Universidade Federal de Pernambuco
Centro de Ciências da Saúde
Departamento de Nutrição
Programa de Pós-Graduação em Nutrição
Doutorado em Nutrição

Tereza Cristina Bomfim de Jesus Deiró

DESENVOLVIMENTO SOMÁTICO E SENSÓRIO-MOTOR E COMPORTAMENTO ALIMENTAR EM RATOS NEONATOS: EFEITOS DO TRATAMENTO COM AGENTES SEROTONINÉRGICOS DURANTE O PERÍODO DE CRESCIMENTO RÁPIDO DO ENCÉFALO

Recife – 2004

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Tese apresentada para obtenção do título de Doutor em Nutrição do Programa de Pós-Graduação em Nutrição da Universidade Federal de Pernambuco.

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RESUMO

O presente trabalho se baseia em possíveis efeitos da manipulação serotoninérgica, após a administração de inibidores da recaptação da serotonina – SSRIs. O trabalho apresenta como objetivo geral, investigar a maturação somática, o desenvolvimento sensório-motor e o comportamento alimentar neonatal, em ratos Wistar tratados ou não com inibidores seletivos da recaptação da serotonina – SSRIs. De acordo com o objetivo geral, algumas hipóteses foram levantadas: 1- Tratamento neonatal com SSRIs citalopram, fluoxetina e sertralina, em diferentes doses retarda o crescimento somático, através das medidas do peso corporal, das medidas dos eixos craniais e da maturação de características físicas, bem como provoca retardos no desenvolvimento sensório motor. 2- O peso corporal, a ingestão alimentar dos filhotes e o comportamento alimentar neonatal sofrem atrasos após administração de fluoxetina -SSRI em animais neonatos, em diferentes doses. Durante o desenvolvimento deste estudo, as hipóteses foram testadas e os resultados obtidos. Tais resultados estão apresentados em artigos aceitos ou submetidos para publicação e conduziram às seguintes conclusões: De maneira geral, o tratamento com citalopram, fluoxetina e sertralina, em particular nas maiores doses, provocou significantes retardos no ganho de peso corporal e nos eixos craniais, modificando a estrutura craniofacial até o final do experimento e provocou retardo na maturação da maioria das características físicas. Outrossim, observou-se retardo na maioria dos reflexos estudados. O tratamento neonatal com fluoxetina em diferentes doses retardou o ganho de peso corporal e reduziu a ingestão alimentar nos filhotes, em todas as doses utilizadas. Os comportamentos de interação mãe-filho e o comportamento de limpeza tiveram a freqüência aumentada nos animais tratados na maior dose. Os resultados demonstrados neste trabalho tornam evidente que tratamento crônico com SSRIs no período de rápido crescimento do encéfalo, de alguma maneira causa danos ao crescimento e ao desenvolvimento de animais, alterando ainda respostas comportamentais. É, portanto sugestiva a participação do sistema serotoninérgico em mecanismos fisiológicos que determinam a maturação de determinadas funções neurais e o crescimento de tecidos não neurais no inicio da vida.

Palavras-chave: Crescimento. Citalopram. Fluoxetina. Sertralina. Serotonina.

ABSTRACT

The present work if base on a possible effect of the serotoninergic manipulation, after administration of the selective serotonin reuptake inhibitor - SSRIs. The work presents as general objective, to investigate the somatic maturation, the development sensory- motor and the neonatal feeding behavior, in Wistar rats that had a treated or not with selective serotonin reuptake inhibitor. According the general objective, some hypotheses had been raised: 1-Neonatal treatment with SSRIs citalopram, fluoxetine and sertraline, in different doses delays the somatic growth, through the measures of the body weight, the measures of the cranial axis and the physical features maturation , as well as provokes retards in the sensory- motor development. 2- The body weight, the pups ingestion and the neonatal feeding behavior, suffer to delays after administration from fluoxetine in neonates rats, in different doses. During the development of this study, the hypotheses had been tested and the results was presented in this study. Such results are presented in accepted or submitted articles for publication and had lead to the following conclusions: In general way, the treatment with citalopram, fluoxetine and sertraline, in particular in the higgest doses, provoked significant retards in the body weight and the cranial axis, modifying the craniofacial structure until the finished of experiment and also provoked retardation in the maturation of the majority of the physical characteristics. Also, was observed retard in the majority of the reflex studied The neonatal treatment with fluoxetine in different doses delayed the body weight and reduced the pups ingestion in all the used doses. The interaction of the mother-pups and the grooming had the frequency increased in the animals, treated in the highest dose. The results demonstrated in this work become evident that, chronic treatment with SSRIs in the period of brain growth spurt of in some way cause damages to the growth and the development of animals, modifying still mannering answers. it is, therefore, suggestive the participation of the serotoninergic system in physiological mechanisms that determine the maturation of definitive neural functions and the no neural tissues growth in the early in life.

Keywords: Development. Growth. Citalopram. Fluoxetine. Sertraline. Food behavior. Neonate. Serotonin.

LISTA DE ABREVIATURAS E SIGLAS

APHA	Antero-posterior head axis
5-HT	5-hidroxitriptamina, serotonina
5,7 – DHT	5,7 dihidroxitriptamina
5-HIAA	Ácido 5-hidroxiindolacético
8-OH-DPAT	8-hydroxy-2-(di-n-propylamino)tetralin
CEEA	Comissão de Ética em Experimentação Animal
Cit	Citalopram
Cit5	Grupo citalopram de 5 mg/kg
Cit10	Grupo citalopram de 10 mg/kg
EP	Erro padrão
Flu	Fluoxetina
Flu1	Grupo fluoxetina de 1 mg/kg
Flu5	Grupo fluoxetina de 5 mg/kg
Flu10	Grupo fluoxetina de 10 mg/kg
ISRS	Inibidor seletivo de recaptação de serotonina
MLHA	Medio-lateral head axis
NaCl	Cloreto de sódio
pCPA	para-clorofenilalanina
SN	Sistema nervoso
SNC	Sistema nervoso central
SSRI = ISRS	Inibidor seletivo de recaptação de serotonina
Sert 5	Grupo sertralina 5 mg/kg
Sert 10	Grupo sertralina 10 mg/kg
Sert 15	Grupo sertralina de 15 mg/kg

SUMÁRIO

1 INTRODUÇÃO

O presente estudo foi realizado no laboratório de Fisiologia da Nutrição Dra. Naide Teodósio do Departamento de Nutrição da Universidade Federal de Pernambuco, tendo como orientador o Professor Raul Manhães de Castro, Doutor em Farmacologia Experimental e neste estudo foram investigados os efeitos da manipulação farmacológica neonatal com três agentes serotoninérgicos, antidepressivos - inibidores da recaptação da serotonina (SSRIs) – sobre o crescimento somático e sensório motor de ratos neonatos, além do estudo de parâmetros do comportamento alimentar neonatal. Este trabalho de pesquisa gerou cinco artigos científicos: O primeiro intitulado **Neonatal administration of citalopram delays somatic maturation in rats,** aceito para publicação como artigo original na revista: Brazilian Journal of Medical and Bilogical Research em maio de 2004. Neste estudo observou-se que o tratamento com citalopram durante o período de aleitamento retarda o ganho de peso corporal, altera o crescimento do crânio e provoca retardo na maturação de características físicas. O segundo manuscrito intitulado**: Neonatal treatment with fluoxetine delays the somatic maturation and reflex ontogeny,** submetido para publicação como artigo original na revista: Pharmacology Biochemistry and Behavior - julho de 2004.

Neste estudo observou-se que o tratamento com fluoxetina durante o período critico de desenvolvimento do SN, retarda a evolução ponderal, a maturação de características físicas, o crescimento corporal, altera o crescimento do crânio, e atrasa a maturação da maioria dos reflexos estudados. O terceiro artigo sob o título: **Sertraline delays the somatic growth and reflex ontogeny in neonate rats,** submetido para publicação como artigo original na revista: Physiology and Behavior julho de 2004. Neste trabalho observou-se que o tratamento com sertralina durante o período crítico de desenvolvimento do cérebro, retarda o crescimento corporal dos animais e o ganho de peso, altera o crescimento da estrutura cranial, retarda a maturação de características físicas e atrasos na maturação de reflexos. O quarto artigo intitulado: **Neonatal treatment with the serotonin selective reuptake inhibitor citalopram, delays reflex ontogeny,** submetido para publicação como artigo original na revista: Archives General Psychiatry - julho de 2004. Este estudo mostrou que o tratamento com citalopram durante o período vulnerável do SN, retarda a evolução ponderal e atrasa a maturação da maioria dos reflexos estudados. E finalmente o quinto artigo intitulado, **Neonatal feeding behavior: effect of the treatment with SSRI fluoxetine, s**ubmetido para publicação como artigo original na revista: Brazilian Journal of Medical and Biological Research - julho de 2004. O tratamento com fluoxetina durante o período de aleitamento retardou a evolução ponderal e reduziu a ingestão alimentar neonatal.

1.1 Crescimento, desenvolvimento e sistema nervoso.

Em mamíferos, de acordo com cada espécie, o crescimento estrutural e o desenvolvimento cumprem as etapas de maturação de forma muito semelhante (Morgane et al 1978; Morgane et al 1993). Em particular, os estudos dos aspectos anatômicos, bioquímicos e fisiológicos desses organismos, contribuem sobremodo para melhor compreender a ontogênese do sistema nervoso (SN). O desenvolvimento morfofuncional adequado da estrutura nervosa é a base para a expressão comportamental nesses indivíduos (Morgane et al., 1993). Evidentemente, dentre os vários fatores necessários, um adequado suprimento de nutrientes é indispensável para manter o crescimento em nível celular bem como, em última instância, as distintas funções inerentes a cada um dos sistemas fisiológicos, inclusive o sistema nervoso (SN) (Morgane, 1993; Wainwright et al, 1999; Perez-Torrero et al, 2001).

O crescimento celular em qualquer sistema, inclusive o sistema nervoso central (SNC), é caracterizado por importantes etapas classicamente conhecidas como fase de hiperplasia, hiperplasia com hipertrofia e fase de hipertrofia respectivamente (Winick, 1972; Morgane et al., 1993). Durante a ontogênese do SNC, tanto no homem como no rato, é particularmente decisiva para a determinação das características morfo-funcionais adultas, a fase que envolve processos de diferenciação neuronal, migração, sinaptogênese, multiplicação glial e mielinização (Morgane et al 1978). Este período de rápido crescimento, assim denominado por Smart J. L. e Dobbing (1971a), se constitui em fase mais sensível às agressões, sendo, portanto considerado como período crítico do desenvolvimento do SN (Winick, 1972; Morgane et al., 1993).

Em humanos, este período coincide com o último trimestre de gestação, podendo se estender até o terceiro ou quarto anos de vida (Morgane et al., 1978). No rato, ocorre nas três primeiras semanas após o nascimento e corresponde ao período de aleitamento (Smart J. L. e Dobbing, 1971b). Neste período, de acordo com a idade, portanto, a grande velocidade com que ocorrem os eventos celulares torna o SN altamente vulnerável às agressões, estas podem alterar o

desenvolvimento normal, afetar a estrutura nervosa e induzir conseqüências imprevisíveis sobre, por exemplo, a ontogênese de reflexos (Otellin et al, 2002).

Eventos seqüenciais apresentados pelo SN durante o desenvolvimento pré e pós-natal, determinam a composição neuroquímica e a estrutura morfofuncional definitivas, presentes no adulto (Morgane et al., 1993; 2002). Como a estrutura do SN não é homogênea, a proliferação celular varia em intensidade, de acordo com a região, o tipo celular e a etapa do desenvolvimento (Winick, 1972; Morgane et al., 1993). No cérebro de ratos, a divisão celular vai até os 21 dias após o nascimento; já no cerebelo, não ultrapassa os 16 ou 17 dias (Morgane et al., 1978).

Os eventos do crescimento e desenvolvimento, até aqui mencionados, são observados em todas as regiões do SN e podem ser modificados por fatores exógenos, tais como alterações nutricionais e manipulações farmacológicas dos sistemas de neurotransmissores. A desnutrição no período perinatal altera a forma dos neurônios (Borba et al., 2000), modifica a excitabilidade cerebral, aumentando a velocidade da "depressão alastrante" cortical (Guedes et al, 1992) e a resposta agressiva em ratos adultos (Barreto Medeiros et al., 2002). Já a manipulação farmacológica neonatal com inibidor seletivo da recaptação da serotonina (SSRI, causa redução da agressividade, altera o tempo de imobilização e as tentativas de fuga em modelo de depressão experimental no adulto e causa retardo na evolução ponderal, sobretudo

durante o período do tratamento (Manhães de Castro et al., 1995; Manhães de Castro et al., 1996; Manhães de Castro et al., 2001; Barreto Medeiros et al., 2004) (Figura 1- fotos de avaliações do crescimento corporal). Ratos submetidos ao uso de etanol durante a vida intra-uterina apresentaram anormalidades específicas que alteraram os componentes do sistema serotoninérgico (Kim et al,1996). Assim, a depender da magnitude, insultos ao SN durante o período rápido de crescimento podem acarretar deficiências permanentes no cérebro e em outros tecidos (Winick, 1972; Morgane et al., 1993).

O desenvolvimento e a plasticidade são determinantes da organização morfofuncional do sistema nervoso. As alterações dessas propriedades podem ser a base de processos fisiopatológicos (envolvendo eventos neuroquímicos, mudanças neuro-estruturais, alterações da excitabilidade neural e alterações do comportamento), encontrados em importantes doenças humanas. Estudos que interpretem a relação entre os distintos eventos do desenvolvimento, constitui-se ferramentas utilizadas para a compreensão dos efeitos das agressões, ocorridas durante a maturação do SN (Morgane et al., 1993).

A neurotransmissão constitui-se na função precípua das células nervosas. Contudo, as moléculas neurotransmissoras têm outras funções, em particular, no desenvolvimento pré (Buznikov et al., 1967; 1993) e pós-natal (Lidov e Molliver, 1982), servindo também como sinais transitórios que modulam a proliferação celular em diversos tecidos. Outrossim, os neurotransmissores (Morgane et al, 2002) são em muitos dos casos, sintetizados a partir de aminoácidos provenientes dos alimentos e que por vezes atuam como o próprio neurotransmissor. Dentre os neurotransmissores cujos precursores são aminoácidos essenciais, está a serotonina. A serotonina tem se revelado uma molécula fundamental na filogênese e no desenvolvimento dos seres vivos (Turlejsky, 1996).

1.2 Crescimento, desenvolvimento e sistema serotoninérgico

A serotonina juntamente com os neurônios que a liberam e todos seus receptores constituem o sistema de neurotransmissão serotoninérgico. A 5-hidroxitriptamina ou 5-HT tem como precursor o aminoácido essencial triptofano que origina a 5HT após duas reaões enzimáticas, cujos os níveis hipotalâmicos quando elevados, aumenta os níveis dessa indolamina nos tecidos e sua liberação, ocorrendo o oposto quando os níveis do triptofano se encontra reduzido (Schaechter e Wurtman,1990), A 5-HT , está presente em várias espécies desde as mais primitivas como os artrópodes moluscos e insetos, até as mais evoluídas como

o homem (Turlejsky, 1996). As inervações serotoninérgicas aparecem muito precocemente, sendo provavelmente as primeiras projeções a longa distancia no cérebro (Lauder e Bloom, 1971). Em ratos, os primeiros neurônios serotoninérgicos aparecem entre 12^{o} e 14^{o} dias da gestação (Lauder e Bloom, 1971). Os núcleos da rafe no tronco cerebral, onde muitos corpos celulares dos neurônios serotoninérgicos estão localizados (Figura 2), projetam axônios para a maioria das estruturas do SNC (Jacobs e Azmitia, 1992). As múltiplas intervenções da 5-HT em nível central no animal adulto teriam, portanto, esse primeiro suporte anatômico.

Estruturas moleculares protéicas denominadas receptores são as responsáveis pelas ações da serotonina em nível celular, estas ações resultam mais precisamente da interação da amina com seus receptores especificos. Estes, apresentam uma heterogeneidade surpreendente e localização pré e pós-sináptica (neste último caso, em neurônios serotoninérgicos ou não). Já foram identificados 14 tipos e subtipos de receptores serotoninérgicos (Manhães de Castro, 1996, Hoyer et al., 1994).

Durante o desenvolvimento do cérebro, a serotonina, atuando em seus múltiplos receptores, é possivelmente um fator neuronal trófico (Hamon e Emerit, 1989). Estimulação da glia, para produção de fatores tróficos, foi observada por Whitaker-Azmitia (1989; Liu e Lauder 1992) em estudos *in vitro*. A regulação serotoninérgica das interações mesenquima-epitélio com importante papel na morfogênese craniofacial foi observada por Shuei et al., (1993).

Em mamíferos, a densidade final e localização de terminais serotoninérgicos são estabelecidas durante a maturação pós-natal do sistema nervoso central que, em ratos, pode durar semanas ou meses (Lidov e Molliver 1982; Azmitia et al., 1983; Wallace e Lauder 1983). No período de desenvolvimento pós-natal, grandes variações na medida das ligações especificas de agonistas dos receptores 5-HT ocorrem em várias regiões cerebrais (Daval et al., 1987). Ratos neonatos apresentam um progressivo aumento dos sítios 5-HT_{1A} desde o período pós-natal até a fase adulta; neste caso, no girus denteado, no hipocampo e no córtex cerebral (Daval et al., 1987).

Altas densidades de receptores serotoninérgicos 5-HT_{1A} são observados no lobo occipital de cérebros de fetos de macacos; estes receptores parecem estar envolvidos com o estímulo à proliferação de neurônios corticais durante o período de crescimento nestes animais (Lidow et Rakic, 1995). Assim, parecem atuar como sinalizadores do desenvolvimento de diversas estruturas do sistema nervoso, particularmente aquelas associadas a funções nas quais a serotonina desempenha um papel como neurotransmissor ou neuromodulador, como é o caso do comportamento alimentar.

1.3 Sistema serotoninérgico e comportamento alimentar neonatal

Os comportamentos são disparados e guiados por eventos neurais, porém cada ato comportamental envolve, nos sistemas fisiológicos periféricos, uma resposta que é traduzida na atividade neuroquímica do cérebro (Blundell, 1993). A ingestão alimentar é regulada por uma complexa interação de mecanismos periféricos e centrais (York, 1999). O controle do apetite reflete, no homem, a operação sincrônica entre: 1- eventos psicológicos (percepção da fome, desejos) e operações comportamentais (refeição, ingestão de alimentos); 2- Eventos metabólicos e fisiológicos periféricos; 3- Interações metabólicas e de neurotransmissores no cérebro (Blundell, 1993). Esta atividade cerebral é a base da motivação para ativar ou inibir a ingestão (Blundell, 1993).

As experiências alimentares no início da vida têm implicações para o crescimento e desenvolvimento e para a formação dos traços comportamentais (Selling *et al.*,1947). No ser humano, a interação materno-infantil diária, em particular durante a alimentação, parece ter impacto psicológico (Keren e Tyano, 1998). Os sentimentos em torno de experiências alimentares precoces desenvolvem a relação da criança com o ambiente (Selling *et al*., 1947). Assim, distúrbios alimentares no adulto poderiam, muitas vezes, refletir transtornos ocorridos na infância (Keren e Tyano, 1998). Entretanto, as bases experimentais para esta proposição ainda não estão consolidadas. No rato, este período, corresponde ao aleitamento e apresenta intenso crescimento e

diferenciação do SN. Ao nascimento, o repertório comportamental murino é primitivo e limitado, caracterizando-se por reflexo de enrolar-se em resposta a estímulos nocivos, locomoção dianteira rude e sucção (Hall *et al*., 1977).

O comportamento de sucção em mamíferos é uma atividade sob múltiplos controles, originando e recebendo diferentes influências através do desenvolvimento. (Friedman, 1975; Hall *et al*., 1977; Smortherman e Robinson, 1994). Do ponto de vista nutricional, a sucção é um análogo primitivo do comportamento adulto, sofrendo alterações graduais até o padrão alimentar definitivo (Houpt e Epstein, 1973; Hall *et al*., 1977).

Durante a ontogênese, o comportamento alimentar dos mamíferos sofre uma intensa mudança. Ao nascimento, ele é simples e automático, transformando-se, em pouco tempo, no comportamento adulto complexo (Houpt e Epstein, 1973). No rato, até o 18^{o} dia de vida o controle do comportamento alimentar não apresenta características do animal adulto (Weller, 2000). Nesse mamífero, a maturidade comportamental parece ser atingida quando a sucção termina em resposta a sinais de saciedade; os sinais periféricos principalmente de distensão gastrointestinal preponderam antes dos 14 dias de vida (Hall e Rosenblatt, 1978). À medida que o desenvolvimento ocorre, esses animais iniciam respostas aos reguladores nervosos centrais. A colecistocinina mostra-se ineficiente em ratos com 10 dias de idade, contudo, reduz a ingestão em ratos a partir dos 20 dias (60%) até a idade adulta (Antin *et al*.; 1975; Weller *et al*.; 1990). A partir desta mesma idade, a seqüência comportamental de saciedade é também similar àquela do adulto, com concomitante redução do consumo de leite materno (Hall e Rosenblett, 1977). Quanto à privação alimentar, ratos respondem no primeiro dia de vida, de forma similar aos adultos, aumentando a ingestão subseqüente (Moorcroft, 1971; Houpt e Epstein, 1973; Henning *et al*., 1979).

O rato neonato depende de sua mãe para uma variedade de funções fisiológicas, como manutenção da temperatura corporal, excreção urinária e alimentação (Friedman, 1975).

Aproximadamente até o 14º dia de idade, eles têm suas mães como fonte única de alimento, e a disponibilidade de leite varia assim com a presença ou ausência materna no ninho, com seu estado emocional e nutricional (Friedman, 1975). Até os filhotes alcançarem maturidade para autocontrole da ingestão, é a mãe quem os estimula a iniciar a sucção (Rosenblatt, 1969). A ritmicidade biológica materna impõe-se sobre a dos filhotes, pois o padrão de ganho ponderal destes varia em função da disponibilidade do alimento para a mãe (Levin e Stern, 1975). É provável que os filhotes, a partir do 14º dia também auxiliados pela abertura dos olhos, sejam estimulados a comer alimento sólido quando a mãe também o fizer (Galef e Clark, 1971a). Ademais, a presença da mãe influencia o tipo de alimento selecionado e o momento inicial da alimentação (Galef e Clark, 1972 b).

Estudos de Blundell, (1994), Halford e Blundell (2000) e Rodgers et al., (2002), demonstram a essencialidade da atividade cerebral como sendo a base da motivação para ativar ou inibir a ingestão. A participação de neurotransmissores, em particular a serotonina (5-HT) em estudos comportamentais com animais adultos, são abundantes na literatura (Blundell, 1991; Lightowler, 1994; Chopin et al., 1994; Halford e Blundell, 1996a; Halford e Blundell, 1996b).

Em animais adultos, injeção de pequenas doses de serotonina no núcleo paraventricular do hipotálamo leva a uma redução no consumo de carboidratos. Entretanto, quando altas doses de serotonina são injetadas, em geral uma anorexia pode ocorrer. (Toornvliet, 1996). Outros estudos indicam que a maioria dos tratamentos que aumentam a disponibilidade da 5-HT na fenda sináptica resultam numa redução do consumo alimentar em mamíferos. Outrossim, eventos que direta ou indiretamente diminuem a disponibilidade da 5-HT na fenda sináptica, causam o efeito oposto (Blundel, 1984; 1986; Simansky, 1996). A alteração na disponibilidade sináptica do neurotransmissor afetará a sua interação com os receptores a nível celular. No animal adulto, através da interação com os receptores serotoninérgicos, a serotonina elicia ou modula uma ampla variedade de funções do SNC,

dentre as quais, o comportamento alimentar (Chopin et al., 1994). Dos subtipos de receptores estudados até o momento, as evidências sugerem que 5-HT_{1A}, 5-HT_{1B} e 5-HT_{2C} têm papel importante no controle da ingestão alimentar (Halford e Blundel, 1996a, 1996b). Os agonistas não seletivos dos receptores serotoninérgicos e drogas que independente de sua ação terapêutica aumentam a disponibilidade sináptica de serotonina como precursores, agentes liberadores ou inibidores de recaptação, reduzem o consumo alimentar (Samanin et al., 1972, 1979; Goudie et al., 1976; Sugrue, 1987; Lucki et al., 1988; Clifton et al, 1989). Estudos de Garattini (1995) mostraram que d-fenfluramina, cuja ação farmacológica é aumentar a disponibilidade sináptica da serotonina, causa redução da ingestão alimentar em ratos. Achados semelhantes foram observados após administração de fluoxetina em animais pré-tratados com pCPA, um inibidor de síntese da 5-HT (Lightowler et al, 1996). Embora o pré-tratamento com pCPA tenha causado uma redução de 90% da 5-HT e 5-HIAA, esses autores observaram que o efeito hipofágico da fluoxetina não foi alterado.

1.4 Outros neurotransmissores envolvidos no comportamento alimentar

Estudos demonstram que tratamento agudo com inibidor da recaptação da noradrenalina (NE) da 5-HT, sibutramina, reduz a ingestão alimentar (Jackson et al., 1997a; Jackson et., al 1997b), podendo essa perda de peso estar relacionada com a dose dessa substancia (Bray et al., 1999). Estudo de Leibowitz, 1970, propõe que NE endógena reduz alimentação quando sua ação ocorre através de receptores adrenérgicos hipotalâmicos lateral, mas aumenta a alimentação através de receptores adrenérgicos no hipotálamo ventro-medial. Aumento da liberação de NE tem sido observada no hipotálamo total durante alimentação ou

tratamento com doses que induzem a saciedade com o octapepídeo colecistocinina, (CCK-8) administradas perifericamente (Kaneyuky, Morimasa e Shomori, 1986). Estudo mais recente, entretanto (Cannon e Palmiter 2003), é controverso, demonstrando que o tratamento com CCK-8 é importante no controle do tamanho da alimentação, mas a NE não interferiu na saciedade induzida pela CCK.

As evidências acima demonstram a complexidade dos eventos que envolvem o comportamento alimentar, em especial do animal neonato. Alguns comportamentos do filhote no processo alimentar, se tornam mais bem elaborados com a idade e independentes da mãe, enquanto outros, desaparecem. Ademais, é evidente, mas ainda meritória de estudos, a participação do sistema serotoninérgico no desenvolvimento do comportamento alimentar.

1.5 Abordagem científica e perguntas importantes

À luz das evidências acima referidas, os componentes do sistema serotoninérgico, parecem estar envolvidos no desenvolvimento do sistema nervoso, além de desempenharem importante função reguladora no comportamento alimentar do animal adulto. Entretanto, no que concerne ao desenvolvimento do comportamento alimentar, ainda são insipientes estudos sobre o papel direto ou indireto da serotonina. Estamos, portanto diante de questões científicas que precisam ser elucidadas e assim fornecer contribuição decisiva para o entendimento da estreita relação entre o sistema nervoso e a nutrição em mamíferos. Uma primeira pergunta, focalizando em particular, o desenvolvimento do comportamento neonatal, de sua fase inicial até aquela muito semelhante a do animal adulto, seria: Como fatores epigenéticos, a exemplo da manipulação farmacológica, precisa e específica do sistema serotoninérgico, poderiam influenciar no desenvolvimento do comportamento alimentar? A interferência na expressão

desses fenômenos que constituem a inteireza do comportamento alimentar, muito dos quais pré-estabelecidos, podem comprometer a estrutura comportamental definitiva do adulto? Outra questão, não menos importante, ainda não totalmente esclarecida, mas certamente mais estudada é: Como o crescimento de tecidos neurais ou não neurais, que expressam um padrão determinado, seriam modificados, direta ou indiretamente, por alterações de componentes serotoninérgicos durante o período critico de desenvolvimento.

A expressão do crescimento somático e de reflexos compõe aspectos cruciais do desenvolvimento no mundo animal e em particular nos mamíferos. O reflexo, produto da função nervosa, é um comportamento provocado por estimulação preestabelecida e precisa (Smart e Dobbing, 1971a). Os reflexos aparecem em períodos determinados durante o desenvolvimento ontogenético (Fox, 1965). Os diversos reflexos (Figura 3 e 4 a-g) superpõem-se uns aos outros, caracterizando também a ocorrência simultânea de vários eventos estruturais do desenvolvimento do SNC. O aparecimento dos reflexos parece assim obedecer a uma seqüência pré-determinada de acordo com a idade dos animais (Fox, 1965; Smart e Dobbing, 1971a). Isso é uma constatação surpreendente, especialmente para aquela seqüência de reflexos que são a base para os movimentos da cabeça e influenciam concomitantemente a posição das patas. Isto pode ser visto com clareza nos reflexos de recuperação do decúbito e da geotaxia negativa em ratos. Assim, muitos reflexos menos complexos expressam atividades labirínticas e constituem comportamentos com alto grau de complexidade relacionados à sobrevivência do animal, por exemplo: a conservação da temperatura, a alimentação e outros (Fox, 1965). Pode-se inferir, portanto, a existência de uma estreita relação entre os fatores genéticos, ambientais, o desenvolvimento estrutural, bioquímico, funcional e o aparecimento da atividade reflexa, da mais simples até aquela mais complexa. Isto tudo indicando o desenvolvimento harmonioso do funcionamento paulatino do sistema nervoso. O fato da desnutrição pós-natal, uma perturbação

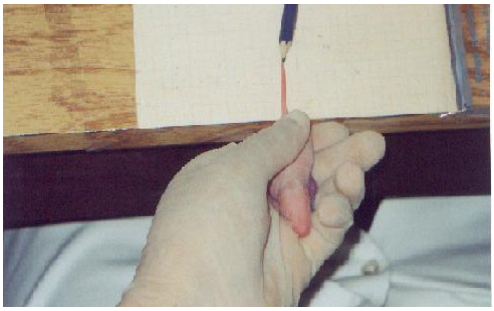
ambiental, acarretar retardo na ontogênese de reflexos (Adlard e Dobbing, 1971) é um claro exemplo.

Atualmente, com o avanço da farmacologia, é possível dispor de substâncias altamente seletivas que atuam especificamente em determinados sistemas de neurotransmissores. Para aumentar a disponibilidade sináptica de serotonina e assim observar o papel do sistema serotoninérgico nos parâmetros a serem estudados, utilizaremos portanto, no presente trabalho, os inibidores seletivos de recaptação da 5-HT (SSRIs). Citalopram, fluoxetina e sertralina são potentes SSRIs, são substâncias que interferem na transmissão sináptica da serotonina, porém diferem em sua estrutura química, metabolismo e farmacocinética, apresentando mecanismos comuns de ação (Figura 5) (Baumman e Rochat, 1995). Fluoxetina e citalopram estão disponíveis quimicamente como racematos. Estudo realizado com ratos, *in vitro*, demonstrou que o citalopram nas suas formas isômeras, S-citalopram e S-demetilcitalopram, são potentes SSRIs (Hyttel, 1994; Baumman e Rochat, 1995). Citalopram é metabolizado a demetilcitalopram e a N-didemetilcitalopram. Sertralina também possui seu metabólito ativo que é formado por N-dimetilação (norsertralina), bem como a fluoxetina que também é demetilada a norfluoxetina. Todos estes metabólitos são também SSRIs, menos potentes (Baumman, 1996; Eap e Baumann 1996). Além disso, apresentam alta afinidade por sítios de recaptação da serotonina, o que as tornam drogas modelo, para estudos que envolvam esse neurotransmissor (Arranz e Marcusson, 1994). Portanto, todos estes SSRIs são a princípio ferramentas farmacológicas altamente específicas e relativamente precisas que podem ser usadas para manipulação em nível celular. Já há um acúmulo razoável de conhecimento a partir dos estudos do papel do sistema serotoninérgico em diversas funções. O que permite a utilização por especialistas de agentes serotoninérgicos no tratamento de diversos transtornos, inclusive aqueles que envolvem o comportamento alimentar. Contudo, de modo menos criterioso, estes mesmos produtos vêm sendo utilizados em vários organismos

em idades cada vez mais precoces (Oberlander 2000). À exceção dos trabalhos de poucos grupos, dentre estes os nossos, não têm sido encontrados na literatura, estudos sobre as conseqüências de manipulações diretas ou indiretas do sistema serotoninérgico ocorridas durante o período crítico de desenvolvimento do SN sobre o comportamento. Entre os estudos realizados em nosso laboratório, um deles demonstrou que em ratos neonatos desnutridos há atraso no padrão de desenvolvimento do comportamento alimentar (Souza, 2001) (Figura 6 a,b,c). Em outro estudo de nossa equipe, foi observado que o tratamento neonatal com tianeptina (um incentivador da recaptação da serotonina) não produz alterações no desenvolvimento do comportamento alimentar (Freitas Silva, 2002). Pretendemos com a presente abordagem avançar nessa linha de pesquisa.

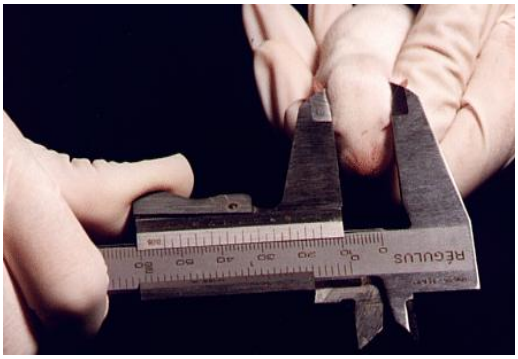
1.6 Figuras

Figura 1 - Fotos dos procedimentos de avaliações.



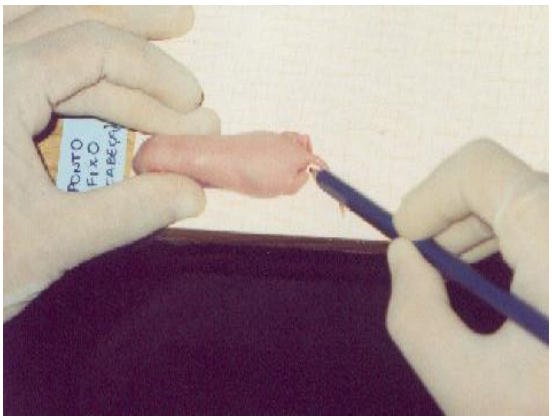
A

B



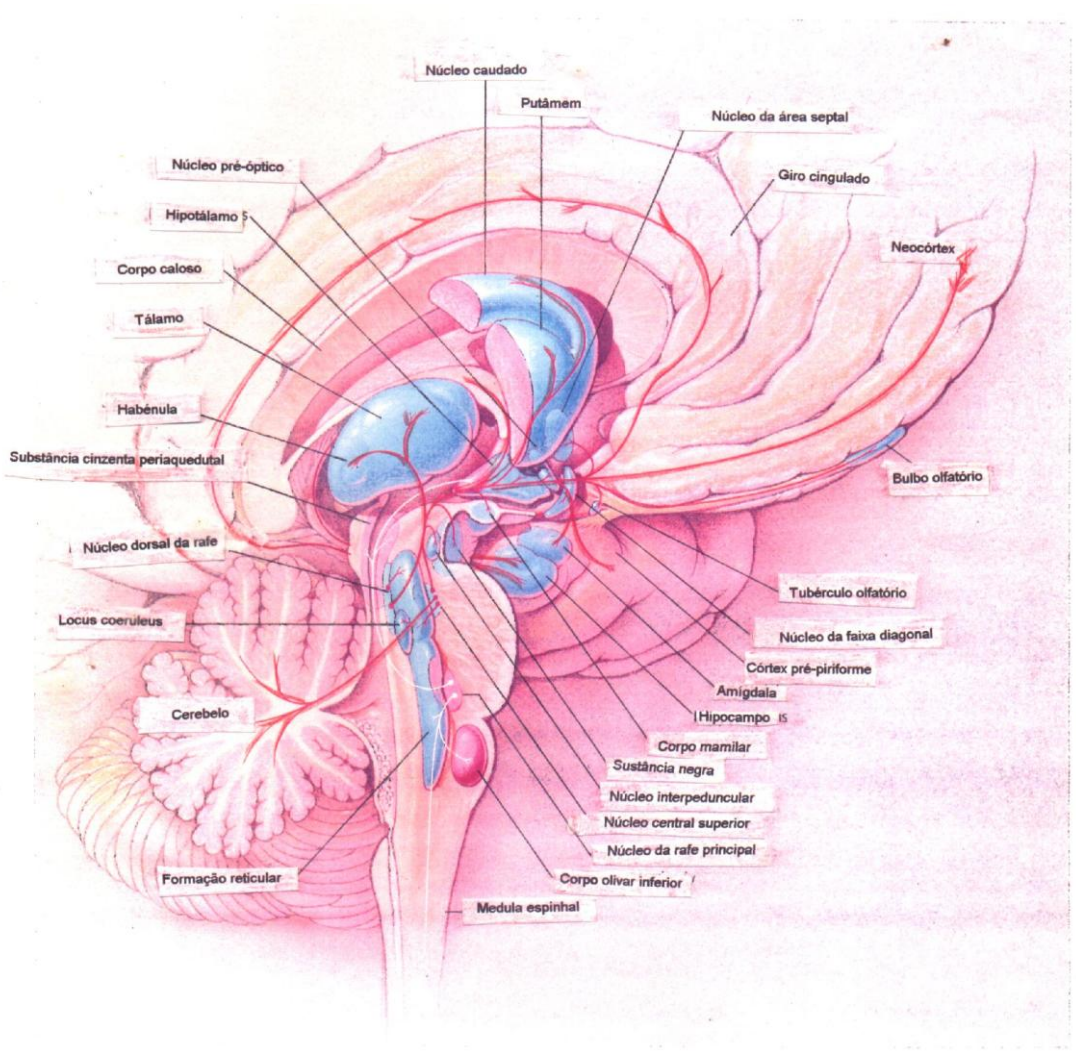
C

D



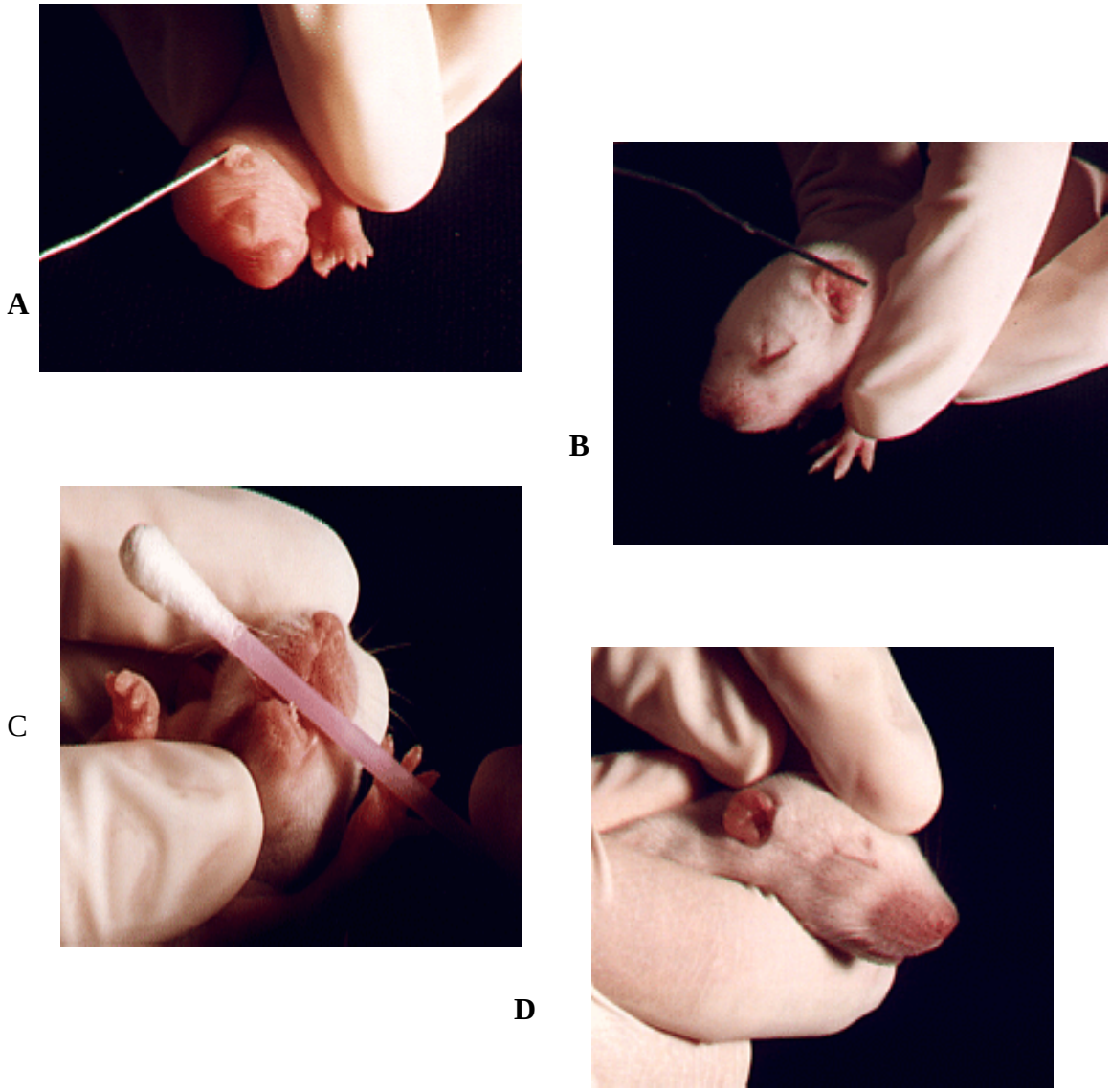
Fonte: Fotos da autora: A- comprimento da cauda; B- eixo látero-lateral do crânio; C- eixo antero-posterior do crânio; D- eixo longitudinal do corpo.

Figura 2. Distribuição das vias serotoninérgicas no cérebro.



Fonte: Modificado de Snyder, S.H. in: Drugs and the Brain. Scientific American Libery, 1996.

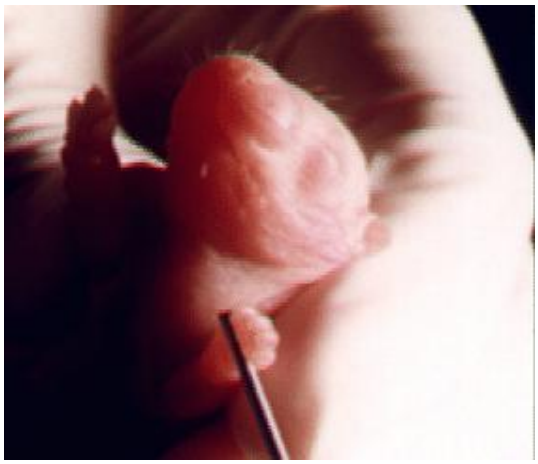
Figura 3. Fotos da avaliação das características físicas. A- abertura do pavilhão auditivo, B- abertura do conduto auditivo, C- Irrupção dos incisivos inferiores; D- Abertura dos olhos.



Fonte: Fotos da autora: A- abertura do pavilhão auditivo, B- abertura do conduto auditivo, C- Irrupção dos incisivos inferiores; D- Abertura dos olhos.

Figura 4a - Estudo do reflexo de preensão palmar. Ao leve contato com bastão metálico, ocorre rápida flexão dos dedos

.



A **B** **C**

D

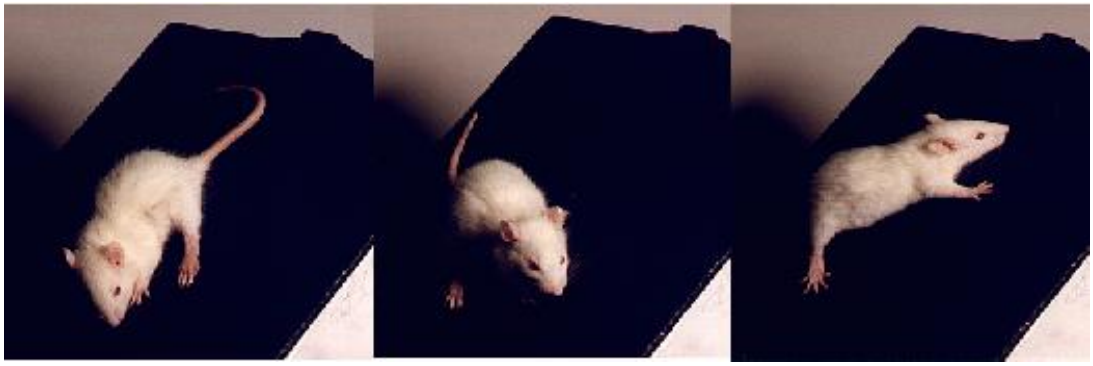
Figura 4b. Estudo do reflexo de recuperação de decúbito. A – O pesquisador mantém o rato em decúbito dorsal; B e C- retirada a contenção, o rato gira o tronco sobre o próprio eixo; D- Rato alcança o decúbito ventral, apoiado sobre as quatro patas.

A  **B**

Figura 4c. Estudo do reflexo de colocação pelas vibrissas. A -O pesquisador segura o rato pela cauda de modo a permitir que suas vibrissas toquem a borda de uma mesa, assim, o rato inclina seu corpo em direção à mesa; B- o rato apóia as patas anteriores e realiza movimentos de marcha.

A **B** **C**

Figura 4d. Estudo do reflexo de aversão ao precipício; A- o rato é colocado próximo à borda da mesa, de forma que suas patas anteriores toquem esta borda, B- ele retrai o corpo, fazendo uma rotação lateral do tronco, C- se afasta da mesa.



A **B** **C**

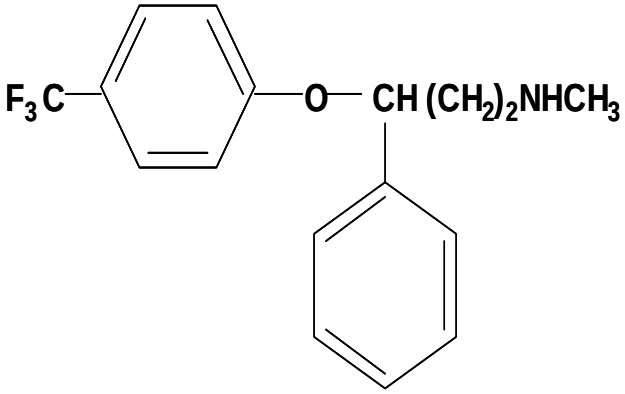
Figura 4e. Estudo do reflexo de geotaxia negativa; A - o rato é colocado numa rampa inclinada (45º) de cabeça para baixo, B- animal realizando retorno com torção lateral do tronco, C- posicionando sua cabeça para o plano mais elevado e inicio de marcha.

Figura 4f. Estudo do reflexo de resposta ao susto. Com a batida de um bastão sobre um recipiente metálico, produz-se um ruído súbito e o animal se retrai e apresenta tremor.

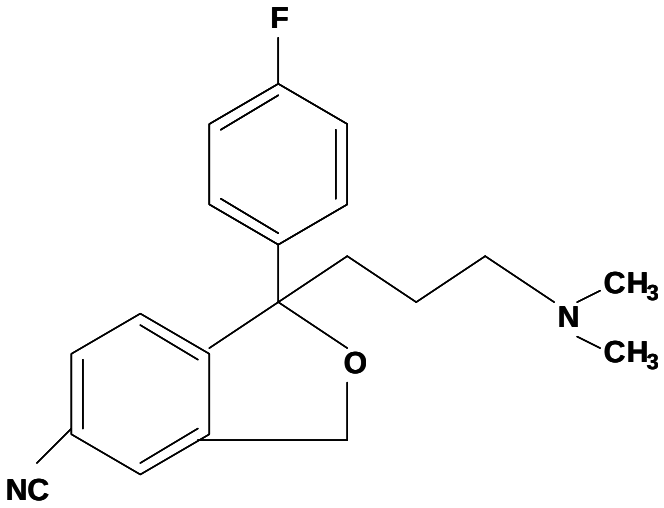
Figura 4g. Estudo do reflexo de recuperação de decúbito. A – O rato é solto de uma altura de 30cm preensão, B – gira sobre o próprio eixo durante a queda, apoiando-se nas 4 patas ao atingir o solo.

A **B**

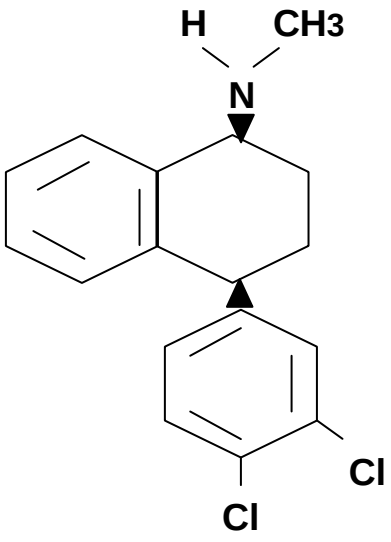
Figura 5. Estrutura química dos Inibidores Seletivos de Recaptação da Serotonina - SSRIs.



Fluoxetina



Citalopram



Sertralina

Figura 6a – Comportamento de limpeza materno. A- a lactante lambendo os pelos. B- a lactante lambendo as patas.

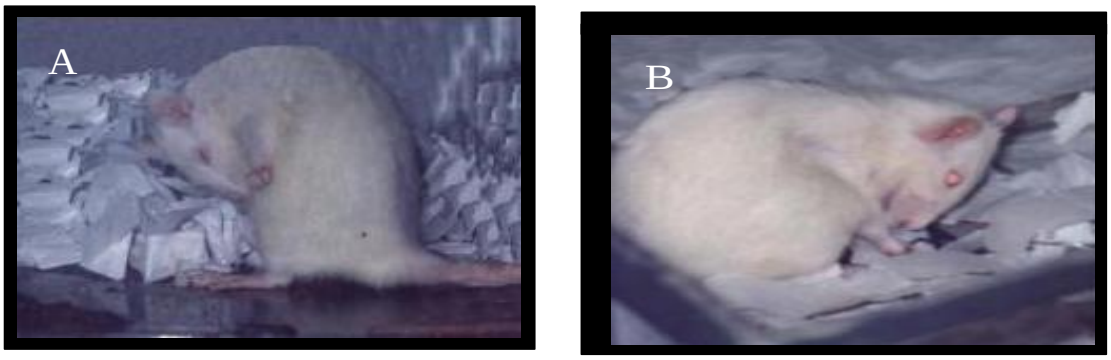
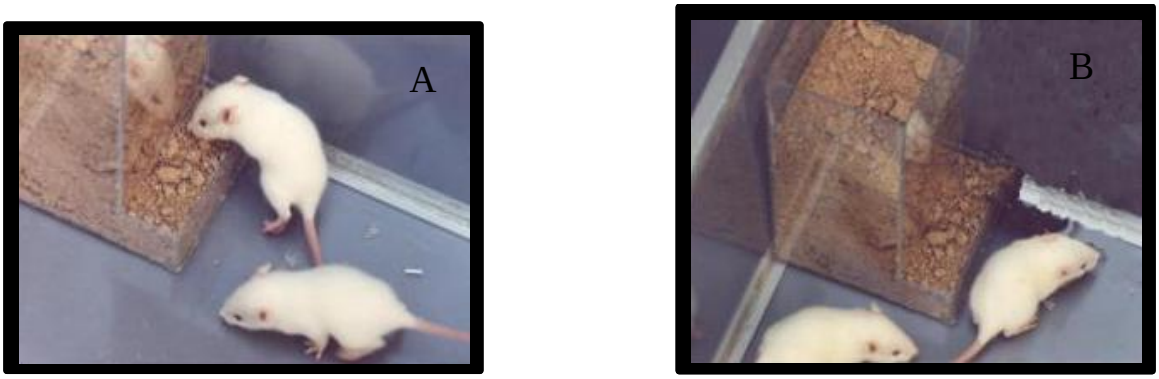


Figura 6b – Comportamento de sucção em ratos. A – O comportamento é iniciado quando o primeiro filhote fixa-se ao mamilo materno. B- Sucção em conjunto. C- O comportamento finaliza quando o último filhote deixa o mamilo.

Figura 6c – Comportamento de ingestão de ração dos filhotes. A- Inicia-se quando o primeiro filhote chega ao comedouro. B – Finaliza quando o último filhote abandona o comedouro.



2 JUSTIFICATIVA

O presente estudo, utilizando o rato, como modelo experimental, para avaliação do desenvolvimento de reflexos e indicadores da maturação de caracteres somáticos, poderá fornecer subsídios para melhor compreensão dos efeitos de agressões farmacológicas durante o período crítico de desenvolvimento do SNC. Outrossim, neste trabalho, respostas à agressões farmacológicas, contribuirão de forma seletiva e específica para esclarecer o possível envolvimento do sistema serotoninérgico no crescimento corporal e desenvolvimento do SNC. O estudo de parâmetros do comportamento alimentar neonatal durante o período de rápido crescimento do encéfalo, será de grande relevância para melhor compreensão do efeito da manipulação neonatal do sistema serotoninérgico e sua repercussão em parâmetros comportamentais. A essência dos estudos experimentais em paradigmas comportamentais darão outrossim, relevância a este trabalho, como parte dos estudos realizados em nosso laboratório. Por fim, considerando a utilização de drogas antipressivas - inibidores da recaptação da serotonina- durante o período gestacional e em crianças e adolescentes, ressaltamos a relevância clínica da realização deste trabalho. Os aspectos a serem avaliados (até o presente momento pouco estudados), sem dúvida contribuirão, para uma nova visão da utilização desses fármacos, auxiliando os especialistas (psiquiatras, nutricionistas, nutrólogos, endocrinologistas, e demais profissionais de áreas afins), que os utilizam na terapêutica clínica, quanto aos possíveis efeitos destas drogas em seus pacientes.

3 OBJETIVO

3.1 GERAL

Investigar em ratos neonatos, as repercussões do tratamento durante o período de rápido crescimento do SNC, com diferentes inibidores seletivos da recaptação da serotonina (SSRI), sobre a maturação somática, o desenvolvimento sensório-motor e o comportamento alimentar neonatal.

4 HIPÓTESES

De acordo com os objetivos apresentados, levantamos as seguintes hipóteses

1-Tratamento crônico com inibidor seletivo de recaptação da serotonina (SSRI)-citalopram - retarda a maturação somática.

2- Ratos neonatos tratados com fluoxetina, um inibidor seletivo da recaptação da serotonina, retardam o crescimento somático e a maturação de reflexos.

3- Tratamento crônico com sertralina um SSRI- retarda a maturação somática e de reflexos em ratos neonatos.

4- Tratamento crônico com inibidor seletivo de recaptação da serotonina – citalopram em ratos neonatos, retarda a ontogenia de reflexos.

5 – Fluoxetina um SSRI, reduz parâmetros do comportamento alimentar em ratos neonatos

5 APRESENTAÇÃO DOS ARTIGOS

O presente trabalho é estruturado em cinco artigos científicos, onde foram avaliados os efeitos do tratamento com três diferentes SSRIs sobre o crescimento, através de medidas corporais diárias e da maturação de características físicas; sobre o desenvolvimento sensório-motor, através do estudo da ontogenia de reflexos; sobre o comportamento alimentar neonatal, utilizando-se a observação direta (duração e freqüência de parâmetros comportamentais) em animais submetidos à privação alimentar, em quatro dias intercalados do período de aleitamento. Dos resultados obtidos das pesquisas, foram extraidos artigos científicos, dos quais um foi aceito para publicação e quatro estão submetidos.

5.1 Primeiro artigo

Título: **NEONATAL ADMINISTRATION OF CITALOPRAM DELAYS SOMATIC MATURATION IN RATS.**

Aceito para publicação como artigo original na revista: Brazilian Journal of Medical and Bilogical Research em maio de 2004.

Neste artigo foram estudados os efeitos do tratamento neonatal com o inibidor seletivo da recaptação da serotonina – citalopram - sobre o crescimento somático e a maturação de características físicas em ratos. Neste estudo observou-se que o tratamento com citalopram durante o período de aleitamento-periodo critico de desenvolvimento do cérebro - retarda o ganho de peso corporal, altera o crescimento do crânio e provoca retardo na maturação de características físicas.

Brazilian Juornal of Medical and biological Research (Aceito para Publicação)

Neonatal administration of citalopram delays somatic maturation in rats

Abstract

We investigated the somatic maturation of neonate rats treated during the suckling period with citalopram, a selective serotonin reuptake inhibitor. Groups with 6 male neonates were randomly assigned to different treatments 24 h after birth. Each litter was suckled by one of the dams until the 21st postnatal day. Body weight, head axis and tail length were measured daily from the 1st to the 21st postnatal day. Time of ear unfolding, auditory conduit opening, incisor eruption and eye opening was determined. Pups received 5 mg (Cit5), 10 mg (Cit10) or 20 mg/kg (Cit20) citalopram *sc*, or saline (Sal; 0.9% NaCl *sc*). Compared to Sal, body weight was lower (24.04%), for Cit10 from the 10th to the 21st day ($P < 0.01$) and for Cit20 from the 6th to the 21st day (38.19%), ($P < 0.01$). Tail length was reduced in the Cit20 group (15.48%), from the 8th to the 21st day ($P < 0.001$). A reduction in mediolateral head axis (10.53%), was observed from the 11th to the 21st day in Cit10 ($P < 0.05$) and from the 6th to the 21st day in Cit20 (13.16%), ($P < 0.001$). A reduction in anteroposterior head axis was also observed in the Cit20 group (5.28%), from the 13th to the 21st day ($P < 0.05$). Conversely, this axis showed accelerated growth from the 12th to the 21st day in the Cit5 group (13.05%), ($P < 0.05$). Auditory conduit opening was delayed in the Cit5 and Cit20 groups and incisor eruption was delayed in all citalpram. These findings show that citalopram injected during suckling to rats induces body alterations and suggest that the activity of the serotoninergic system participates in growth mechanisms.

Key words

Serotonin
Growth indicators
Selective serotonin reuptake inhibitor
Citalopram
Craniofacial
Skull

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Introduction

Selective serotonin reuptake inhibitors (SSRI) such as citalopram have been used to induce increased serotoninergic activity in the brain (1,2). Experimental evidence indicates that serotonin can influence embryogenesis and growth (3,4) presumably by acting as a developmental signal (5) or as a neurotrophic factor as well (6). Furthermore, serotonin seems to play a role in regulating the development of the mammalian brain through its action on the production of self serotonin neurons (4,7,8) and on target tissues innervated by serotoninergic neurons (9-11). Pharmacological or nutritional manipulations during pregnancy or the suckling period can induce drastic morphological and functional changes in the growth and

development of the nervous system (12-16). Several studies have shown that treatment with SSRI reduces food intake and body weight in rats (17,18). A hypophagic effect was observed following fluoxetine administration (19). As a consequence, the possibility exists that the use of serotoninergic agents in the early phase of life could influence other specific maturation processes in the body. Therefore, it is desirable to investigate the possible effects of manipulation of the serotoninergic system early in life.

The objective of the present study was to test the hypothesis that the administration of citalopram - a highly selective serotonin reuptake inhibitor (20) - to rats during the suckling period induces changes in somatic development and in body growth.

Material and Methods

Animals

Litters obtained from dams mated to male Wistar rats from the colony of the Nutrition Department, Federal University of Pernambuco, Recife, PE, Brazil, were used in the present study. During gestation and until the end of the experiment, the animals were housed in polyethylene cages. Male pups from different mothers (N = 18) were randomly divided into groups of 6 neonates 24 h after birth. Each pup was marked with methyl violet solution on the skin for identification during the experiment. Each litter was suckled by one of the dams until the 21st postnatal day (birth day was considered to be zero day). The animals were maintained at a room temperature of 23 ± 1ºC, on a 12/12-h light-dark cycle (lights from 6:00 am to 6:00 pm) with free access to ration (Labina-Purina - São Lourenço da Mata, PE, Brazil) and water.

Citalopram

The experiment was performed blind to prevent identification of the experimental groups. The animals of the different groups were evaluated simultaneously. For the experimental treatment, four groups (N = 27 each) of suckling rats were distributed as follows: three groups received citalopram: group Cit5 (5 mg/kg, *sc*), group Cit10 (10 mg/kg, *sc*), and group Cit20 (20 mg/kg, *sc*), and a control group received an equivalent volume of 0.9% (w/v) saline solution, *sc*. During the experiment, one neonate rat in the Cit20 group and one in the Cit5 group died. Therefore, 106 rats were evaluated. The time of physical feature maturation and somatic growth was recorded. Citalopram (H. Lundbeck A/S, Copenhagen-Valby, Denmark), was dissolved in saline and 1 ml/100 g body weight was injected *sc*. The treatment was applied daily from the 1st to the 21st postnatal day (suckling period) at 0:30 pm - 1:30 pm.

Physical features maturation

The following physical features were observed: unfolding of the external pinnae of both ears to the fully erect position; auditory conduit opening is internal auditory conduit opening of both ears; incisor eruption – the first visible and palpable crest of lower incisors; and eye opening, i.e., when any visible break in the covering membrane of both eyes was detected. The features were evaluated daily between 10:00 and 12:00 a.m. by the method of Smart and Dobbing (21)during the suckling period until maturation of the variable. The maturation age of a particular feature was defined as the day it was first observed.

Somatic growth

Somatic growth was assessed in terms of body weight, tail length, and mediolateral and anteroposterior head axis measurements made from the 1st to 21st postnatal day between 1:00 and 2:00 p.m. as follows: body weight was measured with a FANEM (São Paulo, SP, Brazil) scale with 100 mg precision. Tail length (distance from tail tip to tail base) and length of the mediolateral skull axis, MLHA (distance between the ear holes), anteroposterior axis of the head, and APHA (distance between snout and head-neck articulation) were measured with a Starret caliper with 0.05 mm precision.

Statistical analysis

Statistical analysis was performed after preliminary testing to identify normality of distribution and homogeneity of variance among the groups. Two-way ANOVA for repeated measures (from the 1st to the 21st day for body weight, head axis and tail length) followed by the post-hoc Tukey test were used to compare growth indicators between each citalopram group and the saline control. Kruskal-Wallis analysis of variance followed by the Dunn test was used to compare physical features between each citalopram group and the saline control. The level of significance was set at $P < 0.05$.

The experimental protocol of the study was approved by the Ethics Committee for Animal Experimentation (CEEA) of the Federal University of Pernambuco, and was consistent with the National Institute of Health Guide for Care and Uses of Laboratory Animals (Publication no. 85-23, revised, 1985).

Results

ANOVA identified main effects of time and treatment in addition to interaction between these factors regarding somatic growth. For body weight, statistical analysis showed an effect of citalopram ($F_{3, 102} = 52.9$, $P < 0.001$), and day of life ($F_{20, 2040} = 2340.6$, $P < 0.001$) as well as a citalopram versus day of life interaction ($F_{60, 2040} = 69.5$, $P < 0.001$). Body weight gain (Figure 1A) was reduced from the 10th to the 21st day (24.04%), ($P < 0.01$) in Cit10 and from the 6th to the 21st day (38.19%),($P < 0.01$) in Cit20 when compared to saline. This lower weight gain was more marked in the Cit20 than in the Cit10 group. The Cit 5 group did not differ from saline (Figure 1A).

For tail length (Figure 1B), statistical analysis showed a main effect of citalopram ($F_{3, 102} = 13.7$, $P < 0.001$) and day of life ($F_{20, 2040} = 3198.0$, $P < 0.001$) in addition to an interaction between citalopram and day of life ($F_{60, 2040} = 18.7$, $P < 0.001$). A reduced growth of tail length was observed in the Cit 20 group from the 8th to the 21st day (15.48%), ($P < 0.01$); however, the Cit10 and Cit5 groups did not differ from the saline control.

ANOVA showed an effect of citalopram ($F_{3, 102} = 23.8$, $P < 0.001$) and day of life ($F_{20, 2040} = 1622.1$, $P < 0.001$), as well as an interaction between citalopram and day of life ($F_{60, 2040} = 15.3$, $P < 0.001$) on the mediolateral head axis. A reduction in mediolateral head axis growth was observed from the 11th to the 21st day in the Cit10 group (10.53%) ($P < 0.05$), and from the 6^{th} to the 21^{st} day in the Cit20 (13.16%), ($P < 0.001$) group compared to the saline control. Moreover, the reduced growth of this axis was more marked for the 20 mg dose than for the 10 mg dose (Figure 2A).

There was also a significant change in growth of the anteroposterior head axis length. There were effects of citalopram ($F_{3, 102} = 15.1$, $P < 0.001$), day of life ($F_{20, 2040} = 2770.6$, $P < 0.001$) and interaction between citalopram and day of life ($F_{60, 2040} = 14.7$, $P < 0.001$). In the

Cit20 group a reduction of this axis occurred from the 13th to the 21st day (5.28%),($P < 0.01$), whereas the growth was accelerated from the 12th to the 21st day in the Cit5 group (13.05%), ($P < 0.05$). There was no difference for the Cit10 group (Figure 2B).

Physical features

For physical feature maturation (Table 1), Kruskal-Wallis analysis indicated significant changes in auditory conduit opening ($H = 10.59$, $P < 0.05$) and incisor eruption ($H = 19.33$, $P < 0.01$) in the citalopram groups. Multiple comparisons of these features among treatment groups (Dunn test) showed that auditory conduit opening was delayed in the Cit5 ($P < 0.05$) and Cit 20 groups ($P < 0.05$). Incisor eruption was delayed in the Cit5 ($P < 0.05$), Cit10 ($P < 0.01$) and Cit20 ($P < 0.05$) groups. It is noteworthy that a wide variation (11-18 days for auditory conduit opening and 10-17 days for incisor eruption) was observed in the Cit20 group but not in groups receiving the lowest citalopram doses or saline. For eye opening and ear unfolding there was no significant difference.

Discussion

The present results show that chronic administration of citalopram during the critical neonatal period of rat brain development induces changes in growth. In general the drug impaired somatic growth and physical body maturation. As observed for body weight, increasing doses of citalopram resulted in equivalent growth alteration of tail and head. Regarding craniofacial development, the heads of the animals became longer with the lowest citalopram dose, narrower with the middle dose and both shorter and narrower with the highest dose. Therefore, the head suffered a shape distortion during development that was

dependent on the dose of citalopram. The sensitivity of vertebrate head growth to epigenetic manipulations has been well demonstrated (22,23). Changes in the craniofacial skeleton of developing rats submitted to a low protein diet were shown by Miller and Germann (24). Some of these changes consisted of shortening of skull dimensions, a result similar to that experienced by the animals receiving 20 mg of citalopram in the present study. It is noteworthy that early protein malnutrition such as studied by Miller and Germann (24) is known to increase brain serotonin (5-HT) levels (13). Therefore it is appropriate to consider the delayed development of the structures inside the skull (auditory conduit) and face (lower incisors) observed in the present study, because the delays might be associated with reduced head growth. Furthermore, the interaction between citalopram and time indicates that the highest citalopram dose caused not only a reduction of body weight gain and of tail growth, but also that these reductions in rats receiving 20 mg began earlier than in animals receiving the two lowest doses. These changes are important because they indicate the existence of growth mechanisms in body tissues which are sensitive to the dose of citalopram. Since this drug increases serotonin release (20) we suggest that serotoninergic mechanisms may be responsible for the effects of citalopram.

In addition, there is evidence that SSRI and some serotoninergic agonists also increase 5-HT release not only in neuronal tissues (10,11,25) but also in non-neuronal tissues (26,27). Interestingly, serotonin has a trophic action on the differentiation of several tissues during the prenatal and postnatal periods (27,28) by acting as a neurotrophic factor (6). Some studies on mouse embryo cultures treated with serotonin suggest an improved development of serotoninergic cells in the nervous tissue and of cells of the nasal prominence, epithelium covering the eye, optic vesicle and oral cavity (10,29-33) These findings indicate that serotonin plays a role in the control of the epithelial-mesenchymal interactions during craniofacial morphogenesis (26). Serotoninergic treatment also caused malformation of both

nerve and bone structures of mouse embryos (10). Several of these events begin in the second week of life and the first serotoninergic neurons appear as early as on the 12th to the 14th day of gestation (29). Therefore, during suckling the tissue may be able to respond to the challenge with citalopram. Moreover, a trophic role of 5-HT in the formation of craniofacial structures, including the maturation of the tooth germ, has been reported (28).

Our findings support the data in the literature because the delayed tooth eruption observed in the citalopram groups. However, the participation of citalopram in this maturation process appears to be complex since the highest dose induces a wider variation of the physical feature than the lowest doses. On the other hand, the possibility exists that the effects observed in this experiment may be induced by the action of 5-HT on anorectic mechanisms and not by a direct of 5-HT on tissues. In fact, it has been well documented that hypophagia induced by citalopram reduces body weight in adult rats (17,34) and body weight gain in young rats (16). Therefore, it cannot be ruled out in the present experiment that the hypophagia induced by citalopram and the resulting malnutrition were capable of causing growth disorders in animals that received citalopram.

We conclude that citalopram administered during the period of rapid brain development causes important morphological body alterations. These data support the view that growth mechanisms are highly susceptible to the manipulation of the serotoninergic system during this period.

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Table 1. Maturation (in days) of the physical features of suckling rats treated with citalopram from the 1st to the 21st day of life; d = days.* P <0.05; ** P <0.001

TABLE 1

	GROUPS			
	SALINE	CITALOPRAM		
		5 mg	10 mg	20 mg
PHYSICAL FEATURES	Median (range)	Median (range)	Median (range)	Median (range)
Ear unfolding	3.0 (1-4)	3.0 (2-6)	3.0 (2-4)	3.0 (2-5)
Auditory conduit opening	12.0 (11-14)	13.0 (12-14) *	12.5 (12-14)	13.0 (11-18) *
Irruption of the lower incisors	12.0 (9-13)	13.0 (12-14) *	12.0 (11-15) **	12.0 (10-17) *
Eyes opening	14.0 (12-15)	14.0 (12-16)	14.0 (12-14)	14.0 (12-17)

Kruskal-Wallis analysis of variance followed by the Dunn test was used to compare physical features between each citalopram dose and the saline control group. The number of animals is given in paremtheses. A significant change in auditory conduit opening ($H = 10.59$, $P < 0.05$) and incisor eruption ($H = 19.33$, $P < 0.01$) was observed in the citalopram groups.

Figure 1. Effect of citalopram on suckling rats weight and tail length. *A*, Body weight from the 1st to the 21st day of life of suckling rats treated with solutions (1 ml/100 g body weigh, *sc*) of citalopram (Cit) 5 mg (N = 26), Cit 10 mg (N = 27) and Cit 20mg/kg, *sc* (N = 26) or saline (Sal) (N = 27). Comparisons were made by two-way ANOVA for repeated measures: citalopram ($F_{3, 102} = 52.9$, $P < 0.001$), day of life ($F_{20, 2040} = 2340.6$, $P < 0.001$), and citalopram versus day of life interaction ($F_{60, 2040} = 69.5$, $P < 0.001$). Post-hoc multiple comparisons (Tukey test) between each citalopram dose and saline: *$P < 0.001$. Each point represents the mean values according to the dose. The asterisks (*) indicate the beginning of significant differences ($P < 0.01$) that persisted through the end of the experiment. *B*, Tail length. Comparisons were made by two-way ANOVA for repeated measures: citalopram ($F_{3, 102} =$

13.7, P < 0.001), day of life ($F_{20,\ 2040}$ = 3198.0, P < 0.001), and citalopram versus day of life interaction ($F_{60,\ 2040}$ =18.7, P < 0.001). *Post hoc* multiple comparisons (Tukey test) between each citalopram dose and saline: Each point represents the mean values according to the dose. The asterisks (*) indicate the beginning of significant differences (P < 0.01) which persisted to the end of the experiment according to the dose.

Figure 1A- Body weight

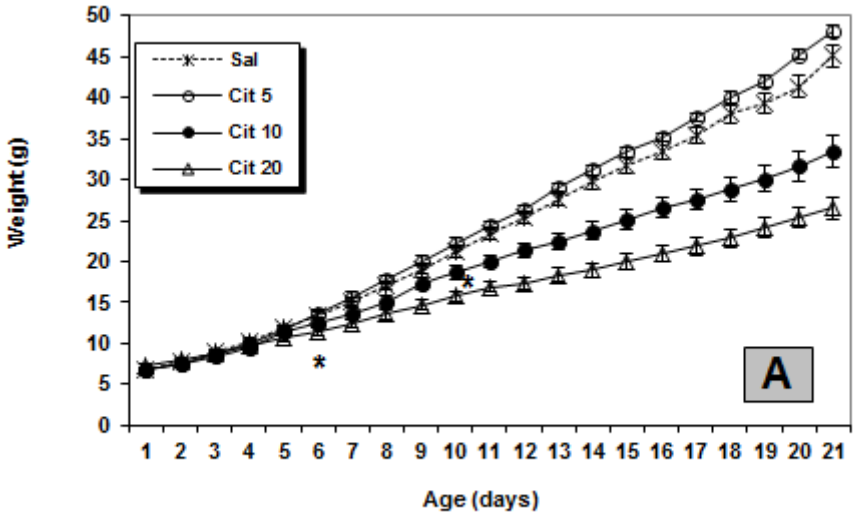


Figura 1 B - Tail Length

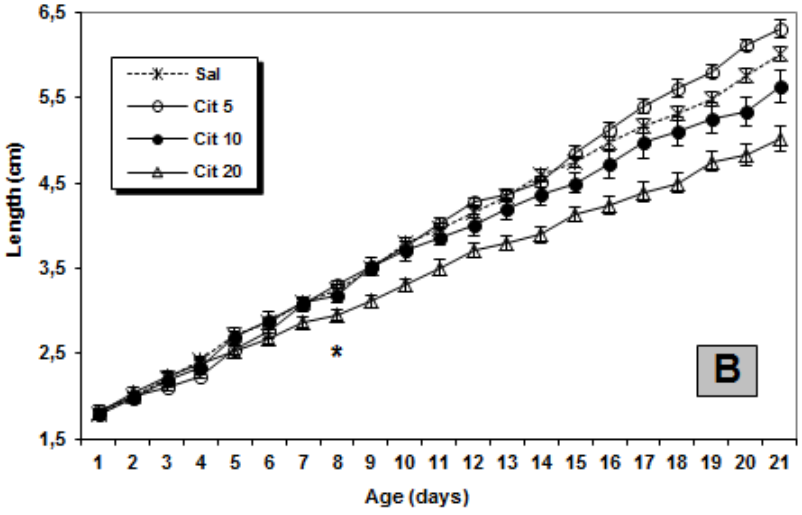


Figure 2. Effect of citalopram on suckling rat mediolateral skull axis length and distance between snout and head-neck articulation. *A*, Mediolateral skull axis from the 1st to 21st day of life of suckling rats treated with solutions (1 ml/100 g body weigh, *sc*) of Cit 5 mg (N = 26), Cit 10 mg (N = 27) and Cit 20 mg/kg, *sc* (N = 26) or Sal (N = 27). Comparisons were made by two-way ANOVA for repeated measures for citalopram ($F_{3,\ 102}$ = 23.8, P < 0.001) and day of life ($F_{20,\ 2040}$ = 1622.1, P < 0.001) and citalopram versus day of life interaction ($F_{60,\ 2040}$ = 15.3, P < 0.001)folowed by *Post hoc* multiple comparisons (Tukey test) between each citalopram dose and saline: The asterisks (*) indicate the beginning of significant difference in the Cit10 group (P < 0.05) and Cit20 group (P < 0.001) that persisted to the end of the experiment. *B*, Distance between snout and head-neck articulation. Comparisons were made by two-way ANOVA for repeated measures: Citalopram ($F_{3,\ 102}$ = 15.1, P < 0.001), day of life ($F_{20,\ 2040}$ = 2770.6 P < 0.001), and citalopram versus day of life interaction ($F_{60,\ 2040}$ = 14.7, P < 0.001). *Post-hoc* multiple comparisons (Tukey test) between each citalopram dose and saline: The asterisks (*) indicate the beginning of significant differences for Cit20 (P < 0.01) and Cit5 (P < 0.05) that persisted to the end of the experiment.

Figure 2 A – Mediolateral head axis

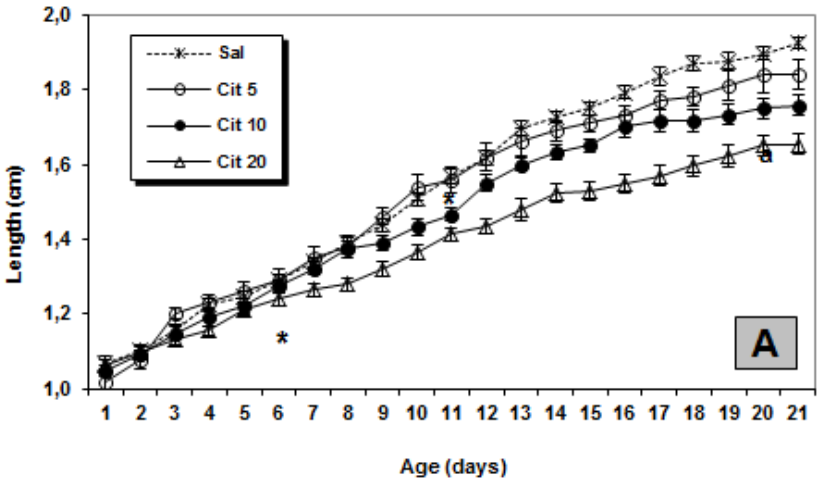
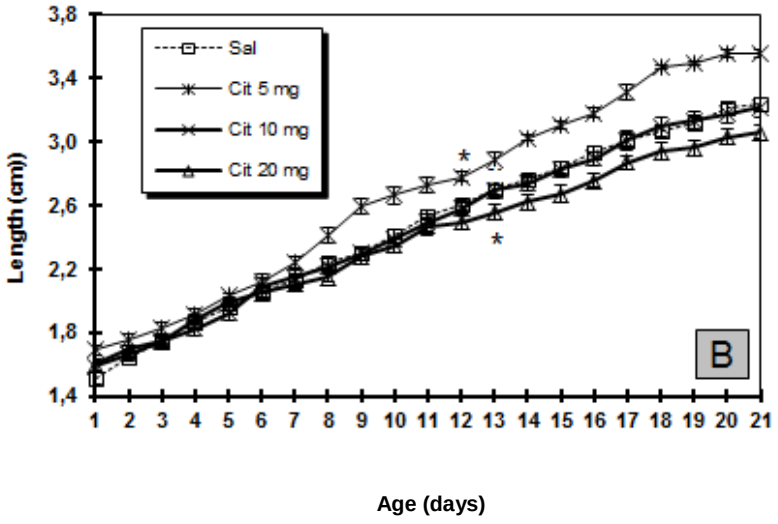


Figure 2 B - Anteroposterior head axis



5. 2 Segundo artigo

Título: **NEONATAL TREATMENT WITH FLUOXETINE DELAYS THE SOMATIC MATURATION AND REFLEX ONTOGENY**

Submetido para publicação como artigo original na revista: Pharmacology Biochemistry and Behavior-julho de 2004.

Neste segundo estudo, o objetivo foi investigar em ratos os efeitos do tratamento ou não, com o inibidor seletivo da recaptação da serotonina – fluoxetina - sobre o crescimento somático, a maturação de características físicas e o desenvolvimento de reflexos em ratos. Neste estudo observou-se que o tratamento com fluoxetina durante o periodo critico de desenvolvimento do cérebro, retarda a evolução ponderal, o crescimento corporal, altera o crescimento do crânio, retarda a maturação de características físicas e provoca atraso na maturação da maioria dos reflexos estudados.

Running Title: SSRI and ontogeny of the somatic maturation

NEONATAL TREATMENT WITH FLUOXETINE DELAYS THE SOMATIC MATURATION AND REFLEX ONTOGENY

Abstract

This study investigated the somatic maturation (growth indicators and time for appearance of physical features) and reflex ontogeny of treated neonate rats during the suckling period with different doses of fluoxetine (1mg, 5mg or 10mg/kg, sc, daily). Growth indicators measurements (body weight, axis of the head, longitudinal body length and tail length) were taken daily, from the 1st to the 21st postnatal day. The time of ear unfolding, auditory conduit opening, irruption of lower and upper incisors; and eye opening, was recorded..Besides the time for appearance (disappearance in the case of palmar grasp) of the reflexes righting, negative geotaxis, cliff avoidance, auditory startle response, vibrissa placing

free-fall righting and palm grasp was recorded. Body weight gain reduction and delay of physical features maturation as well a craniofacial anatomic distortion, were observed. A delay in the reflexes development was observed as well. The findings suggest that un increase 5-HT avaiability early in life could play a role in the retardation of the body growth and in weight gain as well as a delay in the maturation of the most reflexes responses.

Keywords: serotonin, growth indicators, SSRI, fluoxetine, reflexes.

1. Introduction

A number of selective serotonin reuptake inhibitors (SSRIs), have been described including femoxetine, zimelidine, citalopram and fluoxetine (Fuller, 1994). Evidence is consistent with the view that these SSRIs increases the concentrations of serotonin within the synaptic cleft by blocking its removal via the membrane-transporters (Fuller, 1995). Based on this evidence SSRIs have been routinely used to enhance serotoninergic activity in the brain (Toornivliet, 1996; Baumman, 1996). Experimental findings indicate that serotonin can influence embryogenesis and growth (Palén et al., 1979; Whitaker-Azmitia, 1991) by acting presumably as a developmental signal (Liu and Lauder, 1992) or as a neurotrophic factor as well (Yan et al., 1997). Furthermore, serotonin seems to play a role in regulating the development of mammalian brain through its action on producing serotonin neurons (Whitaker-Azmitia and Azmitia, 1986; Shemer et al., 1991; Whitaker-Azmitia, 1991) and in target tissues, innervated by serotonergic neurons (Lauder and Krebs, 1976; Lauder, 1990). During the pregnancy or the suckling period pharmacological manipulations can induce morphological and functional changes in the growth and development of nervous system (Manhães de Castro et al., 1993; Manhães de Castro et al., 2001. Studies have showed that treatment with SSRIs, reduces food intake and body weight in rats (Halford and Blundell,

1996; McCann et al., 1997). A hypophagic effect was observed following fluoxetine administration (Lightowler et al, 1996). As a consequence the possibility exists that the use of serotoninergic agents in the initial phase of life could influence other specific maturation processes duryng early development of the body. Therefore, investigating possible effects of serotoninergic system manipulation early in life is desirable. The objective of this study was to test the hypothesis that administration of fluoxetine – one of the most selective serotonin inhibitor (Hytell, 1994) - to rats during the suckling period induces changes on in the somatic development, in the physical features maturation and in reflex ontogeny.

2. Material and methods

2.1. Animals

Wistar rats coming from the colony of the Nutrition Department – Federal University of Pernambuco - Brazil were coupled, for obtaining litters. During gestation and until the end of the experiment, the animals were housed in polyethylene cages. Male pups from different mothers (n=18) were randomly distributed in litters of 6 neonates, 24 hours after the birth. Each litter was labeled with a mark of methyl violet solution in the skin, for identification during the whole experiment. Each litter was breastfed by one of the dams until the 21st post-natal day (birth day was considered as zero day). The animals were maintained at a room temperature of 23±1^{o}C, on a light-dark cycle of 12/12 hours (light on 6:00 a.m. to 6:00 p.m.) with free access to meal (Labina-Purina of Brazil) and water.

2.2. Pharmacological treatment and experimental groups

A blind study was performed so that the groups, were not identified during the experiment. The animals of the different groups were simultaneously appraised. According to the experimental treatment, four groups of suckling rats were designed as follows: Three groups received fluoxetine: group flu10 (n=34) (10mg/kg, sc); group flu5 (n=30) (5mg/kg, sc); group flu1 (n=30),

(1mg/kg, sc); and one control group received an equivalent volume of saline solution (n=23) (NaCl 0.9%, sc.). During the experiment, four neonate rats of the 10mg group and two of the flu5 group died. Therefore, 111 rats were evaluated. Fluoxetine hidrochloride (Sigma-USA) was dissolved in saline and injected in the concentration of 1ml/100g b.w. The treatment was applied daily from the 1st to the 21st postnatal day (suckling period) from 0:30 pm to 1:30 pm.

2.3 - Physical features and reflexes

The following physical features were observed daily from the 1º to 21º postuled day (suckling period): unfolding of the external pinnae of both ears to the fully erect position; auditory conduit opening – internal auditory conduit opening of both ears; incisor eruption – the first visible and palpable crest of upper and lower incisors; and eye opening – when any visible break in the covering membrane eyes was detected. The maturation age of a particular feature was defined as the day when it occured for first time. The reflex testing (according to Smart and Dobbing, 1971) was carried out daily also from the 1st to the 21st (box 1). Testing and observation were conducted between 10:00 a.m. and 1:00 p.m. The progress of the individuals was followed throughout the experiment**.** The time of appearance of each reflex was defined as the first day of its occurrence during a period of three consecutive days. The maturation age of a particular feature was defined as the day when it occurred for first time. Box 1 – Reflex tests (modified by Smart and Dobbing, 1971)

REFLEX	STIMULUS	RESPONSE
Palmar grasp - PG	Palm of forepaw stroked gently with a paper clip.	Flexion of digits. As maturation response, any or a very slight flexion must be seen. To this reflex, the disappearance date is registered.
Righting - R	Rat placed on back on a flat surface.	It turns over, to rest in ventral decubitus, with the four paws on the surface, in 10 seconds.
Vibrissa placing – VP	Rat held by the tail, head facing an edge of bench, vibrissa just touching vertical surface.	Lifts head and extends forepaws in direction of the bench, making oriented "walking" movements to go far from the edge, in 10 seconds.
Cliff avoidance - CA	Rat put on edge of bench, with nose and forefeet just over edge.	Withdrawal of head and both forefeet from edge, moving away from "cliff", in 10 seconds.
Auditory startle - AS	Sudden sound stimulus by percussion with a metalic stick in a metal surface.	Body retraction, with a transitory immobility. The stimulus was given twice in each test, with 1 minute interval.
Negative Geotaxis - NG	Rat placed with head downwards, on a 45 slope.	Turns to face up the slope, , at least ≥ 130º, in 10 seconds.
Free-fall righting - FR	Rat held by the paws, back downwards, is dropped from 30 cm on to cotton wool pad.	Turns body in mid-air, to land on all fours. All legs must be free of body on landing.

2.4 - Somatic growth

Somatic growth was assessed by body weight, body length, tail length, medio-lateral and anteroposterior head axis dimensions. These measurements were accomplished daily, from the 1st to the 21st life day between 8:00 a.m. and 9:00 a.m. as follows: the body weight was measured with a Fanem scale (São Paulo-SP, precision 100mg). The body length (distance between snout and the tail base). The tail length - (distance between tail tip and its base) as well as the cranium medio-lateral axis of the head-MLHA (distance between the ear holes) and the anteroposterior axis of the head- APHA (distance between the snout and the head-neck articulation) were measured with a Starret caliper rule (precision .05mm).

3 - Statistical analysis

Following preliminary testing to identify the normality of the distributions (Kolmogorov – Smirnov test) and variance homogeneity among the groups (Levene´s test), statistical analyses were performed. A two-way ANOVA for repeated measures followed by the post-hoc Tukey test were used to compare the growth indicators between each fluoxetine group and saline. The Kruskal-Wallis analysis of variance followed by the Dunn test was used to compare physical features and reflex development between each fluoxetine dose and saline group. The level of significance was $p < .05$. The Ethical Committee for Animal Experimentation (CEEA) for Federal University of Pernambuco-UFPE, being in agreement with National Institute of Health Guide for Care and Uses of Laboratory Animals (Publication no. 85-23, revised, 1985) approved the experimental protocol.

4 – Results

For somatic growth, the observed effects corresponded to different increases of all variables (body weight, body length, tail length, MLHA, and APHA), betweeen each fluoxetine group and the saline ones. For body weight it was found an effect of fluoxetine ($F_{3,107} = 36.23$; $p < .001$), day of life ($F_{20, 2140} = 3224.15$; $p < .001$) in addition to an interaction between these two factors ($F_{60, 2140} = 29.23$; $p < .001$). The body weight was lower for Flu 10 from 3rd to 21st day ($p <. 05$), for Flu5 from 4th to 21st day and for Flu1 from 4th day to 21st ($p <. 05$). This lower weight gain was larger for Flu10 than for Flu5 group and Flu1 group respectively, compared saline group (figure 1A).

For the tail length the statistical analysis shown un effect of fluoxetine ($F3,107=18.67$; $p < .001$) day of life ($F\ 20, 2140 =5204.21$; $p <. 001$) and interaction between fluoxetine and day of life ($F60, 2140 = 20.29$; $p <. 001$). Flu 10 group, showed a reduction from the 8th day until the 21st day ($p <.001$), but no difference in the Flu 5mg and Flu 1mg groups (Fig. 1B).

Fig. 1 A

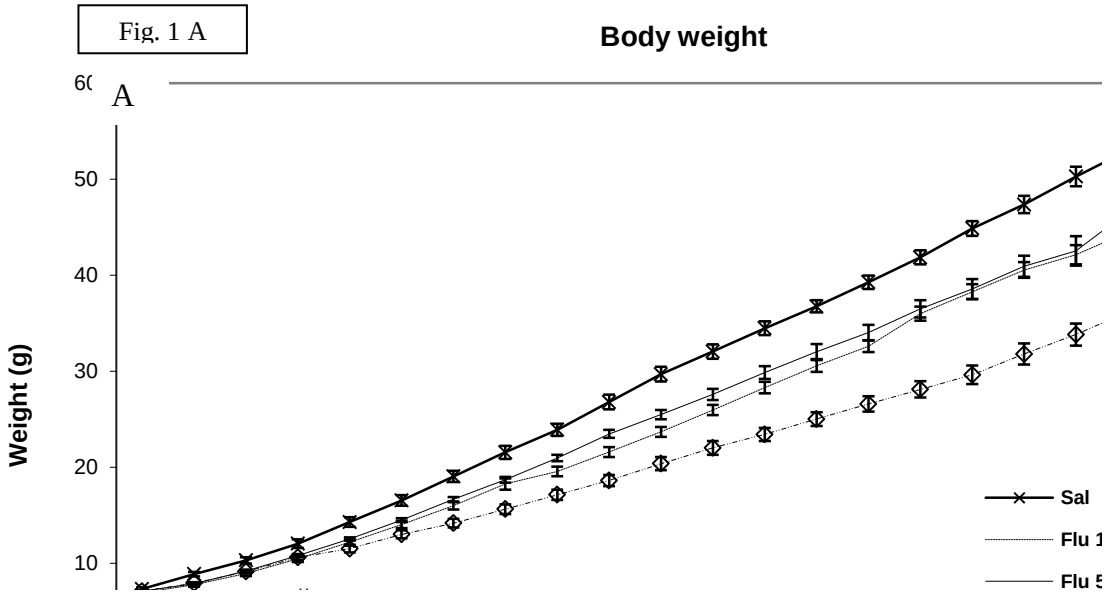
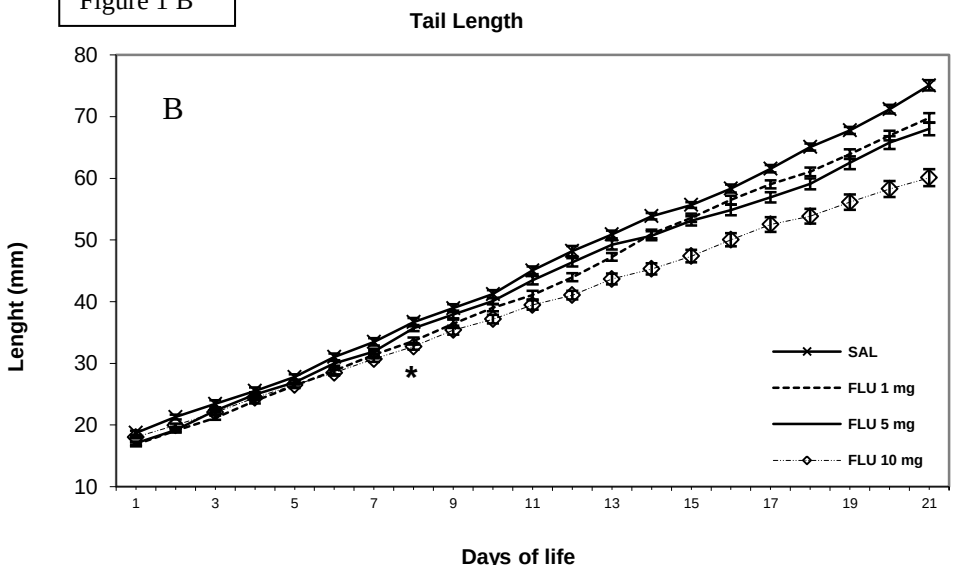


Figure 1 B



For body length an effect of fluoxetine ($F_{3,107}$ = 47.97; p< .001), day of life ($F_{20,\ 2140}$ = 4982.64; p< .001) and an interaction between fluoxetine and day of life ($F_{60,\ 2140}$ = 27.62; p< .001) were observed. Flu 10 and Flu 5 groups, showed a reduction from the 3rd day until the 21st day (p<.001). Flu 1 group retard from 5st day until the end of the experiment (Figure 2A).

For the medio-lateral head axis the statistical analysis found a effect of fluoxetine ($F_{3,\ 107}$ = 24.00; p< .001), day of life ($F_{20,\ 2140}$ = 3753.77; p<. 001) in addition to an interaction between fluoxetine and day of life ($F_{60,\ 2140}$ = 24.09; p< .001). Growth reduction of the medio-lateral

head axis in Flu10 group, starting at the 5th day until the 21st day, (p<.05). Flu5 and Flu1 groups presented reduction in this axis from the 11th until 21st day (p<. 05), (Figure 2B).

For anteroposterior head axis the statistical analysis indicated an effect of fluoxetine ($F_{3, 107}$ = 3.58; p< .016), day of life ($F_{20, 2140}$ = 5114.75; p< .001) in addition to an interaction between these factors ($F_{60, 2140}$ = 6.52 p< .001). A significant growth reduction of anteroposterior head axis in Flu 10 group, starting at the 13th day until the 21st day (p< .05) was observed. There was no difference for Flu1 and Flu 5 groups,compared to saline (Figure 2C).

Figure 2 A

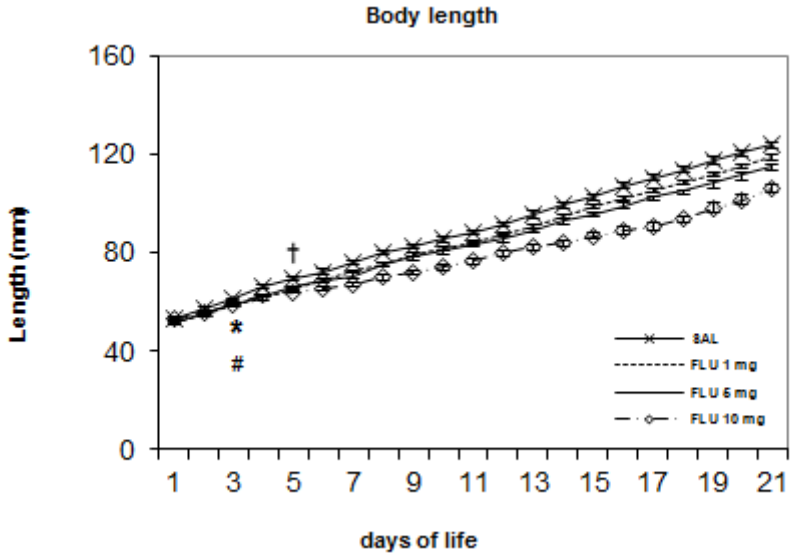
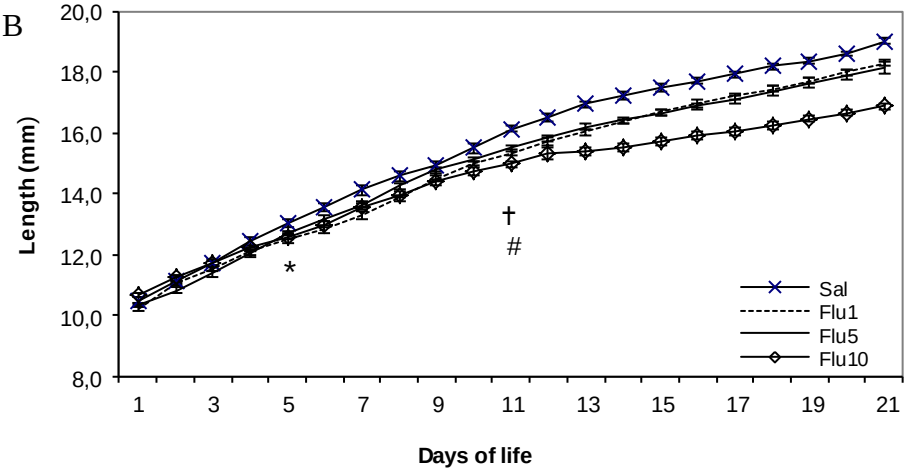
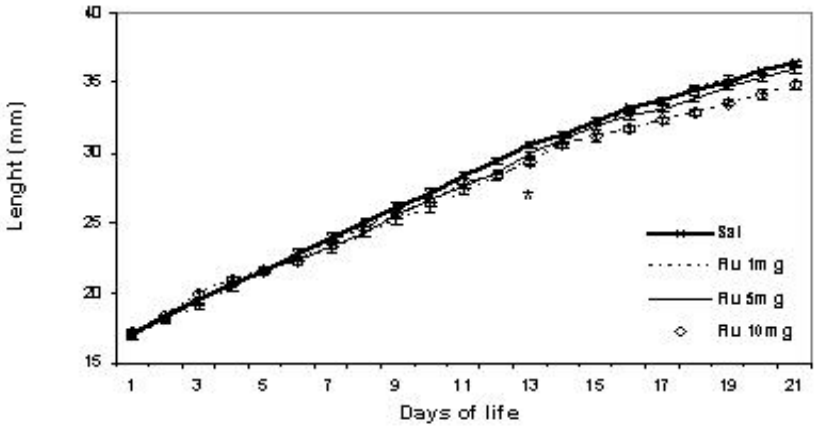


Figure 2B



Anteroposterior Head Axis

Figure 2C



The (*) indicate the begining of significant differences for Flu10 (P < 0.01): (#) for Flu 5 and (†) for Flu 1 (P < 0.05) that persisted to the end of the experiment.

The time for physical features maturation (Table 1) suffered statistically significant alterations in the ear unfolding (H=25.11, p<0.001); this physical feature delayed in flu5 group (p<0.05) compared to saline group. The eyes opening was changed between groups (H=24.91; p<0.001); a delay in this physical characteristic ocurring in the Flu5 and Flu10 group (p<0.05). The upper incisors irruption presented statistical difference between groups (H=38.99; p<.001); a retard was observed in Flu1, and Flu5 groups (p<0.05); flu10 group was antecipated in this physical characteristic (P<0.05). The lower incisors irruption and auditory conduit opening there was not a statistically signifficant difference among groups.

Reflex data are showed in table 2. The delay in time of appearance for palmar grasp was changed between groups (H= 39.69; p<0.001); a delay in Flu10 (p<0.05) was found; the righting presented statistical difference among groups H=22.26; p<0.001); Flu5 and Flu10 groups were antecipated (p<0.05); negative-geotaxis suffered change between groups (H= 22.18; p<0.001); this reflex delayed in Flu10 group (p<0.05); statistical difference among groups was observed in the vibrissa-placing reflex compared to saline (H=16.316; p<0.001); in this case a retard in flu10

group was verified (p<.05). The auditory-startle-response changed between groups (H=20.099; p<0.001); delays in Flu10, Flu5 and Flu1 groups were observed (p<0.05) when compared to saline. Free-fall righting did not show statistically signifficant difference among groups. Finally, the cliff-avoidance didn`t retard in anyone treated group compared to saline.

Table 1. Maturation (in days) of the physical features of suckling rats treated with fluoxetina from the 1st to the 21st day of life; d = days.* P <0.05; ** P <0.001.

PHYSICAL FEATURES	GROUPS							
	SALINE		FLUOXETINE					
			1 mg (d)		5 mg (d)		10 mg (d)	
	Median	(range)	Mediana	(range)	Median	(range)	Median	(range)
Eyes opening	14,0	(11,0-16,0)	13,0	(11,0-15,0)	14,0*	(13,0-17,0)	14,0*	(12,0-18,0)
Ear unfolding	2,0	(1,0-4,0)	3,0	(1,0-4,0)	3,0*	(2,0-4,0)	2,0	(1,0-5,0)
Auditory conduit opening	11,0	(10,0-14,0)	12,0	(11,0-13,0)	12,0	(12,0-13,0)	12,0	(9,0-16,0)
Irruption of the upper incisors	9,0	(6,0-12,0)	8,0*	(6,0-10,0)	9,0*	(8,0-11,0)	7,5*	(6,0-10,0)
Irruption of the lower incisors	11,0	(9,0-13,0)	11,0	(9,0-13,0)	12,0	(10,0-15,0)	11,0	(8,0-15,0)

Table 2. Maturation (in days) of the reflexes of suckling rats treated with fluoxetina from the 1st to the 21st day of life; d = days.* P <0.05; ** P <0.001.

REFLEXES	GRUPOS							
	SALINE		FLUOXETINE					
			1 mg		5 mg		10 mg	
	Median	(range)	Mediana	(range)	Median	(range)	Median	(range)
Palmar grasp	5,0	(3,0-8,0)	6,0	(4,0-10,0)	5,0	(4,0-8,0)	3,5*	(2,0-5,0)
Righting	9,0	(5,0-13,0)	9,0	(7,0-13,0)	6,5*	(3,0-11,0)	7,0*	(3,0-11,0)
Vibrissa placing	11,0	(8,0-14,0)	11,0	(9,0-14,0)	11,0	(7,0-14,0)	12,0*	(8,0-15,0)
Cliff avoidance	10,5	(6,0-16,0)	11,0	(7,0-15,0)	10,0	(8,0-15,0)	11,0	(7,0-18,0)
Negative geotaxis	12,5	(10,0-16,0)	12,0	(9,0-15,0)	13,0	(11,0-18,0)	15,0*	(9,0-19,0)
Auditory startle	11,0	(10,0-14,0)	13,0*	(12,0-16,0)	12,0*	(12,0-14,0)	12,0*	(11,0-15,0)
Free-fall righting	14,0	(11,0-17,0)	15,0	(10,0-17,0)	14,0	(12,0-17,0)	14,0	(12,0-17,0)

Discusion

Our study showed that chronic administration of fluoxetine during the critical period of rat brain development induced changes in the body growth and in reflexes development. Concerning body weight, the increase of drug's doses resulted in equivalent growth reduction. For craniofacial measures the head of the animals became wider in the lowest fluoxetine dose, unaltered in the midle dose and both shorter and narrower in the highest dose. Therefore, the head suffered shape distortions during development related to the dose administered. The sensitivity of vertebrate head growth to epigenetic manipulations has been well demonstrated (Herring, 1993; Dressino and Pucciarelli, 1997). Particularly, changes in craniofacial skeleton of developing rats submitted to a low protein diet were shown by Miller et al., (1999). These changes consisted of the shortening of skull dimensions, a result similar to the one experienced by the animals receiving the highest dose of fluoxetine in this present experiment. It is noteworthy that early protein malnutrition such as that studied by Miller et.al, (1999) is a condition known to increase the brain 5-HT levels (Morgane et al., 1993). Therefore it is pertinent to considerer the delayed development of the structures inside the skull (auditory conduit and lower incisors), observed in the present work, since such a delay could be associated to the irregular growth of animal head. Furthermore, the interaction effect between fluoxetine and time, indicates that the highest fluoxetine dose caused not only a reduction in the body weight gain, in the body length and the tail growth but this reduction began earlier than that observed in the two lowest doses groups. These changes deserve attention because they reveal the existence of growth mechanisms in body tissues, which are dose-sensitive to fluoxetine. Since this drug increases the serotonin release (Hytel, 1994) it may be suggested that serotoninergic mechanisms could be related to the observed effects. In addition, there is evidence that SSRIs and some serotoninergic agonists increase 5-HT release not only in neural tissues (Lauder, 1988; 1990; Buznikov, 1990) but also in non-neural ones (Shuey, 1992; Lauder, 1993). Interestingly, serotonin has a trophic action in the differentiation of several tissues

during prenatal and postnatal periods (Lauder, 1993; Moiseiwitsch and Lauder, 1996). Some studies in culture of mouse embryos treated with serotonin suggest an improved development of serotoninergic cells in the nervous tissue, as well as in cells of nasal prominence, epithelium covering the eye, optic vesicle and oral cavity (Lidov and Molliver, 1982; Wallace and Lauder, 1983; Hall, 1980, 1981; Thorogood, 1981; Lauder et al, 1988). These findings support the view that this amine plays a role in the control of epithelial-mesenchymal interactions during craniofacial morphogenesis (Shuey et al., 1993). Several of these events begin in the second week of life. In addition it was already demonstrated that the first serotoninergic neurons appear as soon as the 12^{th} to14^{th} pregnancy day (Lidov and Moliver, 1982). Therefore in that period the tissue may be able to respond to the fluoxetine challenge. Moreover, a 5-HT trophic role in the formation of this craniofacial structure, including the maturation of the tooth germ has been suggested (Moiseiwitsch and Lauder, 1996). Considering the teeth irruption acelerated in Flu 10 group, our findings are supported by these studies.

Alterations in the appearance of reflexes of early malnourished rats are supposed to indicate a correlation between biochemical and structural development during the ontogenesis of the nervous system (Adlard and Dobbing, 1971). Besides protein malnutrition induce an increase brain serotonin level (Morgane et al., 1992; Resnick et al., 1993). Serotonin immunoreactive neurons were observed in hypothalamus of embryos and neonate rats following pargillin and l-tryptophan pre-treatment, supposed to be related to 5-HT reuptake from extracelular enviroment (Ugromov et al, 1988). Reduced motor activity and running velocity of young rats treated with sertraline (another SSRI), was showed by Stenford et al., (2002). Our data agree with these findings since reflex activity is related with motor behaviors. Therefore we could suggest that in our study an improved 5-HT brain release provoked by fluoxetine, caused the delay of the reflexes. This could indicate that even in very young rats the 5-HT system is responsive to SSRI.

Althoug SSRIs are frequently used to treat maternal depression during pregnancy, the

effect on increased 5-HT agonists in the fetal humain brain remains unknown (Oberlander et al, 2002). However the repercussions of other neonatal manipulations - as for example, early malnutrition - on the development of the structural, neurochemical and functional integrity of the nervous cells are well known (Morgane et al., 1993). Undernourished rats present a delay in the development of reflexes, such as palm grasp, startle response and free-fall righting (Smart and Dobbing, 1971). Increased latency of the startle response in adult rats treated during the neonatal period with fluoxetine, was shown by Dow-Edwards (1996). This effect was reduced by m-chlorophenylpiperazine, a 5-$HT_{1B/2C}$ agent. This finding suggests the involvement of the receptors in the reflex elicitation.

However, the possibility exists that the effects observed in this experiment could be induced by malnutrition dependent on 5-HT anoretic mechanisms and not by the direct 5-HT action on the tissues. In fact, it is well demonstrated that hipophagia induced by fluoxetine, reduces body weight in adult rats (Halford and Blundell, 1996; Halford and Blundell, 2000) and body weight gain in young rats (Manhães de Castro et al., 2001).

Therefore we cannot rule out the hypothesis that malnutrition resulting from fluoxetine hipophagia induced the growth delay.

In conclusion, the present findings demonstrate that fluoxetine administered during brain growth spurt in rats, induces damages to body and head growth as well as alteration in the maturation time of reflexes and physical features. The magnitude of these alterations depend on fluoxetine dose.

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5.3 Terceiro artigo

Título: **SERTRALINE DELAYS THE SOMATIC GROWTH AND REFLEX ONTOGENY IN NEONATE RATS.**

Submetido para publicação como artigo original na revista: Physiology and Behavior - julho de 2004.

O objetivo deste terceiro trabalho, foi investigar em ratos neonatos os efeitos do tratamento ou não, com o inibidor seletivo da recaptação da serotonina - sertralina sobre o crescimento somático, a maturação de características físicas e o desenvolvimento de reflexos em ratos. Neste estudo observou-se que o tratamento com setralina durante o período critico de desenvolvimento do cérebro, retarda o crescimento do corpo dos animais, o ganho de peso corporal, altera o crescimento da estrutura cranial, retarda a maturação de características físicas e provoca atraso na maturação da maioria dos reflexos estudados.

Running Title: Sertraline, somatic growth, reflexes and skull

SERTRALINE DELAYS THE SOMATIC GROWTH AND REFLEX ONTOGENY IN NEONATE RATS.

Abstract

This study investigated the somatic maturation and ontogeny of reflexes in neonate rats treated during the suckling period with sertraline (Sert) in the doses (5mg, 10mg ou 15mg/kg s.c, daily) and distilled water (Dw) (1ml/kg/bw). Growth indicators (body weight, brain weight, axis of the head and tail length), were measured daily, from the 1st to the 21st postnatal day. The reflexes (righting, free-fall righting, negative geotaxis, cliff avoidance, auditory startle response, vibrissa placing and palm grasp) and physical features maturation (ear unfolding; auditory conduit opening, eruption of the lower incisors and eye opening), were recorded in days of life. All the groups were compared to Dw. The body weight gain reduced in all groups. A delay was observed in the growth of the body lenght in all groups as

well. The speed of growth in the tail length was reduced in higher doses. The medio-lateral head axis reduced in Sert 15mg and Sert5mg doses. Otherwise, Sert10mg had a temporary acceleration in this growth. The anteroposterior head axis had a delay in the growth in all groups as well. The physical features maturation delayed in highest doses. The palmar grasp reflex (disappearance) was retarded in Sert10mg dose; cliff avoidance advanced in Sert10 group; negative-geotaxis and free-fall righting retarded in Sert15 group. The findings suggest that altered serotoninergic system activity induced by sertraline early in life could play a role in the retard of the somatic growth ontogeny as well as a delay in the maturation of some reflexes.

Keywords:, 5-HT, somatic growth, SSRI, sertraline, reflex development, and physical features.

1. Introduction

Serotonin influences embryogenesis and growth (Palén et al., 1979; Whitaker-Azmitia, 1991) by acting presumably as a developmental signal (Liu and Lauder, 1992) or as a neurotrophic factor (Yan et al., 1997). Serotonin (5-HT) is among the early neurotransmitters ocurring in mammal embryos (Lauder, 1990, 1993). The tryptophan hydroxylase, an enzyme of the 5-HT synthesis system, has been detected in 15 days embryos, after serotonin was identified in the organism (Lauder et al, 1981). There is a fast increase of serotonin during prenatal and postnatal periods, reaching adult levels at the end of the third week of life (Lidov and Molliver, 1982). The time course of these events is intimately related to synaptogenesis (Jacobs and Azmitia, 1992). Besides, serotonin participates of multiple functions by exercising modulatory effect as a neurotransmiter (Jacobs and Azmitia, 1992; Aghajanian and Marek, 1997). A recent study demonstrate that neonatal rats treated with 5,7 dihydroxytryptamine (5,7-DHT) presented reduced levels of 5-HT and 5-HIAA in frontal cortex, striatum and hyppocampus when adults. However, did not affect dopaminergic neurons. (Kostowsky and Krzáscik, 2003).

Environmental insults such as early malnutrition (Morgane et al 1993) and neonatal pharmacological manipulation (Manhães de Castro et al., 1993; Manhães de Castro et al., 2001) may provoke alterations in the 5-HT neurotransmission system with several consequences for the organism. Many antidepressants, such as fluoxetine, citalopram and clomipramine interfere with food intake behavior and perform their actions through the inhibition of the 5-HT synaptic reuptake (Blundell and Lathan 1980; Sugrue in revision: 1987; Baumann et al 1996). Chronic administration of clomipramine to rats, during the neonatal period, resulted in adult behaviors similar to that observed in human depression (Mirmiran et al., 1981; Vogel et al., 1990). In our laboratory, neonate rats treated with citalopram reduced the body weight gain during the drug administration period and aggressiveness in adult life. (Manhães de Castro et al, 2001). These evidences sustain the hypothesis that pharmacological insults early in life can modify the maturation of serotoninergic neurotransmission, inducing neurobehavioral changes (Vogel et al., 1990).

Among the selective serotonin reuptake inhibitors, sertraline is one of the most potent (Koe et al,1983). The main objective in this study was to test the hypothesis that administration of sertraline to rats, during the suckling period, induces changes on somatic and sensory-motor development.

2. Material and methods

2.1. Animals

Wistar male rats coming from the colony of the Nutrition Department – Federal University of Pernambuco - Brazil were coupled, to obtain litters. During gestation until the end of the experiment, the animals were housed in polyethylene cages. Male pups from different mothers (n=19) were randomly distributed in litters of 6 neonates, 24 hours after birth. Each pup was labeled with a mark of methyl violet solution on the skin, for identification during the

experiment. Each litter was breastfed by one of the dams until the 21st postnatal day (birth day was considered as zero day). When necessary, female rats were included to litters to complete 6 pups but they were not used in the tests. The animals were maintained at a room temperature of 23±1°C, on a light-dark cycle of 12/12 hours (lights on 6:00 a.m. to 6:00 p.m.) with free access to food (Labina-Purina of Brazil) and water.

2.2 Pharmacological treatment and experimental groups

A blind experiment was performed to prevent identification of the experimental groups. The animals of the different groups were simultaneously evaluated. According to the experimental treatment, four sertraline groups (Sert) of suckling rats were distributed as following: Three groups received different doses of sertraline: group Sert5 (5mg/kg, sc, n=25); group Sert10 (10mg/kg, sc, n=27); group Sert 15, (15mg/kg, sc, n=17); one control group received an equivalent volume of distilled water (Dw, n=28). During the experiment, eight rats of the Sert 15 group and three of the Sert10 group were females; Sert 5 group had four female and one rat died; Two rats of Dw group were female Therefore, 97 rats were evaluated during the complete experiment. Time of physical features maturation and somatic growth was studied. Sertraline (hydrochloride, Pfizer) was dissolved in distilled water and injected in the concentration of 1ml/100g b.w. The treatment was done daily from the 1st to the 21st postnatal day (suckling period).

2.3 Phisycal features and somatic growth

The observation of the physical features was made according to Smart and Dobbing (1971a) and carried out daily in the suckling period between 10:00 a.m. and 12:00 a.m. until maturation of the variables. The following physical features were observed: unfolding of the external pinnae of both ears to the fully erect position; auditory conduit opening – internal auditory conduit opening of both ears; incisor eruption – the first visible and palpable crest of lower incisors; and eye opening – when any visible break in the covering membrane of both eyes

was detected. Maturation age of a particular feature was defined as the day when it occurred for the first time.

2.4 Somatic growth

Somatic growth was assessed by body weight, body length, tail length, medio-lateral and anteroposterior head axis measurements. These measurements were done in each animal, from the 1st to 21st postnatal day between 1:00 p.m. and 2:00 p.m. as it follows: the body weight was measured with a *Marte* scale (accuracy 100mg). The longitudinal body growth (distance between the snout and the base tail), tail length (distance between tail tip and its base) as well as medio-lateral head axis, MLHA (distance between the ear holes) and the anteroposterior head axis, APHA (distance between snout and head-neck articulation) were measured with a Starret caliper rule (.05mm precision).

2.5 Reflex testing

The reflex tests (according to Smart and Dobbing, 1971) were carried out daily from the 1st to the 21st postnatal day (Figure 1). Testing and observation were conducted between 10:00 a.m. and 1:00 p.m. The progress of the individuals was followed throughout the experiment**.** The time of appearance of each reflex was defined as the first day of its occurrence during a period of three consecutive days.

Figure 1

REFLEX	STIMULUS	RESPONSE
Palmar grasp - PG	Palm of forepaw stroked gently with a paper clip.	Flexion of digits. As maturation response, any or a very slight flexion must be seen. To this reflex, the disappearance date is registered.
Righting - R	Rat placed on back on a flat surface.	It turns over, to rest in ventral decubitus, with the four paws on the surface, in 10 seconds.
Vibrissa placing – VP	Rat held by the tail, head facing an edge of bench, vibrissa just touching vertical surface.	Lifts head and extends forepaws in direction of the bench, making oriented “walking” movements to go far from the edge, in 10 seconds.

Cliff avoidance - CA	Rat put on edge of bench, with nose and forefeet just over edge.	Withdrawal of head and both forefeet from edge, moving away from "cliff", in 10 seconds.
Auditory startle - AS	Sudden sound stimulus by percussion with a metalic stick in a metal surface.	Body retraction, with a transitory immobility. The stimulus was given twice in each test, with 1 minute interval.
Negative Geotaxis - NG	Rat placed with head downwards, on a 45 slope.	Turns to face up the slope, , at least ≥ 130º, in 10 seconds.
Free-fall righting - FR	Rat held by the paws, back downwards, is dropped from 30 cm on to cotton wool pad.	Turns body in mid-air, to land on all fours. All legs must be free of body on landing.

(reflex tests modified from Smart and Dobbing, 1971)

3 - Statistical analysis

Following preliminary testing to identify distributions normality and variance homogeneity among the groups, statistical analyses were performed. A two-way ANOVA for repeated measures (from 1st to 21st day for body weight, head axis and tail length), followed by the post-hoc Dunn test, was used to compare growth indicators between each sertraline group and distilled water group. Kruskal-Wallis analysis of variance followed by the Dunn test were used to compare physical features between each sertraline dose and saline group. The level of significance was $p<.05$.

The experimental protocol of this paper was approved by the Ethical Committee for Animal Experimentation (CEEA) of Federal University of Pernambuco, being in agreement with the National Institute of Health guide for Care and Uses of Laboratory Animals (Publication no. 85-23, revised, 1985).

4. Results

4.1 Somatic growth

Regarding to somatic growth, ANOVA identified main effects for time and treatment in addition to interaction between these factors. For body weight the statistical analysis showed an effect of sertraline ($F_{3, 92}$ = 11,62, p<.001), and day of life ($F_{20, 1840}$ = 3890,74 p<.001) as well as a sertraline versus day of life interaction ($F_{60, 1840}$ = 8.63, p<.001). Body weight gain (Figure 1A) was reduced in sert15 and sert10 from 4th to 21st day (p<. 001) when compared to distilled water from 6th to 21st group. This lower weight gain was more pronounced for Sert15 and Sert10.

For body lenght (Fig. 1B), statistical analysis indicated a main effect of sertraline ($F_{3,92}$ =25.87, p<. 05) and day of life ($F_{20, 1840}$ = 4622.97, p<. 001) in addition to an interaction between sertraline versus day of life ($F_{60, 1840}$ = 9.19, p<. 001). An unsatisfactory body lenght was observed in Sert15 group, from the 4st day until the 21st day (p<. 001); as well in Sert10 and Sert5 group from 6 th to 21st day compared with Dw.

For the tail length (Fig.1C), a main effect of sertraline ($F_{3, 92}$ =5.60, p<. 001) and day of life ($F_{20, 1840}$ =6059.8, p<. 001) as well as an interaction between sertraline versus day of life ($F_{60, 1840}$ =5.31, p<. 001) were observed. An unsatisfactory growth of the tail length in Sert15 group was found, from the 11st until the 21st day (p<. 001); however Sert10 and Sert5 groups did not differ from distiled water.

Figure 1A

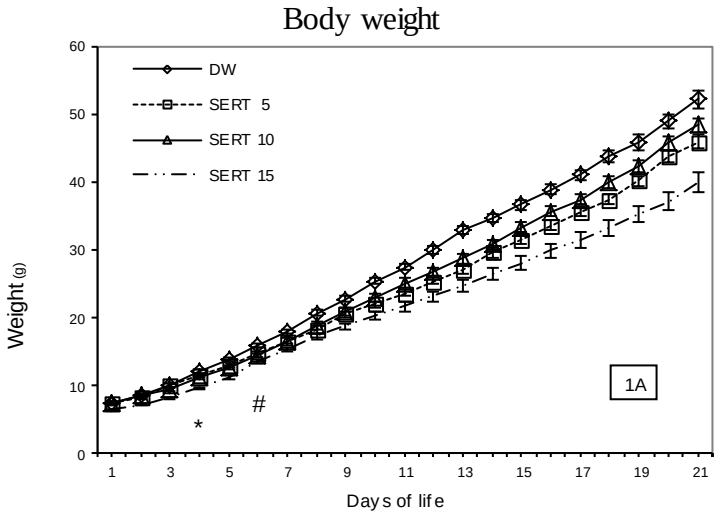


Figure 1B

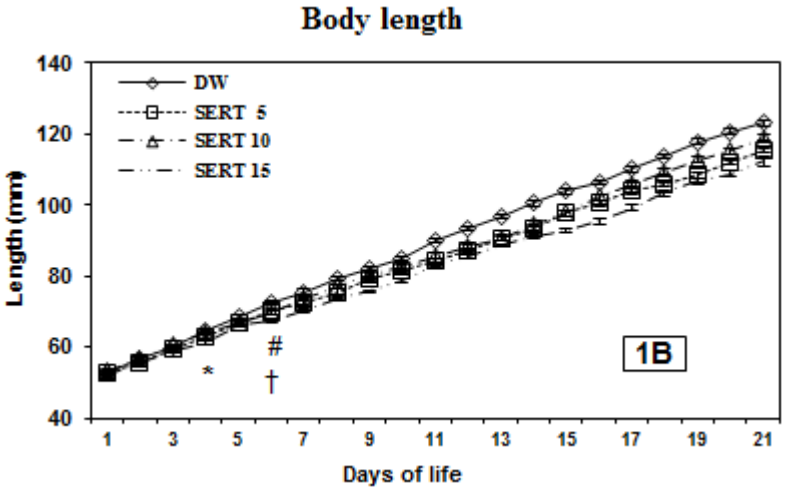
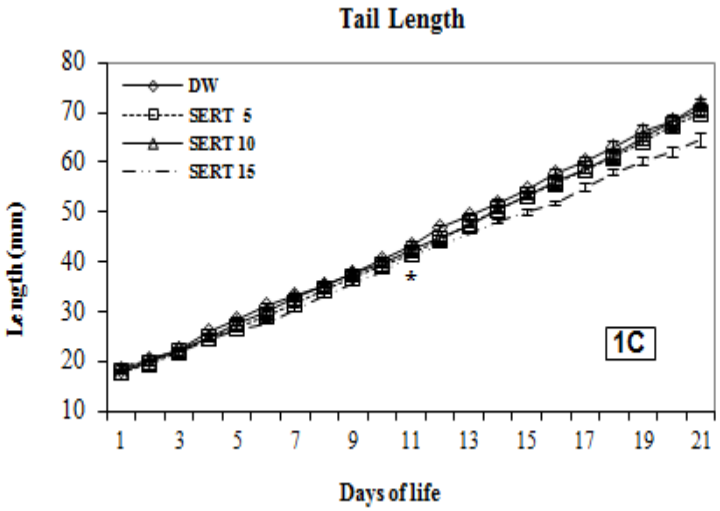
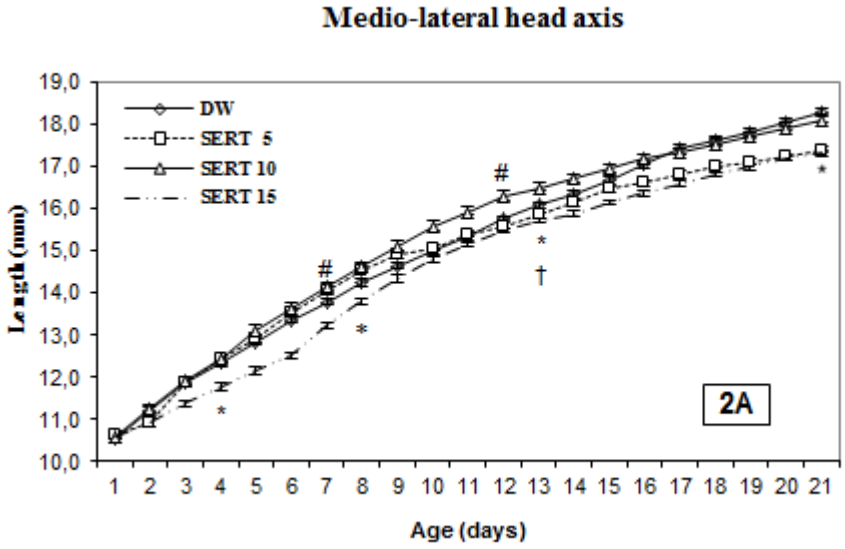


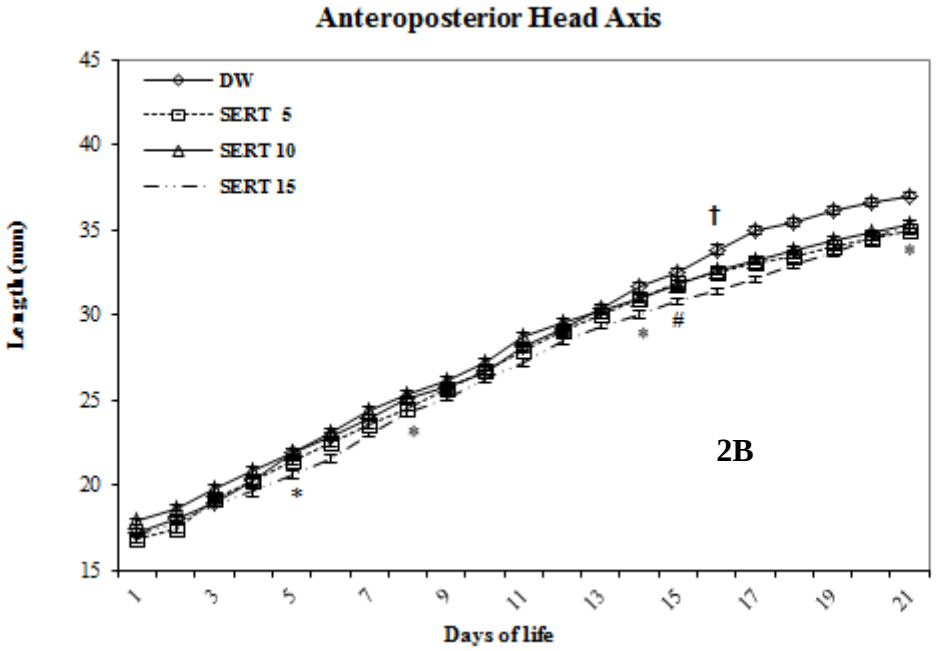
Figure 1C



Concerning the medio-lateral head axis, ANOVA, showed an effect of sertraline ($F_{3,92} = 3.56$, $p<.01$) and of day of life ($F_{20, 1840} = 146.90$, $p<.001$); an interaction sertraline versus day of life ($F_{60, 1840} = 1.8097$, $p<.001$) was observed as well. Growth reduction of the medio-lateral head axis in Sert15 group, starting at the 4rd to 8th and 13th until 21st day ($p<.05$), and in Sert5 group, starting at 13nd day until 21st day ($p<.001$) were detected. Otherwise, Sert10 acelerated the growth from 7th until 12th remaining such as the distiled water group in the other days. Moreover, the reduced growth of this axis was more pronounced for the 15mg dose than for the 5mg dose. (Fig. 2A).

There was also a significant (ANOVA following Dunnet test growth change of the anteroposterior head axis - APHA: sertraline ($F_{3,92} = 6.32$, $p<.001$), day of life ($F_{20, 1840} = 1247.12$, $p<.001$); interaction between sertraline and day of life ($F_{60, 1840} = 2.90$, $p<.001$). Comparing the sert15 group with Dw group, a reduction of this axis began on the 5th day until the 8st day ($p<. 05$) and from14th at 21st ($p<.05$) was observed. A reduction of this axis in Sert10 group from 15th to 21st day and in Sert 5 group from 16th to 21 th ocurred ($p<. 05$). (Fig. 2B).


Medio-lateral head axis
DW
SERT 5
SERT 10
SERT 15
Length (mm)
Age (days)
2A


Anteroposterior Head Axis
DW
SERT 5
SERT 10
SERT 15
Length (mm)
Days of life
2B

4.2 Physical features maturation and reflex testing

For physical features maturation, Kruskal-Wallis analysis indicated significant changes for the ear unfolding (H =32,763, p < 0.001); and auditory conduit opening (H =31,139, p < 0.001) as well in the lower (H = 13.256, p < 0.004) and upper incisors irruption retarded (H = 16.630, p< 0.004) in sertraline group in all doses when compared to Dw. Multiple comparisons of these features among treatment groups (Dunn test) showed that ear unfolding delayed in Sert5, Sert10 and Sert 15 groups (P < 0.05); as well the auditory conduit opening was delayed in the Sert5 Sert10 and Sert 15 groups (P < 0.05). Incisor irruption was delayed in the Sert 10 and sert15 group (p < 0.05) groups. For eye opening (H = 14.027, p < 0.004) there was no significant difference (Fig. 3A).

Figure 3A

PHYSICAL FEATURES	GROUPS							
	DISTILED WATER		SERTRALINE					
			5 mg (d)		**10 mg** (d)		**15 mg** (d)	
	Median	(range)	Median	(range)	Median	(range)	Median	(range)
Ear unfolding	2,0	(1-3)	3,0	(2-4)*****	3,0	(2-3)*****	3,0	(2-4)*****
Auditory conduit opening	11,0	(10-12)	12,0	(10-13)*****	13,0	(11-14)*****	12,0	(10-13)*****
Irruption of the upper incisors	8,5	(6-9)	9,0	(7-10)	9,0	(7-10)	9,0	(8-13)*****
Irruption of the lower incisors	11,5	(8-13)	11,0	(10-14)	12,0	(10-14)*****	12,0	(10-14)*
Eyes opening	13,0	(12-14)	13,0	(12-16)	13,0	(11-14)	14,0	(11-15)

d =days of life

For reflex ontogeny, Kruskal-Wallis analysis indicated significant changes in the cliff avoidance (H=22.385, p < 0,001), negative geotaxis (H= 39.997, p < 0,001) and free- fall righting (H=13.044, p < 0,005). Multiple comparisons of these reflex among treated groups (Dunn test) showed that the cliff avoidance advanced in Sert10 (p<.05), negative-geotaxis (p<.05) delay in Sert15 group and free-fall righting retarded in Sert15 group (p<.05). The clif avoidance

antecipated in Sert10 ($p < .05$). Do not observed delay in palmar grasp, righting, startle response and vibrissa-placing reflex in any doses, compared to Dw. (Fig. 3B).

Figure 3B

REFLEXES	GROUPS							
	DISTILED WATER		SERTRALINE					
			5 mg (d)		10 mg (d)		15 mg (d)	
	Median	(range)	Median	(range)	Median	(range)	Median	(range)
Palm grasp	4,0	(3-6)	5,0	(3-8)	6,0	(3-8)*	5,0	(3-6)
Righting	9,0	(6-12)	9,0	(7-13)	9,0	(5-12)	9,0	(7-14)
Vibrissa placing	12,0	(10-15)	11,0	(8-14)	11,0	(9-15)	12,0	(10-15)
Cliff avoidance	12,0	(7-15)	12,0	(8-14)	10,0	(5-13)*	13,0	(9-15)
Negative Geotaxis	12,0	(10-13)	13,0	(9-17)	12,0	(10-14)	15,0	(11-16)*
Startle response	12,0	(11-13)	12,0	(12-16)	13,0	(10-16)	13,0	(12-13)
Free fall righting	14,0	(11-17)	14,5	(12-17)	14,0	(12-17)	15,0	(13-17)*

DISCUSSION

Our findings showed that chronic administration of sertraline during the critical period of rat brain development induced changes in the growth and in the development. In general the drug impaired the somatic growth measures and body weight. As for body weight the increase of drug's doses resulted in equivalent growth alteration of the body length, of the tail length and axis of the head. In relation to the craniofacial measures, the treatment with sertraline provoked a distortion in the head's growth depending on the dose. Since the treatment with sertraline committed the maturation of some reflexes it is likely that structural alterations have affected the nervous function

The sensitivity of vertebrate head growth to epigenetic manipulations has been well demonstrated (Herring 1993; Dressino & Pucciarelli (1997). The action of the drug upon the serotoninergic mechanisms might be responsible for these effects since SSRIs increase the

serotonin release in the synaptic cleft (Hytel, 1994). Shuey et al (1993) demonstrated alterations in the craniofacial development in embryos, manipulating 5-HT_{1A} receptor with the specific serotoninergic agonist 8-OH-DPAT. Some of these changes consisted of shortening of skull dimensions, a result similar to that experienced by the animals receiving 15 mg of sertraline in the present experiment.

Therefore it is pertinent to considerer the delayed development of the structures inside the skull (ear unfolding and auditory conduit) and face (upper and lower incisors), in this study, because such a delay might be associated to the head growth reduction also here observed. In addition, the interaction between sertraline and time, indicates that the highest sertraline dose caused not only a reduction of body weight gain, of body growth and of tail growth but also that this reduction began earlier than in the two lowest doses. These changes deserve attention because they reveal the existence of growth mechanisms in body tissues, which are dose-sensitive to sertraline. Since this drug increases serotonin release (Hyttel 1994), it may be suggested that serotoninergic mechanisms may be responsible for the observed effects. Therefore during sukling period, the tissue may be able to respond to the sertraline challenge. Moreover, a 5-HT trophic role in the formation of the craniofacial structure, including the maturation of the tooth germ, was also already described (Moiseiwitsh and Lauder 1996). Our findings are supported by these studies considering teeth eruption delayed in the sertraline groups. Also, there is evidence that SSRIs and some serotoninergic agonists as well increase 5-HT release not only in neuronal tissues (Lauder et al, 1988; Lauder 1990; Buznikov 1990) but in non-neuronal ones as well (Shuey et al 1993; Lauder 1993). Interestingly, serotonin has a trophic action in the differentiation of several tissues during the prenatal and postnatal periods (Lauder 1993; Moiseiwitsh and Lauder, 1996) by acting as a neurotrophic factor (Yan et al., 1997). Some studies in culture of mouse embryos treated with serotonin suggest an improved development of serotoninergic cells in the nervous tissue, as well as cells of the nasal prominence, epithelium covering the eye, optic vesicle and oral

cavity (Lauder et al, 1988; Lidov and Molliver 1982) These findings indicate that this amine play a role in the control of the epithelial-mesenchymal interactions during craniofacial morphogenesis (Hall 1981; Shuey et al 1993). Serotoninergic treatment also caused malformation both in nerve and bone structures of mice embryo (Lauder and Krebs 1993). Several of these events begin in the second week of life and the first serotoninergic neurons appear soon as the 12^{th} to 14^{th} days of gestation (Lidov and Molliver 1982) Thus during suckling the tissue may be able to respond to the sertraline challenge. Moreover, a 5-HT trophic role in the formation of craniofacial structures, including maturation of the tooth germ, was also observed (Moiseiwitsh and Lauder, 1996). Our findings are supported by these studies considering teeth eruption delayed in the sertraline groups.

In the present study, besides the damages in growth parameters, the possible increased serotonin level induced to sertraline, provoked a delay in the reflexes. Reduced levels of 5HIAA were found in the brain of rats after chronic treatment with sertraline (Kelly and Leonard, 1994) and other SSRIS (Fuller et al., 1988; Koe et al., 1983). These points out what suggest an increased 5-HT following sertaline treatment. It is noteworthy that early protein malnutrition is a condition known to increase brain's 5-HT levels (Morgane et al., 1993). It is known that alterations in the appearance of reflexes indicate a correlation between biochemical and structural development and ontogenesis of the nervous system (Adlard and Dobbing, 1971; Dow-Edwards 1996). Undernourished rats present a delay in the development of reflexes, such as palm grasp; startle response and free-fall righting (Smart and Dobbing, 1971b). Besides, the neonatal treatment of rats with a another SSRI, LU 10-134-C, induced an increased immobility time and an altered forced swimming behavior (Hansen et al.,1997). On the other hand, it is known that the 5-HT system has an important role in the regulation of appetite and that drugs which increase extracellular concentration of the 5-HT, like SSRIs, are effective anorexic agents in adult rats (Kelly and Leonard, 1994); Ishii et al., 2003). Thus, the persistent reduction in the weight gain observed in this study, in all sertraline doses, seems to

indicate that permanent alterations in the serotoninergic system have induced this damage. However, we can not exclude the effect of Sertraline *per se* since that recent studies measuring the time of imobillity in forced swimming test found a reduction that was not antagonized by depletion of 5-HT induce by 5,7- DHT (Kostowski and Krzáscik, 2003). So, we cannot rule out a similar action of sertraline since the delayed the gain of body weight and reflex ontogenesis. In our experiment deserve attention the fact that changes in appearance of some reflexes`, occurred at the same time or even before changes in body measurements. This suggests that influence of 5-HT in these distinct processes.

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Legends

Figure 1A- Body weight of suckling rats from the 1st to 21st day of life, treated with solutions (1ml/100g b.w., s.c.) sertraline (Sert): Sert 5mg/kg s.c. (n=25): Sert 10mg (n=27), Sert 15mg (n=17) or distiled water (Dw, n=27). Comparisons were made by two-way ANOVA for repeated measures: sertraline ($F_{3, 92}$ = 11,62, p<. 001), day of life ($F_{20, 1840}$ = 3890,74 p<. 001) interaction sertraline versus day of life ($F_{60, 1840}$ = 8.63, p<.001). Comparisons (Dunnett test) between each sertraline dose and saline: each point represents mean, value. The simbols * = Sert15 , # = Sert10 and † = Sert5 indicate beginning of significant difference (p<.05) until the end of the experiment.

Figure 1B- Body length of suckling rats from the 1st to 21st day of life, treated with solutions (1ml/100g b.w., s.c.) sertraline (Sert): Sert 5mg/kg s.c. (n=25): Sert 10mg (n=27), Sert 15mg (n=17) or distiled water (Dw, n=27). Comparisons were made by two-way ANOVA for repeated measures: ($F_{3,92}$ =25.87, p<. 001) and day of life ($F_{20, 1840}$ = 4622.97, p<. 001) in addition to an interaction between sertraline versus day of life ($F_{60, 1840}$ = 9.19, p<. 001).

Comparisons (Dunnett test) between each sertraline dose and Dw: each point represents mean, value. Each point represents mean value. The simbols * = Sert15 , # = Sert10 and † = Sert5 indicate beginning of significant difference (p<. 05) until the end of the experiment.

Figure 1C- The tail length (TL) of suckling rats from the 1st to 21st day of life, treated with solutions (1ml/100g b.w., s.c.) sertraline (Sert): Sert 5mg/kg (n=25): Sert 10mg (n=27), Sert 15mg (n=17) or distilled water (Dw, n=27). Comparisons were made by two-way ANOVA for repeated measures: sertraline ($F_{3, 92}$ =5.60, p<. 001) and day of life ($F_{20, 1840}$ =6059.8, p<. 001) in addition to an interaction between sertraline versus day of life ($F_{60, 1840}$ =5.31, p<. 001). Comparisons (Dunnett test) between each sertraline dose and saline: each point represents mean, value. The simbol * = Sert15, indicate beginning of significant difference (p<. 05) until the end of the experiment (Figure 2A).

Figure 2A- Medio-lateral head axis (MLHA), of suckling rats from the 1st to 21st day of life, treated with solutions (1ml/100g b.w., s.c.) sertraline (Sert): Sert 5mg/kg (n=25): Sert 10mg (n=27), Sert 15mg (n=17) or distiled water (Dw, n=27). Comparisons were made by two-way ANOVA for repeated measures: sertraline ($F_{3,92}$ = 3.56, p<.001) and day of life ($F_{20, 1840}$ = 146.90, p<.001); an interaction sertraline versus day of life ($F_{60, 1840}$ = 1.809, p<. 001). Comparisons (Dunnett test) between each sertraline dose and saline: each point represents mean, value. each point represents mean, value. The simbols * = Sert15 (4th to 8th and 13th to 21stdays) and † = Sert5 indicate beginning of significant difference (p<. 05) until the end of the experiment or statistical significance interval.

Figure 2B-Anteroposterior head axis (APHA) of suckling rats from the 1st to 21st day of life, treated with solutions (1ml/100g b.w., s.c.) sertraline (Sert): Sert 5mg/kg (n=25): Sert 10mg (n=27), Sert 15mg (n=17) or distiled water (Dw, n=27). Comparisons were made by two-way ANOVA for

repeated measures: sertraline ($F_{3,95}$ = 6.32, p <. 001), day of life ($F_{20, 1900}$ = 1247.12, p <. 001); interaction between sertraline and day of life ($F_{60, 1900}$ = 2.90, p<.001). Comparisons (Dunnett test) between each sertraline dose and saline: each point represents mean, value. (p<. 05). The simbols * = Sert15 , # = Sert10 and † = Sert5 indicate beginning of significant difference (p<.05) until the end of the experiment.

Figure 3A- Maturation of physical features of the suckling rats treated from the 1st to the 21st day of life with solutions (1ml/100g b.w. s.c) Sertraline (Sert) 5mg (n=25), 10mg (n=27) and 15 mg/kg s.c. (n=17) or distiled water (Dw, n=27). Comparisons were made by the Kruskal-Wallis analysis of variance followed by the Dunn´ test to compare physical features between each sertraline dose and Dw group. Significant difference, *p < .05.

Figure 3B. Reflex maturation of suckling rats from the 1st to 21st day of life, treated with solutions (1ml/100g b.w., s.c.) sertraline (Sert): Sert 5mg/kg (n=25): Sert 10mg (n=27), Sert 15mg (n=17) or distiled water (Dw, n=27): Comparisons were made by the Kruskal-Wallis analysis of variance followed by the Dunn´ test to compare physical features between each sertraline dose and Dw group. Significant difference, *p< .05.

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5.4 Quarto artigo

Título: **NEONATAL TREATMENT WITH THE SEROTONIN SELECTIVE REUPTAKE INHIBITOR, CITALOPRAM, DELAYS REFLEX ONTOGENY.**

Submetido para publicação como artigo original na revista: Archives General Psychiatry- julho de 2004.

O objetivo deste trabalho foi investigar efeito da administração neonatal, com o inibidor seletivo da recaptação da serotonina - citalopram sobre o peso corporal, e o desenvolvimento de reflexos em ratos. Os resultados obtidos mostram que os tratamentos com citalopram durante o período critico de desenvolvimento do cérebro, retarda a evolução ponderal e provoca atrasos na maturação da maioria dos reflexos estudados.

Running Title: SSRI and ontogeny of reflexes

REFLEX ONTOGENY RETARD AFTER TREATMENT WITH THE SEROTONIN SELECTIVE REUPTAKE INHIBITOR, CITALOPRAM

Abstract

Serotonin influences growth and development of the nervous tissue including serotoninergic neurons. The possibility exists that increased brain serotonin availability in young animals facilitates the neuro-behavioral responses. This study investigated the body

weight gain and reflexes ontogeny of neonate rats treated during the suckling period with different doses of citalopram (5mg, or 10mg/kg, sc, daily). The time for appearance of reflexes (palm grasp righting, free-fall righting, vibrissa placing, auditory startle response, negative geotaxis and cliff avoidance) as well as the body weight evolution were recorded. A delay in time of reflex development and a reduced weight gain were observed. The findings suggest that altered serotoninergic system activity induced by citalopram administered early in life could play a role in the body weight gain and in the maturation of the most reflex responses.

Keywords: serotonin, SSRI, citalopram, reflex development.

1. Introduction

Serotonin has been shown to have multiple functions as a neurotransmitter by exerting modulating effects on neural excitability (Aghajanian and Marek, 1997). There is large evidence of its participation in pain sensitivity, motor activity, body thermal-regulation, sleep, feeding behavior and mood (Chopin et al., 1994; Olivier et al., 1995). Encephalic areas known to be involved in psychomotor processes, such as brainstem, cerebellum, diencephalon, basal ganglia and cerebral cortex, are innervated by serotoninergic pathways from raphe nuclei (Jacob and Azmitia, 1992).

Experimental evidence indicates that also serotonin can influence embryogenesis and growth (Palén et al., 1979; Whitaker-Azmitia, 1991) by acting presumably as a developmental signal (Liu and Lauder, 1992) or as a neurotrophic factor as well (Yan et al., 1997). Furthermore, serotonin seems to play a role in regulating the development of mammalian brain through an action on the serotoninergic neurons itselves (Whitaker-Azmitia and Azmitia, 1986; Shemer et al., 1991; Whitaker-Azmitia, 1991).

By the other hand, the growth and development of the central nervous system occur

with great intensity during the gestation and suckling period in the rat (Morgane et al., 1993). In these phases brain structures are is highly vulnerable to several types of aggression. In the rat, the first serotoninergic neurons appear between the12th and the 14th day of gestation (Lidov and Molliver, 1982). The second and third week of postnatal life are distinguished for an abundant dendritic arborization of serotoninergic axons in the cerebral and cerebelar cortex, in the hippocampus and in striatum (Lidov and Molliver, 1982). The final density and location of the serotoninergic neurons terminals occur only during postnatal maturation (Lidov and Molliver, 1982; Wallace and Lauder, 1983)

During pregnancy or suckling period, pharmacological or nutritional manipulations can induce drastic morphological and functional changes in the growth and development of the nervous system (Noback and Eisenman, 1981; Manhães de Castro et al., 1993; Manhães de Castro et al., 2001). Furthermore, the reflexes maturation constitutes an indicator of the development nervous system (Fox, 1965). Retard in reflex ontogeny in malnourished rats was observed by Smart and Dobbing (1971). High levels of 5-HT and 5-HIAA were found in brains of undernourished animals up to 300 days old (Stern et al., 1975). In addition the selective serotnin reuptake inhibitor in general have anorexic properties (Moses and Wurtman, 1984; Clifton and Lee, 1997)

Thus, the possibility exists that the use of serotoninergic agents in the initial phase of life could have some effects on the body growth and sensory motor functions. Since there is no data about this issue, the investigation of the possible effects of early serotoninergic system manipulation on reflex development is highly desirable.

The objective of this study was to test the hypothesis that the administration of citalopram, a highly selective serotonin reuptake inhibitor (Baumman 1996), in rats during the suckling period - the so called brain growth spurt - induces changes in the appearance time of congenital reflexes.

2 Material and methods

2.1 Animals

Wistar male rats from the colony of the Nutrition Department – Federal University of Pernambuco – Brazil were coupled to obtain litters. During gestation until the end of the experiment, the animals were housed in polyethylene cages (30 x 27 x 47). Male pups from different mothers were randomly distributed in litters of 6 neonates, 24 hours following the birth. Each pup was labeled with a mark of methyl violet solution in the skin, for identification during the experiment. Each litter was breastfed by one of the dams until the 21st post-natal day (day of birth considered as zero day). The animals were maintained at a room temperature of 23±1^{o}C, on a light-dark cycle of 12/12 hours (light on 6:00 a.m. to 6:00 p.m.) with free access to meal (Labina-Purina of Brazil) and water.

2.2. Pharmacological treatment and experimental groups

A blind experiment was performed to prevent identification of the experimental groups. The animals of the different groups were simultaneously appraised. According to the experimental treatment, three groups (n=27 each one) of suckling rats were distributed as follows: group Cit5 (5mg/kg, sc); group Cit10 (10mg/kg, sc); and one control group receiving an equivalent volume of saline solution (NaCl 0.9%, sc.). During the experiment one neonate of the Cit5 group died. Therefore, 80 rats were evaluated during the whole experiment. The time for reflexes maturation and the body weight were determined. Citalopram (H.Lundbeck A/S, Copenhagen-Valby, Denmarck), was dissolved a saline solution and injected in the concentration of 1ml/100g b.w. The treatment was applied daily from the 1st to the 21st postnatal day (suckling period).

2.3 -Body weight

The measurement of body weight was accomplished at the 3,7,14 and 21 day, between 10:00 p.m. and 1:00 p.m, by using a Marte scale, Brazil-São Paulo, SP (100mg precision).

2.4 - Reflex testing

The reflex tests (according Smart and Dobbing, 1971) were carried out daily from the 1st to the 21st postnatal day (Figure 1) and were conducted between 11:00 a.m. and 1:00 p.m. The progress of the individuals was followed throughout the experiment**.** The time of appearance of each reflex was defined as the first day of its occurrence during a period of three consecutive days.

Figura 1

REFLEX	STIMULUS	RESPONSE
Palmar grasp - PG	Palm of forepaw stroked gently with a paper clip.	Flexion of digits. As maturation response, any or a very slight flexion must be seen. To this reflex, the disappearance date is registered.
Righting - R	Rat placed on back on a flat surface.	It turns over, to rest in ventral decubitus, with the four paws on the surface, in 10 seconds.
Vibrissa placing – VP	Rat held by the tail, head facing an edge of bench, vibrissa just touching vertical surface.	Lifts head and extends forepaws in direction of the bench, making oriented "walking" movements to go far from the edge, in 10 seconds.
Cliff avoidance - CA	Rat put on edge of bench, with nose and forefeet just over edge.	Withdrawal of head and both forefeet from edge, moving away from "cliff", in 10 seconds.
Auditory startle - AS	Sudden sound stimulus by percussion with a metalic stick in a metal surface.	Body retraction, with a transitory immobility. The stimulus was given twice in each test, with 1 minute interval.
Negative Geotaxis - NG	Rat placed with head downwards, on a 45 slope.	Turns to face up the slope, , at least ≥ 130º, in 10 seconds.
Free-fall righting - FR	Rat held by the paws, back downwards, is dropped from 30 cm on to cotton wool pad.	Turns body in mid-air, to land on all fours. All legs must be free of body on landing.

3 - Statistical analysis

After preliminary testing to identify the distribution normality and homogenity among the groups, statistical analysis were done: One-way ANOVA in 1st, 3th, 7 th and 21st day for body weight followed by post-hoc Dunnett test were used to compare each citalopram group with saline. The Kruskal-Wallis analysis of variance followed by Dunn test was used to compare the time appearance of the reflexes between each citalopram dose and saline. The level of significance was $p<.05$.

The experimental protocol of this paper was approved by the Ethical Committee for Animal Experimentation (CEEA) of the Federal University Pernambuco-UFPE and is in agreement with the National Institute of Health guide for Care and Uses of Laboratory Animals (Publication no. 85-23, revised, 1985).

4 – Results

Compared with saline, ANOVA found a lower body weight gain in the Cit10 group from 7th to 21st day ($p<.01$) and the Cit5mg dose did not differ (figure 2).

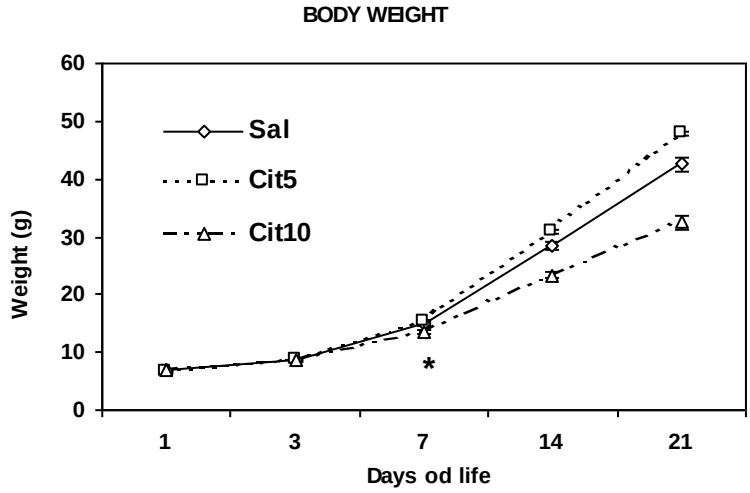

Figure 2 – Body weight of suckling rats from the 1st , 3 th , 7 th to 21st day of life, treated with solutions (1ml/100g b.w., s.c.) Cit 5mg (n=26), Cit 10mg (n=26) or Sal (n=28). Comparisons were made by One-way ANOVA follwed Dunnett test: citalopram F=48.5 ($p<.001$); between each citalopram dose and saline: *: $p<.05$. Each point

.

The disappearance of the palmar grasp and the vibrissa-placing were significantly retarded in the two citalopram groups . Cit5 ($p<.05$) and Cit10 ($p<.01$); negative-geotaxis in Cit10 ($p<.01$) and Cit5 groups ($p<.05$) Free-fall righting retarded in the two citalopram doses ($p<.001$). The auditory-startle-response delayed in Cit10 group ($p<.01$) but not in the Cit5 group. Finally, the cliff-avoidance in citalopram groups did not statistically differ from the saline one.

REFLEX	GROUPS					
			CITALOPRAM			
	SALINE					
			5 mg		10 mg	
Palmar grasp	2.5	(1-9)	6.5	(3-5) [b]	6.0	(5-9) [b]
Righting	5.0	(3-9)	8.0	(4-9) [a]	9.0	(5-10) [b]
Vibrissas placing	11.0	(8-15)	13.0	(11-16) [b]	13.0	(11-17) [b]
Cliff avoidance	8.0	(7-14)	10.0	(8-13)	11.0	(7-18)
Negative geotaxis	12.0	(8-16)	12.0	(9-16) [a]	13.0	(12-18) [b]
Auditory startle	11.0	(11-14)	12.0	(12-18)	13.0	(12-18) [b]
Free-fall righting	14.0	(12-18)	17.0	(16-19) [c]	16.0	(14-21) [c]

Figure 3-Effect of the treatment with citalopram 5mg and 10mg/kg once day. Evaluations were realized of the 1st to 21th day of life on ontogeny reflex. Kruskal Wallis followed Dunnet´s . [a]$p<0.05$; [b]$p<0.01$; [c]$p<0.001$

5. Discussion

The present study showed that chronic administration of citalopram during the critical period of brain development in rats delays the body weight gain and the appearance time of reflexes. The action of the drug upon serotoninergic mechanisms might be responsible for these effects since SSRIs increase the serotonin release in the synaptic cleft (Hytel, 1994).

Although there is no direct evidence for this release, some studies observed the use of SSRIs in young children (Riddle et al., 2001) or in neonate rats (Hansen et al., (1997), suggesting a depression-like effect by neonatal manipulation of the 5-HT system with SSRI. In this study the anorexic effect of citalopram was observed since a reduced delay in body weight gain occurred. Recently, we showed a body weight gain decrease and aggressiveness reduction in adult rats treated, early in life, with citalopram (Manhães de Castro et al., 2001; Barreto Medeiros, 2002).

These findings were supposed to be associated to serotoninergic mechanisms because of the serotonin inhibitory action on food intake, as shown by Clifton and Lee, 1997; Halford and Blundell (1996, 2000).

The repercussions of other neonatal manipulations - as for example, early malnutrition - on the development of the structural, neurochemical and functional integrity of the nervous cells are well known (Morgane et al., 1993). Alterations in the appearance of reflexes indicate a correlation between biochemical and structural development and ontogenesis of the nervous system (Adlard and Dobbing, 1971). The content of the brain monoamines during development increase more quickly after birth (Loizou and Salt, 1970). Rats submitted early in life to low protein diets reveal altered brain levels of noradrenaline, dopamine and serotonin (Resnik et al., 1979; Wiggins et al., 1984; Chen et al., 1995). Therefore, a increased serotonin availability provoked by the SSRI treatment could have caused the observed delay in the reflexes

Our data are supported by others: undernourished rats present a delay in the development of reflexes, such as palm grasp, startle response and free-fall righting (Smart and Dobbing, 1971). Increase on the latency of the startle response in adult rats treated with fluoxetine, during the neonatal period was shown by Dow-Edwards (1996), this effect being reduced by m-chlorophenylpiperazine, a 5-$HT_{1B/2C}$ agent, suggesting the involvement of these receptors in the reflex elicitation. In addition the treatment with fluoxetine diminished the lomotor activity in young rats (Stanford et al., 2002). So, we could suggest a similar action of citalopram in the delay of the reflexes ontogenesis.

Furthermore, chronic treatment with fluoxetine, another SSRI, reduced the synthesis, dose-dependent maner of 5-HT, in the encephalon of mice indicated a reduction of the 5-HIAA/5-HT rate (Stenfors and Boss, 2002). This finding reinforce the assumption of increased serotoninergic neurotransmission after chronic treatment with citalopram in the present study.

The fact that citalopram administrated during the suckling period affect the body weight gain and the development of the nervous system and their early behavioral expressions support the view that: permanent morphological or functional alterations were produced during the period of fast brain development, indicating the participation of the serotoninergic system in this events.

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5.5 Quinto artigo

Título: **NEONATAL FEEDING BEHAVIOR: EFFECT OF THE TREATMENT WITH FLUOXETINE.**

Submetido para publicação como artigo original na revista: Brazilian Journal of Medical and Biological Research - julho de 2004.

Neste trabalho investigou-se o efeito da administração neonatal em ratos, com o inibidor seletivo da recaptação da serotonina – fluoxetina em diferentes doses sobre o peso corporal, a ingestão dos filhotes e o comportamento alimentar. Os resultados mostram que o tratamento com fluoxetina durante o período critico de desenvolvimento do cérebro, retarda a evolução ponderal, reduz a ingestão alimentar dos filhotes e reduz a freqüência de comportamentos.

Running Title: SSRI and neonatal behavior

NEONATAL FEEDING BEHAVIOR: EFFECTS OF TREATMENT WITH FLUOXETINE

Abstract

This study investigated the effect of fluoxetine treatment on the body weight and neonatal feeding behaviors. Male and female pups from different mothers were randomly distributed in litters of 6 neonates, 24 hours after the birth (n= 8-10 litters). Three groups (litters) received fluoxetine: group flu10 (n= 8) (10mg/kg, sc); group flu5 (n=8) (5mg/kg, sc); group flu1 (n=8), (1mg/kg, sc); and one control group received an equivalent volume of saline solution (n=10) (NaCl 0.9%, sc.). Therefore, litters were evaluated: Fluoxetine hidrochloride (Sigma-USA) was dissolved in saline and injected in the concentration of 1ml/100g b.w. The treatment was applied daily from the 1st to the 21st postnatal day (suckling period). Our results establish that the fluoxetine – reduce the frequency of mother-pups interaction and the maternal grooming behavior; The time influenced the behaviors studied in all treatments. In conclusion, fluoxetine - drug which increases the serotoninergic function by inhibiting its reuptake - when administered during the suckling period, may reduce the frequency of mother-pups interaction and the maternal grooming behavior, which involves several another behaviors. Maternal behaviors are modified according to the age, but they were not influenced by the treatment. Fluoxetine provokes a reduction in the body weight gain and in the maternal

milk ingestion in the doses considered as moderately anorexic or even in lower doses.

1. Introduction

During the NS ontogenesis, the feedings behaviour of mammals goes through some expressive changes. At birth, it is simple and automatic, becoming, in a short time, the complex adult behaviour. In the rat, until the 18th day of life, the adult control of alimentary behaviour in not effective. In this mammal, the behavioural maturity seems to be reached when suction ends in response to satisfaction signs; before 14 days of life, peripherical signs of gastrointestinal distension prevail. From this age on, the behavioural sequence of satisfaction is also similar to the adult one, with a simultaneous reduction of mother milk consumption. Rats deprived of suckling have a response similar to adults, in their first day of life, increasing subsequent ingestion.

In mammals, the organical systems, particularly the NS, obey to a developmental chronogram, in which different phases occur in a genetically pre-defined temporal sequence. In the rat, the critical period of development corresponds to suckling and shows intense growing and differentiation of the NS.

Until pups reach the maturity of ingestion auto control, the mother plays the role of stimulating the begin of suction. Studies with malnourished animals show that mother-pup interaction is modified, changing the maternal behaviour.

Besides the aspects referred above about pups development and the modifications due to environmental factors, the period of the NS growth spurt has as a characteristic the neurotransmitters system development, among them the serotoninergic system (5-HT). In the rat, this monoamine, derived from tryptophan, is presente in the encephalon of rats between the 12nd and 14th days of gestation. Thus, besides of a growth and differentiation factor, as serotonina (5-HT) seems to be, among other epigenetic factors, the adequate supply of

essential nutrients during the critical period of brain growth is also highly relevant in men as in rats.

Thus, the studies which hypothesis is the 5-HT system involvement in behavioral features of mother-pup interactions, during suckling period, are scarce. The aim of this study was to investigate, in neonate rats subjected to alimentary deprivation, the effects of SSRI (fluoxetine) neonatal treatment on the behaviour of mothers and pups.

2. Material and methods

2.2 - Animals

Albine Wistar rats were obtained from the colony of Nutrition Department of Universidade Federal de Pernambuco. Males and females were matched (between 90 and 120 days of life) in a room with temperature ranging between 23 $\pm$ 2 ºC, light /dark of 12/12h (light 6:00 am to 6:00 pm) with food and water *ad libitum* during gestation, confirmed through the body weight.

Pregnant females were housed individually in transparent polycarbonate cages (47 x 32 x 20cm) which allowed free observation. Male and female pups from different mothers were randomly distributed in litters of 6 neonates, 24 hours after the birth (n= 8-10 litters). Each pup was labeled with a mark of methyl violet solution in the skin, for identification during the whole experiment. Each litter was breastfed by one of the dams until the 21st post-natal day (birth day was considered as zero day). The animals were maintained at a room temperature of 23$\pm$1^{o}C, on a light-dark cycle of 12/12 hours (light on 6:00 a.m. to 6:00 p.m.) with free access to meal (Labina-Purina of Brazil) and water.

3. Pharmacological treatment and experimental groups

A blind study was performed so that the groups, were not identified during the experiment. The animals of the different groups were simultaneously appraised. According to the experimental treatment, four groups of suckling rats were designed as follows: Three groups (litters) received fluoxetine: group flu10 (n= 8) (10mg/kg, sc); group flu5 (n=8) (5mg/kg, sc); group flu1 (n=8), (1mg/kg, sc); and one control group received an equivalent volume of saline solution (n=10) (NaCl 0.9%, sc.). Therefore, litters were evaluated: Fluoxetine hidrochloride (Sigma-USA) was dissolved in saline and injected in the concentration of 1ml/100g b.w. The treatment was applied daily from the 1st to the 21st postnatal day (suckling period). The pups received the Pharmacological treatment after evaluation behaviors. The feeding deprivation and behavioral observation was realized on 3rd, 7th, 14th e 21st days of life. The body weight was verified also in the 1st day for verifying weight homogeneity . The pups were deprived by 4h from theirs dams, without access to milk of this mother, water and food in the mentioned experiment days. At this time (12:00 to 16:00h) the litters housed in cages with same characteristics of the biotery, including of the nest by way to let the body temperature of pups at $\pm$31 ºC.

4. Behavioral Analyses

The behaviors were registered for direct observation during 60 minutes. on 3rd, 7th, 14th e 21st suckling days. A experimental protocol and digital Watch to register the duration in seconds and the frequency (numbers of times of occurrence) of the evaluated at 12:00 pm 1h pm. The observator stayed about 1,5m to distance of cage. By according the birth`s day were changed to the table`s observation a day before of the test , to facilitate the behavioral analysis of the animals.

The behaviors were evaluated for direct observations (Riul et al., 1999) and registered the duration and frequency of mentioned behaviors.

4.1 - Suckling

Was registered when at least one pup was observed attachment maternal ´s nipple .The behavior was finished When the last pup left the nipple.

4.2 - Ingestion

The individual feeding ingestion in grams (g) was verified in all tests days (3^{rd}, 7^{th}, 14^{th} and 21^{st}). It was quantified through of the body weight of the pups (HOUPT, 1973) during the period of behavioral observation. This one was calculated by the subtraction of the final weight of every pup by the initial one.

4.3 - Mother-pup Interaction

This behavior was registered when the observer verified active`s interaction between mother and one or more of her pups , sniffing, licking or carrying the pups.

When at least one pup interacting with her mother, sniffing, together or in top of her was also registered.

4.4- Building construction

The activity where the mother was observed pushing or carrying with her mount the material of the nest. This behavior was evaluated on 3rd, 7th and 14th postnatal day.

4.5 - Grooming

The mother licking the previous paws with movements of this one on the head go on licking the ventral body region, back and subsequent paws. This is a stereotyped behavior constituted a previous behavior.

4.6 - Feeding pups behavior (21^{st} day)

This behavior was registered only, when one of pups was observed in tunnel and eating ration. The register was started when the observator saw one or more pups feeding and finished when the last of them left the tunnel.

5.0 - Body weight

The pups were weighed individually on 1st postnatal day (to verify if it would not have difference in the mean or measured of its weight in the start of the treatment)and the other days of the test. The weighted was realized before and after of the behavioral observation. Before weighted, It was carried through a genital stimulation in the pups for eliminations (Hall e Epstein et al., 1977).before weights them. This method consists in a light friction with a swab in the genital region of the pups until that the excretion of urine or excrements occurred. This procedure guarantied a higher confiability to the quantifying of the feeding ingestion.

In the realization of the weighted was used electronic balance with precision of 100 mg (MARTE, model 1001).

6.0 - Statistical analysis

Following preliminary testing to identify the normality of the distributions (Kolmogorov – Smirnov test) and variance homogeneity among the groups (Levene´s test), statistical analyses were performed. The results of the behaviors (in seconds) were changed in Log_{10}. A two-way ANOVA for repeated measures followed by the post-hoc Bonferroni test was utilized for the mother behaviors and interaction mother-pups behaviors; ANOVA followed Dunnett test for body weight; Kruskal Wallis (Normality test failed) for analysis the pups ingestion. The level of significance was $p < 0.05$.

The Ethical Committee for Animal Experimentation (CEEA) for Federal University of Pernambuco-UFPE, being in agreement with National Institute of Health Guide for Care and Uses of Laboratory Animals (Publication no. 85-23, revised, 1985) approved the experimental protocol. For analysis of the collected information was used the program GMASTAT 2.0.

7 - Results

7.1 - Body weight

For body weight gain ANOVA showed significant effect of fluoxetine ($F_{3,140}$ = 16,982, p < 0.001) days of life ($F_{4, 560}$ = 4626,87, p< 0,001 as well a an interaction between fluoxetine and days of life ($F_{12, 560}$ = 14,982, p<0.001). Dunnett test, demonstrate significant difference in fluoxetine by the end of the lactation period (21st day): An increase in body weight of Flu1 (50.27±1.10g) and a decrease in Flu10 (40.46± 0.90g) was observed compared to saline group (46.50±1.20). No difference in Flu5 group was observed compared to saline group (p<0.05) (Figure 1).

7.2 - Pups ingestion

As illustrated in Figure 2, ANOVA indicated an significant overall effect for fluoxetine ($F_{3,188}$ = 92,4380, p< 0.001), significant effect for day of life ($F_{3, 564}$ = 145,7268, p<0,001) and in the interaction between these two factors ($F_{9, 564}$ = 3,6227, p<0,001). Kruskal Wallis test showed significant difference in the three fluoxetine doses compared to saline: by the end of the lactation period (21st day) the ingestion of the saline group 1.45 (0.70-2.90)g compared with fluoxetine groups: 0.90 (0.70-1.20) g Flu1; 0.90 (0.70-1.20) g Flu5; and 1.20 (0.20- 4,10) g Flu10 (p<0.05).

7.3 - Suckling

For duration of the suckling statistical analysis showed significant effect for days of life ($F_{3,102}$ = 16.1964; p< 0.001). Did not significant effect of fluoxetine as well as interaction between these two factors. A significant increase in duraction of the suckling in 3rd day when compared to 14th and 21st days (p<0.05) (figure 3).

For frequency during the suckling period observed the statistical analysis showed an significant effect for day of life ($F_{3, 102}$ = 5.3816; p<0,001); and not significant for fluoxetine and

interaction between fluoxetine and the time. Occurred a higher frequency in the 14th compared to 21stday ($p<0.05$) (figure 1).

7.4 - Building construction

For duration of building construction, ANOVA showed significant effect for days of life ($F_{2,68} = 10.3300$; $p< 0.001$); did not occurred difference in the treatment and in the interaction between the factors treatment and time.

A higher frequency in the 3rd and 7th compared 14th day was observed ($p<0.05$) (figure 4). For these behavior the frequency presented significant difference for day of life ($F_{2, 68} = 7,9098$; $p< 0,001$). An effect did not significant for fluoxetine and interaction between these factors was observed. A lower frequency in the 3rd day compared 14th day was observed ($p<0.05$) (figure 4).

7.5 - Mother-pup interaction

For mother-pup interaction the duration of this behavior presented did not significant effect for any of the factors analyses (figure 5).

Statistical analysis showed significant effect of the frequency of mother-pup interaction for fluoxetine groups ($F_{3,34} = 5,8063$, $p < 0.01$); However did not significant effect was showed for day of life and the interaction between treatment and time. A higher frequency in Flu10 and Flu5 groups compared to Sal group was found ($p<0,05$) (figure 5)

7.6 - Grooming

For duration of the grooming behavior, significant effect in days of life ($F_{3, 102} = 3,6514$, $p < 0,01$) was showed. Statistical analysis found did not significant effect of fluoxetine as well in the interaction between fluoxetine and days of life. A higher duration in day of life in these behavior was identified ($p<0,05$) (figure 6).

Statistical analysis showed significant effect for fluoxetine ($F_{3,34}$ = 5,1865, p< 0.01) and for day of life ($F_{3,\ 102}$ = 7,5155, p<0,001) for frequency of the grooming**.** significant effect in the interaction between these factors did not observed. The higher frequency was observed in the 3rd day compared to14th and 21st day as well as a greater increase in the frequency of mother-grooming in Flu10 group compared with Sal group in all days observed. (p<0,05) (Table 4).

7.7 - Feeding behavior of pups (21st day)

The duration of pups feeding behavior (21st day) statistical analysis showed did not significant difference between treatments as well as in frequency of feeding behavior of the pups in fluoxetine groups compared to saline group (figure 7)

8.0 - DISCUSSION

In the present study, it has been observed that the fluoxetine (SSRI) neonatal treatment delayed the body weight gain in the highest doses and increased body weight gain in the lowest dose. Besides, the fluoxetine treatment provoked a reduction in mother milk ingestion for all used doses in most of the studied days. Recently, some studies by our labs verified that a reduction in the body weight gain in young rats treated with citalopram (another SSRI) (Manhães de Castro et al., 2001). The highest dose used here was considerate as moderately anorexic (Clifton et al., 1989).

Among the behaviors studied, the fluoxetine in highest doses provoked an increase in the frequency of mother-pups interaction and in the frequency of mother grooming behavior. The increase of the frequency of mother-pups interaction observed in the present study characterizes higher mother-pups interaction, for the execution of other subjacent behavior, which, in this case, seems to have suffered the influence of pups treatment, which were approaching for more times their mothers. Moreover, neonate rats are unable to keep body temperature. The vascular

response of the animal for body heating becomes effective after 12 days of life and only after the 21st day of life, the rat acquires the capacity to keep stable body temperature (Couklin and Heggeness, 1971). Thus, the increase of the mother-pups interaction, here observed, may be viewed as an addition of maternal care to keep pups temperature, since fluoxetine treatment did not alter neither the duration, nor the frequency of suckling.

In addition, the observation that the relationship of mother-pups interaction with the duration and the frequency of suction, which suffered time influence, is not depending on the drug is interesting. The first week of life was outstanding in the expression of this behavior duration, while the frequency increased in the second week of life when the pups begin to interact with their mothers in an autonomous and independent way. Thus, the influence of age, observed in this study, would be justified. In this way, during suckling, the mother enjoyed the interaction with its pups in a simultaneous way (by licking, even cleaning and approaching them to itself), which does not occur to other behavior depending generally on the distance of the pups. The cleaning behavior had its frequency increased in the litters which were treated with fluoxetine in the highest dose, also being observed the influence of age on this behavior, which was more frequent in the two initial weeks of life.

The behavioral synchrony between mother and pups, during the suckling period, requires a continuous maternal adaptation to the development of the pups (Crnic, 1980). In rats, several maternal behavioral settings play an important role in the determination of energy availability for the pups (Crnic, 1980). Some studies have been demonstrating that mother-pup interaction, the quantity/quality of maternal cares and the interaction between brothers, during the critical period of development, seem to interfere on the CNS ontogenesis. The first hours of mother-pup interaction are considered decisive for the behavioral development (Galler e Propert, 1982).

The changers observed in the present study may indicate the participation of 5-HT in the maturation of early post-natal behaviors. Since the pre-natal phase, the serotonina would be

present in development stages, taking part in early processes of cellular control involved in the development and acting as a neuronal growth factor (Buznikov 1984; Lauder 1988; Whitaker-Azmitia 1987). High densities of 5-HT receptors may be found in the brainstein and in the forebrain in the post-natal period (Whitaker -Azmitia *et al.*,1987).

On the other hand, the feeding provides energy and nutrients necessary to maturation and functional development of the NS (Morgane *et al*., 1993). Nutritional deficiency is able to alter morphogenetic events with harmful consequences to development and acquisition of mature physiological patterns (Dobbing 1964; Winick e Nobel, 1966; Noback e Eisenman, 1981). The neonates subjected to protein malnutrition present a disorganization of hypothalamic centers which regulate body weight and the metabolism (Plagemann, 2000). A study suggests that nutrients deficiency during early life have influences on the neurotransmission and, in consequence, could affect the behavioral expression (Wauben e Wainwright, 1999). The alterations in mother-pups interaction, as those derived from the malnourished, may cause a delay in the development and an increased dependence of the pups to their mothers (Riul *et al*., 1999). Moreover, behaviors are triggered and guided by neural events, but each behavioral act involves a response of peripheral physiological system which is translated to the brain neurochemical activity (Blundell, 1993).

A significant reduction in body weight of pups treated with fluoxetine in the highest dose has been observed in this study. Besides of the weight loss, pups ingestion was also reduced in all used doses, being influenced by the time (days of life of the animals) and by the interaction between both these factors. Thus, the anorexic effect provoked by fluoxetine seems clear in this study, independent of changes in other behaviors. Kalia *et al.,* (2000) observed dramatic anorexic effect and weigh loss after fluoxetine treatment. A study by Simansky and Vaidya (1990) showed that sertraline reduce milk ingestion, after alimentary deprivation in adult rats, through modifications of behavioral mechanisms. The expressive weight loss of animals treated with

fluoxetine in the highest dose observed in this study, may be explained this way, causing a possible malnutrition to the pups. The permanency next to the mother may not be an exclusive condition for suckling, since the neonate depends on its mother for maintaining temperature, among other physiological functions (Friedman, 1975). An interesting fact was the increase of weight observed in the lowest dose. Chronic treatment with SSRIs may provoke receptors desensitization, causing hyperphagia and increase of the weight (Gardier *et al.*, 1996; Hamon and Gozlan 1993).

In conclusion, our results establish that the fluoxetine – a drug which increases the serotoninergic function by inhibiting its reuptake - when administered during the suckling period, may reduce the frequency of mother-pups interaction and the maternal grooming behavior, which involves several sub-behaviors. Other maternal behaviors are modified according to the age, but they were not influenced by the treatment. Fluoxetine provokes a reduction in the body weight gain and in the maternal milk ingestion in the doses considered as moderately anorexic or even in lower doses.

Figure 1 Body Weigth

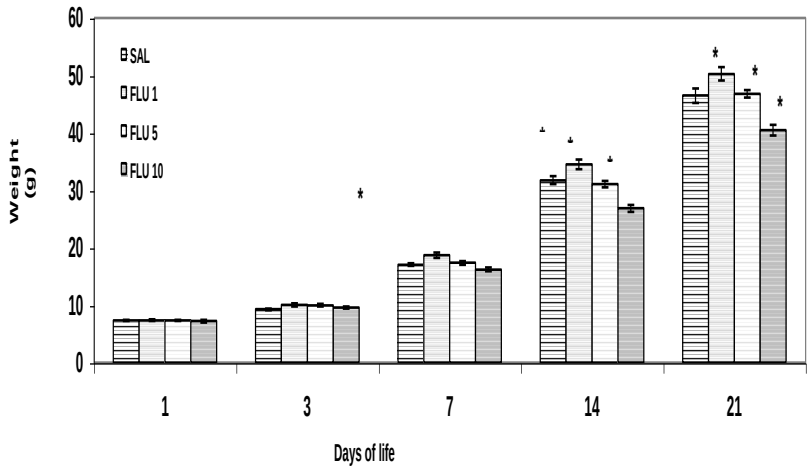

Fig.1. Effect of the fluoxetine treatment on the body weight. The pups were weighted throughout the lactation in 3rd, 7th, 14th and 21st days. Each bar represents the mean ± SEM; Comparisons by Anova One-Way. Dunnett test was used for multiple comparisons (*p<0.05).

Figure 2
Pups Ingestion

Days of life	N	GROUPS			
		Saline	Fluoxetine		
			1 mg	5 mg	10 mg
3	36	0.50 (0.0 - 2.4)	0.20 (0.1 - 0.5)*	0.20 (0.0 - 0.7)*	0.50 (0.0 - 1.3)
7	36	1.10 (0.1 - 1.6)	0.40 (0.0 - 0.7)*	0.30 (0.1 - 1.4)*	0.90 (0.2 - 1.7)*
14	36	1.40 (0.6 - 2.7)	0.70 (0.4 - 0.9)*	0.65 (0.2 - 1.2)*	1.10 (0.3 - 3.2)*
21	36	1.45 (0.7 - 2.9)	0.90 (0.7 - 1.2)*	0.90 (0.2 - 2.0)*	1.20 (0.2 - 4.1)*

Fig.2. Effect of the treatment with fluoxetine on the ingestion of the pups. The ingestion of the milk and/or diet (g) was evaluated during 1h, in 3rd, 7th, 14th and 21st days. Values represented in median, maximum and minimums (parenthesis). *p< 0,05, Kruskal Wallis followed Dunnett test.

Figure 3

Days Of Life	GROUPS							
	Saline (n = 10)		Fluoxetine					
			1 mg (n = 8)		**5 mg (n = 10)**		**10 mg (n = 10)**	
	Duration (s)	**Frequency**	Duration (s)	**Frequency**	Duration (s)	**Frequency**	Duration (s)	**Frequency**
3	3.51 ± 0.02[a]	0.45 ± 0.13[a]	3.42 ± 0.04[a]	0.53 ± 0.06[a]	3.39 ± 0.05[a]	0.46 ± 0.07[a]	3.47 ± 0.02[a]	0.51 ± 0.11[a]
7	3.43 ± 0.03[a]	0.53 ± 0.14[a]	3.45 ± 0.03[a]	0.44 ± 0.04[a]	3.40 ± 0.04[a]	0.47 ± 0.05[a]	3.42 ± 0.03[a]	0.58 ± 0.06[a]
14	3.36 ± 0.03[b]	0.67 ± 0.11[a]	3.30 ± 0.06[b]	0.58 ± 0.07[a]	3.34 ± 0.04[b]	0.43 ± 0.08[a]	3.22 ± 0.07b	0.58 ± 0.05[a]
21	3.31 ± 0.03[c]	0.47 ± 0.16[c]	3.31 ± 0.04[c]	0.29 ± 0.10[c]	3.30 ± 0.04[c]	0.341 ± 0.08[c]	3.31 ± 0.05[c]	0.34 ± 0.08[c]

Fig.3. Effect of the treatment with fluoxetine on the suckling pups. The duration and the frequency of this behavior was registered during 1h, in 3rd, 7th, 14th e 21st suckling days. Different letters represents statistical difference of the mean ± SEM; Comparisons by Anova Two-Way. Bonferroni t-test was used for multiple comparisons ([a, b, c,] p<0.05). [s = seconds]

Figure 4
Building construction

Days of life	GROUPS							
	Saline (n = 8)		Fluoxetine					
			1 mg (n = 8)		5 mg (n = 11)		10 mg (n = 10)	
	Duration (s)	**Frequency**	Duration (s)	**Frequency**	Duration (s)	**Frequency**	Duration (s)	**Frequency**
3	1.73±0.27[a]	0.38±0.10[a]	2.31±0.18[a]	0.69±0.13[a]	1.39±0.29[a]	0.24±0.08[a]	1.79±0.20[a]	0.57±0.11[a]
7	1.51±0.25[a]	0.39±0.10[a]	1.72±0.31[a]	0.36±0.12[a]	1.50±0.25[a]	0.40±0.09[a]	1.23±0.35[a]	0.45±0.12[a]
14	0.45±0.30[b]	0.04±0.04[b]	0.92±0.35[b]	0.27±0.12[b]	1.33±0.29[b]	0.23±0.08[b]	0.92±0.31[b]	0.29±0.10[b]

S = seconds

Fig.4. Effect of the treatment with fluoxetine on the nest construction. The duration and the frequency of the behavior was registered during 1h, in 3rd, 7th and 14th suckling days. Different letters represents statistical difference of the mean ± SEM; Comparisons by Anova Two-Way. Bonferroni t-test was used for multiple comparisons ([a, b,] $p<0.05$).

Figure 5
Mother-pups interaction

Days Of Life	GROUPS							
	Saline (n = 8)		Fluoxetine					
			1 mg (n = 8)		5 mg (n = 11)		10 mg (n = 10)	
	Duration (s)	**Frequency**	Duration (s)	**Frequency**	Duration (s)	**Frequency**	Duration (s)	**Frequency**
3	2.45±0.36	0.76±0.13	2.89±0.08	1.05±0.07	2.43±0.25	0.79±0.10*	2.97±0.05	1.28±0.05*
7	2.64±0.38	0.88±0.14	2.95±0.10	1.01± 0.12	2.81±0.09	0.86±0.09*	2.98±0.05	1.22±0.06*
14	3.00±0.07	1.02±0.02	2.68±0.16	0.84±0.10	2.89±0.04	0.98±0.06*	2.84±0.11	1.04±0.11*
21	2.83±0.09	0.95±0.07	2.88±0.08	0.93±0.11	2.72±0.11	0.99±0.07*	2.74±0.07	1.03±0.07*

S = seconds

Fig.5. Effect of the fluoxetine treatment on the mother-pups interaction (seconds transformed in log10). The duration and the frequency of the behavior was registered during 1h, in 3rd, 7th, 14th e 21st suckling days. Values represented in parenthesis by mean ± SEM;*$p<0.05$. Values represented in parenthesis by mean ± SEM; Comparisons by Anova Two-Way. Bonferroni t-test was used for multiple comparisons (*$p<0.05$).

Figure 6
Grooming

Days Of Life	GROUPS							
	Saline (n = 8)		Fluoxetine					
			1 mg (n = 8)		5 mg (n = 11)		10 mg (n = 10)	
	Duration (s)	Frequency	Duration (s)	Frequency	Duration (s)	Frequency	Duration (s)	Frequency
3	2.06 ± 0.30	0.90 ± 0.09	2.34 ± 0.12	0.94 ± 0.15	2.51 ± 0.14	0.91 ± 0.05	2.50 ± 0.11	1.26 ± 0.05
7	2.42 ± 0.17*	0.88 ± 0.14	2.41 ± 0.08*	0.85 ± 0.10	2.48 ± 0.11*	0.87 ± 0.07	2.43 ± 0.11*	0.93 ± 0.06
14	1.94 ± 0.28*	0.73 ± 0.09	1.87 ± 0.42*	0.54 ± 0.13	2.13 ± 0.16*	0.79 ± 0.07	2.26 ± 0.15*	1.03 ± 0.07
21	2.35 ± 0.13	0.75 ± 0.05	2.01 ± 0.12	0.54 ± 0.13	2.42 ± 0.05	0.85 ± 0.07	2.04 ± 0.28	0.99 ± 0.13

S = seconds

Fig.6. Effect of the fluoxetine treatment on the grooming. (seconds transformed in log10). The duration and the frequency of this behavior was registered during 1h, at 3rd, 7th, 14th e 21st suckling days. Values represented in mean ± SEM; Comparisons Anova Two-Way, Bonferroni t-test was used for multiple comparisons (*p<0.05).

Figure 7A

Feeding behavior of pups

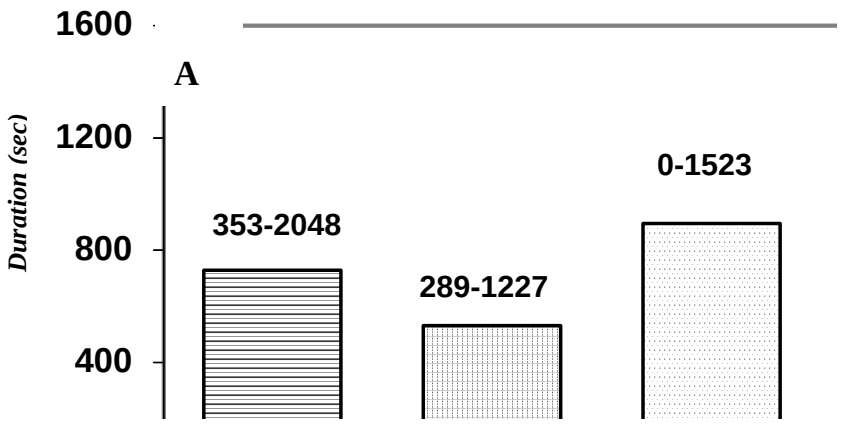

Figure 7 B

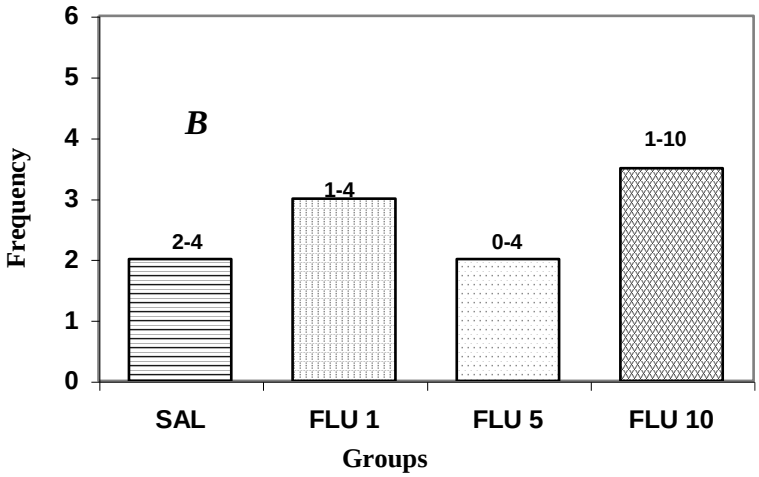

Fig.7. Duration of the feeding behavior of the littres (A)and mean frequency of this behavior (B) during 1h, at 21st day of suckling period. Each bar represents by median, maximum and minimum. Kruskal Wallis followed Dunnett test ($p<0.05$); there is not a statistically significant difference.

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6 DISCUSSÃO GERAL

De maneira geral, os dados obtidos no presente trabalho demonstram que o tratamento crônico durante o período crítico de desenvolvimento do SNC, com inibidores seletivos de recaptação da serotonina, em diferentes doses, causa várias alterações em parâmetros do crescimento e desenvolvimento, bem como do comportamento alimentar neonatal, em ratos. A magnitude das alterações de muitos dos aspectos estudados, parece ter dependido da dose administrada.

Em princípio, este estudo revelou que o tratamento com os SSRIs citalopram, fluoxetina e sertralina, em particular nas doses mais altas, durante o chamado período vulnerável do desenvolvimento do encéfalo, foi determinante para causar alterações importantes no desenvolvimento de reflexos e de características físicas do animal neonato. Sendo as características físicas estudadas, peculiarmente imaturas, em grande parte dos mamíferos ao nascimento (MORGANE et al., 1978). Durante esse período, as alterações observadas no peso corporal, nas demais medidas murinométricas e em parâmetros do comportamento alimentar neonatal, trazem relevantes informações acerca das hipóteses levantadas para o desenvolvimento deste trabalho e que originaram os artigos que dele fazem parte.

A abordagem utilizada no presente trabalho assemelha-se àquela referida em um estudo de HANSEN et al., (1997) e corroborada por KOSTOWSKY e KRZASCIKI, (2003). HANSEN et al., (1997) demonstraram em ratos, que tratamento com LU10-134-C, um SSRI, no período pós-natal, provoca alterações comportamentais na idade adulta. Assim, eles observaram que o tratamento neonatal com LU10-134-C aumenta o tempo de imobilidade no teste de nado forçado (depressão experimental) embora não mostrasse alterações no "Open-field". Com estes experimentos, eles sugeriram um hipotético modelo animal de depressão originado no período neonatal. A manipulação farmacológica com SSRI permitiu também a esses autores considerar o envolvimento do sistema serotoninérgico na fase em que o cérebro é mais vulnerável a agentes epigenéticos.

No presente trabalho, procurou-se aprofundar o estudo no período crítico de desenvolvimento do SN, analisando-se também o desenvolvimento do animal durante a fase neonatal quando da manipulação do sistema serotoninérgico. Os testes de reflexos e a observação de características físicas empregados permitiram obter informações importantes a respeito da ontogênese do SNC no rato. Várias hipóteses de trabalho para os resultados obtidos podem ser

aventadas. Duas delas, todavia, merecem destaque, uma vez que podem fornecer uma explicação mais abrangente para os fenômenos aqui observados.

Uma das hipóteses aqui levantadas para as alterações encontradas neste estudo seria do efeito hipofágico em neonatos, causado pela administração de SSRI durante o aleitamento. Assim, o aumento da serotonina na sinapse induzido por SSRI, intimamente relacionado a hipofagia, poderia ter ocasionado redução na ingestão diária do leite materno pelos filhotes. As repercussões da desnutrição neonatal sobre a integridade estrutural, neuroquímica e funcional da célula nervosa, responsável pelas funções cerebrais são bem conhecidas (MORGANE et al., 1993). Neste caso, a ausência dos elementos energéticos e plásticos fornecidos através do leite seria fator predisponente de desnutrição.

A segunda hipótese aqui discutida é de que a manipulação farmacológica com SSRI durante o período de vulnerabilidade do encéfalo altera o efeito trófico da serotonina em tecidos neurais e não neurais, levando aos resultados observados no presente trabalho. Nesse particular, tem sido cada vez mais salientada na literatura, a influência trófica da serotonina no período perinatal sobre a diferenciação de diversos tecidos, em vertebrados (LAUDER, 1983; 1990; MOISEIWITSCH e LAUDER, 1996). Assim, logo após o nascimento, ratos exibem agregações transitórias de axônios serotoninérgicos, em áreas do córtex somatosensório e do córtex visual (DORI et al., 1996). Essas agregações transitórias parecem contribuir para o desenvolvimento do padrão adulto, alcançado na 3ª semana de vida, dessas regiões cerebrais (DORI et al., 1996). Evidentemente, mudanças no desenvolvimento de padrões estruturais influenciados pelo sistema serotoninérgico, podem ser compatíveis com mudanças também na velocidade do desenvolvimento e nas resultantes funções. A grande maioria dos resultados do presente trabalho parece corroborar essa hipótese.

Doravante, com o intuito de uma melhor compreensão, considerando que este estudo se pauta nos artigos científicos aqui apresentados, a discussão manterá uma abordagem, sob a luz, principalmente das hipóteses testadas nesses artigos: a) Os danos causados ao crescimento, e também os funcionais, provocados durante o tratamento neonatal com cada um dos SSRIs, em diferentes doses; b) As repercussões do tratamento neonatal com um dos SSRIs, em diferentes doses, sobre o comportamento alimentar neonatal. Outrossim, as avaliações sensório-motoras e de maturação somática realizada, junto às medidas do crescimento ponderal e corporal, durante o período de aleitamento, fornecem neste trabalho

um conjunto de idéias acerca de possíveis danos causados pela manipulação do sistema serotoninérgico.

Diante dessa abordagem mais ampla, é pertinente agora discutir de maneira mais direta os achados do presente trabalho: A administração crônica de inibidores seletivos da recaptação da serotonina (SSRIs) durante o período crítico de desenvolvimento do cérebro do rato, induziu modificações no crescimento e no desenvolvimento. Em geral, os SSRIs utilizados citalopram, fluoxetina e sertralina, provocaram retardos no crescimento somático e na maturação de caracteres físicos. Além desses danos, aqueles antidepressivos provocaram atrasos na maturação de reflexos, alteraram o comportamento alimentar neonatal e a interação mãe-filhote. O tratamento com estes fármacos resultou em alterações dose-dependente no crescimennto corporal, no crescimento da cauda e do crânio. Assim a depender da dose, observou-se que o tratamento neonatal com os SSRIs provoca distorções na estrutura da cabeça do animal experimental na maioria dos casos. Ademais, foram também observados retardos na maturação de características físicas constituintes do desenvolvimento normal do arcabouço craniofacial; tais como: a abertura do conduto auditivo e a irrupção dos dentes. Os atrasos provocados na maturação de reflexos podem ter estreita relação com a estrutura corporal, visto que envolvem respostas tanto muscular-esqueléticas quanto nervosas.

A sensibilidade do crescimento da cabeça de vertebrados às manipulações epigenéticas foi bem demonstrada (HERRING 1993; DRESSINO e PUCCIARELLI (1997) em ratos submetidos à outra manipulação durante o período crítico de desenvolvimento, neste caso, à desnutrição. No caso das manipulações farmacológicas empregadas no presente estudo, a ação sobre mecanismos serotoninérgicos pode ser responsável pelos efeitos no crescimento e desenvolvimento, já que os SRSIs aumentam a disponibilidade da serotonina na fenda sináptica (HYTEL, 1994). Isto incrementaria, próximo aos tecidos em desenvolvimento, a interação da bioamina com seus receptores específicos. Há evidências de que os SSRIs e alguns agonistas serotoninérgicos aumentam a liberação de 5-HT não somente em tecidos nervosos (LAUDER et al, 1988; LAUDER 1990; Buznikov 1990) mas também em tecidos não-nervosos (SHUEY et al., 1993; LAUDER 1993). Neste particular, SHUEY et al., (1993) demonstraram alterações no desenvolvimento craniofacial de embriões, manipulando o receptor $5HT_{1A}$ com o agonista serotoninérgico específico 8-OH-DPAT. Algumas dessas mudanças consistiram do encurtamento das dimensões do crânio, resultado semelhante ao observado por animais que receberam sertralina, ou fluoxetina ou citalopram nos distintos experimentos do presente estudo.

Portanto, é pertinente considerar o atraso no desenvolvimento de estruturas do crânio (pavilhão auricular e conduto auditivo) e da face (incisivos superiores e inferiores), nessa série de estudos, análogo à redução no crescimento da cabeça. Ademais, a relação entre cada SSRI e o tempo indica que as reduções do ganho de peso corporal, do crescimento do corpo e da cauda eram mais precoces quanto maior era a dose de cada droga empregada. Essas mudanças merecem atenção porque revelam a existência de mecanismos de crescimento em tecidos do corpo, que são dose-sensíveis aos inibidores seletivos de recaptação da serotonina. Portanto, durante o período de aleitamento, período critico do desenvolvimento nervoso no rato, outros tecidos são também influenciados pelos efeitos dos SSRIs, muito provavelmente no sistema de neurotransmissão serotoninérgica, já presente no rato na fase pós-natal (LIDOV AND MOLLIVER, 1982). Além disso, um papel trófico da 5-HT na formação da estrutura craniofacial, incluindo a maturação do germe dos dentes, também já foi descrita (MOISEIWITCH AND LAUDER 1996). O atraso da irrupção dos dentes nos grupos tratados com os SSRIs aqui utilizados, corroboram estes estudos.

A serotonina possui ação trófica na diferenciação de vários tecidos durante os períodos pré e pós-natal (LAUDER 1993; MOISEIWITSH e LAUDER, 1996) por agir como um fator neurotrófico (YAN et al., 1997). Alguns estudos com culturas de embriões de camundongos tratados com serotonina sugerem um desenvolvimento aumentado das células serotoninérgicas no tecido nervoso, bem como células da proeminência nasal, do epitélio que recobre o olho, da vesícula ótica e da cavidade oral (LAUDER et al, 1988; LIDOV e MOLLIVER 1982). Esses achados indicam que essa amina desempenha um papel no controle das interações mesênquima-epitélio durante a morfogênese craniofacial (HALL 1981; SHUEY et al 1993). O tratamento serotoninérgico também causou malformações em estruturas nervosas e ósseas de embriões de camundongos (LAUDER e KREBS 1993). Muitos desses eventos começam na 2ª semana de vida e os primeiros neurônios sertoninérgicos aparecem entre o 12º e o 14º dias de gestação (LIDOV e MOLLIVER 1982). Assim, durante o aleitamento, o tecido pode ser capaz de responder aos SSRIs.

Alterações no aparecimento dos reflexos indicam uma correlação entre desenvolvimento bioquímico e estrutural e ontogênese do sistema nervoso. Ratos desnutridos apresentaram retardo no desenvolvimento dos reflexos, como preensão palmar; resposta ao susto e endireitamento e aversão ao precipício. Curiosamente, a desnutrição protéica precoce é uma condição que aumenta os níveis de 5-HT no cérebro (RESNIK e MORGANE 1984). A administração pós-natal de 5,7 –DHT (KOSTOWSKY e KRZASCIKI, 2003), levou a níveis reduzidos de 5-HT e 5HIAA, indicando atividade da 5-HT nessa fase precoce da vida. No

presente estudo, além dos prejuízos nos parâmetros de crescimento, o possível aumento no nível de serotonina induzido pelos SSRIs provocou atraso nos reflexos.

Por outro lado, é sabido que o sistema serotoninérgico tem um importante papel na regulação do apetite e que drogas que aumentam a concentração extracelular de 5-HT, a exemplo dos SSRIs, são agentes anoréxicos efetivos e provocam perda de peso em ratos adultos (HALFORD e BLUNDEL, 1996). Assim, a redução persistente no ganho de peso observada neste trabalho, em todas as doses de cada SSRI utilizado, parece indicar que alterações persistentes no sistema serotoninérgico induziram tal dano. Em nossos experimentos, merece atenção o fato de que mudanças no aparecimento de alguns reflexos nem sempre aconteceram ao mesmo tempo das mudanças nas medidas corporais. Isso sugere uma influência distinta e específica da 5-HT em cada um dos processos de desenvolvimento dos tecidos.

Uma das hipóteses levantadas em perspectivas de trabalhos anteriores realizados em nosso laboratório, seria aquela do possível efeito hipofágico de SSRIs no período pós - natal, o que também originou um dos artigos aqui apresentados, tendo como premissa estudar, utilizando um dos SSRIs – em diferentes doses, o efeito sobre o comportamento alimentar neonatal através de parâmetros utilizados em outros estudos (RIUL et al., 1999). Observou-se, portanto no presente estudo que o tratamento neonatal com o SSRI retardou o ganho de peso. Além disso, o tratamento com o SSRI provocou redução na ingestão alimentar em todas as doses utilizadas na maioria dos dias estudados. A maior dose do SSRI aqui utilizada, foi considerada como moderadamente anoréxica por CLIFTON et al., (1989).

Entre os comportamentos aqui estudados, o SSRI provocou aumento na freqüência da interação mãe-filhotes e na freqüência do comportamento de limpeza materno. O aumento da freqüência na interação entre a mãe e sua prole observada no presente estudo, caracteriza maior interação mãe-filho, para realização de outros comportamentos subjacentes, o que neste caso, parece ter sofrido a influência do tratamento nos filhotes que se mantinham mais vezes próximos de suas mães. Ademais, ratos neonatos são inábeis para manter a temperatura corporal. A resposta vascular para o aquecimento do corpo do animal se torna efetiva após 12 dias e, só após o 21º dia do nascimento o rato adquire a capacidade de manter a temperatura corporal estável (COUKLIN e HEGGENESS, 1971). Assim, é possível que o aumento da freqüência da interação mãe-filho, aqui observada, seja visto como acréscimo de cuidados maternos para a manutenção da temperatura dos filhotes, visto que, o tratamento com o SSRI não alterou a duração nem a freqüência para o aleitamento.

Em adição, é interessante a observação, da relação entre a interação mãe-filho com a duração e a freqüência da sucção. Esta última, sofreu influência do tempo independente do tratamento. A primeira semana de vida foi marcante na expressão da duração desse comportamento, enquanto a freqüência aumentou na segunda semana de vida, quando os filhotes começam interagir com sua mãe de forma autônoma e independente desta. Assim, estaria justificada a influência da idade, observada no presente estudo. Dessa forma, durante a amamentação a mãe usufrui desta interação com a sua prole de forma simultânea (lambendo-os, carregando-os para junto de si e até limpando-se), o que não ocorre com outros comportamentos que dependem geralmente do seu afastamento dos filhotes. O comportamento de limpeza teve a freqüência aumentada nas ninhadas tratadas com o SSRI, observando-se também a influência da idade, que se apresentou de maneira mais freqüente nas duas primeiras semanas de vida.

A sincronia comportamental entre mãe e filho, durante o período de amamentação, requer adaptação materna contínua ao desenvolvimento das crias (CRNIC, 1980). Em ratos, vários ajustes comportamentais maternos exercem importante papel na determinação da disponibilidade de energia para a prole (CRNIC, 1980). Estudos têm demonstrado que a interação entre mãe e filho, a quantidade/qualidade dos cuidados maternos e a interação entre irmãos, durante o período crítico do desenvolvimento, parecem interferir na ontogênese do SNC (LAVIOLA e TERRANOVA, 1998). As primeiras horas da interação mãe-filho são consideradas cruciais para o desenvolvimento comportamental (GALLER e PROPERT, 1982).

As mudanças observadas nos resultados aqui apresentados podem indicar a participação da 5-HT na maturação de comportamentos no inicio da vida pós-natal. Desde a fase pré-natal a serotonina estaria presente em estágios do desenvolvimento, participando de processos precoces do controle celular envolvidos no desenvolvimento e agindo como fator de crescimento neuronal (BUZNIKOV 1984; LAUDER 1988; WHITAKER-AZMITIA 1987). Altas densidades de receptores 5-HT são encontradas no tronco cerebral e cérebro anterior no período pós-natal (WHITAKER -AZMITIA et al., 1987).

Por outro lado, a alimentação fornece energia e nutrientes necessários à maturação e desenvolvimento funcional do SN (MORGANE et al., 1993). A deficiência nutricional é capaz de alterar os eventos morfogenéticos com conseqüências, deletérias para o desenvolvimento e aquisição de padrões fisiológicos maduros (DOBBING 1964; WINICK e NOBEL, 1966; NOBACK e EISENMAN, 1981). Neonatos submetidos a desnutrição protéica apresentam desorganização de centros hipotalâmicos reguladores do peso corporal e do metabolismo (Plagemann, 2000). Estudo sugere que deficiências de nutrientes durante a vida precoce influenciam a neurotransmissão e, em conseqüência, poderiam afetar a expressão comportamental

(WAUBEN e WAINWRIGHT, 1999). Alterações na interação mãe-filho a exemplo daquelas decorrentes da desnutrição podem acarretar retardo no desenvolvimento e elevada dependência do filhote em relação à mãe (RIUL et al., 1999). Ademais, os comportamentos são disparados e guiados por eventos neurais, porém cada ato comportamental envolve uma resposta que é traduzida nos sistemas fisiológicos periféricos, na atividade neuroquímica do cérebro (BLUNDELL, 1993).

Observe-se neste estudo a significante redução do peso corporal dos filhotes tratados com o SSRI. Além da perda de peso, a ingestão dos filhotes mostrou-se reduzida em todas as doses utilizadas sendo influenciada pelo tempo (dias de vida dos animais) e pela interação entre estes dois fatores. Assim parece ficar claro o efeito anoréxico provocado pela fluoxetina no presente estudo, independente de mudanças em outros comportamentos. Efeito anorético dramático e perda de peso após tratamento com fluoxetina foi observado por Kalia et al., (2000). Estudo de Simansky e Vaidya (1990) mostrou que sertralina, outro SSRI, reduziu o consumo de leite, após privação alimentar em ratos adultos, através de mudanças de mecanismos comportamentais. A perda expressiva de peso dos animais tratados com o SSRI na dose mais alta neste estudo pode ser explicada por esse caminho, provocando possível desnutrição nos filhotes. A permanência junto à mãe pode não ser condição exclusiva para amamentação, desde que o neonato é dependente de sua mãe para manutenção da temperatura entre outras funções fisiológicas (FRIEDMAN, 1975. Fato interessante foi o aumento de peso observado na dose menor. Tratamento crônico com SSRIs, pode provocar desenssibilização de receptores provocando hiperfagia e aumento de peso (GARDIER et al., 1996; HAMON e GOZLAN 1993)

Em conclusão, nossos resultados respondem a relevantes indagações cientificas, provocando outras novas, acerca do possível papel da serotonina, durante o período de aleitamento, no desenvolvimento corporal (através dos retardos nas medidas do peso, do crescimento e dos eixos craniais e da maturação de características físicas); no desenvolvimento neural (através dos atrasos na ontogenia de reflexos) provocados pela administração dos três SSRIs utilizados; a administração de um desses, causou aumento nas freqüências da interação mãe-filho e do comportamento de limpeza materno, que por sua vez, envolve outros sub-comportamentos, bem como, provoca redução no ganho de peso corporal e na ingestão do filhote até em doses consideradas como baixas e moderadamente anoréxicas.

Estes achados suportam a hipótese de que os mecanismos do crescimento e do desenvolvimento em ratos neonatos são altamente suscetíveis à manipulação do sistema serotoninérgico. Ademais, na fase pós-natal, a serotonina teria papel modulador

comportamental e na ontogênese de tecidos neurais e não neurais

7 CONCLUSÃO E PERSPECTIVAS

CONCLUSÃO

Numa visão geral, os resultados apresentados neste trabalho, conduzem às seguintes conclusões:

1- Tratamento neonatal com inibidor seletivo da recaptação da serotonina (SSRI)- citalopram- retarda o crescimento ponderal, o crescimento dos eixos do crânio a maturação de características físicas e a ontogenia de reflexos em ratos.

2- A administração crônica de fluoxetina um SSRI atasa o crescimento corporal e altera a estrutura craniofacial de ratos neonatos além de retardar a maturação de reflexos.

3- O tratamento com SSRI- sertralina provoca retardos tanto no ganho ponderal como no crescimento corporal, além de modificar o crescimento da estrutura craniofacial de animais neonatos.

4- Ratos neonatos submetidos ao tratamento com fluoxetina apresentam mudanças na freqüência de parâmetros do comportamento e da ingestão alimentar neonatal.

5- Ademais as conclusões apresentadas neste estudo, visam contribuir com os profissionais que prescrevem esses fármacos na rotina clinica do tratamento da depressão, na fase da vida em que o encéfalo é mais vulnerável a agressões.

PERSPECTIVAS

1. Diante dos resultados obtidos no presente trabalho, é possível delinear as seguintes perspectivas:

2. Estudar a composição de tecidos corporais muscular, ósseo e adiposo de animais tratados com SSRIs durante o período de aleitamento, em diferentes idades, durante e após cessado o tratamento.

3. Investigar através de raio "X" e outros recursos de imagens, possíveis alterações no tecido ósseo e outros tecidos do arcabouço craniofacial de animais neonatos tratados com SSRIs.

4. Estudar em animais neonatos e adultos, repercussões do tratamento com SSRIs em determinadas regiões do encéfalo, em particular aquelas onde o sistema serotoninérgico tem função destacada e, relacionada ao comportamento alimentar.

5. Investigar o efeito do tratamento com SSRIs durante a pré-pubescência e adolescência, bem como repercussões na idade idade adulta, em animais fêmeas normais e geneticamente obesas.

6. Estudar possíveis mudanças na morfologia e morfometria de órgãos de animais tratados com SSRIs no período tanto pré como pós –natal.

7. Utilizando técnicas mais avançadas, investigar a participação de receptores serotoninérgicos no crescimento de tecidos neurais e não neurais, em animais tratados com SSRIs durante o período de aleitamento.

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APÊNDICE A - Neonatal administration of citalopram delays somatic maturation in rats

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Neonatal administration of citalopram delays somatic maturation in rats

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Abstract

We investigated the somatic maturation of neonate rats treated during the suckling period with citalopram, a selective serotonin reuptake inhibitor. Groups with 6 male neonates were randomly assigned to different treatments 24 h after birth. Each litter was suckled by one of the dams until the 21st postnatal day. Body weight, head axis and tail length were measured daily from the 1st to the 21st postnatal day. Time of ear unfolding, auditory conduit opening, incisor eruption, and eye opening was determined. Pups received 5 mg (Cit5), 10 mg (Cit10) or 20 mg/kg (Cit20) citalopram *sc*, or saline (0.9% NaCl, w/v, *sc*). Compared to saline, body weight was lower (24.04%, $P < 0.01$) for Cit10 from the 10th to the 21st day and for Cit20 from the 6th to the 21st day (38.19%, $P < 0.01$). Tail length was reduced in the Cit20 group (15.48%, $P < 0.001$) from the 8th to the 21st day. A reduction in mediolateral head axis (10.53%, $P < 0.05$) was observed from the 11th to the 21st day in Cit10 and from the 6th to the 21st day in Cit20 (13.16%, $P < 0.001$). A reduction in anteroposterior head axis was also observed in the Cit20 group (5.28%, $P < 0.05$) from the 13th to the 21st day. Conversely, this axis showed accelerated growth from the 12th to the 21st day in the Cit5 group (13.05%, $P < 0.05$). Auditory conduit opening was delayed in the Cit5 and Cit20 groups and incisor eruption was delayed in all citalopram groups. These findings show that citalopram injected during suckling to rats induces body alterations and suggest that the activity of the serotoninergic system participates in growth mechanisms.

Key words
- Serotonin
- Growth indicators
- Selective serotonin reuptake inhibitor
- Citalopram
- Craniofacial
- Skull

Introduction

Selective serotonin reuptake inhibitors (SSRI) such as citalopram have been used to induce increased serotoninergic activity in the brain (1,2). Experimental evidence indicates that serotonin can influence embryogenesis and growth (3,4) presumably by acting as a developmental signal (5) or as a neurotrophic factor as well (6). Furthermore, serotonin seems to play a role in regulating the development of the mammalian brain through its action on the production of self serotonin neurons (4,7,8) and on target tis-

sues innervated by serotoninergic neurons (9-11). Pharmacological or nutritional manipulations during pregnancy or the suckling period can induce drastic morphological and functional changes in the growth and development of the nervous system (12-16). Several studies have shown that treatment with SSRI reduces food intake and body weight in rats (17,18). A hypophagic effect was observed following fluoxetine administration (19). As a consequence, the possibility exists that the use of serotoninergic agents in the early phase of life could influence other specific maturation processes in the body. Therefore, it is desirable to investigate the possible effects of manipulation of the serotoninergic system early in life.

The objective of the present study was to test the hypothesis that the administration of citalopram - a highly SSRI (20) - to rats during the suckling period induces changes in somatic development and in body growth.

Material and Methods

Animals

Litters obtained from dams mated to male Wistar rats from the colony of the Nutrition Department, Federal University of Pernambuco, Recife, PE, Brazil, were used in the present study. During gestation and until the end of the experiment, the animals were housed in polyethylene cages. Male pups from different mothers (N = 18) were randomly divided into groups of 6 neonates 24 h after birth. Each pup was marked with methyl violet solution on the skin for identification during the experiment. Each litter was suckled by one of the dams until the 21st postnatal day (birth day was considered to be zero day). The animals were maintained at a room temperature of 23 ± 1ºC, on a 12/12-h light-dark cycle (lights from 6:00 am to 6:00 pm) with free access to ration (Labina-Purina, São Lourenço da Mata, PE, Brazil) and water.

Citalopram

The experiment was performed blind to prevent identification of the experimental groups. The animals of the different groups were evaluated simultaneously. For the experimental treatment, four groups (N = 27 each) of suckling rats were distributed as follows: three groups received citalopram: group Cit5 (5 mg/kg, *sc*), group Cit10 (10 mg/kg, *sc*), and group Cit20 (20 mg/kg, *sc*), and a control group received an equivalent volume of saline (0.9% NaCl, w/v) *sc*. During the experiment, one neonate rat in the Cit20 group and one in the Cit5 group died. Therefore, 106 rats were evaluated. The time of physical feature maturation and somatic growth was recorded. Citalopram (H. Lundbeck A/S, Copenhagen-Valby, Denmark), was dissolved in saline and 1 ml/100 g body weight was injected *sc*. The treatment was applied daily from the 1st to the 21st postnatal day (suckling period) at 0:30-1:30 pm.

Physical feature maturation

The following physical features were observed: unfolding of the external pinnae of both ears to the fully erect position; internal auditory conduit opening of both ears; incisor eruption, i.e., the first visible and palpable crest of lower incisors, and eye opening, i.e., when any visible break in the covering membrane of both eyes was detected. The features were evaluated daily between 10:00 and 12:00 am by the method of Smart and Dobbing (21) during the suckling period until maturation of the variable. The maturation age of a particular feature was defined as the day it was first observed.

Somatic growth

Somatic growth was assessed in terms of body weight, tail length, and mediolateral and anteroposterior head axis measurements made from the 1st to 21st postnatal day

between 1:00 and 2:00 pm as follows: body weight was measured with a FANEM scale (São Paulo, SP, Brazil) with 100 mg precision. Tail length (distance from tail tip to tail base), length of the mediolateral skull axis (distance between the ear holes), and length of the anteroposterior axis of the head (distance between snout and head-neck articulation) were measured with a Starret caliper with 0.05 mm precision.

Statistical analysis

Statistical analysis was performed after preliminary testing to identify normality of distribution and homogeneity of variance among the groups. Two-way ANOVA for repeated measures (from the 1st to the 21st day for body weight, head axis and tail length) followed by the *post hoc* Tukey test were used to compare growth indicators between each citalopram group and the saline control. Kruskal-Wallis analysis of variance followed by the Dunn test was used to compare physical features between each citalopram group and the saline control. The level of significance was set at $P < 0.05$.

The experimental protocol of the study was approved by the Ethics Committee for Animal Experimentation of the Federal University of Pernambuco, and was consistent with the National Institute of Health Guide for Care and Uses of Laboratory Animals (Publication No. 85-23, revised, 1985).

Results

ANOVA identified main effects of time and treatment in addition to interaction between these factors regarding somatic growth. For body weight, statistical analysis showed an effect of citalopram ($F_{3,102} = 52.9$, $P < 0.001$), and day of life ($F_{20,2040} = 2340.6$, $P < 0.001$) as well as a citalopram versus day of life interaction ($F_{60,2040} = 69.5$, $P < 0.001$). Body weight gain (Figure 1A) was reduced from the 10th to the 21st day (24.04%, $P < 0.01$) in Cit10 and from the 6th to the 21st day (38.19%, $P < 0.01$) in Cit20 when compared to saline. This lower weight gain was more marked in the Cit20 than in the Cit10

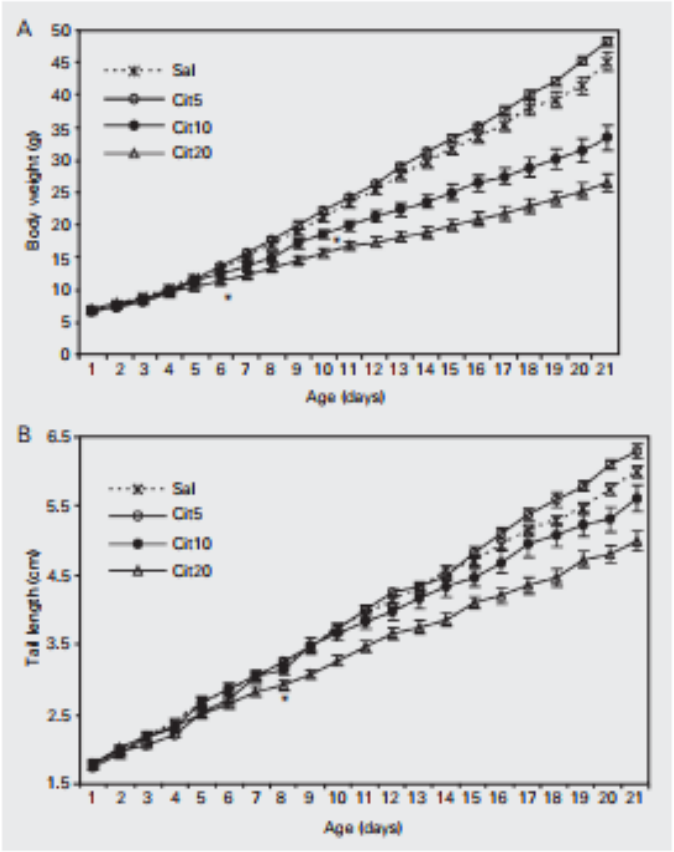

Figure 1. Effect of citalopram on suckling rat weight and tail length. *A*, Body weight from the 1st to the 21st day of life of suckling rats treated with solutions (1 ml/100 g body weight, *sc*) of citalopram: 5 mg (Cit5, N = 26), 10 mg (Cit10, N = 27) and 20 mg/kg (Cit20, N = 26) or saline (Sal, N = 27). Comparisons were made by two-way ANOVA for repeated measures: citalopram ($F_{3,102} = 52.9$, $P < 0.001$), day of life ($F_{20,2040} = 2340.6$, $P < 0.001$), and citalopram versus day of life interaction ($F_{60,2040} = 69.5$, $P < 0.001$) followed by *post hoc* multiple comparisons (Tukey test) between each citalopram dose and saline: *$P < 0.001$. Each point represents the mean values according to the dose. The asterisks indicate the beginning of significant differences ($P < 0.01$) that persisted through the end of the experiment. *B*, Tail length. Comparisons were made by two-way ANOVA for repeated measures: citalopram ($F_{3,102} = 13.7$, $P < 0.001$), day of life ($F_{20,2040} = 3198.0$, $P < 0.001$), and citalopram versus day of life interaction ($F_{60,2040} = 18.7$, $P < 0.001$) followed by *post hoc* multiple comparisons (Tukey test) between each citalopram dose and saline: each point represents the mean values according to the dose. The asterisk indicates the beginning of significant differences ($P < 0.01$) which persisted to the end of the experiment according to the dose.

group. The Cit5 group did not differ from saline (Figure 1A).

For tail length (Figure 1B), statistical analysis showed a main effect of citalopram ($F_{3,102}$ = 13.7, P < 0.001) and day of life ($F_{20,2040}$ = 3198.0, P < 0.001) in addition to an interaction between citalopram and day of life ($F_{60,2040}$ = 18.7, P < 0.001). A reduced growth of tail length was observed in the Cit20 group from the 8th to the 21st day (15.48%, P < 0.01); however, the Cit10 and Cit5 groups did not differ from the saline control.

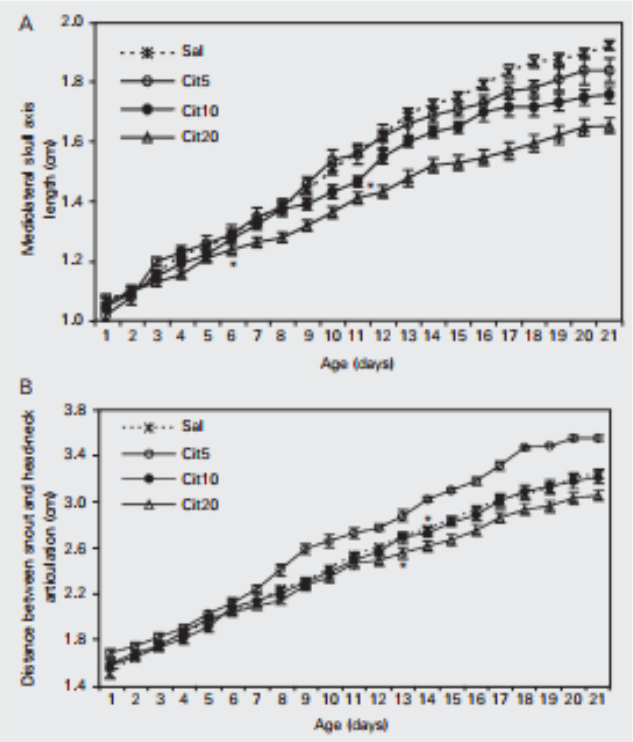

Figure 2. Effect of citalopram on suckling rat mediolateral skull axis length and distance between snout and head-neck articulation. *A*, Mediolateral skull axis from the 1st to 21st day of life of suckling rats treated with solutions (1 ml/100 g body weight, sc) of citalopram: 5 mg (Cit5, N = 26), 10 mg (Cit10, N = 27) and 20 mg/kg (Cit20, N = 26) or saline (Sal, N = 27). Comparisons were made by two-way ANOVA for repeated measures for citalopram ($F_{3,102}$ = 23.8, P < 0.001) and day of life ($F_{20,2040}$ = 1622.1, P < 0.001) and citalopram versus day of life interaction ($F_{60,2040}$ = 15.3, P < 0.001) followed by *post hoc* multiple comparisons (Tukey test) between each citalopram dose and saline. The asterisks indicate the beginning of significant difference in the Cit10 group (P < 0.05) and Cit20 group (P < 0.001) that persisted to the end of the experiment. *B*, Distance between snout and head-neck articulation. Comparisons were made by two-way ANOVA for repeated measures: citalopram ($F_{3,102}$ = 15.1, P < 0.001), day of life ($F_{20,2040}$ = 2770.6, P < 0.001), and citalopram versus day of life interaction ($F_{60,2040}$ = 14.7, P < 0.001) followed by *post hoc* multiple comparisons (Tukey test) between each citalopram dose and saline. The asterisks indicate the beginning of significant differences for Cit20 (P < 0.01) and Cit5 (P < 0.05) that persisted to the end of the experiment.

ANOVA showed an effect of citalopram ($F_{3,102}$ = 23.8, P < 0.001) and day of life ($F_{20,2040}$ = 1622.1, P < 0.001), as well as an interaction between citalopram and day of life ($F_{60,2040}$ = 15.3, P < 0.001) on the mediolateral head axis. A reduction in mediolateral head axis growth was observed from the 11th to the 21st day in the Cit10 group (10.53%, P < 0.05), and from the 6th to the 21st day in the Cit20 group (13.16%, P < 0.001) compared to the saline control. Moreover, the reduced growth of this axis was more marked for the 20 mg dose than for the 10 mg dose (Figure 2A).

There was also a significant change in growth of the anteroposterior head axis length. There were effects of citalopram ($F_{3,102}$ = 15.1, P < 0.001), day of life ($F_{20,2040}$ = 2770.6, P < 0.001) and interaction between citalopram and day of life ($F_{60,2040}$ = 14.7, P < 0.001). In the Cit20 group a reduction of this axis occurred from the 13th to the 21st day (5.28%, P < 0.01), whereas the growth was accelerated from the 12th to the 21st day in the Cit5 group (13.05%, P < 0.05). There was no difference for the Cit10 group (Figure 2B).

Physical features

For physical feature maturation (Table 1), Kruskal-Wallis analysis indicated significant changes in auditory conduit opening (H = 10.59, P < 0.05) and incisor eruption (H = 19.33, P < 0.01) in the citalopram groups. Multiple comparisons of these features among treatment groups (Dunn test) showed that auditory conduit opening was delayed in the Cit5 (P < 0.05) and Cit20 groups (P < 0.05). Incisor eruption was delayed in the

Cit5 ($P < 0.05$), Cit10 ($P < 0.01$) and Cit20 ($P < 0.05$) groups. It is noteworthy that a wide variation (11-18 days for auditory conduit opening and 10-17 days for incisor eruption) was observed in the Cit20 group but not in groups receiving the lowest citalopram doses or saline. For eye opening and ear unfolding there was no significant difference.

Discussion

The present results show that chronic administration of citalopram during the critical neonatal period of rat brain development induces changes in growth. In general the drug impaired somatic growth and physical body maturation. As observed for body weight, increasing doses of citalopram resulted in equivalent growth alteration of tail and head. Regarding craniofacial development, the heads of the animals became longer with the lowest citalopram dose, narrower with the middle dose and both shorter and narrower with the highest dose. Therefore, the head suffered a shape distortion during development that was dependent on the dose of citalopram. The sensitivity of vertebrate head growth to epigenetic manipulations has been well demonstrated (22,23). Changes in the craniofacial skeleton of developing rats submitted to a low protein diet were shown by Miller and German (24). Some of these changes consisted of shortening of skull dimensions, a result similar to that experienced by the animals receiving 20 mg citalopram in the present study. It is noteworthy that early protein malnutrition such as studied by Miller and German (24) is known to increase brain serotonin (5-HT) levels (13). Therefore, it is appropriate to consider the delayed development of the structures inside the skull (auditory conduit) and face (lower incisors) observed in the present study, because the delays might be associated with reduced head growth. Furthermore, the interaction between citalopram and time indicates that the highest citalopram dose caused not only a reduction of body weight gain and of tail growth, but also that these reductions in rats receiving 20 mg began earlier than in animals receiving the two lowest doses. These changes are important because they indicate the existence of growth mechanisms in body tissues which are sensitive to the dose of citalopram. Since this drug increases serotonin release (20) we suggest that serotoninergic mechanisms may be responsible for the effects of citalopram.

In addition, there is evidence that SSRI and some serotoninergic agonists also increase 5-HT release not only in neuronal tissues (10,11,25) but also in non-neuronal tissues (26,27). Interestingly, serotonin has a trophic action on the differentiation of sev-

Table 1. Maturation (in days) of the physical features of suckling rats treated with citalopram from the 1st to the 21st day of life.

Physical features	Saline (N = 27)	Citalopram (mg/kg)		
		5 (N = 26)	10 (N = 27)	20 (N = 26)
Ear unfolding	3.0 (1-4)	3.0 (2-6)	3.0 (2-4)	3.0 (2-5)
Auditory conduit opening	12.0 (11-14)	13.0 (12-14)*	12.5 (12-14)	13.0 (11-18)*
Eruption of the lower incisors	12.0 (9-13)	13.0 (12-14)*	12.0 (11-15)*	12.0 (10-17)*
Eyes opening	14.0 (12-15)	14.0 (12-16)	14.0 (12-14)	14.0 (12-17)

Kruskal-Wallis analysis of variance followed by the Dunn test were used to compare physical features between each citalopram dose and the saline control group. A significant change in auditory conduit opening ($H = 10.59$, $P < 0.05$) and incisor eruption ($H = 19.33$, $P < 0.01$) was observed in the citalopram groups. Data are reported as median and range (in parentheses).
*$P < 0.05$ compared to control.

eral tissues during the prenatal and postnatal periods (27,28) by acting as a neurotrophic factor (6). Some studies on mouse embryo cultures treated with serotonin suggest an improved development of serotoninergic cells in the nervous tissue and of cells of the nasal prominence, epithelium covering the eye, optic vesicle, and oral cavity (10,29-33). These findings indicate that serotonin plays a role in the control of the epithelial-mesenchymal interactions during craniofacial morphogenesis (26). Serotoninergic treatment also caused malformation of both nerve and bone structures of mouse embryos (10). Several of these events begin in the second week of life and the first serotoninergic neurons appear as early as on the 12th to the 14th day of gestation (29). Therefore, during suckling the tissue may be able to respond to the challenge with citalopram. Moreover, a trophic role of 5-HT in the formation of craniofacial structures, including the maturation of the tooth germ, has been reported (28).

Our findings support the data in the literature because the delayed tooth eruption observed in the citalopram groups. However, the participation of citalopram in this maturation process appears to be complex since the highest dose induces a wider variation of the physical feature than the lowest doses. On the other hand, the possibility exists that the effects observed in this experiment may be induced by the action of 5-HT on anorectic mechanisms and not by a direct action of 5-HT on tissues. In fact, it has been well documented that hypophagia induced by citalopram reduces body weight in adult rats (17,34) and body weight gain in young rats (16). Therefore, in the present experiment we could not rule out that the hypophagia induced by citalopram and the resulting malnutrition were capable of causing growth disorders in animals that received citalopram.

We conclude that citalopram administered during the period of rapid brain development causes important morphological body alterations. These data support the view that growth mechanisms are highly susceptible to the manipulation of the serotoninergic system during this period.

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