maternal" é crucial para um desenvolvimento adequado dos filhotes, alteração nestas características podem influenciar as respostas comportamentais e neuroendócrinas da prole na vida adulta (Cirulli *et al*., 2003).

As variações no cuidado materno têm sido utilizadas como influência crítica no desenvolvimento do indivíduo. Em ratos, as variações do comportamento materno, particularmente os comportamentos de lamber e limpar (linking/grooming) os filhotes são responsáveis por regular o desenvolvimento das respostas endócrinas, emocionas e cognitivas em resposta ao estresse (Fairbanks & McGuire, 1988; Plotsky & Meaney, 1993; Liu *et al*., 1997; Van Der Oers *et al*., 1998; Caldji *et al*., 2000; Pryce *et al*., 2001; Champagne & Meaney, 2001, Champagne *et al*., 2003b; Macri *et al*., 2004).

Analisando populações de ratos observam-se mães muito cuidadosas e mães que cuidam pouco de seus filhotes. Os parâmetros adotados para analisar as mães são:

lamber/limpar e amamentar os filhotes com o dorso arqueado (Liu *et al*., 1997; Caldji *et al*., 1998; Francis *et al*., 1999b).

Considerando esta característica natural e peculiar de cada rata primípara, de ser uma mãe muito cuidadosa ou pouco atenciosa com a sua ninhada (Champagne & Meaney, 2001), passou-se a estudar as possíveis variações no comportamento materno, verificando que as variações naturais que ocorrem no cuidado materno influenciam no desenvolvimento neural das ninhadas subsequentes e estas variações são transmitidas de mãe para filhas.

Porém, nos experimentos em que os filhotes de mães pouco cuidadosas foram adotados por mães muito cuidadosas, verificou-se na idade adulta que os animais apresentaram os mesmos padrões comportamentais dos filhotes biológicos dessas mães muito cuidadosas, ou seja, agiram como se não fossem adotados, indicando que há o fator não genético na transmissão dessas características e possivelmente seja pelo estímulo táctil da mãe em lamber a região anogenital e o corpo dos filhotes (Francis *et al*., 1999a, Champagne, 2008).

Apesar dos fatores genômicos serem importantes para o comportamento materno em geral, alguns pesquisadores (Caldji *et al*., 1998, 2000; Champagne, 2008) têm mostrado evidências não genômicas consistentes para a transmissão das diferenças no comportamento de ratas, evidenciando que o comportamento materno tem efeito que se mantém, ou seja, apresenta padrão nas respostas de medo ou ansiedade nos animais adultos e na transmissão das características maternais de cuidado com a prole da mãe em relação às filhas biológicas ou de criação (Champagne & Meaney, 2001).

Efeitos do cuidado materno na neurobiologia e comportamento da prole

Diversos estudos demonstram uma íntima relação entre o cuidado maternal da mãe e o comportamento maternal da prole na vida adulta. Estudos transgeracionais realizados em primatas e roedores demonstram que estas características não são de origem genética, pois não são mediadas por variações em seqüencias de DNA, mas sim comportamental, sendo exclusivamente dependente da qualidade da interação materno-infantil no período pós-natal (Champagne, 2008).

O impacto da variação natural do comportamento materno na expressão de genes e nas funções neuroendócrinas tem sido extensivamente explorado em roedores. Trabalhos iniciais demonstraram as conseqüências do baixo cuidado materno para a fisiologia e resposta comportamental ao estresse. Filhotes que recebem baixo cuidado de suas mães apresentam uma elevação prolongada de adrenocorticotropina (ACTH) e corticosterona quando submetidos à situação de estresse. Esses animais possuem redução hipocampal de receptores de glucocorticóides e elevação do hormônio corticotrófico hipotalâmico (Caldji *et al*., 1998.; Liu *et al*., 1997), e redução no número de neurônios em áreas cerebrais envolvidas com o estresse e reprodução (Lucion *et al*., 2003).

Por outro lado, ninhadas oriundas de mães muito cuidadosas apresentam aumento na densidade sináptica no hipocampo e melhor desempenho no aprendizado e memória espacial (Liu *et al*., 2000; Bredy *et al*., 2003).

Influencia materna na função reprodutiva da prole feminina

Experiências nos primeiros dias pós-natal podem influenciar o comportamento sexual e a função reprodutiva em ratos adultos. Ratas neonatas que recebem pouco cuidado

materno, quando adultas apresentam um aumento da freqüência de ciclos estrais anovulatórios (Uriarte *et al*., 2007) e diminuição da receptividade sexual (Gomes *et al*., 1999, 2005, 2006).

Variações naturais do comportamento materno em ratos são uma importante fonte de informação para compreender as diferenças individuais no desenvolvimento do sistema neuroendócrino (Cameron *et al*., 2005). Variações no cuidado materno influenciam a estabilidade da expressão de receptor de estrógeno (ER-α) na prole feminina, em regiões encefálicas que controlam os comportamentos reprodutivos (Champagne *et al*., 2003b, 2006). Mães que oferecem alto cuidado materno, bem com suas filhas, demonstram um aumento na expressão de ER-α na área pré-optica medial do hipotálamo (mPOA), esta diferença de ER-α na mPOA é responsável por implicações no comportamento materno, porém a mPOA também coordena o comportamento sexual feminino inibindo a lordose ou facilitando a cópula (Champagne *et al*., 2003b., Erskine *et al*., 2004).

Outros trabalhos revelam o atraso no desenvolvimento sexual, bem como no tempo de instalação da puberdade em fêmeas filhas de mães de alto cuidado quando comparadas com filhas de mães de baixo cuidado materno (Cameron *et al*., 2008).

Alcoolismo e a relação materno-infantil

Estudo realizado com mães dependentes de substâncias químicas (principalmente etanol), cujos filhos possuíam até quatro anos de idade, demonstrou que essas mães apresentavam maiores dificuldades em cuidar de suas crianças comparadas às mães não dependentes (Savonlahti *et al*., 2004), evidenciando, dessa forma, distúrbio no comportamento materno durante o período pós-natal. Encontrando dados semelhantes,

Brudenell (2000) enfatizou que os primeiros três meses após o nascimento é o período mais crítico para a maternidade. Sendo período especialmente difícil para a mãe alcoólica, que mesmo tentando abster-se da ingestão de etanol, acaba suspendendo atividades de ajuda na recuperação. Concluindo que o uso do etanol pela mãe, após o nascimento, pode aumentar os riscos para a saúde da criança, causando efeitos sociais e psicológicos como abuso e negligência infantil.

Lieberman (2000), ao rever diversos trabalhos sobre filhos de alcoólicos, observou que estes apresentavam alto risco de dependência alcoólica, não somente devido às características genéticas ou às congênitas, mas também aos fatores do convívio familiar. Salientou que, mesmo após reestruturação familiar, os problemas comportamentais dos filhos de alcoólicos persistiam, sugerindo que os problemas iniciavam precocemente.

Experiências traumáticas na infância precoce estão associadas ao aumento do risco de abuso de etanol e substâncias tóxicas na adolescência e no adulto. Crianças e adolescentes maltratados manifestam distúrbios do sistema biológico de resposta ao estresse, incluindo efeitos prejudiciais no desenvolvimento cerebral. Esses distúrbios podem ser a causa do aumento do risco de abuso de etanol e drogas durante a adolescência e na vida adulta (De Bellis, 2002; Roman *et al*., 2004; Jaworski *et al*., 2005; Moffett *et al*., 2006).

Diferenças individuais nas características da personalidade são transmitidas para a prole. Estudos com gêmeos têm provido evidências de mecanismos genéticos envolvidos nos mais complexos padrões de comportamento e personalidade, como o alcoolismo. Por outro lado, transmissões não genéticas de comportamento também foram descritas (Francis *et al*., 1999b; Jablonka & Lamb, 2005).

Segundo Pryce & Feldon (2003), são de fundamental importância estudos experimentais que consideram os efeitos de impactos crônicos, ocorridos no ambiente pós-natal, sobre o neurocomportamento da prole na vida adulta. Estes estudos constituem exemplos de uma abordagem interdisciplinar, considerando as interfaces entre as ciências biológicas, sociais e médicas.

Nas diferentes espécies de mamíferos, evidências experimentais sobre a importância do cuidado materno é frequentemente realizada em modelos de privação (separação materna). Assim, tanto em primatas quanto em roedores, os infantes privados de cuidado materno por um determinado período de tempo exibem um aumento no comportamento agressivo no ambiente social, medo e prejuízo no desenvolvimento cognitivo (Ladd *et al*., 2000; Champagne *et al*., 2003a). Entretanto, poucos estudos têm reportado a influência da variação normal no comportamento materno sobre o desenvolvimento e fisiologia da prole.

O modelo experimental

Devido às dificuldades e implicações éticas em pesquisas com seres humanos sobre o alcoolismo, a grande parte do conhecimento, descrito na literatura especializada, provém de estudos com roedores (Martinez *et al*., 2001).

Modelos animais têm contribuído para explicar alguns aspectos biológicos: bioquímicos, fisiológicos e morfológicos envolvendo o consumo de bebidas alcoólicas por humanos (Cândido *et al*., 2007). Li *et al*. (1987) menciona a existência de três pares de linhagens de ratos de alto e baixo consumo de etanol. Os ratos UChA e UChB (UCh=Universidade do Chile) os mais antigos, os de Helsing iniciados por Eriksson &

Pikkarainen (1968) e os de Lumeng & Li em Indianápolis. As linhagens AA (*Alko Alcohol*) e ANA (*Alko Nonalcohol*) de Helsink foram obtidas por cruzamentos alternados de *inbreeding* e *outbreeding*. As linhagens P (*Alcohol Prefering* – 5-8g/Kg/dia) e NP (*Non-Alcohol Prefering* – menos de 0,5g/Kg/dia) de Lumeng & Li tem sido obtidas por cruzamentos *outbreeding*. Além dessas linhagens de ratos, existem raças de camundongos, tanto de alto consumo, a C57BL/6, como de baixo consumo de etanol, a BALB e PBA. Dos três pares descritos, os únicos que se mantém em *inbreeding* são os ratos UCh.

As variedades de ratos UChA (baixo consumo de etanol - menor que 1,9g/Kg/dia) e UChB (alto consumo de etanol - maior que 4,0g/Kg/dia) constituem modelos raros para estudos relacionados aos fatores genéticos, bioquímicos, fisiológicos, nutricionais e farmacológicos dos efeitos do etanol, além do apetite e a tolerância que são importantes fatores do alcoolismo humano (Mardones & Segovia-Riquelme, 1983; Tampier *et al*., 2000; Martinez *et al*., 2002).

Justificativas

Sabe-se que no período neonatal o organismo é sensível aos estímulos ambientais e que é nesse período que as primeiras relações sociais são formadas. Há anos têm-se despertado grande interesse em compreender quais os fatores do ambiente neonatal e como esses fatores interferem no comportamento, fisiologia e reprodução do animal durante a vida adulta. Dentre os vários fatores, sabe-se que as intervenções na relação mãe-filhote induzem a variações do comportamento materno e que essas variações são consideradas

como principal mediador das respostas comportamentais e neuroendócrinas dos animais quando adultos. Mais recentemente, alguns desses pontos têm sido alvos da curiosidade dos investigadores. Porém, para que as dúvidas ainda existentes possam ser esclarecidas são necessárias maiores informações sobre o assunto. Nesse contexto, insere-se a caracterização do comportamento materno da variedade UChA e UChB (bebedores voluntários de etanol) e suas consequências no desenvolvimento físico e sexual, na função reprodutiva e no perfil hormonal da prole feminina.

Objetivos

Sabendo que o comportamento materno é crucial para o desenvolvimento adequado da prole e que alterações dessas características podem levar a complexas e duradouras mudanças comportamentais, neuroendócrinas e reprodutivas da prole, e que existem diferenças naturais e individuais entre os animais no cuidado materno, o presente trabalho tem por objetivos:

- Caracterizar o comportamento materno, sem intervenção ou privação dos filhotes, nas ratas da variedade UChA e UChB (consumidoras voluntárias de etanol).
- Avaliar os impactos da variação do comportamento materno no desenvolvimento físico, na instalação da puberdade, no ciclo estral, na atividade ovariana e no perfil hormonal da prole feminina de ratas UCh.

Artigos

O presente trabalho originou dois artigos que serão apresentados a seguir:

Artigo I. "Maternal care alters physical development and sexual maturation in female UCh rats offspring".

Artigo II. "Variations in maternal care alters the corticosterone and 17β-estradiol levels, estrous cycle, folliculogenesis and stimulates the expression of estrogen receptors alpha and beta in the ovaries of UCh rats", publicado no periódico *Reproductive Biology and Endocrinology*.

Artigo I

Maternal care alters physical development and sexual maturation in female UCh rats offspring

João PA Amorim[1,2], Luiz GA Chuffa[1,2], Giovana R Teixeira[2], Leonardo O Mendes[1,2], Beatriz A Fioruci[1,2], Otávio A Martins[2], Wilson Mello Júnior[2], Janete A Anselmo-Franci[4], Patricia FF Pinheiro[2], Marcelo Martinez[3], Francisco E Martinez*[2].

[1]Department of Structural and Cellular Biology, Institute of Biology, Universidade Estadual de Campinas – UNICAMP, Campinas-SP 13083-863, Brazil
[2]Department of Anatomy, Bioscience Institute, UNESP – Univ. Estadual Paulista, Botucatu-SP 18618-970, Brazil
[3]Department of Morphology and Pathology, UFSCar – Universidade Federal de São Carlos, São Carlos-SP 13565-905, Brazil
[4]Department of Morphology, Stomatology and Physiology, USP – Universidade de São Paulo, Ribeirão Preto-SP 14040-900, Brazil

***Corresponding author**: Francisco Eduardo Martinez, Department of Anatomy, Biosciences Institute, UNESP - Univ Estadual Paulista, P.O. Box 510, Postal Code: 18618-000, Rubião Júnior, s/n, Botucatu, SP – Brazil, Telephone number: +55 (14) 3811-6040, Fax: +55 (14) 3811-6361.

E-mail: martinez@ibb.unesp.br

Abstract

Background: Studies focused on drug-dependent mothers (mainly ethanol-dependent mothers) have demonstrated that there is a wide difference in the care of their children compared to non-dependent mothers. Variations in maternal care induce lasting and complex behavioral, neuroendocrine and reproductive changes in offspring which appears during adulthood. The aim of this work is to analyze and characterize the maternal behavior of lactating rats from two groups designated as ethanol-preferring rats (UChA and UChB). Furthermore, this work evaluates maternal care the consequent impact on physical development, the onset of puberty and the reproductive function of female offspring. UCh rats were derived from original Wistar rats and were selected for ethanol consumption at the University of Chile over almost 60 years.

Methods: Twenty-four male and female UCh rats, 120 days of age, were randomly divided into two groups (UChA and UChB) and mated. The maternal behavior was assessed from birth (0 day) to the 10th postnatal day (PND).

Results: UChB rats (10% (v/v) high ethanol consumption) had difficulty caring for their offspring and exhibited poor maternal care. In contrast, UChA rats (10 % (v/v) low ethanol consumption) showed extreme dedication to their offspring with effective maternal care. The UChA offspring showed greater body weight gain, accelerated eye opening and vaginal opening. Additionally, the female UChA rats enhanced the installation and synchronization of estrous cycle.

Conclusions: The results indicate that UCh rats show variations during maternal care, and the lack of affinity with their offspring directly affects the physical development and the onset of puberty and estrous cycle in the UChB female offspring.

Key words: Maternal care; physical development; sexual maturation; UCh rats.

Background

The maternal behavior effects have been demonstrated across many species and serve as an important cue to offspring development. In rats, maternal care includes several integrated elements relating to nutrition and pup's care, and these elements appear to be spontaneously enacted by primiparous females [1, 2]. The "maternal environment" is crucial for the adequate development of pups, and alterations in such interactions could affect the process of offspring development in adulthood [3]. In the postpartum period, the onset of maternal care is mediated by hormones as estradiol, but is sustained by recent sensorial experiences interacting with the pups [4].

Moreover, there may be transmission of these effects to subsequent generations through alterations in the offspring reproductive behavior. The mechanisms mediating this transmission have been explored in rodents and involve epigenetic alterations to steroid receptor genes that produce long-term changes in gene expression and behavior [2].

Studies focused on drug-dependent mothers (mainly ethanol-dependent mothers) have demonstrated that there is an enormous difference in the care of their children compared to non-dependent mothers. Children and adolescents who are not cared while developing may be changing their biological systems in response to stress, and these factors could be contributing to the risk of ethanol dependence or other abuse substances during adulthood [5,6].

UCh rats were derived from original Wistar rats and were selected for ethanol consumption at the University of Chile over almost 60 years [7]. These ethanol-preferring

rats are considered a special model for understanding of the basis of alcoholism-linked characteristics, such as those found in alcohol-related human diseases.

Maternal behavior is essential to the development of pups, and when proper maternal behavior is not maintained, serious irreversible impairments due to less-than-optimal maternal behavior could lead to permanent behavioral, neuroendocrine and reproductive changes in pups. In the literature, little is known about the natural influence of maternal care upon the development, physiology and reproduction of female offspring in adulthood.

The aim of the present work is to evaluate maternal care in UCh ethanol-preferring rats and its effects on physical development and sexual maturation in female offspring.

Methods

Animals

Forty-eight adult male and female UChA and UChB rats, aged 60 days (225-240 g), were obtained from the Department of Anatomy, Bioscience Institute/Campus of Botucatu, IBB/UNESP – Univ Estadual Paulista. The animals were randomly divided into two groups (n=24/group). All animals were housed in polypropylene cages (43 cm×30 cm×15 cm) with laboratory-grade pine shavings as bedding and maintained under controlled temperature settings (23 ± 1 °C) and lighting conditions (12-h L, 12-h D photoperiod, lights switched off at 0700 h). The animals were handled in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA)

and approved by the IBB/UNESP Ethical Committee for Animal Research, Protocol 01/08-CEEA.

After UCh rats were individually housed (aged 60 days), they were given a choice between two bottles containing either water or 10% (v/v) ethanol *ad libitum* for 15 days. After this period, 12 animals per group displaying ethanol consumption less than 1.9 g ethanol/kg BW/day (UChA strain) and higher than 2.0 g ethanol/kg BW/day (UChB strain; ranging from 4 to 5 g ethanol/kg/day) were selected for the experiment [7]. For this study, the preference ratio associated with ethanol-seeking care was approximately 50%. In addition, to ensure better efficiency and prevent against damage arising from ethanol consumption during pregnancy, the animals were maintained without access to ethanol after preference determination.

Experimental groups

Twenty-four male and female rats (120 days old) derived from UChA and UChB lineages were allowed to mate. At night, mature females were housed together with males to encourage copulation. The confirmation of mating was seen in early morning by the presence of sperm on the slides. This finding was designated as day 0 of pregnancy. The rats were monitored once per day, and near the end of pregnancy, rats were monitored twice per day in order to determine time of pup's birth. The time and date of birth was fixed as the postnatal day 0 (PND 0), and the sexing and standardization in 8 pups/litter, with proper balance between male and female being ensured in order to avoiding any interference on maternal preference, also occurred at this time. During the mate, pregnancy

and lactation, the following groups were formed: UChA mothers (n= 12) and UChB mothers (n= 12).

Importantly, the mothers of both UCh lineages did not receive ethanol solution during mating, pregnancy or lactation, in order to prevent the effects of Fetal Alcohol Syndrome and alcohol withdrawal.

Evaluation of maternal care

Mothers and offspring were housed in individual home-cages for evaluation of maternal care. The animals were monitored by one experimenter. Food and water were provided *ad libitum*. The maternal care was evaluated from birth (PND 0) until the 10th postnatal day (PND 10) during 60 minutes of observation four times a day. This observation amounted to 1056 h of observations. During the 60 minutes observations, each female was observed every 3 minutes for a total of 80 observations/mother/day. The observations occurred at regular times each day with three periods during the light phase (0800, 1200 and 1600 h) and one period during the dark phase (2000 h) [1, 8]. The following categories of maternal care were achieved: carry, licking/grooming, arched-back nursing and licking/grooming, arched-back nursing, passive nursing, contact with the pups or not. No contact with the pups occurred when mothers were engaged in nest building, environmental exploration, self-grooming and feeding.

Evaluation of external physical signals during female development

After birth, the female offspring was daily observed during physical development from the 1st until the 21st PND throughout the following parameters: body weight, age of

eye opening, appearance of pelage and detachment of the ears. To avoid maternal rejection, the offspring body mass was measured from PND 0, and monitored every 3 days until weaning. UChA (n = 12) and UChB (n = 12) female offspring.

Onset of puberty and estrous cycle

After the vaginal opening occurred indicating the onset of prepuberty (started at 30st PND) and to investigate the age of first and second estrus, the cycles were monitored by colpocytological examination (vaginal smears) every day during 21 consecutive days. Cells detaching from the vaginal epithelium were removed with a pipette (Lab Mate 0.5-10 μL, UK). The filter tips containing 10 μL 0.9% saline were discarded after the vaginal secretion had been transferred to clean slides. Colpocytological examination time was set at 9:00 am. Each slide was analyzed under a Zeiss Axiophot II microscope (Carl Zeiss, Germany) at 10X and 25 X magnifications.

Estradiol measurements

After decapitation, blood samples were collected into heparinized tubes from the ruptured cervical vessels of UCh mothers (approximately 170 days of age) after variation of maternal care in the postpartum period. Afterwards, plasma was obtained by centrifugation at 1,200 x g for 15 min at 4ºC and stored at - 20ºC until assayed by radioimmunoassay (RIA). The plasma concentration of estradiol (E2) was determined using Estradiol Maia kits (Biochem Immunosystems, Serotec, Italy). The lower detection limit and the intra-assay coefficient of variation was 7.5 pg/mL and 2.5% for E2. All samples were measured

in duplicate and at different dilutions, if necessary. In order to prevent interassay variation, all samples were assayed in the same RIA.

Statistical analysis

For maternal care comparison, the non-parametric Mann–Whitney test was used and the results are given as median (min; max) values. Student t test was applied to hormones and other parameters and results expressed as mean ± SEM. Differences were considered significant when $p<0.05$. The statistical software used was *GraphPad Instat version 4.0* and *Sigma Plot version 11.0* for graphic design.

Results

Maternal care

Significant differences between the UChA and UChB lines were observed in carrying, licking/grooming, arched-back nursing and licking/grooming, arched-back nursing and non-contact during the postpartum period. There were no significant differences in passive nursing and contact during the postpartum period (Table 1). UChB mothers showed deficiencies in the behaviors assessed.

The maternal care duration showed alterations during the first 10 days. The greatest variation occurred on the first day postpartum which were gradually declined towards time progressed postpartum. The differences in behavior between lactating UChA and UChB females were significant on most days during experiment (Figure 1).

Physical development

On the day of birth, the UChA offspring's weight was not significantly different compared to UChB offspring (Table 2). Moreover, the two groups of offspring had significant differences in body weight gain from day 3 postpartum onward, and the difference remained until day 21 postpartum (Table 2).

The UChA mother's offspring opened their eyes before UChB offspring. There was no significant difference between the groups comparing hair growth and detachment of the ears (Table 2).

Sexual maturation

The UChB mother's offspring showed delayed vaginal opening and occurrence of the first and second estrus (Table 2).

Estradiol levels

Mothers providing high maternal care presented an elevation in E2 levels compared to those of low maternal care (UChA 20.67±4.05 pg/mL vs. UChB 9.15±0.53 pg/mL). Values are expressed as mean ± SEM (N=10 animals/group, $p<0.05$, Student *t* test).

Discussion

Variations in maternal care are associated with marked differences in the reproductive physiology and behavior of the adult female offspring [9]. Observations of maternal behavior during the first 10 days postpartum revealed significant differences in

maternal-infant interaction between the UCh lactating rats. These UCh rats showed high (UChA mother) and low maternal care (UChB mother), similar to other rodent species [1, 8].

According to these results, two distinct strategies of maternal care were categorized: first, UChA lactating females offered high mother-offspring contact, exhibited high infant excitement frequency (licking/grooming) and showed dedication to offspring (nutrition and heating); second, UChB mothers showed low levels of excitement and dedication to offspring, occurring occasionally by the mother rejecting their offspring.

Mothers providing low maternal care (UChB rats) presented a decrease in E2 levels, during the lactation period, resulting in the absence of affinity with their offspring. The influence of E2 mediated by ER-α on maternal care is, in turn, regulated by an estradiol-induced increase on oxytocin (OT) receptors which activates OT mRNA receptor in the medial preoptic area of the hypothalamus (MPOA) [10]. In the rat, central OT receptors are obligatory for the expression of maternal care [11], and variations in OT receptor levels at MPOA are functionally linked to differences in maternal care [12].

The maternal effect on ER-α expression in the MPOA may serve as a mechanism for differences during maternal care of female offspring. The activation of ER-α in the MPOA enhances OT receptor binding, and this effect is essential for the onset of maternal care in the rat [11]. Infusions of E2 [13] directly into the MPOA facilitate the expression of maternal care. OT receptor binding in the MPOA of lactating high LG mothers is significantly greater than low LG mothers and central infusion of an OT receptor antagonist eliminates the difference in the pup LG [12]. The difference in OT receptor binding as regarding to MPOA is estrogen-dependent [11].

Differences in maternal care provided to offspring from the 1st to 10th days postpartum were related to changes in sexual maturation of female offspring during puberty. The UChB offspring who received low maternal care showed delayed vaginal opening (early puberty) and installation of estrous cycle.

Puberty is initiated in the CNS by an activation of GnRH neurons. Once activated, GnRH neurons release neuropeptides through high-frequency pulses into the hypophysial portal vessels, which leads to the stimulation of gonadotropin secretion from the anterior pituitary thus activating the gonadal function [14]. Kisspeptin neurons are needed to turn on the GnRH pulse generator, which determines the awakening of the hypothalamus-pituitary-gonadal axis, marking the onset of puberty [15].

Two main populations of kisspeptin neurons are present in the mammalian brain: one located in the ARN/infundibular nucleus; and another one in the MPOA, known as RP3V in rodents. Several studies point to the RP3V kisspeptin neurons as being the population that directly innervates GnRH neuron cell bodies [16].

Variations in maternal care influence the activity of neurons in the MPOA, most specifically the oxytocin receptors, which are sensitive to estrogen [17]. Similarly, low maternal care is related to changes in oxytocin neuronal activity at the MPOA, and probably the GnRH neurons, which belong to the same brain region, are also affected, justifying the delay in early puberty found in UChB female offspring.

The pups that received high maternal care (UChA rats) showed significant anticipation in the age of eye opening. UChA offspring established within the enriched environment supported by high maternal care, influencing the hippocampus function and modulating molecules that are crucial for phenotypic plasticity, such as N-methyl D-

aspartate (NMDA) and brain-derived neurotrophic factor (BDNF) receptors in the visual cortex, responsible for accelerating the eye opening [18,19].

The UChB mother's offspring gained less weight than those of UChA mothers during lactation, possibly related to stress condition in the postnatal environment, where the UChB pups were subjected to mistreated, neglected and abandoned by their mothers [20]. Another important factor is linked to the nutrition of animals. We believed that intense maternal care provided by the UChA rats resulted in a better thermoregulation, cleanliness and nutrition of their pups.

Conclusion

We conclude that UCh rats show variations maternal care. The UChB mothers exhibited low maternal care. The lack of affinity to their offspring directly affects the physical development, the installation of puberty and the early estrous cycle in the UChB female offspring.

Competing of interests

The authors declare that they have no competing interest.

Acknowledgments

We are grateful to Mr. Wanderley Thiago da Silva from Central Biotherium, IBB/UNESP, Botucatu, SP, for animal care and Mr. Gelson Rodrigues from Department of Anatomy, IBB/UNESP, for technical assistance. We would like to special thanks to

FAPESP (Procs. 07/59355-1 and 08/56229-8) and CAPES by providing financial support. This study is part of the PhD Thesis presented by Amorim JPA to the State University of Campinas - UNICAMP, Brazil.

References

1. Champagne FA, Francis DD, Mar A, Meaney MJ: **Variations in maternal care in the rat as a mediating influence for the effects of environment on development**. *Physiol Behav* 2003a; **79:** 359-371.
2. Champagne FA: **Epigenetic mechanisms and the transgenerational effects of maternal care**. *Front Neuroendocrinol* 2008; **29:** 386-397.
3. Macrí S, Mason G, Wurbel H: **Dissociation in the effects of neonatal maternal separations on maternal care and the offspring's HPA and fear responses in rats**. *Eur Journal Neurosci* 2004; **20:** 1017-1024.
4. Numan M: **Maternal Behavior**. In: Neill JD, Knobil E. and Neill JD (Editors). *The Physiology of Reproduction*, Raven Press (New York), 1994. p221-302.
5. Jaworski JN, Francis DD, Brommer CL, Morgan ET, Kuhar MJ: **Effects of early maternal separation on ethanol intake, GABA receptor and metabolizing enzymes in adult rats**. *Psychopharmacology* 2005; **181:** 8-15.
6. Moffett M, Harley J, Francis D, Sanghani S, Davis W, Kuhar M: **Maternal separation and handling effects cocaine self-administration in both the treated pups as adults and the dams**. *J Pharmacol Exp Ther* 2006; **317:** 1210-1218.

7. Mardones J, Segovia-Riquelme N: **Thirty-two years of selection of rats by ethanol preference: UChA and UChB strains**. *Neurobehav Toxicol Teratol* 1983; **5:** 171-178.

8. Uriarte N, Breigeiron MK, Benetti F, Rosa XF, Lucion AB: **Effects of maternal care on the development, emotionality, and reproductive function in male and female rats**. *Dev Psychobiol* 2007; **49:** 451-462.

9. Cameron NM, Fish EW, Meaney MJ: **Maternal influences on the sexual and reproductive success of the female rat**. *Horm Behav* 2008; **54:** 178-184.

10. Young LJ, Muns S, Wang Z, Insel TR: **Changes in oxytocin receptor mRNA in rat brain during pregnancy and the effects of estrogen and interleukin-6**. *J Neuroendocrinol* 1997; **9:** 859-865.

11. Pedersen CA: **Oxytocin control of maternal behavior. Regulation by sex steroids and offspring stimuli**. *Ann N Y Acad Sci* 1997; **807:** 126-145.

12. Champagne F, Diorio J, Sharma S, Meaney MJ: **Variations in maternal care in the rat are associated with differences in estrogen-related changes in oxytocin receptor levels.** *Proc Nat Acad Sci USA* 2001; **98:**12736–12741.

13. Fahrbach SE, Morrell JI, Pfaff DW: **Possible role for endogenous oxytocin in estrogen-facilitated maternal behavior in rats**. *Neuroendocrinology* 1985; **40:** 526-535.

14. Ojeda SR, Skinner MK: **Puberty in the rat. In: Neill JD, Knobil E. and Neill JD (Editors)** ***Physiology of reproduction***. 3rd ed. *San Diego: Academic Press*; 2006. p2061–2126.

15. Clarkson J, Han SK, Liu X, Lee K, Herbison AE: **Neurobiological mechanisms underlying kisspeptin activation of gonadotropin-releasing hormone (GnRH) neurons at puberty**. *Mol Cell Endocrinol* 2010; **324:** 45-50.

16. Clarkson J, Herbison AE: **Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropinreleasing hormone neurons**. *Endocrinology* 2006; **147:** 5817-5825.

17. Champagne FA, Weaver ICG; Diorio J, Sharma S, Meaney MJ: **Natural variations in maternal care are associated with estrogen receptor alpha expression and estrogen sensitivity in the medial preoptic area**. *Endocrinology* 2003b; **144:** 4720-4724.

18. Liu D, Diorio J, Day JC, Francis D, Meaney MJ: **Maternal care, hippocampus synaptogenesis and cognitive development in rats**. *Nat Neurosci* 2000; **3:** 799-806.

19. Landi S, Sale A, Berardi N, Viegi A, Maffei L, Cenni MC: **Retinal functional development is sensitive to environmental enrichment: a role for BDNF**. *FASEB J* 2007; **21:** 130-139.

20. Baker S, Rees S, Chebli M, Lemarec N, Godbout R; Huta V, Bielajew C. **Effects of gestational stress: 2. Evaluation of male and female adult offspring**. *Brain Research* 2009; **1302:**194-204.

Tables

Table 1. Duration (%) of observed maternal care over days 0-10 postpartum exhibited by UCh lactating female. (n=12/group).

Behaviors	**UChA Mothers**	**UChB Mothers**
Carrying	0.37 (0.28 - 1.59)	0.18 (0.09 - 0.46) *
Licking/Grooming - L/G	1.55 (0.50 - 3.13)	0.63 (0.26 - 0.83) *
Arched-back nursing and L/G	4.33 (2.75 - 6.55)	1.43 (0.56 - 3.10) **
Arched-back nursing	56.53 (52.28 - 67.59)	32.73 (15.81 - 40.63) **
Passive nursing	5.87 (0 - 14.17)	9.28 (1.0 - 17.77)
Contact with pups	5.07 (2.84 - 10.42)	5.22 (1.68 - 8.32)
No contact with pups	26.27 (14.25 - 36.83)	50.52 (45.16 - 67.75) **

*$p < 0.001$, **$p < 0.0001$, Mann-Whitney test. Values are expressed as Median (min - max) values.

Table 2. Body weight, age of developmental physical and onset puberty of UCh pups. (n=12/group).

Parameters	**UChA Offspring**	**UChB Offspring**
Pup weight on day 0 (g)	6.35±0.07	6.45±0.09
Pup weight on day 3 (g)	9.44±0.15	8.24±0.19**
Pup weight on day 9 (g)	19.41±0.33	16.86±0.28***
Pup weight on day 15 (g)	31.35±0.43	29.67±0.59*
Pup weight on day 21 (g)	45.68±0.56	40.88±0.83***
Age of eye opening (days)	13.41±0.15	14.43±0.24*
Age of fur birth (days)	4.37±0.20	4.13±0.07
Age of ear detachment (days)	2.24±0.11	2.51±0.10
Age of vaginal opening (days)	32.15±0.29	42.25±0.80***
Age of first estrus (days)	39.47±0.69	43.95±0.64**
Age of second estrus (days)	42.19±0.58	49.92±1.21**

*$p < 0.05$, **$p < 0.001$, ***$p < 0.0005$, Student's *t*-test. Values are expressed as mean ± SEM.

Figure 1. Duration of maternal care: (A) duration of carrying; (B) duration of licking/grooming (L/G); (C) duration of arched-back nursing and licking/grooming; (D) duration of arched-back nursing; (E) duration of passive nursing and (F) duration of contact with pups. Values are expressed as means ± SEM. UChA and UChB rats (n=12/group). Mann-Whitney test; *$p < 0.05$. (PND) Postpartum day.

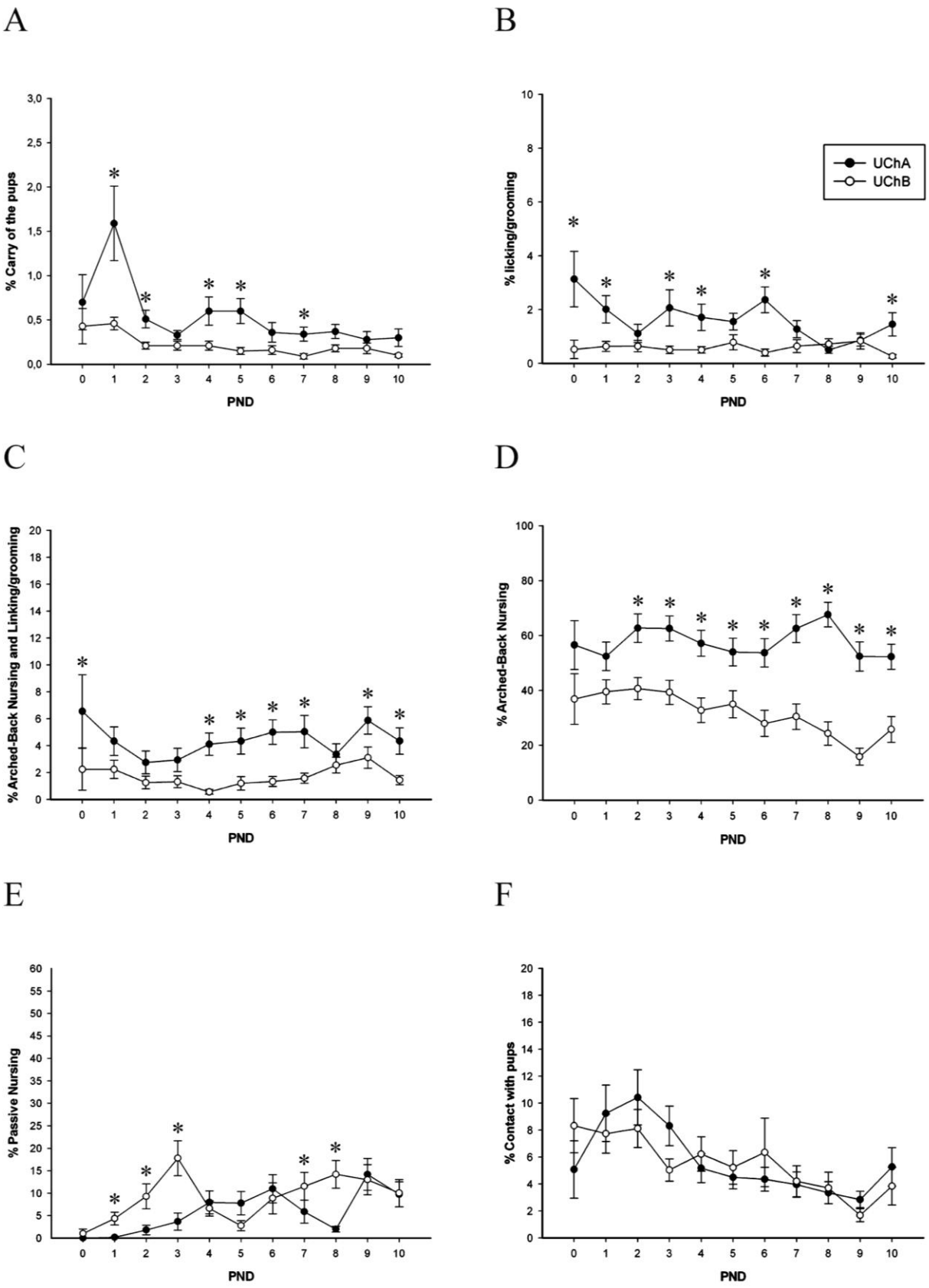
A
B
C
D
E
F
% Carry of the pups
% licking/grooming
% Arched-Back Nursing and Linking/grooming
% Arched-Back Nursing
% Passive Nursing
% Contact with pups
PND
UChA
UChB

FIGURE 1

Artigo II. Publicado no periódico *Reproductive Biology and Endocrinology,* 2011 **9**:160.

Variations in maternal care alters the corticosterone and 17beta-estradiol levels, estrous cycle, folliculogenesis and stimulates the expression of estrogen receptors alpha and beta in the ovaries of UCh rats

João PA Amorim[1,2], Luiz GA Chuffa[1,2], Giovana R Teixeira[2], Leonardo O Mendes[1,2], Beatriz A Fioruci[1,2], Otávio A Martins[2], Wilson Mello Júnior[2], Janete A Anselmo-Franci[4], Patricia FF Pinheiro[2], Marcelo Martinez[3], Francisco E Martinez*[2].

[1]Department of Structural and Cellular Biology, Institute of Biology, Universidade Estadual de Campinas – UNICAMP, Campinas-SP 13083-863, Brazil
[2]Department of Anatomy, Bioscience Institute, UNESP – Univ. Estadual Paulista, Botucatu-SP 18618-970, Brazil
[3]Department of Morphology and Pathology, UFSCar – Universidade Federal de São Carlos, São Carlos-SP 13565-905, Brazil
[4]Department of Morphology, Stomatology and Physiology, USP – Universidade de São Paulo, Ribeirão Preto-SP 14040-900, Brazil

***Corresponding author**: Francisco Eduardo Martinez, Department of Anatomy, Biosciences Institute, UNESP - Univ Estadual Paulista, P.O. Box 510, Postal Code: 18618-000, Rubião Júnior, s/n, Botucatu, SP – Brazil, Telephone number: +55 (14) 3811-6040, Fax: +55 (14) 3811-6361.

E-mail: martinez@ibb.unesp.br

Abstract

Background: Variations in maternal care are associated with neonatal stress, hormonal disturbances and reproductive injuries during adulthood. However, the effects of these variations on sex hormones and steroid receptors during ovary development remain undetermined. This study aimed to investigate whether variations in maternal care are able to influence the hormonal profile, follicular dynamics and expression of AR, ER-alpha and ER-beta in the ovaries of UCh rat offspring.

Methods: Twenty-four adult UCh rats, aged 120 days, were randomly divided into two groups (UChA and UChB) and mated. Maternal care was assessed from birth (day 0) to the 10th postnatal day (PND). In adulthood, twenty adult female rats (UChA and UChB offspring; n=10/group), aged 120 days, were euthanized by decapitation during the morning estrus.

Results: UChA females (providing high maternal care) more frequently displayed the behaviors of carrying pups, as well as licking/grooming and arched back nursing cares. Also, mothers providing high care had elevated corticosterone levels. Additionally, offspring receiving low maternal care showed the highest estrous cycle duration, increased corticosterone and 17beta-estradiol levels, overexpression of receptors ER-alpha and ER-beta, increased numbers of primordial, antral and mature follicles and accentuated granulosa cell proliferation.

Conclusions: Our study suggests that low maternal care alters corticosterone and 17beta-estradiol levels, disrupting the estrous cycle and folliculogenesis and differentially regulating the expression of ER-alpha and ER-beta in the ovaries of adult rats.

Keywords: maternal care, sex steroid receptors, corticosterone, E2, ovary.

Background

In mammals, physical and psychological development depends on the relationship established between the mothers and their offspring. Any disturbance during maternal care represents an important factor affecting the regulation of hypothalamic-pituitary-adrenal axis (HPA) in addition to the pups' care [1]. HPA activation is a central physiological event that is triggered in response to stress. Deficiency in maternal care leads to neonatal injuries, which are subsequently related to disease susceptibility, hormonal imbalances, reproductive damage and social problems in adulthood [2-6].

In adult rats, maternal care includes several integrated elements relating to nutrition and pup care, and these elements appear to be spontaneously enacted by primiparous females [7, 8]. After birth, essential hormones, such as prolactin, oxytocin, estrogen and corticosterone may be associated with maternal interaction [9-11], behavioral and hormonal changes stimulate the female to protect their litters [12, 13]. However, once the mother-pup relationship is established, the pup's activities signal to the mother to stimulate maternal care. The major stimulus is the presence of pups that attract the attention of the mother with vocalizations, body movements and smell [14-17]. Alterations in maternal care might cause deleterious effects during development, and they seem to be detrimental to female reproduction.

Ovarian steroid hormones, such as estradiol (E2), strongly influence neural circuits that regulate sexual behavior and estrous cycle [18]. The action of E2 and androgens is mediated through estrogen receptors (ER), composed of ER-α and ER-β subunits, and androgen receptors (AR), respectively. These receptors belong to a family of steroid

nuclear receptors with tissue-specific functions [19, 20].

Mothers who offer low maternal care, as well as their daughters, tend to exhibit a reduced level of estrogen receptor (ERα) expression in the brain regions that regulate maternal care and the hypothalamic-pituitary-gonadal axis [21-23], but little is known about the influence of maternal care on the expression of ER-α, ER-β and AR receptors in the ovarian tissue. Interestingly, this study is the first to report the impact of maternal care on ovarian ER expression. This study also demonstrates that increases in luteinizing hormone (LH) and follicle stimulating hormone (FSH) are necessary for ovulation to occur. The preovulatory LH surge is triggered by LHRH activity, which, in turn, is dependent on increased E2 levels [24-26].

UCh rats were derived from original Wistar rats and were selected for ethanol consumption at the University of Chile over almost 60 years [27]. These ethanol-preferring rats are considered a special model for understanding of the basis of alcoholism-linked characteristics, such as those found in alcohol-related human diseases.

Despite growing evidence of the consequences of maternal care on offspring development, no study has yet evaluated the effect of maternal care on ovarian activity. Therefore, this study aimed to investigate whether variation of maternal care can alter hormonal levels and estrous-cycle duration, as well as the cell proliferation index, during folliculogenesis and expression of ER-α, ER-β and AR in the UCh rat ovary.

Methods

Animals

Forty-eight adult male and female UChA and UChB rats, aged 60 days (225-240 g), were obtained from the Department of Anatomy, Bioscience Institute/Campus of Botucatu, IBB/UNESP – Univ Estadual Paulista. The animals were randomly divided into two groups (n=24/group). All animals were housed in polypropylene cages (43 cm×30 cm×15 cm) with laboratory-grade pine shavings as bedding and maintained under controlled temperature settings (23 ± 1 °C) and lighting conditions (12-h L, 12-h D photoperiod, lights switched off at 0700 h). The animals were handled in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by the IBB/UNESP Ethical Committee for Animal Research, Protocol 01/08-CEEA.

After UCh rats were individually housed (aged 60 days), they were given a choice between two bottles containing either water or 10% (v/v) ethanol *ad libitum* for 15 days. After this period, 12 animals per group displaying ethanol consumption less than 1.9 g ethanol/kg BW/day (UChA strain) and higher than 2.0 g ethanol/kg BW/day (UChB strain; ranging from 4 to 5 g ethanol/kg/day) were selected for the experiment [27]. For this study, the preference ratio associated with ethanol-seeking care was approximately 50%. In addition, to ensure better efficiency and prevent against damage arising from ethanol consumption during pregnancy, the animals were maintained without access to ethanol after preference determination.

Experimental groups

Twenty-four male and female rats (120 days old) derived from UChA and UChB lineages were allowed to mate. At night, mature females were housed together with males to encourage copulation. The confirmation of mating was seen in early morning by the presence of sperm on the slides. This finding was designated as day 0 of pregnancy. The rats were monitored once per day, and near the end of pregnancy, rats were monitored twice per day in order to determine time of pup's birth. The time and date of birth was fixed as the postnatal day 0 (PND 0), and the sexing and standardization in 8 pups/litter, with proper balance between male and female being ensured in order to avoiding any interference on maternal preference, also occurred at this time. During mate, pregnancy and lactation, the following groups were formed: UChA mothers (n= 12) and UChB mothers (n= 12).

The mothers of both UCh lineages did not receive ethanol during mating, pregnancy or lactation in order to prevent the effects of Fetal Alcohol Syndrome.

Evaluation of maternal care

Mothers and offspring were housed in individual home-cages for evaluation of maternal care. The animals were monitored by one experimenter. Food and water were provided *ad libitum*. The maternal care was evaluated from birth (PND 0) until the 10th postnatal day (PND 10) during 60 minutes of observation four times a day. This observation amounted to 1056 h of observations. During the 60 minutes observations, each female was observed every 3 minutes for a total of 80 observations/mother/day. The observations occurred at regular times each day with three periods during the light phase

(0800, 1200 and 1600 h) and one period during the dark phase (2000 h) [5, 7]. The measured categories of maternal care were adopted from previous work [28-31]: carrying, licking/grooming, arched-back nursing and licking/grooming, arched-back nursing, passive nursing and contact. No contact with the pups occurred when mothers were engaged in nest building, environmental exploration, self-grooming and feeding.

Estrous cycle analysis

At 90 days of age, the estrous cycles of female offspring were monitored by colpocytological examination (vaginal smears) every day for 21 consecutive days. Cells detaching from the vaginal epithelium were removed with a pipette (Lab Mate 0.5-10 μL, UK). Filter tips containing 10 μL 0.9% saline were discarded after the vaginal secretions had been transferred to clean slides [32]. Colpocytological examination time was set at 0900 h. Each slide was analyzed under a Zeiss Axiophot II microscope (Carl Zeiss, Germany) at 10X and 25X magnifications.

Biological sample collection

At 120 days of age, all UChA and UChB rats in estrous were weighed and euthanized by decapitation. Blood samples were collected and stored at - 80° C for further analysis. Ovaries were dissected and weighed using analytical balance (Owalabor) and were fixed by immersion in 10% buffered formalin.

Follicle counts

These analyses were performed using 5-µm-thick slices under light microscopy, adopting one section and discarding ten sections in sequence and finally resulting in 12 repetitions/ovary [33]. The primordial, primary, growing (more than two layers until the antral cavity appear), preantral, antral and mature follicles were each counted. All data were analyzed under a Zeiss Axiophot II microscope (Carl Zeiss, Germany) using 20X magnification for primordial and primary follicles and 10X for others.

Immunohistochemistry for Ki-67

Sections of paraplast-embedded ovaries (5 µm) from each offspring were collected on silanized glass slides and pretreated with 2 N HCl for 30 min at 37 ºC. Antigen retrieval was achieved by incubating the slides with 0.1% trypsin for 15 min at 37 ºC. After washing, the slides were blocked with 3% hydrogen peroxide in methanol for 20 min and 3% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Next, slides were incubated with monoclonal anti-Rat-Ki-67 antibody (clone MIB-5, Dako, Carpinteria, CA) at a 1:50 dilution in 1% BSA in PBS and incubated overnight at 4 ºC. After washing with PBS, the slides were incubated for 1 h at room temperature with biotinylated goat anti-mouse IgG antibody (Santa Cruz Biotechnology, CA) diluted 1:100 in 1% BSA in PBS. After washing, the sections were incubated with avidin-biotin-peroxidase solution (diluted 1:50) for 45 min (Elite ABC kit, Vector Laboratory, Burlingame, CA, EUA). Chromogen color development was carried out with 3,3'-diaminobenzidine tetrahydrochloride. Slides were counterstained with Harris's hematoxylin. A negative control was performed by

omitting the primary antibody incubation step. The data were analyzed under a Zeiss Axiophot II microscope (Carl Zeiss, Germany).

To quantitatively evaluate Ki-67-immunostained nuclei (proliferation index), the total number of positive granulosa cells in 10 randomly selected follicles was counted at 40X magnification for each follicular development stage per animal. The results were expressed as a percentage of total cells counted (number of labeled nuclei X 100/total number of cells).

Western blotting analysis and protein quantification

After euthanasia, the ovaries from UCh offspring were rapidly removed, and tissue samples of 50 mg were immediately frozen in liquid nitrogen and stored at -80 ºC. All tissues were homogenized with RIPA lysis buffer (Pierce Biotechnology, Rockford, IL, USA), using a homogenizer (IKA® T10 basic Ultra, Staufen, Germany). Aliquots of 10% Triton were added to homogenates, and samples were placed on dry ice for 2 h for optimal extraction. These suspensions were centrifuged at 21,912 x g for 20 min at 4 ºC, and the pellet was discarded. Protein concentrations were measured by the Bradford method. Total proteins were dissolved in 3X sample buffer used for SDS-PAGE (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein (70 µg) were loaded per well onto preformed gradient gels, 4-12% acrylamide (Amersham Biosciences, Uppsala, Sweden) with a Tris-glycine running buffer system for electrophoresis (60 mA fixed during 2 h). After electrophoresis, total proteins were electro-transferred (400 mA fixed by 1 h 30 min) onto 0.2 µm nitrocellulose membranes in a Tris-glycine-methanol buffer. Prestained standards were used as molecular weight markers. Thereafter, the membranes were blocked

with TBS-T solution containing 3% BSA at room temperature (RT) for 60 min and subsequently incubated at 4 ºC overnight with rabbit primary antibody AR anti-androgen receptor (AR); rabbit clone E115 anti-ERα; and rabbit clone 68-4 anti-ERβ (dilutions of 1:1000; 1:250; 1:500 in 1% BSA, respectively). This step was followed by washing 3 x 5 min in TBS-T solution and incubation for 2 h at RT with rabbit HRP-conjugated secondary antibodies (diluted 1:1000 in 1% BSA; Sigma, St. Louis, MO, USA). After sequential washing with TBS-T, signals were enhanced by mixing 10mL PBS, 8µl H_2O_2 and 0.02g diaminobenzidine (DAB) as chromogen. Immunoreactive bands of each protein were obtained from blots of six rats per group using image analysis software (NIS-Elements, Advanced Research, Nikon). β-actin was used as an endogenous control, and all results were expressed as means ± SEM. Immunoblotting concentrations were represented as optical densitometry values (band intensity /β-actin ratio).

Hormone assay

Blood samples were collected into heparinized tubes from the trunk of decapitated UCh mothers and female offspring after variation of maternal care in the postpartum period. Afterwards, plasma was obtained by centrifugation at 1,200 x g for 15 min at 4 ºC and stored at -20 ºC until assayed by radioimmunoassay (RIA). Plasma samples were assayed for FSH and LH by double-antibody RIA with specific kits provided by the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases (NIADDK, Baltimore, MD, USA). The FSH primary antibody was anti-rat FSH-S11, and the standard was FSH-RP2. The antiserum for LH was LH-S10 using RP3 as reference. The lower limit of detection for FSH and LH was 0.2 ng/mL and the intra-assay coefficients of variation

were 3% and 4%, respectively. Plasma concentrations of E2 and P4 were determined using Estradiol and Progesterone Maia kits (Biochem Immunosystems, Serotec, Italy). The lower detection limit and the intra-assay coefficient of variation were, respectively, 7.5 pg/mL and 2.5% for E2 and 4.1 ng/mL and 3.7% for P4. Plasma concentrations of corticosterone were determined using specific kits provided by Sigma-Aldrich, Steinheim, Germany. Assay sensitivity was 0.023 ng/mL, and the intra-assay coefficient of variation was 4.5%.

All samples were measured in duplicate and at different dilutions when necessary. In order to prevent interassay variation, all samples were assayed in the same RIA.

Statistical analysis

The non-parametric Mann–Whitney test was used for general maternal care comparisons. Two Way Repeated Measures ANOVA was performed to evaluate the influence of time (days) along the period of observation (based on two independent factors: time and UCh varieties). Data of follicle counts and percentages were expressed as median followed by quartiles [Q1–Q3]. Student's t-test was applied to other parameters, and the results were expressed as means ± SEM. Differences were considered significant when $p < 0.05$. The statistical software used was *GraphPad Instat version 4 and Sigma Plot version 11.0* for graphic design.

Results

Maternal care

UChA females showed greater frequencies of care behaviors than the UChB females, including carrying pups, licking/grooming (L/G) the offspring's anogenital region, arched-back nursing with L/G and arched-back nursing alone. There was no significant difference with regard to passive nursing, although the UChA lactating females presented this care less frequently than did the UChB females. The UChA females displayed additional contact care with their offspring more frequently than did the UChB females, but this difference was not statistically significant (Table 1).

In addition, maternal care showed considerable during the first 10 days postpartum, with the highest care levels being observed at birth followed by a gradual decrease over time. Two-way repeated measures ANOVA indicated a main effect of both day ($F_{10,24}$=5.1, P<0.001) and UCh varieties ($F_{1,24}$=5.8, P<0.05) on the frequency of carrying (Figure 1A). Analysis of licking/grooming over the first 10 days postpartum indicated a main effect of both day ($F_{10,24}$=2.1, p<0.05) and UCh varieties ($F_{1,24}$=15.2, p<0.001) (Figure 1B). Analysis of arched-back nursing with L/G indicated of days ($F_{10,24}$=1.6, p=0.12) and UCh varieties ($F_{1,24}$=34.8, p<0.001) (Figure 1C). Analysis of arched-back nursing over the first 10 days postpartum indicated a main effect of both day ($F_{10,24}$=2.5, p<0.01) and varieties ($F_{1,24}$=99.5, p<0.001), and a significant interaction between day and varieties ($F_{10,24}$=2.8, p<0.01) (Figure 1D) . In frequency of passive nursing the repeated measures ANOVA indicate of both days ($F_{10,24}$=7.1, p<0.001) and varieties ($F_{1,24}$=4.3, p<0.05) and a significant interaction between day and strain ($F_{10,24}$=2.1, p<0.05) (Figure 1E). Analysis of

contact indicated a main effect of day ($F_{10,24}$=4.3, $p<0.001$) and UCh varieties ($F_{1,24}$=1.0, p=0.34), with females of all varieties decreasing their levels of contact over successive days (Figure 1F). The differences in maternal care between UChA and UChB rats were notably significant during the experiment (Figure 1).

Analysis of the estrous cycle of female offspring

Female offspring who received low maternal care (UChB rats) exhibited the longest estrous cycle duration with prolonged metestrus stage (3-4 days arrested; Table 2).

Ovary weight and follicles counts of female offspring

The absolute and relative ovary weights of rats receiving low maternal care (UChB) were significantly higher than those of UChA rats. Also, the UChB rat ovaries had a higher number of primordial, antral and mature follicles, while the primary and growing follicles were predominant in the ovaries of rats receiving high maternal care (Table 3).

Plasma corticosterone and sex hormones

Mothers providing high maternal care (UChA rats) presented an elevation in corticosterone levels, and conversely, the low maternal care increased both corticosterone and E2 levels in the UChB offspring (Figures 2 A, B, E). Plasma LH, FSH and P4 were not significantly different, due to the variations in maternal care during early postnatal life (Figures 2 C, D, F).

Analysis of ovarian AR, ER-α, ER-β in UCh offspring

Variations in maternal care resulted in different expression patterns of ovarian sex-steroid receptors. Similarly, ovarian ER-α and ER-β were overexpressed in rats that received low maternal care, whereas AR levels did not differ between the groups (Figures 3 A, B).

Cell proliferation index (Ki-67) in UCh offspring

Immunoreactivity for Ki-67 in the granulosa cells of the primary, preantral, antral and mature follicles was significantly higher in animals receiving low maternal care during early postnatal life. In contrast, growing follicles did not show significant differences between the groups (Table 3; Figure 4).

Discussion

In the rat, variations in maternal care are associated with individual differences during the development of the neuroendocrine and reproductive system, most prominently in the females [34-36]. Evaluation of maternal care during the first 10 days postpartum revealed significant differences in maternal-infant interaction between the UCh lactating rats. UChA mothers showed higher frequencies of carrying, licking/grooming, arched-back nursing with L/G and arched-back nursing cares. However, the analysis of passive nursing and simple contact with offspring did not show significant differences. Two distinct strategies of maternal care were categorized: first, lactating UChA females offered high levels of mother-offspring contact, exhibited high infant excitement frequency

(licking/grooming) and showed devotion to offspring (nutrition and heating); second, UChB mothers showed low levels of excitement and dedication to offspring and high frequencies of maternal rejection. Therefore, female UCh may be high-care (UChA mother) or low-care (UChB mother), similar to other rodent species [7, 37, 38].

The onset of maternal care in postpartum lactating females reflects hormone concentrations and the densities of hypothalamic receptors that stimulate and prepare the animal, beginning during the prenatal period [39]. Differently from UChB mothers, UChA mothers were more dedicated and busy during the execution of maternal care, evidencing the highest plasma corticosterone levels at the end of lactation. It is well known that mothers treated with moderate concentrations of exogenous corticosterone have increased frequency of licking/grooming and arched-back nursing care activities [40, 41]. This moderate increase in maternal corticosterone is fundamental to the offspring, as it induces the appropriate development of the aminoacidergic and serotoninergic systems and maturation of hypothalamic-pituitary-adrenal axis, thereby regulating the corticosterone levels in basal conditions and under stress during adulthood [42-44]. Curiously, the UChA offspring that received high maternal care exhibited the lowest concentrations of corticosterone in adulthood. The positive effect of corticosterone on the expression of rat maternal care occurs through its action in the medial preoptic area via E2-ER activation [21]. Female rats receiving low maternal care showed high E2 concentrations, which stimulate mRNA transcription for corticotropin-releasing hormone (CRH) in the hypothalamic paraventricular nucleus (PVN) when mediated by ER-α and ER-β, thereby increasing the corticosterone secretion [45,46]. E2 also exerts a negative regulation on the neurons secreting gonadotropin-releasing hormone (GnRH). Taken together, these results

point to the mutual and bidirectional interaction between the hypothalamic-pituitary-adrenal and hypothalamic-pituitary-ovarian axis [47, 48].

Although FSH levels remained unchanged during the experiment, the UChB offspring had the highest E2 concentrations, as well as overexpression of their receptors. It has already been demonstrated that E2-bound ER induces multiple actions; in particular, ER-α and ER-β play different roles during ovarian activity [49]. Interestingly, E2 seems to promote its effects through activation of both ERs, mainly through ER-α in the estrus period. Our data are consistent with the previous report [50] in which ovarian ER-α was upregulated in animals that received low maternal care. To date, nuclear ER-α levels were exclusively documented in extracts from specific brain regions, being significantly elevated in the female offspring of high LG dams [21, 51, 36].

In UChB rats, the increase in E2 did not induce an LH surge during ovulation, arresting the estrous cycle in these rats. This result was previously described by [52] in which a prolonged estrous phase was followed by a fall in LH levels but not a lack of ovulation. Evidence suggests that the LH produced by thecal cells stimulates testosterone synthesis [53], while granulosa cells produce aromatase, an enzyme required for conversion of androgens into estrogens [54]. In our study, it is likely that androgens have been converted to estrogen because variations of stressor agents can modulate aromatase activity [55]. In addition, concentrations of plasma LH and the AR expression did not differ between the groups. Ultimately, the ovarian AR was upregulated regardless of the maternal care received. The AR responsiveness could partially explain our findings because low androgen availability leads to upregulation of its receptor. It has been proposed that androgens may act on granulosa cells throughout folliculogenesis by preventing follicular

atresia and improving follicle development and maintenance of fertility [50, 56]. In this regard, the AR-mediated activities are not affected during differential maternal care.

The concentrations of FSH and LH did not vary after maternal care, unlike the concentrations of E2 in UChB rats during estrus. Additionally, these rats showed higher ovarian weight and increased number of primordial, antral and mature follicles. It has recently been proposed that the androgen-AR complex is essential to promote the expression of FSH-RH during follicular growth, which stimulates the synthesis of E2 via FSH receptor activation [57]. Furthermore, androgen is responsible for stimulating early follicular growth until preantral development [58]. This regulation through E2 signaling or E2-ER binding was remarkably high in those animals receiving low maternal care, thereby contributing to follicular development. This function appears to be essentially related to primordial and primary follicles [59]. Conversely, the UChA rats exhibited a greater number of primary and growing follicles. These differences in follicular dynamics may be due to changes caused by the specific type and intensity of maternal-infant interaction beside the pattern of response mediated by activation of the HPA on the HPG axis and disturbances of the female sex hormones.

The rats that received low maternal care had higher rates of granulosa cell proliferation at most stages during follicular development. This condition is due to the mitogenic effect produced by E2, probably through an activation pathway initiated by the cyclin D2 in the granulosa cells [60]. In contrast, the UChA offspring showed a reduced granulosa cell proliferation index. It has been recently established that moderate maternal corticosterone levels during the postpartum period are responsible for attenuating cell proliferation in several tissues in adulthood [61].

Conclusion

We conclude that low maternal care increases the plasma corticosterone and E2 levels, cell proliferation in ovarian follicles and duration of the estrous cycle and also differentially regulates the expression of ER-α and ER-β in the ovaries of the offspring during adulthood. Further studies are needed to elucidate the negative effects of low maternal care in the mother-infant interface, especially focusing on the female reproductive system.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JPAA, FEM collected and analyzed the data and drafted the manuscript beyond conceiving the main idea of the study. LGAC and GRT: western blotting analysis and substantial interpretation of data. LOM, BAF, MM, OAM, WMJ and PFFP: participated in the acquisition of data, in the design of the study and in the intellectual conception of the study. JAAF participated in all RIA dosages and in interpretation of these data. All authors helped to perform the statistical analyses. All authors read and approved the final version of the manuscript.

Acknowledgments

We are grateful to Mr. Wanderley Thiago da Silva from Central Biotherium, IBB-UNESP, Botucatu-SP for animal care and Mr. Gelson Rodrigues and Dr. Wagner José Fávaro from the Department of Anatomy, IBB/UNESP for technical assistance. We would

like to thank FAPESP (Procs. 07/59355-1 and 08/56229-8) and CAPES specially for providing financial support. This manuscript was edited by American Journal Experts.

This study is part of the PhD Thesis presented by Amorim JPA to the State University of Campinas - UNICAMP, Brazil.

References

1.Walker CD, Deschamps S, Proulx K, Tu M, Salzman C, Woodside B, Lupien S, Gallo-Payet N, Richard D: **Mother to infant or infant to mother? Reciprocal regulation of responsiveness to stress in rodents and the implications for humans.** *J. Psychiatry Neurosci* 2004, **29**:364-382.

2. Liu D, Dioro J, Tannenbaum B, Caldji C, Francis D, Freedman A, Shaema S, Pearson D, Plotsky PM, Meaney MJ: **Maternal care, hipocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal response to stress.** *Science* 1997, **277:** 1659-1662.

3. Francis DD, Diorio J, Plotsky PM, Meaney MJ: **Environmental enrichment reverses the effects of maternal separation on stress reactivity**. *J Neurosci* 2002, **22:**7840-7843.

4. Jaworski JN, Francis DD, Brommer CL, Morgan ET, Kuhar MJ: **Effects of early maternal separation on ethanol intake, GABA receptor and metabolizing enzymes in adult rats**. *Psychopharmacology* 2005, **181:** 8-15.

5. Uriarte R, Breigeiron MK, Benetti F, Rosa XF, Lucion AB: **Effects of maternal care on the development, emotionality, and reproductive function in male and female rats.** *Dev Psychobiol* 2007, **49:** 451-462.

6. Champagne FA, Meaney MJ: **Transgenerational effects of social environment on variations in maternal care and behavioral response to novelty**. *Behav Neurosci* 2007, **121:** 1353-1363.

7. Champagne FA, Francis DD, Mar A, Meaney MJ: **Variations in maternal care in the rat as a mediating influence for the effects of environment on development.** *Physiol Behav* 2003a, **79:** 359-371.

8. Numan M: **Maternal Behavior**. (2nd ed). In.: Edited by KNOBIL E, NEILL, JD. The Physiology of Reproduction Raven Press (New York), 1994: 221-302.

9. Macrí S, Mason G, Wurbel H. **Dissociation in the effects of neonatal maternal separations on maternal care and the offspring's HPA and fear responses in rats.** *Eur J Neurosci* 2004, **20:**1017-1024.

10. Petersen CA, Boccia ML: **Oxytocin antagonism alters rat dams' oral grooming and upright posturing over pups**. *Physiol Behav* 2003, **80:** 233-241.

11.Catalani A, Alemà GS, Cinque C, Zuena AR, Casolini P: **Maternal corticosterone effects on hypothalamus-pituitary-adrenal axis regulation and behavior of the offspring in rodents.** *Neurosci Biobehav Rev* 2011, **35:**1502-1517.

12. Michel GF, Tyler AN: **Can Knowledge of developmental processes illuminate the evolution of parental care?** *Dev Psychobiol* 2007, **49:** 33-44.

13. Stern JM, Johnson SK: Ventral somatosensory determinants of nursing care in Norway rats. **Effects of variations in quality and quality of pup´s stimuli**. *Physiol Behav* 1990, **47:** 993-1011.

14. Rosenblatt JS, Siegel HI, Mayer AD: **Progress in the study of maternal behavior in the rat: hormonal, nonhormonal, sensory, and developmental aspects**. Vol. 10. Edited by Rosenblatt JS, Hinde RA, Beer C, Busnel MC. In Advances in the study of care, Acad. Press, New York; 1979: 225-311

15. Rosenblatt JS: **Hormonal and nonhormonal regulation of maternal behavior: A theoretical survey**. *Reprod Nutr Dev* 1980, **20**: 791-800.

16. Polan HJ, Hofer MA: **Maternally directed orienting behaviors of newborn rats.** *Dev Psychobiol* 1999, **34:** 269-279.

17. Coutellier L, Friedrich A, Failing K, Wurbel H: **Variations in the postnatal environment in mice: Effects on maternal behaviour and behavioural and endocrine responses in the adult offspring**. *Physiol Behav* 2008, **93:** 395-407.

18. Auger AP: **Ligand-independent activation of progestin receptors: relevance for female sexual care**. *Reproduction* 2001, **122:** 847-855.

19. Koike S, Nii A, Sakai M, Muramatsu M: **The steroid binding domain of porcine estrogen receptor.** *Biochemistry* 1987, **5:** 2563-2568.

20. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA: **Cloning of a novel receptor expressed in rat prostate and ovary.** *Proc Natl Acad Sci U S A* 1996, **11:** 5925-5930.

21. Champagne FA, Weaver ICG, Diorio J, Sharma S, Meaney MJ: **Natural variations in maternal care are associated with estrogen receptor alpha expression and estrogen sensitivity in the medial preoptic area**. *Endocrinology* 2003b, **144:** 4720-4724.

22. Erskine MS, Lehmann ML, Cameron NM, Polston EK: **Co-regulation of female sexual behavior and pregnancy induction: an exploratory synthesis.** *Behav Brain Res* 2004, **153:** 295-315.

23. Cameron N, Del Corpo A, Diorio J, McAllister K, Sharma S, Meaney MJ: **Maternal programming of sexual behavior and hypothalamic-pituitary-gonadal function in the female rat**. *PLoS One* 2008, **21**:e2210.

24. Levine JE, Chappell LM, Bauer-Dantoin AC, Besecke LM, Conaghan LA, Legan SJ, Meredith JM, Strobl FJ, Urban JH, Vogelsong KM, Wolfe AM: **Neuroendocrine regulation of luteinizing hormone pulse generator in the rat**. *Recent Prog Horm Res* 1991, **47:** 97-153.

25. Herbison AE: **Multimodal influence of estrogen upon gonadotropin releasing hormone neurons**. *Endocrinol Rev* 1998, **19:** 302-330.

26. Mahesh VB, Brann DW: **Regulation of the preovulatory gonadotropin surge by endogenous steroids**. *Steroids* 1998, **63:** 616-629.

27. Mardones J, Segovia-Riquelme N: **Thirty-two years of selection of rats by ethanol preference: UChA and UChB strains.** *Neurobehav Toxicol Teratol* 1983, **5:**171-178.

28. Rosenblatt JS: **Nonhormonal basis of maternal behavior in the rat**. *Science* 1967, **16:**1512-1514.

29. Fleming AS, Rosenblatt JS: **Maternal behavior in the virgin and lactating rat**. *J Comp Physiol Psychol* 1974, **86**:957-972.

30. Myers MM, Brunelli SA, Squire JM, Shindeldecker RD, Hofer MA: **Maternal behavior of SHR rats and its relationship to offspring blood pressures**. *Dev Psychobiol* 1989, **22**:29-53.

31. Pryce CR, Bettschen D, Feldon J: **Comparison of the effects of early handling and early deprivation on maternal care in the rat**. *Dev Psychobiol* 2001, **38:** 239 -251.

32. Marcondes FK, Bianchi FJ, Tanno AP: **Determination of the estrous cycle phases of rats: some helpful considerations**. *Braz J Biol* 2002, **62:** 609-614.

33. Plowchalk DR, Smith BJ, Mattison DR: **Assessment of toxicity to the ovary using follicle quantitation and morphometrics.** *Methods Toxicol* 1993, **3:**57-68.

34. Cameron NM, Champagne FA, Parent C, Fish EW, Ozaki-Kuroda K, Meaney MJ: **The programming of individual differences in defensive responses and reproductive strategies in the rat through variations in maternal care.** *Neurosci Biobehav Rev* 2005, **29:** 843-865.

35. Cameron NM, Fish EW, Meaney MJ: **Maternal influences on the sexual behavior and reproductive success of the female rat**. *Horm Behav* 2008, **54:** 178-184.

36. Cameron NM, Soehngen E, Meaney MJ: **Variation in maternal care influences ventromedial hypothalamus activation in the rat**. *J Neuroendocrinol* 2011, **23**:393-400.

37. Caldji C, Tannenbaum B, Sharm S, Francis D, Plotsky PM, Meaney MJ: **Maternal care during infancy regulates the development of neural systems mediating the expression of fearfulness in the rat**. *Proc Natl Acad Sci USA* 1998, **95**: 5335-5340.

38. Meaney MJ, Szyf M: **Environmental programming of stress responses through DNA methylation: life at the interface between a dynamic environment and a fixed genome.** *Dialogues Clin Neurosci* 2005, **7:**103-123.

39. Rosenblatt JS, Mayer AD, Giordano AL: **Hormonal basis during pregnancy for the onset of maternal behaviour in the rat.** *Psychoneuroendocrinology* 1988, **13**: 29-46.

40. Casolini P, Domenici MR, Cinque C, Alema GS, Chiodi V, Galluzzo M, Musumeci M, Mairesse J, Zuena AR, Matteucci P, Marano G, Maccari S, Nicoletti F, Catalani A: **Maternal exposure to low levels of corticosterone during lactation protects the adult offspring against ischemic brain damage.** *J Neurosci* 2007, **27:** 7041–7046.

41. Graham MD, Rees SL, Steiner M, Fleming AS: **The effects of adrenalectomy and corticosterone replacement on maternal memory in postpartum rats.** *Horm Behav* 2006, **49:** 353-361.

42. Catalani A, Casolini P, Scaccianoce S, Patacchioli FR, Spinozzi P, Angelucci L: **Maternal corticosterone during lactation permanently affects brain corticosteroid receptors, stress response and behaviour in rat progeny.** *Neuroscience* 2000, **100:** 319-325.

43. Leret ML, Peinado V, Suarez LM, Tecedor L, Gamallo A, Gonzalez JC: **Role of maternal adrenal glands on the developing serotoninergic and aminoacidergic systems of the postnatal rat brain**. *Int J Dev Neurosci* 2004, **22:** 87-93.

44. Wilcoxon JS, Redei EE: **Maternal glucocorticoid deficit affects hypothalamic–pituitary–adrenal function and behavior of rat offspring**. *Horm Behav* 2007**, 51:**321-327.

45. Lalmansingh AS, Uht RM: **Estradiol regulates corticotropin-releasing hormone gene (crh) expression in a rapid and phasic manner that parallels estrogen receptor-alpha and -beta recruitment to a 3′,5′-cyclic adenosine 5′-monophosphate regulatory region of the proximal crh promoter**. *Endocrinology* 2008, **149:** 346-357.

46. Miller WJ, Suzuki S, Miller LK, Handa R, Uht RM: **Estrogen receptor (ER) beta isoforms rather than ERalpha regulate corticotropin-releasing hormone promoter activity through an alternate pathway**. *J Neurosci* 2004, **24:**10628–10635.

47. Yen SSC, Lein A: **The apparent paradox of the negative and positive feedback control system on gonadotropin secretion.** *Am J Obstet Gynecol* 1976, **126:** 942-954.

48. Shivers BD, Harlan RE, Morrell JI, Pfaff DW: **Absence of estradiol concentration in cell nuclei of LHRH-immunoreactive neurons**. *Nature* 1983, **304:** 345-347.

49. Zhao L, Watanabe M, Yano T, Yanagisawa J, Nakagawa S, Oishi H, Wada-Hiraike O, Oda K, Minaguchi T, Yasugi T, Kato S, Taketani Y: **Analysis of the status of the novel estrogen receptor α (ERα) coactivator p72 in endometrial cancer and its cross talk with erbB-2 in the transactivation of ERα**. *Mol Med Report* 2008, 1:387-390.

50. Pelletier G, Labrie C, Labrie F: **Localization of oestrogen receptor alfa, oestrogen beta receptor and androgen receptors in the rat reproductive organs**. *J Endocrinol* 2000, **165:** 359-370.

51. Champagne FA, Weaver ICG, Diorio J, Dymov S, Szyf M, Meaney JM: **Maternal care associated with methylation of the oestrogen receptoralpha1b promoter and oestrogen receptor-alpha expression in the medial preoptic area of female offspring**. *Endocrinology* 2006, **147**: 2909-2915.

52. Chuffa LG, Padovani CR, Martinez FE: **Ovarian structure and hormonal status of the UChA and UChB adult rats in response to ethanol**. *Maturitas* 2009, **62**: 21-29.

53. Couse JF, Yates MM, Walker VR, Korach KS: **Characterization of the hypothalamic-pituitary-gonadal axis in estrogen receptor (ER) Null mice reveals hypergonadism and endocrine sex reversal in females lacking ERα but not ERβ.** *Mol Endocrinol* 2003, **17:**1039-1053.

54. Strauss JF, Hsueh AJW: **Ovarian hormone synthesis**. *Endocrinology,* Volume **3.** *Fourth Edition*. Edited by DeGroot LJ, Jameson JL. Philadelphia: Saunders Company; 2001: 2043-2052.

55. Liu J, Hu P, Qi XR, Meng FT, Kalsbeek A, Zhou JN: **Acute restraint stress increases intrahypothalamic oestradiol concentrations in conjunction with increased hypothalamic oestrogen receptor β and aromatase mRNA expression in female rats.** *J Neuroendocrinol* 2011, **23:** 435-443.

56. Sen A, Hammes SR. **Granulosa cell-specific androgen receptors are critical regulators of ovarian development and function.** *Mol Endocrinol* 2010, **24:**1393-1403.

57. Nielsen ME, Rasmussen IA, Kristensen SG, Christensen ST, MØllgard K, Wreford Andersen E, Byskov AG, Yding Andersen C: **In human granulosa cells from small antral follicles, androgen receptor mRNA and androgen levels in follicular fluid correlate with FSH receptor mRNA**. *Mol Hum Reprod* 2011, **17:** 63-70.

58. Weil SJ, Vendola K, Zhou J, Adesanya OO, Wang J, Okafor J, Bondy CA. **Androgen receptor gene expression in the primate ovary: cellular localization, regulation, and functional correlations**. *J Clin Endocrinol Metab*1998, **83:**2479-2485.

59. Kolibianakis EM, Papanikolaou EG, Fatemi HM, Devroey P: **Estrogen and folliculogenesis: is one necessary for the other?** *Curr Opin Obstet Gynecol* 2005, **17:** 249-253.

60. Robker RL, Richards JS: **Hormone-induced proliferation and differentiation of granulosa cells: A coordinated balance of the cell cycle regulators cyclin D2 and p27^{kip1}**. *Mol Endocrinol* 1998, **12:**924-940.

61. Brummelte S, Galea LA: **Chronic corticosterone during pregnancy and postpartum affects maternal care, cell proliferation and depressive-like behavior in the dam.** *Horm Behav* 2010, **58**: 769-79.

Tables

Table 1. Frequency of maternal behaviors during the first 10 days postpartum in UCh lactating females (n=12/group).

Behaviors	**UChA Mothers**	**UChB Mothers**
Carrying	11.21±1.83	4.97±0.70**
Licking/Grooming (L/G)	1.99±0.24	1.01±0.08*
Arched-Back Nursing and L/G	2.30±0.19	0.98±0.09***
Arched-Back Nursing	8.38±0.17	5.05±0.40***
Passive Nursing	1.28±0.27	1.50±0.22
Contact with the pups	3.67±0.34	3.17±0.47

*****p<0.005; **p<0.001; ***p<0.0001. Mann-Whitney test. Values are expressed as means ± SEM.

Table 2. Duration of estrous cycles in the UCh female offspring (n=10/group).

Parameters	**UChA Offspring**	**UChB Offspring**
Estrous cycle duration (days)	4.00±0.10	5.86±0.30*
Frequency in proestrus (days)	5.30±0.15	3.63±0.15*
Frequency in estrus (days)	5.50±0.16	4.83±0.56
Frequency in metestrus (days)	0.70±0.26	3.36±0.43*
Frequency in diestrus (days)	9.50±0.16	9.18±0.67

*$p<0.05$. Student's *t*-test. Values are expressed as means ± SEM.

Table 3. Body and ovary weights. Frequency and cell proliferation index of the ovarian follicles in the UCh offspring (n=10/group).

Parameters	**UChA Offspring**	**UChB Offspring**
Body weight (g)	233.50±3.16	240.0±8.26*
Absolute ovary weight (g)	0.059±0.001	0.074±0.004*
Relative ovary weight (g/100 g)	0.025±0.001	0.030±0.001*
Primordial follicle	76 (59 - 113)	108 (84 - 133)*
Primary follicle	39 (14 - 70)	21.5 (15 - 27) *
Ki67 index (%)	30 (20-36)	50(34-75) *
Growth follicle	89 (49 - 106)	63.5 (46 - 73) *
Ki67 index (%)	40 (20-48)	41 (31-58)
Preantral follicle	57 (28 - 74)	53.5 (41 - 59)
Ki67 index (%)	41 (12-53)	64 (42-90) *
Antral follicle	58.5 (47 - 74)	72.5 (59 - 86)*
Ki67 index (%)	2.4 (2.1-3.5)	8.6 (7.3-9.8) *
Mature follicle	4.5 (4 - 7)	6 (2 - 11)
Ki67 index (%)	3.7 (1.6-10)	15.5 (9.09-21.87) *

Body weight data are expressed as means ± SEM. Values of frequency and cell proliferation index are expressed as median (Q1 - Q3). Student's *t*-test; *$p<0.05$.

Figure 1. Frequency of maternal care: low care (UChB mothers) and high care (UChA mothers). (A) frequency of carrying; (B) frequency of licking/grooming (L/G); (C) frequency of arched-back nursing and L/G; (D) frequency of arched-back nursing; (E) frequency of passive nursing and (F) frequency of contact with pups. Values are expressed as means ± SEM. UChA and UChB rats (n=12/group). Two Way Repeated Measures ANOVA; *$p < 0.05$.

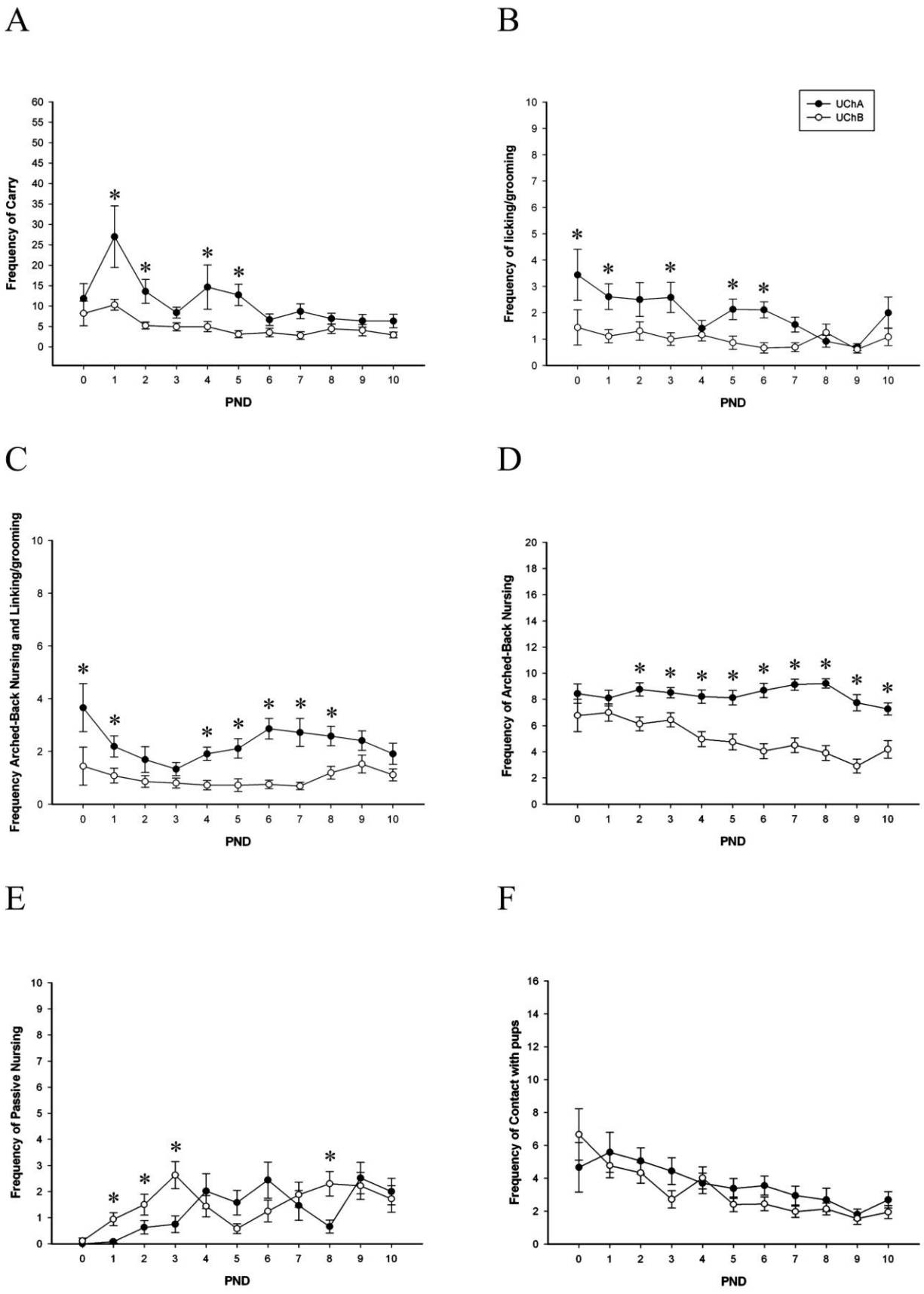
A
B
C
D
E
F
Frequency of Carry
Frequency of licking/grooming
Frequency Arched-Back Nursing and Linking/grooming
Frequency of Arched-Back Nursing
Frequency of Passive Nursing
Frequency of Contact with pups
PND
UChA
UChB

FIGURE 1

Figure 2. Hormonal profile of UCh mothers and offspring after variation of maternal care in the postpartum period. (A) Plasma corticosterone levels in the UCh mothers (ng/mL); (B) Plasma corticosterone levels in the UCh offspring (ng/mL); (C) Plasma FSH levels in the UCh offspring (ng/mL); (D) Plasma LH levels in the UCh offspring (ng/mL); (E) Plasma E2 levels in the UCh offspring (pg/mL) and (F) Plasma P4 levels in the UCh offspring (ng/mL). Values are expressed as means ± SEM. n=10 animals/group. Student's *t*-test; * $p < 0.05$. (PND) Postpartum day.

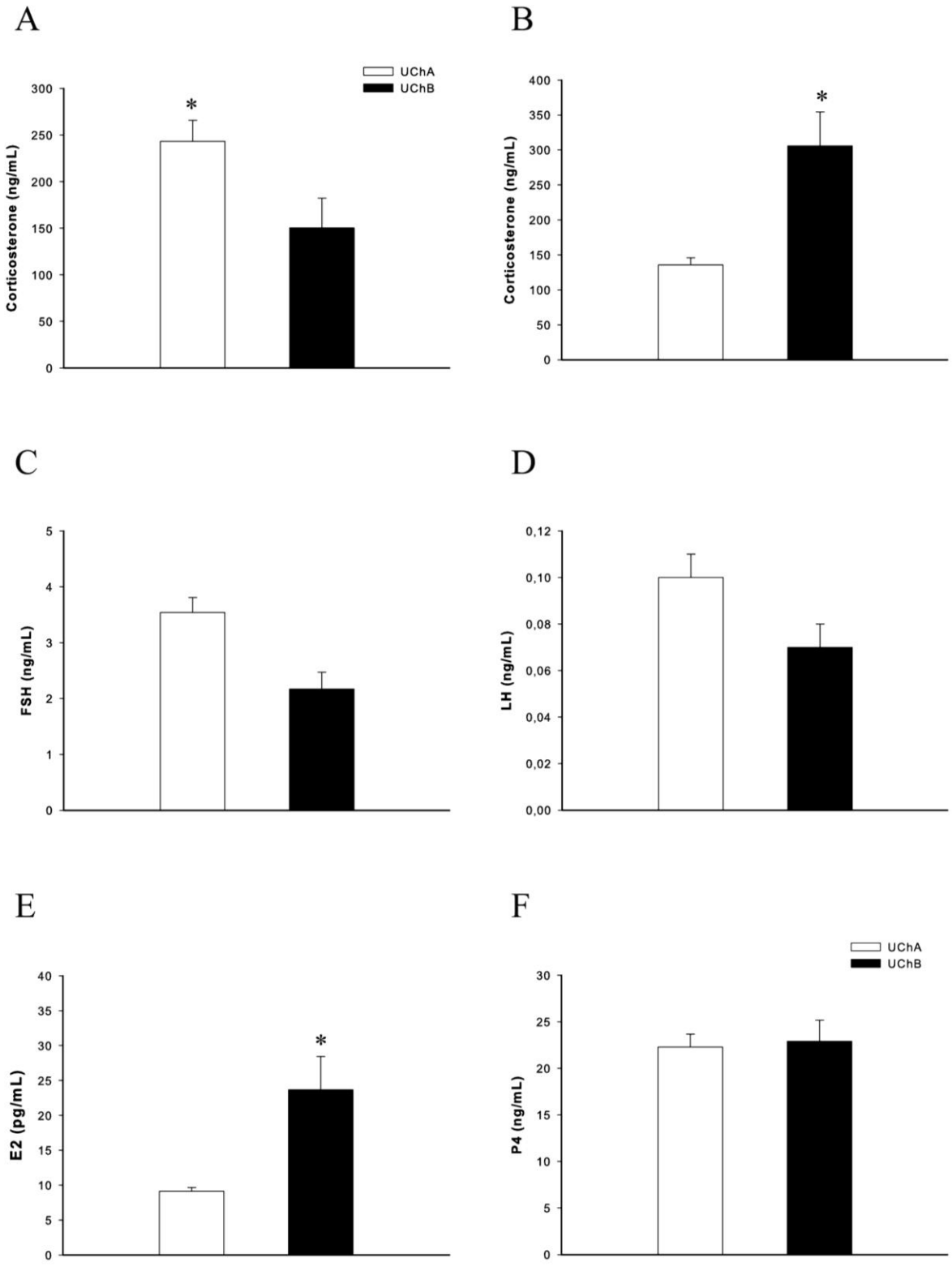
A
B
C
D
E
F
UChA
UChB
Corticosterone (ng/mL)
FSH (ng/mL)
LH (ng/mL)
E2 (pg/mL)
P4 (ng/mL)
*

FIGURE 2

Figure 3. Analysis of ovarian receptors. (A) Representative western blotting analysis of androgen receptor (AR), estrogen receptor subunits (ER-α and ER-β) in rat ovaries after variation of maternal care. Indicated concentrations of each total protein (70 µg extracted from a pool of 6 organs/group) were used to detect specific protein expression levels in the blots (upper panel). (B) Densitometry values for AR, ER-α and ER-β levels were studied following normalization to the housekeeping gene β-actin. All results are expressed as means ± SEM (n=6 animals/group). Student's *t*-test; * $p < 0.05$.

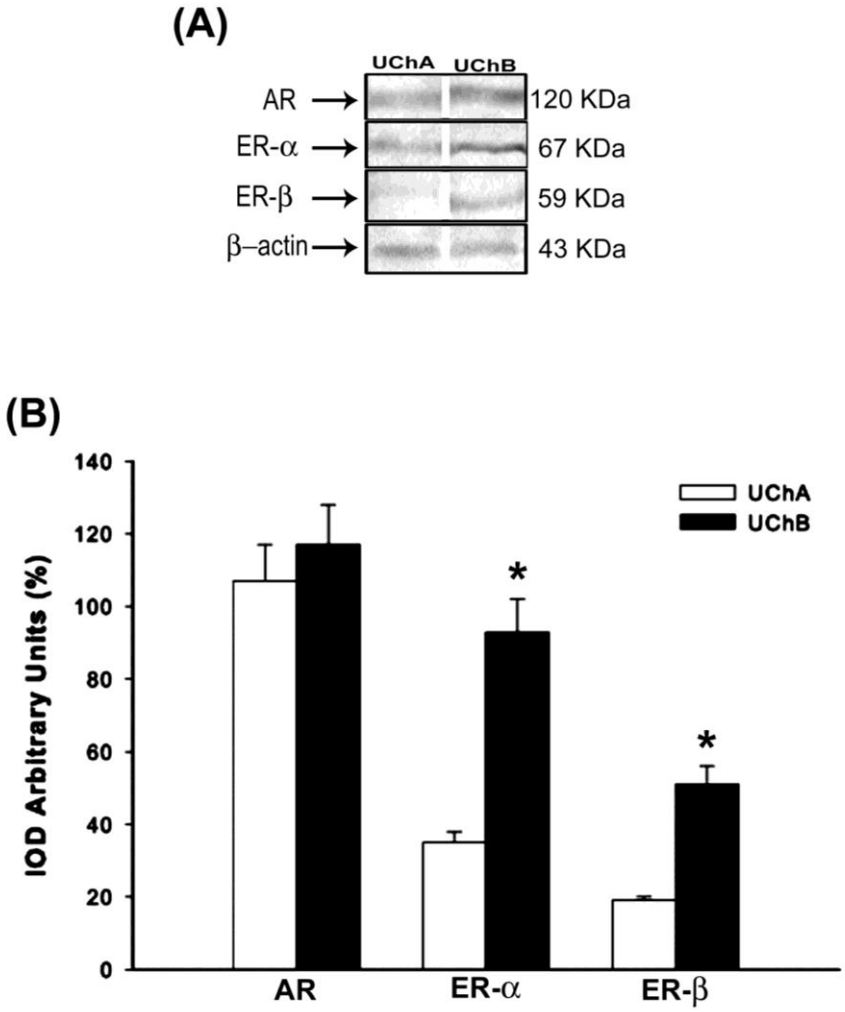
(A)
UChA
UChB
AR
ER-α
ER-β
β–actin
120 KDa
67 KDa
59 KDa
43 KDa
(B)
IOD Arbitrary Units (%)
140
120
100
80
60
40
20
0
UChA
UChB
*
*
AR
ER-α
ER-β

FIGURE 3

Figure 4. Immunostaining for Ki-67 in different stages of ovarian follicular development in UCh offspring. (A, B) primary follicle; (C, D) growth follicle; (E, F) preantral follicle and (G, H) antral follicle. Arrows show the positive nuclei for Ki-67.

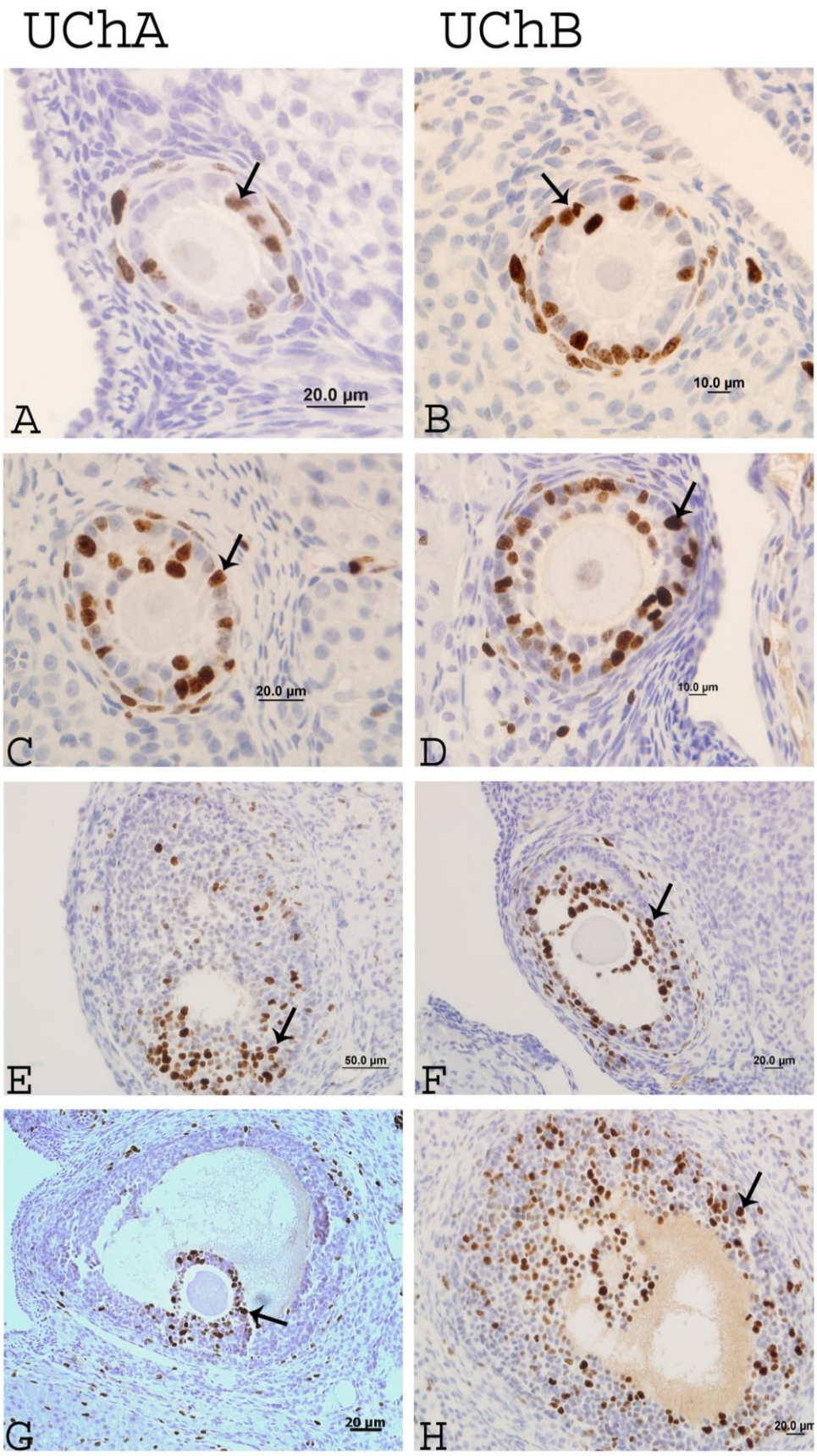
UChA
UChB
A
20.0 µm
B
10.0 µm
C
20.0 µm
D
10.0 µm
E
50.0 µm
F
20.0 µm
G
20 µm
H
20.0 µm

FIGURE 4

Conclusões Finais

Concluímos que a variedade de ratas UChB, apresenta acentuada variação do comportamento materno, sendo classificada como mãe pouco cuidadosa e essa variação do cuidado materno afeta diretamente o desenvolvimento físico, a instalação da puberdade, os níveis hormonais, desregula o ciclo estral e a foliculogênese e regula diferencialmente os níveis protéicos de receptores ER-α e ER-β nos ovários de ratas adultas.

Referências da Introdução Geral

AFONSO, V.M.; KING, S.; CHATTERJEE, D.; FLEMING, A. S. Hormones that increase maternal responsiveness affect accumbal dopaminergic responses to pup- and food-stimuli in the female rat. Horm Behav., v. 54, p.178-184, 2009.

ALBERT, D. J.; WALSH, M.H. Agression in the lacting female rat: the declive is not dependent on the physical development of the pups. Physiol Behav., v.58, p.477-481, 1995.

BIRKE, L.I.A.; SADLER, D. Differences in maternal behavior of rats and the sociosexual development of the offspring. Dev Phychobiol., v. 20, p.85-99, 1987.

BREDY, T.W.; GRANT, R.J.; CHAMPAGNE, D.L.; MEANEY, M.J. Maternal care influences neuronal survival in the hippocampus of the rat. Eur J Neurosci., v. 18, p.2903-2909, 2003.

BRUDENELL, I. Parenting an infant during alcohol recovery. J. Pediatric. Nurs., v.15, p.82-88, 2000.

CALDJI, C.; FRANCIS, D.D.; SHARA, S.; PLOTSKY, P.M.; MEANEY, M. J. The effects of early reating environment on the development of GABA and central benzodiazepine receptor levels and novelty-induced fearfulness in the rat. Neuropsychopharmacology, v,22, p.219-229, 2000.

CALDJI, C.; TANNENBAUM, B.; SHARM, S.; FRANCIS, D.; PLOTSKY, P.M.; MEANEY, M.J. Maternal care during infancy regulates the development of neural systems mediating the expression of fearfulness in the rat. Proc Natl Acad Sci USA., v. 95, p.5335-5340, 1998.

CAMERON, N.M.; CHAMPAGNE, F.A.; PARENT, C.; FISH, E.W.; OZAKI-KURODA, K.; MEANEY, M.J. The programming of individual differences in defensive responses and reproductive strategies in the rat through variations in maternal care. Animal Models Depression antidepressant Activity. Neurosci Rev., v.29, p. 843-865, 2005.

CAMERON, N.M.; FISH, E.W.; MEANEY, M.J. Maternal influences on the sexual and reproductive success of the female rat. Horm Behav., v. 54, p. 178-184, 2008.

CÂNDIDO, E.M.; CARVALHO, C.A.; MARTINEZ, F.E.; CAGNON, V.H. Experimental alcoholism and pathogenesis of prostatic diseases in UChB rats. Cell Biol Int., v.31, p.459-472, 2007.

CHAMPAGNE, F.A. Epigenetic mechanisms and the transgenerational effects of maternal care. Front Neuroendocrinol., v.29, p.386-397, 2008.

CHAMPAGNE, F.A.; FRANCIS, D.D.; MAR, A.; MEANEY, M.J. Variations in maternal care in the rat as a mediating influence for the effects of environment on development. Physiol Behav., v.79, p.359-371, 2003a.

CHAMPAGNE, F.A.; WEAVER, I.C.G.; DIORIO, J.; SHARMA, S.; MEANEY, M.J. Natural variations in maternal care are associated with estrogen receptor alpha expression and estrogen sensitivity in the medial preoptic area. Endocrinology, v.144, p.4720-4724, 2003b.

CHAMPAGNE, F.A.;WEAVER, I.C.G.; DIORIO, J.; SHARMA, S.; MEANEY, M.J. Maternal care associated with methylation of the estrogen receptor-alpha 1b promoter and estrogen receptor-alpha expression in the medial preoptic area of female offspring. Endocrinology, v.147, p.2909-2915, 2006.

CHAMPAGNE, F.; MEANEY, M.J. Like mother, like daughter: evidence for non-genomic transmission of parental behavior and stress responsivisity. Prog Brain Res., v.133, p.287-302, 2001.

CIRULLI, F.; BERRY, A.; ALLEVA, E. Early disruption of the mother-infant relationship: Effects on brain plasticity and implication for psychopathology. Neurosci Biobehav Rev., v. 27, p. 73-82, 2003.

COUTELLIER, L.; FRIEDRICH, A.; FAILING, K.; WURBEL, H. Variations in the postnatal environment in mice: Effects on maternal behavior anf behavioural and endocrine responses in the adult offspring. Physiol Behav., v.93, p.395-407, 2008.

DE BELLIS, M.D. Developamental traumatology: a contributory mechanism for alcohol and substance use desorders. Psychoneuroendocrinology, v.27, p.155-170, 2002.

ERIKSSON, K.; PIKKARAINEN, P.H. Differences between the sexes in voluntary alcohol consumption and liver ADH-activity in inbred strains of mice. Metabolism., v.17, p.1037-1042, 1968.

ERSKINE, M.S.; LEHMANN, M.L.; CAMERON, N.M.; POLSTON, E.K. Co-regulation of female sexual behavior and pregnancy induction: an exploratory synthesis. Behav Brain Res., v. 153, p.295-315, 2004.

FAIRBANKS, L.A.; McGUIRE, M.T. Long-term effects of early mothering behavior on responsiveness to the environment in ver-vet monkeys. Dev Psychobiol., v. 21, p.711-724, 1988.

FRANCIS, D.D.; CHAMPAGNE, F.A.; LIU, D.; MEANEY, M. J. Maternal care, gene expression, and the development of individual differences in stress reactivity. Ann N Y Acad Sci., v.296, p.66-84, 1999a.

FRANCIS, D.D.; DIORIO, J.; LIU, D.; MEANEY, M.J. Nongenomic transmission across generations of maternal behavior and stress responses in the rat. Science, v.286, p.1155-1158, 1999b.

GOMES, C.M.; ANSELMO-FRANCI, J.A.; FRANCI, C.R.; LUCION, A.B.; SANVITTO, G. L. Neonatal handling induces alteration in progesterone secretion after sexual behavior but not in angiotensin II receptor density in the medial amygdale: implications for reproductive success. Life Sci., v.78, p.2897-2871, 2006.

GOMES, C.M.; FRANTZ, P.J.; SANVITTO, G.L.; ANSELMO-FRANCI, J.A.; LUCION, A. B. Neonatal handling induces anovulatory estrous cycles in rats. Braz J Med Biol Res., v. 32, p.1239-1242, 1999.

GOMES, C.M.; RAINEKI, C.; DE PAULA, P.R.; SEVERINO, G.S.; HELENA, C.V.; ANSELMO-FRANCI, J.A.; FRANCI, C. R.; SANVITTO, G. L.; LUCION, A. B. Neonatal handling and reproductive function in female rats. J Endocrinol., v. 184, p.435-445, 2005.

GROTA, L.J.; ADER, R. Continuous recording of maternal behaviour in Rattus norvegicus. Anim Behav., v.17, p.722-729, 1969.

JABLONKA, E.; LAMB, M.J. Evolution in Four Dimensions. Genetic, Epigenetic, Behavioral, and Symbolic Variation in the History of Life. Cambridge , MA: MIT Press. 2005.

JAWORSKI, J.N.; FRANCIS, D.D.; BROMMER, C.L.; MORGAN, E.T.; KUHAR, M. J. Effects of early maternal separation on ethanol intake, GABA receptor and metabolizing enzyms in adult rats. Psychopharmacology, v. 181, p.8-15, 2005.

KITTRELL, E.M.W.; SATINOFF, E. diurnal rhythms of body temperature, drinking and activity over reproductive cycles. Physiol Behav., v.42, p.477-484, 1988.

LADD, C.O.; HUOT, R.L.; THRIVIKRAMAN, K.V.; NEMEROFF, C.B.; Meaney, M.J.; PLOTSKY, P.M. Long-term behavioral and neuroendocrine adaptations to adverse early experience. Prog Brain Res., v.122, p.81-103, 2000.

LI T.K.; LUMENG, L.; MCBRIDE, W.J.; MURPHY, J.M. Rodent lines selected for factors affecting alcohol consumption. Alcohol Alcohol Suppl., v.1, p.91-96, 1987.

LIEBERMAN, D. Z. Children of alcoholics: an update. Curr Opin Pediatr., v. 12, p. 336-340, 2000.

LIU, D.; DIORIO, J.; DAY, J. C.; FRANCIS, D.; MEANEY, M.J. Maternal care, hippocampus synaptogenesis and cognitive development in rats. Nat Neurosci., v. 3, p.799-806, 2000.

LIU, D.; DIORO, J.; TANNENBAUM, B.; CALDJI, C.; FRANCIS, D.; FREEDMAN, A.; SHAEMA, S.; PEARSON, D.; PLOTSKY, P.M.; MEANEY, M.J. Maternal care, hippcampal glucocorticoid receptores, and hypothalamic-pituitary-adrenal response to stress. Science, v. 277, p.1659-1662, 1997.

LUCION, A.B.; PEREIRA, F.M.; WINKELMAN, E.C.; SANVITTO, G.L.; ANSELMO-FRANCI, J.A. Neonatal handling reduces the number of cells in the locus coeruleus of rats. Behav Neurosci., v. 117, p.894-903, 2003.

MACRÍ, S.; MASON, G.; WURBEL, H. Dissocioation in the effects of neonatal maternal separations on maternal care and the offspring's HPA and fear responses in rats. Eur J Neurosci., v.20, p.1017-1024, 2004.

MARDONES, J.; SEGOVIA-RIQUELME, N. Thirty-two years of selection of rats by ethanol preference: UChA and UChB strains. Neurobehav Toxicol Teratol., v.5, p.171-178, 1983.

MARTINEZ, F.E.; LAURA, I.A.; MARTINEZ, M.; PADOVANI, C.R.; BUSTOS-OBREGÓN, E. Morphology of the ventral lobe of the prostate and seminal vesicles in an ethanol-drinking strain of rats (UChA and UChB). J Submicrosc Cytol Pathol., v.33, p.99-106, 2001.

MARTINEZ, M.; MARTINEZ, F.E.; DA CUNHA, M.R.; SEGATELLI, T.M.; PINHEIRO, P.F.; ALMEIDA, C. Morphological effects on the hard palatine mucosa of Calomys callosus submitted to experimental chronic alcoholism. J Submicrosc Cytol Pathol., v.34, p.77-83, 2002.

MICHEL, G.F.; TYLER, A.N. Can Knowledge of developmental processes illuminate the evolution of parental care? Dev Psychobiol., v.49, p.33-44, 2007.

MOFFETT, M.; HARLEY, J.; FRANCIS, D.; SANGHANI, S.; DAVIS, W.; KUHAR, M. Maternal separation and handling effects cocaine self-administration in both the treated pups as adults and the dams. J Pharmacol Exp Ther., v. 317, p. 1210-1218, 2006.

MOORE, C.L. Sex differences in urinary odors produced by young laboratory rats (Rattus novergicus). J Comp Psychol., v.99, p.336-341, 1985.

MOORE, C.L.; POWER, K.L. Variations in maternal care and individual differences in play, exploration and grooming of juvenile Norway rat offspring. Dev Psychobiol., v.25, p.165-182, 1992.

NELSON, R.J. Parental behavior. In: An introduction to Behavioral Endocrinology. Sinauer Associates, Inc. 1995.

PEDERSEN, C.A.; BOCCIA, M.L. Oxytocin antagonism alters rat dams' oral grooming and upright posturing over pups. Physiol Behav., v.80, p.233-241, 2003.

PLOTSKY, P.M.; MEANEY, M.J. Early, postnatal experience alterns hypothalamic-corticotrophin releasing factor (CRF) mRNA, median eminence CRF sontents and stress-induced release in adults rats. Brain Res Mol Brain Res., v.18, p. 195-200, 1993.

POLAN, H.J.; HOFER, M.A. Maternal directed orienting behaviors of newborn rats. Dev Psychobiol., v.34, p.269-279, 1999.

PRYCE, C.R.; BETTSCHEN, D.; FELDON, J. Comparison of the effects of early handling and early deprivation on maternal care in the rat. Dev Psychobiol., v.38(4), p. 239 – 251, 2001.

PRYCE, C.R.; FELDON, J. Long-Term neurobehavioral impact of the postnatal environment in rats: manipulations, effects and mediating mechanisms. Neurosci Biobehav Rev., v.27, p.57-71, 2003.

REISBICK, S.; ROSENBLATT, J.S.; MAYER, A.D. Decline of maternal behavior in the virgin lactating rat. J Comp Physiol Psychol., v.89, p.722-732, 1975.

ROMAN, E.; PLOJ, K.; NYLANDER, I. Maternal separation has no effect on voluntary ethanol intake in female Wistar rats. Alcohol, v.33, p.31-39, 2004.

SAVONLAHI, E.; PAJULO, M.; HELENIUS, H.; KORVENRANTA, H.; PIHA, J. Children younger than 4 years and their substance-dependent mothers in the child welfare clinic. Acta. Paediatr., v.93, p.989-995, 2004.

STERN, J.M.; JOHNSON, S.K. Ventral somatosensory determinants of nursing behavior in Norway rats. Effects of variations in quality and quality of pup´s stimuli. Physiol Behav., v.47, p.993-1011, 1990.

TAMPIER, L.; QUINTANILLA, M.E.; MARDONES, J. Acute tolerance, alcohol sensitivity and drinking pattern in the F2 generation of UChA and UChB rats. J Stud Alcohol., v.61, p.647-651, 2000.

TODESCHINI, S.A. Efeitos da manipulação e da separação dos filhotes no período neonatal sobre o comportamento da mãe. Dissertação de mestrado apresentada a Universidade Federal do Rio Grande do Sul. Porto Alegre, 2002.

URIARTE, R.; BREIGEIRON, M.K.; BENETTI, F.; ROSA, X.F.; LUCION, A.B. Effects of maternal care on the development, emotionality, and reproductive function in male and female rats. Dev Psychobiol., v.49, p.451-462, 2007.

VAN OERS, H.J.J.; DE KLOET, E.R.; WHELAN, T.; LEVINE, S. Maternal deprivation effect on the infant´s neural stress markers is reversed by tactile stimulation and feeding but not by suppressing corticosterona. J Neurosci., v.18, p.10171-10176, 1998.

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha **Tese de Doutorado** intitulada **"Caracterização do comportamento materno e suas implicações no desenvolvimento físico, na função reprodutiva e no perfil hormonal da prole feminina de ratas UChA e UChB (consumidoras voluntárias de etanol a 10%)"**.

( ) não se enquadra no § 3º do Artigo 1º da Informação CCPG 01/08, referente a bioética e biossegurança.

Tem autorização da(s) seguinte(s) Comissão(ões):

( ) CIBio - Comissão Interna de Biossegurança , projeto nº _____, Instituição: _____.

(**X**) CEUA - Comissão de Ética no Uso de Animais , projeto nº. **01/08**, Instituição: COMISSÃO DE ÉTICA NA EXPERIMENTAÇÃO ANIMAL (CEEA) – IBB/UNESP.

( ) CEP - Comissão de Ética em Pesquisa, protocolo nº _____, Instituição: _____.

** Caso a Comissão seja externa ao IB/UNICAMP, anexar o comprovante de autorização dada ao trabalho. Se a autorização não tiver sido dada diretamente ao trabalho de tese ou dissertação, deverá ser anexado também um comprovante do vínculo do trabalho do aluno com o que constar no documento de autorização apresentado.*

Aluno: João Paulo de Arruda Amorim

Orientador: Prof. Dr. Francisco Eduardo Martinez

Para uso da Comissão ou Comitê pertinente:
(X) Deferido ( ) Indeferido

Carimbo e assinatura

Profa. Dra. ANA MARIA APARECIDA GUARALDO
Presidente da Comissão de Ética no Uso de Animais
CEUA/UNICAMP

Para uso da Comissão ou Comitê pertinente:
( ) Deferido ( ) Indeferido

Carimbo e assinatura

unesp UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

CERTIFICADO

Certificamos que o Protocolo nº **01/08-CEEA**, sobre "**Caracterização do comportamento materno e suas implicações no desenvolvimento físico, sexual inicial, reprodutivo e hormonal da prole feminina de ratas UChA e UChB (consumidoras voluntárias de etanol a 10%)**", sob a responsabilidade de **FRANCISCO EDUARDO MARTINEZ**, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado pela ***COMISSÃO DE ÉTICA NA EXPERIMENTAÇÃO ANIMAL*** (CEEA), em reunião de **07/03/2008**.

Botucatu, 7 de março de 2008.

Prof. Dr. MARCELO RAZERA BARUFFI
Presidente - CEEA

NADIA JOVÊNCIO COTRIM
Secretária – CEEA

Instituto de Biociências – Diretoria Técnica Acadêmica
Distrito de Rubião Júnior s/n CEP 18618-000 Botucatu SP Brasil
Tel 14 3811 6013/6014 fax 14 3815 3744 e-mail: dta@ibb.unesp.br