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Pode a Exposição Humana ao Inseticida Clorpirifós Alterar o Desenvolvimento do Sistema Nervoso Central? Contribuições de Experimentos em Animais.

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Esta tese é dedicada

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Abreviações

βAR	Receptor β-Adrenérgico
AC	Adenilato ciclase ou adenylyl cyclase
ACh	Acetilcolina
AChE	Acetilcolinesterase
AMPc	adenosina 3', 5'-monofosfato cíclico
BHE	Barreira hematencefálica
Bs	Brainstem (tronco cerebral)
Cb	Cerebellum (cerebelo)
CPF	Clorpirifós ou Chlorpyrifos
CPF-oxon	Clorpirifós oxon ou Chlorpyrifos oxon
Cx	Cerebral Cortex (cortex cerebral)
FSK	Forskolin
GABA	Ácido Gama Amino Butírico
GD	Gestational day (dia gestacional)
GLU	Glucagon
Hp	Hippocampus (hipocampo)
Isso	Isoproterenol
LHRH	Hormônio de Liberação do Hormônio Luteinizante
Mb	Midbrain (mesencéfalo)
PBB	Bifenila Polibramada
PCB	Bifenila Policlorada

PN	Postnatal day (dia pósnatal)
POP	Poluente Orgânico-Persistente
Rx	Treatment (tratamento)
SN	Sistema Nervoso
SNC	Sistema Nervoso Central
St	Striatum (estriato)
TCP	Tricloropiridinol

Índice

Resumo

É cada vez mais evidente que o mecanismo de ação pelo qual o inseticida organofosforado clorpirifós (CPF) exerce sua toxicidade durante o período de desenvolvimento não é determinado primariamente pelo clássico modelo da inibição colinesterásica. De fato, diversos estudos têem demonstrado que a exposição a CPF altera os processos fundamentais do desenvolvimento do sistema nervoso e que a exposição neonatal a esse inseticida altera a atividade da adenilato ciclase (AC), enzima que cataliza a produção do segundo mensageiro AMP cíclico, que por sua vez tem papel primordial durante o desenvolvimento. Neste trabalho, estudamos os efeitos imediatos da exposição a CPF em dois momentos específicos do período gestacional sobre a atividade da AC e sobre receptores β-adrenérgicos (βAR) no cérebro de ratos. Além disso, investigamos se a exposição gestacional e neonatal a CPF produz alterações de longo prazo sobre a atividade dessa enzima. Por fim, buscamos avaliar qual o impacto da exposição a CPF fora do sistema nervoso, durante o desenvolvimento, utilizando o coração e o fígado. Nos estudos sobre os efeitos da exposição gestacioanal, ratas grávidas foram expostas a CPF em GD9-12 e GD17-20. Já para os estudos no período pós-natal, os animais foram tratados em PN1-4 e PN11-14. A atividade da AC foi avaliada utilizando diferentes estimulantes. Além da atividade basal, avaliamos as respostas a dois estimulantes diretos da AC (Forskolina e Mn^{2+}), ao Isoproterenol, para avaliar a sinalização via βAR, ao fluoreto de sódio, que ativa proteína G, que por sua vez acopla o receptor à AC. Além desses, nos estudos envolvendo coração e fígado, avaliamos também a resposta ao glucagon, que estimula a AC através de receptores específicos. Os resultados demonstraram que a exposição gestacional a CPF também produz alterações na atividade da AC, que foram mais evidentes após a exposição no final da gestação (GD17-20) e sobre a região do tronco cerebral. Quanto aos efeitos de longo prazo, a exposição a CPF em todos os 4 períodos do desenvolvimento avaliados neste estudo (GD9-12, GD17-20,

PN1-4 e PN11-14) produziu alterações na atividade da AC em diversas regiões cerebrais de ratos adutos (PN60). As exposição ocorridas em GD17-20 em diante produziram efeitos diferenciados entre machos e fêmeas. Os efeitos da exposição gestacional e neonatal a clorpirifós foram ainda mais expressivos no coração e no fígado. Embora os efeitos imediatos tenham sido pouco significativos, as exposições em GD9-12, GD17-20 e PN1-4 causaram importantes alterações de longo prazo em PN60. Já o período de exposição PN11-14 produziu efeitos de menor monta, indicando o final do período de vulnerabilidade. Da mesma forma que no cérebro, os efeitos de longo prazo sobre a atividade da AC no coração e no fígado foram diferentes entre machos e fêmeas. Ainda mais importante, os resultados mostraram uma clara janela de vulnerabilidade diferenciada para coração e fígado. Enquanto que a exposição gestacional (GD9-12 e GD17-20) afetou principalmente o coração, a mudança do período de exposição para o período neonatal (PN1-4), teve como alvo principal o fígado. Este trabalho fornece novas contribuições para o entendimento dos efeitos do CPF sobre o período de desenvolvimento. De uma forma geral, os efeitos observados em todos os estudos não foram receptor específico e desta forma podem ser compartilhados por diversos neurotransmissores e hormônios, que estimulam a via de sinalização celular da AC. As alterações observadas no coração e no fígado expandem os efeitos do CPF, durante o desenvolvimento, para além da neurotoxicidade e suas possíveis repercussões sobre distúrbios metabólicos e cardiovasculares não podem ser desprezados.

Abstract

There is increasing evidence that the mechanism for systemic toxicity, named cholinergic hyperstimulation consequent to inhibition of cholinesterase, is not the primary mechanism for adverse effects of chlorpyrifos (CPF) during development. In fact, many studies have shown that CPF disrupts the fundamental processes of brain development. In addition, neonatal exposure to CPF alters cell signaling mediated by adenylyl cyclase, the enzyme responsible for cyclic AMP production, a major controller of cell replication and differentiation. In the present study, we evaluated the immediate effects of gestational CPF exposure on the adenylyl cyclase (AC) signaling cascade and β-adrenergic receptors (βAR) in rat brain. We also investigated whether exposure to CPF during specific gestational or neonatal periods would lead to long term alterations on AC activity. In addition, we evaluated the immediate and long-term effects of CPF exposure of rats during, gestational (GD) and postnatal (PN) days, on the adenylyl cyclase (AC) signaling cascade in the heart and liver. For gestational studies, dams were injected during GD9-12 or GD17-20. For postnatal studies, animals were treated during PN1-4 or PN11-14. AC activity was assessed using different stimulants. In addition to basal activity, we evaluated the responses to direct AC stimulants (forskolin, Mn2+); to isoproterenol, which activates signaling through β-adrenoceptors coupled to stimulatory G-proteins, to sodium fluoride, which activates G-proteins that couple the receptor to AC. Besides, the studies using heart and liver, we also probed AC response to glucagon, which activate signaling through specific membrane receptors. CPF administered to pregnant rats on gestational days (GD) 9–12 elicited little or no change in any components of AC activity or βARs. However, shifting the treatment window to GD17–20 produced regionally selective augmentation of AC activity. In the brainstem, the response to forskolin or Mn^{2+} was markedly stimulated by doses at or below the threshold for observable toxicity of CPF or for inhibition of fetal brain cholinesterase, whereas comparable effects

were seen in the forebrain only at higher doses. In addition, low doses of CPF reduced βAR binding without impairing receptor-mediated stimulation of AC. CPF exposure in any of the four periods (GD9-12, GD17-20, PN1-4, PN11-14) elicited significant long term changes in AC signaling in a wide variety of brain regions in adulthood (PN60). In general, GD9–12 was the least sensitive stage, requiring doses above the threshold for impaired maternal weight gain, whereas effects were obtained at subtoxic doses for all other regimens. Most of the effects were heterologous, involving signaling elements downstream from the receptors, and thus shared by multiple stimulants. Superimposed on this basic pattern, there were also selective alterations in receptor-mediated responses, in G-protein function, and in AC expression and subtypes. Exposures conducted at GD17–20 and later all produced sex-selective alterations. On the AC signaling in the heart and liver, few immediate effects were apparent when CPF doses remained below the threshold for systemic toxicity. Nevertheless, CPF exposures on GD9–12, GD17–20, or PN1–4 elicited sex-selective effects that emerged by adulthood (PN60), whereas later exposure (PN11–14) elicited smaller, nonsignificant effects, indicative of closure of the window of vulnerability. These results indicate that signal transduction through the AC cascade is a target for CPF during a discrete developmental period in late gestation, an effect that is likely to contribute to the noncholinergic component of CPF's developmental neurotoxicity. Results also show that developmental exposure to CPF elicits long-lasting alterations in cell-signaling cascades in the brain that are shared by multiple neurotransmitter and hormonal inputs; the resultant abnormalities of synaptic communication are thus likely to occur in widespread neural circuits and their corresponding behaviors. At last, developmental toxicity of CPF extends beyond the nervous system, to include cell signaling cascades that are vital to cardiac and hepatic homeostasis. Future work needs to address the potential implications of these effects for cardiovascular and metabolic disorders that may emerge long after the end of CPF exposure.

1 – INTRODUÇÃO

No Brasil, os termos empregados para se designar o conjunto de substâncias utilizadas no controle de pragas e vetores de doenças, seja na agricultura ou meio urbano, são variados. Agrotóxicos, defensivos agrícolas, praguicidas, pesticidas ou mesmo remédios de planta e venenos são alguns desses termos que são utilizados por diferentes segmentos da sociedade, os setores produtivos e da academia (Peres et al. 2003). Nesta tese, a opção por pesticidas é de inteira responsabilidade do autor.

1.1 – O uso de pesticidas no Brasil e no mundo

As vendas de pesticidas no mundo quase dobraram entre as décadas de 80 e 90. Entretanto, o uso de culturas mais resistentes ao ataque de pragas e os esforços de países industrializados em reduzir o uso destes produtos vem contribuindo para uma pequena queda nestas vendas desde então (Yudelman et al. 1998)(Figura 1).

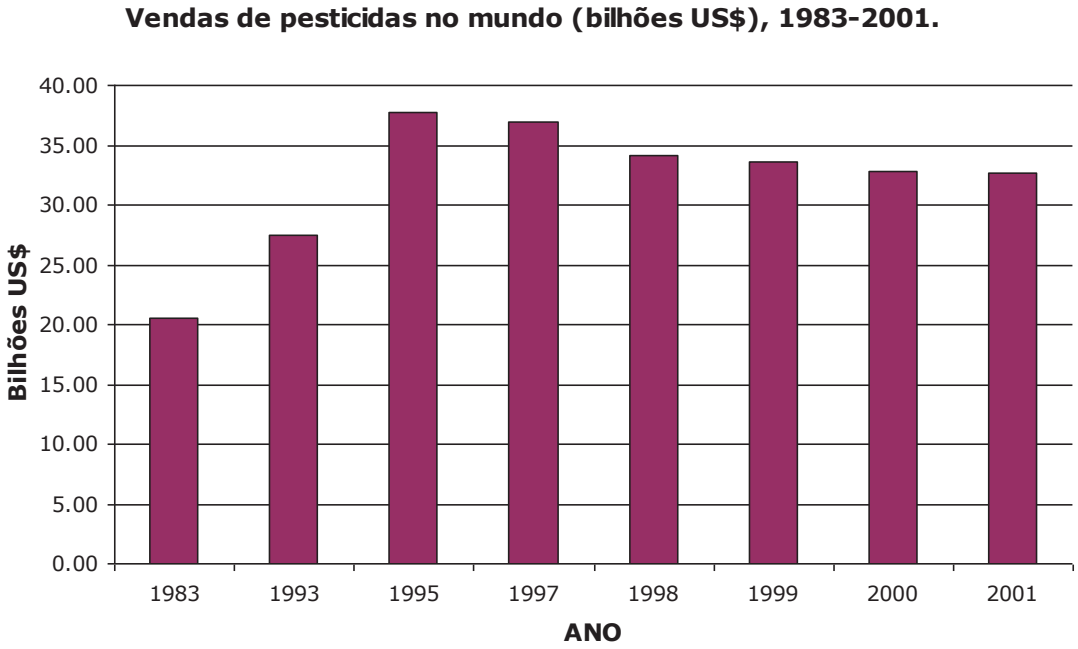

Figura 1 – Vendas de pesticidas no mundo entre os anos de 1983 e 2001, em bilhões de US$. Fonte dos dados: para os anos de 1983, 1993 e 1998 (Yudelman et al., 1998), e para os anos de 1995, 1997, 1999, 2000 e 2001 (Aspelin 1997; Aspelin and Grube 1999; Donaldson et al. 2002; Kiely et al. 2004).

Ainda assim, as exportações de pesticidas a partir de portos dos EUA, um dos maiores produtores mundiais, cresceu consideravelmente na década de 90 (Figura 2). Fato preocupante sobre essas exportações é que

uma fração considerável dos pesticidas exportados é de produtos banidos, ou que não receberam registro para venda nos EUA, produtos de uso altamente restrito, ou ainda pesticidas da classe toxicológica I (altamente tóxicos). O principal destino destes pesticidas foram os países emergentes, como os da América Latina (FASE 1996a; FASE 1998a; Smith 2001).

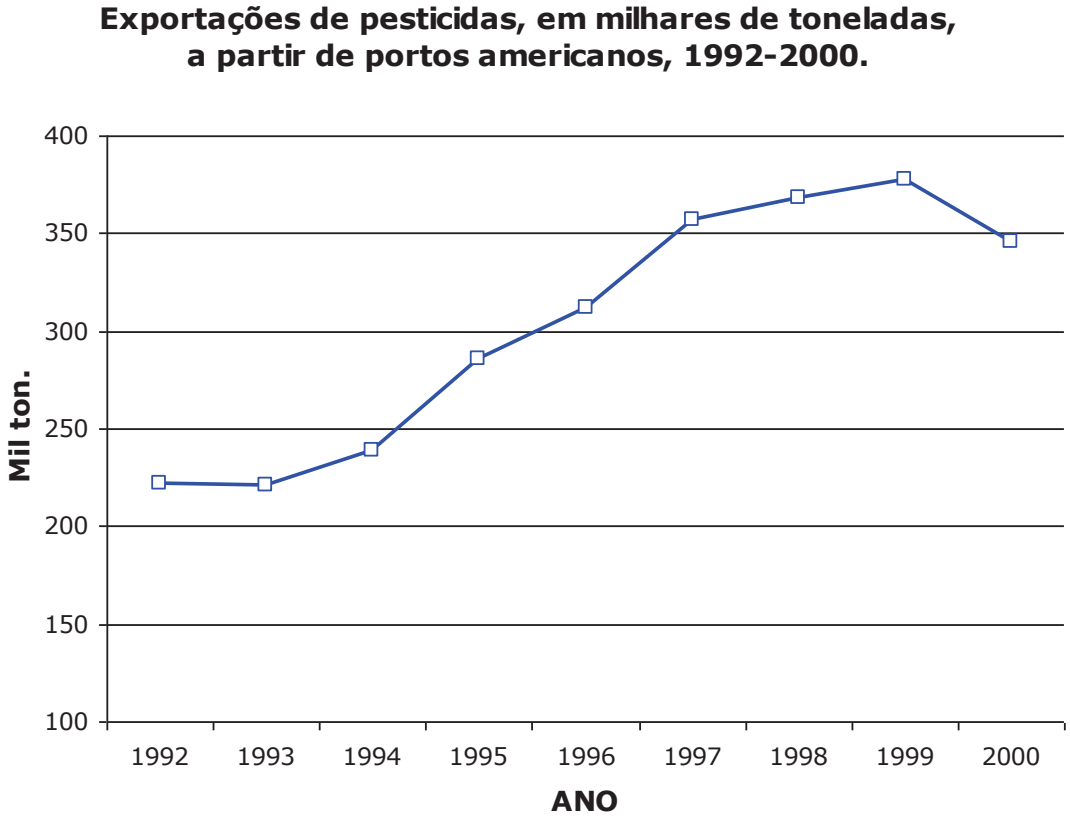

Figura 2 – Exportações de pesticidas a partir de portos americanos. Fonte dos dados: (FASE 1996a; FASE 1998a; Smith 2001)

O Brasil, por exemplo, recebeu 4960 ton destes pesticidas entre os anos de 1992 e 1994 e em apenas 2 anos (1995-1996) essas importações mais que triplicaram (18650 ton) (FASE 1996b; FASE 1998b). Somando-se a isso, a produção brasileira de pesticidas também cresceu de forma expressiva nas últimas décadas. Só entre os anos de 1989 e 2003, as vendas destes produtos no mercado brasileiro saltaram de 0,98 para 3,14

bilhões de dólares, o que representou um crescimento de 220% neste período (Figura 3).

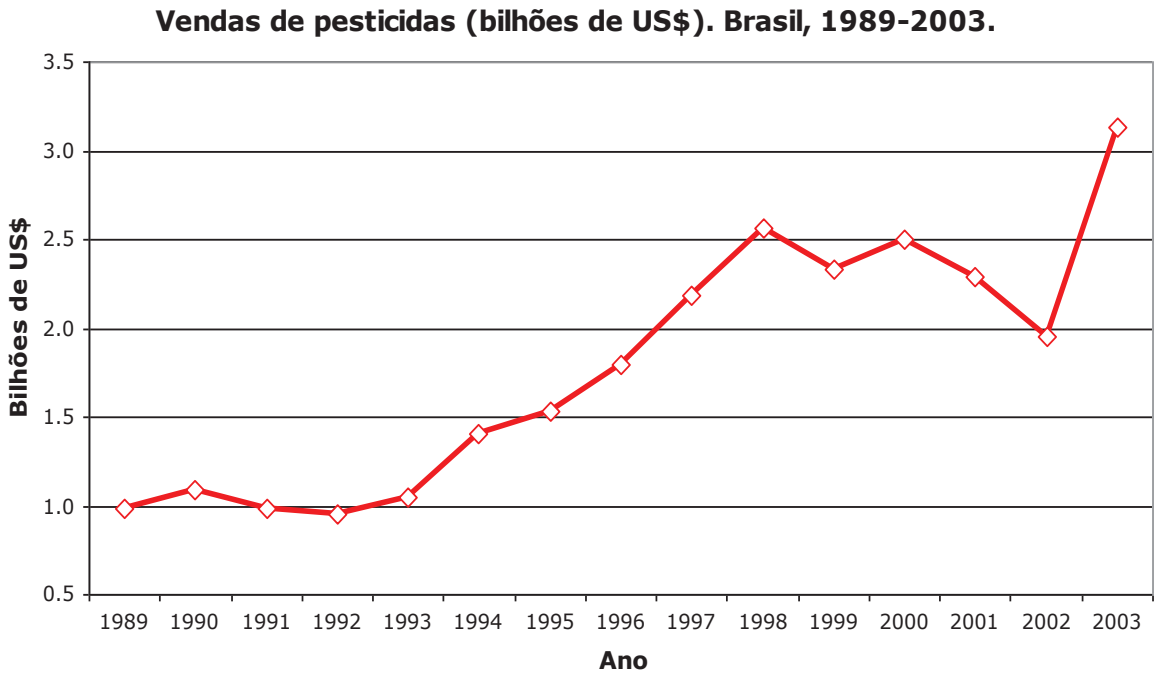

Figura 3 – Vendas de pesticidas no Brasil, entre os anos de 1989 e 2003, em bilhões de US$. Adaptado de Ministério da Agricultura, Pecuária e Abastecimento (www.agricultura.gov.br). Fonte dos dados: SINDAG – Sindicato Nacional das Indústrias de Produtos para Defesa Agrícola (www.sindag.com.br).

1.2 – O impacto do uso dos pesticidas sobre a saúde humana.

A preocupação com o uso intensivo de pesticidas no mundo não é recente. Já na década de 60, o celebre livro de Rachel Carson, silent spring (Carson 1962), teve profundo impacto, não só sobre a comunidade científica, mas também sobre sociedade e os órgãos de regulamentação, principalmente nos países mais desenvolvidos. Nele, a autora reuniu uma série de evidências sobre os efeitos deletérios do uso de pesticidas sobre o ambiente, a vida selvagem e o homem. Assim, o inseticida DDT, um dos ícones do milagre químico e talvez o pesticida mais utilizado na história da humanidade, que chegou a render um premio Nobel a Paul Miller em 1939, por estudar suas propriedades inseticidas, foi banido anos mais tarde, em 1972, pela então recém-criada Agência de Proteção Ambiental americana (USEPA) (EPA 1972; EPA 2000). Na verdade, o primeiro pesticida a ter seu

uso banido por esta agência (EPA 2000). Desde então, a avaliação do impacto do uso de pesticidas sobre a saúde ambiental e humana tem tido cada vez mais representatividade nas agendas científico-políticas dos países de economia consolidada e, mais recentemente, dos países em desenvolvimento também (Pimentel 1996; Ecobichon 2001a).

De forma meramente didática, os efeitos sobre a saúde humana podem ser de dois tipos. Os efeitos agudos são caracterizados por sinais e sintomas que podem ser específicos para cada substância ou conjunto de substâncias, mas que essencialmente se tornam aparentes em período curto de tempo, em geral 24h após a exposição a níveis elevados do agente ou agentes em questão. Já os efeitos crônicos são resultantes de exposições repetidas e a doses relativamente baixas de uma ou mais substâncias. Podem se tornar evidentes desde dias até anos após o período de exposição e, por isso mesmo, mais difíceis de serem relacionados ao agente tóxico.

Enquanto que os efeitos agudos da exposição a pesticidas são bem conhecidos (Reigart and Roberts 1999; Ecobichon 2001b), efeitos crônicos como câncer, distúrbios reprodutivos, genotoxicidade, alterações no sistema imunológico e distúrbios neurocomportamentais ainda representam um desafio, mesmo para países desenvolvidos (Ecobichon 2001a; Ecobichon 2001b).

No Brasil, o cenário não é diferente. A avaliação dos níveis de intoxicação aguda de diferentes populações, em sua grande maioria agricultores, tem sido um dos aspectos mais estudados pelos grupos que se dedicam a este tema (Carvalho et al. 1990; Gonzaga and Santos 1992; Oliveira-Silva et al. 2001; Moreira et al. 2002; Delgado and Paumgartten 2004; Faria et al. 2004). Entretanto, já é possível perceber algumas iniciativas em se avaliar de que forma o perfil de morbi-mortalidade de uma série de doenças crônicas no Brasil pode ser afetado pela exposição continuada a baixas doses de pesticidas. Meyer e colaboradores (Meyer et al. 2003a) demonstraram que a mortalidade por alguns tipos específicos de câncer foi maior entre agricultores da região serrana do estado do Rio de Janeiro do que em algumas populações de referência. Alguns tipos específicos de câncer, bem como alguns distúrbios reprodutivos, também apresentaram correlação positiva com o consumo de pesticidas no Brasil

(Koifman et al. 2002). Além disso, uma elevada prevalência de distúrbios psiquiátricos menores foi observada em agricultores da região sul país (Faria et al. 1999). Outro tema que vem freqüentando a agenda da saúde pública brasileira é a controvertida hipótese da desregulação endócrina ambiental, através da qual uma série de poluentes ambientais, dentre os quais diversos pesticidas, poderiam alterar a homeostase do sistema endócrino (Safe 2004; Sharpe and Irvine 2004). Este tema, cujo debate se acentuou, com grande espaço na literatura científica internacional desde meados da década de 90, começa a aparecer também em publicações brasileiras (Meyer et al. 1999; Koifman and Paumgartten 2002; Meyer et al. 2003b).

Grande também tem sido a preocupação sobre como a exposição a agentes químicos, em especial pesticidas, afeta o desenvolvimento pré- e pós-natal e que conseqüências as alterações ocorridas neste período podem ter na vida adulta, especialmente no que diz respeito ao desenvolvimento do sistema nervoso (SN) (Rodier 1994; Rodier 1995; Landrigan et al. 1999; Slotkin 1999; Barone et al. 2000; Eriksson and Talts 2000; Weiss 2000; Berkowitz et al. 2003; Bowers et al. 2004; Rodier 2004; Slotkin 2004a).

1.3 - O Sistema Nervoso em Desenvolvimento é Mais Vulnerável à Ação de Agentes Químicos

O SN em adultos é comumente visto como um dos sistemas mais bem protegidos do corpo humano. Ele é fisicamente protegido contra choques e traumas pelos ossos da caixa craniana, e a barreira hemato-encefálica (BHE), com sua permeabilidade seletiva, se encarrega de evitar que compostos químicos atinjam de forma aleatória o cérebro. Tanto é assim, que os principais alvos da toxicidade de agentes químicos são tecidos e órgãos que mantém contato direto com o meio externo, como o sistema respiratório, o trato gastrintestinal e a pele, além de órgãos internos com grande exposição a xenobióticos presentes na corrente sanguínea, como fígado e rins (Rodier 1995).

Entretanto, durante o período de desenvolvimento, o SN é particularmente vulnerável à ação tóxica de diversos agentes químicos (Rodier 1994; Rodier 1995; May 2000; Rice and Barone 2000; Costa et al. 2004). A lista de xenobióticos que afetam preferencialmente o cérebro em

desenvolvimento é extensa e inclui compostos bastante conhecidos como metilmercúrio (Choi 1989; Myers et al. 1998), chumbo (Davis and Svendsgaard 1987; Gomaa et al. 2002; Canfield et al. 2003), álcool (Streissguth et al. 1980a; Streissguth et al. 1980b; Spohr et al. 1993), bifenilas policloradas (Jacobson et al. 1990; Seegal and Shain 1992; Chen and Hsu 1994; May 2000; Landrigan 2001; Winneke et al. 2002), além de diversos agrotóxicos (Thiruchelvam et al. 2002; Bowers et al. 2004; Slotkin 2004a).

Não é verdade, entretanto, que todo órgão imaturo seja mais vulnerável à ação de substâncias químicas. O lítio e o mercúrio inorgânico, por exemplo, são dois metais que causam danos aos rins, primordialmente após a função de filtração renal se tornar efetiva (Christensen et al. 1982; Daston et al. 1983). Assim, devem existir outros fatores que concorrem para a vulnerabilidade do cérebro em desenvolvimento. Sem dúvida, um aspecto importante e diferencial é a formação da BHE, que em seres humanos, não estará completamente formada até os 6 meses de idade (Adinolfi 1985; Engelhardt 2003). Contudo, a ausência de uma BHE completamente formada também não explica a vulnerabilidade do cérebro em desenvolvimento. Mesmo alguns compostos que atravessam livremente a BHE, e que, portanto, têm livre acesso ao cérebro adulto, são mais tóxicos ao cérebro em desenvolvimento (Rodier 1994). Ainda mais marcante é o fato de agentes que nem são transportados pelo sangue, como por exemplo a radiação ionizante, que não encontra na BHE nenhum obstáculo para atingir livremente o cérebro e ainda assim é mais danosa durante o desenvolvimento (Schull et al. 1990).

Aparentemente, existe uma combinação de fatores que são inerentes ao próprio processo de desenvolvimento do SN que o torna particularmente vulnerável. Assim, o conhecimento dos processos biológicos que ocorrem durante esse período é fundamental para entendermos tal vulnerabilidade.

1.4 - Processos Vulneráveis Durante o Desenvolvimento do Sistema Nervoso

O desenvolvimento do cérebro é o resultado de uma série de eventos coordenados, que ocorrem em diferentes momentos e em diferentes regiões cerebrais, seguindo um estrito cronograma biológico (Barone et al.

2000; Rice and Barone 2000). É justamente a perturbação deste cronograma e destes processos, que incluem, por exemplo, proliferação, migração, diferenciação e morte celulares, além da formação da BHE que torna o cérebro em desenvolvimento particularmente susceptível à ação de poluentes ambientais (Rodier 1995; Costa et al. 2004).

1.4.1 – Proliferação Neuronal

A proliferação de precursores neuronais é um dos processos funcionais envolvidos no desenvolvimento do cérebro. A geração de novos neurônios (neurogênese), ocorre no cérebro, durante o desenvolvimento, mesmo antes do fechamento do tubo neural, e continua após o nascimento em uma grande variedade de mamíferos, inclusive no homem (Alvarez-Buylla et al. 2001; Kintner 2002).

Ao contrário das células de diversos outros órgãos, onde somente alguns poucos tipos celulares são replicados a exaustão ao longo de toda a vida, o Sistema Nervoso Central (SNC) é composto de uma grande variedade de tipos celulares. De uma forma geral, neurônios com morfologia e função similares são gerados em um curto período de tempo. Em muitas estruturas do SNC, a proliferação celular gera um número de neurônios muito maior do que será necessário na vida adulta. Assim, o período de proliferação é seguido por uma onda de morte celular que irá estabelecer o número final apropriado de neurônios (veja abaixo no tópico 1.4.5).

1.4.2 Migração Neuronal

A posição que um neurônio ocupa no cérebro nem sempre coincide com o local onde o mesmo foi gerado. Faz parte do processo natural de desenvolvimento do cérebro a migração de neurônios de seu "lugar de nascimento", as zonas germinativas, até as posições que ocuparão definitivamente no cérebro, onde desempenharão suas funções específicas (Nadarajah et al. 2001; Hatten 2002). Em estruturas como o córtex cerebral, onde as células são geradas na superfície interna do tubo neural e movem-se então para a superfície externa, a distância percorrida pode ser substancial (Nadarajah et al. 2001; Rao and Wu 2001).

Embora a migração neuronal ocorra durante todo o desenvolvimento do SN, este processo foi mais bem estudado no cerebelo e no córtex cerebral (Nadarajah and Parnavelas 2002). Duas modalidades principais de migração foram identificadas até então: a migração radial e a migração tangencial. A migração radial é a modalidade mais estudada e a principal forma observada no córtex cerebral em desenvolvimento (Hatten 1999). Sua principal característica é que os neurônios, gerados nas zonas proliferativas, movem-se para a superfície do cérebro, em sentido perpendicular, ao longo de fibras de glia radial. Já na migração tangencial, o movimento dos neurônios se dá paralelo à superfície do cérebro ao longo dos axônios ou mesmo de outros neurônios e freqüentemente ultrapassa limites regionais. Um exemplo desta modalidade de migração é o movimento de interneurônios corticais a partir de sua origem no telencéfalo ventral para o córtex cerebral em desenvolvimento (Marin and Rubenstein 2001)

A migração celular não ocorre a uma taxa constante durante todo o desenvolvimento. Assim como na proliferação neuronal, a migração ocorre em ondas associadas aos diferentes tipos celulares. A maior parte da migração neuronal ocorre ainda no período de gestação, quando as distâncias dentro do cérebro são pequenas. As longas migrações de células pequenas do córtex cerebral, do hipocampo, e do cerebelo continuam por vários meses após o nascimento (Hatten 1999).

1.4.3 – Diferenciação Neuronal

A diferenciação de neuroblastos pode ser definida como o processo de expressão dedicada a um fenótipo final. Mesmo que a proliferação, a migração, e a diferenciação sejam investigadas e aqui discutidas separadamente, a fase inicial do processo de diferenciação começa assim que os precursores neuronais terminam sua última divisão e estão prontos para a migração para a placa cortical, num processo dinâmico e continuado. Enquanto os neurônios migram, há um constante processo de troca de informações entre o meio extra- e o meio intracelular, que inicia a expressão de genes específicos que influenciarão o fenótipo neuronal ou glial. As características fenotípicas tais como a forma, o tamanho, a

polaridade, e a expressão de neurotransmissores e receptores, permitem a distinção entre os diversos tipos neuronais e gliais.

1.4.4 – Conexões, neurotransmissores e receptores.

De forma a exercer sua função primordial de condução do impulso nervoso, os neurônios precisam estabelecer conexões com outras células. Isto requer o desenvolvimento de estruturas especializadas, como os dendritos e axônios. O ponto de contato entre estas estruturas é a sinapse e o processo de formação destas sinapses, ou sinaptogênese, consiste em mudanças morfológicas e bioquímicas tanto no neurônio pré-sináptico como no neurônio pós-sináptico (Haydon and Drapeau 1995; Munno and Syed 2003). Dentre as diversas mudanças observadas, tem especial relevância a formação e interação entre neurotransmissores e seus receptores específicos ainda durante os estágios iniciais do desenvolvimento do SNC (Spencer et al. 2000; Lovell et al. 2002). Acredita-se que esta comunicação precoce entre neurotransmissores e receptores desempenhe um papel distinto daquele que se conhece durante a vida adulta de um indivíduo, que é o de controlar as funções corpóreas vitais através da propagação do impulso nervoso. Esta interação, durante o desenvolvimento, teria o papel de modular o próprio processo de desenvolvimento (Lauder 1993; Cameron et al. 1998; Nguyen et al. 2001; Herlenius and Lagercrantz 2004). Por exemplo, sabe-se que o ácido gama aminobutírico (GABA), o principal neurotransmissor inibitório (Petroff 2002) e o glutamato, principal neurotransmissor excitatório (Petroff 2002) presentes no SNC agem como agentes quimiotáticos para neurônios corticais em migração (Behar et al. 1998; Behar et al. 1999; Owens and Kriegstein 2002).

1.4.5 – Morte celular programada durante o desenvolvimento

Acredita-se que metade dos neurônios produzidos durante o processo de proliferação celular seja ativamente eliminado, durante momentos específicos do desenvolvimento cerebral, por um processo de morte celular programada denominado apoptose (Henderson 1996; Meier et al. 2000; Nijhawan et al. 2000). Importantes avanços têm sido feitos em compreender de que forma as células morrem no curso normal do

desenvolvimento do SNC. Sabe-se que fatores importantes são o número e o tipo de conexão que um neurônio faz durante seu período de sinaptogênese. Assim, o objetivo deste processo de remoção agressiva e extremante eficiente seria produzir um número otimizado de neurônios bem conectados. A apoptose pode ser diferenciada da necrose, outra categoria de morte celular, por características específicas que incluem a manutenção da membrana celular, a condensação de cromatina, redução do volume celular e a ausência de um processo inflamatório (Rice and Barone 2000).

1.4.6 – Gliogênese e mielinização

O termo glia, evidência a pouca importância que este conjunto de diferentes tipos celulares recebeu dos primeiros microscopistas. Glia é uma palavra derivada do termo grego "gliok" que é comumente utilizado para designar cola (Nedergaard et al. 2003). Assim, o termo reflete bem o conceito que perdurou durante quase todo o século XX de que a essas células cabia um papel secundário de meros coadjuvantes, preenchendo os espaços entre seus parceiros mais famosos do SN, os neurônios. Entretanto, numerosos estudos vêm contribuindo para mudar este conceito, atribuindo às células de glia um papel de destaque no funcionamento, adoecimento e, principalmente, desenvolvimento do SN (Nagler et al. 2001; Ullian et al. 2001; Campbell and Gotz 2002a; Kretzschmar and Pflugfelder 2002; Pfrieger 2002; Stevens 2003). Glia, que no cérebro humano excede o número de neurônios em cerca de dez vezes, na verdade são um conjunto de diferentes tipos celulares que incluem oligodendrócitos, células de Schwann, microglia, astrócitos e glia radial (Rodier 1995; Stevens 2003). De forma bem resumida, aos oligodendrócitos e às células de schwann cabe a mielinização axonal do SNC e periférico, respectivamente. Já os astrócitos, que também compõem a berreira hematencefáfica, são responsáveis pela manutenção do equilíbrio iônico e trófico no ambiente extracelular e na regulação da própria sinaptogênese (Slezak and Pfrieger 2003). Microglia é considerada a célula análoga aos macrófagos no SNC (van Rossum and Hanisch 2004), buscando microrganismos invasores e células mortas e sinalizando para estimulação da resposta imune. Por fim, mas não menos importante, temos

as células de glia radial, que participam ativamente do processo de migração radial de neurônios recém-formados, das zonas ventriculares para as zonas mais externas, durante o período de desenvolvimento do SNC (Campbell and Gotz 2002b). Existem evidências importantes que apontam para o fato de as células de glia radial serem precursores neurais (Parnavelas and Nadarajah 2001).

1.4.7 - Formação da Barreira Hematencefálica (BHE)

Nos primeiros estudos sobre o tema, observou-se que a injeção do composto hidrofílico azul de Tripam não se difundia de fora para dentro ou de dentro para fora do cérebro (de Boer et al. 2003; Ballabh et al. 2004). Hoje sabe-se que os capilares cerebrais exercem um controle estrito do influxo e efluxo de substâncias biologicamente ativas através de uma série de mecanismos. Alguns nutrientes como, por exemplo, a glicose e os aminoácidos entram no cérebro utilizando-se de transportadores especializados, já moléculas maiores como a insulina e a leptina recorrem a endocitose mediada por receptores. Moléculas lipofílicas pequenas como O_2 e CO_2 difundem-se livremente pela membrana plasmática, o que não ocorre com facilidade para substâncias hidrofílicas (Ballabh et al. 2004).

Assim, a formação da BHE é sempre um ponto importante a se considerar quando o assunto é vulnerabilidade no SNC em desenvolvimento. A ausência de tal barreira durante o desenvolvimento permite que, uma vez absorvidos por um organismo, poluentes ambientais tenham livre acesso ao cérebro exatamente durante o período em que o desenvolvimento do encéfalo está em pleno curso.

1.5 - Alguns Agentes Químicos e Suas Ações Sobre o Desenvolvimento do Sistema Nervoso

Antes de uma breve descrição da ação de alguns agentes tóxicos sobre o cérebro imaturo, é importante abordar um aspecto essencial sobre a neurotoxicidade durante o desenvolvimento. Uma vez que os processos que ocorrem durante esse período obedecem a um "cronograma" específico, exposições em diferentes períodos do desenvolvimento irão afetar os processos em curso e as estruturas em formação naquele período

específico, que é o que se convencionou chamar de janelas de vulnerabilidade ou períodos críticos (Rodier 1994; Rodier 1995; Rice and Barone 2000). A figura 4 ilustra este conceito. Nela é possível observar o perfil temporal de proliferação neuronal (hachuradas em azul) em três diferentes regiões cerebrais de roedores. O hipocampo, no topo, tem o seu primeiro pico de proliferação celular no período médio de gestação, quando se formam basicamente células maiores (células piramidais). Já as células menores, do giro denteado, formam-se basicamente no período neonatal. Situação semelhante pode ser observada no cerebelo (na base da figura), onde nota-se um primeiro pico, ou onda, de proliferação celular referente às células de Purkinje ocorrendo em torno do 12º dia de gestação (GD), portanto, no período médio da gestação de ratos (período total de gestação de 21 dias). Assim como no hipocampo, é possível também observar um segundo período de importante proliferação celular no cerebelo no período pós-natal. O terceiro perfil de proliferação neuronal representado na Figura 1 diz respeito a um conjunto bastante específico de células do hipotálamo, os neurônios que sintetizam e secretam o hormônio de liberação do hormônio luteinizante (LHRH, no centro da figura). É possível notar que a formação destes neurônios ocorre em um período bastante restrito da gestação. Assim, ao expor ratos a agentes com reconhecida ação sobre a proliferação celular em GD15 (linha lilás semitransparente) observa-se que estes animais não produzem a quantidade normal de neurônios piramidais do hipocampo, com conseqüente repercussão sobre as funções de memória e aprendizado (Rodier et al. 1975), mas aparentemente sem prejuízos a produção das células de Purkinje, no cerebelo, e dos neurônios do hipotálamo que liberam LHRH. Entretanto, se anteciparmos ligeiramente o período de exposição (linha verde semitransparente), o que se observa são alterações especificamente relacionadas às funções do cerebelo (Rodier 1986), mas a produção de neurônios que expressam LHRH também será afetada, como demonstrado por Gavin e cols (Gavin et al. 1994).

Este exemplo demonstra a importância do tempo de exposição para os estudos sobre neurotoxicidade de um agente durante o desenvolvimento, além de esclarecer como um mesmo agente tóxico pode produzir efeitos diferenciados, ou mesmo não produzir nenhum efeito, sobre o cérebro em formação.

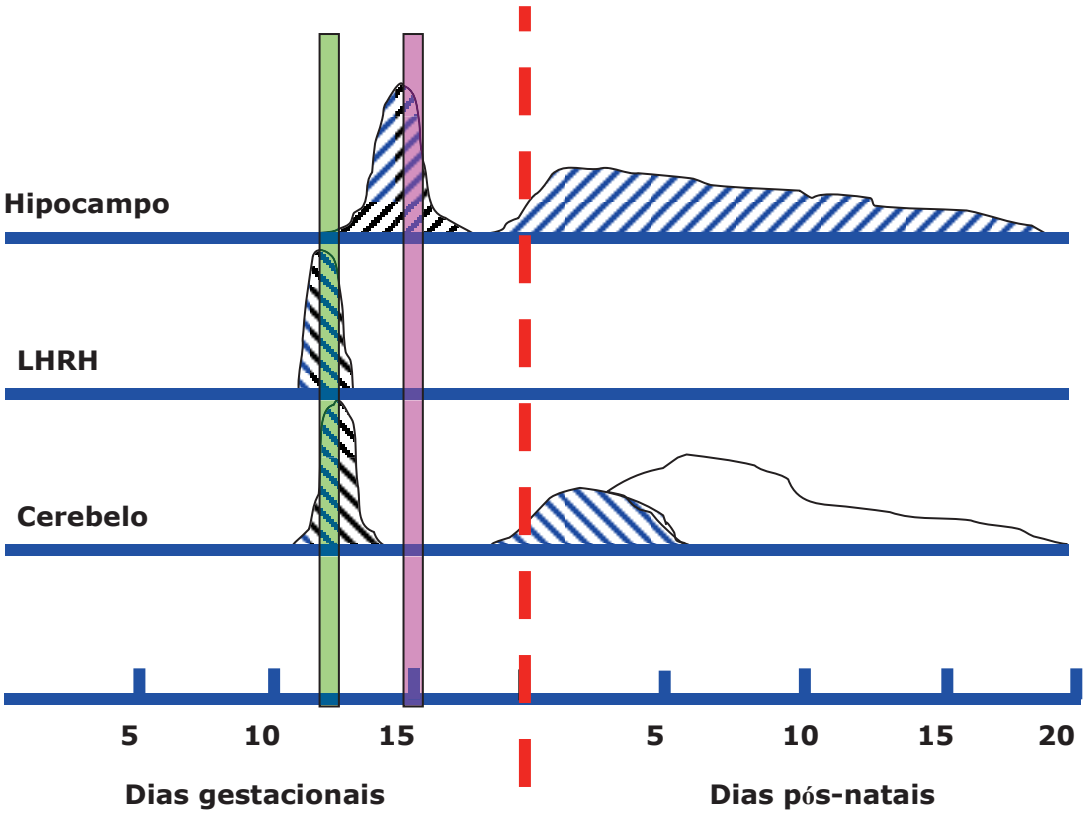

Figura 4 – Período de proliferação de alguns tipos neuronais em três regiões cerebrais. Adaptado de Rodier (Rodier 1994).

A literatura científica está repleta de exemplos de compostos químicos que exercem sua neurotoxicidade preferencialmente sobre o cérebro em desenvolvimento, ou ainda, que agem por mecanismos diferenciados em relação ao cérebro maduro e imaturo (Slotkin 1999; Andersen et al. 2000; Landrigan 2001; Costa et al. 2004; Rodier 2004; Slotkin 2004a). Dentre os metais pesados, por exemplo, sabe-se que o chumbo altera a diferenciação neuronal e a sinaptogênese, mas parece exercer um efeito mais intenso sobre estágios mais avançados do desenvolvimento, provavelmente interferindo com a etapa de remoção/otimização das conexões sinápticas formadas em excesso, além de induzir apoptose neuronal (Crumpton et al. 2001; Deng et al. 2001; Costa et al. 2004). O chumbo também promove significativa redução na formação de mielina, retardando a diferenciação de oligodendrócitos (Deng et al. 2001; Deng and Poretz 2003) e tem a formação da BHE como um dos processos particularmente afetados durante o desenvolvimento do SNC (Zheng et al. 2003). Além de um acúmulo

expressivo de evidências em estudos animais acerca da neurotoxicidade do chumbo durante o desenvolvimento cerebral, estudos epidemiológicos também têm contribuído de forma significativa nesse sentido. Para se ter uma idéia da importância desses estudos, seus resultados contribuíram para que, somente nos últimos 50 anos, o Centro de Controle de Doenças dos Estados Unidos diminuíssem por 4 vezes o nível de chumbo em sangue que se considera "seguro", que caiu de 60 para atuais 10 μg/dl (Rogan and Ware 2003). Do ponto vista da epidemiologia, existe uma grande discussão na literatura científica atual sobre os possíveis efeitos de exposições a baixíssimas doses de chumbo sobre o desenvolvimento cognitivo de crianças (Kaufman 2001b; Kaufman 2001a; Nation and Gleaves 2001; Needleman and Bellinger 2001; Rogan and Ware 2003). Embora ainda encontrem alguma resistência (Kaufman 2001b; Kaufman 2001a), inúmeros estudos têm demonstrado que existe uma correlação negativa entre os níveis de chumbo em sangue e o quociente de inteligência em crianças de diversas idades (Bellinger and Dietrich 1994; Pocock et al. 1994; Tong et al. 1996; Wasserman et al. 1997), inclusive com níveis de chumbo em sangue abaixo de 10 μg/dl (Canfield et al. 2003; Emory et al. 2003; Bellinger 2004; Koller et al. 2004). Outro metal bastante estudado sob o ponto de vista de sua neurotoxicidade durante o desenvolvimento é o mercúrio (Myers et al. 1998; Costa et al. 2004). O ser humano pode se expor tanto à formas orgânicas como inorgânicas de mercúrio. Diferente dos adultos, cuja exposição ao mercúrio inorgânico (mercúrio elementar e vapor de mercúrio) pode ocorrer com relativa freqüência em determinadas atividades profissionais, crianças são mais freqüentemente expostas ao metilmercúrio, forma orgânica do mercúrio cuja fonte mais comum é o consumo de peixes (Counter and Buchanan 2004). Entretanto, exposições materno-infantis ao mercúrio inorgânico podem ocorrem em situações especiais como em populações circunvizinhas à atividade garimpeira de ouro, mas principalmente através de amálgamas dentários (Davidson et al. 2004). Grande parte do que se conhece acerca dos efeitos da exposição ao mercúrio sobre o cérebro em desenvolvimento em populações humanas se deve à estudos que sucederam grandes desastres ambientais envolvendo esta substância, como aqueles ocorridos em Minamata e Niigata, no Japão (Weiss et al. 2002; Davidson et al. 2004) e na área rural do Iraque (Amin-Zaki et al. 1974; Myers et al. 2000; Counter and Buchanan 2004). Em

ambos os casos foi possível observar efeitos sobre o sistema nervoso de crianças expostas ainda durante a gestação ou no período neonatal, que apresentaram diferenças marcantes em relação aos efeitos observados em populações adultas. Enquanto que em adultos, os danos foram mais evidentes em áreas cerebrais específicas, como a camada granular do cerebelo e no córtex visual; em crianças, os danos foram mais difusos e quanto mais cedo a exposição, mais generalizados foram esses danos (Choi 1989). Assim como no caso do chumbo, a exposição ao mercúrio altera diversos dos processos que ocorrem durante o período de desenvolvimento do SNC. Desde a proliferação (Faustman et al. 2002), a migração (Kunimoto and Suzuki 1997) e a diferenciação neuronal (Parran et al. 2003), além de induzir apoptose (Kunimoto M. 1994; Kunimoto and Suzuki 1997) e alterar o processo de formação da BHE (Bertossi et al. 2004). Além dos dois exemplos descritos acima, diversos outros metais produzem efeitos neurotóxicos sobre o cérebro em desenvolvimento. Por exemplo, o manganês, metal essencial ao metabolismo humano, mas que em excesso ou déficit pode causar sérios danos ao SNC (May 2000; Takser et al. 2003).

Além dos metais, diversos outros compostos podem interferir com o desenvolvimento normal do SN. Alguns poluentes orgânico-persistentes (POP), como as bifenilas policloradas (PCB), por exemplo, são amplamente estudas quanto a sua neurotoxicidade ante a exposições pré- e neonatais (Tilson et al. 1990; Morse et al. 1993; Huisman et al. 1995; Landrigan 2001; Winneke et al. 2002; Bowers et al. 2004). Ainda entre os POP, sabe-se também que as bifenilas polibromadas (PBB) (Eriksson et al. 2001; Branchi et al. 2003; Branchi et al. 2004), as dioxinas (Golub and Jacobson 1995; Porterfield 2000; Hill et al. 2003; Williamson et al. 2005) e diversos pesticidas organoclorados (Eriksson 1997; Massol et al. 2000; Buznikov et al. 2001; Moser et al. 2001; Bowers et al. 2004) também podem interferir no curso normal do desenvolvimento cerebral.

No caso dos organoclorados, é interessante notar que este grupo químico é quase que exclusivamente composto de inseticidas, que são agentes neurotóxicos por natureza (Ecobichon 2001b). Assim, não surpreende a extensa lista de pesticidas com ação neurotóxica também durante o desenvolvimento (Andersen et al. 2000). Neste sentido, os pesticidas organofosforados têm merecido grande destaque na literatura

científica (Carr et al. 1999; Pope 1999; Ray and Richards 2001; Slotkin 2004b; Slotkin 2004a).

1.6 - O Inseticida Clorpirifós

Dentre os organofosforados, o composto que mais tem sido estudado, sob a perspectiva da neurotoxicidade durante o desenvolvimento, é sem dúvida o inseticida clorpirifós (CPF; Figura 2) (National Research Council 1993; Physicians for Social Responsibility 1995; Campbell et al. 1997; Landrigan et al. 1999; Pope 1999; Slotkin 1999; May 2000; Abdel-Rahman et al. 2002; Slotkin 2004a; Slotkin 2004b). O organofosforado CPF, *0,0-diethyl 0-(3,5,6-trichloro-2-pyridyl) phosphorothioate*, tem sido largamente utilizado tanto no meio urbano, no controle de baratas, cupins e outros insetos domésticos, como no meio rural para controle de carrapatos em rebanhos e diversas pragas na agricultura (ATSDR 1997). Seu uso em grande escala deve-se principalmente ao fato de ser relativamente menos tóxico e mais persistente que alguns de seus antecessores consagrados no mercado, como o paration. Além disso, CPF não induz a síndrome de neurotoxicidade tardia, comum a outros fosforados, salvo se a dose for superior à DL_{50} (Richardson et al. 1993).

Como a maioria dos organofosforados, CPF pode inibir a atividade da enzima acetilcolinesterase (AChE), presente nas fendas sinápticas e junções neuromusculares. Sendo CPF um membro da subclasse dos fosforotioatos, essa inibição só ocorre de forma eficaz após sua biotransformação em seu oxon análogo, o clorpirifós-oxon (CPF-oxon) (Jeyaratnam and Maroni 1994). Com a inibição da AChE, enzima responsável pela degradação do neurotransmissor acetilcolina (ACh), ocorre o acúmulo de ACh na fenda sináptica, com conseqüente estimulação excessiva dos receptores colinérgicos nicotínicos e muscarínicos. Assim, exposições agudas, a altas doses de organofosforados, podem resultar em sintomas relacionados aos efeitos muscarínicos como aumento da sudorese, salivação e lacrimejação, broncoconstricção, espasmos abdominais com vômito, diarréia e bradicardia. Mas também sintomas relacionados aos efeitos nicotínicos como taquicardia, fasciculação muscular e em casos mais severos, parada respiratória. Por fim, ainda é possível observar sintomas que ficam restritos ao nível do sistema nervoso central,

como dores de cabeça, ansiedade, confusão, convulsões e até o coma (Sultatos 1994; Jamal 1997).

O mecanismo de ação descrito acima tem sido o paradigma que norteou grande parte das pesquisas sobre os efeitos causados pela exposição aos pesticidas organofosforados de uma forma geral. Entretanto, diversos estudos, publicados principalmente ao longo da década de 90, apontaram importantes lacunas nesse modelo, colocando em dúvida se este mecanismo de ação seria capaz de explicar toda a gama de efeitos biológicos observados face à exposição aos organofosforados, lacunas essas que se tornaram mais evidentes em exposições ocorridas durante o desenvolvimento do SN (Slotkin 1999).

Estas questões ganham singular importância, um vez que, segundo diversos estudos, o CPF está largamente difundido nos meios rural e urbano e a exposição/contaminação de gestantes e crianças apresenta níveis alarmantes (Fenske et al. 1990; Gurunathan et al. 1998; Lemus and Abdelghani 2000; Adgate et al. 2001; Fenske et al. 2002). Para se ter uma idéia, em estudo recente sobre o nível de exposição infantil a diversos pesticidas em uma determinada região dos EUA, 93% das crianças com idades entre 3 e 13 anos apresentaram níveis entre 1,4 e 9,2 μg/L de 3,5,6-trichloro-2-pyridinol (tricloropiridinol (TCP) - o produto final do metabolismo do CPF) na urina (Adgate et al. 2001). Em outro estudo realizado também nos EUA, Fenske e colaboradores (Fenske et al. 2002)avaliaram os níveis de CPF e paration na poeira de residências de uma região agrícola. Enquanto que 41% das residências apresentaram resíduos do metabólito do paration, 100% das amostras foram positivas para o metabólito do CPF. O CPF também foi encontrado, junto com outros agentes tóxicos, no mecônio de recém nascidos (Ostrea et al. 2002). Esta amostra biológica tem sido utilizada com grande interesse nos estudos de avaliação da exposição materno-infantil a agentes químicos, pois além de ser uma amostra não invasiva, retrata a exposição do feto por intermédio da mãe ao longo da gravidez (Ostrea et al. 1994; Ostrea 2001; Whyatt and Barr 2001).

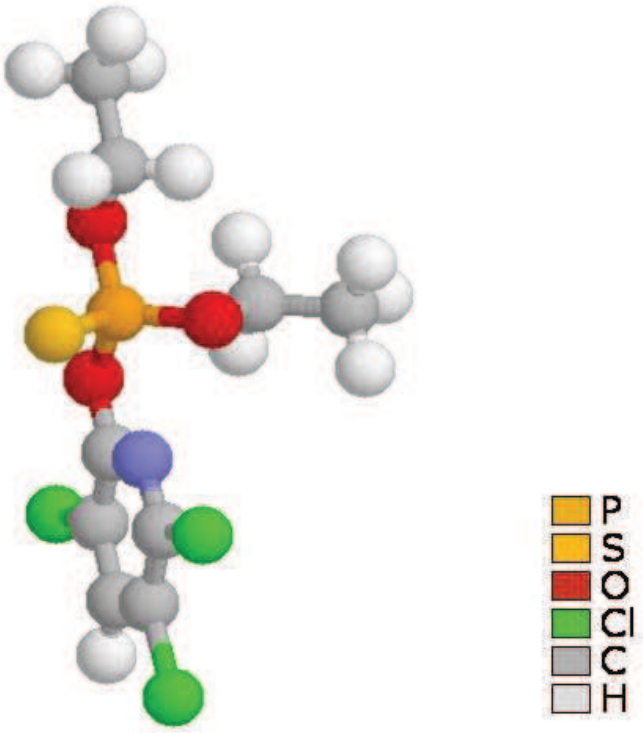

Figura 5 – Representação, em três dimensões, da molécula de CPF.

1.7 - Os Efeitos do Clorpirifós Sobre o Desenvolvimento do Sistema Nervoso Não São Mediados Exclusivamente Pela Inibição da AChE

Das e Barone (Das and Barone 1999), por exemplo, demonstraram que expor células PC12, em meio de cultura, à baixas doses de CPF, CPF-oxon e TCP inibe o crescimento de neuritos, que é um marcador morfológico clássico de diferenciação neuronal. As células PC12 são obtidas, em ratos, a partir de um tumor da glândula suprarenal, denominado feocromocitoma. São consideradas células neuronotípicas, pois além de apresentarem catecolaminas, a estimulação delas com fator de crescimento neural (NGF) resulta em crescimento de neuritos, que podem até formar sinapses colinérgicas (Fujita et al. 1989). Neste caso é importante notar que a capacidade do CPF-oxon de inibir a diferenciação neuronal em células PC12 pode estar relacionada à sua habilidade de inibir a atividade da AChE, uma vez que esta enzima parece participar ativamente do processo de diferenciação (Small et al. 1996; Koenigsberger et al. 1997; Brimijoin and Koenigsberger 1999). Entretanto, como explicar a capacidade do CPF e TCP

em inibir o crescimento de neuritos em doses que não provocam nenhuma inibição colinesterásica (Das and Barone 1999)?

CPF também altera a proliferação neuronal, como evidenciado através da inibição da síntese de DNA. Ratos expostos, por via subcutânea, durante o período neonatal, a doses de clorpirifós que não causam dano sistêmico, apresentaram importante redução da síntese de DNA e proteína. Além disso, a injeção direta de CPF no cérebro, minimizando assim o metabolismo hepático e conseqüentemente a produção de CPF-oxon, produziu resultados semelhantes (Whitney et al. 1995). Este resultado aponta fortemente para o fato de que é o próprio CPF, um inibidor muito menos potente da AChE quando comparado com o seu metabólito ativo CPF-oxon, o principal responsável pela inibição da síntese de DNA. Mais uma evidência importante sobre efeito do CPF sobre a síntese de DNA é a de que células PC12 e C6, que são células de glioma e, portanto também chamadas de gliotípicas, expostas a CPF, CPF-oxon ou TCP apresentaram uma robusta inibição da síntese de DNA. Neste caso, os autores demonstraram que o CPF é um inibidor mais potente da síntese de DNA que seu metabólito anticolinesterásico CPF-oxon, tanto em células PC12 como em células C6, apontando mais uma vez para um possível mecanismo não-colinérgico de distúrbio dos processos de desenvolvimento cerebral (Qiao et al. 2001).

Outro desses processos afetados pelo CPF, em que o mecanismo de ação parece não ser exclusivamente colinérgico, é a sinaptogênese (Dam et al. 1999). Neste estudo, ratos foram expostos a doses de CPF que não causam dano sistêmico durante dois períodos neonatais distintos, dia pós-natal (PN) 1 a 4 e PN11 a 14, e foram avaliados marcadores responsivos e constitutivos para sinapses colinérgicas e catecolaminérgicas em três regiões cerebrais (prosencéfalo, tronco e cerebelo). Os marcadores utilizados no estudo foram a colina acetiltransferase, a enzima responsável pela biossíntese de ACh, que é um excelente marcador para inervação colinérgica, mas cuja atividade não responde adequadamente a mudanças na atividade colinérgica. Foi medida a recaptação de colina, através da ligação ao hemicolinium-3 ao transportador pré-sináptico, que é o marcador mais indicado para este fim. Para a avaliação da inervação e atividade catecolaminérgicas foram utilizados os níveis das catecolaminas

(dopamina e norepinefrina) e seu "turnover", respectivamente. A estratégia de se usar regiões distintas, permitiu testar indiretamente a hipótese de que tais alterações poderiam ser mediadas pelo mecanismo de inibição da AChE. Isso por que estas três regiões apresentam inervação colinérgica diferenciada; prosencéfalo e tronco desenvolvem rica inervação colinérgica, mas o cerebelo não. Tanto os sistemas colinérgico quanto catecolaminérgico apresentaram alterações na sinaptogênese. Porém, talvez ainda mais importante seja o fato de que tais alterações tenham ocorrido tanto no prosencéfalo e tronco, regiões ricas em inervações colinérgicas, quanto no cerebelo, região sem inervação colinérgica importante (Dam et al. 1999).

A lista de evidências que apontam para a inadequação do mecanismo de inibição da acetilcolinesterase como um modelo que explique todos os efeitos da exposição a organofosforados, principalmente durante o desenvolvimento, é bem mais extensa do que os exemplos apresentados acima e é objeto de três excelentes revisões (Pope 1999; Slotkin 1999; Barone et al. 2000). Tais evidências têm despertado a atenção da comunidade científica e diversos estudos vêm sendo realizados com o objetivo de buscar novos alvos biológicos ou mesmo novos mecanismos pelos quais o clorpirifós e outros organofosforados exerçam sua neurotoxicidade durante o desenvolvimento.

1.8 - AMP Cíclico: Um Possível Alvo da Ação do Clorpirifós e Diversos Outros Agentes Tóxicos

Um dos alvos biológicos mais proeminentes e que tem recebido grande atenção por parte da comunidade científica é a adenosina 3', 5'-monofosfato cíclico (AMPc). O AMPc é um segundo mensageiro, cujo mecanismo de atuação é uma das vias bioquímicas mais bem estudas no mundo (para uma revisão detalhada, leia Shaywitz and Greenberg 1999; Hanoune and Defer 2001; Chin et al. 2002; Stachowiak et al. 2003). Na figura 6, podemos ver um diagrama resumido da via bioquímica do AMPc. Essencialmente, sua indispensável participação nos processos de crescimento e diferenciação celulares, apoptose, além da regulação do metabolismo e da expressão de diversos genes e principalmente seu papel crítico em coordenar a transição entre a proliferação e a diferenciação

celular em virtualmente todas as células eucarióticas e procarióticas (Guidotti 1972; Van Wijk et al. 1973; Claycomb 1976; Bhat et al. 1983; Hultgårdh-Nilsson et al. 1994), confere a essa molécula um papel bastante importante e diferenciado durante o período de desenvolvimento e portanto um alvo potencial de agentes neurotóxicos.

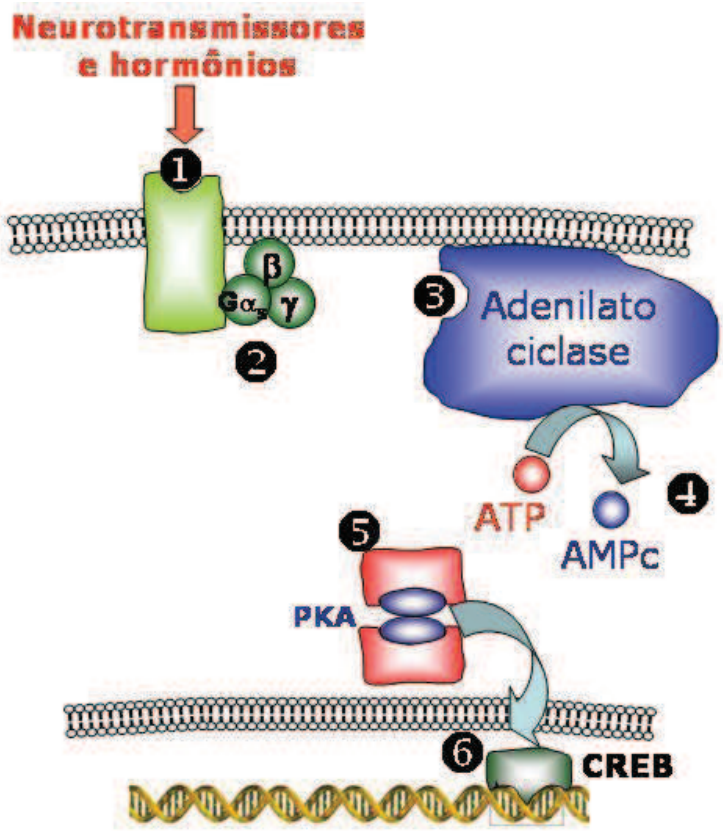

Figura 6 – Ativação do CREB: um fator de transcrição responsivo ao aumento de AMPc intracelular. A ligação de neurotransmissores e hormônios a seus respectivos receptores ❶, ativa isoformas específicas de proteínas G ❷, que por sua vez vão acoplar-se à enzima adenilato ciclase ❸. A ativação desta enzima irá catalisar a produção de AMPc, a partir de ATP ❹. Os efeitos do AMPc são mediados pela fosforilação de seu receptor, a proteína quinase A (PKA) ❺. A PKA, que é formada de duas ades catalíticas (PKAc) e duas subunidades regulatórias (PKAr), permanece inativa sem a ligação ao AMPc. Entretanto, quando os níveis intracelulares de AMPc se elevam, quatro destas moléculas se ligam as duas subunidades PKAr e liberam as 2 subunidades PKAc. PKAc por sua vez, é capaz de fosforilar uma série de proteínas intracelulares, tanto no citoplasma quanto no compartimento nuclear. Como exemplo, dentre essas proteínas alvo da fosforilação da PKA, temos o fator de transcrição CREB (cAMP response element binding protein), que se liga e regula a expressão de genes contendo CRE ❻ (cAMP response element). Adaptado de Shaywitz e Greenberg (1999)

1.9 - A Enzima Adenilato Ciclase Catalisa a Produção de AMPc

Como visto na figura 6, a enzima que catalisa a produção de AMPc é a adenilato ciclase (AC) (Shaywitz and Greenberg 1999; Hanoune and Defer

2001). Cerca de nove genes são responsáveis pela expressão da grande família das AC em mamíferos que, até o momento, apresenta 9 isoformas (AC1 a AC9).

Alterações na atividade desta enzima, provocadas pela exposição a agentes tóxicos, como o CPF, por exemplo, se refletem nos níveis de AMPc. Em 1994, Huff e colaboradores (Huff et al. 1994) demonstraram que o CPF-oxon é capaz de se ligar diretamente ao subtipo m2 do receptor colinérgico muscarínico (m2AChR) do striato de ratos. Além disso, os autores verificaram que o CPF-oxon inibiu a atividade da AC. Estes resultados sugerem um mecanismo de neurotoxicidade adicional desvinculado da inibição colinesterásica. Além disso, o uso de atropina, um agonista clássico de mAChR, não bloqueou o efeito do CPF-oxon em inibir a atividade da AC, indicando que o efeito observado sobre a atividade desta enzima não foi uma conseqüência do efeito sobre o receptor, mas sim uma provável ação direta sobre a enzima. Os próprios Huff e Abou-Donia (Huff and Abou-Donia 1995), em um estudo subseqüente utilizando cultura de células NG108-15 (neuroblastoma) e de células de ovário de hamister transfectadas com cDNA de receptores colinérgicos muscarínicos m2 e m4 (m2AChR e m4AChR) humanos, confirmaram que o CPF-oxon é capaz de interagir diretamente com m2AChR, mas também com m4AChR. Além disso, mais uma vez, a atividade da AC foi inibida por este metabólito e a adição de atropina não bloqueou este efeito. Desta forma, os autores demonstraram que os efeitos observados anteriormente não só podiam ser replicados, mas também se aplicavam a outros tipos celulares. Em 1996, outra evidência da ação do CPF-oxon sobre a atividade da AC dissociada da inibição da acetilcolinesterase veio de um estudo sobre o efeito de organofosforados sobre segundos mensageiros acoplados a mAChR. (Ward and Mundy 1996) Estes receptores, que apresentam cinco subtipos (m1 a m5), utilizam pelo menos dois sistemas distintos de segundos mensageiros. Enquanto que m2AChR e m4AChR sinalizam através da inibição da formação de AMPc, m1AChR e m3AChR utilizam a hidrólise de fosfatidilinositol-4,5-bifosfato para formar diacilglicerol (DAG) e inositol-1,4,5-trifosfato (IP3). Neste estudo, os autores demonstraram que além do CPF-oxon, dois outros metabólitos ativos, paraoxon e malaoxon, inibiram a formação de AMPc. Entretanto, nenhum efeito foi observado sobre a

hidrólise de fosfatidilinositol, um efeito que seria esperado como resultado do acumulo de ACh em resposta a inibição da AChE pelos oxons análogos.

Entretanto, foram mesmo Song e colaboradores (Song et al. 1997) que investigaram de forma mais detalhada os efeitos do CPF sobre a atividade da AC. Por todos os motivos já aqui apresentados, os autores formularam a hipótese de que o próprio CPF seria capaz de interferir com a sinalização celular mediada pela AC durante o período de desenvolvimento do SN, o que poderia explicar, ao menos em parte, alguns dos efeitos descritos acima. Para isso, eles expuseram ratos a diferentes doses de CPF durante dois períodos distintos do desenvolvimento neonatal (PN1-4 e PN11-14) e avaliaram a atividade da AC, densidade de mAChR e densidade de receptores β-adrenérgicos (β-AR) em duas regiões cerebrais distintas, prosencéfalo e o cerebelo, que diferem tanto em inervação colinérgica como também no "cronograma" de desenvolvimento, além do coração, um tecido não neuronal. Para os animais expostos em PN1-4, as medições foram feitas em PN5 e PN10 e aqueles expostos em PN11-14, foram avaliados em PN15 e PN20. Como a via de sinalização do AMPc apresenta várias etapas até a produção do AMPc em si (ver Figura 6), os autores utilizaram diversas ferramentas moleculares para avaliar detalhadamente que etapas desta via poderiam estar sendo afetadas pelo CPF. Assim, além da atividade basal, avaliada na ausência de guanosina trifosfato (GTP), eles avaliaram a atividade estimulada por fluoreto de sódio (NaF), que na presença de GTP, apresenta máxima estimulação da proteína G. Para detectar alterações diretamente sobre AC, utilizaram forskolina e Mn^{2+} combinados. Assim, detectaram alterações na atividade da AC em ambas as regiões cerebrais, prosencéfalo rico em inervação colinérgica, mas também no cerebelo, de inervação colinérgica esparsa, além do coração. Além disso, o prosencéfalo, que seria um alvo preferencial da "superestimulação" colinérgica, apresentou efeitos mais acentuados sobre a AC em doses mais baixas de CPF. Grande parte dos efeitos sobre a atividade da AC foi mais evidente fora do pico máximo de inibição colinesterásica; ou seja, para animais expostos em PN1-4, os efeitos foram maiores em PN10 e para aqueles expostos em PN11-14, efeitos mais pronunciados foram observados em PN20. A estratégia de avaliar a atividade de AC na presença de diferentes estimulantes permitiu identificar

que o CPF é capaz de interagir em todos os níveis da via de produção de AMPc.

Mais recentemente, Schuh e colaboradores (Schuh et al. 2002) testaram a hipótese de que se o CPF altera os níveis de AMPc, tal alteração deve se refletir nos níveis subseqüentes da cascata de sinalização. Assim, expuseram neurônios corticais e do hipocampo, em cultura primária, a variadas doses de CPF, CPF-oxon e TCP e avaliaram os níveis de CREB, uma molécula essencial para o desenvolvimento e a função cognitiva do cérebro, além de ser um dos alvos possíveis da via de sinalização do AMPc (ver Figura 6). Verificaram que CPF causou significativa (até 400% em relação ao controle) indução nos níveis de CREB fosforilado, CREB ativado, mesmo em doses de CPF onde não se observou nenhuma inibição colinesterásica. Os resultados deste estudo demonstraram claramente que a exposição a CPF altera a sinalização celular, provavelmente com repercussões importantes sobre a expressão gênica de proteínas essenciais ao processo de desenvolvimento do SN e que podem explicar em parte os danos comportamentais observados em animais expostos a CPF durante o desenvolvimento (Jett et al. 2001; Levin et al. 2001; Levin et al. 2002; Icenogle et al. 2004).

Dos estudos apresentados acima, é possível depreender que a exposição a CPF altera os processos fundamentais do desenvolvimento do SN, com possíveis repercussões sobre funções cerebrais importantes como memória, aprendizado, atenção, coordenação motora, entre outras. Além disso, é cada vez mais evidente que a alteração desses processos fundamentais se dá por mecanismos dissociados da inibição da AChE, provavelmente envolvendo a "desregulação" da sinalização celular mediada pelo AMPc. Entretanto, existem algumas lacunas importantes na construção deste conhecimento e que foram objetos de estudo dessa tese. Primeiro, embora tenha sido evidenciado que a exposição neonatal a CPF altera a atividade da AC, nada se conhece sobre os efeitos da exposição pré-natal a este inseticida sobre a atividade desta enzima. Como visto anteriormente, no período pré-natal ocorrem uma série de processos vitais ao desenvolvimento cerebral e a sinalização celular do AMPc poderia ser afetada também neste período. Assim, o primeiro objetivo desta tese foi investigar se a exposição de ratos a CPF, no período de desenvolvimento

pré-natal, altera a atividade da enzima adenilato ciclase. Além disso, neste primeiro estudo, foi investigado também que período de desenvolvimento pré-natal é mais afetado pelo CPF. Assim, ratas grávidas foram expostas a CPF em dois períodos distintos, dias gestacional 9 a 12 (GD9-12) e GD17-20. Foram utilizadas diferentes doses de CPF. Desde doses que não produzem inibição colinesterásica importante até doses mais elevadas, onde se observa efeito sistêmico.

1.10 - Origem Fetal das Doenças: A Hipótese de Barker

O segundo objetivo desta tese teve como base teórica a hipótese de que diversas doenças que emergem na vida adulta, têm origem ainda durante a gestação e o período neonatal (Barker et al. 2002; Barker 2003; Barker 2004). Esta hipótese, também denominada "Hipótese de Barker" (Gram et al. 1995; Paneth and Susser 1995), como crédito ao seu propositor e principal divulgador, o professor David Barker, tem como eixo central evidências de que o risco de algumas doenças como diabetes do tipo 2 e doença coronariana na vida adulta aumenta em função de alguns eventos que ocorram durante o período fetal e neonatal, como por exemplo retardo no crescimento e baixo peso ao nascer (Byrne and Phillips 2000; Barker 2003). Entretanto, atualmente essa linha de investigação tem incorporado também questões relacionadas a exposição química durante o desenvolvimento e o aparecimento de doenças na vida adulta (Power and Jefferis 2002; Toschke et al. 2002; Slikker and Schwetz 2003). Assim, de acordo com esta hipótese, buscamos verificar se a exposição pré- ou pós-natal a CPF produz efeitos de longa duração sobre a atividade da AC e a sinalização celular em diferentes regiões cerebrais do rato, seja na forma de alterações que persistem até a vida adulta do animal ou mesmo alterações que irão emergir nestes durante a vida adulta.

Por fim, embora alguns autores tenham demonstrado que a exposição a CPF durante o desenvolvimento também altera a atividade da AC em tecidos não neurais como o do coração (Song et al. 1997) e do fígado (Auman et al. 2000), eles também o fizeram expondo animais somente durante o período neonatal do desenvolvimento cerebral do rato. Assim, ainda que estes autores tenham contribuído para a compreensão de que as alterações causadas pela exposição a CPF durante o desenvolvimento

podem ir além dos efeitos neurotóxicos, as mesmas lacunas observadas anteriormente em relação aos efeitos sobre o cérebro ainda existem. Primeiro, a exposição fetal a CPF também altera a atividade da AC no coração e no fígado? Segundo, estas alterações estão presentes na vida adulta? Terceiro, estes dois órgãos são igualmente afetados pela exposição a CPF em diferentes períodos do desenvolvimento? Por fim, as alterações observadas na vida adulta são uma continuação dos efeitos presentes no período de desenvolvimento ou são conseqüência da "desregulação" da programação celular e dessa forma emergem posteriormente?

Os resultados desta tese foram publicados em três artigos científicos, que correspondem aos três objetivos descritos acima. A formatação original dos artigos, ou seja, o formato em que os artigos foram publicados na revista foi modificado de forma a acompanhar a formatação deste texto e tornar a leitura mais agradável. Ainda assim, se o leitor preferir examinar os artigos em seu formato original, é possível acessá-los nos endereços abaixo:

Meyer A, Seidler FJ, Cousins MM, Slotkin TA (2003) Developmental neurotoxicity elicited by gestational exposure to chlorpyrifos: when is adenylyl cyclase a target? Environ. Health Perspect. 111: 1871–1876. Disponível em: http://ehp.niehs.nih.gov/members/2003/6468/6468.html

Meyer A, Seidler FJ, Aldridge JE, Tate CA, Cousins MM, Slotkin TA (2004a) Critical Periods for Chlorpyrifos-Induced Developmental Neurotoxicity:Alterations in Adenylyl Cyclase Signaling in Adult Rat Brain Regions after Gestational or Neonatal Exposure. Environmental Health Perspectives 112: 295-301. Disponível em: http://ehp.niehs.nih.gov/members/2003/6755/6755.html

. Disponível em: http://ehp.niehs.nih.gov/members/2003/6690/6690.html

Embora cada artigo apresente sua própria introdução e discussão, o texto introdutório acima se faz necessário, uma vez que busca harmonizar os objetivos e experimentos da cada estudo/artigo, mas também permitiu abordar uma série de conceitos básicos necessários ao completo entendimento desta tese e que a seção de introdução de um artigo, por

limitações editoriais, não permite. Entretanto, não é possível prescindir da leitura da introdução de cada artigo, uma vez que cada um deles traz informações adicionais pertinentes a cada um dos objetivos específicos.

2 - Artigo 1

Research | Article

Developmental Neurotoxicity Elicited by Gestational Exposure to Chlorpyrifos: When Is Adenylyl Cyclase a Target?

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The developmental neurotoxicity of chlorpyrifos (CPF) involves mechanisms over and above cholinesterase inhibition. In the present study, we evaluated the effects of gestational CPF exposure on the adenylyl cyclase (AC) signaling cascade, which regulates the production of cyclic AMP, a major controller of cell replication and differentiation. In addition to basal AC activity, we assessed the AC response to direct enzymatic stimulants [forskolin, manganese (Mn^{2+})]; the response to isoproterenol, which activates signaling through β-adrenoceptors (βARs); and the concentration of βAR binding sites. CPF administered to pregnant rats on gestational days (GD) 9–12 elicited little or no change in any components of AC activity or βARs. However, shifting the treatment window to GD17–20 produced regionally selective augmentation of AC activity. In the brainstem, the response to forskolin or Mn^{2+} was markedly stimulated by doses at or below the threshold for observable toxicity of CPF or for inhibition of fetal brain cholinesterase, whereas comparable effects were seen in the forebrain only at higher doses. In addition, low doses of CPF reduced βAR binding without impairing receptor-mediated stimulation of AC. These results indicate that signal transduction through the AC cascade is a target for CPF during a discrete developmental period in late gestation, an effect that is likely to contribute to the noncholinergic component of CPF's developmental neurotoxicity. *Key words:* adenylyl cyclase, β-adrenoceptor, brain development, chlorpyrifos, organophosphate insecticides. *Environ Health Perspect* 111:1871–1876 (2003). doi:10.1289/ehp.6468 available via *http://dx.doi.org/* [Online 29 August 2003]

Introduction

Along with other widely used organophosphate insecticides, chlorpyrifos (CPF) is undergoing increasing scrutiny because of its developmental neurotoxicity (Barone et al. 2000; Landrigan 2001; Landrigan et al. 1999; May 2000; Physicians for Social Responsibility 1995; Pope 1999; Rice and Barone 2000; Slotkin 1999.). Although originally all organophosphates were thought to elicit neurodevelopmental damage through inhibition of cholinesterase (Mileson et al. 1998; Pope 1999), it is now apparent that other mechanisms play an important, perhaps predominating role, involving concentrations below the threshold for the systemic toxicity associated with cholinergic hyperstimulation (Barone et al. 2000; Das and Barone 1999; Pope 1999; Schuh et al. 2002; Slotkin 2004). CPF itself, as distinct from CPF oxon, the active metabolite that inhibits cholinesterase, disrupts the fundamental processes of brain development, such as DNA synthesis (Dam et al. 1998; Whitney et al. 1995), expression and function of macromolecular constituents and transcription factors that control cell differentiation (Crumpton et al. 2000; Garcia et al. 2001; Johnson et al. 1998; Schuh et al. 2002), and expression and function of neurotransmitters and their receptors that act as neurotrophins in the developing brain (Buznikov et al. 2001; Dam et al. 1999a, 1999b; Howard and Pope 2002; Huff et al. 2001; Liu et al.2002; Yanai et al. 2002; Zhang et al. 2002). Although these studies provide a reasonable doubt as to the importance of cholinesterase inhibition for developmental neurotoxicity of CPF, they leave open the issue of which cellular targets are the most critical, most sensitive, or primary in eliciting long-term changes in nervous system development. One pathway that has received much attention is that mediated by the intracellular second messenger cyclic AMP (cAMP), which ubiquitously coordinates the critical transition from cell replication to cell differentiation (Bhat et al. 1983; Claycomb 1976; Guidotti 1972; Hultgårdh-Nilsson et al. 1994; Van Wijk et al. 1973). In brain development, cAMP ultimately influences cell division, differentiation, axonal outgrowth, neural plasticity, and programmed cell death (Shaywitz and Greenberg 1999; Stachowiak et al. 2003), events

known to be targeted by CPF (Barone et al. 2000; Das and Barone 1999; Pope 1999; Schuh et al. 2002; Slotkin 2004). Furthermore, neurotransmitter receptors that control adenylyl cyclase (AC), the enzyme responsible for cAMP production, and AC itself have been found to be targets for CPF (Auman et al. 2000; Huff and Abou-Donia 1995; Huff et al. 1994, 2001; Olivier et al. 2001; Schuh et al. 2002; Song et al. 1997; Ward and Mundy 1996; Yanai et al. 2002; Zhang et al. 2002). One of the most sensitive effects involves changes in the transcription factors that are downstream targets for cAMP and that are known to participate in the activation of the genes necessary for cell differentiation (Schuh et al. 2002). These findings thus raise the possibility that actions on the AC pathway are among the critical targets of CPF in the developing brain. In the present study we explore this prospect with an *in vivo* exposure model, using CPF regimens that bracket the threshold for cholinesterase inhibition and resultant maternal/fetal toxicity (Qiao et al. 2002, 2003). We concentrated on two phases of development, an early stage involving formation of the neural tube, gestational days (GD) 9–12, and a later stage involving the transition from replication to differentiation of major neuronal cell populations (GD17–20). In both periods, CPF elicits mitotic abnormalities, apoptosis, and architectural anomalies in the developing brain at exposures that are not otherwise embryotoxic (Lassiter et al. 2002; Roy et al. 1998; White et al. 2002). At lower exposure levels, CPF-induced damage is not immediately apparent, but synaptic and functional abnormalities appear later, in adolescence and adulthood (Levin et al. 2002; Qiao et al. 2002, 2003). Thus, if the production of cAMP is involved in the adverse effects of CPF on brain development, effects on the AC signaling pathway should be evident immediately upon exposure to these lower exposures, preceding the delayed-onset anomalies. The potential effects of CPF on AC were assessed in several ways. First, we evaluated basal enzymatic activity. Second, we determined the response to two AC stimulants, forskolin and manganese (Mn2+). Because the two stimulants act at different epitopes on the AC molecule, the preference for one over the other reflects shifts in molecular conformation,

primarily influenced by the AC isoform (Zeiders et al. 1999b). Third, we probed the AC response to specific receptor-mediated activation with isoproterenol, a β-adrenoceptor (βAR) agonist that links to AC by activating the stimulatory G-protein, Gs. This receptor has defined neurotrophic roles in brain cell development and is a postulated target for CPF (Auman et al. 2000; Dreyfus 1998; Garcia et al. 2001; Kasamatsu 1985; Kulkarni et al. 2002; Kwon et al. 1996; Morris et al. 1983; Popovik and Haynes 2000; Schwartz and Nishiyama 1994; Slotkin et al. 1989; Song et al. 1997; Yanai et al. 2002).

Materials and Methods

Materials.

Animals were purchased from Zivic Laboratories (Pittsburgh, PA, USA). CPF was purchased from Chem Service (West Chester, PA, USA). [125I]Iodopindolol (specific activity, 2,200 Ci/mmol) was obtained from Perkin-Elmer Life Sciences (Boston, MA, USA), and cAMP radioimmunoassay kits were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). All other chemicals were purchased from Sigma Chemical Corp. (St. Louis, MO, USA).

Animal treatments

All experiments using live animals were carried out in accordance with the declaration of Helsinki and with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources 1996). Timed-pregnant Sprague–Dawley rats were housed in breeding cages, with a 12-hr light–dark cycle and with free access to food and water. CPF was dissolved in dimethyl sulfoxide to provide rapid and complete absorption (Whitney et al. 1995) and was injected subcutaneously in a volume of 1 mL/kg body weight. For exposure during neurulation, dams were injected daily with CPF at 1 or 5

mg/kg body weight on GD9–12. Dams were decapitated, and fetal tissues were harvested without distinction by sex on GD17 and GD21. For later gestational exposure (GD17–20), dams were given CPF daily at 1, 2, 5, 10, 20, or 40 mg/kg, and tissues were collected on GD21. Control animals received DMSO injections on the same schedules. For samples collected on GD17, we analyzed the whole brain, whereas in GD21 samples the forebrain was separated from the rest of the brain by making a cut rostral to the thalamus; because the cerebellum represents an inappreciable proportion of brain weight on GD21, the rest of the brain was considered to represent primarily the brainstem. This dissection, which follows the natural planes of the fetal and neonatal rat brain, includes the corpus striatum, hippocampal formation, and neocortex within the area designated as "forebrain." The region designated as "brainstem" includes the midbrain, colliculi, pons, and medulla oblongata (but not cervical spinal cord), as well as the thalamus. All tissues were frozen with liquid nitrogen and maintained at –45°C until assayed.

Membrane preparation.

Tissues were thawed and homogenized (Polytron, Brinkmann Instruments, Westbury, NY, USA) in 39 volumes of ice-cold buffer containing 145 mM NaCl, 2 mM MgCl2, and 20 mM Tris (pH 7.5), and the homogenates were sedimented at 40,000 . *g* for 15 min.The pellets were washed twice by resuspension (Polytron) in homogenization buffer, followed by resedimentation, and were then dispersed with a homogenizer (smooth glass fitted with Teflon pestle) in a buffer consisting of 250 mM sucrose, 2 mM MgCl2, and 50 mM Tris (pH 7.5).

Assays.

To evaluate βAR binding, aliquots of membrane preparation were incubated with [125I]iodopindolol (final concentration, 67 pM), in 145 mM

NaCl, 2 mM MgCl2, 1 mM sodium ascorbate, and 20 mM Tris (pH 7.5), for 20 min at room temperature in a total volume of 250 µL. Incubations were stopped by dilution with 3 mL of ice-cold buffer, and the labeled membranes were trapped by rapid vacuum filtration onto Whatman GF/C filters, which were then washed with additional buffer and counted by liquid scintillation spectrometry. Nonspecific binding was assessed by displacement with 100 µM isoproterenol. Iodopindolol binds to both β1ARs and β2ARs equally, which is important in light of the presence of both subtypes in the developing brain and their effective linkage to AC (Erdtsieck-Ernste et al. 1991; Pittman et al. 1980; Slotkin et al. 1994, 2001). For assessment of AC activity, aliquots of the same membrane preparation were incubated for 10 min at 30°C with final concentrations of 100 mM Tris-HCl (pH 7.4), 10 mM theophylline, 1 mM ATP, 2 mM MgCl2, 1 mg/mL bovine serum albumin, and a creatine phosphokinase-ATP–regenerating system consisting of 10 mM sodium phosphocreatine and 8 IU/mL phosphocreatine kinase, with 10 µM guanosine triphosphate (GTP) in a total volume of 250 µL. The enzymatic reaction was stopped by placing the samples in a 90–100°C water bath for 5 min, followed by sedimentation at 3,000 . *g* for 15 min, and the supernatant solution was assayed for cAMP using radioimmunoassay. Preliminary experiments showed that the enzymatic reaction was linear well beyond the assay period and was linear with membrane protein concentration; concentrations of cofactors were optimal, and in particular, higher concentrations of GTP produced no further augmentation of activity. In addition to measuring basal AC activity, we assessed the response to βAR stimulation (100 µM isoproterenol), as well as the response to the direct AC stimulants forskolin (100 µM) and Mn2+ (10 mM). These concentrations of each stimulant produce maximal responses, as assessed in previous studies (Auman et al. 2000, 2001; Zeiders et al. 1997, 1999a).

Data analysis.

Because the treatments were given to the dams, only one fetus was used from each dam, so the number of determinations represents the number of dams. The fetuses were derived from the same litters as those used in two previous studies on cell damage and cholinergic biomarkers; therefore effects on cholinesterase activity, maternal and fetal body weights, and other litter characteristics have been published elsewhere (Garcia et al. 2002; Qiao et al. 2002). Data are presented as mean ± SE. For convenience, some results are given as the percent change from control values, but statistical evaluations were always conducted on the original data. To establish treatment differences in receptor binding or AC activity, a global analysis of variance (ANOVA; data log transformed whenever variance was heterogeneous) was first conducted across *in vivo* treatment groups, age, brain region, and the five types of measurements made on the membranes (βAR binding, AC activity under four different conditions); the AC measurements were considered to be repeated measures because each membrane preparation was used for the multiple types of determinations. As justified by significant interactions of treatment with the other variables, data were then subdivided to permit testing of individual treatments and AC measures that differed from control values; these were conducted by lower-order ANOVAs followed, where appropriate, by Fisher's protected least significant difference to identify individual values for which the CPF groups differed from the corresponding control. For all tests, we assumed significance for main treatment effects at $p < 0.05$; however, for interactions at $p < 0.1$, we also examined whether lower-order main effects were detectable after subdivision of the interactive variables (Snedecor and Cochran 1967). For presentation, control values from GD21 samples were combined across both cohorts (controls used for CPF administration on GD9–12 and GD17–20). However, statistical comparisons of the effects of CPF were made only with the appropriately matched control cohort.

Results

Development of βAR binding and AC in controls

βARs in both the forebrain and brainstem were higher than the values in whole brain in samples collected on GD17, and regional differences were apparent, with higher binding in the brainstem (Table 1).

Table 1. Development of βAR binding and AC activities in controls.

Measure	GD17 whole brain (*n* = 6)	GD21 forebrain (*n* = 17)	GD21 brainstem (*n* = 18)
βAR binding[b]	4.7 ± 0.3	8.0 ± 0.3	10.9 ± 0.5[a]
Basal AC[c]	83 ± 5	92 ± 3	589 ± 28[a]
Isoproterenol-stimulated AC[c]	91 ± 6	94 ± 4	636 ± 27[a]
Forskolin-stimulated AC[c]	177 ± 15	226 ± 11	1,175 ± 59[a]
Mn2+-stimulated AC[c]	484 ± 41	526 ± 11	1,814 ± 78[a]

Values were combined across both cohorts (controls used for CPF administration on GD9–12 and on GD17–20); however, statistical comparisons of the effects of CPF were made only with the appropriately matched control cohort.
a. Signficant difference between GD21 forebrain and brainstem
b. (fmol/mg protein)
c. (pmol/min/mg protein)

Similarly, AC activities in GD21 samples were much higher in the brainstem than in the forebrain. To assess whether the differences in βARs corresponded to enhanced AC sensitivity to receptor stimulation, we assessed the response to the isoproterenol relative to basal activity and to the maximum Gs-sensitive AC response as assessed with forskolin (Figure 1A). Across all regions, isoproterenol caused a small but significant stimulation over basal activity (ratio > 1, $p < 0.03$). However, the response was significant only for GD17 whole brain ($p < 0.008$) and GD21 brainstem ($p < 0.002$) and not for GD21 forebrain. Similarly, although GD17 whole brain and GD21 brainstem showed an equivalent proportion of isoproterenol

response relative to forskolin, the value was significantly lower for GD21 forebrain. Thus, the absolute concentration of βARs did not provide the primary determinant of the response to isoproterenol. The higher AC activity seen in the brainstem was also accompanied by differential effects on the response to the two direct AC stimulants, forskolin and Mn2+ (Figure 1B). Although forskolin stimulation was relatively consistent as a proportion to basal activity, the Mn^{2+}-mediated response in the brainstem was 50% lower. Calculated as the forskolin/Mn2+ ratio, values were 0.37 ± 0.01 in whole brain collected on GD17 and 0.43 ± 0.02 in the GD21 forebrain, whereas it was significantly higher in the GD21 brainstem (0.65 ± 0.02, $p < 0.0001$ vs. brainstem).

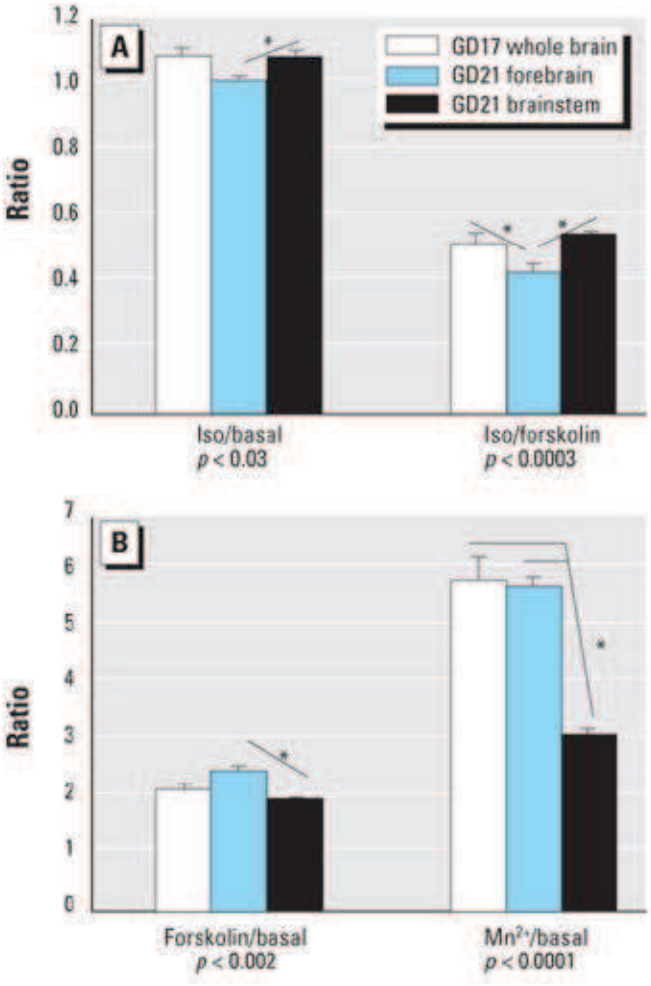

Figure 1. Development of AC responses to isoproterenol (*A*) and to forskolin and Mn2+ (*B*) relative to basal AC activity in controls. Iso, isoproterenol. Data are presented as mean ± SE of six determinations on samples collected on GD17 and 17–18 determinations for those collected on GD21. ANOVA results for each measure appear within the figure, and lines and asterisks denote individual groups that differ significantly from each other.

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Systemic toxicity of CPF

As reported previously (Garcia et al. 2002; Qiao et al. 2002), the threshold for CPF-induced impairment of maternal growth was 5 mg/kg with treatment on either GD9–12 or GD17–20, but fetal brain growth was unaffected even at the highest doses (data not shown). Neither the early nor the late treatment paradigm affected the number of fetuses or fetal viability. Fetal brain cholinesterase showed significant inhibition at ≥ 5 mg/kg (Qiao et al. 2002).

CPF exposure on GD17–20

Before examining the effects of CPF on each variable and each brain region, a global ANOVA was performed across both regions and all measurements so as to avoid type 1 statistical errors that would otherwise result from multiple tests on the same data set. The overall test indicated a significant main effect of CPF ($p < 0.003$) and interactions of treatment with region and type of measurement: $p < 0.007$ for treatment . region, $p < 0.0001$ for treatment x measure, and $p < 0.03$ for treatment x region x measure. Accordingly, the results were separated into the two regions for further analysis of treatment effects on each measure. In the forebrain, animals treated with CPF from GD17 through GD20 displayed robust (> 40%) βAR decreases at 2 mg/kg, a dose below the threshold for systemic toxicity and at which cholinesterase inhibition is barely detectable (Qiao et al. 2002) (Figure 2A). However, the response displayed distinct hormesis (i.e., was nonmonotonic), disappearing as the dose was raised above the toxicity threshold. Across all AC measures, CPF elicited a net increase in activity (main effect), but the magnitude of enhancement differed among the various stimulants (treatment x measure interaction). Basal and isoproterenol-stimulated AC activity showed no significant changes overall, whereas the responses to forskolin and Mn2+ showed major increases only at doses of > 10 mg/kg. When activities were determined relative to basal AC, there were

some specific differences from the pattern seen for absolute AC activity, but the overall pattern was similar (Figure 2B). Isoproterenol-mediated responses were significantly elevated by small amounts, and the enhanced responses to the two direct AC stimulants were fully evident. Nevertheless, all these effects involved CPF doses of ≥ 5 mg/kg. There were no changes in the forskolin/Mn2+ response ratio that would have accompanied a shift in the AC isoform (Zeiders et al. 1999b).

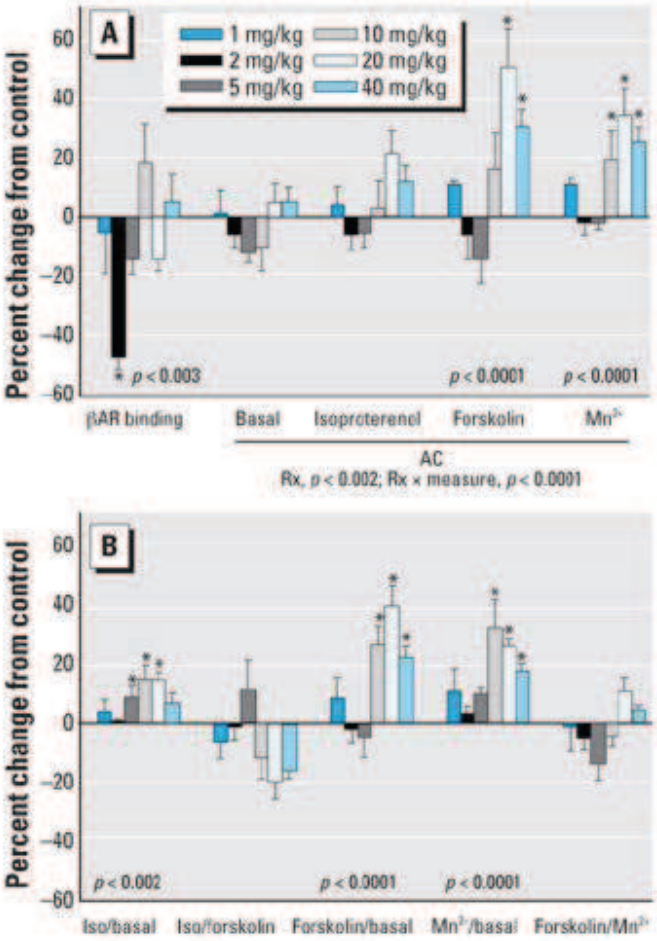

Figure 2. Effects of CPF exposure on GD17–20 on forebrain βAR binding and AC activity measured on GD21. Abbreviations: Iso, isoproterenol; Rx, treatment. (*A*) Effects on absolute activities. (*B*) Activity ratios. Data represent mean ± SE obtained from five to seven animals for each group. ANOVA results for each measure appear within the figure, and asterisks denote individual groups that differ significantly from the corresponding control.

In the brainstem, CPF elicited alterations in βAR binding and AC activities that were in the same direction as those seen in the forebrain, but the dose–effect relationships were distinctly different (Figure 3A). The decrement of βAR binding was evident even at the lowest dose of CPF, which lies below the threshold for detectable cholinesterase inhibition (Qiao et al. 2002); again, the response was hormetic and disappeared once the dose was raised above the toxicity threshold. Overall stimulation of AC displayed differential effects depending on the test stimulant (treatment x measure interaction). In this case, unlike in the forebrain, every single measure of AC showed significant augmentation after CPF treatment. Responses displayed hormesis for basal and isoproterenol-stimulated AC. For forskolin and Mn2+, the enhancement was evident at 2 mg/kg, a lower dose than that required for effects in the forebrain. Because of the differential effects on disparate measures of AC activity, we reexamined the responses as relative ratios (Figure 3B). Although the absolute response to isoproterenol was augmented, the effect was actually no greater than the change in basal AC; accordingly, the isoproterenol/basal activity ratio was unaffected. In contrast, the isoproterenol/forskolin response ratio showed significant decrements, indicating that βAR-mediated responses were suboptimal after CPF treatment. Forskolin- and Mn2+-stimulated AC activities remained significantly elevated after correction for basal AC, but the effects were not as robust until the dose was raised above 5 mg/kg. As was true in the forebrain, the brainstem also showed no change in the forskolin/Mn2+ activity ratio.

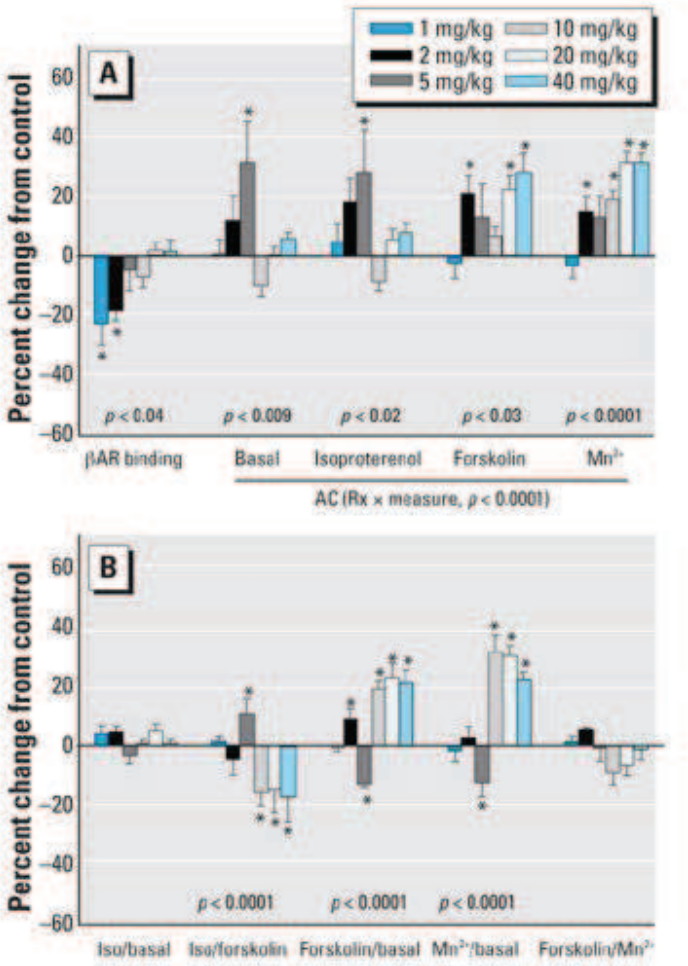

Figure 3. Effects of CPF exposure on GD17–20 on brainstem βAR binding and AC activity measured on GD21. Abbreviations: Iso, isoproterenol; Rx, treatment. (*A*) Effects on absolute activities. (*B*) Activity ratios. Data represent mean ± SE obtained from five to seven animals for each group. ANOVA results for each measure appear within the figure, and asterisks denote individual groups that differ significantly from the corresponding control.

CPF exposure on GD9–12.

For examination of the effects of CPF during neurulation, the dose range was more restricted, encompassing exposures below and up to the threshold for systemic toxicity (Qiao et al. 2002). Across all measures and the three different tissues (GD17 whole brain, GD21 forebrain, GD21 brainstem), global ANOVA indicated a significant interaction of treatment . measure (p < 0.03), and accordingly, we then assessed each measurement separately. This

lower-order test indicated significant effects on βAR binding ($p < 0.04$ for main treatment effect, $p < 0.09$ for treatment x tissue) but not for AC activities. The absence of significant overall effects on AC should be interpreted with caution, however, because it mixes together the effects in whole brain on GD17 with those of the two separate regions on GD21. Restricting the analysis to the latter measurements, we detected a significant overall decrement in AC at the highest CPF dose ($p < 0.0006$ for main effect). In any case, the direction of change with this regimen was opposite that obtained with treatment on GD17–20 and was statistically distinguishable from those effects ($p < 0.06$ for treatment . region . regimen). Examining each age and tissue independently, the effects of CPF on GD17 were relatively minor and did not achieve statistical significance for any of the measurements (Figure 4A). By GD21, there was significant augmentation of βAR binding in the forebrain Figure 4B), with effects fully evident at 2 mg/kg, a dose below the threshold for systemic toxicity (Qiao et al. 2002). No such effect was seen in the brainstem (Figure 4C), and the regional difference was statistically robust ($p < 0.02$ for treatment x region). As noted above, AC activities were significantly decreased overall across the two regions on GD21 at the highest dose, although the absence of a treatment x measure interaction did not permit us to examine the significance of each measurement separately.

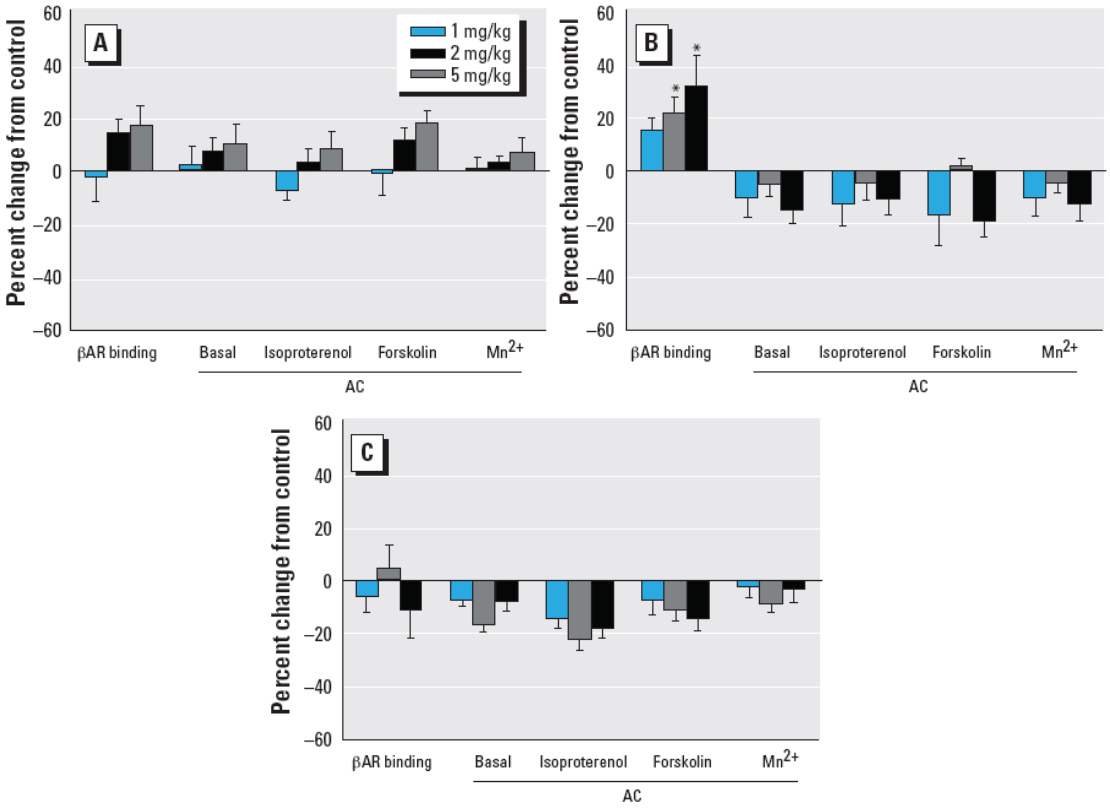

Figure 4. Effects of CPF exposure on GD9–12 assessed in whole brain on GD17 (*A*) and in forebrain (*B*) and brainstem (*C*) on GD21. Data represent mean ± SE of five or six determinations for each group at each age.
*Individual groups differ significantly from the corresponding control ($p < 0.04$; ANOVA).

Discussion

Results of the present study indicate that gestational exposure to CPF evokes immediate alterations in AC-mediated cell signaling in the developing brain, with a distinct regional hierarchy and critical window of vulnerability. It is highly unlikely that CPF interacts directly with the signaling proteins of this intracellular transduction cascade or that it simply causes global alterations in the expression or function of the proteins, because in those situations, effects would have been temporally and spatially uniform. Because cAMP is a pivotal control point for the trophic control of cell replication and differentiation by neurotransmitters and hormones (Bhat et al. 1983;

Claycomb 1976; Guidotti 1972; Hultgårdh-Nilsson et al. 1994; Van Wijk et al. 1973), the complex series of changes in AC signaling elicited by developmental exposure to CPF provides a mechanism for deleterious outcomes. By far the greatest period of sensitivity was late gestation after CPF exposure on GD17–20. We observed significant βAR deficits at doses below the threshold for maternal or fetal systemic toxicity and, indeed, below the level at which significant cholinesterase inhibition can be detected in the fetal brain (Qiao et al. 2002). Nevertheless, the AC response mediated by βARs, isoproterenol-stimulated AC activity, was unaffected or even increased, indicating that receptor binding is not the primary determinant of the receptor-mediated signaling response. These results reinforce the idea that the expression and function of signaling proteins downstream from the receptor provide the primary determinants of the net cellular response to receptor activation (Gao et al. 1998, 1999; Navarro et al. 1991a, 1991b; Slotkin et al. 2001, 2003). Accordingly, we evaluated AC responses mediated by direct stimulants, which test the inherent responsiveness of AC itself. CPF exposure on GD17–20 elicited marked increases in AC responses to forskolin or Mn2+ but with a distinct regional hierarchy: the brainstem was far more sensitive than the forebrain. Indeed, in the brainstem, AC induction was evident with doses as low as 2 mg/kg using either AC stimulant. Because forskolin and Mn2+ operate through different epitopes of the AC molecule (Limbird et al. 1979; Seamon and Daly 1986; Zeiders et al. 1999b), the parallel effect of CPF on the responses to the two agents, unaccompanied by a shift in their relative activity (i.e., no change in the forskolin/Mn2+ response ratio), implies that CPF treatment increases (induces) the concentration of AC molecules. A closer examination of the effects on AC in the brainstem indicates that the enzyme induction caused by CPF exposure actually masks deficits in βAR-mediated responses. If there were no changes in the receptormediated component, then the isoproterenol response would simply mimic the effect seen on total AC activity. Instead, the proportion of AC capable of responding to isoproterenol declined in the CPF group, evidenced by a drop in the isoproterenol/forskolin response ratio. Unlike the

effects on AC itself, however, this deficit in the relative βAR response was detectable only at doses above the threshold for cholinesterase inhibition. In contrast to the prominent effects of the GD17–20 CPF regimen on AC signaling, similar treatment on GD9–12 elicited little or no effect; if anything, AC activities tended to be reduced slightly, rather than increased. Accordingly, the window of vulnerability for CPF's effects on AC signaling appears to be concentrated in late gestation. Although our studies do not address the specific reasons for the higher liability of late gestational exposure, there are certainly major developmental differences in the two stages. Within the brain itself, the basic processes of neurogenesis, gliogenesis, axonogenesis, cell migration, and architectural organization are completely distinct in mid- versus late gestation (Rodier 1988). Further, the later period corresponds to the onset of sexual differentiation of the brain. Although CPF is only weakly estrogenic (Andersen et al. 2002; Vinggaard et al. 2000), effects on neural development are likely to influence the ontogeny of sexual dimorphism, endocrine responses, or even hormonal levels, and CPF intoxication in adults is known to have secondary endocrine effects (Guven et al. 1999). In the present study, we did not examine male and female fetuses separately. However, in previous work we found that CPF treatment on GD17–20 produces sex-dependent neurobehavioral differences that emerge in adolescence and adulthood (Levin et al. 2002). If sexual differentiation is a component of CPF's targeted effects on brain development, then we would predict that the effects of earlier exposure on GD9–12 might not show sex dependence; these studies are currently under way. Regardless of the mechanisms underlying the critical period for effects of CPF on AC, it is important to note that CPF exerts other types of developmental neurotoxicant effects in the earlier phases of development. These include abnormal patterns of cell replication and cell death during CPF exposure at the neural tube stage (Roy et al. 1998), as well as lasting neurobehavioral effects of such exposure (Icenogle et al. In Press). Our results indicate that those effects are not mediated through initial alterations in the AC cascade, but rather through other mechanisms. Similarly, the

window for targeted effects on AC components shows postnatal closure. CPF treatment of neonatal rats does not augment brainstem or forebrain AC activity as was seen here for the late gestational treatment regimen (Song et al. 1997). Instead, the postnatal exposures cause delayed-onset deterioration of AC signaling that likely represents a consequence of other mechanisms contributing to altered cell development (Campbell et al. 1997; Dam et al. 1998, 1999a). Findings in the cerebellum, a region that develops much later than the brainstem or forebrain (Rodier 1988), reinforce the concept of a critical period of cell maturation in which AC is vulnerable to CPF; postnatal CPF exposure elicits the same type of immediate increase in cerebellar AC activity as seen here for the earlier-developing regions with gestational CPF treatment (Song et al. 1997). Finally, it is interesting to note that several effects of CPF displayed distinct hormesis (i.e., the effects were nonmonotonic), with alterations apparent at low doses but disappearing once the dose was raised above the threshold for cholinesterase inhibition and systemic toxicity. A similar phenomenon has been noted for effects on biomarkers of synaptic development (Qiao et al. 2002, 2003) and for behavioral consequences of gestational or neonatal CPF treatment (Levin et al. 2001, 2002; Icenogle et al. In Press). Cholinergic input provides a positive trophic effect on brain development at the levels of cell maturation and regional architecture (Hohmann and Berger-Sweeney 1998; Lauder and Schambra 1999), and it is thus possible that raising the dose of CPF above the threshold for cholinesterase inhibition can partially offset deleterious effects mediated by noncholinergic mechanisms. Consequently, the dose–effect curve for the developmental neurotoxicity of CPF can be expected to display multiple phases, not a monotonic relationship. This also points out an inherent difficulty in ascribing any effects of CPF in an *in vivo* treatment model to a definitive "cholinergic" or "noncholinergic" mechanism. Effects on signaling pathways, such as the AC pathway, no doubt have an influence on responses mediated by cholinergic signals, which operate in part through cAMP. In turn, cholinergic effects influence AC and cAMP formation. Resolution of these issues thus ultimately requires simplified systems such as

cell cultures or lower organisms (Buznikov et al. 2001; Schuh et al. 2002; Song et al. 1998). The present study thus reinforces the idea that CPF elicits developmental neurotoxicity through mechanisms independent of, and at doses below the threshold for, cholinesterase inhibition. The AC signaling cascade represents a major control point for brain cell replication and differentiation, and CPF targets this intracellular pathway with discrete temporal and regional selectivity. In addition to immediate changes in AC signaling, CPF also has the potential to evoke delayed-onset alterations (Song et al. 1997) that may influence later maturational events such as axonogenesis, synaptogenesis, and synaptic function (Barone et al. 2000; Das and Barone 1999; Pope 1999; Schuh et al. 2002; Slotkin 1999. In press). Accordingly, future studies will need to address the issue of the long-term effects of gestational CPF exposure on the AC pathway.

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3 - Artigo 2

Research | Article

Critical Periods for Chlorpyrifos-Induced Developmental Neurotoxicity: Alterations in Adenylyl Cyclase Signaling in Adult Rat Brain Regions after Gestational or Neonatal Exposure

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Developmental exposure to chlorpyrifos (CPF) alters the function of a wide variety of neural systems. In the present study we evaluated the effects in adulthood of CPF exposure of rats during different developmental windows, using the adenylyl cyclase (AC) signaling cascade, which mediates the cellular responses to numerous neurotransmitters. Animals were exposed on gestational days (GD) 9–12 or 17–20 or on postnatal days (PN) 1–4 or 11–14 and assessed at PN60. In addition to basal AC activity, we evaluated the responses to direct AC stimulants (forskolin, Mn^{2+}) and to isoproterenol, which activates signaling through β-adrenoceptors coupled to stimulatory G-proteins. CPF exposure in any of the four periods elicited significant changes in AC signaling in a wide variety of brain regions in adulthood. In general, GD9–12 was the least sensitive stage, requiring doses above the threshold for impaired maternal weight gain, whereas effects were obtained at subtoxic doses for all other regimens. Most of the effects were heterologous, involving signaling elements downstream from the receptors, and thus shared by multiple stimulants; superimposed on this basic pattern, there were also selective alterations in receptor-mediated responses, in G-protein function, and in AC expression and subtypes. Exposures conducted at GD17–20 and later all produced sex-selective alterations. These results suggest that developmental exposure to CPF elicits long-lasting alterations in cell-signaling cascades that are shared by multiple neurotransmitter and hormonal inputs; the resultant abnormalities of synaptic communication are thus likely to occur in widespread neural circuits and their corresponding behaviors. *Key words:* adenylyl cyclase, β-adrenoceptor, brain development, chlorpyrifos, organophosphate insecticides. *Environ Health Perspect* 112:295–301 (2004). doi:10.1289/ehp.6755 available via *http://dx.doi.org/* [Online 24 November 2003]

Introduction

The ability of organophosphate insecticides such as chlorpyrifos (CPF) to elicit developmental neurotoxicity has received a great deal of attention in the last decade (Barone et al. 2000; Landrigan 2001; Landrigan et al. 1999; May 2000; Physicians for Social Responsibility 1995; Pope 1999; Rice and Barone 2000; Slotkin 1999, in press b), resulting in its restricted domestic use [U.S. Environmental Protection Agency (EPA) 2000a, 2000b, 2001]. It is now evident that CPF alters brain development by a panoply of mechanisms in addition to cholinesterase inhibition (Barone et al. 2000; Das and Barone 1999; Pope 1999; Schuh et al. 2002; Slotkin 1999, in press b), compromising neural cell replication and differentiation, axonogenesis and synaptogenesis, and the programming of synaptic function (Buznikov et al. 2001; Crumpton et al. 2000; Dam et al. 1998, 1999a, 1999b; Garcia et al. 2001; Howard and Pope 2002; Huff et al. 2001; Johnson et al. 1998; Liu et al. 2002; Whitney et al. 1995; Yanai et al. 2002; Zhang et al. 2002). One prominent mechanism by which CPF acts on the developing brain is through its ability to alter the expression and function of cell-signaling proteins that control the generation of cyclic AMP (cAMP) (Crumpton et al. 2000; Garcia et al. 2001; Meyer et al. 2003; Olivier et al. 2001; Schuh et al. 2002; Song et al. 1997; Zhang et al. 2002). In turn, cAMP coordinates the critical transition from cell replication to cell differentiation (Bhat et al. 1983; Claycomb 1976; Guidotti 1972; Hultgårdh-Nilsson et al. 1994; Van Wijk et al. 1973) and, during brain development, plays a pivotal role in axonal outgrowth, neural plasticity, and apoptosis (Shaywitz and Greenberg 1999; Stachowiak et al. 2003). We recently demonstrated that, during critical developmental periods, CPF alters the activity of adenylyl cyclase (AC), the enzyme responsible for cAMP production, as well as the G-proteins that couple neurotransmitter receptors to AC, along with the receptor-mediated responses to neurotransmitters that serve neurotrophic roles in brain development (Aldridge et al. 2003; Garcia et al. 2001; Meyer et al. 2003; Song et al. 1997).

The question remains as to whether the ability of CPF to alter cell signaling lasts into adulthood, or instead, whether the effects are restricted to developmental stages in which these signals affect neural cell differentiation. Certainly, CPF exposure leads to behavioral anomalies that emerge in adolescence and persist in adults (Levin et al. 2001, 2002; Richardson and Chambers 2003; Slotkin et al. 2001a, 2002). In association with the behavioral deficits, developmental CPF exposure evokes profound alterations in activity of neurotransmitter systems that operate through AC, notably cholinergic and catecholaminergic pathways (Qiao et al. 2003; Slotkin, in press a; Slotkin et al. 2001a, 2002). In the present study, we investigated the long-term impact of gestational or neonatal CPF exposure on the functioning of the AC signaling cascade, focusing on four different critical windows: the formation of the neural tube [gestational days (GD) 9–12], the late gestational period (GD17–20) in which sexual differentiation of the brain is initiated (McCarthy 1994; Mong and McCarthy 1999), and postnatal phases of terminal neuronal differentiation and synaptogenesis [postnatal days (PN) 1–4, PN11–14]; these are the same treatment windows examined for short-term effects in our previous study (Meyer et al. 2003). Doses were chosen to enable us to determine whether the threshold for effects on AC signaling lies below that for systemic toxicity and/or inhibition of cholinesterase (Aldridge et al. 2003; Garcia et al. 2003; Meyer et al. 2003; Qiao et al. 2002; Slotkin 1999, in press a).

In adulthood (PN60), we examined the effects of CPF on AC in several ways. First, we evaluated basal enzymatic activity. Second, we determined the response to two AC stimulants, forskolin and Mn2+. Because the two stimulants act at different epitopes on the AC molecule, the preference for one over the other reflects shifts in molecular conformation, primarily influenced by the AC isoform (Zeiders et al. 1999). Third, we probed the AC response to specific receptor-mediated activation with isoproterenol, a β-adrenoceptor (βAR) agonist linked to AC by the stimulatory G-protein (Gs). This receptor has defined neurotrophic roles in brain cell development and is a postulated target for CPF (Auman et al. 2000; Dreyfus 1998; Garcia et al.

2001; Kasamatsu 1985; Kulkarni et al. 2002; Kwon et al. 1996; Morris et al. 1983; Popovik and Haynes 2000; Schwartz and Nishiyama 1994; Slotkin et al. 1989; Song et al. 1997). Our studies also characterized the regional specificity and sex selectivity of the long-term effects of CPF on cell signaling.

Materials and Methods

Animal treatments.

All experiments using live animals were carried out in accordance with the Declaration of Helsinki (World Medical Association 2002) and with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources 1996) as adopted and promulgated by the National Institutes of Health. Timed pregnant Sprague-Dawley rats were housed in breeding cages, with a 12-hr light/dark cycle and with free access to food and water. CPF was dissolved in DMSO to provide rapid and complete absorption (Whitney et al. 1995) and was injected subcutaneously in a volume of 1 mL/kg body weight; control animals received vehicle (DMSO) injections on the same schedules. For exposure on GD9–12 or GD17–20, dams were injected daily with CPF at 1 or 5 mg/kg body weight. These doses span the threshold for inhibition of fetal brain cholinesterase activity, fetal growth impairment, and reduced maternal weight gain, all of which become evident at ≥ 5 mg/kg (Garcia et al. 2003; Qiao et al. 2002). On the day of birth, all pups were randomized within their respective treatment groups and redistributed to the dams with a litter size of 10 to maintain a standard nutritional status. Randomization was repeated at intervals of several days; in addition, dams were rotated among litters to distribute any maternal caretaking differences randomly across litters and treatment groups. Animals were weaned on PN21. On PN60, animals were decapitated and the brain was dissected into cerebral cortex, hippocampus, striatum, midbrain, brainstem, and cerebellum, which were frozen with liquid nitrogen and stored at –45°C.

For studies of CPF effects in the first few days after birth, animals were given 1 mg/kg by subcutaneous injection daily on PN1–4; for studies in older animals, which tolerate higher doses (Campbell et al. 1997; Pope and Chakraborti 1992; Pope et al. 1991; Whitney et al. 1995), daily treatment with 5 mg/kg was given on PN11–14. The same randomization procedure was followed. Neither regimen evokes weight loss or mortality (Campbell et al. 1997; Dam et al. 1998; Johnson et al. 1998; Song et al. 1997), and in the present study we did not observe any changes in suckling or maternal caretaking. Samples were obtained on PN60 as described above.

None of the prenatal or postnatal treatment regimens evoked a significant change in weight of any of the brain regions on PN60, nor were there any deficits in body weight (data not shown).

Membrane preparation and assays

All of the assay methodologies used in this study have been described previously (Aldridge et al. 2003; Auman et al. 2000, 2001; Meyer et al. 2003; Slotkin et al. 2001b; Zeiders et al. 1997, 1999), so only brief descriptions are provided here. Tissues were thawed and homogenized with a polytron (Brinkmann Instruments, Westbury, NY), and cell membranes were prepared and washed by sequential sedimentation at 40,000 × *g*. The membrane pellets were dispersed with a smooth-glass homogenizer and used for ligand binding and AC assays. [125I]Iodopindolol (67 pM) was used to determine βAR binding, and nonspecific binding was assessed by displacement with 100 μM isoproterenol. AC activity was determined by enzymatic generation of cAMP, which was then measured by radioimmunoassay. In addition to measuring basal AC activity, we assessed the response to βAR stimulation (100 μM isoproterenol), as well as the response to the direct AC stimulants forskolin (100 μM) and Mn2+ (10 mM). These concentrations of each stimulant produce maximal responses, as assessed in previous studies (Auman et al. 2000, 2001; Slotkin et al. 2001b; Zeiders et al. 1997, 1999).

Data analysis.

Data are presented as means and SE obtained from eight animals of each sex for each prenatal treatment group and six animals per sex for each postnatal treatment group. For convenience, results are given as the percent change from control values, but statistical evaluations were always conducted on the original data. To establish treatment differences in receptor binding or AC activity, a global analysis of variance (ANOVA; data log transformed whenever variance was heterogeneous) was first conducted across the *in vivo* treatment groups, sexes, regions, and measurements made on the membranes (βAR binding, AC activity under four different conditions); the latter were considered to be repeated measures because each membrane preparation was used for the multiple conditions under which AC was determined. As justified by significant interactions of treatment with the other variables, data were then subdivided to permit testing of individual treatments and AC measures that differed from control values; these were conducted by lower-order ANOVAs, followed, where appropriate, by Fisher's protected least significant difference to identify individual values for which the CPF groups differed from the corresponding control. For all tests, significance for main treatment effects was assumed at $p < 0.05$; however, for interactions at $p < 0.1$, we also examined whether lowerorder main effects were detectable after subdivision of the interactive variables (Snedecor and Cochran 1967).

For presentation, control values (Table 1) were combined across the multiple cohorts (controls used for administration on GD9–12, GD17–20, PN1–4, PN11–14). However, statistical comparisons of the effects of CPF were made only with the appropriately matched control cohort.

Materials.

Animals were purchased from Zivic Laboratories (Pittsburgh, PA, USA).

CPF was purchased from Chem Service (West Chester, PA, USA). [125I]Iodopindolol (specific activity, 2,200 Ci/mmol) was obtained from Perkin-Elmer Life Sciences (Boston, MA, USA), and cAMP radioimmunoassay kits were purchased from Amersham Biosciences (Piscataway, NJ, USA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Table 1. Binding parameters and AC levels in controls

Measure, sex	Cerebral cortex	Hippocampus	Striatum	Midbrain	Brainstem	Cerebellum
βAR binding (fmol/mg protein)						
Male	39.9 ± 0.9	17.3 ± 0.7	44.1 ± 1.0	15.8 ± 0.3	10.0 ± 0.3	27.2 ± 0.6
Female	41.4 ± 0.9	18.1 ± 0.7	46.3 ± 1.6	16.6 ± 0.3	10.2 ± 0.3	27.1 ± 0.7
Basal AC (pmol/min/mg protein)						
Male	177 ± 11	146 ± 6	141 ± 6	240 ± 9	147 ± 9	267 ± 12
Female	186 ± 10	142 ± 4	137 ± 7	242 ± 10	133 ± 7	248 ± 12
Isoproterenol-stimulated AC (pmol/min/mg protein)						
Male	197 ± 12	153 ± 6	151 ± 6	254 ± 9	153 ± 8	328 ± 14
Female	208 ± 12	148 ± 5	153 ± 8	250 ± 10	141 ± 7	301 ± 12
Forskolin-stimulated AC (pmol/min/mg protein)						
Male	1,206 ± 93	576 ± 27	3,817 ± 194	1,040 ± 51	421 ± 14	997 ± 40
Female	1,340 ± 113	594 ± 21	3,960 ± 199	1,049 ± 41	428 ± 15	1,019 ± 47
Mn^{2+}-stimulated AC (pmol/min/mg protein)						
Male	1,811 ± 119	1,174 ± 60	2,145 ± 72	1,485 ± 60	828 ± 24	1,932 ± 94
Female	2,007 ± 135	1,184 ± 42	2,292 ± 98	1,481 ± 66	817 ± 28	2,015 ± 106

Values were combined across multiple cohorts (controls used for CPF administration on GD9–12, GD17–20, PN1–4, and PN11–14). However, statistical comparisons of the effects of CPF were made only with the appropriately matched control cohort. None of the sex differences is statistically significant.

Results

CPF exposure on GD9–12.

CPF administration during the neural tube stage had no long-term effects on βAR binding in any of the brain regions (Figure 1A). In contrast, there were significant effects on AC activity that depended on the type of AC measurement (significant interaction of treatment × measure). Examining this effect for each dose group, it was apparent that the alterations were confined to those receiving 5 mg/kg ($p < 0.0002$; not significant for 1 mg/kg group). Because the statistics could not distinguish significant selectivity for sex or brain region (i.e., no interaction of treatment × sex or treatment × region), we characterized the differential effect on AC measures by calculating the specific changes elicited by each stimulant, namely, the ratio

of activity with the added stimulant to basal AC (Figure 1B). CPF exposure elicited small but significant increases in the response to isoproterenol, forskolin, and Mn^{2+}. Additionally, the effect on the isoproterenol response was significantly smaller than that for forskolin ($p < 0.03$). There was no change in the forskolin: Mn^{2+} activity ratio (data not shown). In light of the positive findings with this first treatment regimen, the scope of the regions examined was extended to include hippocampus and striatum for the subsequent studies.

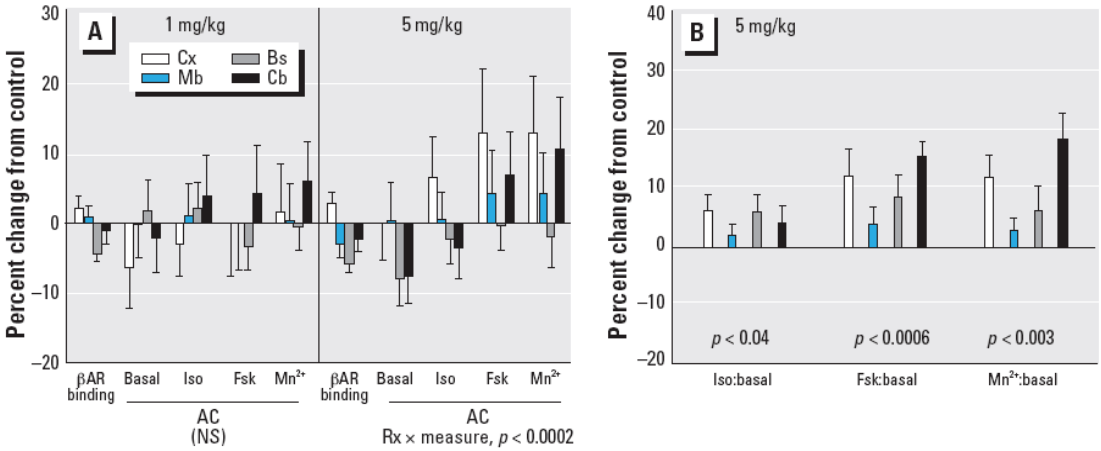

Figure 1. Effects of GD9–12 CPF exposure on PN60 (*A*) βAR binding and AC activity (ANOVA for Rx × measure, $p < 0.002$) and (*B*) AC activity ratios. Abbreviations: Bs, brainstem; Cb, cerebellum; Cx, cerebral cortex; Fsk, forskolin; Iso, isoproterenol; Mb, midbrain; NS, not significant; Rx, treatment. Data are means and SEs of the percent change from corresponding control values (Table 1). Although effects on βAR binding were not significant, ANOVA indicated a significant treatment effect that depended on the specific AC measure, and the effects were restricted to the group exposed to 5 mg/kg CPF. To characterize the differential effects on AC measures, activity ratios were calculated relative to basal AC activity (*B*). ANOVAs for each measure are shown within the figure. Separate statistical evaluations for males and females or for each region were not carried out because of the absence of treatment interactions with these variables.

CPF exposure on GD17–20

In contrast to the effects of GD9–12 CPF exposure, shifting the exposure period to later in gestation had a profound, sex-dependent effect on βAR binding in adulthood. Overall, ANOVA identified treatment interactions with sex and brain region: $p < 0.006$ for treatment × sex, $p < 0.02$ for treatment

× region, $p < 0.02$ for treatment × sex × region. Furthermore, the effects were distinguishable both at the low dose (1 mg/kg) of CPF ($p < 0.002$ for treatment × sex) as well as at the higher (5 mg/kg) dose ($p < 0.03$, $p < 0.004$, $p < 0.004$, for the three interactions, respectively). In light of the consistent sex differences, we subdivided the data into males and females for presentation and further analysis (Figure 2). Males showed a treatment × region interaction, reflecting small elevations in the striatum and midbrain, as well as a reduction in the cerebellum in the high-dose group. In contrast, females showed significant overall reductions (main treatment effect) that were robustly significant even with the lower dose of CPF ($p < 0.02$ across all regions). The largest individual changes were obtained for the striatum and brainstem.

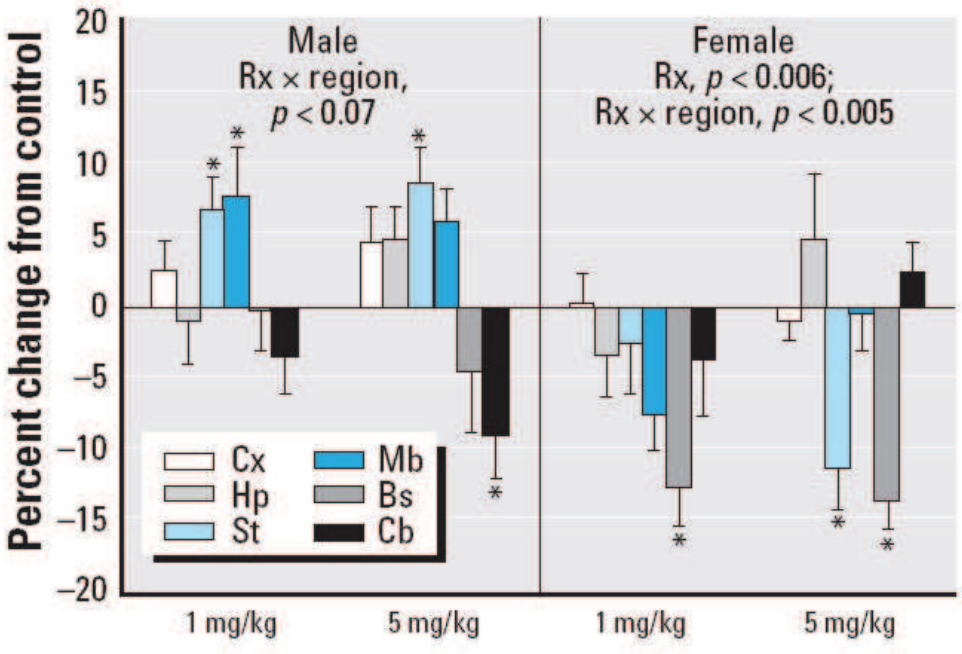

Figure 2. Effects of GD17–20 CPF exposure on PN60 βAR binding. Abbreviations: Bs, brainstem; Cb, cerebellum; Cx, cerebral cortex; Hp, hippocampus; Mb, midbrain; Rx, treatment; St, striatum. Data are means and SEs of the percent change from corresponding control values (Table 1). Across all treatments, all regions, and both sexes, ANOVA indicated a significant treatment effect that differed between the two sexes (Rx × sex, $p < 0.006$; Rx × region, $p < 0.02$; Rx × sex × region, $p < 0.02$), so values were separated for males and females. Lower-order ANOVAs for each sex appear within the figure.
*Individual groups differ significantly from the control.

CPF exposure on GD17–20 also influenced AC measures in a fashion that interacted with brain region (treatment × region × measure, $p < 0.1$), so values were subdivided into the individual regions and reexamined for treatment effects and interactions. CPF effects were identified in the hippocampus (main treatment effect, $p < 0.05$), striatum (treatment × measure, $p < 0.02$; treatment × sex, $p < 0.1$), midbrain (treatment × sex, $p < 0.1$), and cerebellum (treatment × sex, $p < 0.07$), and these are displayed in Figure 3; results for the other regions are not shown. For the hippocampus, CPF treatment evoked an overall elevation of AC, without preferential effects on any of the stimulants (Figure 3A); effects were significant only at 5 mg/kg. In the striatum, there was a sex disparity, with males tending to show increases in AC and females showing decreases. For individual measures, males exposed to 5 mg/kg CPF showed significant elevations in the responses to the two direct AC stimulants forskolin and Mn2+, but the isoproterenol response was unaffected. In the midbrain, CPF exposure similarly tended to elevate AC in a sex-selective manner (treatment × sex interaction), but considered separately, neither sex passed the threshold for statistically significant differences (Figure 3C); the sex difference reflected the relatively greater effect of the lower dose of CPF in males compared with that in females. In the cerebellum, CPF exposure had a preferential effect on AC in females, evoking significant elevations at either 1 or 5 mg/kg (Figure 3D); the effects were exerted across all AC measures, without preference for different stimulants. Males did not show any significant differences.

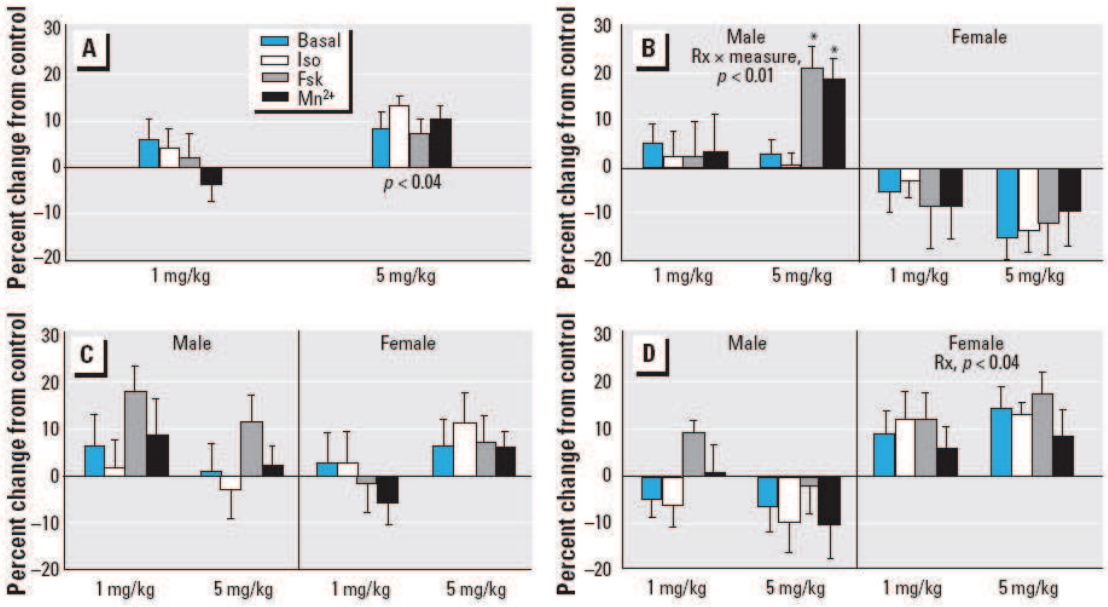

Figure 3. Effects of GD17–20 CPF exposure on PN60 AC activity in (*A*) hippocampus, (*B*) striatum, (*C*) midbrain, and (*D*) cerebellum. Abbreviations: Fsk, forskolin; Iso, isoproterenol; Rx, treatment. Data are means and SEs of the percent change from corresponding control values (Table 1). ANOVA results for each region across all variables are as follows: for (*A*) Rx, $p < 0.05$; for (*B*) Rx × measure, $p < 0.02$; Rx × sex × measure, $p < 0.1$; for (*C*) Rx × sex, $p < 0.1$; for (*D*) Rx × sex, $p < 0.07$; subdivision into the two sexes was carried out only when the ANOVA indicated an interaction of treatment × sex. Lower-order tests are shown within the figure. *Individual groups differ significantly from the control (calculated only when lower-order tests indicated a significant treatment × measure interaction).

CPF exposure on PN1–4

In contrast to the effects of GD17–20 exposure, shifting the period of CPF treatment to the early neonatal period resulted in no significant long-term alterations of βAR binding on PN60 (data not shown). However, there was a significant overall elevation of AC (main treatment effect, $p < 0.05$) that again depended on sex, brain region, and AC measure ($p < 0.03$ for treatment × sex, $p < 0.03$ for treatment × measure, $p < 0.07$ for treatment × sex × measure, $p < 0.009$ for treatment × region × measure). Accordingly, we performed lower-order assessments on each region, looking for main treatment effects and interactions of treatment with other variables. Two of the regions that were targeted by GD17–20 exposure, the

hippocampus and striatum, showed no significant overall effects on AC with the PN1–4 regimen (data not shown). The cerebral cortex and brainstem each showed a significant treatment × measure interaction without sex selectivity (no treatment × sex interaction), so results for males and females were combined for presentation (Figure 4A). Both regions showed significant elevations of AC but with different preference for the various stimulants. In the cerebral cortex, significant elevations were seen for direct AC stimulants (forskolin, Mn2+), whereas in the brainstem, the effects were preferential for isoproterenol. Unlike the other two regions, there were sex-selective effects in the cerebellum, necessitating separate analysis of males and females (Figure 4B). Males displayed significant deficits in basal and isoproterenol-stimulated AC activity, whereas females showed a global elevation of AC.

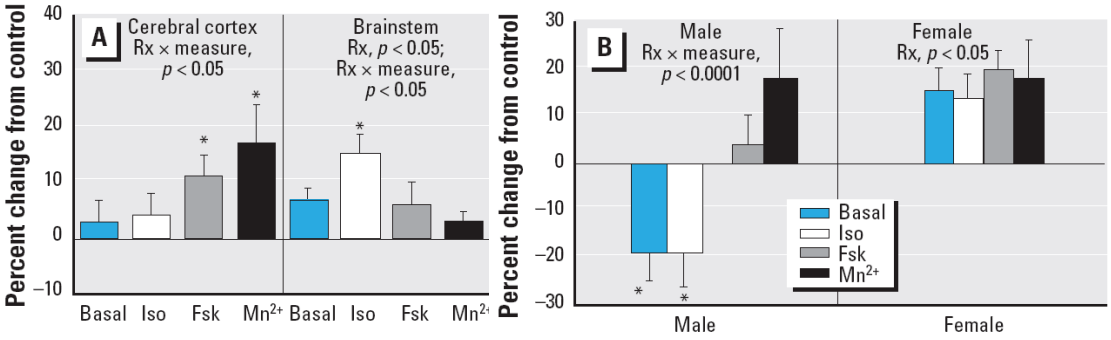

Figure 4. Effects of PN1–4 CPF exposure (1 mg/kg/day) on PN60 AC activity in (*A*) the cerebral cortex and brainstem, and (*B*) the cerebellum. Abbreviations: Fsk, forskolin; Iso, isoproterenol; Rx, treatment. Data are means and SEs of the percent change from corresponding control values (Table 1). ANOVA results across all variables are as follows for (*B*): Rx × sex, $p < 0.09$; Rx × measure, $p < 0.0004$; Rx × sex × measure, $p < 0.007$). For each region, ANOVA is shown across all variables, with subdivision into the two sexes carried out only when the ANOVA indicated an interaction of treatment × sex. Lower-order tests are shown within the figure.
*Individual groups differ significantly from the control (calculated only when lower-order tests indicated a significant treatment × measure interaction).

CPF exposure on PN11–14

With this treatment regimen, βAR binding showed significant overall decreases (main treatment effect, $p < 0.03$) that were distinctly sex selective (treatment × sex, $p < 0.03$). Separating the values for males and females

indicated a small but consistent decrement in females but not males (Figure 5).

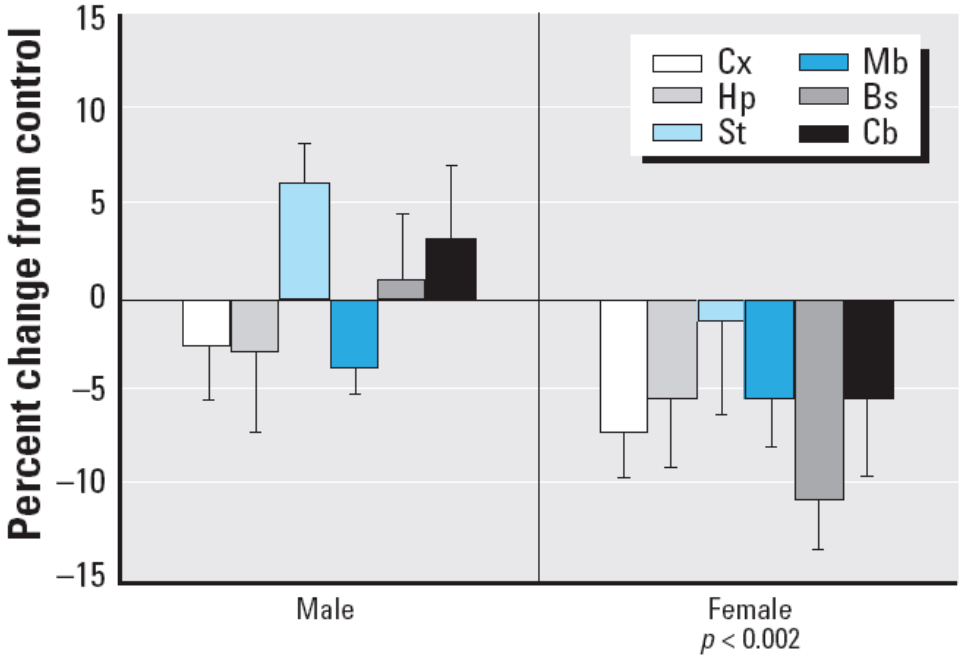

Figure 5. Effects of PN11–14 CPF exposure (5 mg/kg/day) on PN60 βAR binding. Abbreviations: Bs, brainstem; Cb, cerebellum; Cx, cerebral cortex; Hp, hippocampus; Mb, midbrain; Rx, treatment; St, striatum. Data are means and SEs of the percent change from corresponding control values (Table 1). Across all treatments, all regions, and both sexes, ANOVA (Rx, $p < 0.03$; Rx × sex, $p < 0.03$) indicated a significant treatment effect that differed between the two sexes, so values were separated to conduct lower-order ANOVAs for males and females. Individual regions for which the CPF group differs from the control were not tested because of the absence of a treatment × region interaction.

Similarly, AC activities in this treatment group did not indicate regionally selective CPF effects but did indicate the need to examine males and females separately for differential effects on the various AC measures (treatment × measure, $p < 0.07$; treatment × sex × measure, $p < 0.002$). In males, CPF treatment evoked significant, 10–20% decrements in basal and isoproterenol-stimulated AC activity with relatively smaller effects on the responses to forskolin and Mn2+ (Figure 6A). Females showed a more uniform pattern, with a significant overall decrease (main effect of CPF) and specific reductions in basal and forskolin-stimulated activity (Figure 6B); the cerebral cortex showed the greatest overall effect and was the only region in

which the reductions achieved statistical significance individually ($p < 0.02$). Again, to characterize the differences in the effects of CPF directed toward specific AC stimulants, we calculated AC activity ratios (Figure 6C). In males, the response to isoproterenol was suppressed relative to that to forskolin, with significant deficits in striatum and cerebellum. In contrast, the same measurement in females indicated overall increases. Additionally, females showed a significant reduction in the forskolin: Mn^{2+} AC activity ratio.

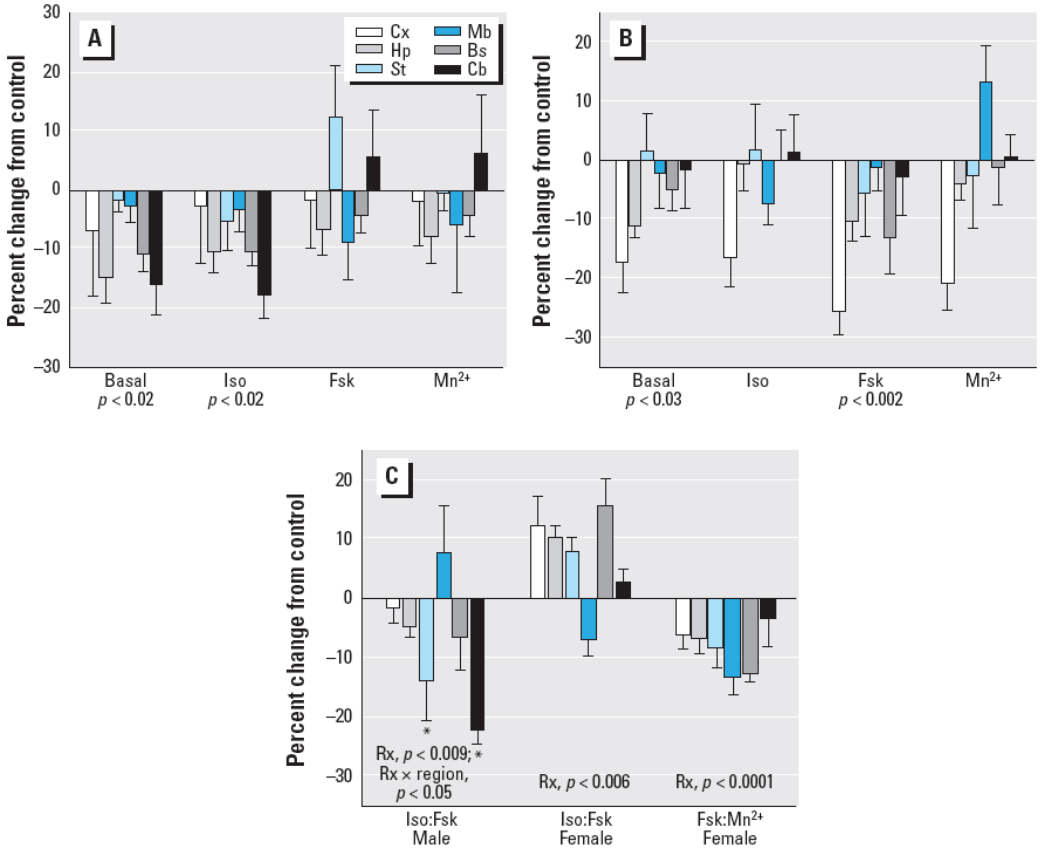

Figure 6. Effects of PN11–14 CPF exposure (5 mg/kg/day) on PN60 AC activity. (*A*) Males. (*B*) Females. (*C*) AC activity ratios. Abbreviations: Bs, brainstem; Cb, cerebellum; Cx, cerebral cortex; Fsk, forskolin; Hp, hippocampus; Iso, isoproterenol; Mb, midbrain; Rx, treatment; St, striatum. Across all regions and both sexes, ANOVA indicated a significant interaction of treatment with sex, so values were separated for males (*A*) and females (*B*). Data are means and SEs of the percent change from corresponding control values (Table 1). ANOVA across all regions and measures is as follows: for (*A*) Rx × measure, $p < 0.02$; for *B*, Rx, $p < 0.04$; Rx × measure, $p < 0.02$). Lower-order tests of the individual AC measures are shown within the figure. Separate tests for each region were not carried out because of the absence of a treatment × region interaction. Activity ratios (*C*) were calculated because of the selective effects of CPF on the various AC stimulants; ANOVA across regions appears within the figure.
*Individual groups differ significantly from the control (calculated only when lower-order tests indicated a significant treatment × region interaction).

Discussion

Results of the present study indicate that exposure to CPF during development elicits longterm alterations in AC-mediated cell signaling in the central nervous system, with differential effects according to sex and brain region that depend upon the exposure period. This effectively rules out the possibility that CPF simply interacts directly with the neurotransmitter receptors or proteins of the AC signaling cascade (Huff and Abou-Donia 1995; Huff et al. 1994; Ward and Mundy 1996), in which case the alterations would have been similar in every region, for both sexes, and for each regimen. Instead, our findings point to disruption of the program for development of cell signaling, with attendant targeting of specific regions for each sex that depend upon maturational phases of vulnerability of various neural cell populations (Garcia et al. 2002, 2003; Rodier 1988; Slotkin 1999, in press a). Indeed, CPF affects replication and differentiation of both neurons and glia, thus eliciting neurotoxicant actions over all the exposure periods studied here (Aldridge et al. 2003; Garcia et al. 2002, 2003; Icenogle et al., in press; Levin et al. 2001, 2002; Meyer et al. 2003; Qiao et al. 2003; Raines et al. 2001; Slotkin 1999, in press a; Slotkin et al. 2001a, 2002). Nevertheless, because AC signaling is a common final pathway for the transduction of diverse neural and hormonal signals involved in neural cell differentiation, axonal outgrowth, synaptic plasticity, and apoptosis (Bhat et al. 1983; Claycomb 1976; Guidotti 1972; Hultgårdh-Nilsson et al. 1994; Shaywitz and Greenberg 1999; Stachowiak et al. 2003; Van Wijk et al. 1973), lasting disruption of this pathway by CPF is likely to contribute directly to neurobehavioral anomalies (Icenogle et al., in press; Levin et al. 2001, 2002). Furthermore, the fact that alterations in AC signaling are heterologous, rather than being confined to the immediate cholinergic target of CPF's actions, implies that effects will be exerted in regions, such as the cerebellum, that are sparse in cholinergic projections, and that alterations will extend to other neurotransmitter pathways. Again, this corresponds to earlier observations of disrupted cell replication and differentiation and synaptic communication in disparate brain regions (Aldridge et al. 2003;

Campbell et al. 1997; Dam et al. 1999a; Garcia et al. 2003; Meyer et al. 2003; Raines et al. 2001; Slotkin 1999; Slotkin et al. 2002; Whitney et al. 1995). There were four major features of the lasting alterations of AC signaling elicited by developmental exposure to CPF: regional selectivity, existence of a peak period of sensitivity, localization of the effects to specific signaling proteins, and preferential effects according to sex. First, the regional targeting changed dramatically with a shift in the CPF exposure period but not in a manner that would be predicted solely from the maturational timetable of each region. In general, neural maturation occurs earliest in the brainstem, later in forebrain areas, and last in the cerebellum (Rodier 1988). In contrast to that pattern, we found effects of CPF on AC signaling components in both early- and late-developing regions with either gestational or postnatal exposures, and without a distinct ontogenetic shift as would be expected from uniform targeting of a specific phase of cell development. Again, this is consistent with CPF's ability to affect different neural cell types by a variety of mechanisms ranging from mitotic inhibition to impairment of differentiation, axonogenesis, and synaptogenesis (Barone et al. 2000; Pope 1999; Rice and Barone 2000; Slotkin 1999, in press a). Nevertheless, our results point to a specific phase in which CPF is most likely to disrupt long-term programming of AC function. With the earliest exposure (GD9–12), effects were seen only when the dose was raised to 5 mg/kg, above the threshold for systemic toxicity as assessed by impaired maternal weight gain (Qiao et al. 2002); even then, the magnitude of effect was only half of that seen with CPF exposure in later periods. With later gestational exposure (GD17–20), significant sex-dependent effects on AC signaling began to emerge at subtoxic exposure, best exemplified by the female cerebellum. Shifting the treatment to the postnatal period intensified the effects, with significant alterations in multiple brain regions at 1 mg/kg in animals treated on PN1–4, a dose that causes no discernible systemic toxicity (Dam et al. 1999a, 2000; Song et al. 1997); similarly, treatment with 5 mg/kg on PN11–14 elicited robust long-term alterations in AC signaling in adulthood. It thus appears that the early neonatal period in the rat, which

approximates neurologic development in the third trimester and perinatal stage of human brain development (Rodier 1988), is particularly sensitive to persistent effects on AC signaling. Interestingly, this is the same conclusion that was reached from evaluations of the short-term effects on AC signaling in the fetal and neonatal brain seen immediately after CPF treatment (Meyer et al. 2003; Song et al. 1997), suggesting that the persistent effects are dependent on the earlier changes. Given the role of cAMP in neural development, it seems likely that there is a mechanistic link between early perturbations and the persistent alterations seen here in adulthood.

CPF exposure evoked alterations in AC signaling at all loci within the pathway, displaying both temporal and regional selectivity for the targeting of specific proteins. With the earliest treatment (GD9–12), the responses to the two direct AC stimulants forskolin and Mn^{2+} were enhanced to the same extent, suggesting augmented expression and/or catalytic activity of AC itself. Because there was no change in the forskolin:Mn2+ response ratio, it is unlikely that there was a shift in the AC isoform, so a global increase in AC expression is probable. A similar effect was seen in the female hippocampus and male striatum after CPF exposure on GD17–20 and in the cerebral cortex after PN1–4 treatment. On the other hand, the PN11–14 treatment did produce a change in the forskolin: Mn^{2+} response ratio across multiple brain regions in females, indicating that isoform shifts can also be elicited, specifically with late postnatal exposure. Effects on the isoproterenol response, both in absolute terms and relative to the changes in the forskolin response, also indicated the targeting of receptor-mediated AC stimulation. With GD9–12 treatment, the βAR-mediated effect was augmented to a smaller extent than that of the direct AC stimulant (decreased isoproterenol: forskolin response ratio), suggesting an impairment of receptor coupling to AC superimposed on the induction of AC itself. The same effect was seen in the striatum when CPF treatment was given on GD17–20, in the cerebral cortex and male cerebellum with the PN1–4 exposure, and in the male striatum and cerebellum for the PN11–14 exposure. We also found one instance of a specific enhancement of the isoproterenol response, in the

brainstem of the PN1–4 group. Again, it is possible to make inferences about the actual locus of CPF's effects on βAR-mediated AC signaling: none of the changes correlated with the alterations in βAR binding sites, which in some cases were opposite to the effects on the isoproterenol AC response. Accordingly, these particular effects of CPF are likely to depend upon alterations in expression or function of the G-proteins that couple βARs to AC activity. Our general conclusion, then, is that the effects of CPF on AC signaling reflect actions exerted at the levels of the signaling components downstream from the receptors, the G-proteins and AC itself; therefore, the changes are heterologous, affecting all inputs that converge on this pathway. This inference is consistent with the view that development of G-protein–coupled receptor-mediated cell signaling is regulated primarily by mechanisms operating at the levels of G-proteins and AC (Gao et al. 1998, 1999; Gaudin et al. 1995; Karoor et al. 1996; Kohout and Lefkowitz 2003; Ostrom et al. 2000; Slotkin et al. 2003; Vatner et al. 1998; Watts 2002).

Finally, as in our previous studies with CPF (Aldridge et al. 2003; Dam et al. 2000; Garcia et al. 2002; Icenogle et al., in press; Levin et al. 2001, 2002; Slotkin et al. 2001a, 2002), we found distinct sex differences for exposures on GD17–20, PN1–4, or PN11–14 but not with the early gestational treatment (GD9–12). As examples, with the GD17–20 regimen, βAR binding was affected in opposite directions in males and females, as were striatal and cerebellar AC activities; for PN1–4 exposure, basal and isoproterenol-stimulated AC activities were reduced in males but enhanced in females; and for the PN11–14 treatment, βARs and forskolin-stimulated AC were reduced only in females, the isoproterenol: forskolin response ratio was affected in opposite directions in the two sexes, and only females showed an AC isoform shift (reduced forskolin:Mn2+ response ratio). Although it is not possible from these data alone to determine a specific mechanism for the sex differences, it is important to note that sexual differentiation of the brain commences toward the latter part of gestation in the rat (McCarthy 1994; Mong and McCarthy 1999) and specifically involves the cAMP pathway (Auger 2003). Although CPF is only weakly estrogenic (Andersen et al. 2002;

Vinggaard et al. 2000), there is new evidence that it interferes with testosterone catabolism (Usmani et al. 2003). Additionally, the effects of CPF on brain development are themselves likely to influence sexual differentiation and resultant endocrine responses or hormonal levels because CPF intoxication in adults has distinct endocrine effects (Guven et al. 1999). In any case, the present findings of a critical developmental period for the lasting, sex-selective effects of CPF on AC signaling are consonant with behavioral assessments that demonstrate the same window of vulnerability (Icenogle et al., in press; Levin et al. 2001, 2002).

In summary, we found lasting alterations in AC signaling in a wide variety of rat brain regions after CPF exposure during developmental windows ranging from the earliest phases of brain development (neurulation) through postnatal stages that are comparable with human brain development in the perinatal period (Rodier 1988). Within this broad window of vulnerability, there were differences in the regional locus, sex selectivity, and the specific signaling proteins targeted by CPF that depended on the period of exposure. Given the pivotal role played by AC signaling as a final common pathway in the response to neuronal and hormonal signals, the persistent alterations seen here are likely to contribute to lasting physiologic and behavioral alterations after developmental exposure to CPF.

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4 - Artigo 3

Research | Article

Developmental Effects of Chlorpyrifos Extend Beyond Neurotoxicity: Critical Periods for Immediate and Delayed-Onset Effects on Cardiac and Hepatic Cell Signaling

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The fetal and neonatal neurotoxicity of chlorpyrifos (CPF) and related insecticides is a major concern. Developmental effects of CPF involve mechanisms over and above cholinesterase inhibition, notably events in cell signaling that are shared by nonneural targets. In the present study, we evaluated the immediate and long-term effects of CPF exposure of rats during different developmental windows [gestational days (GD) 9–12 or 17–20, postnatal days (PN) 1–4 or 11–14] on the adenylyl cyclase (AC) signaling cascade in the heart and liver. In addition to basal AC activity, we assessed the responses to direct AC stimulants (forskolin, Mn^{2+}); to isoproterenol and glucagon, which activate signaling through specific membrane receptors; and to sodium fluoride, which activates the G-proteins that couple the receptors to AC. Few immediate effects on AC were apparent when CPF doses remained below the threshold for systemic toxicity. Nevertheless, CPF exposures on GD9–12, GD17–20, or PN1–4 elicited sex-selective effects that emerged by adulthood (PN60), whereas later exposure (PN11–14) elicited smaller, nonsignificant effects, indicative of closure of the window of vulnerability. Most of the effects were heterologous, involving signaling elements downstream from the receptors, and thus were shared by multiple inputs; superimposed on this basic pattern, there were also selective alterations in receptor-mediated responses. These results suggest that the developmental toxicity of CPF extends beyond the nervous system, to include cell signaling cascades that are vital to cardiac and hepatic homeostasis. Future work needs to address the potential implications of these effects for cardiovascular and metabolic disorders that may emerge long after the end of CPF exposure. *Key words:* adenylyl cyclase, β-adrenoceptor, chlorpyrifos, critical developmental periods, heart development, liver development, organophosphate insecticides. *Environ Health Perspect* 112:170–178 (2004). doi:10.1289/ehp.6690 available via *http://dx.doi.org/* [Online 3 November 2003]

Introduction

The potential for organophosphate insecticides, notably chlorpyrifos (CPF), to elicit developmental neurotoxicity has led to increasing concern and restricted use [Barone et al. 2000; Landrigan 2001; Landrigan et al. 1999; May 2000; Physicians for Social Responsibility 1995; Pope 1999; Rice and Barone 2000; Slotkin 1999, In press a; U.S. EnvironmentalProtection Agency (EPA) 2000a, 2000b]. The systemic toxicity of organophosphates reflects their ability to inhibit cholinesterase, but it is now evident that other mechanisms are at least equally, if not more, important in determining the long-term liability of fetal or neonatal CPF exposure (Barone et al. 2000; Das and Barone 1999; Pope 1999; Schuh et al. 2002; Slotkin 1999, In press a). The cyclic AMP (cAMP) signaling pathway has received particular attention because this second messenger coordinates the critical transition from cell replication to cell differentiation in virtually all prokaryotic and eukaryotic cells (Bhat et al. 1983; Claycomb 1976; Guidotti 1972; Hultgårdh- Nilsson et al. 1994; Van Wijk et al. 1973). A number of studies in the developing brain have centered on the effects of CPF on the receptors and signaling proteins that control the activity of adenylyl cyclase (AC), the enzyme that synthesizes cAMP, as well as on the downstream elements that are targets for cAMP (Crumpton et al. 2000; Garcia et al. 2001; Meyer et al. 2003; Olivier et al. 2001; Schuh et al. 2002; Song et al. 1997; Zhang et al. 2002).

Recent attention has begun to explore the contributions of fetal factors and chemical exposures to the later emergence of cardiovascular and metabolic diseases. The "Barker Hypothesis" originally drew a connection between fetal growth retardation and the subsequent incidence of coronary artery disease and diabetes (Barker 2003; Phillips 2002), and there is also significant literature on the long-term consequences of prenatal stress and the role of glucocorticoid hormones (Dodic et al. 1999, 2001; Nyirenda and Seckl 1998). More recently, there are suggestions that environmental toxicants may play an important contributory role in such disorders as hypertension, diabetes, and obesity, beyond neural contributions (Power and Jefferis 2002; Slikker and Schwetz 2003; Toschke et al. 2002). Thus,

although most studies on the developmental effects of CPF are appropriately directed toward neurotoxicity, the present work instead takes a similar approach with regard to cell signaling in the liver and heart, concentrating on the cAMP cascade and its responses to some of the major inputs that control that pathway, β-adrenoceptors (βARs), and glucagon receptors (Figure 1). CPF is concentrated in developing peripheral tissues, especially the liver (Hunter et al. 1998), and high doses can be hepatotoxic (Goel et al. 2000). Significant effects in the immature liver or heart are elicited at exposures below the threshold for systemic toxicity, and these include effects on signal transduction (Auman et al. 2000; Song et al. 1997) and cell number and size (Qiao et al. 2002).

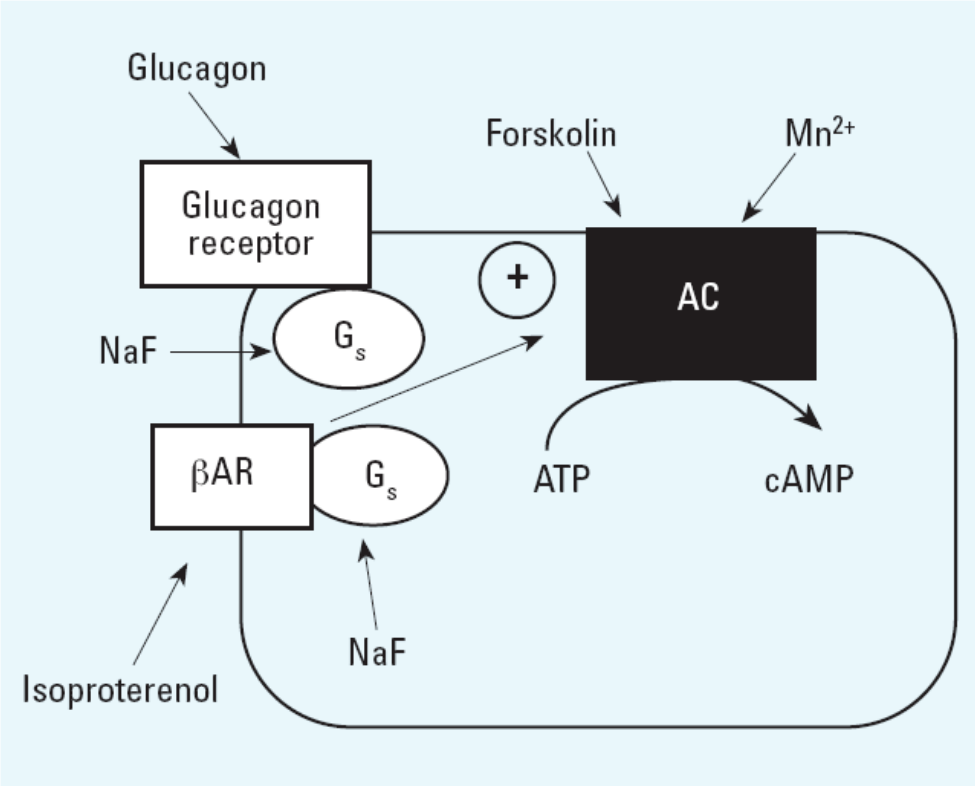


Figure 1. Mechanisms controlling AC activity in developing rat cardiac and hepatic cells. Both βARs and glucagon receptors enhance AC activity through the stimulatory G-protein, Gs. Each step in the pathway can be probed with the appropriate stimulant: isoproterenol for βARs, glucagon for the glucagon receptor, NaF for the G-proteins, and forskolin and Mn2+ for AC itself. The attachment of Gs to AC (+) enhances the response to forskolin while suppressing the response to Mn2+; in addition, different AC isoforms show preferential responses to forskolin versus Mn2+. When alterations involve signaling elements downstream from the receptors (Gs, AC), effects will be shared by multiple receptors and will therefore be "heterologous."

The present study addresses several essential questions: What is the critical period for effects of CPF on signal transduction in the liver and heart? Are CPF-induced signaling abnormalities present in adulthood? If so, do the adult effects represent persistence of alterations that were elicited during the initial CPF exposure, or alternatively, does CPF disrupt the programming of cell signaling so that defects arise only after a delay?

Materials and Methods

Animal treatments.

All experiments using live animals were carried out in accordance with the declaration of Helsinki and with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources 1996) as adopted and promulgated by the National Institutes of Health. Timedpregnant Sprague–Dawley rats were housed in breeding cages, with a 12-hr light/dark cycle and with free access to food and water. CPF was dissolved in DMSO to provide rapid and complete absorption (Whitney et al. 1995) and was injected subcutaneously in a volume of 1 mL/kg body weight; control animals received vehicle (DMSO) injections on the same schedules. For exposure on gestational days (GD) 9–12, dams were injected daily with CPF at 1, 2, or 5 mg/kg body weight, and for later gestational exposure (GD17–20), dams were given CPF daily over a range of 1–40 mg/kg; tissues were harvested on GD21. These doses span the threshold for inhibition of fetal brain cholinesterase activity, fetal growth impairment, or reduced maternal weight gain, all of which become evident at about 5 mg/kg (Garcia et al. 2003; Qiao et al. 2002). Additional dams were treated on GD9–12 or GD17–20 with 1 or 5 mg/kg for measurements in the offspring when they reached adulthood [postnatal day (PN) 60]. On the day of birth, all pups were randomized within their respective treatment groups and redistributed to the dams with a litter size of 10 to maintain a standard nutritional status. Randomization was repeated at intervals of several days; in addition, dams were rotated among

litters to distribute any maternal caretaking differences randomly across litters and treatment groups. Offspring were weaned on PN21.

For studies of CPF effects in the first few days after birth, animals were subcutaneously injected with 1 mg/kg daily on PN1–4; for studies in older animals, which tolerate higher doses (Campbell et al. 1997; Pope and Chakraborti 1992; Pope et al. 1991; Whitney et al. 1995), animals were treated daily with 5 mg/kg on PN11–14. The same randomization procedure was followed. Neither regimen evokes weight loss or mortality (Campbell et al. 1997; Dam et al. 1998; Johnson et al. 1998; Song et al. 1997), and in the present study we did not observe any changes in suckling or maternal caretaking. Animals were selected 24 hr and 6 days after the last CPF injection, and at PN60.

These prenatal and postnatal CPF regimens have been previously shown to alter brain development without eliciting overt systemic toxicity (Slotkin 1999, In press a, In press b). Behavioral differences remain apparent, or may first emerge, after weaning (Dam et al. 2000; Levin et al. 2001, 2002). For each experiment, animals were decapitated and the tissues were frozen with liquid nitrogen and stored at –45°C. Preliminary experiments showed no changes in any of the measures as a result of freezing and storage.

Membrane preparation.

Tissues were thawed and homogenized (Polytron; Brinkmann Instruments, Westbury, NY) in 39 volumes of ice-cold buffer containing 145 mM sodium chloride, 2 mM magnesium chloride, and 20 mM Tris (pH 7.5), strained through several layers of cheesecloth to remove connective tissue, and the homogenates were sedimented at 40,000 × *g* for 15 min. The pellets were washed twice by resuspension (Polytron) in homogenization buffer followed by resedimentation and were then dispersed with a homogenizer (smooth glass fitted with Teflon pestle) in a buffer consisting of 250 mM sucrose, 2 mM MgCl2, and 50 mM Tris.

Assays.

To evaluate βAR binding, aliquots of membrane preparation were incubated with [125I]iodopindolol (final concentration, 67 pM) in 145 mM NaCl, 2 mM MgCl2, 1 mM sodium ascorbate, 20 mM Tris (pH 7.5), for 20 min at room temperature in a total volume of 250 μL. Incubations were stopped by dilution with 3 mL ice-cold buffer, and the labeled membranes were trapped by rapid vacuum filtration onto glass fiber filters, which were then washed with additional buffer and counted by liquid scintillation spectrometry. Nonspecific binding was assessed by displacement with 100 μM isoproterenol.

For assessment of AC activity, aliquots of the same membrane preparation were incubated for 30 min at 30°C with final concentrations of 100 mM Tris-HCl (pH 7.4), 10 mM theophylline, 1 mM ATP, 2 mM MgCl2, 1 mg/mL bovine serum albumin, and a creatine phosphokinase–ATP–regenerating system consisting of 10 mM sodium phosphocreatine and 8 IU/mL phosphocreatine kinase, with 10 μM GTP in a total volume of 250 μL. The enzymatic reaction was stopped by placing the samples in a 90–100°C water bath for 5 min, followed by sedimentation at 3,000 × *g* for 15 min, and the supernatant solution was assayed for cAMP using radioimmunoassay. Preliminary experiments showed that the enzymatic reaction was linear well beyond the assay period and was linear with membrane protein concentration; concentrations of cofactors were optimal, and, in particular, higher concentrations of GTP produced no further augmentation of activity.

AC activity was evaluated in several ways (Figure 1). First, we measured basal AC activity without addition of any stimulants. Next, we compared the responses to activation of two stimulatory G-protein (Gs)-coupled receptors, the βAR, and glucagon receptor, using 100 μM isoproterenol or 3 μM glucagon, respectively. Third, we evaluated the effects of global activation of all G-proteins elicited by sodium fluoride (10 mM). Finally, we compared the responses of two direct AC stimulants, forskolin (100 μM) and Mn2+ (10

mM); these discriminate the effects of Gs–AC association, which selectively enhances the forskolin response (Limbird and Macmillan 1981), as well as allowing for detection of shifts in the AC isoform (Zeiders et al. 1999b). The concentrations of each stimulant produce maximal responses, as assessed in earlier studies (Auman et al. 2000, 2001; Zeiders et al. 1997, 1999a).

Data analysis

For treatments given to dams, only one fetus was used from each dam, so the number of determinations represents the number of dams. The fetuses were derived from the same litters as those used in two earlier studies on cell damage and cholinergic biomarkers (Garcia et al. 2002; Qiao et al. 2002), and effects on cholinesterase activity, maternal and fetal body weights, and other litter characteristics appear in those publications. For postnatal determinations, each litter contributed no more than one male and one female for a given set of determinations.

Data are presented as means and SEs. For convenience, some results are given as the percent change from control values, but statistical evaluations were always conducted on the original data. To establish treatment differences in receptor binding or AC activity, a global analysis of variance (ANOVA; data log transformed whenever variance was heterogeneous) was first conducted across the *in vivo* treatment groups, ages, sexes, tissues, and the measurements made on the membranes (βAR binding, AC activity under six different conditions); the latter were considered to be repeated measures because each membrane preparation was used for the multiple conditions under which AC was determined. As justified by significant interactions of treatment with the other variables, data were then subdivided to permit testing of individual treatments and AC measures that differed from control values; these were conducted by lower order ANOVAs, followed, where appropriate, by Fisher's protected least significant difference test to identify individual values for which the CPF groups differed from the corresponding control. For all tests, significance for main treatment effects was assumed at

$p < 0.05$; however, for interactions at $p < 0.1$, we also examined whether lower-order main effects were detectable after subdivision of the interactive variables (Snedecor and Cochran 1967).

For presentation, control values were combined across the multiple cohorts (controls used for administration on GD9–12, GD17–20, PN1–4, PN11–14). However, statistical comparisons of the effects of CPF were made only with the appropriately matched control cohort.

Materials.

Animals were purchased from Zivic Laboratories (Pittsburgh, PA, USA). CPF was obtained from Chem Service (West Chester, PA, USA). [125I]Iodopindolol (specific activity, 2,200 Ci/mmol) was from Perkin-Elmer Life Sciences (Boston, MA, USA), and cAMP radioimmunoassay kits were purchased from Amersham Biosciences (Piscataway, NJ, USA). All other chemicals were bought from Sigma Chemical Company (St. Louis, MO, USA).

Results

Development of βAR binding and AC in controls

In accordance with earlier results (Auman et al. 2000; McMillian et al. 1983; Navarro et al. 1991), large ontogenetic changes were observed in the development of βAR binding and AC responses to different stimulants in heart and liver over the course from late gestation to adulthood (Table 1). Cardiac βARs decreased about 50% over that period, whereas the liver showed a much more profound decrease that was paralleled by a loss of basal AC activity. The tissues also displayed large disparities in AC activities measured in the presence of specific stimulants. In the heart, activity with addition of isoproterenol, glucagon, NaF, and forskolin all increased with age, but for Mn2+, the effect was diminished in adulthood. In the liver, only the forskolin-stimulated activity was higher in adulthood than in the fetus, and all other measures showed a decrease in AC. There were only minor sex

differences in adulthood, with females showing slightly higher hepatic βAR binding and slightly higher isoproterenol-stimulated cardiac AC.

Table 1. Development of βAR binding and AC activities in controls

	GD21	PN60	
	(n=16)	Male (n=28)	Female (n=28)
Heart			
βAR binding (fmol/mg protein)	19.4±0.8	10.4±0.3*	10.4±0.2*
Basal AC	12.2±0.4	9.1±0.3*	9.6±0.3*
Isoproterenol-stimulated AC	16.2±0.5	25±1*	29±1*,**
Glucagon-stimulated AC	12.6±0.4	23.8±0.9*	26±1*
NaF-stimulated AC	42±2	64±2*	69±3*
Forskolin-stimulated AC	235±12	304±13*	300±9*
Mn2+-stimulated AC	135±7	100±5*	103±4*
Liver			
βAR binding (fmol/mg protein)	27.7±0.7	4.9±0.1*	6.5±0.2*,**
Basal AC	15.1±0.4	3.52±0.07*	3.35±0.06*
Isoproterenol-stimulated AC	27±1	4.7±0.1*	4.91±0.09*
Glucagon-stimulated AC	30±2	27.1±0.4*	26.2±0.5*
NaF-stimulated AC	46±1	28.0±0.6*	27.5±0.5*
Forskolin-stimulated AC	41±3	49±2*	45±1*
Mn2+-stimulated AC	63±2	50±1*	49±1*

Values shown are in picomoles per minute per milligram of protein except where noted. Values were combined across multiple cohorts (controls used for CPF administration on GD9–12, GD17–20, PN1–4, and PN11–14); however, statistical comparisons of the effects of CPF were made only with the appropriately matched control cohort.
*Significantly different from GD21.
**Significantly different from male.

Because the stimulant-associated AC activities were superimposed on large ontogenetic changes in basal AC, the values were reassessed as the specific response, the ratio of AC with stimulant to basal AC. Despite the ontogenetic decline in βAR binding, the cardiac AC response to isoproterenol doubled between GD21 and adulthood, paralleled by a similar rise in the response to glucagon (Figure 2A). In contrast, the hepatic AC response to

isoproterenol declined significantly in adulthood, whereas the response to glucagon was markedly higher than in the fetus. Disparities between the two tissues were similarly displayed by the response to direct AC stimulants (Figure 2B). In the heart on GD21, the response to forskolin was nearly double that to Mn^{2+}, and the forskolin response increased with age, whereas that to Mn2+ did not. Accordingly, the forskolin:Mn2+ preference ratio doubled over the course of development. In the liver, the initial response to Mn2+ was greater than that to forskolin; although both stimulants showed an increased response in adulthood, the preferential effect for Mn2+ was lost, such that nearly equivalent responses were seen for the two stimulants. Accordingly, both tissues showed ontogenetic changes in the forskolin:Mn2+ preference ratio suggestive of an isoform shift (Zeiders et al. 1999b).

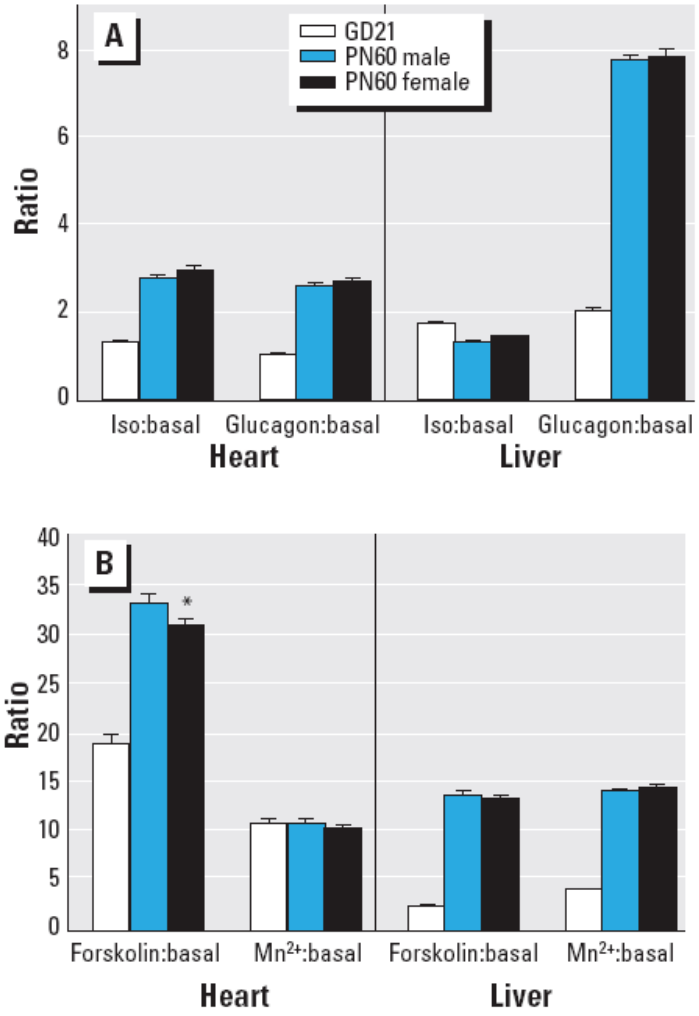


Figure 2. Development of responses to AC stimulants in control animals. (*A*) Responses to receptormediated stimulation by isoproterenol (Iso) or glucagon. (*B*) Responses to direct AC stimulants forskolin and Mn2+. Data represent means and SEs of the ratios of each stimulant to basal AC activity, obtained from 16–28 animals in each group. With the exception of cardiac Mn2+ responses, ANOVA indicates a significant change with age ($p < 0.0001$).
*The only response for which a sex difference was seen on PN60.

Systemic toxicity of CPF

In keeping with earlier reports (Garcia et al. 2002; Qiao et al. 2002), CPF treatment on GD9–12 impaired maternal weight with a threshold at 5 mg/kg, displaying small, transient deficits that resolved completely by parturition (data not shown). Similarly, as found before (Garcia et al. 2002; Qiao et al. 2002), the GD17–20 CPF regimen had a similar threshold, with progressively larger deficits as the dose was raised to 10, 20, or 40 mg/kg (data not shown). With either regimen, GD21 fetal heart weights remained normal at all CPF doses, but liver weights were reduced in animals receiving doses above 5 mg/kg; the effects on tissue weights have already been reported (Garcia et al. 2002; Qiao et al. 2002). Neither regimen affected the number of fetuses or fetal viability, nor were there effects on the number of pups at term or on neonatal viability. As found earlier, the postnatal treatment regimens had no significant effects on body, heart, or liver weights 24 hr or 6 days after the end of CPF treatment (Auman et al. 2000; Song et al. 1997).

In adulthood, animals receiving prenatal or postnatal CPF regimens did not display any consistent differences in body weights (data not shown), in agreement with earlier reports (Dam et al. 1999; Qiao et al. 2003); the group exposed to CPF on GD9–12 showed a small (< 10%) reduction in body weight at 1 mg/kg ($p < 0.006$) but not at 5 mg/kg. Heart weights were similarly unaffected by all treatments except the GD9–12 regimen, which displayed the same inconsistent effect, a 7% reduction at 1 mg/kg ($p < 0.006$) without any significant difference at 5 mg/kg. Comparisons of liver weights were not done because only one lobe was sampled on PN60.

CPF exposure on GD9–12

In the heart, animals treated with CPF on GD9–12 showed immediate (GD21) and long-term (PN60) alterations in βAR binding and AC responses. On GD21, there was significant up-regulation of cardiac βARs at the two lowest CPF doses, but the effect disappeared when the dose was raised to 5 mg/kg, above the threshold for systemic toxicity (Figure 3A). Consistent with

this pattern, AC activities also showed augmentation at subtoxic CPF exposures and loss of the effect at 5 mg/kg. This hormetic effect was statistically significant across all AC measures ($p < 0.02$ for CPF 5 mg/kg vs. 1 mg/kg; $p < 0.01$ for CPF 5 mg/kg vs. CPF 2 mg/kg). For AC activities, the most profound effect was on the response to Mn2+, which showed elevations as high as 60% above control values, effects that far exceeded the smaller induction of the other AC measures. In particular, there was a statistically significant ($p < 0.005$) preferential effect on the Mn2+ response compared with the forskolin response, suggestive of an AC isoform shift. A smaller but significant ($p < 0.03$) shift was seen in the ratio of NaF to basal activity, indicative of a defect in G-protein signal transduction: control, 3.27 ± 0.21; CPF 1 mg/kg, 2.70 ± 0.09 ($p < 0.005$); CPF 2 mg/kg, 2.76 ± 0.05 ($p < 0.02$); CPF 5 mg/kg, 2.93 ± 0.11.

In contrast to the effects seen in the heart, the immediate (GD21) alterations in hepatic βARs and AC were much less notable, and neither set of variables achieved overall statistical significance (Figure 3B). The lack of significant effect in the liver was statistically distinguishable from the changes seen in the heart, as evidenced by interactions of treatment × tissue ($p < 0.0001$) and treatment × tissue × measure ($p < 0.0001$).

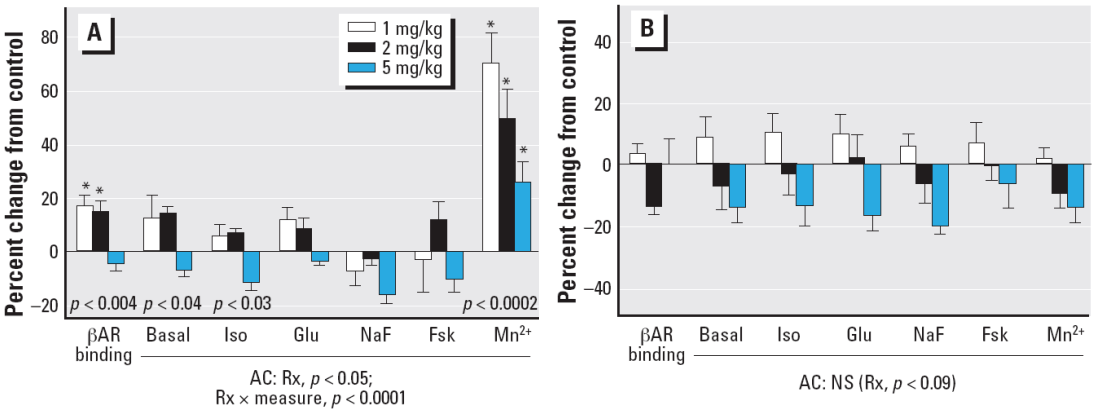


Figure 3. Effects of GD9–12 CPF exposure on (*A*) cardiac and (*B*) hepatic βAR binding and AC activity on GD21. Abbreviations: Fsk, forskolin; Glu, glucagon; Iso, isoproterenol; NS, not significant; Rx, treatment. Data represent means and SEs obtained from six animals in each treatment group, shown as the percent change from corresponding control values (Table 1). Across both tissues, ANOVA indicates significant treatment effects that differed between the two tissues, justifying separate comparisons for heart and liver: βAR binding, $p < 0.004$ for treatment × tissue; AC activities, $p < 0.0001$ for treatment × tissue, $p < 0.0001$ for treatment × tissue × measure. Lower-order ANOVAs for each tissue are shown within the figure. *Individual groups differ significantly from the control (calculated only for variables showing a significant overall effect by ANOVA).

At adulthood (PN60), animals exposed to CPF on GD9–12 showed major alterations in βARs and AC signaling profiles. In the heart (Figure 4A), animals that received the low dose of CPF (1 mg/kg) displayed overall increases in AC measures that were sex selective, with significant elevations in males but not in females. There were no differential effects detected among the various AC measures, implying that activity was enhanced globally (i.e., induction of total AC activity), rather than involving specific changes in receptor-mediated responses, G-protein coupling, or AC isoform shifts. To evaluate whether these effects were secondary to systemic toxicity of CPF, we also evaluated a higher dose (5 mg/kg) that was at the threshold for impairment of maternal weight gain during the treatment period; presumably, effects due to toxicity would show an enhancement at the higher dose. Instead, however, we found the same pattern but with smaller effects: AC elevations in males were no longer statistically significant, and values in females tended to be subnormal. Across both the heart and liver on PN60, the main effect of CPF was statistically significant ($p < 0.04$), but when hepatic values were examined separately, the tendency toward elevated AC activities was only at the margin of significance (Figure 4B). Hepatic βAR binding was significantly elevated only in males.

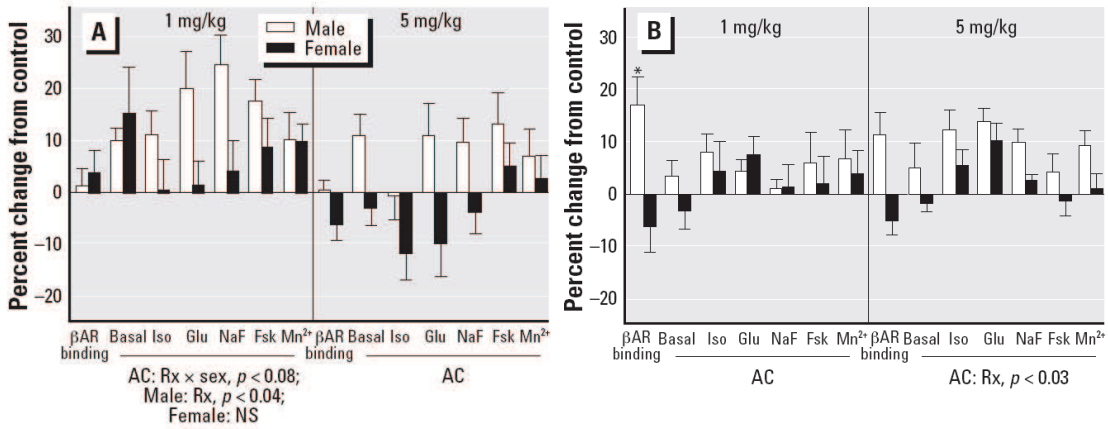
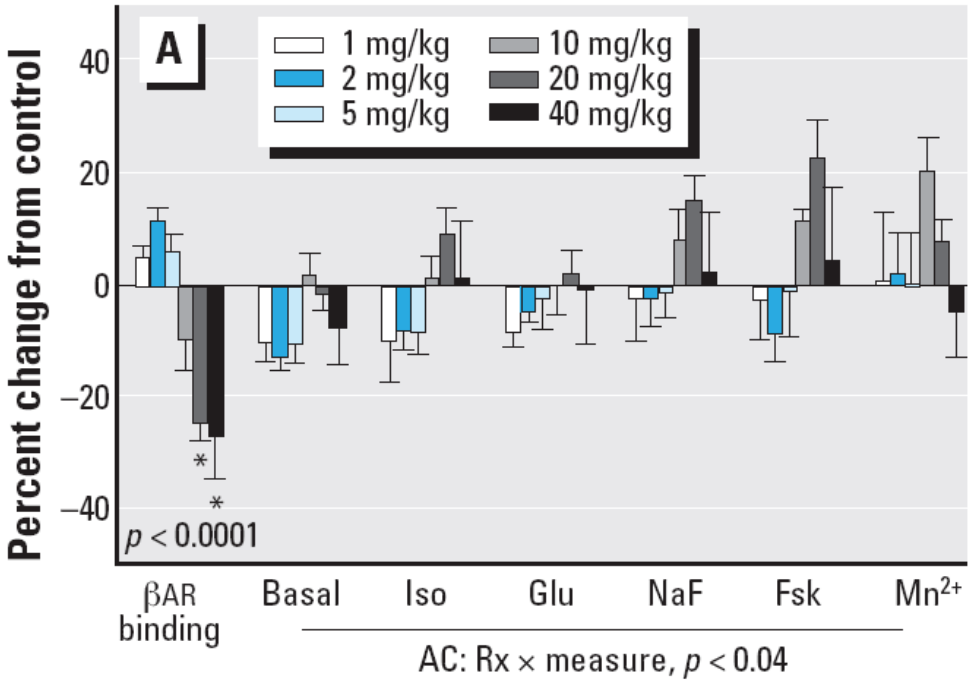


Figure 4. Effects of GD9–12 CPF exposure on (*A*) cardiac and (*B*) hepatic βAR binding and AC activity on PN60. Abbreviations: Fsk, forskolin; Glu, glucagon; Iso, isoproterenol; NS, not significant; Rx, treatment. Data represent means and SEs obtained from eight animals in each treatment group for each sex, shown as the percent change from corresponding control values (Table 1). Across both tissues, ANOVA indicates significant sex-dependent effects on βAR binding ($p < 0.04$ for treatment × sex). For AC activities, the overall ANOVA showed treatment effects that differed between the two tissues and among the different AC measures ($p < 0.04$ for treatment × tissue × measure), necessitating separate comparisons for heart and liver. ANOVAs across both CPF doses are as follows: for (*A*), Rx × measure, $p < 0.1$; Rx × sex × measure, $p < 0.02$; for (*B*), NS ($p < 0.09$). ANOVAs for each dose are shown within the figure. Significance testing for individual AC values was not done because of the absence of treatment × measure interactions after subdivision of the data into the separate tissues and doses. *Individual group differs significantly from control.

CPF exposure on GD17–20

Animals treated with CPF during late gestation (GD17–20) presented a different pattern of immediate and delayed-onset alterations in βAR binding and AC activity from those receiving treatment earlier in gestation. For the immediate effects on GD21, the response patterns for animals receiving CPF on GD17–20 were statistically distinguishable from those treated on GD9–12 ($p < 0.05$ for treatment × regimen). In the heart (Figure 5A), GD17–20 exposure at CPF doses below (1 or 2 mg/kg) and up to the threshold (5 mg/kg) for systemic toxicity failed to cause significant alterations in βAR binding or any of the AC measures on GD21. Raising the dose further elicited significant decrements in cardiac βAR binding but still failed to cause any consistent changes in AC. In the liver, CPF exposures of 1, 2, or 5 mg/kg similarly did not alter any of the parameters, but higher doses suppressed virtually all AC measures in a parallel manner (Figure 5B).



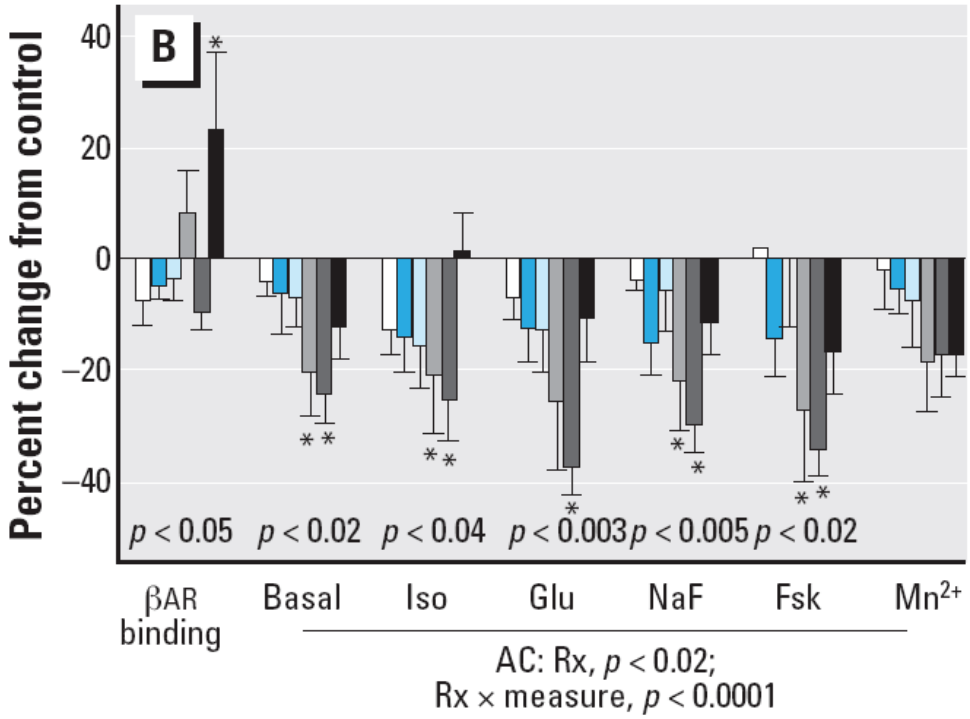


Figure 5. Effects of GD17–20 CPF exposure on (*A*) cardiac and (*B*) hepatic βAR binding and AC activity on GD21. Abbreviations: Fsk, forskolin; Glu, glucagon; Iso, isoproterenol; Rx, treatment. Data represent means and SEs obtained from six animals in each treatment group, shown as the percent change from corresponding control values (Table 1). Across both tissues, ANOVA indicates significant treatment effects that differed between the two tissues, justifying separate comparisons for heart and liver: βAR binding, $p < 0.0001$ for treatment × tissue; AC activities, $p < 0.004$ for treatment tissue, $p < 0.02$ for treatment × tissue × measure. Lower-order ANOVAs for each tissue are shown within the figure. *Individual groups differ significantly (calculated only for variables showing a significant overall effect by ANOVA).

For examination of effects in adulthood (PN60), we again chose two dose groups, one (1 mg/kg) well below the threshold for fetal cholinesterase inhibition or impaired maternal weight gain (Qiao et al. 2002) and one (5 mg/kg) at or above the threshold. Animals exposed to 1 mg/kg CPF on GD17–20 displayed sex-dependent effects on cardiac βARs and AC activities in adulthood (Figure 6A). Males showed elevated βAR binding, although this effect did not correspond to an elevation in the cardiac AC response to isoproterenol. Instead, most of the AC measures found to be increased were in females, with the notable exception of glucagon-stimulated AC, which was unchanged. Because both βARs and glucagon receptors operate through Gs to stimulate AC, we compared the effect on the isoproterenol response with

that on the glucagon response and found a significant reduction ($p < 0.006$) in the relative response to glucagon (glucagon:isoproterenol response ratio, 0.97 ± 0.04 in control females, 0.82 ± 0.03 in females exposed to CPF 1 mg/kg). Again, raising the CPF exposure above the threshold for systemic effects did not produce significant enhancement of the long-term effects on cardiac βARs or AC activity and instead diminished the βAR effect in males; in addition, the higher dose elicited significant reductions in cardiac AC measures that were not seen at the lower exposure. In the liver on PN60 (Figure 6B), animals exposed to 1 mg/kg CPF on GD17–20 displayed significant suppression of glucagons and forskolin-stimulated AC activity, without demonstrable sex selectivity. Raising the dose above the threshold for systemic toxicity (5 mg/kg) did not intensify the effects but rather reduced them.

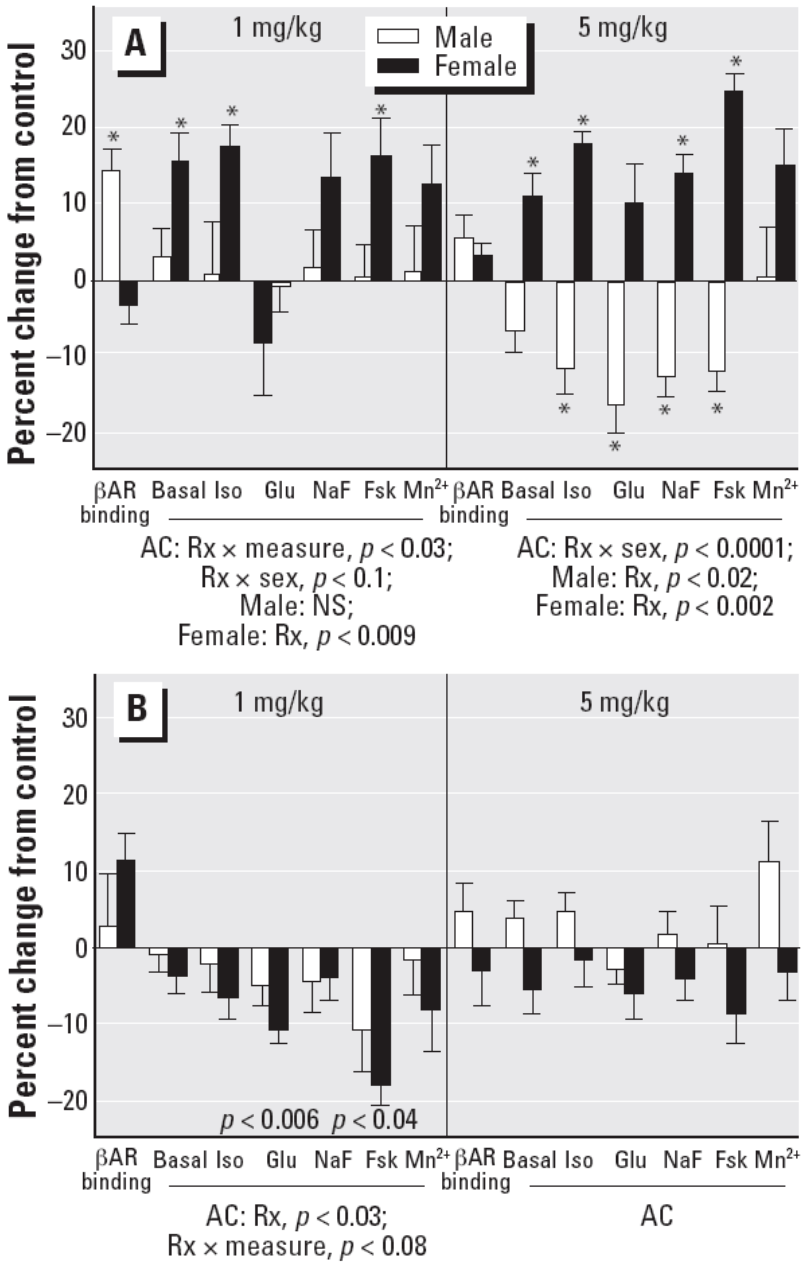


Figure 6. Effects of GD17–20 CPF exposure on (*A*) cardiac and (*B*) hepatic βAR binding and AC activity on PN60. Abbreviations: Fsk, forskolin; Glu, glucagon; Iso, isoproterenol; NS, not significant; Rx, treatment. Data represent means and SEs obtained from eight animals in each treatment group for each sex, shown as the percent change from corresponding control values (Table 1). Across both tissues, ANOVA indicates significant sex- and tissue-dependent effects on βAR binding ($p < 0.05$ for treatment × sex × tissue). For AC activities, the overall ANOVA showed treatment effects that differed between the two tissues, between sexes, and among the different AC measures ($p < 0.02$ for treatment × tissue; $p < 0.007$ for treatment × measure; $p < 0.006$ for treatment × sex × measure), necessitating separate comparisons for heart and liver. ANOVAs across both CPF doses are as follows: for (*A*), Rx × sex, $p < 0.003$; Rx × measure, $p < 0.05$; male: NS; female: Rx, $p < 0.005$; for (*B*), Rx × measure, $p < 0.08$. ANOVAs for each dose are shown within the figure. *Individual groups differ significantly from the control (calculated only for variables showing significant treatment × measure and treatment × sex interactions after separation by dose; otherwise, only the main effects are shown.

CPF exposure on PN1–4

In an earlier study (Song et al. 1997), we found that exposure to 1 mg/kg CPF on PN1–4 evoked short-term (PN5, PN10) deficits in basal cardiac AC activity without significant effects on the response to AC stimulants; there were no effects on βAR binding. Evaluation of these animals in adulthood (PN60) indicated no significant overall effect (Figure 7A), although AC values tended to be decreased by the same magnitude (10%) as that obtained for the immediate post treatment effects.

As reported previously (Auman et al. 2000), CPF treatment on PN1–4 produced transient elevations in hepatic AC responses to glucagons and Mn2+ that disappeared by PN10. In the present study, we also assessed short-term effects on hepatic βAR binding, none of which was statistically significant: PN5—control male, 16.9 ± 0.5 fmol/mg protein (n = 5); control female, 14.5 ± 0.6 (n = 4); CPF male, 15.8 ± 1.1 (n = 4); CPF female, 14.7 ± 0.5 (5); PN10—14.5 ± 0.8 (n = 6), 12.5 ± 0.6 (n = 4), 13.2 ± 0.7 (n = 7), and 12.3 ± 0.8 (n = 7), respectively. When these animals reached adulthood, we still did not detect alterations in βAR binding, but there were major, sex-dependent effects on AC activities (Figure 7B). Basal AC activity was unaffected, but males showed elevations of AC responses to stimulants with the notable exception of Mn2+. In females, AC responses to stimulants were not augmented and tended instead to be reduced. Because of the strong, differential effect on AC responses to specific stimulants, we characterized the pattern by calculation of response ratios (Figure 7C).

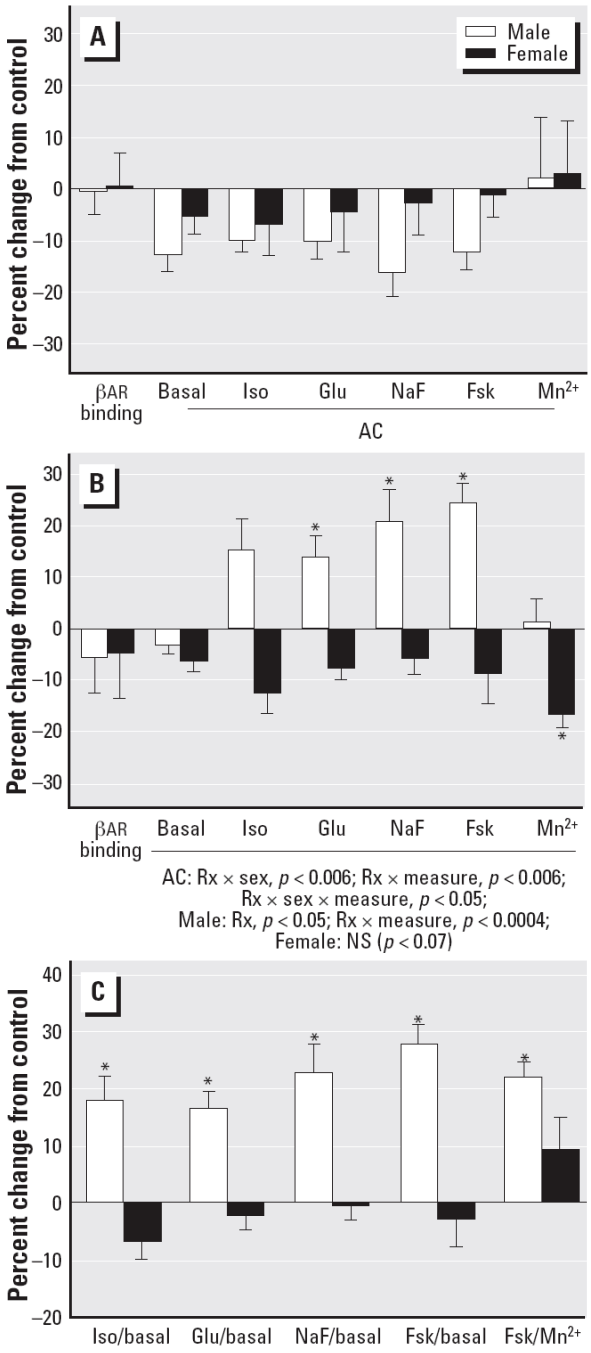


Figure 7. Effects of PN1–4 CPF exposure (1 mg/kg) on (*A*) cardiac and (*B*) hepatic βAR binding and AC activity on PN60, and (*C*) activity ratios for liver values. Abbreviations: Fsk, forskolin; Glu, glucagon; Iso, isoproterenol; NS, not significant; Rx, treatment. Data represent means and SEs obtained from six animals for each sex, shown as the percent change from corresponding control values (Table 1). Across both tissues, ANOVA indicates no significant effects on βAR binding. For AC activities, the overall ANOVA showed treatment effects that differed between the two tissues, between sexes, and among the different AC measures ($p < 0.04$ for treatment × sex × tissue; $p < 0.04$ for treatment × tissue × measure), necessitating separate comparisons for heart and liver. Lower-order ANOVAs are shown within the figure.
*Individual groups differ significantly from the control (calculated only for variables showing significant treatment × measure and treatment × sex interactions).

CPF exposure on PN1–4 elicited specific activation of the responses to stimulants operating through G-proteins (isoproterenol, glucagon, NaF) and preferentially enhanced the response to forskolin as opposed to Mn^{2+}. We also performed an additional experiment to identify whether the delayed-onset effects on hepatic AC signaling appeared earlier (Figure 8). By PN30, the elevations of AC stimulant responses had not yet appeared, and in fact, activities were significantly lower than in controls, again with a preferential effect toward males.

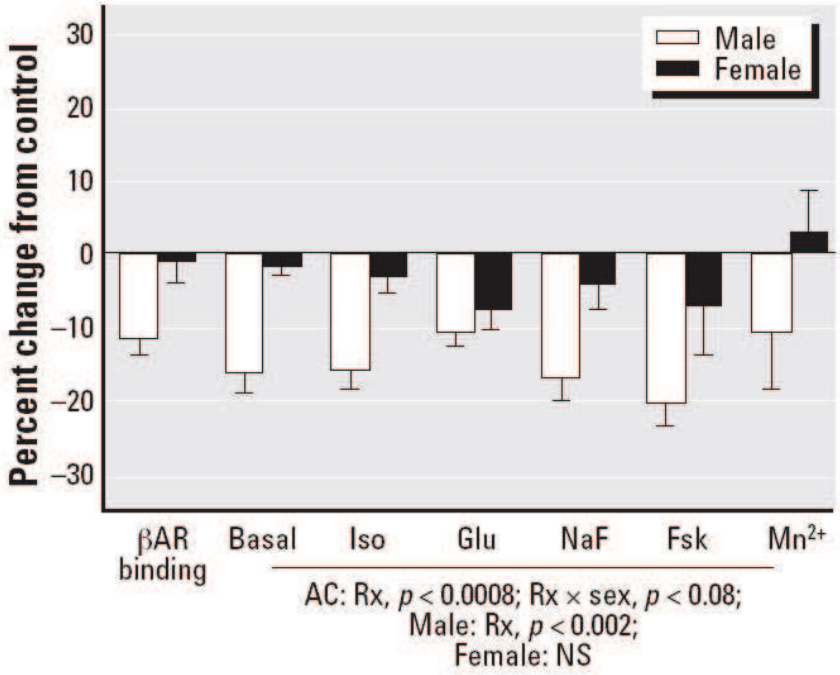


Figure 8. Effects of PN1–4 CPF exposure (1 mg/kg) on hepatic βAR binding and AC activity on PN30. Abbreviations: Fsk, forskolin; Glu, glucagon; Iso, isoproterenol; NS, not significant; Rx, treatment. Data represent means and SEs obtained from six animals for each sex, shown as the percent change from corresponding control values for males and females, respectively: βAR binding, 5.7 ± 0.2 and 6.0 ± 0.3 fmol/mg protein; basal AC, 3.3 ± 0.1 and 2.8 ± 0.1 pmol/min/mg protein; isoproterenolstimulated AC, 5.1 ± 0.2 and 4.7 ± 0.2 pmol/min/mg protein; glucagon-stimulated AC, 27 ± 1 and 25 ± 2 pmol/min/mg protein; NaF-stimulated AC, 33 ± 1 and 30 ± 1 pmol/min/mg protein; forskolin-stimulated AC, 65 ± 1 and 53 ± 2 pmol/min/mg protein; Mn2+-stimulated AC, 51 ± 2 and 41 ± 1 pmol/min/mg protein. ANOVA indicates no significant CPF effects on βAR binding. For AC activities, the overall ANOVA (shown within the figure) displayed treatment effects that differed between the two sexes but not among the different AC measures, so lower-order tests were not done for the individual measures. Main treatment effects for each sex are also shown.

CPF exposure on PN11–14

As evaluated earlier (Song et al. 1997), animals exposed to 5 mg/kg CPF on PN11–14 showed reductions in cardiac βAR binding and AC responses to βAR stimulation on PN20. In the liver, the short-term effects consisted of transient elevations in AC responses to glucagon and Mn2+ that disappeared by PN20 (Auman et al. 2000). We did not find significant effects on hepatic βAR binding on PN15 or PN20: PN15—control male, 9.3 ± 0.5 fmol/mg protein (n = 6); control female, 9.0 ± 1.7 (n = 6); CPF male, 9.8 ± 0.6 (n = 6); CPF female, 6.9 ± 0.8 (n = 6); PN20—4.4 ± 0.5 (n = 6), 4.5 ± 0.3 (n = 6), 4.2 ± 0.4 (n = 6), and 4.0 ± 0.5 (n = 6), respectively.

In adulthood, we found the same magnitude and direction of effect on cardiac AC as was seen previously for short-term (PN20) evaluations (Song et al. 1997), although without achieving statistical significance in the present study (Figure 9A). A similar pattern was seen in the liver (Figure 9B), and ANOVA combined across the two tissues indicated a significant overall AC reduction ($p < 0.05$) despite a lack of significance for either tissue considered separately.

Discussion

Results of this study indicate that developmental CPF exposure elicits immediate and lateemerging alterations in AC-mediated cell signaling in the heart and liver, extending the adverse effects of CPF beyond neurotoxicity. Although CPF at high concentrations can interact directly with neurotransmitter receptors and/or AC (Huff and Abou-Donia 1995; Huff et al. 1994, 2001; Ward and Mundy 1996), our results indicate that such direct actions are unlikely to explain the net effects on the development of AC signaling after *in vivo* exposure: we found distinct sex-, tissue-, and age-selective actions that are incompatible with a unitary, stoichiometric interaction between CPF and the signaling proteins. Indeed, the fact that many of the effects emerged after a prolonged delay (i.e., between PN30 and PN60) indicates instead that CPF exposure alters the programming of the

development of cell signaling. In light of the disparate effects of the different CPF treatment windows, it is worthwhile to examine the characteristics that are shared by, or that differentiate among, the various treatment paradigms before arriving at general conclusions.

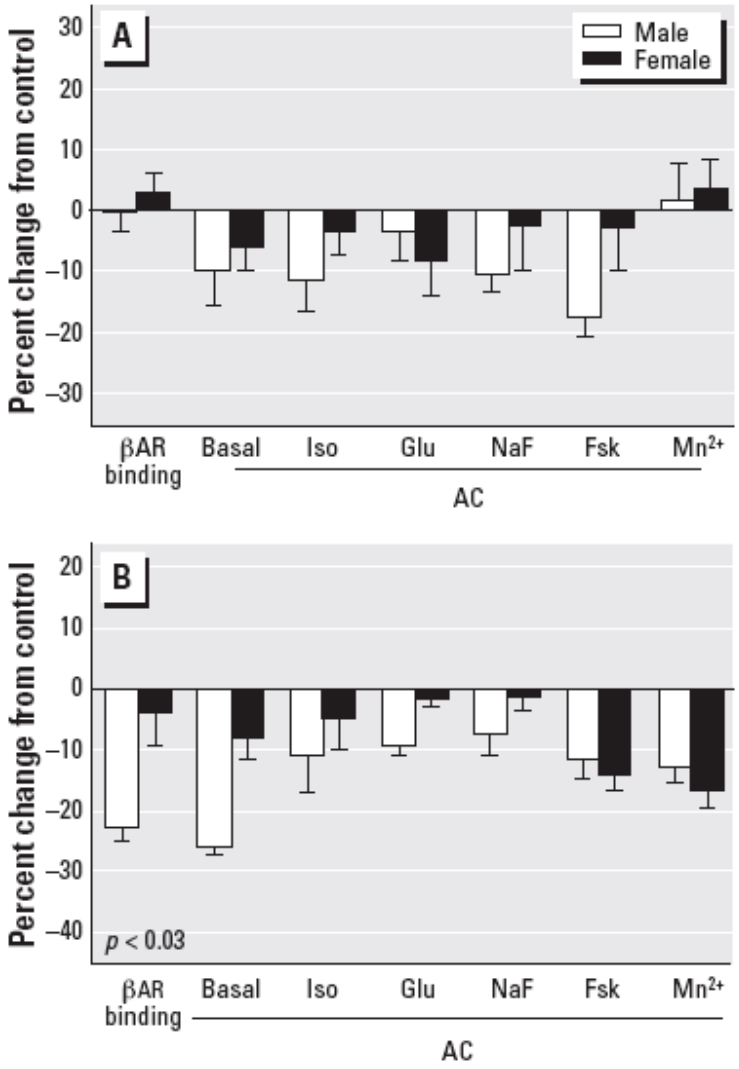


Figure 9. Effects of PN11–14 CPF exposure (5 mg/kg) on (*A*) cardiac and (*B*) hepatic βAR binding and AC activity on PN60. Abbreviations: Fsk, forskolin; Glu, glucagon; Iso, isoproterenol; Rx, treatment. Data represent means and SEs obtained from six animals for each sex, shown as the percent change from corresponding control values (Table 1). Across both tissues, ANOVA indicates effects on βAR binding that differed between tissues and sexes ($p < 0.1$ for treatment × sex; $p < 0.03$ for treatment × tissue), but the sex interaction was not maintained when the values were separated into the two tissues; accordingly, only the main effect is shown (*B*). For AC activities, the overall ANOVA showed only a main treatment effect ($p < 0.05$) without significant interactions of treatment with other variables, so separate tests or subtests were not done for the two tissues, although the values are shown separately.

Although the major effects of CPF emerged in adulthood, some acute effects were detected in the period immediately after exposure. With treatment on GD9–12, there was a shift in the cardiac AC isoform, evidenced by a preferential increase in the response to Mn^{2+} as opposed to forskolin (Zeiders et al. 1999b). For the GD17–20 treatment window, adverse effects on hepatic signaling profiles emerged at CPF doses above the threshold for systemic toxicity. Similarly, our earlier work detailed short-term effects of postnatal CPF treatments on AC (Auman et al. 2000; Song et al. 1997). However, none of these effects was sustained, nor are they likely to explain the long-term changes identified later. For example, there were no immediate effects for the GD17–20 regimen at the subtoxic doses that nevertheless produced robust effects on signaling in adulthood; similar discrepancies between acute and long-term effects have already been noted in the presentation of each set of results. In one case, similar outcomes were obtained over both the immediate and the long-term time frames, namely, the cardiac effects of CPF exposure on PN11–14, but even in this situation the magnitude of impairment was small and nonsignificant. In general, then, the acute effects of CPF on AC signaling were poorly correlated with the subsequent emergence of major, sex-dependent, tissue-selective functional alterations, implying that CPF affects the developmental program for cell signaling.

The second common feature for all the exposure models is the fact that many of the emergent effects on signaling were heterologous; that is, they involved ubiquitous changes in the function of signaling proteins themselves rather than in the specific responses to receptor stimulation. Thus, the effects on AC tended to appear in "clusters," with increases or decreases shared by different receptor stimulants (isoproterenol, glucagon), by a G-protein activator (NaF), or by AC stimulants that bypass receptors (forskolin) or receptors and G-proteins (Mn2+). These heterologous changes thus indicate that CPF alters the ability of the entire signaling cascade to respond to the multiple neuronal and hormonal inputs that elicit cellular responses through AC. Nevertheless, superimposed on these heterologous effects, we

did detect instances where CPF exposure affected a specific input to the transduction pathway. As one example, the effects on cardiac AC signaling after exposure to 1 mg/kg CPF on GD17–20 included specific impairment of the response to glucagon in adulthood, superimposed on heterologous activation of other AC responses. We also found evidence for AC isoform shifts, such as the alteration in the preference ratio for forskolin versus Mn2+ in the liver after PN1–4 CPF exposure; because the various AC isoforms have distinct catalytic properties and differential responses to second messengers, these too may provide a basis for more selective functional alterations despite a common origin in heterologous AC effects. An additional feature common to all the exposure models is the general dissociation of effects on βAR binding from the AC response to the βAR stimulant isoproterenol. Thus, on GD21, the two prenatal CPF exposure regimens affected cardiac βARs but not the corresponding AC response; in the liver, the GD17–20 treatment group displayed no change or an increase in βARs on GD21, but a decrease in the isoproterenol AC response. A similar lack of parallelism was seen for the other treatment paradigms and for the delayed-onset alterations determined in adulthood. These results are consistent with the view that the signaling proteins downstream from the receptors are the major determinants of functional responses (Gao et al. 1998, 1999; Navarro et al. 1991), so evaluations of receptor binding alone may be entirely misleading for the interpretation of physiologic consequences. Despite the fact that all the CPF exposure windows shared major features of their effects on cell signaling, there were distinct differences that depended on the period of exposure. Focusing on the long-term effects of low-dose CPF exposures, cardiac AC showed augmentation with the prenatal treatment windows, with a different critical period for effects on males (GD9–12) versus females (GD17–20). Smaller, nonsignificant inhibitory effects were seen with postnatal exposures; therefore, the critical window of sensitivity for CPF-induced perturbation of cardiac AC signaling must begin to close soon after birth. The liver displayed a different pattern, with sensitivity first emerging late in gestation, characterized by long-term inhibitory effects after GD17–20

exposure. With later treatment on PN1–4, effects were stimulatory and restricted to males, whereas again, a shift to even later treatment (PN11–14) elicited only small, nonsignificant decrements. As a general observation, then, the effects of CPF on the programming of AC cell signaling occur during a distinct developmental window of vulnerability that closes in the second postnatal week.

An additional point of interest is the sex dependence of the effects of CPF on cardiac and hepatic cell signaling, which shifts radically according to the developmental window in which exposure occurs. In the present study, males were preferentially affected by most treatment paradigms, with the notable exception of the selective effects on females seen with the GD17–20 exposure. Although we have no information about the mechanisms underlying sex-selective actions, it is notable that similar shifts have been seen for effects on behavior from these particular treatment windows (Dam et al. 2000; Levin et al. 2001, 2002). The late gestational period is associated with sexual differentiation of the brain (Rodier 1988), and it is tempting to attribute the unique pattern seen with exposure on GD17–20 to effects on sexual dimorphism of neural pathways. CPF is only weakly estrogenic (Andersen et al. 2002; Vinggaard et al. 2000), but certainly, secondary endocrine effects are feasible (Guven et al. 1999). However, the fact that sex-selective effects were seen even earlier, with GD9–12 exposure, makes it unlikely that all the differences can be explained by a primary action on sexual differentiation of the brain. Obviously, this is an issue of considerable importance for future investigation. Finally, there are two elements of the delayed-onset emergence of abnormalities in AC signaling that need to be addressed. First, the alterations in receptor binding and AC biomarkers ranged up to about 20%; this is approximately the magnitude of the changes evoked by CPF in the developing brain (Aldridge et al. 2004; Meyer et al. 2003; Song et al. 1997), by other defined neurotoxicants such as nicotine (Slotkin et al. 1992), or by the loss of function associated with aging (Fraeyman et al. 2000; Kilts et al. 2002). Relatively small changes have important functional consequences because the AC pathway amplifies

receptor signals by orders of magnitude: the activation of a single AC molecule leads to the production of numerous cAMP molecules and consequent activation of protein kinase A, which in turn elicits a plethora of downstream phosphorylation events (Freissmuth et al. 1989; Gilman 1989; Stiles 1989; Weiss et al. 1988). During development, these types of changes produce massive changes in cellular functions linked to cAMP despite modest changes in elements of the signaling cascade itself (Schuh et al. 2002; Song et al. 1997). Second, the dose–response relationships seen here are often complex. For example, although many of the acute effects of CPF show a typical, monotonic relationship to dose, we noted several instances where the effects did not progress beyond those seen at lower doses, and even cases where effects displayed hormesis, with larger alterations at low doses (e.g., Figures 3A, 4A). Indeed, a number of other studies indicate nonmonotonic effects of CPF at the biochemical and behavioral levels (Levin et al. 2001, 2002; Meyer et al. 2003; Qiao et al. 2002, 2003), and we recently found that this extends to AC signaling in the brain (Aldridge et al. 2003, 2004). One important distinction is whether a given dose lies above the threshold for cholinesterase inhibition, because, during fetal development, cholinergic input can lead to lasting improvements in neural performance (Meck and Williams 1997, 1999; Montoya et al. 2000). The doses used here bracket the threshold for fetal cholinesterase inhibition (Qiao et al. 2002). Given the clear involvement of neural input in the cardiac and hepatic effect or mechanisms studied here, similar factors may then produce hormetic responses to AC in these peripheral tissues. The results obtained here indicate that apparently subtoxic developmental exposures to CPF, even at levels that do not produce significant inhibition of cholinesterase (Qiao et al. 2002), alter cardiac and hepatic cellular function in adulthood. There are four important characteristics of these effects. First, the alterations involve heterologous changes in signaling components (G-proteins, AC itself) that are shared by multiple neuronal and hormonal stimulants. Accordingly, there will be global effects on the responses to diverse inputs. Second, these effects are sex selective, so future work with animal models or human populations will need

to take sex differences into account. Third, the effects emerge late in development, thus requiring longitudinal evaluations. Finally, superimposed on the heterologous changes in AC signaling, we found specific alterations in the responses to glucagons and βAR stimulation, both of which play critical roles in cardiovascular and metabolic homeostasis, and with regard to the latter, specifically glucose homeostasis. The secretion of insulin, the counterbalancing hormone for glucagon, proceeds through activation of AC (Gao et al. 2002), so the heterologous augmentation of AC signaling caused by CPF exposure is likely to amplify the physiologic effect of a superimposed deficiency in the glucagons response. It is now recognized that diseases that occupy a distinct cluster—hypertension, obesity, and diabetes—may have significant dependence on prenatal stress and/or toxicant exposures (Dodic et al. 1999, 2001; Nyirenda and Seckl 1998; Power and Jefferis 2002; Slikker and Schwetz 2003; Toschke et al. 2002). The present results point to the possibility that otherwise subtoxic, nonsymptomatic developmental exposure may provide predisposition to these types of diseases, with a specific component of delayed-onset effects. As such, the adverse adult outcomes predicted by the Barker Hypothesis may actually extend to exposures below the threshold for fetal growth impairment. Indeed, a number of the effects on signaling cascades seen here for CPF exposure have been examined in transgenic mice overexpressing the corresponding receptors or downstream transduction proteins, and clearly indicate the emergence of cardiac myopathies as well as hepatic dysfunction and damage (Andre et al. 1999; Asai et al. 1999; Du et al. 2000; Singh et al. 2001; Vatner et al. 1999). Nevertheless, given the multifactorial nature of hypertension and diabetes, it seems unlikely that the magnitude of the effects of developmental CPF exposure on the AC cascade alone would trigger these diseases, but instead might provide a risk factor that acts in concert with other comorbidities. Studies of CPF effects in animal models of hypertension, obesity, and diabetes are likely to resolve this issue, in concert with examination of human cohorts with significant fetal or childhood CPF exposures.

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5 – Considerações Gerais e Conclusão

Cada um dos artigos que compõem esta tese apresenta extensa e detalhada discussão sobre os aspectos específicos de cada experimento e seus respectivos resultados. Assim, neste capítulo irei discutir apenas os aspectos mais gerais de cada um deles. Entretanto, além disso, mas principalmente como os resultados destes três trabalhos

5.1 - Aspectos Gerais Sobre os Resultados da Tese

Os resultados deste trabalho indicam que não só a exposição neonatal, mas também a exposição fetal a CPF produz alterações imediatas sobre a sinalização celular mediada pela AC no sistema nervoso. Além disso, tanto a exposição pré- como a exposição pós-natal produziram alterações de longo prazo na atividade da AC em diversas regiões cerebrais. Estas alterações se mostraram dependentes da região estudada, do sexo do animal e do período de exposição. Os resultados aqui apresentados também demonstraram que a exposição a CPF durante o período de desenvolvimento produz alterações imediatas e de longo prazo na sinalização celular do coração e do fígado, ampliando assim os efeitos deste inseticida para além do efeito neurotóxico. Estas alterações também demonstraram especificidades quanto ao orgão, sexo e período de exposição.

De uma forma geral, a magnitude das alterações na atividade da AC observadas no primeiro artigo situou-se em torno de 40% em relação ao controle. Já nos outros 2 estudos, salvo algumas exceções em que as alterações foram ainda mais elevadas, a magnitude desses efeitos foram ao redor dos 20% em relação ao controle, o que pode ser interpretado por alguns como alterações de pequena monta. Entretanto, é importante ressaltar que estas alterações não se refletem necessariamente em alterações funcionais pequenas. Uma característica marcante das vias de sinalização celular em cascata é a amplificação do sinal, cascata abaixo (Schmelck and Hanoune 1980; Feliciello et al. 2001). No caso do AMPc, uma única molecula de AC pode catalizar a produção de inúmeras moléculas deste

segundo mensageiro, que irão ativar outras tantas moléculas de PKA, que por sua vez possui muitos alvos biológicos intracelulares (Weiss et al. 1988; Freissmuth et al. 1989; Gilman 1989; Stiles 1989). Além disso, a magnitude das alterações apresentadas aqui esta de acordo com relatos anteriores anteriores de efeitos do CPF (Song et al. 1997; Auman et al. 2000) e da nicotina (Slotkin et al. 1992), sobre a atividade da AC.

Uma questão que permeou grande parte dos resultados deste trabalho foi a clara diferença nas alterações observadas em machos e fêmeas, face a regimes específicos de exposição ao CPF. A sensibilidade diferenciada de machos e fêmeas a exposições a diferentes drogas é um fenômeno relativamente bem conhecido, embora pouco explorado nos estudos de toxicidade (Gandhi et al. 2004). Diversos estudos têm demonstrado que o efeito tóxico do CPF durante o desenvolvimento depende do sexo (Moser and Padilla 1998; Levin et al. 2001; Levin et al. 2002; Aldridge et al. 2004; Meyer et al. 2005). Neste trabalho, cabe ressaltar aqui alguns exemplos desse fenômeno. Na figura 2 do segundo artigo, observamos que enquanto o perfil de alterações na densidade de βAR, nas diversas regiões cerebrais de machos adultos (PN60), produzidas pela exposição a CPF (1mg/kg) foi predominantemente indutivo, em fêmeas, tais alterações foram de natureza essencialmente inibitória. Ainda no mesmo artigo 2, a figura 3B mostra que o efeito do CPF sobre a atividade da AC no striato de ratos machos adultos foi a indução dessa atividade, principalmente em resposta ao estimuladores diretos (forskolina e Mn^{2+}). Entretanto em fêmeas, embora não significante, as alterações enzimáticas foram de ordem inibitória. Quanto aos tecidos não cerebrais, coração e fígado objetos de estudo do terceiro artigo, tais efeitos gênero dependentes também foram observados. Na figura 6A do artigo 3, a dose de 5 mg/kg de CPF administrada em GD17-20 causou importante indução da atividade da AC em fêmeas adultas, mas em machos a atividade da enzima foi significativa inibida. Quando a exposição se deu no período de PN1-4 (Figura 7, artigo 3), os resultados no fígado de animais também adultos se apresentaram de maneira oposta, indução em machos e inibição em fêmeas (Figura 3B, no centro).

Dependendo do período de exposição, CPF produziu efeitos diferenciados sobre a atividade da AC em uma mesma região cerebral ou orgão estudados. No primeiro artigo, por exemplo, enquanto que a exposição a CPF em GD17-20 produziu significativa indução imediata da atividade da AC no tronco cerebral (brainstem) e, em menor grau, no prosencéfalo (forebrain), quando a exposição ocorreu em GD9-12, as alterações nestas mesmas regiões não foram estatisticamente significativas e com padrão inibitório ao invés de indutivo. Com as alterações de longo prazo, efeito semelhante pode ser observado. No segundo artigo, a exposição a CPF durante PN1-4 produziu significativa indução da atividade da AC no cortex cerebral em PN60, principalmente a atividade estimulada por forskolina e Mn^{2+}. Já a exposição em GD9-12 não produziu alterações importantes no cortex do animal adulto (PN60). Ainda no segundo artigo, enquanto a exposição em GD9-12 não teve efeito significativo sobre a densidade de βAR, estes receptores foram claramente afetados quando da exposição em GD17-20. Exemplos desta seletividade também puderam ser observados no terceiro artigo. Enquanto que a exposição gestacioanal (GD9-12 e GD17-20) produziu alterações preferencialmente sobre a sinalização da AC no coração do animal adulto, a exposição em PN1-4 afetou principalmente o fígado, poupando o coração. Além disso, a exposição em PN11-14 não produziu alterações significativas em nenhum dos dois orgãos. De fato, como visto anteriormente, o período de desenvolvimento compreende uma série de eventos sequenciais que são afetados de forma diferenciada pela exposição química, o que pode explicar porque exposições a doses toxicologicamente equivalentes de CPF nos diferentes períodos do desenvolvimento empregados aqui produziram efeitos diferenciados em uma mesma região cerebral ou orgão.

Da mesma forma, exposição a CPF em um mesmo período não produziu o mesmo efeito nas diferentes regiões cerebrais ou orgãos utilizados neste estudo. Diversos exemplos podem ser observados nos artigos que compõem esta tese, principalmente no que diz respeito aos efeitos de longo prazo. Na figura 3 do artigo 2, que ilustra as alterações na atividade da AC em quatro regiões cerebrais em PN60 após exposição a CPF em GD17-20, é possível

notar que enquanto no striato (Figura 3B) das ratas fêmeas houve inibição da atividade, no cerebelo (Figura 3D) o padrão de alterações foi primordialmente de indução da atividade. Entre os machos, estas duas regiões comportaram-se de maneira oposta. No striato, a atividade em machos foi induzida em animais expostos, enquanto que no cerebelo predominou a inibição da atividade. Já no terceiro artigo, se observarmos a figura 6 veremos que a exposição a CPF produziu alterações imediatas distintas sobre a atividade da AC no coração (Figura 6A) e no fígado (Figura 6B). Na figura 7 do terceiro artigo é possível observar também que o CPF produziu inibições não significativas na atividade da AC no coração de machos adultos. Já no fígado, o CPF produziu expressiva indução na atividade desta enzima nos mesmo animais. Assim, mais uma vez, é possível observar que os princípios que regulam o período de desenvolvimento se aplicam aos resultados obtidos neste trabalho. É bastante provável que as diferenças nas alterações observadas em diferentes regiões após a exposição a uma mesma dose de CPF, durante o mesmo período, deva-se ao fato de que os processos de desenvolvimento não ocorrem concomitantemente em todas as regiões cerebrais, ou mesmo orgãos do corpo.

5.2 - Alguns pontos específicos.

No primeiro artigo, que teve como principal mérito demonstrar que exposição a CPF durante a gestação também produz efeitos imediatos sobre a sinalização mediada pela AC, ampliando assim resultados anteriores observados por Song e colaboradores (Song et al. 1997), é importante perceber que estes efeitos foram mais pronunciados após a exposição no período final da gestação (GD17-20) e no corte histológico que denominamos de tronco cerebral (brainstem). O encéfalo primitivo ou arquencéfalo dá origem a três vesículas encefálicas primárias: o prosencéfalo (forebrain), mesencéfalo (midbrain) e rombencéfalo (hindbrain). O prosencéfalo dá origem ao telencéfalo (composto pelos dois hemisférios e comissuras) e ao diencéfalo (composto hipotálamo, tálamo, subtálamo e epitálamo). O

mesencéfalo continua com a mesma denominação e o rombencéfalo origina o metencéfalo e o mielencéfalo. O metencéfalo origina o cerebelo e a ponte. O mielencéfalo origina o bulbo. Porque neste período do desenvolvimento (GD17-20) o cerebelo ainda é uma porção inespressiva do cérebro, ao separarmos o prosencéfalo (forebrain) o restante do cérebro foi considerado tronco cerebral (brainstem).

Sabe-se que o período de diferenciação sexual do cérebro de roedores tem início no final da gestação, ao redor de GD18, e estende-se até após o nascimento (Auger 2003). Neste período, os hormônios esteróides imprimem mudanças estruturais e funcionais ao hipotálamo e a algumas outras estruturas cerebrais (McCarthy 1994; McEwen 1999), que irão estabelecer uma organização, gênero-específica, de circuitos neuronais que controlam uma série de funções cognitivas, neuroendócrinas e comportamentais. Foi justamente quando os animais foram expostos no final da gestação (GD17-20) que os efeitos imediatos sobre a atividade da AC foram mais evidentes (artigo 1). Assim, uma questão pendente ao final do primeiro artigo foi se os mecanismos de neurotoxicidade do CPF durante o período de desenvolvimento poderiam influenciar ou serem influenciados pela diferenciação sexual do cérebro. Embora o segundo artigo não tenha sido desenhado para responder esta questão específica, é lógico imaginar que se essa hipótese é verdadeira, somente as exposições em, ou a partir de, GD17-20 em diante produziriam efeitos diferenciados em machos e fêmeas, enquanto que animais adultos expostos em GD9-12 não apresentariam diferenças quanto ao sexo. De fato, ao examinarmos a figura 1 do artigo 2, notamos que os resultados da exposição ao CPF, em GD9-12, sobre a atividade da AC e densidade de βAR em animais adultos (PN60), são apresentados sem diferenciação por sexo. Isso porque a estatística utilizada, ANOVA, não demonstrou interação entre o tratamento (controle e grupos tratados com CPF) e sexo. Em outras palavras, o efeito do CPF neste período não pode ser diferenciado entre machos e fêmeas. Entretanto, ao mudarmos o período de exposição para GD17-20 (Figuras 2 e 3, artigo 2), PN1-4 (Figura 4, artigo 2) e PN11-14 (Figura 5, artigo 2), diferenças entre machos e

fêmeas foram frequentes. Cabe ressaltar que existem evidências de que os níveis de CREB influeciam a diferenciação sexual do cérebro, sendo que machos e fêmeas respondem de forma distinta a ativação desse fator de transcrição (Auger 2003). Por outro lado, uma das vias de ativação de CREB pode ser a elevação dos níveis intracelulares de AMPc (Shaywitz and Greenberg 1999; Hanoune and Defer 2001), reforçado por esta tese como um dos possíveis alvos da neurotoxicidade do CPF durante o desenvolvimento.

5.3 – Conclusões e perspectivas futuras

Por fim, tentando responder a pergunta feita no título desta tese "Pode a Exposição Humana ao Inseticida Clorpirifos Alterar o Desenvolvimento do Sistema Nervoso Central?", as evidências apresentadas ao longo desta tese permitem crer que sim, a exposição ao CPF e possivelmente outros organofosforados podem ter repercussões negativas sobre o desenvolvimento do SNC. Mais importante ainda, os resultados aqui descritos agregam novas contribuições às evidências já existentes. O final da gestação em roedores, que basicamente corresponde, em termos de desenvolvimento do SNC, ao segundo trimestre da gravidez humana (Rodier 1988; Rice and Barone 2000), também é um período vulnerável a ação do CPF sobre os eventos celulares aqui estudados. Além disso, o segundo artigo discute também o fato que de que exposições ao CPF durante o desenvolvimento pré- e pósnatal pode ter também repercussões futuras, como durante a vida adulta. Este também foi um tema abordado no terceiro artigo, mas empregando tecidos não cerebrais, neste caso coração e fígado. O objetivo foi o de investigar os efeitos do CPF sobre o desenvolvimento de outros orgãos, tomando como base a hipótese de que algumas doenças, observadas durante a vida adulta, tem como base fatores genéticos e ambientais do período de desenvolvimento (Barker et al. 2002; Power and Jefferis 2002; Toschke et al. 2002; Barker 2003; Slikker and Schwetz 2003; Barker 2004).

Como perspectivas para a saúde pública, o constante "dialogo" entre os estudos experimentais e a epidemiologia pode possibilitar a ampliação dos resultados obtidos nesta tese, na medida em que, através da elaboração e execução de estudos adequados, é possível verificar se de fato determinados segmentos da população humana, especificamente gestantes, crianças e adolescentes, podem ter seu desenvolvimento cognitivo, afetivo ou motor afetados pela exposição a pesticidas durante períodos críticos. Ou ainda qual o papel destas exposições na etiologia de várias doenças na vida adulta como diabetes, doenças cardiovasculares, doença de parkinson ou alzheimer. Na verdade, a necessidade em se avançar, dentro da saúde pública brasileira, no conhecimento sobre a neurotoxicidade durante o desenvolviemento foi muito bem demonstrada por Sarcinelli (Sarcinelli 2003) em um dos poucos estudos sobre o tema no Brasil. Nele, a autora já apontava para uma série de alterações neurocomportamentais observadas em uma amostra de crianças residentes na área rural do Estado do Rio de Janeiro. Efeitos como alterações de memória, um dos efeitos mais comuns observados também em estudos comportamentais em ratos, dificuldade de concentração, irritabilidade e nervosismo foram mais frequentes no grupo de crianças da região rural que no grupo controle urbano.

No próprio campo da experimentação animal, os resultados desta tese permitem uma série de desdobramentos. Um desses foi o estudo sobre os efeitos da exposição ao CPF e ao fármaco terbutalina, durante momentos específicos do desenvolvimento. A terbutalina é um agonista β-adrenérgico comumente utilizado em trabalhos de parto prematuro para tentar prolongar a gravidez até o período normal de nascimento de feto. Embora eficaz nesta tarefa, diversos autores tem expressado preocupação sobre os possíveis impactos do seu uso no desenvolvimento não só do SNC, mas também de outros orgãos como o coração (Slotkin et al. 1989; Auman et al. 2001; Slotkin et al. 2001; Garofolo et al. 2002; Garofolo et al. 2003; Rhodes et al. 2003a; Rhodes et al. 2003b). Como os efeitos do CPF e da terbutalina podem convergir sobre a sinalização mediada pela AC, Rhodes e colaboradores (Rhodes et al. 2003c) primeiro exploraram a hipótese de que a pré-exposição

à terbutalina sensibilizaria o cérebro a futuras exposições a CPF. Obtendo sucesso ao demonstrar que alguns dos efeitos resultantes da dupla exposição foram diferentes do que a simples soma, ou subtração, dos efeitos separados, este estudo, junto com os artigos que compõem esta tese serviram de base para dois outros estudos. No primeiro, demonstramos que a exposição a terbutalina, seguida da exposição a CPF, ambos ainda no período neonatal, produz alterações na atividade da AC em ratos no final da adolescência (PN45) e no início da vida adulta (PN60), que foram de maior magnitude e afetaram um número maior de regiões cerebrais, quando comparado com os efeitos individuais de cada agente (Meyer et al. 2005). O mesmo desenho experimental não conseguiu evidenciar nenhum efeito interativo entre os dois agentes na densidade de receptores serotonérgicos (5HT), subtipos 1 ($5HT_1$) e 2 ($5HT_2$), além do transportador pré-sináptico de serotonina (5HTT). Os efeitos observados foram, de uma forma geral, apenas aditivos e apresentaram clara distinção entre machos e fêmeas (Aldridge et al. 2005). Além disso, os efeitos observados nesta tese se referem a alterações de natureza pós-sináptica (receptores e sinalização celular). A investigação de possíveis efeitos de longo prazo da exposição pré- e pós-natal a CPF sobre marcadores da atividade pré-sináptica, como os níveis e o "turnover" de diversos neurotransmissores, permitiriam examinar possíveis relações, se existentes, entre as alterações pré- e pós-sinápticas. Avanços neste sentido foram recentemente observados em artigo que descreve alterações de longo prazo sobre o turnover de serotonina, além dos níveis e do turnover de dopamina, em diversas regiões cerebrais de ratos expostos em períodos específicos do desenvolvimento (Aldridge et al. in press).

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