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SENSIBILIDADE SOMESTÉSICA COMO FATOR PARTICIPANTE DO DESENVOLVIMENTO NEURAL: ANÁLISE ELETROFISIOLÓGICA EM RATOS PRECOCEMENTE DESNUTRIDOS

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Sensibilidade somestésica como fator participante do desenvolvimento neural: Análise eletrofisiológica em ratos precocemente desnutridos

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Orientador: Prof. Rubem Carlos Araújo Guedes

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À minha família,

Ao professor Rubem Guedes,

Aos futuros estudiosos da Depressão Alastrante Cortical.

“Não tema avançar lentamente, receie apenas ficar parado”.

(Provérbio chinês)

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RESUMO

A condição nutricional, bem como a interação organismo-ambiente são fatores que influenciam o desenvolvimento do sistema nervoso. Mecanismos de plasticidade neural ocorrem diante de modificações nessas variáveis, podendo resultar em conseqüências funcionais. No rato, a privação da aferência sensorial representado pelas vibrissas pode induzir alterações morfológicas na correspondente área de representação cortical, modificando suas propriedades funcionais. No presente trabalho investigou-se a influência da remoção precoce unilateral das vibrissas sobre as características da Depressão Alastrante Cortical (DAC) analisada em ratos após o desmame e na idade adulta, sob condições favoráveis e desfavoráveis de lactação (respectivamente, grupos bem-nutrido e desnutrido; ninhadas formadas por 6 e 12 filhotes). Os animais foram submetidos à remoção unilateral das vibrissas no 2º. ou 3º. dia de vida e os registros eletrofisiológicos da DAC foram realizados em dois pontos da superfície cortical de ambos os hemisférios cerebrais, aos 30-40 dias (animais jovens) ou aos 90-120 dias (animais adultos). Os pesos corporais e encefálicos foram reduzidos, enquanto que a velocidade de propagação da DAC aumentou nos animais desnutridos, quando comparados aos animais bem-nutridos. A remoção das vibrissas esteve associada a uma redução nos pesos corporais e encefálicos nos animais jovens, mas não nos adultos. Os ratos submetidos à privação sensorial apresentaram velocidades da DAC mais altas no hemisfério contralateral à remoção das vibrissas, quando comparadas com o hemisfério ipsilateral, no entanto, aqueles que foram submetidos à desnutrição apresentaram uma redução significativa da velocidade da DAC no hemisfério ipsilateral à remoção das vibrissas, quando comparados com os hemisférios correspondentes dos desnutridos que permaneceram com as vibrissas intactas. Nos animais controles (mantidos com as vibrissas intactas), nenhuma diferença inter-hemisférica foi encontrada. Os resultados sugerem que (1) a privação sensorial originada nas vibrissas durante o desenvolvimento cerebral facilita a propagação da DAC e (2) a desnutrição pode influenciar esse efeito, o qual (3) parece ser permanente, uma vez que persistiu até a idade adulta.

Palavras chave: Plasticidade neural; Depressão alastrante cortical; Privação sensorial; Desenvolvimento cerebral; Desnutrição

ABSTRACT

The nutritional condition and the interaction organism-environment are factors that influence the mammalian neural development. Mechanisms of neural plasticity occur as a consequence of modifications on these variables, and can result in functional alterations. In the rat, deprivation of the sensory input represented by the vibrissae can induce morphological changes on the corresponding cortical representation fields, modifying their functional properties. In the present work we investigated the influence of early unilateral vibrissae removal on Cortical Spreading Depression (CSD) propagation features, assessed in weaned and in adult rats, previously submitted to favorable and unfavorable lactation conditions (respectively well-nourished and malnourished groups; litters formed by 6 and 12 pups). Unilateral vibrissae removal was carried out at postnatal days (PD) 2-3 and CSD electrophysiological recordings were done on two points of the cortical surface of both cerebral hemispheres, either at PD 30-40 (young animals) or 90-120 (adult animals). Body- and brain weights were reduced, whereas CSD propagation velocities were increased, in the malnourished animals, as compared to the corresponding well-nourished ones. Vibrissae removal was associated to body- and brain weight reduction in the young, but not in the adult animals. Compared to the nutrition- and age-matched controls (intact vibrissae animals), CSD propagation was faster in the hemisphere contralateral to the vibrissae removal side, however, the malnourished lesioned animals showed a significant reduction in the CSD velocity on the hemisphere ipsilateral to the vibrissae removal, as compared to the corresponding hemisphere of the malnourished intact animals. The results suggest that (1) the vibrissae-mediated sensory deprivation during brain development facilitates CSD propagation, and (2) early malnutrition may influence this effect, which (3) seemed to be permanent, since it remained until the adult life.

Key-words: Neural plasticity, Cortical spreading depression; Sensory deprivation, Brain development, Malnutrition.

LISTA DE ABREVIATURAS E SIGLAS

1. Anova – Análise de variância
2. BDNF – Fator neurotrófico derivado do cérebro
3. bFGF – Fator de crescimento fibroblástico básico
4. DAC – Depressão alastrante cortical
5. IGF-I – Fator de crescimento insulínico – I
6. i.p. – Intraperitonial
7. KCl – Cloreto de potássio
8. LAFINNT – "Laboratório de fisiologia da nutrição Naíde Teodósio"
9. NGF – Fator de crescimento neuronal
10. PD – Postnatal day
11. SNC – Sistema Nervoso Central
12. SNP - Sistema Nervoso Periférico
13. TGFB – Fator transformador do crescimento - β
14. VLV – Variação lenta de voltagem.

SUMÁRIO

1. Introdução

O desenvolvimento do sistema nervoso sofre influências de fatores intrínsecos e extrínsecos ao organismo (Kehoe et al., 2001; Morgane et al., 1993; Rice e Barone Jr., 2000; Le Roy et al., 2001). Dentre os fatores extrínsecos, a condição nutricional e a interação somatossensorial com o ambiente em períodos críticos do desenvolvimento exercem grande impacto na organização estrutural e funcional do encéfalo (Fernández et al., 1998; Fox, 1992; Kehoe et al., 2001; Medina-Aguirre et al., 2008; Mierau et al., 2004; Morgane et al., 1993; Resnick et al., 1979).

Vários efeitos da desnutrição sobre a estrutura e a função neural durante o desenvolvimento têm sido estudados em animais de laboratório (Morgane et al., 1978; 1993; Resnick et al., 1979) e em seres humanos (Gordon, 1997; Scrimshaw, 1998), demonstrando-se que a desnutrição pode interferir negativamente em vários aspectos do desenvolvimento do sistema nervoso central (SNC), com conseqüências sobre os aspectos neuromotor, comportamental e cognitivo (Barros et al., 2006; Gordon, 1997; Scrimshaw, 1998).

Por outro lado, as experiências sensoriais obtidas através da interação de receptores periféricos com o ambiente externo participam dos mecanismos de organização estrutural do SNC (Markham e Greenough, 2004; Petersen, 2007), bem como das suas respostas, que irão influenciar os processos de aprendizagem (McGraw et al., 2009) e o comportamento (Renner e Rosenzweig, 1987).

Diante de tais evidências, verifica-se que a desnutrição em períodos iniciais da vida, bem como as manipulações nos sistemas sensoriais, estimulando-os ou restringindo-os, podem interferir nos mecanismos de plasticidade neural – definida como a capacidade de resposta adaptativa do sistema nervoso diante de alterações orgânicas e ambientais (Morgane et al., 1993; Buonomano e Merzenich, 1998; Rema et al., 2006).

Esses mecanismos de plasticidade neural são fundamentais para o desenvolvimento adequado do SNC, a capacidade de aprender novas tarefas e ainda para a recuperação funcional após danos neurológicos de causas diversas (Buonomano e Merzenich, 1998; McGraw et al., 2009; Ramanathan et al., 2006).

Vários estudos, tanto experimentais quanto clínicos, têm buscado elucidar os mecanismos subjacentes à plasticidade neural, aplicando estes conhecimentos ao desenvolvimento de tratamentos de distúrbios neurológicos (Bach-Y-Rita, 1990; Fox, 2009; Gauthier, 2008; Kolb e Whishaw, 1989).

As vibrissas dos roedores oferecem várias vantagens para estudar os mecanismos de desenvolvimento neural e de plasticidade dependentes da experiência, tendo em vista a sua ampla representação no córtex somatossensorial (Diamond et al., 1993; Fox e Wong, 2005). Esse fato, aliado à facilidade de manipulação das vibrissas (tanto para estimulação quanto para sua remoção), possibilita intervir sobre esta via sensorial de forma relativamente simples e com grande especificidade de respostas (Mierau et al., 2004).

Estudos eletrofisiológicos realizados no "Laboratório de fisiologia da nutrição Naíde Teodósio" (LAFINNT) têm mostrado que manipulações nutricionais (Guedes et al., 1987) e sensoriais (Santos-Monteiro et al., 2000; Santos-Monteiro, 2002; Monte-Silva et al., 2007) durante o período crítico de desenvolvimento cerebral influenciam a propagação do fenômeno da Depressão Alastrante Cortical (DAC), o qual se constitui num modelo interessante para a compreensão do funcionamento do cérebro (Leão, 1944; Martins-Ferreira et al., 2000; Guedes, 2005).

A linha de pesquisa sobre a relação entre desnutrição, sistemas sensoriais e DAC tem abordado diferentes tipos de manipulações sensoriais durante o desenvolvimento neural. Inicialmente, em estudos de Santos-Monteiro et al. (2000), avaliou-se a influência do aumento ou redução de estímulos multisensoriais, no início da vida. Em seguida, foram estudados os efeitos da privação de uma via sensorial específica (a visual), isoladamente, através da enucleação unilateral (Santos-Monteiro, 2002). Em etapa posterior, Monte-Silva et al. (2007) analisaram as conseqüências da hiperativação de *input* sensorial através de estimulação elétrica periférica (Monte-Silva et al., 2007). Estes estudos prévios demonstraram haver influências das alterações sensoriais aplicadas aos animais sobre as características da DAC, sendo tais efeitos variáveis de acordo com o tipo de manipulação sensorial, com o período do desenvolvimento em que ela foi realizada e com o estado nutricional dos animais no início da vida.

O presente trabalho foi desenvolvido para dar continuidade à investigação da participação das vias sensoriais no desenvolvimento neurofisiológico sob diferentes estados nutricionais, utilizando a DAC como ferramenta de estudo. Nessa perspectiva, estudou-se em ratos Wistar, por meio da deaferentação unilateral das vibrissas, o papel da privação do seu *input* sensorial, associada à desnutrição, sobre o desenvolvimento neural. Para isso foram analisadas características eletrofisiológicas da DAC em animais jovens (30-40 dias) e adultos (90-120 dias). Esta proposta é considerada inovadora, uma vez que os efeitos, sobre a DAC, da interação entre nutrição e informações somatossensoriais das vibrissas não têm sido objeto de estudo na literatura.

Objetivos

Objetivo Geral:

Investigar os efeitos da supressão, durante o desenvolvimento cerebral, de informações somatossensoriais originadas nas vibrissas sobre o fenômeno da DAC em ratos submetidos a condições favoráveis e desfavoráveis de nutrição durante o aleitamento.

Objetivos Específicos:

- Estudar os efeitos da remoção unilateral precoce das vibrissas mistaciais sobre a susceptibilidade cortical à Depressão Alastrante, através da análise da sua velocidade de propagação em ambos os hemisférios cerebrais;

- Avaliar estes efeitos logo após o período crítico de desenvolvimento cerebral (pós-desmame imediato) e a longo prazo (na idade adulta);

- Analisar a influência, sobre tais efeitos, das condições nutricionais favoráveis e desfavoráveis, induzidas pela variação do tamanho da ninhada, durante a lactação;

- Avaliar o impacto, sobre o crescimento do corpo e do cérebro, da deficiência nutricional e da remoção das vibrissas, por meio das medidas dos pesos corporais e encefálicos.

Hipóteses

As hipóteses levantadas neste estudo são as de que:

- A supressão de informações somatossensoriais providas pelas vibrissas no início da vida influenciaria a propagação da DAC;

- Os efeitos dessa privação sensorial sobre a DAC seriam de longa duração, persistindo até a idade adulta;

- O estado nutricional pregresso (durante o aleitamento) a que os lactentes foram submetidos poderia modificar os efeitos acima postulados.

2. Revisão da Literatura

Períodos críticos de desenvolvimento neural e a influência de fatores nutricionais

O desenvolvimento do sistema nervoso é um processo influenciado por fatores genéticos e ambientais, havendo períodos de maior vulnerabilidade a tais influências. Isto ocorre em uma etapa da ontogenia neural conhecida como "período crítico" de desenvolvimento, caracterizado por um pico na intensidade de processos como a neurogênese, a migração e a diferenciação celular, os quais são acompanhados de alta taxa de síntese protéica. Nesse período, as diversas áreas cerebrais se desenvolvem mais rapidamente em relação a outros períodos (Morgane et al., 1978; 1993). Como uma das conseqüências, o encéfalo tem seu peso e tamanho aumentados de maneira particularmente acelerada (Dobbing e Smart, 1974).

Este período de intenso desenvolvimento cerebral ocorre em épocas distintas nas diversas espécies de mamíferos (Rice e Barone Jr., 2000). Nos seres humanos inicia-se no período pré-natal (terceiro trimestre da gestação), continuando-se até os primeiros anos de vida (Scrimshaw e Gordon, 1968), enquanto que no rato, corresponde às três primeiras semanas de vida pós-natal, ou seja, o período de lactação (Dobbing, 1968; Rice e Barone Jr., 2000).

Os efeitos da desnutrição sobre o sistema nervoso irão depender da sua gravidade e duração, bem como do período em que o insulto ocorre (Rocha-de-Melo e Guedes, 1997), tendo sido demonstrada uma maior gravidade destes efeitos quando a injúria ocorre no período crítico do crescimento rápido cerebral (Dobbing, 1968).

Assim, episódios de desnutrição nessa fase induzem alterações na gênese dos microneurônios, bem como nos processos de diferenciação celular, sinaptogênese, gliogênese e mielinização (Ballabriga, 1990; Morgane et al., 1978; Resnick et al., 1979).

Durante o aleitamento, a desnutrição leva a uma diminuição do peso do cerebelo, do hipocampo e do córtex cerebral, evidenciando-se uma maior vulnerabilidade dessas estruturas à agressão nutricional, em comparação com outras regiões do encéfalo (Morgane et al., 1978). Além dessas alterações morfológicas, mudanças comportamentais e eletrofisiológicas também foram demonstradas (Gordon, 1997; Prasad, 1996; Ranade et al., 2008; Resnick et al., 1979; Resnick e Morgane, 1984; Ruiz et al., 1985), bem como retardo na maturação reflexa e somática (Barros et al., 2006; Smart e Dobbing, 1971).

Nos seres humanos, as conseqüências da desnutrição precoce não se limitam aos altos índices de morbi-mortalidade, tendo em vista que muitos dos que sobrevivem à desnutrição podem sofrer conseqüências funcionais que irão repercutir no potencial de desenvolvimento intelectual e sócio-cultural (Nwuga, 1977; Walker et al, 2007). Apesar da melhoria demonstrada pelos indicadores populacionais, em várias partes do mundo, incluindo o Brasil, a desnutrição protéico-energética continua sendo um problema de saúde pública (Onis et al., 2000).

Os aspectos ora mencionados justificam a importância de se aprofundar os estudos relativos à influência da nutrição sobre o desenvolvimento do sistema nervoso, apresentando-se como um aspecto de grande interesse de estudos no campo da fisiologia da nutrição.

Informação sensorial e plasticidade neural: Importância para o desenvolvimento do sistema nervoso

O sistema nervoso dos mamíferos possui a capacidade de modificar sua estratégia de desenvolvimento e/ou de funcionamento em resposta a alterações orgânicas e ambientais, sendo esta característica denominada de plasticidade neural (Buonomano e Merzenich, 1998; Markham e Greenough, 2004). Devido a essa importante característica, a experiência produzida pela interação organismo-ambiente influencia a organização anatômica do encéfalo durante o período de desenvolvimento (Markham e Greenough, 2004; Petersen, 2007), bem como a sua reorganização frente a demandas ambientais (Buonomano e Merzenich, 1998). Em termos funcionais, a plasticidade pode ser relacionada à capacidade de aprendizagem de novas tarefas (McGraw et al., 2009) e à possibilidade de reorganização neural após lesões do sistema nervoso central ou periférico (Chowdhury et al., 2004; Johnston et al., 2009; Kolb e Whishaw, 1989; Ramanathan et al., 2006; Werhahn, 2002).

O encéfalo em desenvolvimento possui mais plasticidade do que o encéfalo adulto, o que está relacionado com a maior facilidade das crianças, em comparação aos adultos, para aprender novas habilidades motoras e cognitivas, tais como o desempenho na prática de esportes e a aprendizagem de línguas estrangeiras, e ainda com o melhor prognóstico de recuperação após danos neurológicos (Johnston et al., 2009). Todavia, estudos realizados tanto em animais (Ramanathan et al., 2006; Tailby et al., 2005) como em humanos (Gauthier et al., 2008), revelam que o encéfalo adulto também apresenta certo grau de plasticidade, o que tem sido objeto de estudo de muitas pesquisas na atualidade (Llorens-Martín et al., 2009).

Os mecanismos subjacentes à neuroplasticidade são múltiplos e incluem aspectos morfológicos, bioquímicos e funcionais (Buonomano e Merzenich, 1998; Gu, 2002; Naka et al., 2002).

Dentre tais mecanismos, estão a neurogênese, a sinaptogênese, a axogênese compensatória e o brotamento neurítico colateral, além de fatores neurotróficos, tais como o fator de crescimento insulínico-I (IGF-I), o fator de crescimento neuronal (NGF), o fator neurotrófico derivado do cérebro (BDNF), o fator transformador do crescimento-β (TGFB) e o fator de crescimento fibroblástico básico (bFGF) (Fuxe et al., 1996; Goldman e Plum, 1997; Gomide e Chadi, 1999).

O sistema somatossensorial é amplamente utilizado para estudos relacionados à plasticidade neural, tendo em vista a organização anatômica do córtex somatossensorial em

mapas somatotópicos (Fox, 1992; Fox e Wong, 2005; Fox, 2009; Rema et al., 2006), os quais são estruturas dinâmicas, que se adaptam aos graus variados de estimulação periférica (Merzenich et al., 1990). Além dessas mudanças estruturais, a distribuição de sinapses excitatórias e inibitórias também é influenciada pelo fluxo de informações entre a periferia e o SNC (Cheetham et al., 2007; Finnerty e Connors, 2000; Micheva e Beaulieu, 1995; Mierau et al., 2004).

Segundo Gu (2002), uma característica importante dos neurônios do neocórtex é a capacidade para modificar suas propriedades de resposta em decorrência de alterações prolongadas de impulsos aferentes, sendo esta forma de plasticidade neuronal principalmente observada nas áreas corticais sensoriais, sobretudo durante períodos precoces da vida.

As modificações no desenvolvimento cerebral, concernentes à função plástica do tecido nervoso, têm sido demonstradas em ratos criados em ambientes com diferentes graus de complexidade. Quando esses animais são submetidos, durante o seu desenvolvimento, a um ambiente "rico" em estímulos, verifica-se aumento no peso e na espessura de estruturas corticais; no teor de aminas cerebrais; na atividade de enzimas neuronais; no número e diâmetro dos capilares corticais e diminuição na densidade celular, em comparação com animais criados em ambientes "pobres" em estímulos. O aumento do número de contatos sinápticos e de ramificações dendríticas nesses animais evidencia também plasticidade sináptica (Diamond, 1964; Coleman e Riessen, 1968; Naka et al., 2002; Rosenzweig et al., 1969; Renner e Rosenzweig, 1987; Sirevaag et al., 1988).

Também têm sido descritas alterações comportamentais associadas à estimulação ambiental ou ao isolamento, como resposta do SNC a níveis diferentes de experiências sensoriais (Levitsky e Barnes, 1972; 1989; Renner e Rosenzweig, 1987; Hilakivi et al., 1989; Mohammed et al., 1990; Van Den Berg, et al., 1999).

Além do córtex somatossensorial, outras estruturas do SNC também têm sido bastante investigadas com relação à neuroplasticidade, tais como o hipocampo (Laplagne et al., 2006; Llorens-Martín et al., 2009) e o córtex visual (Fox e Wong, 2005; Renner e Rosenzweig, 1987; Rosenzweig et al., 1969; Sirevaag et al., 1988).

Tais evidências demonstram a importância da plasticidade para o desenvolvimento adequado do sistema nervoso, bem como as suas repercussões em etapas posteriores da vida, tendo em vista as consequências desses mecanismos adaptativos sobre a aquisição e manutenção das funções neurais.

Influência de fatores nutricionais sobre a Plasticidade Neural

Conforme os comentários já mencionados acerca da importância da nutrição para o desenvolvimento neural, verifica-se que ela se apresenta como um fator capaz de desencadear inúmeros mecanismos de plasticidade neural, sendo estes passíveis de modificações com a interferência dos estímulos ambientais.

Na década de 70, Levitsky e Barnes (1972), divulgaram a hipótese do "isolamento funcional", na qual consideraram a existência de uma adaptação do organismo à desnutrição precoce, adaptação esta envolvida com a conservação de energia. De acordo com essa hipótese, o indivíduo utilizaria a sua quota energética dando prioridade à manutenção corporal; em segundo lugar, ao crescimento e por último, à atividade comportamental ou cognitiva. Assim, a energia necessária para a sobrevivência seria poupada, apresentando esse indivíduo, em relação à sua idade, além de redução no tamanho e peso corporal, uma menor exploração do ambiente, o que provocaria uma redução na aferência sensorial, influenciando a plasticidade dependente da experiência.

Conforme descrito em tópico anterior, as adaptações do sistema nervoso diante da desnutrição crônica envolvem alterações na gênese celular, nos arranjos arquitetônicos das células e em mudanças na conectividade sináptica (Morgane et al., 1978; 1993), apresentando-se estes efeitos como alterações plásticas, que podem ser avaliadas por estudos anatômicos quantitativos e por análises funcionais (Borba et al., 2000; Guedes, 2005).

Diversos autores mostraram que alguns desses prejuízos ao sistema nervoso decorrentes da desnutrição, podem ser parcialmente revertidos (ou compensados) pela estimulação ambiental. A partir desses resultados, inferiu-se que a estimulação ambiental pode contribuir na recuperação dos efeitos deletérios da desnutrição sobre a função cerebral, tanto em animais de laboratório (Levitsky e Barnes, 1972; Fernández et al., 1998; Fernández-Teruel et. al., 1997; Almeida et al., 1998; Scrimshaw, 1998), como em humanos (Barret e Radke-Yarrow, 1985; Grantham-McGregor et al., 1991; Walker et al., 2000; Hall et al., 2001). Esta recuperação é um dos exemplos relacionados à capacidade plástica do tecido nervoso em face de situações adversas (Crutcher, 1991).

As evidências de que a estimulação psicossocial é capaz de contribuir para a recuperação de funções neuromotoras e cognitivas de indivíduos previamente desnutridos são de interesse clínico crescente (Barret e Radke-Yarrow, 1985; Gratham-McGregor et al., 1991; Walker et al., 2000; Hall et al., 2001). Entretanto, os estudos indicam que a estimulação não é

suficiente por si só para se obter esse efeito, sendo necessária também a concomitante reabilitação nutricional dos indivíduos (Grantham McGregor et al.,1991).

O Sistema somatossensorial das vibrissas, no rato albino: um modelo para estudos de plasticidade neural

A manipulação das condições de estimulação sensorial em um organismo em desenvolvimento, com a finalidade de exacerbá-las ou restringi-las, constitui uma abordagem frequentemente utilizada no estudo da plasticidade neural (Fox, 1992; Naka et al., 2002; Renner e Rosenzweig, 1987). No rato, a abolição das sensações providas pelas vibrissas é um procedimento muito utilizado em estudos sobre o desenvolvimento neural (Diamond et al., 1993; Fox e Wong, 2005). As vibrissas são importantes receptores de estimulações sensoriais do ambiente, e a sua mobilidade constitui um componente básico da resposta de exploração nesses animais (Diamond, 2008), sendo sua função comparada à habilidade tátil das pontas dos dedos em primatas (Carvell e Simons, 1990; Qi e Kaas, 2004). As vibrissas são utilizadas para orientação espacial, discriminação da forma e tamanho de objetos, além da textura de suas superfícies (Von Heimendahl et al., 2007).

Tais receptores periféricos têm projeções para regiões específicas do córtex somatossensorial contralateral, onde possuem uma representação topográfica, com áreas em proporções correspondentes à sua importância funcional (Welker et al., 1991), com a mesma organização espacial das vibrissas na face, resultando em um mapa somatotópico de colunas corticais (Diamond et al., 1993).

Os folículos das vibrissas são inervados por células do nervo trigêmeo, esses neurônios são capazes de codificar localização, deflexão, direção, início, término, amplitude, duração, velocidade de repetição e padrões temporais dos estímulos mecânicos aplicados a cada vibrissa, os quais são convertidos em potenciais de ação. Esses sinais aferentes passam pelos corpos celulares do gânglio trigeminal e continuam ao longo do ramo central do nervo para formar sinapses nos núcleos trigeminais do tronco encefálico. Em seguida, as informações são transmitidas para o tálamo e então, são projetadas para o córtex somatossensorial, onde são formados agregados de terminações na camada celular IV, denominadas colunas ou barris corticais (Diamond et al., 2008; Van der Loos e Woolsey, 1973; Woolsey e Van der Loos, 1970).

Os barris corticais se desenvolvem nos primeiros dias de vida pós-natal (Agmon et al., 1993; Jhaveri et al. 1991; O'Leary et al., 1994). Porém, a privação da experiência sensorial das vibrissas pode prejudicar a formação dessas estruturas (Wong-Riley e Welt, 1980) e esta alteração morfológica pode induzir a modificações da função sináptica com conseqüências sobre a excitabilidade cortical (Mierau et al., 2004; Cheetham et al., 2007). Tais alterações

são mais evidentes quanto mais precocemente ocorrer a privação sensorial, porém, na idade adulta ainda permanece certo grau de plasticidade sináptica (Fox, 1992; Wong-Riley e Welt, 1980).

Além da privação sensorial, outros fatores têm sido relacionados com alterações na formação dos barris corticais, tais como a desnutrição (Medina-Aguirre et al., 2008) e a exposição ao álcool (Oladehin et al., 2007) durante o período crítico do desenvolvimento neural.

Diante de tais evidências, é possível concluir que a via sensorial iniciada nas vibrissas dos roedores oferece várias vantagens para estudar os mecanismos de plasticidade cortical dependente da experiência, durante o desenvolvimento do sistema nervoso. A manipulação dessa via em um paradigma eletrofisiológico é o objeto principal deste trabalho.

Depressão Alastrante Cortical: Conceito, Histórico e Aplicações

O fenômeno da DAC foi primeiramente observado e descrito pelo pesquisador brasileiro Aristides Leão, na década de 40 (Leão, 1944), durante estudos experimentais sobre epilepsia, utilizando registros da atividade elétrica cortical cerebral em coelhos anestesiados.

Este fenômeno pode ser descrito como uma diminuição (depressão) reversível e acentuada da atividade elétrica espontânea ou provocada, em resposta à estimulação (mecânica, química ou elétrica) de um ponto cortical. A DAC propaga-se, a partir daí, de forma concêntrica e com velocidade aproximadamente uniforme (da ordem de 2 a 5 mm/min; no rato adulto jovem, é de 3-3,5 mm/min.) por todo o córtex. Enquanto o local inicialmente deprimido se recupera, a depressão da atividade se alastra por regiões mais distantes. A recuperação completa se dá em torno de 10 a 15 minutos (Leão, 1944; Leão, 1947).

Simultaneamente à depressão eletrocorticográfica, ocorre uma variação lenta de voltagem (VLV) na região cortical invadida pelo fenômeno. Com isto, o córtex torna-se negativo em relação a um ponto de voltagem fixa. Essa variação negativa de amplitude entre 5 e 20 mV, é eventualmente precedida, e freqüentemente seguida por uma fase positiva de menor amplitude (Leão, 1951).

Várias hipóteses e evidências clínicas têm sugerido a associação da DAC com algumas doenças neurológicas humanas, incluindo a epilepsia (Leão, 1944), a enxaqueca com aura (Parsons e Strijbos, 2003), doenças cerebrovasculares, traumatismo craniano e amnésia transitória global (Gorji, 2001). Além das pesquisas com modelos animais, este fenômeno também tem sido demonstrado em humanos (Mayevsky et al, 1996; Dohmen et al., 2008).

A influência de diversos fatores, associados ao estado nutricional, sobre a plasticidade cerebral tem sido estudada em animais de laboratório utilizando-se a DAC (Santos-Monteiro, 2002), a qual tem sua propagação facilitada em situações de desnutrição (Amâncio-dos-Santos et al., 2006; Guedes et al., 1987). Esse importante fenômeno tem sido utilizado, como modelo, em diversos estudos no LAFINNT, visando investigar o papel da desnutrição, associado a outras variáveis, incluindo as ambientais, sobre o desenvolvimento do sistema nervoso (Para uma revisão, vide Guedes, 2005).

Estudos eletrofisiológicos desenvolvidos no LAFINNT têm mostrado que manipulações nutricionais (Guedes et al., 1987; Rocha-de-Melo et al, 2006) e multi-sensoriais (Santos-Monteiro et al., 2000; Santos-Monteiro, 2002) durante o desenvolvimento influenciam a propagação da DAC no córtex cerebral do rato adulto. A DAC vem sendo estudada como fenômeno potencialmente relevante para o melhor conhecimento dos

mecanismos das patologias neurais humanas anteriormente mencionadas. O fenômeno é, complementarmente, investigado como indicador de alterações eletrofisiológicas cerebrais. A sua relevância para a compreensão do funcionamento do cérebro pode ser encontrada na literatura (Leão, 1944; Martins-Ferreira et al., 2000; Gorji, 2001; Guedes, 2005).

De acordo com estudos acerca da DAC, verificou-se que algumas condições podem modificar a susceptibilidade cortical ao fenômeno, e dentre elas destacam-se: a estimulação ambiental, modificação da atividade de sistemas neurotransmissores, alterações hormonais e metabólicas, e o estado nutricional (Guedes et al., 1987; 2002; Santos-Monteiro et al., 2000; Guedes e Pereira-da-Silva, 1993; Guedes et al., 1996; Guedes e Cavalheiro, 1997; Rocha-de-Melo e Guedes, 1997; Costa-Cruz e Guedes, 2001).

Em estudo anterior realizado no LAFINNT, foi averiguada a influência da estimulação sensorial, associada a diferentes estados nutricionais, sobre a DAC. Ratos bem nutridos e desnutridos foram submetidos a graus variados de estimulação sensorial, através de "enriquecimento" ou "empobrecimento" ambiental, durante períodos precoces de suas vidas. Observou-se que animais desnutridos mantidos em ambientes ricos em estímulos, desde a lactação até a idade adulta, apresentaram redução da velocidade de propagação da DAC. Os grupos desnutridos e submetidos à estimulação ambiental apenas após o desmame não apresentaram diferenças significativas em relação ao grupo desnutrido não estimulado (Santos-Monteiro et al., 2000). Esses dados sugerem que há um efeito diferencial da estimulação ambiental sobre a DAC, dependendo do estado nutricional e do período em que a estimulação ocorre (Guedes et al., 1996; Santos-Monteiro et al., 2000).

Em estudo seguinte, foram averiguados os efeitos da privação da via sensorial visual, através da enucleação durante o período de lactação, sobre as características da DAC em ratos bem nutridos e desnutridos. Verificou-se, na idade adulta, um efeito facilitador para a incidência e propagação da DAC no hemisfério contralateral à privação visual, quando comparado ao hemisfério ipsilateral, sendo este efeito abolido nos animais desnutridos (Santos-Monteiro et al., 2002).

Dando seqüência a abordagens sensoriais, Monte-Silva et al. (2007) analisaram a influência da estimulação elétrica periférica sobre a velocidade de propagação da DAC, em diferentes condições nutricionais. Para isso, ratos em desenvolvimento foram submetidos a sessões diárias de estimulação elétrica repetitiva das patas (anterior e posterior) do lado esquerdo do corpo. Com idade entre 35-45 dias, estes animais apresentaram uma redução na velocidade da DAC no hemisfério contralateral às patas estimuladas, em relação ao hemisfério ipsilateral e este efeito foi modificado pela desnutrição.

Os dados desses estudos sugerem que diferentes tipos de manipulações das vias sensoriais, durante o desenvolvimento cerebral, influenciam a incidência e propagação do fenômeno da DAC, podendo tais efeitos ser modificados pela desnutrição precoce. Os mecanismos subjacentes a esses resultados necessitam ser investigados através de novos estudos na mesma linha de investigação.

3. Métodos

Animais:

Ratos Wistar machos (n=80), provenientes da colônia mantida pelo Departamento de Nutrição da UFPE foram utilizados neste estudo. Todos os procedimentos metodológicos foram iniciados após aprovação pela comissão de ética para pesquisa com animais da Universidade Federal de Pernambuco (UFPE) (Anexo A).

Os animais foram mantidos sob condições padrão de biotério, com temperatura de 23 ± 2^{o}C e submetidos a um ciclo claro/escuro de 12/12 horas (luz das 06 às 18:00 horas).

Foram estabelecidos grupos experimentais de acordo com o estado nutricional (Bem-nutridos e Desnutridos), a condição sensorial das vibrissas (Controle e Lesado) e a idade de realização do registro eletrofisiológico (Jovem e Adulto), desta forma, os animais foram distribuídos nos seguintes grupos:

Jovem		Adulto	
Bem-nutrido	Controle (vibrissa) (n=10)	Bem-nutrido	Controle (vibrissa) (n=10)
	Lesado (vibrissa) (n=10)		Lesado (vibrissa) (n=10)
Desnutrido	Controle (vibrissa) (n=10)	Desnutrido	Controle (vibrissa) (n=10)
	Lesado (vibrissa) (n=10)		Lesado (vibrissa) (n=10)

Condições nutricionais:

A intervenção sobre o estado nutricional dos animais foi realizada durante toda a lactação através de variações no tamanho das ninhadas (De Luca et al., 1977; Fernández et al., 1993; Rocha-de-Melo et al., 2006), criando-se condições diferenciadas de aleitamento, a saber:

- Animais Bem-nutridos (n=40): Amamentados em ninhadas de 6 filhotes (condições favoráveis de lactação);
- Animais Desnutridos (n=40): Amamentados em ninhadas de 12 filhotes (condições desfavoráveis de lactação).

Após o desmame (aos 21 dias), os animais foram distribuídos em grupos de 4 a 5 ratos por gaiola (51 x 35,5 x 18,5 cm). A dieta de manutenção de todos os grupos experimentais foi

a mesma dieta materna, Labina®, contendo 23% de proteína, a qual foi utilizada durante todo o protocolo experimental, tendo os animais livre acesso à mesma, bem como à água filtrada.

Peso Corporal:

Para se estabelecer a evolução ponderal, os animais tiveram o peso corporal registrado em protocolo individual aos 7, 14, 21, 30, 60 e 90 dias de idade, utilizando-se balança digital Marte, modelo 1001, com capacidade para 1610 g e sensibilidade 0,1 g.

Condições sensoriais:

Em cada um dos grupos nutricionais (Bem-nutrido e Desnutrido), metade dos animais (n=20) foi submetida à remoção unilateral permanente das vibrissas (Grupo Lesado; ver descrição abaixo), sob crioanestesia, enquanto a outra metade (n=20; Grupo Controle) permaneceu com as vibrissas intactas, sendo submetidos apenas à crioanestesia.

O procedimento para remoção das vibrissas foi realizado aos 2-3 dias de vida pós-natal. Os animais foram submetidos à crioanestesia (Danneman e Mandrell, 1997), técnica através da qual os filhotes foram mantidos a uma temperatura de -20 ^{o}C, durante 10 minutos. Assim anestesiados, eles tiveram as vibrissas mistaciais do lado direito retiradas com uma pinça, seguida de eletrocauterização dos seus folículos, conforme descrito por Woolsey e Van der Loos (1970).

Os animais eram examinados, a cada dois dias, para verificar se havia crescimento das vibrissas, e assim confirmar a eficácia da eletrocauterização.

Registros eletrofisiológicos:

Tanto os grupos que sofreram remoção das vibrissas, quanto os seus respectivos controles, compuseram dois subgrupos, de acordo com a idade em que foram submetidos ao registro da DAC: em um deles, o registro foi realizado aos 30-40 dias de idade (grupo jovem), e no outro, os registros ocorreram aos 90-120 dias (grupo adulto).

Esses registros foram efetuados em um polígrafo MODELO 7D (Grass Medical Instruments). Inicialmente, os animais foram anestesiados com uma mistura de 1 g/Kg de uretana + 40 mg/Kg de cloralose (i.p.), suas cabeças foram fixadas em um aparelho estereotáxico, e 3 orifícios (medindo 3 mm de diâmetro) foram trepanados em cada hemisfério cerebral, os

quais eram alinhados na direção anteroposterior e paralelos à linha média (Fig. 1). Os orifícios mais posteriores de cada hemisfério cerebral eram utilizados para aplicar os estímulos para elicitação da DAC (solução de KCl a 2%). Nos outros orifícios eram efetuados os registros da variação lenta de voltagem, característica da propagação da DAC, através de 4 eletrodos de Ag-AgCl Agar-Ringer (2 eletrodos em cada hemisfério, um em cada orifício de registro) contra um eletrodo de referência comum, do mesmo tipo, colocado no osso nasal. Entre os dois pontos de registro estava localizada a área cortical de representação topográfica das vibrissas (coordenadas estereotáxicas compreendidas de AP -0.3mm para -3.3mm em relação ao bregma e de ML 4.5 para 6.5 mm em relação à linha média), definida segundo o Atlas Estereotáxico de Paxinos e Watson (1998). Em cada hemisfério cerebral, a DAC foi provocada a intervalos de 20 minutos, através da aplicação de uma bola de algodão (1-2 mm de diâmetro) umedecido com uma solução de KCl a 2% (270 mM). A variação lenta de voltagem foi continuamente registrada durante 4 horas. A temperatura retal era monitorada continuamente e mantida a 37 ± 1^{o}C através de uma placa aquecedora.

A velocidade de propagação da DAC foi calculada com base na distância entre os dois eletrodos registradores e no tempo gasto pela DAC para percorrer esta distância. A partir das medidas individuais, foi calculada a velocidade média de propagação do fenômeno, para cada animal, seguida do cálculo da velocidade média para cada grupo.

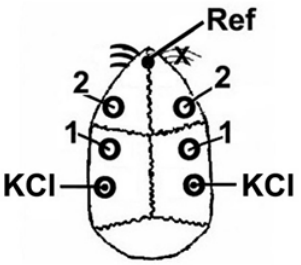

Fig. 1: Esquema ilustrativo do crânio do rato, apresentando a localização dos orifícios utilizados para aplicação da solução de KCl, bem como do eletrodo referência (Ref) e dos pontos de registro (1 e 2) em cada hemisfério cerebral. As vibrissas do lado direito da face (que foram removidas) estão marcadas com um "X".

Pesos dos encéfalos:

Em seguida aos registros eletrofisiológicos, os animais, ainda anestesiados, foram submetidos à eutanásia por lesão tronco-bulbar, através da introdução de agulha na cisterna

magna, o que provocou imediata parada cárdio-respiratória, e seus encéfalos foram retirados. Para isso, o neuro-eixo foi seccionado transversalmente em dois níveis: caudalmente, ao nível da borda inferior do cerebelo, incluindo-o; rostralmente, no limite entre os pólos frontais e o bulbo olfatório, excluindo-se esse último. O peso absoluto do encéfalo úmido foi determinado utilizando-se uma balança analítica (modelo Bosch, S-2000, com sensibilidade até 0,1 mg), após essa pesagem, os mesmos foram mantidos em estufa a 100º C e pesados a cada 1-2 dias até atingirem peso constante, o qual foi considerado o peso do encéfalo seco.

Análise Estatística:

As varáveis peso corporal, peso encefálico e velocidade de propagação da DAC foram comparadas entre os grupos usando análise de variância (Anova), incluindo, como fatores, o estado nutricional (Bem-nutrido e Desnutrido), a condição sensorial (Controle e lesado) e o hemisfério cerebral (ipsilateral e contralateral à remoção da vibrissa), seguida de um teste *post-hoc* (Tukey) quando indicado. Em cada grupo, o teste-t pareado foi utilizado para comparar as velocidades de propagação da DAC entre os hemisférios (ipsi- e contralateral à remoção das vibrissas), no mesmo animal. As diferenças foram consideradas significativas nos casos em que $p \leq 0,05$.

4. Resultados

Com os resultados deste estudo foram produzidos dois artigos científicos originais, os quais foram submetidos a periódicos de circulação internacional.

Adiante estão apresentados os referidos artigos em suas versões que foram encaminhadas para as revistas.

O primeiro destes artigos é intitulado: "Early vibrissae removal facilitates cortical spreading depression propagation in the brain of well-nourished and malnourished developing rats". Foi submetido como artigo original à revista *International Journal of Developmental Neuroscience* (Anexo B), a qual explora as áreas de Anatomia, Embriologia, Neurofisiologia, Neuropsicofarmacologia e Neurologia e é classificada como qualis internacional A pela CAPES, possui o fator de impacto 3,608.

O segundo artigo deste estudo é intitulado: "Lasting facilitatory effects of neonatal vibrissae removal on the propagation of Cortical Spreading Depression: an electrophysiological study in well-nourished and early-malnourished adult rats". Foi submetido como artigo original à revista *Nutritional Neuroscience* (Anexo C), classificada como qualis internacional A pela CAPES, com fator de impacto 1,493, divulga artigos relativos à interface entre Nutrição, Dieta, Neurofisiologia, Neuropsicofarmacologia e Neurologia.

Artigo 1:

Title:

Early vibrissae removal facilitates cortical spreading depression propagation in the brain of well-nourished and malnourished developing rats

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Abbreviations: CSD, cortical spreading depression; DC, direct current; W, well-nourished; M, malnourished; GABA, gamma amino butyric acid.

Running title:
Sensory deprivation, nutrition and spreading depression

Abstract

Reduced sensory input activity during brain development can induce morphological and physiological changes in the cerebral cortex, altering their response properties. Malnutrition delays the formation of somatosensory pathways. Here we used cortical spreading depression as a neurophysiological parameter to investigate electrophysiological changes after vibrissae removal in well-nourished and malnourished rats. Male Wistar rat pups had the right mystacial vibrissae removed at postnatal days 2-3, and were submitted to spreading depression recording at 30-40 days of life. In both nutritional conditions, spreading depression velocities were increased in the hemisphere contralateral to the vibrissae removal, as compared to age- and nutrition-matched non-lesioned controls, in which no inter-hemispheric differences were found. In contrast to the well-nourished rats, in the vibrissae-removed malnourished animals the spreading depression propagation in the ipsilateral hemisphere decreased as compared to the corresponding hemisphere of the non-lesioned malnourished rats. It is concluded that deprivation of sensory input from whiskers during brain development facilitates spreading depression propagation, and early malnutrition seems to influence this effect. Since the effect persisted until 40 days, it is tempting to suggest that it is permanent, or at least long-lasting. Data might contribute to the understanding of sensory input deprivation-induced plasticity mechanisms underlying cerebral electrophysiological changes in the developing brain.

Key words: Brain plasticity; Cortical spreading depression; Sensory deafferentation; Brain development; Malnutrition

INTRODUCTION

Different environmental factors such as sensory deprivation and malnutrition, by acting either individually or combined, can induce morphological, biochemical and electrophysiological changes during the period of brain development (Bonatto et al., 2006; Santos-Monteiro et al., 2000). Depending on their intensity and duration, these manipulations early in life can modify the patterns of developmental processes in the brain, influencing its functions and mechanisms of neural plasticity (Buonomano and Merzenich, 1998; Morgane et al., 1978; 1993;; Rema et al., 2006).

Studies concerning the influences of nutritional and environmental factors on neural functions are important to improve understanding about the development of neural system and its strategies for adaptation to insults. It is already well established that the effects of malnutrition are more severe when nutrition-deficiency occurs during the "brain growth spurt period". In the rat, this corresponds to the first three weeks of postnatal life (suckling period), when the brain presents its maximal vulnerability to many types of insults (Dobbing and Smart, 1974). Nutritional status can be impaired during this period by increasing the litter size, i.e., augmenting the number of pups to be suckled by one dam. This condition represents a useful model of malnutrition, implying that each pup receives an insufficient amount of milk, which results in nutritional deficiency (De Luca et al., 1977; Fernández et al., 1993; Rocha-de-Melo et al., 2006).

The somatosensory system shows advantages for investigating the role of sensory experience on development and plasticity of brain circuits, since the activity along these sensory pathways can be manipulated in a relatively easy way (Brecht, 2007; Fox, 1992; Fox and Wong, 2005). It is already well known that, in the somatosensory cortex, maps of the body surface are somatotopic (Buonomano and Merzenich, 1998; Diamond et al. 2003; Woolsey and Van der Loos, 1970) and that in the rat a large proportion of the peripheral

somatosensory information stems from the facial whiskers arrayed on the snout (Bureal et al., 2004; Inan and Crair, 2007).These whiskers transduce sensory stimuli and are capable of activate cells in the somatosensory cortical region known as whisker barrel field cortex through well described sensory pathways (Bureal et al., 2004; Daw et al., 2007; Rema and Ebner, 2003; Van der Loos and Woolsey, 1973; Welker and Sinha, 1972; Woolsey and Van der Loos, 1970).

Several studies report that sensory cortical maps are dynamic representations whose developmental refinement depends on sensory experience (Buonomano and Merzenich, 1998; Hubel and Wiesel, 1970; Quairiaux et al., 2007). In rodents, the barrel cortex develop during postnatal days 0 – 5 (Agmon et al., 1993; Jhaveri et al. 1991; O'Leary et al., 1994) and it is a useful model system for studying experience-dependent cortical development, because there is a simple mapping of individual whiskers to the corresponding barrel columns in the cortex (Diamond et al., 1993; Mierau et al., 2004). However, insults such as malnutrition can delay the emergence of barrels through mechanisms that have not been well established yet (Medina-Aguirre et al., 2008).

Deprivation of sensory input from whiskers, at various stages of development, can induce physiological, and often structural, changes that modify the circuitry of this sensory system (Fox and Wong, 2005; Shepherd et al., 2003; Wong-Riley and Welt, 1980). This is possible because neurons in mammalian neocortex have ability to modify their response properties following prolonged alterations in input activity and this form of neuronal plasticity is mostly documented during early postnatal life (Gu, 2002). Alterations in nervous system ability to change its developmental strategy and/or functioning in front of organic and environmental alterations (neural plasticity) can be ascertained by means of electrophysiological functional analyses, such as that of the present study employing the

phenomenon known as cortical spreading depression (CSD; Guedes, 2005; Santos-Monteiro et al., 2000).

CSD has been first described as a cortical response elicited by electrical, mechanical or chemical stimulation of the tissue surface. This response consists of a reversible and slowly propagating "wave" of reduction of spontaneous and evoked cortical electrical activity, with a simultaneous DC slow potential change of the tissue (Leão, 1944, 1947). During CSD while the spontaneous activity is depressed epileptiform-like waves usually appear and propagate, indicating a possible relationship between brain excitability changes and CSD (Leão, 1944). This possibility has been further investigated by several authors in rats submitted to environmental, pharmacological and nutritional manipulations (Amâncio-dos-Santos et al., 2006; Costa-Cruz et al., 2006; Fregni et al., 2007; Guedes, 2005). These reports show that CSD incidence and propagation can be modified by some conditions that influence brain excitability.

Our laboratory has employed CSD as an experimental model in order to study several conditions of neurological interest, including the effects of environmental complexity on brain development (Santos-Monteiro et al., 2000). The results have shown that multi-sensory manipulation during early stages of neural development in rat may influence the CSD propagation (Santos-Monteiro et al., 2000). However, no data are available on the relationship between CSD and deprivation of sensory input from whiskers.

Thus, the aim of the present study is to investigate the role of deprivation of peripheral somatosensory information, achieved by whiskers deafferentation, on CSD features in the cerebral cortex of developing rats reared under different nutritional conditions. The hypotheses were raised that (1) changes in the development of the cerebral cortex, consequent to early-in-life elimination of the somatosensory input carried by vibrissae, would

alter the cortical susceptibility to CSD, and (2) these alterations could be influenced by the prevailing nutritional status.

EXPERIMENTAL PROCEDURES

Animals

Male Wistar newborn rats (n=40) from the colony of the Department of Nutrition of the Universidade Federal de Pernambuco, Brazil, were used in this study. All experiments were carried out in accordance with the "Principles of Laboratory Animal Care" (National Institutes of Health, Bethesda, USA) and were approved by the Ethics Committee for Animal Research of our university.

The pups and their dams were maintained in a room with controlled temperature (23±2^{o}C) and a 12-12 h light-dark cycle (lights on at 6:00 am). Two nutritional groups – well-nourished (W; n=20) and malnourished (M; n=20) were prepared, as a result of submitting the pups respectively to favorable (litters formed by 6 pups) or unfavorable (12 pups per litter) lactation conditions. The 12-pups-litter procedure has been previously shown to provoke a relatively moderate nutritional deficiency (Rocha-de-Melo et al., 2006). During the lactation period, all dams were fed a standard rodent laboratory chow diet (Purina do Brazil Ltd.) with 23% protein. After weaning, pups had free access to water and food (the same maternal lab chow diet). Their body weights were determined on postnatal days 7, 14, 21 and 30.

Unilateral vibrissae removal

Half of each nutritional pup-group was submitted to removal of the mystacial whiskers from the right side ("Lesioned" groups; 10 W- and 10 M pups). In the remained pups, the whiskers were kept intact (control groups; 10 W- and 10 M pups). Procedures to

whiskers removal were carried out on between postnatal days 2 and 3, as described by Woolsey and Van der Loos (1970). Briefly, under cryoanesthesia and low-power microscope magnification, vibrissae were plucked out with fine tweezers, followed by electrical cauterization of follicles. The non-lesioned, control pups were equally submitted to cryoanesthesia, but the vibrissae were not removed.

CSD recording

On postnatal day 30-40 the animal was intraperitoneally anesthetized with a mixture of 1 g/kg urethane plus 40mg/kg chloralose and three holes (each measuring 3 mm in diameter) were drilled on each side of the skull (total of six holes). In each hemisphere, the holes were aligned in the anteroposterior direction, and parallel to the mid-line. The most posterior hole was used to apply the CSD-eliciting stimulus: a small (1-2 mm diameter) cotton ball soaked with 2% KCl solution and left over the intact dura-mater for 1min. The other two holes were used to position the two CSD recording electrodes; the centers of these holes were between the AP stereotaxic coordinates +0.8mm and -3.3mm in relation to the bregma, and their medio-lateral limits were between ML 3.5 to 6.5 mm from the midline), so that the cortical area of representation of the vibrissae were included in the CSD propagation trajectory between the two recording electrodes, according to Paxinos and Watson (1998). Rectal temperature was continuously monitored and maintained at 37±1^{o}C by means of a heating blanket. In each hemisphere, CSD was elicited at 20 min intervals. The CSD slow potential change was continuously recorded for 4 hours, by using four Ag–AgCl agar-Ringer electrodes (two electrodes on each hemisphere; one in each recording hole) against a common reference electrode of the same type, placed on the nasal bones. These electrodes consisted of 5 cm-long plastic conic pipettes (0.5 mm tip inner diameter), filled with Ringer solution, solidified with the addition of 0.5% agar, into which a chlorided silver wire was

inserted. The DC-potential changes were recorded by connecting the electrodes to GRASS DC-amplifiers in a model 7-D GRASS chart recorder.

In each cerebral hemisphere, ten to fifteen CSD waves were elicited in each rat over the 240-min recording period. No intra-group significant difference was seen in CSD propagation velocities of repeatedly induced CSDs. The CSD velocity of propagation was calculated, in each hemisphere, from the time required for a CSD wave to pass the distance between the two cortical electrodes. The initial point of each DC negative rising phase was taken as the reference point to calculate the CSD velocities, as previously employed (Abadie-Guedes et al., 2008).

At the end of the electrophysiological recordings the animals, while still anesthetized, were killed by lesioning the bulbar region with a sharp needle, inserted through the *cisterna magna*, promptly provoking cardio-respiratory arrest.

The brain was immediately removed and weighed (wet brain weight) using an analytical balance (Model: Bosch, S-2000). After weighing, brains were kept in a stove at 100^{o}C and were weighed at intervals of 1-2 days until reaching invariable weight (dry brain weight).

Statistics

Body- and brain weights, as well as CSD propagation rates, were compared between groups by using ANOVA including as factors: nutritional status (W and M), vibrissae condition (intact and removed) and hemisphere side (ipsilateral and contralateral to the vibrissae removal side) followed by a post-hoc test (Tukey) when indicated. Within each group, a paired T-test was used to compare CSD propagation rates between hemispheres (ipsi and contralateral to vibrissae removal) of each rat. The differences were accepted as

significant at the 95% confidence level ($p \leq 0.05$). All values were presented as means ± standard deviations.

RESULTS

Body Weight

Fig. 1 presents the body weight at the different postnatal days, as described in methods. Malnourished animals (suckled in litters formed by 12 pups) always displayed lower body weights, as compared to the controls (maintained in litters with 6 pups). In the intact, well-nourished animals, the mean body-weights (in grams) between postnatal days 7 to 30 ranged from 18.6±2.0 (at 7days) to 94.7±9.7g (at 30 days) whereas in the malnourished age-matched animals, the mean body weights ranged respectively from 13.5±1.7 to 71.1±8.4g ($P<0.05$). In each nutritional condition, vibrissae removal was also associated to a slight, but significant, reduction in body weights, as compared to the corresponding intact animals.

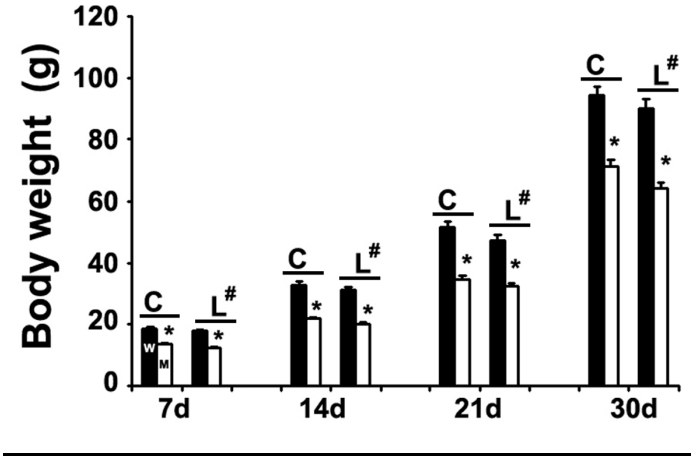

Figure 1: Body weight (mean±standard error of the mean) of well-nourished (W; litters formed by 6 pups; black bars) and malnourished (M; litters formed by 12 pups, white bars) rats, from the groups: Control (C; animals with intact vibrissae), and lesioned (L; animals submitted to lesion of the right facial vibrissae). The weights were measured on postnatal days 7, 14, 21 and 30. The asterisks indicate that all malnourished values are significantly different from the corresponding well-nourished controls and the # symbol indicates that lesioned animals had significantly lower body weights than the corresponding intact controls, at all time-points ($p \leq 0.05$; ANOVA plus Tukey test).

Brain Weights

Fig. 2 presents the wet- and dry brain weights, measured on the day of the CSD recording. The previously malnourished animals displayed lower brain weights than the respective controls. The mean wet-brain weights in well-nourished animals ranged from 1.517±0.041g (Lesioned group) to 1.620±0.033g (Control group) and in malnourished animals, the weights ranged from 1.349±0.026g (Lesioned group) to 1.410±0.026g (Control group). The mean dry-brain weights in well-nourished animals ranged from 0.300±0.007g (Lesioned group) to 0.320±0.006g (Control group) and in malnourished rats, this values ranged from 0.244±0.004g (Lesioned group) to 0.257±0.005g (Control group). As found for body weight, vibrissae removal also resulted in a small, but significant decrease in the brain weights, both in well-nourished and in malnourished groups, as compared with the corresponding controls.

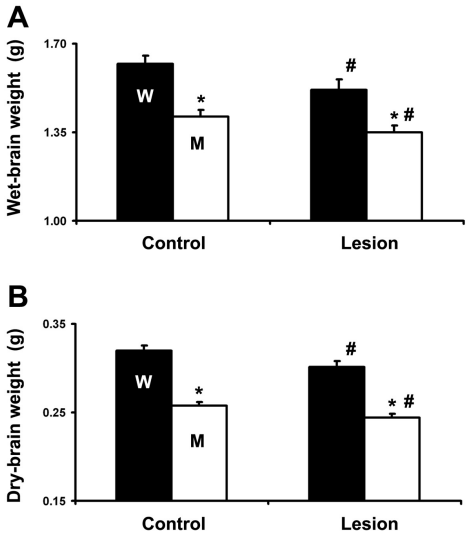

Figure 2: Weights of the wet- (left panel) and dry-brain (right panel) of 30-40 days-old rats from the groups: Control (C; animals with intact vibrissae), and lesioned (L; animals submitted to the removal of facial vibrissae from the right side). The asterisks indicate that all malnourished values (M; white bars) are significantly lower than the corresponding well-nourished ones (W, black bars) and the # symbols indicate that lesioned animals had significantly lower brain weights than the corresponding intact controls ($p \leq 0.05$; ANOVA plus Tukey test).

CSD elicitation

In all groups, most of the topical applications of 2% KCl for 1min at the parietal cortex of one hemisphere consistently elicited one CSD wave, which propagated and was recorded in the same hemisphere by the two recording electrodes located more anteriorly (see methods). In some animals, at a few occasions (usually 1-2 occasions per rat), after a KCl stimulation two or three CSD episodes appeared, instead of one. In the vibrissae-removal groups, this was seen in a slightly higher number of animals at the cerebral hemisphere contrateral to the vibrissae removal, (Figure 3), as compared to the ipsilateral hemisphere (9 and 7 rats, respectively). In the control group, no inter-hemisphere difference was found (7 rats in each group). However, the intergroup difference was not significant. Measurement of the rise- and recovery time, as well as duration of the CSD DC-potential change, did not reveal any intergroup significant difference. The CSD-amplitude however, was higher ($p<0.001$) at the contralateral hemisphere of the well-nourished vibrissae-removal group, as compared to the corresponding control group (Table 1).

Fig. 3 presents electrophysiological recordings showing the slow potential change, confirming the presence of CSD after each KCl-stimulation, on the cortical surface of well-nourished (panel A) and malnourished (panel B) animals.

Group	AMPLITUDE (mV)		DURATION (s)		RISE-TIME (s)		RECOVERY TIME (s)	
	IPSI	CONTRA	IPSI	CONTRA	IPSI	CONTRA	IPSI	CONTRA
W-C	9.1 ± 3.1	9.7 ± 3.3	69.6 ± 17.8	64.8 ± 12.5	36.4 ± 11.4	31.0 ± 5.9	33.2 ± 15.2	33.8 ± 8.5
W-L	12.6 ± 3.7	16.3 ± 3.8*	74.7 ± 19.9	71.6 ± 20.5	33.8 ± 6.3	34.6 ± 6.4	40.9 ± 15.1	37.0 ± 15.8
M-C	11.5 ± 2.3	11.2 ± 2.9	67.6 ± 38.9	61.7 ± 34.2	49.3 ± 14.9	42.8 ± 11.8	31.8 ± 8.3	31.2 ± 7.3
M-L	13.0 ± 3.4	13.0 ± 3.3	76.6 ± 15.5	69.2 ± 20.7	42.5 ± 8.6	42.2 ± 8.3	34.2 ± 10.3	27.0 ± 13.2

Table 1- Amplitudes, duration and rise- and recovery times of the CSD slow potential shifts in the 4 groups (2 well-nourished [W] and 2 malnourished [M] groups). C and L are control and lesioned (vibrissae-removal) groups, respectively. Data are expressed as mean±standard deviation. The asterisk indicates a value significantly different from the corresponding IPSI well-nourished (WL) group.

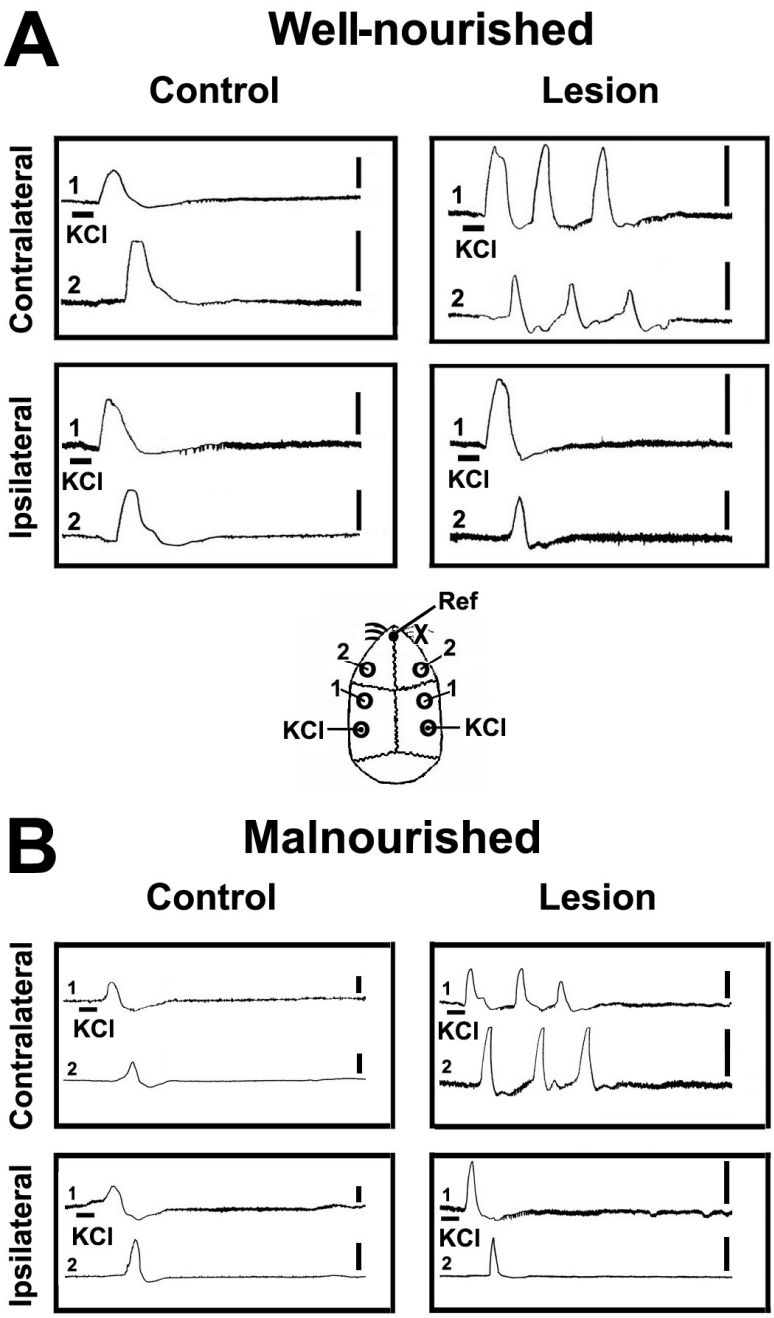

Figure 3: Slow potential change recorded during cortical spreading depression (CSD), in 30-40 days-old, well-nourished (panel A) and malnourished (panel B), control, and lesioned rats (respectively with intact vibrissae and submitted to removal of the right facial vibrissae). The horizontal bars under trace 1 show the period (1 min) of stimulation with 2% KCl, necessary to elicit CSD. The vertical bars equal -10mV (negativity is upwards). The place of KCl application and of the reference electrode are indicated in the inset, which also shows the recording points 1 and 2 in both brain hemispheres. Ipsilateral and contralateral refer to the side (right) of vibrissae lesion. In the contralateral hemisphere of the lesioned groups, sometimes a single 1-min KCl stimulation elicited more than one CSD event, as can be seen in this figure.

CSD propagation

Malnourished rats showed higher CSD velocities of propagation than the well-nourished controls ($p \leq 0.05$; Fig. 4). In the well-nourished animals, which have been submitted to vibrissae removal, CSD velocities were significantly higher in the hemisphere contralateral to the lesion, as compared to the ipsilateral hemisphere of the same animals (Fig. 4, left panel). In the well-nourished and malnourished intact controls, no inter-hemispheric differences in CSD propagation velocities were seen. The lesion-related CSD propagation enhancement was also present in the contralateral hemisphere of the malnourished animals. Unexpectedly however, a small but significant CSD-propagation decrease was found in the ipsilateral hemisphere of the lesioned animals, compared to the corresponding hemisphere of the intact malnourished rats (Fig. 4, right panel).

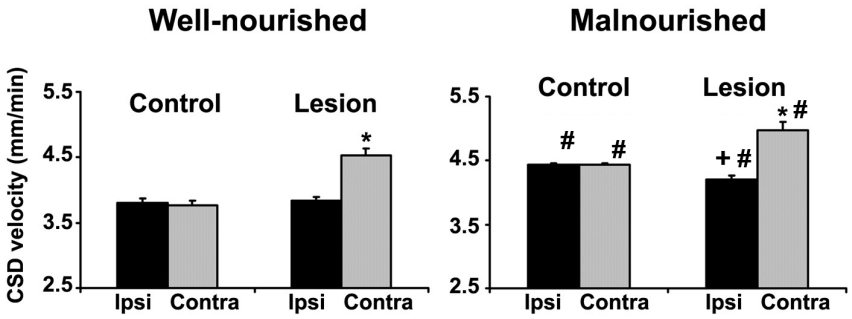

Figure 4: Velocity of propagation, as mean±standard error of the mean, of cortical spreading depression (CSD) in well-nourished (litters formed by 6 pups) and malnourished (litters formed by 12 pups) 30-40 days-old rats. Control and lesion are groups with intact vibrissae and with removal of the right facial vibrissae, respectively. The asterisks in the lesion groups indicate that the CSD velocities in the hemisphere contralateral to the side of vibrissae removal are significantly increased in relation to the ipsilateral hemisphere of the same animals. The # symbol indicates that all malnourished groups displayed CSD velocities significantly higher than the corresponding well-nourished ones. The + symbol indicates a significant CSD-propagation decrease in the ipsilateral hemisphere of the lesioned malnourished animals, compared to the corresponding hemisphere of the intact malnourished ones ($p \leq 0.05$; ANOVA plus Tukey test).

DISCUSSION

As the main finding of the present study, it can be stated that the early sensory deprivation produced by vibrissae removal during the critical period of brain development

facilitated CSD propagation in the cortical tissue of the weaned, young rats, as evaluated by the increases in its propagation velocity. This effect was seen only on the hemisphere contralateral to the side of vibrissae removal, which implies that there was a laterality relationship between the peripheral sensory lesion and the contralateral cortical CSD propagation changes, reinforcing the here postulated causal link. Furthermore, the CSD-effect was induced during the phase of fast brain development (the lactation period) and was detected after weaning (up to 40 days of life), suggesting that (i) it might have affected developmental processes in the brain and (ii) it might be a long-lasting effect. This last suggestion shall be confirmed in future experiments by analyzing the CSD features at a later age.

Vibrissae removal and CSD propagation

Several mechanisms could be implicated in the CSD effects presently reported. One of them would be based on plasticity-dependent changes in cortical excitability. The sensory deprivation by neonatal vibrissae removal represents in the rat the suppression of an important afferent input. This early suppression can result in plastic morphological and functional alteration of cortical maps, including impairment in the formation of the cortical barrels (Shepherd et al., 2003; Van der Loos and Woolsey, 1973; Wong-Riley and Welt, 1980). Plastic structural alterations produced in the cortex following sensory deprivation may also be involved, at least in part, in the here described CSD-effects. Such alterations could influence the distribution of inhibitory and excitatory synaptic contacts in the cortex (Finnerty and Connors, 2000; Micheva and Beaulieu, 1995; Mierau et al., 2004) with subsequent imbalance between excitatory and inhibitory neurotransmitter mechanisms, and changes on cortical excitability (Cheetham et al., 2007; Finnerty and Connors, 2000; Micheva and Beaulieu, 1995; Mierau et al., 2004). The increase in the amplitudes of the CSD DC-potential

changes seen in the contralateral hemisphere of the vibrissae-removal group (Table 1) supports this hypothesis.

A small, but significant decrease in body- and brain weights was found to be associated with vibrissae removal. This could be considered as an expected finding, as the newborn rat pups are very somatosensory, and can use information from the whiskers for nipple attachment. In fact, Sullivan et al (2003) reported that both nipple attachment and huddling behaviors were disrupted in whisker-clipped pups at postnatal (PN) days 3–5 but only marginally altered in PN11–12 pups, suggesting a fast behavioral adaptation of the pups to the absence of the whiskers. Our data do not allow excluding the possibility that the lesion condition may contain a confounding extent of malnutrition. However, if the vibrissae removal has provoked any degree of malnutrition, this is surely very small in comparison to the large-litter-technique. In fact, unpublished data from our laboratory revealed that the body- and brain weight differences between intact- and vibrissae removed groups disappeared in animals older than 30 days, whereas the well-nourished versus malnourished weight differences did not. So, it is reasonable to postulate that the presently reported CSD-effects are due, at least in part, to the brain developmental consequences of the early somatosensory suppression produced by the vibrissae removal.

If this hypothesis has merit, then one should predict that sensory manipulations on the opposite direction (i.e. peripheral sensory activation instead of suppression) would lead to CSD propagation changes that would be contrary (i.e., decrease in CSD propagation velocity) to the presently reported increased CSD propagation. In fact, this has actually been observed by Monte-Silva et al. (2007). These authors submitted developing rats to daily sessions of repetitive peripheral electrical stimulation of the left paws delivered by two electrodes attached to them. At postnatal days 35 to 45 stimulated animals presented slower CSD

propagation in the hemisphere contralateral to the stimulated body-side, as compared to the ipsilateral hemisphere, and also as compared to the non-stimulated control group.

Similar to what has often been demonstrated for different degrees and types of malnutrition, it has also been reported that distinct levels of environmental complexity can influence brain development and function (Coleman and Riesen, 1968; Naka et al., 2002; Rema et al., 2006; Santos-Monteiro et al., 2000; Seo, 1992). Concerning the relationship between sensory input manipulation during brain development and CSD, few studies have so far been conducted. Santos-Monteiro et al. (2000) first reported in developing rats that environmental-dependent multi-sensory activation was effective in antagonizing CSD. In contrast, the unilateral suppression of the visual input by enucleation increased the CSD susceptibility in the contralateral cortex (Santos-Monteiro, 2002). The present study unequivocally showed that vibrissae removal enhances CSD propagation. Both in well nourished and in malnourished groups the increased CSD velocities were associated with the contralateral surgical lesion and not with the cryoanesthesia, since the control groups received the same anesthetic procedure (without vibrissae removal) and did not present any inter-hemispheric CSD-changes. So, our data can be considered as a novel piece of evidence demonstrating that the removal of a sensory input in developing animals is causally linked to CSD enhancement.

Modifications in cortical and sub-cortical excitability can occur as a consequence of peripheral sensory activity changes (Chowdhury et al., 2004; Werhahn et al, 2002). Considering that conditions that change cerebral excitability can also modify CSD incidence and propagation (Amâncio-dos-Santos et al., 2006; Costa-Cruz et al., 2006; Van den Maagdenberg et al., 2004), it is reasonable to postulate that the modification in the cortical excitability status subsequent to the plasticity changes provoked by vibrissae pathway interruption participates in the effects on CSD propagation. The involvement, as possible

contributing mechanisms, of cerebral blood-flow changes, consequent to somatosensory deafferentation (Greenberg et al., 1999), as well as lesion-induced imbalance of cortically-based GABAergic inhibition (Neumann-Haefelin et al., 1995; Pinto and Guedes, 2008; Werhahn et al., 2002), or other-transmitter-based mechanism, are hypotheses whose direct confirmation warrants further investigation.

Influence of Malnutrition and sensory suppression on CSD

The widespread importance of studying the brain effects of malnutrition can become evident if one considers the restricted economic possibility of a large part of human populations to buy nutritionally adequate foods (due to the high costs of these foods). Furthermore, the effects of deficient food ingestion, either quantitative or qualitative, can sometimes be permanent in the nervous system (Morgane et al, 1978; 1993). In the present study, the animals suckled in large litters showed lower body and brain weights than well-nourished ones (Fig. 1 and 2), confirming previous studies concerning the effectiveness of this technique in provoking early malnutrition (De Luca et al., 1977; Fernández et al., 1993; Rocha-de-Melo et al., 2006). This condition alters the development of vital organs such as the brain, resulting in reduction of its weight that might be associated with functional alterations (Morgane et al. 1978, 1993). As commented above, the groups subjected to vibrissae removal presented lower body and brain weights as compared to the corresponding controls (Fig. 1 and 2). This could be related to some degree of deficiency in the suckling activity induced by the sensory deprivation consequent to the vibrissae removal, as suggested by Sullivan et al. (2003). According to some reports (Kuhn and Schanberg, 1998; Laviola and Terranova, 1998), a decrease in mother–infant interaction during the lactation period would also modify the brain development, leading to a number of behavioral and neurophysiological changes. This is one factor that could influence the results achieved in the present study. It is also

important to mention that while the well-nourished lesion-ipsilateral condition seems to show essentially similar propagation to the control condition (Figure 4, left panel), the malnourished lesion-ipsilateral condition showed a decreased CSD propagation, compared to the control malnourished condition (Figure 4, right panel). This indicates that the nutrition factor can influence the CSD-effects of the vibrissae removal, suggesting a kind of interaction between early malnutrition and the sensory deprivation, possibly involving interhemispheric modulation, a process that has previously been demonstrated to influence CSD propagation in both well-nourished and malnourished rats (Pinto and Guedes, 2008). Concerning the influences of nutritional manipulation on CSD, in the present study the malnourished animals displayed a significant increase in CSD velocity of propagation, as compared to the well-nourished ones (Fig. 4), reinforcing previous results about the effects of early malnutrition on CSD (Guedes et al. 1987; Guedes et al., 1992). De Luca et al. (1977) proposed that malnutrition-induced impairment of brain myelin would contribute in facilitating CSD propagation, since myelin between the cells would be an obstacle for the propagation of a diffusion-based phenomenon, like CSD. Furthermore, central hypomyelination would lead to a high cell density, by increasing the cell packing (Morgane et al., 1993), a factor that is also known to facilitate CSD propagation by enhancing the cell–cell interaction at the level of neuropiles or neuropile-like structures (Guedes et al., 1987). In addition, other studies postulate that the malnutrition-induced modifications in neurotransmitter systems (Stern et al., 1974) could be implicated in the mechanisms that facilitate CSD propagation in early-malnourished animals (Guedes et al., 1992; Rocha-de-Melo and Guedes, 1997).

Regarding the relevance of our data for the human brain development and function, it is interesting to note that, in malnourished infants, the inadequate amount of social and psychological stimulation from the environment seems to be related to the subjects' deficiencies in behavioral or intellectual competence (Nwuga, 1977; Stoch et al., 1982).

These data indicate negative effects of sensory activation deficiency on malnourished brain. On the other hand, malnourished organisms under nutritional therapy recover better when environmental stimulation is added to that therapy (Celedon and De Andraca, 1979; Grantham-McGregor et al., 1991), and the same seems to be true in laboratory animals (Lima et al., 1999; Pascual et al, 1996). The presumption that changes in CSD features, under the present experimental conditions, can reflect plastic modifications of neural function (Cheetham et al, 2007) seems to us an interesting issue to be further addressed, concerning the susceptibility of the human brain to development-dependent and energy-consuming diseases.

In conclusion, our data demonstrated an enhancing effect of unilateral vibrissae removal on CSD propagation in the contralateral developing rat cortex, which seems to be long lasting, and is influenced by early malnutrition. Our findings advance the knowledge on the understanding of the mechanisms of plastic cerebral electrophysiological alterations induced by peripheral sensory input elimination during brain development and thus might be useful to shed light on certain brain diseases that might be associated with the here investigated variables.

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Artigo 2:

Title:

Lasting facilitatory effects of neonatal vibrissae removal on the propagation of Cortical Spreading Depression: an electrophysiological study in well-nourished and early-malnourished adult rats

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Abstract

Early malnutrition interferes with the formation of somatosensory pathways and reduced sensory input activity during brain development can induce morphological and physiological changes in the cerebral cortex, long-lastingly altering their response properties. Here we investigated cortical spreading depression (CSD) propagation in male adult rats submitted to unilateral vibrissae removal, at postnatal days 2-3, and malnourished during lactation followed by nutritional recovery until adulthood (90-120 days), when CSD was recorded. Compared to nutrition-matched non-lesioned controls, CSD-propagation was increased in the hemisphere contralateral to the vibrissae removal. The findings indicate that vibrissae removal during brain development enhances CSD-propagation. In contrast to the well-nourished rats, in the vibrissae-removed malnourished animals the spreading depression propagation in the ipsilateral hemisphere decreased as compared to the corresponding hemisphere of the non-lesioned malnourished rats. Considering that CSD-facilitation persisted until adulthood, we suggest that this effect is permanent. Data might contribute to the understanding of the mechanisms by which malnutrition and sensory input deprivation-induced plasticity modifies cerebral electrophysiological responses in the developing brain.

Key words:

Neural plasticity; Cortical spreading depression; Sensory deprivation; Brain development; Malnutrition, Lasting brain alteration.

Introduction

It is already well established that the mammalian nervous system modifies its strategies of development and function in response to internal and external changes [1, 2]. It has been also demonstrated that complex mechanisms of neural plasticity underlying the development of the cerebral cortex are influenced by social, nutritional and environmental factors [3, 4]. The higher degree of brain plasticity in the young human brain is promptly recognized in the higher ability of children to learn new tasks, or to recover from brain injuries [2], as compared to the adult brain. However, some degree of plasticity still remains in adult cortex [5-8] and a variety of studies to elucidate the underlying mechanisms of neural plasticity are leading to better understanding neurological disorders both in the developing and in the adult brain [9-12]. Furthermore, the knowledge about basic mechanisms of neural plasticity is important for developing successful treatments for neurological diseases [13].

The manipulation of the sensory conditions in developing organisms, either enhancing or depriving them, is an approach often employed in studies on neural plasticity [4, 14-16]. The rodent facial vibrissae system in particular has been object of several studies dedicated to this issue [6, 17-19], providing valuable information to understand plasticity in the adult cortex [7, 8]. The vibrissae (whiskers) are important sensory receptors of environmental stimulus [20, 21], that remains highly segregated in the barrel fields of the primary somatosensory cortex and provides tactile skills that are, in some ways, similar to modules of finger representation in primates [20, 22, 23]. The mechanosensory receptors attached to vibrissae are somatotopically represented in the contralateral primary somatosensory cortex by cylindrical arrangements of neurons, located in layer IV of the cortex [23, 24]. The cortical circuits activated by sensory receptors change during the brain development in response to sensory experience [25]. They are dynamic representations of the sensory periphery, since the frequency of the whiskers movement modifies the maps and this fact is an evidence of the experience-dependent

cortical plasticity [26]. The reverse experience of this phenomenon is induced by the deprivation of sensory input by removing vibrissae early-in-life [21, 27], leading to impairment of the circuitry of the whisker sensory system [6, 28].

On the other hand, nutritional conditions in early periods of life are one of the determining factors for the brain development [29, 30]. Depending on the intensity and duration of both environmental and nutritional alterations in the critical period of development, some of their effects can remain until the adulthood, resulting in functional consequences for the adult organism [30, 31]. In the rat, malnutrition on the first three weeks of life (lactation period) induces several modifications in morphological [32] and biochemical [33] patterns of developmental processes in the brain [30, 34, 35] and in particular early malnutrition can induce a delay on the formation of the whisker barrel cortex [36].

A variety of experimental approaches have been applied to investigate the experience-dependent brain plasticity and that includes histological [27], behavioral [37] and imaging techniques [26], but few employed in vivo electrophysiological approaches [18] and none of them addressed this issue by using the cortical spreading depression (CSD). CSD was first described by Leão [38] as a reversible and propagated "wave" of depression of spontaneous neuronal activity in response to electrical, mechanical or chemical stimulation that spreads across the entire cortical surface, with propagation velocities of the order of a few mm/min [39]. Simultaneous with the electrocorticogram depression, a DC slow potential change of the tissue has been described [40]. The phenomenon has been studied in several animal species [39], and has already been demonstrated in the human brain [41, 42]. Since CSD is considered a phenomenon related to brain excitability, it has been causally associated to some human diseases, as for example brain ischemia [43], migraine [44] and epilepsy [38, 45-46].

The brain susceptibility to CSD is considered increased when the CSD-velocity of propagation becomes higher as compared to the control situation, and vice-versa. CSD has

been characterized in laboratory animals under conditions of pharmacological, nutritional and environmental manipulations [48]. Concerning the environmental factors, it has been demonstrated that environmental stimulation decreases CSD propagation [16], but no information is available so far regarding the long lasting effects of sensory deprivation, on CSD, by removing the mystacial vibrissae combined to nutritional changes early in life.

By electrophysiologically recording CSD and measuring its propagation velocity, three questions in the brain of adult rats, subjected to neonatal unilateral mystacial vibrissae removal, and malnutrition during lactation followed by nutritional recovery, have been presently addressed: (1) Does elimination of the vibrissae sensory pathway during the brain development affects CSD propagation, and if so to what extent?, (2) would possible CSD propagation changes persist until adulthood and (3) would this possible effect be influenced by the previous brain nutritional condition?

It was hypothesized that (i) changes in the emergence of cortical barrel fields region, induced by postnatal vibrissae removal, would influence the cortical propagation of CSD; (ii) this influence would remain until the adult life and (iii) early malnutrition would modify this effect. The present findings support these hypotheses.

METHODS

Animals and nutritional conditions

The experiments were performed on male Wistar rats (n = 40) from the colony of the Department of Nutrition of Universidade Federal de Pernambuco (UFPE), Brazil. All experiments were carried out in accordance with the “Principles of Laboratory Animal Care” (National Institutes of Health, Bethesda, USA) and were approved by the Ethics Committee for Animal Research of the Universidade Federal de Pernambuco, Brazil, where the experiments have been conducted.

Following 24 h after birth, newborn rats from several dams were pooled and then randomly distributed to form litters with 6 pups per nurse (W - Well-nourished group, n=20) or with 12 pups per nurse (M - Malnourished group, n=20). The dams were fed a rodent laboratory chow diet (Purina do Brazil Ltd.) with 23% protein. After weaning (21 days), the pups were housed in groups of 4-5 per cage (51 x 35.5 x 18.5 cm) and received the same maternal lab chow diet, with free access to water and food. Housing conditions included controlled temperature (23±2°C) and a standard 12/12 h light/dark cycle (lights on at 6:00 am).

Vibrissae removal

Both nutritional groups (well-nourished and malnourished) were subdivided in two experimental groups, respectively, submitted to removal of mystacial vibrissae from the right side ("Lesioned groups", 10 W- and 10 M pups) and maintained with intact vibrissae (control groups; 10 W- and 10 M pups).

Procedures for vibrissae removal were carried out between postnatal days 2-3. Under cryoanesthesia and low-power microscope magnification, the mystacial vibrissae from the right side were pucked out with tweezers, followed by electrical cauterization of follicles [23]. Daily examination confirmed the effectiveness of the electrical cauterization, since the removed vibrissae did not regrow until the adult life. The control pups (non-lesioned) were equally submitted to cryoanesthesia, but without being followed by vibrissae removal.

CSD recording

The CSD electrophysiological experiments were performed on postnatal day 90-120. The animals were intraperitoneally anesthetized with a mixture of 1 g/kg urethane plus 40mg/kg chloralose, their heads were secured in a stereotaxic apparatus and six trephine

holes, each measuring 3 mm diameter, (three holes in each hemisphere) were drilled. In each hemisphere, the holes were aligned in the anteroposterior direction and parallel to the midline. The most posterior hole was used to apply the CSD-eliciting stimulus (2% KCl solution) to elicit CSD. The other two holes in each hemisphere were used to record the propagating CSD wave, and were positioned over the cortical area of representation of the vibrissae (from AP -0.3mm to -3.3mm in relation to the bregma and from ML 4.5 to 6.5 mm from the midline), according to Paxinos and Watson [49]. Rectal temperature was continuously monitored and maintained at 37±1^{o}C by means of a heating blanket. In each hemisphere, CSD was elicited at 20 min intervals by 1-min application of a cotton ball (1–2 mm in diameter) soaked with 2% KCl solution (approximately 270 mM). The CSD slow potential change was continuously recorded for 4 hours, by using four Ag–AgCl agar-Ringer electrodes (two electrodes on each hemisphere; one in each recording hole) against a common reference electrode of the same type, placed on the nasal bones. The CSD velocity of propagation was calculated, in each hemisphere, from the time required for a CSD wave to pass the distance between the two cortical electrodes. The initial point of each DC negative rising phase was taken as the reference point to calculate the CSD velocities, as previously employed [50].

Body and brain weights

Body weights were measured at postnatal days 7, 14, 21, 30, 60 and 90, as well as on the day of CSD recording. At the end of the recording session, the animals, while still anesthetized, were killed by lesioning the bulbar region with a sharp needle, promptly provoking cardio-respiratory arrest. The brain was immediately removed and weighed (wet brain weight) using an analytical balance (Model: Bosch, S-2000). After that, brains were kept in a stove at 100^{o}C and were weighed at intervals of 1-2 days until reaching constant weight (dry brain weight).

Statistics

Body- and brain weights, as well as CSD propagation rates, were compared between groups by using ANOVA including as factors: nutritional status (W and M), vibrissae condition (intact and removed) and hemisphere side (ipsilateral and contralateral to the vibrissae removal side) followed by a post-hoc test (Tukey) when indicated. Within each group, a paired t-test was used to compare CSD propagation rates between hemispheres (ipsi and contralateral to vibrissae removal) of each rat. The differences were accepted as significant at the 95% confidence level ($p \leq 0.05$). All values were presented as means ± standard deviations.

RESULTS

Body weight

As shown in Fig. 1, early-malnourished rats (suckled in litters with 12 pups) presented lower body weight at the different time-points (7, 14, 21, 30, 60 and 90 days) , as compared to the well-nourished ones (suckled in litters with 6 pups).

In the intact, well-nourished animals, the mean body-weights (in grams) between postnatal days 7 to 90 ranged from 18.6±2.0 (at 7days) to 304.7±34.5g (at 90 days) whereas in the malnourished age-matched animals, the mean body weights ranged respectively from 13.5±1.7 to 277.2±20.6g ($P<0.05$).

In the well-nourished lesion group, these values ranged from 17.7±1.4 (at 7 days) to 299.05±18.8 g (at 90 days) and in malnourished age-matched rats, the mean body weights ranged respectively from 12.41±1.7 to 279.11± 19.7g ($P<0.05$).

In both nutritional conditions, a slight, but significant weight reduction associated to the removal of vibrissae was seen only until 30 days of age, as compared to the

corresponding controls (animals kept with intact vibrissae). In the other ages (60 and 90 days), no weight differences related to the vibrissae removal were seen.

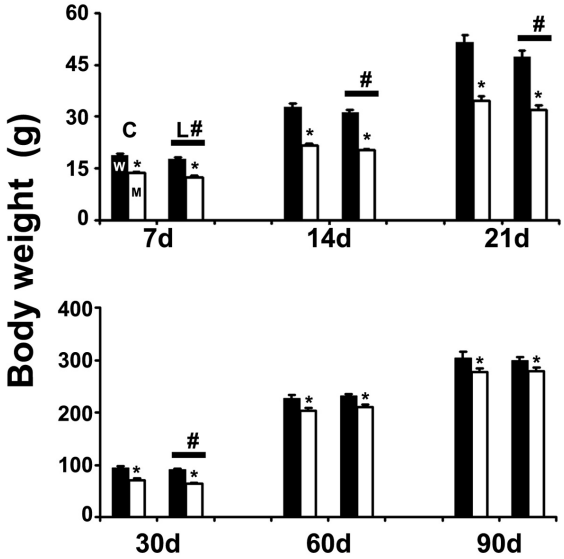

Fig. 1 - Body weight (mean±standard error of the mean) of well-nourished (W; litters formed by 6 pups; black bars) and malnourished (M; litters formed by 12 pups, white bars) rats, from the groups: Control (C; animals with intact vibrissae), and lesioned (L; animals submitted to lesion of facial vibrissae from right side). The weights were measured on 7, 14, 21, 30, 60 and 90 postnatal days. The asterisk indicates that all malnourished values are significantly different from the corresponding well-nourished controls and the # symbol indicates that lesioned animals had significantly lower body weight than corresponding intact controls, at 7, 14, 21 and 30 postnatal days ($p \leq 0.05$; ANOVA plus Tukey test).

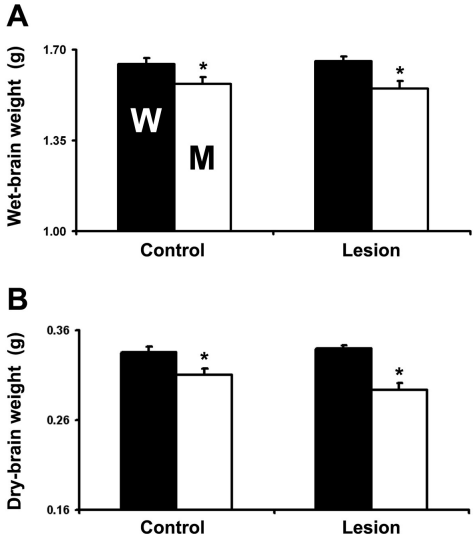

Fig. 2: Weights of the wet- (panel A) and dry-brain (panel B) of 90-120 days-old rats from the groups: Control (C; animals with intact vibrissae), and lesioned (L; animals submitted to the removal of facial vibrissae from the right side). The asterisks indicate that all malnourished values (M; white bars) are significantly lower than the corresponding well-nourished ones (W, black bars) ($p \leq 0.05$; ANOVA plus Tukey test).

Brain Weights

Similar to the body weights, the malnourished animals (suckled in litters of 12 pups) showed significantly lower wet- and dry brain weights, measured on the day of the CSD recording, as compared to the respective well-nourished controls (Fig. 2). No brain weight differences associated to vibrissae removal were found.

The mean wet-brain weights (in grams) in well-nourished rats ranged from 1.644±0.06g (Control group) to 1.655±0.05g (Lesion group), whereas in malnourished animals, the weights ranged from 1.550±0.08g (Lesion group) to 1.567±0.07g (Control group). The mean dry-brain weights in well-nourished animals ranged from 0.335±0.02 g (Control group) to 0.339±0.01 g (Lesion group) and in malnourished rats, these values ranged from 0.294±0.02 g (Lesion group) to 0.310±0.02g (Control group).

CSD elicitation

In all groups, topical application of 270 mM KCl for 1min at the parietal cortex reproducibly elicited a single CSD wave, which was recorded by the two electrodes located more anteriorly in the stimulated hemisphere. Fig. 3 presents electrophysiological recordings showing the slow potential change, accompanying the CSD after each KCl-stimulation, on the cortical surface of well-nourished (panel A) and malnourished (panel B) animals, in both brain hemispheres (ipsi and contralateral to the vibrissae removal).

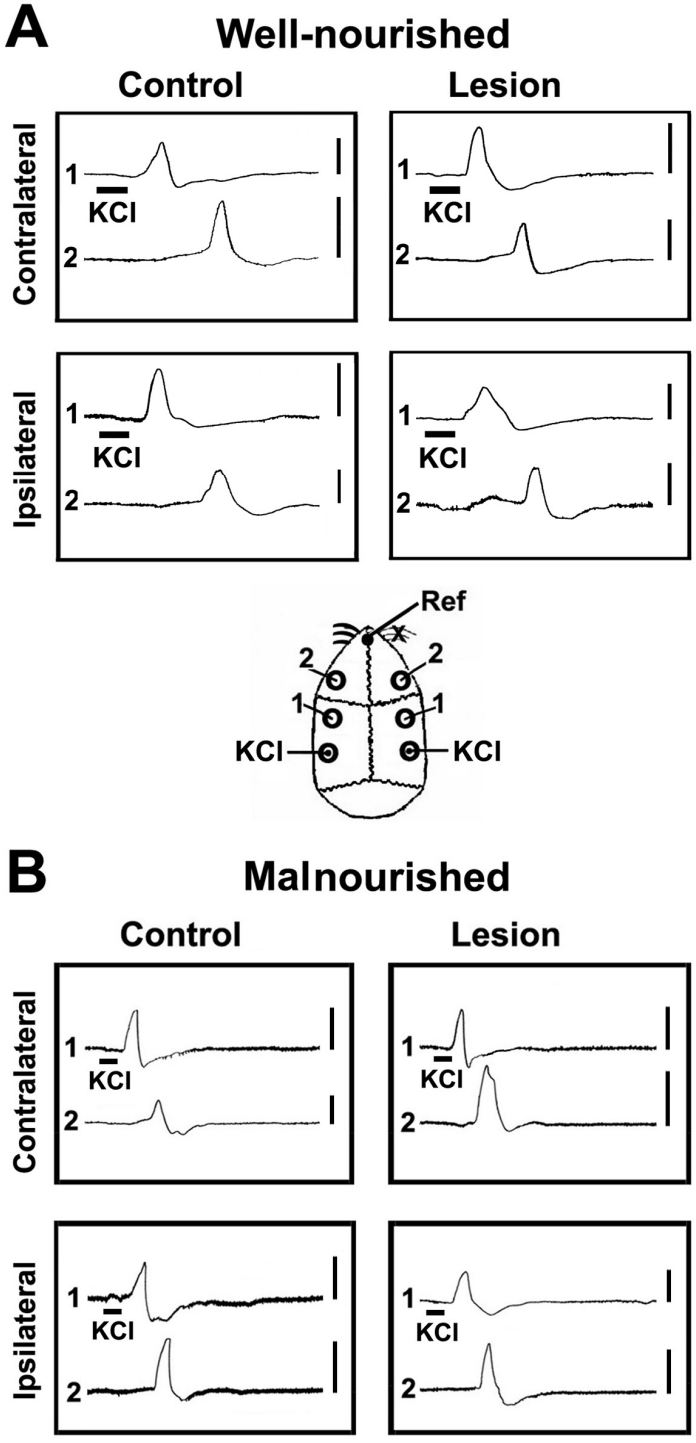

Fig. 3: Slow potential change recorded during cortical spreading depression (CSD), in 90-120 days-old, well-nourished (panel A) and malnourished (panel B), control, and lesioned rats (respectively with intact vibrissae and submitted to removal of the right facial whiskers). The horizontal bars under trace 1 show the period (1 min) of stimulation with 2% KCl, necessary to elicit CSD. The vertical bars equal -10mV (negativity is upwards). The place of KCl application and of the reference electrode are indicated in the inset, which also shows the recording points 1 and 2 in both brain hemispheres. Ipsilateral and contralateral refer to the side (right) of vibrissae removal.

CSD propagation

Malnourished animals displayed higher CSD velocities of propagation than the corresponding well-nourished controls ($p \leq 0.05$; ANOVA plus Tukey test). In the groups submitted to vibrissae removal, CSD velocities were significantly higher in the hemisphere contralateral to the lesion, as compared to the ipsilateral hemisphere of the same animals ($p \leq 0.05$; paired test-T), while in the intact controls no inter-hemispheric differences in CSD propagation velocities were seen. Malnutrition did not modify this CSD effect associated to the vibrissae removal. These results are presented in Fig. 4.

In well-nourished, lesioned animals, the mean CSD (in mm/min) was 3.35±0.06 and 4.13±0.12 respectively in the ipsilateral and contralateral hemispheres in relation to the lesion side ($p \leq 0.05$; paired t-test). In well-nourished, intact animals, these values were 3.41±0.10 (right hemisphere) and 3.40±0.10 mm/min (left hemisphere).

In malnourished, lesioned animals, the mean CSD (in mm/min) was 3.84±0.07 in the hemisphere ipsilateral to the lesion and 4.36±0.09 in the hemisphere contralateral to the lesion ($p \leq 0.05$; paired t-test). In malnourished, intact animals, these values were 4.08±0.04 (right hemisphere) and 4.09±0.05 mm/min (left hemisphere).

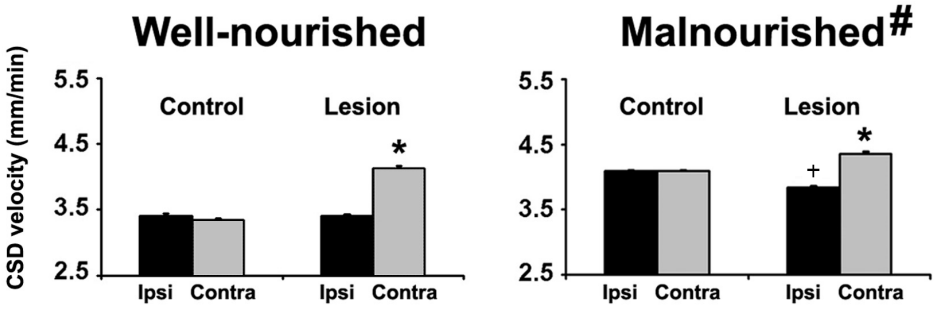

Fig. 4: Velocity of propagation, as mean±standard error of the mean, of cortical spreading depression (CSD) in well-nourished (litters formed by 6 pups) and malnourished (litters formed by 12 pups) 90-120 days-old rats. Control and lesion are groups with intact vibrissae and with removal of the right facial vibrissae, respectively. The asterisks in the lesion groups indicate that the CSD velocities in the hemisphere contralateral to the side of vibrissae removal are significantly increased in relation to the ipsilateral hemisphere of the same animals ($p \leq 0.05$; paired test-T). The # symbol indicates that all malnourished groups displayed CSD velocities significantly higher than the corresponding well-nourished ones. ($p \leq 0.05$; ANOVA plus Tukey test) The + symbol indicates a significant CSD-propagation decrease in the ipsilateral hemisphere of the lesioned malnourished animals, compared to the corresponding hemisphere of the intact malnourished ones.

DISCUSSION

In the present study we demonstrated in adult rats that previous peripheral sensory deprivation, induced by early postnatal vibrissae removal, was related to an enhancement in the CSD susceptibility on the hemisphere contralateral to the side of vibrissae removal. This long-lasting effect was expressed by increases in the CSD velocity of propagation, as compared to the velocities in the ipsilateral hemisphere of the same animals, and to the age- and nutrition-matched controls. The malnutrition seems to influence the CSD-propagation effect of the sensory deprivation.

Although the effect of the sensory deprivation on CSD velocity can be explained by several mechanisms, those related to plasticity-dependent changes induced by suppression of an important afferent input carried by mystacial vibrissae seem to us a tempting possibility. The vibrissae removal on the first week of life may result in morphological impairments in the cortical barrel field [27, 51], that might be associated with electrophysiological alterations [18, 52] and these changes can persist until the adult life, mainly when the sensory deprivation is permanent [27].

Since the brain plasticity is driven by experience achieved throughout the organism-environment interaction and the brain structure reorganizes itself in response to environmental demands [53, 54], the lack of afferent input due to permanent whisker removal can abolish the interaction through this sensory pathway, leading to long-lasting effects, without recovery of the plastic modifications induced in the developing brain.

On the other hand, several pieces of evidence have pointed that sensory stimulation can help in recovering the effects of neural injuries consequent to malnutrition or sensory deprivation in laboratory animals [3, 55] and even in humans [56, 57]. Nevertheless, in the present study, the animals were submitted to chronic sensory deprivation, and the CSD enhancement

observed in the hemisphere contralateral to the vibrissae removal persisted until the adulthood.

By this logic, perhaps an opposite experimental action, e.g., the stimulation of the somatosensory pathways could result in a CSD-effect contrary to that of vibrissae removal on CSD features. This idea is supported by some evidence showing that peripheral sensory activation decreases CSD susceptibility [58]. These authors submitted developing rats to sessions of repetitive peripheral electrical stimulation of the left paws. At postnatal days 35–45 stimulated animals presented slower CSD propagation velocities in the hemisphere contralateral to the peripheral stimulation, as compared to the ipsilateral hemisphere, and also as compared to the non-stimulated control group.

In the same line of investigation as of the present report, previous studies assessed the relationship between manipulation of the visual sensory pathway and CSD features [59]. This author investigated, in adult Wistar rats, the effect of early sensory visual deprivation (produced by unilateral enucleation during lactation) on CSD features and described an increase in CSD velocity of propagation on the hemisphere contralateral to the enucleation, as compared to the ipsilateral hemisphere. Though focusing another sensory pathway, these results are qualitatively similar to the present ones, suggesting some evidence in favor of the existence of a long-lasting inverse relationship between peripheral sensory input and the propagation velocity of CSD. Whether this effect is related to the deprivation-induced modifications of synaptic activity, or to some other plasticity-dependent factor, remains to be further investigated.

Concerning the influence of the early nutritional status on the CSD-effects of vibrissae removal, the results are controversial: while in the present study malnutrition had a small influence on the effects of sensory deprivation on CSD features (only in the ipsilateral hemisphere of the lesioned animals), in the report by Santos-Monteiro [59] malnutrition

abolished the CSD-effects of enucleation. This contrast illustrates the complexity of the interaction among nutritional condition, brain development and plasticity, and environment.

In the present study, the reduced body weights found in the animals reared in large litters allowed us to conclude that this technique was effective in provoking malnutrition in the pups. These data confirm previous studies in which large litters are used to induce unfavorable conditions of nutrition[3, 60, 61]. It is probable that in this experimental malnutrition model maternal milk quantity was insufficient to supply the developmental demands of the pups suckled in the large litters. This nutritional condition induced long-lasting impairment of body development, since the reduced body weight was observed in all ages assessed.

Another important observation in this study was a slight, but significant reduction in body weight in the animals submitted to early vibrissae removal, as compared to the intact vibrissae control animals, until 30 days of life (Figure 1). This result could be related to a decreasing in the dam-pups interaction during the lactation period and consequent reduction in the suckling activity. Such impairment in the dam-pups interaction induced by vibrissae removal has been described by Sullivan et al[37].

When occurring during the “brain growth spurt period”, malnutrition usually induces reduction in brain weight, in addition to the decrease in body weight. This impairment can remain until the adult life, leading to several consequences, including on neural functional aspects [30, 35]. In agreement with these authors, the present results also revealed a lower brain weight in the animals reared in large litters, when compared with the corresponding well-nourished ones. According to morphological studies, such brain weight reduction could be associated to the lesser number and/or size of glial and neuronal cell elements, as well as to delays in the neuronal maturation. These alterations imply in reduction of processes like dendritic development, synapse formation and myelination [30, 62].

Concerning to the effect of the nutritional status on CSD features, our data showed a significant increase in CSD velocity of propagation in animals submitted to early malnutrition as compared with well-nourished controls, which is in agreement with previous studies [61, 63, 64]. This facilitation of CSD propagation could be related to the above-mentioned nutrition-dependent developmental alterations in brain structure, but morphological analyses would be necessary to confirm this hypothesis. Moreover, malnutrition-induced alterations in some neurotransmitter systems [65] constitute another factor that could contribute to the facilitation in CSD propagation in malnourished animals [64, 66].

In the present study, unexpectedly, a small but significant CSD-propagation decrease was found in the ipsilateral hemisphere of the lesioned animals, compared to the corresponding hemisphere of the intact malnourished rats This indicates that the nutrition factor can influence the CSD-effects of the vibrissae removal, suggesting a kind of interaction between early malnutrition and the sensory deprivation, possibly involving interhemispheric modulation, a process that has previously been demonstrated to influence CSD propagation in both well-nourished and malnourished rats[67].

In conclusion, this study reports a long-lasting facilitatory effect of the early deprivation of sensory pathway of vibrissae on CSD propagation, analyzing the three following novel aspects: first, vibrissae removal enhances CSD propagation in the contralateral cerebral cortex; second, the effect of this lesion, carried out early in life, lasts until adulthood; third, early malnutrition seems to influence this CSD-effect. Data may shed some light on the understanding of sensory experience-dependent mechanisms mediated by cortical plasticity, and involved in excitability-related electrophysiological phenomena in the cerebral cortex.

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5. Considerações Finais

De acordo com os resultados obtidos neste estudo, pode-se concluir que a privação de estímulos sensoriais provenientes das vibrissas durante o período crítico de desenvolvimento encefálico é capaz de modificar a velocidade de propagação da DAC, até a idade adulta, podendo este efeito ser influenciado pela desnutrição vivenciada no período de lactação. Nesse sentido, a aferência sensorial originada nas vibrissas parece ter um importante papel no desenvolvimento e funcionamento neurais.

São múltiplos os mecanismos de adaptação do SNC às condições nutricionais e sensoriais vivenciadas pelo indivíduo, sobretudo no início da vida. A compreensão completa de tais mecanismos e de suas repercussões tardias é indispensável para o desenvolvimento de abordagens sobre esses aspectos, no sentido de favorecer o desenvolvimento adequado do sistema nervoso, bem como a sua recuperação frente a situações adversas.

Nesse contexto, o presente estudo traz uma contribuição original para o entendimento das relações entre o sistema somestésico, a nutrição e a DAC. Este fenômeno parece prestar-se muito bem como instrumento para o estudo, por um lado, de alterações neurais como as aqui descritas e, por outro, para investigações visando esclarecer os seus próprios mecanismos, o que levará à compreensão do seu real papel no funcionamento do SNC.

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ANEXO A

Parecer de Aprovação pelo Comitê de Ética em Pesquisa

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Ofício nº 30/06 Recife, 30 de junho de 2006

Da Comissão de Ética em Experimentação Animal (CEEA) da UFPE
Para: **Rubem Carlos Araújo Guedes**
Departamento de Nutrição – UFPE
Processo nº 006772/2006-71

Os membros da Comissão de Ética em Experimentação Animal do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEEA-UFPE) avaliaram a resposta de V. Sa. referente ao primeiro parecer da CEEA sobre o projeto de pesquisa intitulado **"Sensibilidade somestésica como fator participante do desenvolvimento neural: análise eletrofisiológica em ratos precocemente desnutridos".**

Concluímos que os procedimentos descritos para a utilização experimental dos animais encontram-se de acordo com as normas sugeridas pelo Colégio Brasileiro para Experimentação Animal e com as normas internacionais estabelecidas pelo National Institute of Health Guide for Care and Use of Laboratory Animals as quais são adotadas como critérios de avaliação e julgamento pela CEEA-UFPE.

Encontra-se de acordo com as normas vigentes no Brasil, especialmente a Lei 9.605 – art. 32 e Decreto 3.179-art 17, de 21/09/1999, que trata da questão do uso de animais para fins científicos.

Diante do exposto, emitimos **parecer favorável** aos protocolos experimentais realizados.

Atenciosamente,

Prof. Silene Carneiro do Nascimento
Presidente CEEA

CCB: Integrar para desenvolver

ANEXO B

Documentação de encaminhamento do artigo “Early vibrissae removal facilitates cortical spreading depression propagation in the brain of well-nourished and malnourished developing rats” ao periódico International Journal of Developmental Neuroscience.

Date: Mar 22, 2009

To: "Rubem C.A. Guedes" rc.guedes@terra.com.br

From: "Int. J. Developmental Neuroscience " regino.perez-polo@utmb.edu

Subject: Your Submission

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Title: Early vibrissae removal facilitates cortical spreading depression propagation in the brain of well-nourished and malnourished developing rats.

International Journal of Developmental Neuroscience

Dear Rubem,

Reviewers have now commented on your paper. You will see that they are advising that you revise your manuscript. If you are prepared to undertake the work required, I would be pleased to reconsider my decision.

For your guidance, reviewers' comments are appended below.

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Yours sincerely,

J. Regino Perez-Polo Editor-in-Chief

International Journal of Developmental Neuroscience

ANEXO C

Documentação de encaminhamento do artigo "Lasting facilitatory effects of neonatal vibrissae removal on the propagation of Cortical Spreading Depression: an electrophysiological study in well-nourished and early-malnourished adult rats" ao periódico Nutritional Neuroscience

April 5, 2009, 2009

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TITLE: Lasting facilitatory effects of neonatal vibrissae removal on the propagation of Cortical Spreading Depression: an electrophysiological study in well-nourished and early-malnourished adult rats

Dear Author:

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Best regards,

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