Adenylyl Cyclase Cell Signaling as a Target and Underlying Mechanism for Persistent Effects of Early-Life Organophosphate Exposure

by

Abayomi A. Adigun

Department of Pharmacology and Cancer Biology
Duke University

Date:_____

Approved:

_____
Theodore Slotkin, Ph.D., Supervisor

_____
Mohamed Abou Donia, Ph.D.

_____
Edward Levin, Ph.D.

_____
Frederic Seidler, Ph.D.

_____
Sidney Simon, Ph.D.

_____
Richard Whorton, Ph.D.

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in the Department of
Pharmacology and Cancer Biology in the Graduate School
of Duke University

2010

ABSTRACT

Adenylyl Cyclase Cell Signaling as a Target and Underlying Mechanism for Persistent Effects of Early-Life Organophosphate Exposure

by

Abayomi A. Adigun

Department of Pharmacology and Cancer Biology
Duke University

Date:_____
Approved:

_____
Theodore Slotkin, Ph.D., Supervisor

_____
Mohamed Abou Donia, Ph.D.

_____
Edward Levin, Ph.D.

_____
Frederic Seidler, Ph.D.

_____
Sidney Simon, Ph.D.

_____
Richard Whorton, Ph.D.

An abstract of a dissertation submitted in partial
fulfillment of the requirements for the degree
of Doctor of Philosophy in the Department of
Pharmacology and Cancer Biology in the Graduate School
of Duke University

2010

Abstract

Organophosphates (OPs) are developmental neurotoxicants but also produce lasting effects on metabolism. This dissertation examines the cellular mechanisms underlying metabolic dysfunction after early-life OP exposure. We administered diazinon (DZN) or parathion (PRT) to rats on postnatal days (PN) 1-4 at doses straddling the threshold for cholinesterase inhibition and assessed the longitudinal effects on hepatic and cardiac cell function mediated through the adenylyl cyclase (AC) signaling cascade, which controls neuronal and hormonal inputs that regulate hepatic glucose metabolism and cardiac contractility. Specifically, we investigated if outcomes of metabolic dysfunction are related to hepatic AC dysregulation. In the liver, DZN elicited parallel upregulation of AC activity itself and of the responses to AC stimulants acting at β-adrenergic receptors (βARs), glucagon receptors, or G-proteins. The effects intensified from adolescence to adulthood. In contrast, PRT elicited upregulation in adolescence that waned by adulthood. Effects on the liver were more substantial than those in the heart and a brain region (cerebellum) that shares similar AC cascade responses. These findings indicate that OPs produce lasting hepatic AC gain-of-function and alter the trajectory of hepatic cell signaling in a manner consistent with the observed emergence of prediabetes-like metabolic dysfunction. Since the effects are unrelated to cholinesterase inhibition, the various OPs differ in their net impact on AC signaling.

We then examined whether OPs directly affect the expression or function of AC signaling elements, using PC12 cells to evaluate effects on transcription of AC pathway genes and on protein function. Whereas different OPs had disparate effects on gene

transcription, they had nearly identical effects at the protein level, suggesting that programming occurs post-transcriptionally. We further found that otherwise unrelated developmental toxicants (OPs, dieldrin, nickel) can nevertheless converge on the AC pathway, providing a common pathway by which diverse agents can lead to metabolic dysfunction.

The standard view of OPs as developmental toxicants that exclusively target the nervous system requires substantial revision. Through their effects on hepatic cell signaling and other metabolic processes, early-life chemical exposures may play an important role in the worldwide increase in obesity and diabetes.

To Christie,

for her unending love, dedication and support.

Table of Contents

List of Tables

List of Figures

Abbreviations

AC……..	adenylyl cyclase
ANOVA…….	analysis of variance
βAR…….	β-adrenergic receptor
cAMP……. .	cyclic AMP
CPF……..	chlorpyrifos
DEX…….....	dexamethasone
DZN…….	diazinon
GRs …….	glucocorticoid receptors
HPA…….	hypothalamic–pituitary–adrenal
m_2AChR……..	m_2-muscarinic acetylcholine receptor
NGF…….	nerve growth factor
OP……	organophosphate
PN……	postnatal day
PRT……..	parathion

Acknowledgements

First and foremost, I must acknowledge my advisor/mentor, Ted Slotkin. I first met him as a student in Pharmacology 233 and knew right away that I wanted to join his lab. He has truly mastered the art of teaching and has a way of making the "story" of science easy to understand. His jokes and stories are always memorable and actually have helped me to grasp many scientific concepts. Ted's work ethic and enthusiasm for science are contagious and helped mold me into a productive student. Importantly, I thank Ted for not only showing commitment to my project, but also to my personal growth and well being as a student.

Secondly, I must thank Fred Seidler for his masterful planning and coordination of all of my experiments. I appreciated his candor during one-on-one meetings and discussions of my scientific progress. I will also miss our occasional trip to Costco for browsing, shopping and their delicious hot dogs.

I also thank the other members of my committee: Edward Levin, Mohamed Abou Donia, Richard Whorton and Sidney Simon. During committee meetings, their comments and questions were instrumental in solidifying my project and forced me to think as an independent scientist. I thank Dr. Patton for his editorial help.

I must also acknowledge the steadfast support of my wonderful family. I thank my mother and father for always believing in me and providing words of encouragement, my wife for seeing my dreams as her own and helping me achieve my goals, my son for the happiness he has brought me and my siblings for cheering me on to success.

Summary

Organophosphates (OPs) are developmental neurotoxicants but recent evidence points to additional adverse effects on metabolism and cardiovascular function. This dissertation examines the cellular mechanisms underlying metabolic dysfunction after early-life OP exposure. Contrary to earlier views, it is now clear that OPs act through mechanisms other than their shared property as cholinesterase inhibitors, and recent work points to disruption of cell signaling mediated through cyclic AMP as a pivotal, non-cholinesterase mechanism: OPs target G-protein-coupled receptors, G-proteins and adenylyl cyclase (AC) itself. Just as important, the AC cascade operates outside the central nervous system to control neuronal and hormonal inputs that regulate hepatic glucose metabolism and cardiac contractility. With this in mind, we hypothesized that metabolic dysfunction caused by early-life OP exposures is related to hepatic AC dysregulation.

To establish a benchmark, we first studied the effects of the glucocorticoid, dexamethasone (DEX), on AC signaling in the liver and heart. Fetal and/or neonatal DEX exposure is known to produce prediabetes, hypothalamic–pituitary–adrenal (HPA) axis dysregulation and cardiovascular abnormalities. We administered DEX to neonatal rats on postnatal (PN) days 1-3 or 7-9, using doses spanning the threshold for somatic growth impairment. In adulthood, we assessed the effects on hepatic and cardiac cell function mediated through the AC signaling cascade. Treatment on PN1-3 produced heterologous sensitization of hepatic signaling, with upregulation of AC itself leading to parallel increases in the responses to β-adrenergic or glucagon receptor stimulation, or to

activation of G-proteins by fluoride. The effects were prominent at the lowest dose but when the dose was increased past the point of somatic growth impairment, there was a loss of the effect in females. Shifting the exposure to PN7-9 still elicited AC sensitization but with a greater offsetting contribution at the higher doses. Our findings show that dexamethasone elicits a hepatic AC gain-of-function that may be an important contributor to the cellular abnormalities underlying subsequent metabolic and cardiovascular dysfunction. Importantly, we then moved on to see how OPs effects on AC signaling would compare to those of DEX.

We administered diazinon (DZN) or parathion (PRT) to rats on PN 1-4 at doses straddling the threshold for cholinesterase inhibition and assessed the longitudinal effects (PN30, PN60 and PN100) on hepatic and cardiac cell function mediated through the AC signaling cascade. DZN elicited heterologous sensitization in the liver and the effects intensified over the course from adolescence to adulthood. In contrast, PRT elicited upregulation of AC in adolescence that waned by adulthood. Superimposed on these general patterns, there were effects on glucagon receptor coupling to AC, and the effects on the liver were more substantial than those in the heart or the cerebellum. To make certain that later-emerging changes were not occurring with PRT exposure, we performed an additional set of determinations at 5 months of age and again found no persistent effects on AC. These findings indicate that OPs alter the trajectory of hepatic cell signaling in a tissue selective manner consistent with the observed emergence of prediabetes-like metabolic dysfunction. Furthermore, since the effects occur at low exposures and are unrelated to cholinesterase inhibition, the various OPs differ in their

net impact on the peripheral AC signaling, and in a manner divergent from their effects on neurobehavioral outcomes.

Next, we investigated whether metabolic challenge imposed by consuming a high-fat diet for 7 weeks would exacerbate neonatal PRT's effects on AC signaling. Although there were no significant interactions between neonatal PRT exposure and a high-fat diet, their convergent effects on the same signaling cascade indicate that early OP exposure, separately or combination with dietary factors, may contribute to the worldwide increase in the incidence of obesity and diabetes.

We then examined whether OPs directly affect the expression or function of AC signaling elements, using PC12 cells to evaluate effects on transcription of AC pathway genes and on protein function. For the transcription studies, we used microarrays to compare two OPs, chlorpyrifos (CPF) and DZN, to an organochlorine (dieldrin) and a metal (Ni^{2+}), with each agent introduced during differentiation. We assessed genes encoding AC isoforms and regulators, G-protein α- and β,γ-subunits, protein kinase A subtypes and the phosphodiesterase family. CPF and DZN were surprisingly the least alike, whereas there was strong concordance of dieldrin and Ni^{2+} with each other and with each individual organophosphate. Further, when we compared CPF effects in undifferentiated cells to those in differentiating cells, we again saw little or no similarities for transcriptional profiles. These findings indicate that the effects of OPs on AC signaling are not likely to represent direct effects on gene transcription, since in that case, we would have expected similar profiles for CPF and DZN, and for CPF in the undifferentiated and differentiating states. On the other hand, the unexpected similarities

seen for diverse toxicants suggest that otherwise unrelated agents can converge on the cAMP pathway, thereby leading to similar developmental outcomes. We therefore went on to examine if the different OPs instead acted through common mechanisms at the post-transcriptional level.

We exposed PC12 cells to CPF, DZN or PRT in the undifferentiated state and during differentiation and assessed the functioning of AC pathway proteins. In undifferentiated cells, a 2-day exposure to the OPs had no significant effect on AC signaling but the same treatment in differentiating cells produced deficits in all AC measures when exposure commenced at the initiation of differentiation. However, when exposure of the differentiating cells was continued for 6 days, AC activities then became supranormal. The same increase was obtained if cells were exposed only for the first two days of differentiation, followed by four subsequent days without the OPs. Importantly, these results show that whereas different OPs had disparate effects on gene transcription, they had nearly identical effects at the level of protein function, suggesting that their effect converge on the AC pathway post-transcriptionally. Additionally, the results indicate that OP exposure reprograms the AC pathway during a discrete developmental stage at the commencement of differentiation, with effects that continue to emerge after OP exposure is discontinued.

In conclusion, we have shown that developmental OP exposure produces lasting hepatic AC gain-of-function that likely contributes to the eventual emergence of metabolic abnormalities resembling prediabetes. Furthermore, the different OPs share similarities as well as differences: all OPs caused AC gain-of-function but with different

temporal trajectories and sex selectivity, all unrelated to anticholinesterase mechanisms. The programming of AC function proceeds through post-transcriptional mechanisms; whereas transcription is also affected by the OPs, these do not account for the similar outcomes for AC signaling, and indeed, the transcriptional effects were shared by unrelated toxicants. The standard view of OPs as developmental toxicants that exclusively target the nervous system requires substantial revision. Through their effects on hepatic cell signaling and other metabolic processes, early-life chemical exposures may play an important role in the worldwide increase in obesity and diabetes.

Chapter 1: Early-life Toxicant Exposures, Cell-Signaling Cascades and Metabolic and Cardiac Dysfunction in Adulthood

It is increasingly clear that adverse events during fetal development contribute to subsequent metabolic and cardiovascular disease in adulthood. In the early 1990s, a novel hypothesis was advanced by Barker and associates to link stress sufficient to cause growth retardation during embryonic and fetal development not only to impaired maturation of physiological functions, but also to latent diseases in adulthood [19,71]. The "Barker hypothesis" thus postulates that a number of organ structures and associated functions undergo programming during critical periods in embryonic and fetal development, and this programming determines the set point of physiological and metabolic responses that persist into adulthood [95].

The biological basis for these associations appears to be rooted in developmental plasticity [20]. Humans are both 'plastic' and able to adapt to their environment. Furthermore, for many of the body's organs and systems there is a critical period when they are plastic and sensitive to the environment. These critical periods have been found to mainly occur *in utero*, and then are followed by a loss of plasticity. Developmental plasticity is defined as the phenomenon by which one genotype can give rise to a range of different physiological or morphological states in response to different environmental conditions during ontogenesis. It enables the production of phenotypes of a fixed functional capacity that are better matched to their environment than would be possible if the same phenotype were produced in all environments [21].

It is surprisingly easy in animals to experimentally produce permanent changes in

the metabolism and blood pressure of a fetus by minor modifications to the diet of the mother before and during pregnancy [21,95]. Furthermore, data from numerous epidemiological studies have indicated strong inverse associations between intrauterine stress (e.g. maternal undernutrition, maternal obesity, corticosteroid therapy, uteroplacental insufficiency, or hypoxia) that is sufficient to cause growth retardation and the subsequent development of hypertension, insulin resistance, Type 2 diabetes and hyperlipidemia, a cluster of cardiovascular risk factors that are termed the metabolic syndrome [22]. Therefore, alterations in embryonic and fetal environment, as well as endocrine status during gestational and perinatal periods, can result in developmental adaptations that produce permanent structural, physiological and metabolic changes, thereby predisposing an individual to cardiovascular, metabolic and endocrine diseases in adulthood [18,95]. Even though data from both human and animal studies [22,91] suggest that many diseases in the adult can be induced by manipulating the environment of the fetus, the molecular mechanisms underlying the association between intrauterine stress sufficient to cause growth retardation and later disease are not yet fully elucidated. Currently, glucocorticoids released by stress are hypothesized to be among the most prominent features that are likely to contribute to the 'Barker' outcomes. Therefore, much effort has now gone into examining glucocorticoids and their effects on the fetus and later adult outcomes.

The programming effects of glucocorticoids have been extensively investigated. The ultimate secretion of glucocorticoids from the adrenal cortex is controlled in the HPA axis by an endocrine negative-feedback loop (Figure 1).

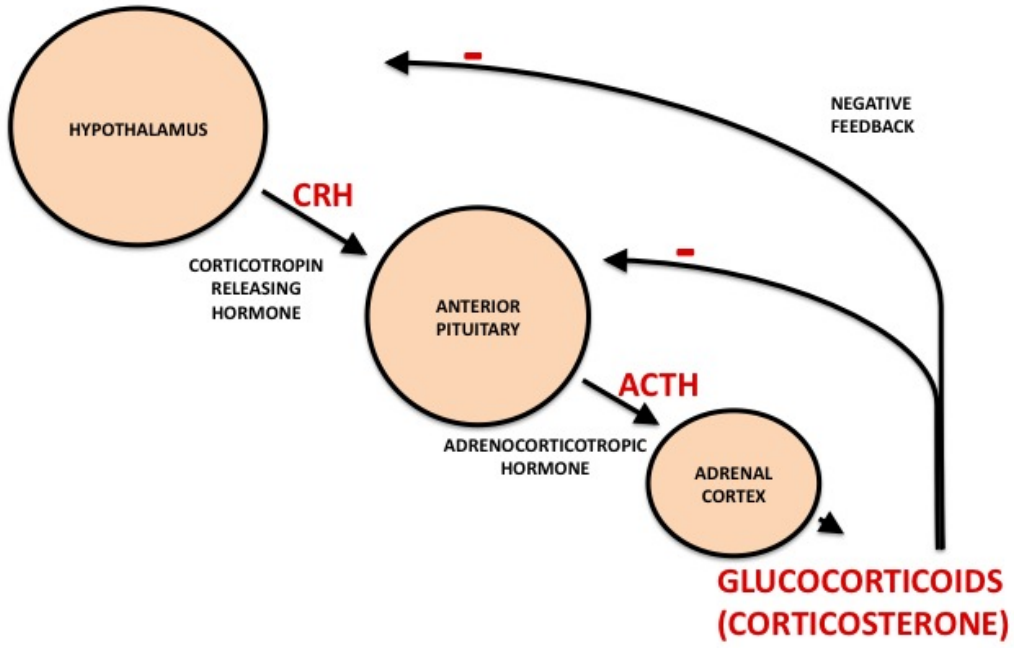

Figure 1: Schematic diagram of the HPA axis. Corticotropin-releasing hormone (CRH) is synthesized in neurons of the paraventricular nucleus of the hypothalamus and released into the hypophysial portal circulation. CRH stimulates corticotropin synthesis and release from corticotrophs in the anterior pituitary gland. Corticotropin, in turn, stimulates production of glucocorticoids from the adrenal cortex. Glucocorticoids are important for maintenance of homoeostasis and signal a variety of metabolic changes that allow the body to respond to stress. They also provide negative feedback by inhibiting CRH and corticotropin secretion at numerous levels in the HPA axis. The HPA axis is also modulated by higher centers, notably the hippocampus and amygdala.

First, glucocorticoids bind to intracellular glucocorticoid receptors (GRs). GRs are members of the nuclear hormone superfamily of ligand-activated transcription factors. In some tissues, glucocorticoids also bind with high affinity to mineralocorticoid receptors. Both receptors are initially activated upon ligand binding, which leads to formation of a receptor-ligand complex. The receptor–ligand complex then translocates to the nucleus. In the nucleus, the complex binds to glucocorticoid response elements in the promoter region of target genes, thereby influencing gene transcription (Figure 2, [200]).

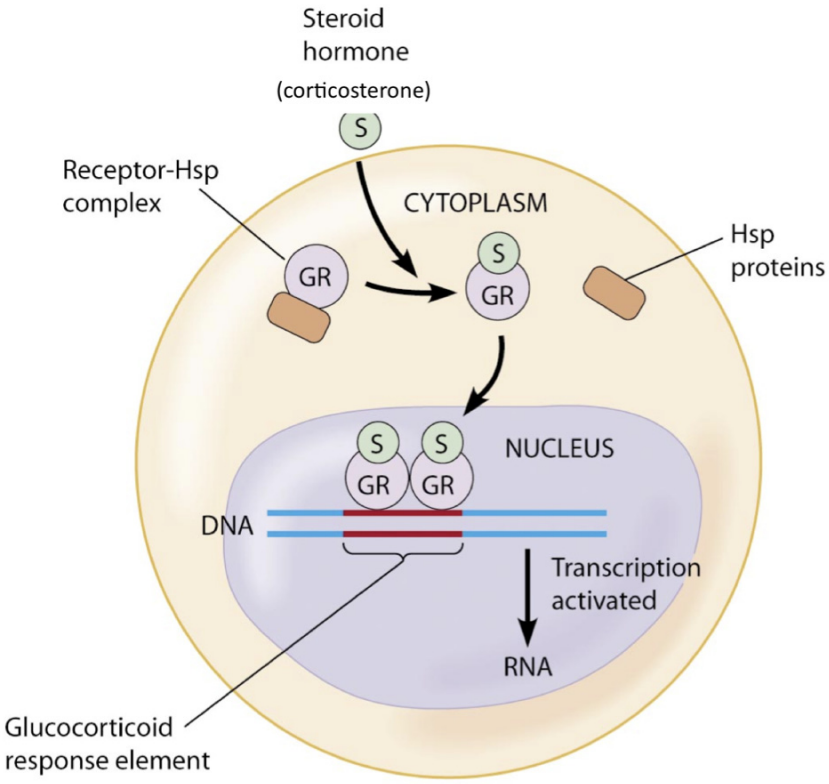

Figure 2: Schematic representation of action of glucocorticoid hormones. The GRs activate gene transcription. Corticosterone, a hydrophobic steroid hormone, can diffuse through a plasma membrane then bind to the intracellular glucocorticoid receptor. Binding the steroid causes the release of an inhibitory protein and activates the glucocorticoid receptor molecule's DNA binding site. The glucocorticoid receptor molecule then enters the nucleus and binds to a glucocorticoid response element in DNA, which causes a second glucocorticoid receptor molecule to bind to the same response element. The resulting glucocorticoid receptor dimer activates transcription of the target gene, adapted from [200].

Glucocorticoids are present in most fetal tissues by midgestation [38] and are also found in the placenta [179]. Many of the significant maturational changes in organ systems, such as the lungs, heart, liver, gut and kidneys [27,28,33], are glucocorticoid dependent and can be induced prematurely by exogenous glucocorticoid administration [17,54]. For example, glucocorticoid receptor-null mice die within the first few hours after birth of respiratory failure due to severe lung atelectasis, and have severely retarded maturation of the adrenergic chromaffin cells and hepatic gluconeogenic enzymes [37].

Additionally, the societal impact of prenatal glucocorticoid exposure, notably to dexamethasone (DEX), is increasingly important because of the expanded use of these agents in preterm labor [64]. This treatment is currently utilized in one in every 10 U.S. pregnancies [104], thus potentially affecting hundreds of thousands of infants born each year [88,104]. It is also important to note that the effects of fetal exposure to endogenous glucocorticoids and those of synthetic glucocorticoids may pose different outcomes. Although endogenous glucocorticoids can bind to both glucocorticoid and mineralocorticoid receptors and the effects in tissues may be mediated by both of these receptors, synthetic glucocorticoids are more selective for GRs. Similarly, there may be differences in local concentrations in specific types of tissues, which may be governed by differences in transport (e.g. across the blood–brain barrier) and differential metabolism of endogenous and synthetic glucocorticoids [47,105,136].

GRs importantly enhance lung maturation and thus prevent neonatal respiratory distress syndrome, a major factor contributing to adverse perinatal outcomes [64]. But, unfortunately, multiple glucocorticoid courses used during pregnancy are increasingly suspected to contribute to subsequent metabolic, cardiovascular and behavioral anomalies, all of which can emerge throughout the lifespan after periods of apparent normality [120,143]. Notwithstanding these epidemiological relationships, few studies have provided a mechanistic understanding of how early glucocorticoid exposure leads to such metabolic or cardiovascular dysfunction. Furthermore, animal studies of glucocorticoid effects on neurodevelopment involve doses sufficient to produce persistent stunting of somatic growth, outright cerebral atrophy, altered behavior and

neuroendocrine disruption [29,57,58,63,66,101,103,104,106,108,152,195]. Therefore, such dose regimens leave major uncertainties as to specific glucocorticoid effects at or below the threshold for typical therapeutic use.

Recent studies have asked whether low-dose DEX administration during late pregnancy modifies adult cardiovascular and metabolic physiology. These studies have shown that glucocorticoid exposure in late rat gestation permanently programs cardiovascular and metabolic physiology, leading to hypertension and decreased insulin sensitivity in adulthood [43,74,120,121,199]. Importantly, in a recent human double-blind placebo controlled randomized trial of antenatal betamethasone for the prevention of neonatal respiratory distress syndrome, 534 individuals were followed for over 30 years. The betamethasone-exposed participants in this study also had evidence of increased insulin resistance, which might signify an increased risk of diabetes and cardiovascular disease as this cohort ages. Equally important, several animal studies have shown that prenatal glucocorticoid excess, from endogenous overproduction with maternal stress, reduces birth weight and causes lifelong hypertension, hyperglycemia and behavioral abnormality in offspring [47].

Although Barker originally showed that intrauterine stress which was sufficient to cause growth retardation could contribute substantial risk for subsequent development of metabolic and cardiovascular disease [21], it is also apparent that programming effects may be seen in the absence of changes in birth weight. In fact, the programming effects of prenatal glucocorticoid excess are not dependent on alterations in fetal growth. For example, short-term prenatal exposure (2 days) of rats to DEX is associated with

programmed effects on blood pressure and renal development [48,125,126,144]; in sheep, short-term exposure to DEX resulted in hypertension even in the absence of changes in birth weight [45,46]. Such studies suggest that birth weight is a crude measure of exposure to an adverse environment *in utero,* and that disease risk may be increased in the absence of changes in weight at birth [47]. Importantly, glucocorticoids are not the only route to later-life disorders. There is emerging evidence that perinatal chemical exposures can reprogram development and metabolic function without the precondition of growth restriction.

There are also suggestions in the literature that environmental contaminants, drugs and chemicals may play an important role in such disorders as hypertension, diabetes and obesity. For example, it is clear that maternal smoking during pregnancy produces metabolic reprogramming that leads to subsequent risk of obesity in the offspring [8,34,61,131,185,191]. Furthermore, perinatal bisphenol A exposure has been shown to increase the expression of genes involved in adipogenesis and lipogenesis in adipose tissue at weaning [174]. Serious attention has also been given to exposure to common pesticides as a similar route to later-emerging disorders, in light of their widespread use in the home and in agriculture.

The potential contribution of pesticides to cardiac and metabolic dysfunction has garnered little interest in comparison to factors such as diet, lifestyle [178], genetics [55], race/ethnicity and socioeconomic status [192]. It is undeniable, however, that we are constantly exposed to inadequately-tested synthetic chemicals in our air, water and food, with pesticides representing the most common type of chemical of concern for human

health. There is also evidence that developmental exposure to pesticides produces lasting changes in cardiovascular and hepatic function [113,145], and induces excess weight gain in rats and humans [51,92,94,138].

The Centers for Disease Control and Prevention have tracked the increase in obesity through National Health and Nutrition Examination Surveys, and two out of three adults in the US are considered clinically overweight or obese [122]. Among children 34% are overweight or on the border of being overweight. Moreover, the prevalence and severity of obesity is increasing [53], and increased adiposity is apparent across ethnicities [122].

Cardiovascular disease (CVD) is the leading killer in many developed countries, and is soon expected to be the leading killer world-wide [100]. Substantial evidence now exists that exposure to chemicals and other environmental substances, including pesticides, have a profound impact on cardiac health [193]. Several government agencies, such as the National Heart, Lung and Blood Institute, have begun to address the links between environmental agents and CVD. Furthermore, advocacy organizations such as the American Lung Association and the Natural Resources Defense Council have increased their efforts to explore this area of research.

Finally, pesticides may also be related to cardiovascular disorders, both indirectly through promoting obesity, and directly pesticide exposure may also be implicated in the increasing prevalence of diabetes. The International Diabetes Federation estimates that 230 million people worldwide have diabetes and more than 300 million people are prediabetic. The outlook suggests that 500 million people will have diabetes or be

prediabetic by 2025, and the American Diabetes Association estimates that 75 million Americans are already in that category. This obviously entails an enormous human and financial cost to society [89], and it is therefore notable that the increases in both types 1 [124] and type 2 diabetes [208] are related to the obesity epidemic. Both obesity and diabetes likely are related, in part, to chemical exposures. For our purposes, it is notable simply that there exist epidemiological links between pesticide exposure and diabetes [115].

Here, we will focus our attention on the organophosphate (OP) pesticides as specific contributors to later-life metabolic and cardiovascular disorders. Restrictions on the use of most of the persistent organochlorine insecticides imposed in the 1970s led to the widespread use of less persistent but highly effective OP agents. OP compounds have been utilized as pesticides, lubricants, flame retardants, and most notoriously, as chemical warfare agents [50]. They are the major class of pesticides used in the world today, and represent 50% of pesticides use worldwide [32]. The majority of these compounds initiate acute toxicity through inhibition of the enzyme acetylcholinesterase in the central and peripheral nervous system. Signs of cholinergic toxicity include symptoms such as salivation, lacrimation, urination and defecation (SLUD), miosis, nausea and heart and respiratory dysfunction [49]. Specifically, OPs phosphorylate the active site's serine residue on acetylcholinesterase and thereby inhibit the catalytic degradation of acetylcholine in the synapse (Figure 3) [59]. For OP anticholinesterase overt toxicity to occur in an organism, at least four steps must be involved: (1) binding to and inhibition of an extensive number of acetylcholinesterase molecules with substantial impairment of

acetylcholine degradation, (2) accumulation of acetylcholine in the synapses of the central and/or peripheral nervous systems, (3) excessive stimulation of postsynaptic cholinergic receptors and/or end organs and (4) altered function in response to excessive stimulation of those receptors (Figure 4). Modulation of any of the neurochemical processes involved in acetylcholine synthesis, acetylcholine release, cholinergic receptor binding, or signal transduction concurrent with anticholinesterase exposure could influence the progression of events from target enzyme acetylcholinesterase inhibition to the expression of toxicity [130]. It is generally accepted that some degree of acetylcholinesterase inhibition can be tolerated without substantial alteration of cholinergic transmission, and overt symptoms of poisoning are not apparent until 70% inhibition is reached [32]. Notably, the OPs examined in this study are all weak reversible acetylcholinesterase inhibitors that are converted to the active oxon form by the cytochrome P450 enzymes. In fact, these agents are actually OP precursors; their native form is an organophosphorothioate; however, in common parlance and throughout the research literature, these are all referred to as OPs, a convention that will be used throughout this dissertation.

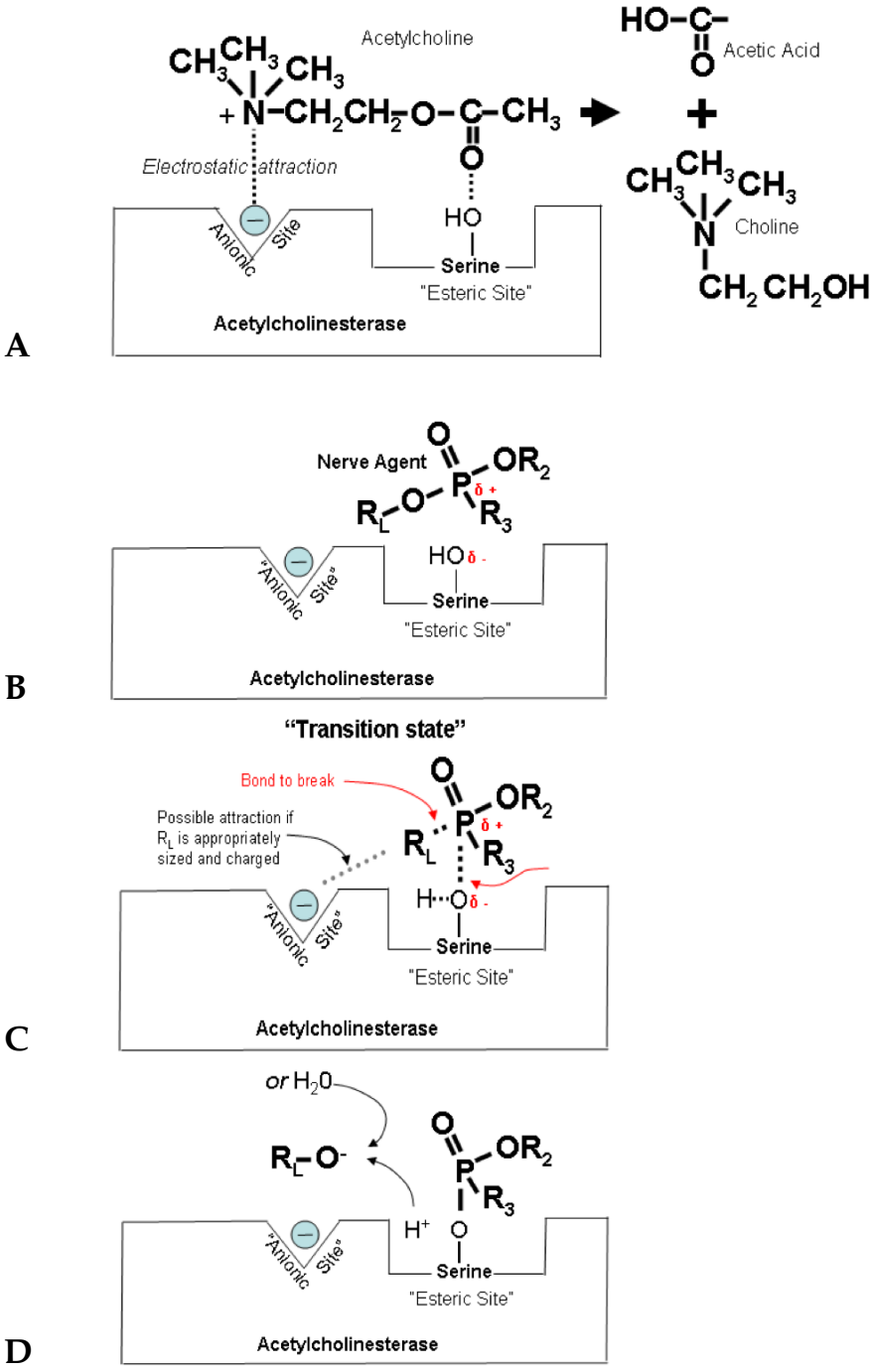

Figure 3: Chemical mechanism for OP inactivation of acetylcholinesterase. 3a) Breakdown of acetylcholine, 3b) Partially electropositive phosphorus is attracted to partially electronegative serine, 3c) Transition state showing which bonds break and which ones form and 3d) OP attached to acetylcholinesterase preventing the attachment of acetylcholine.

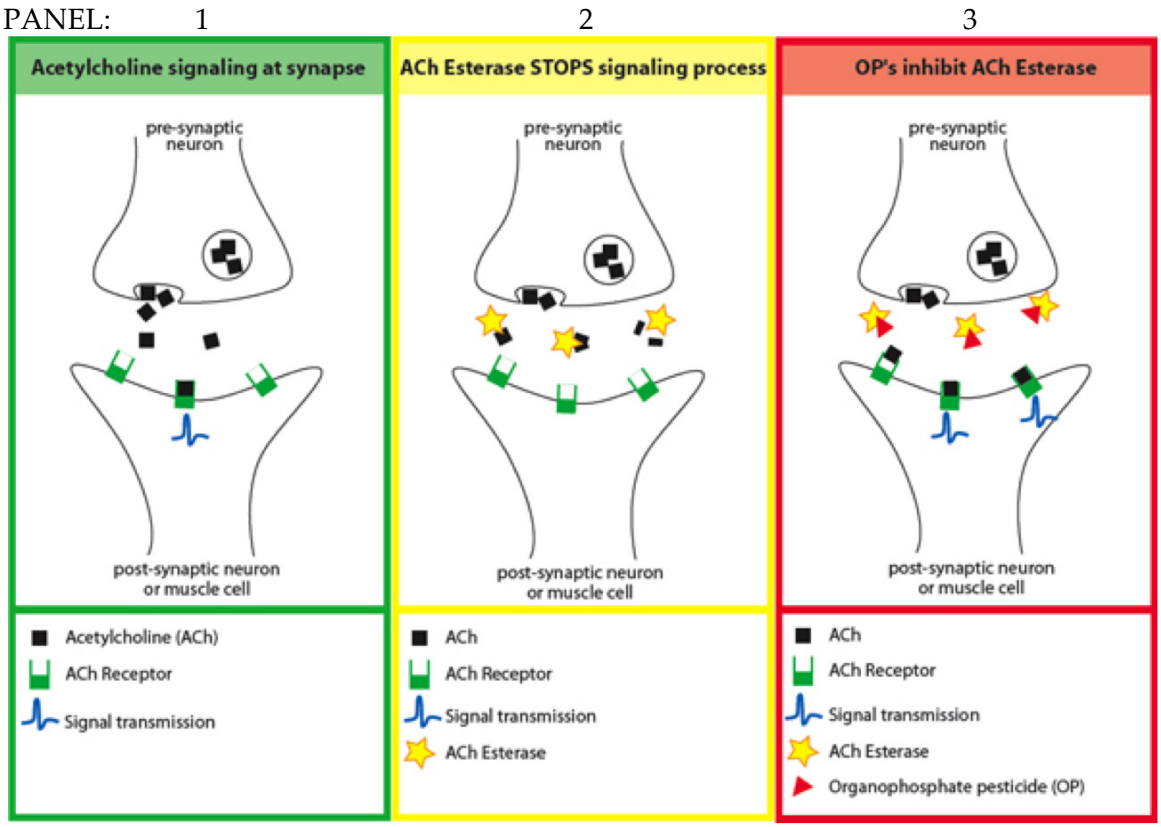

Figure 4: Chemical mechanism for OP inactivation of acetylcholinesterase. OPs are cholinesterase-inhibiting insecticides, and exert their acute effects by causing overstimulation at cholinergic nerve terminals. This process occurs in both insects and humans. Normally, acetylcholinesterase catalyzes the degradation of the neurotransmitter acetylcholine in the synapse (panel 2). OP pesticides phosphorylate acetylcholine, thereby reducing the ability of the enzyme to break down the neurotransmitter (panel 3). This produces an accumulation of acetylcholine in the central and peripheral nervous systems, resulting in an acute cholinergic syndrome via continuous neurotransmission. The clinical onset of cholinergic over-stimulation can vary from almost instantaneous to several hours after exposure.

It is important to note, while some of the OP compounds are direct-acting acetylcholinesterase inhibitors, most require biotransformation to the ultimate toxicant (Figure 5). In some cases, requirement for activation as well as reactivity with detoxification systems contributes to the wide range of acute toxicity with these toxicants [130].

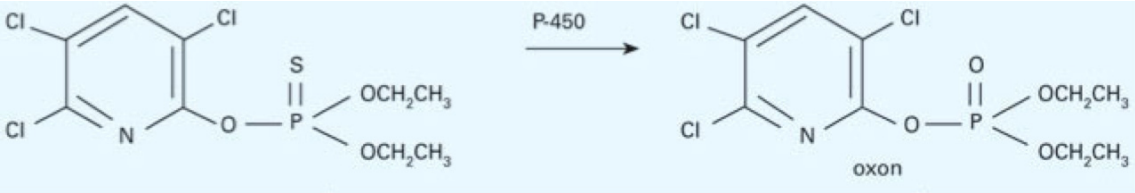

Figure 5: Human metabolism of chlorpyrifos. It is important to understand the human metabolism of organophosphorus insecticides. Chlorpyrifos is used as an example. Chlorpyrifos is bioactivated by cytochrome P450-dependent desulfuration in the liver to chlorpyrifos-oxon.

As pesticides, OPs are unique contaminants in that they are intentionally released into the environment to cause toxicity in certain 'pest' species. Unfortunately, a lack of selectivity often leads to problems of toxicity in humans and other non-target species [77]. The systemic toxicity of OPs reflects their ability to inhibit acetylcholinesterase. While all OP insecticides are thought to act through a common mechanism initiated by inhibition of acetylcholinesterase, differential expression of toxicity following similar changes in acetylcholinesterase activity have been reported [99]. Moreover, animal models have shown that developmental neurotoxicity results from OP exposures at doses that do not elicit any signs of systemic intoxication and even at exposures below the threshold for inhibition of acetylcholinesterase [145-147,154]. Since pregnant women are likely to be exposed to OPs under circumstances that do not elicit outward signs of intoxication [44,69,127], and in light of recent findings that such exposures can produce long-term cognitive impairment in children [137,139], the mechanisms and consequences of OP-induced developmental neurotoxicity remain major environmental concerns [23,42,130,141,146]. However, the brain is not the only target: the same signaling cascades involved in brain development are also involved in the control of cardiovascular and metabolic processes in the periphery.

Indeed, OP exposures have proven to alter metabolic processes outside the central nervous system. In adult rats, chlorpyrifos (CPF) exposures exceeding the threshold for cholinesterase inhibition lead to enhanced weight gain [109] and diabetes-like changes in hepatic energy metabolism [2]. At lower doses administered during development, CPF also produces excess weight gain and dysregulation of leptin [92], along with a metabolic profile resembling prediabetes [150]. Similarly, when neonatal rats were exposed to PRT at doses straddling the threshold for barely-detectable cholinesterase inhibition, they showed later emergence of a prediabetes-like state, involving excessive weight gain, hyperglycemia, abnormalities of lipid metabolism and adipose tissue inflammation [93,94]. Further, many of the metabolic effects of early-life OP exposure were exacerbated when animals were switched to a high-fat diet in adulthood, including a much greater fat-induced weight gain than that with the equivalent dietary change in controls [93,94,138]. It is also notable that there are epidemiological links between the amount of pesticide exposure of pesticide applicators and diabetes [115]. Importantly, it is now evident that other mechanisms are at least equally, if not more important in determining the long-term liability of human exposure. These findings bring attention to the inadequacy of acetylcholinesterase inhibition as the exclusive biomarker for assessing exposure or outcome related to developmental effects. Although the systemic toxicity and signs of OP intoxication reflect their shared ability to inhibit cholinesterase [114,130], other important mechanisms of toxicity clearly exist.

Recent data point to dysregulation of cell signaling cascades as one of the critical targets that contribute to the adverse outcomes seen at lower exposures [68,145,146,157].

Chief among these is the pathway that generates cyclic AMP (cAMP). In 1959, cAMP was first identified as a critical second messenger that mediates numerous neurotransmitter and hormonal receptor signals. Since then it has been discovered that cAMP is also essential in coordinating the critical transition from cell replication to cell differentiation in virtually all prokaryotic and eukaryotic cells [25,35,67,78,189]. In order for cAMP to function as an intracellular mediator, its intracellular concentration must be able to increase and decrease in response to extracellular signals. Such responsiveness requires that rapid synthesis of the molecule be balanced by rapid breakdown or removal. Cyclic AMP is generated from G-proteins that regulate plasma-membrane-bound AC, the enzyme that synthesizes cAMP from ATP. Furthermore, cAMP is rapidly and continuously destroyed by one or more cAMP phosphodiesterases, which hydrolyze cAMP to adenosine 5'-monophosphate (Figure 6).

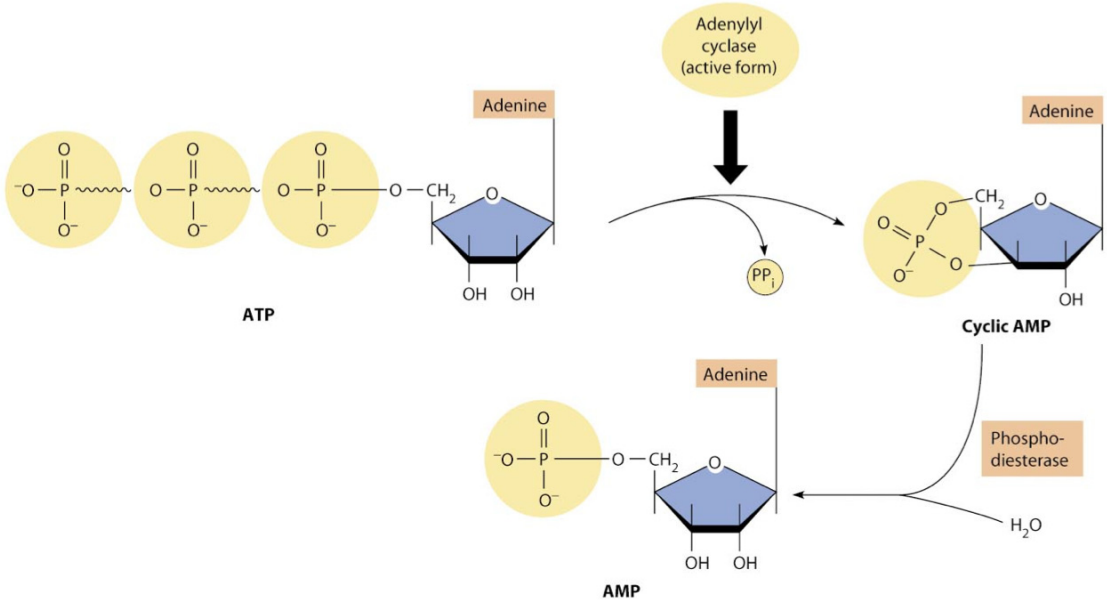

Figure 6: Cyclic AMP is generated from ATP

For our purposes it is important to note that hepatic and cardiac cell function is mediated through the activation of AC. Specifically, AC synthesizes cAMP and controls neuronal and hormonal inputs that regulate liver gluconeogenesis and lipolysis, as well as, heart rate and heart contractility.

The activation of AC is initiated by the binding of hormones to cell surface receptors [142,181]. Epinephrine, dopamine, adenosine and glucagon are a few examples of the many hormones that activate AC through membrane-bound receptors [181]. Glucagon, for example, a hormone that regulates glycogen metabolism in liver and skeletal muscle, recognizes membrane receptors in these tissues, markedly stimulates AC to produce intracellular cAMP. Glucagon-bound receptors communicate with an intracellular, membrane-associated heterotrimeric G protein [129,181] composed of a guanosine diphosphate (GDP)–bound α-subunit and an obligate $\beta\gamma$ heterodimer. Hormone-dependent activation of receptors leads to the exchange of GDP for guanosine triphosphate (GTP). Conformational changes due to GTP binding result in the dissociation of the heterotrimeric G protein into α and $\beta\gamma$ subunits, which then interact with their respective effectors [180,181]. There are multiple classes of α-subunits that regulate AC. Binding of agonists to an appropriate receptor cause activation of a G protein, G_s, which in turn, stimulates AC. Notably, there are also receptor –G protein pathways that inhibit AC directly, as well as more complex mechanisms for both stimulation and inhibition [180].

To date, molecular cloning techniques have identified nine mammalian genes that encode membrane-bound ACs [73,128,171,180], and one gene encoding a soluble isoform [30,181]. AC is an integral membrane protein composed of twelve transmembrane segments. The protein can be visualized as two tandemly repeated hydrophobic domains, each containing six transmembrane segments and a large cytoplasmic (catalytic) loop. The cytoplasmic domains (C1 and C2), which constitute the catalytic site, are subject to intracellular regulations specific for each subtype. In particular the catalytic activity, as well as the sites for interaction with forskolin (FSK) and Gαs, require both cytoplasmic moieties [72].

Numerous studies of the developing brain have focused on the effects of the most extensively studied OP, CPF. These studies have shown that cell signaling cascades that control neural cell replication and differentiation appear to be among the most sensitive targets for OP developmental neurotoxicity and in particular, the pathway regulating cAMP levels appears to be particularly important in mediating cholinesterase-independent mechanisms [39,40,62,112,123,141,175,207]. Importantly, exposure of developing neurons to OP pesticides result in lasting changes in the expression and/or function of the key signaling proteins in the pathway, including the number and activity of G-protein-coupled receptors, the concentration and function of the stimulatory (G_s) and inhibitory (G_i) G-proteins and subtype expression and catalytic efficiency of AC itself [7,110-112,175]. However few studies have looked at AC effects outside the brain, even though cAMP functions to coordinate processes like the metabolic breakdown of glycogen in mammalian liver as well as heart rate and contractility.

There are key epidemiological and animal studies that point to perturbations of AC as critical to the development of cardiovascular and metabolic diseases, given cAMP's important peripheral cardiac, metabolic and hormonal functions. Recently, AC gene polymorphisms have been linked to obesity and diabetes susceptibility [119]. The insulinotropic effect of forskolin in Goto–Kakizaki rats was found to yield an enhanced cAMP generation and overexpression of AC mRNA [1]. Moreover, liver AC activity was increased in the membranes of male ob/ob mice in comparison to lean control mice [24]. It is thus important that neonatal OP exposure leads to a similar augmentation of hepatic cAMP production in adulthood, specifically at the level of AC itself and its gluconeogenic inputs via beta-adrenergic receptors and glucagon [111]; indeed the potential for long-term disruption of hepatic function is evident from the fact that CPF concentrates in the developing liver [79]. Importantly, it has also been shown that neonatal CPF exposure leads to disruption of cardiac cell signaling in adulthood [111]. Furthermore, significant effects in the immature liver and heart are elicited at exposures below the threshold of systemic toxicity, including effects on signal transduction [13] and cell number and size [132]. These effects are sex selective [111], so future work with animal models or human populations will need to consider sex differences. In addition, these effects have been shown to emerge late in development, thus requiring longitudinal evaluations [111,150].

It is clear that further investigation is needed to understand the AC signaling cascade as a target and underlying mechanism for persistent effects of early-life OP exposure. This study will explore how developmental exposures to the OPs, DZN and

PRT; contribute to increased risk of cardiac and metabolic dysfunction through lasting effects on AC pathway function. **Our overarching hypothesis is that developmental exposures to DZN and PRT will produce AC signaling alterations that will result in a phenotype predisposed to metabolic and cardiac dysfunction.** In order to test the hypothesis of the present dissertation, we separated our investigation into the following five studies:

Neonatal Dexamethasone Treatment Leads to Alterations in Cell Signaling Cascades Controlling Hepatic and Cardiac Function in Adulthood

1) In Chapter 2, we set out to determine the effects of early glucocorticoid exposure on AC signaling so as to define the magnitude of effect associated with a treatment known to lead to prediabetes later in life [43,74,120,121,199]**.** In our earlier work with developmental exposure to OPs [13,113], we found that gain-of-function of AC resulted in a change in hepatic cellular function that led to prediabetes [93,150], the same outcome as found for AC polymorphisms in humans [119]. Given the known connection between early-life glucocorticoid exposure and later emergence of similar metabolic disorders [43,74,120,121,199], and the fact that AC signaling cascade controls neuronal and hormonal inputs that regulate hepatic glucose metabolism and cardiac contractility, we hypothesized that the treatments would produce sensitization of AC signaling to gluconeogenic and glycolytic signals.

Neonatal Organophosphate Exposure Alters the Developmental Trajectory of Cell Signaling Cascades Controlling Metabolism: Differential Effects of Diazinon and Parathion in Liver, Heart and Cerebellum

2) In Chapter 3, we looked at the effects of low-level, early-life exposure of two OPs, DZN and PRT, on the trajectory of AC signaling development in heart and liver from adolescence to adulthood, with the specific idea of comparing these outcomes to those seen with DEX. Furthermore, the effects of each OP on the trajectory of development of hepatic and cardiac AC signaling were compared to the those in the cerebellum, a brain region that shares the same, high degree of AC response to βAR stimulation [170]. Our purpose was to see whether the divers outcomes of early-life exposure to different OPs

are associated with differences in their long-term effects on AC signaling. Notably, recent data indicate that low-level OP exposures, below the threshold for anticholinesterase actions, disrupt cell-signaling cascades that control cell differentiation and other critical regulatory functions [68,145-147]. Therefore, we hypothesized that since cell signaling is targeted by OP mechanisms other than their shared property as cholinesterase inhibitors, there is no reason to presuppose that all OPs will act in the same way. Thus, the effects reported in extensive studies of CPF may not be predictive of the actions of all OPs. Furthermore, since AC is a shared mechanism for cell function outside the nervous system, we hypothesized AC would prove to be a target for OP developmental toxicity and an underlying mechanism for the long-term consequences of OP exposure in the liver and heart, as is the case in the brain. As a result, the effects on signaling would involve tissues other than the brain.

Neonatal Parathion Exposure and Interactions with a High-Fat Diet in Adulthood: Adenylyl Cyclase-Mediated Cell Signaling in Heart, Liver and Cerebellum

3) Our results in Chapter 3 showed that neonatal PRT evokes later upregulation of the hepatic AC pathway in adolescence but that the effect wanes by young adulthood; nevertheless metabolic changes resembling prediabetes persist. In Chapter 4, we administered PRT to neonatal rats, straddling the threshold for cholinesterase inhibition, but we extended the studies to 5 months of age and implemented a high-fat diet for 7 weeks. Our goal was to determine whether metabolic challenge imposed by consuming a high-fat diet for 7 weeks would exacerbate neonatal PRT's effects. Alternatively, even in the absence of an interaction between neonatal PRT exposure and a high-fat diet, the diet

alone might produce converging effects on the AC signaling cascade resembling those of early OP exposure; this would in turn suggest that OP exposures could contribute to the worldwide increase in the incidence of obesity and diabetes just as does a high fat diet.

Disparate Developmental Neurotoxicants Converge On The Cyclic AMP Signaling Cascade, Revealed By Transcriptional Profiles *IN VITRO* and *IN VIVO*

4) Our results for OP effects on AC signaling could reflect either direct effects on the pathway, or indirect effects mediated through any of the myriad changes evoked in whole animal physiology by the early-life OP exposure. In Chapter 5, we set out to clarify the extent to which OPs alter cAMP signaling through direct effects on activation or repression of genes encoding the key elements of the pathway using neuronotypic PC12 cells. To determine the specificity of effects towards OPs, we compared CPF and DZN to an organochlorine (dieldrin) and a metal (Ni^{2+}), assessing gene transcription involved in the cAMP pathway. Each agent was introduced during neurodifferentiation for 24 or 72 hr and we mined data from a preexisting database, evaluating 69 genes that encode AC isoforms and regulators, G-protein α- and β,γ-subunits, protein kinase A subtypes and the phosphodiesterase family. Indeed, disparities in the targeting of cell signaling pathways likely account for divergent neurochemical and behavioral outcomes after exposure to different members of the OP class [3,7,9,10,97,138,149,151,158,159,183]. However, we hypothesized that since cell signaling pathways are shared by diverse neurotransmitter and hormonal systems, they may serve as points of convergence for the effects of otherwise unrelated developmental neurotoxicants.

Organophosphate Exposure During a Critical Developmental Stage Reprograms Adenylyl Cyclase Signaling in PC12 Cells

5) The studies in Chapter 5 involved the transcriptional level. We then investigated how the mRNA findings might tie into function at the level of protein. We again used PC12 cells, a well-characterized neurodevelopmental model [182] that reproduces many of the key mechanisms and features of the adverse effects of OPs *in vivo* [15,16,39,40,42,52,83,98,117,133,134,145-147,176,187,201], to examine the effects of different OPs (CPF, DZN and PRT) on AC signaling under conditions spanning different developmental stages from the undifferentiated state, at the initiation of differentiation and after a more prolonged period of differentiation. We planned to determine whether programming of AC function is established by a direct effect during development (i.e. OP effects directly on AC function or expression), or represents a 'downstream' event consequent to disruption of earlier events in differentiation. The following experimental observations led to the solidification of our hypothesis: In control cells, we noted that the first two days after the start of NGF-induced neurodifferentiation had a critical effect on AC activity profiles. Although there was a drop in total activity, stimulants acting at the level of G-proteins or AC itself then elicited larger proportional increases over basal activity than in the undifferentiated state. Further, differentiation produced a drop in the Mn^{2+}/forskolin response ratio, indicative of a shift in AC isoform expression [205]. Over the ensuing four days, most of the effects of NGF on AC signaling leveled off, whereas those on cell growth parameters (DNA, membrane protein/DNA) showed a clear

progression over time. Thus, we hypothesized that the initial stages of neurodifferentiation would be especially sensitive for disruption of AC signaling by OPs.

Chapter 2: Neonatal Dexamethasone Treatment Leads to Alterations in Cell Signaling Cascades Controlling Hepatic and Cardiac Function in Adulthood

Our decision to use DEX to provide a framework for understanding lasting changes in AC was based on the known connection between early-life glucocorticoid exposure and later emergence of metabolic disorders [43,74,120,121,199]. Furthermore, our earlier work with developmental exposure to OPs [13,113] showed gain-of-function of AC resulted in a change in hepatic cellular function that led to prediabetes [93,150].

In a series of recent studies, it was shown that, even at subtherapeutic doses, DEX exposure in fetal and neonatal rats compromises key aspects of brain development when given during specific critical periods corresponding to the selfsame developmental stages recommended for preterm infants [86-88,152]. In this chapter, we have used the same approach to address cell signaling in peripheral tissues that are the likely targets for the emergence of metabolic and cardiovascular disorders, the liver and heart.

We focused on the key role played by the AC cascade, which governs the formation of cAMP. cAMP is the second messenger that controls hepatic gluconeogenesis and glycogenolysis, modulates insulin function and regulates heart rate and contractility. In the liver, βARs and glucagon receptors act through the stimulatory G-protein, G_s, to activate AC, thus eliciting gluconeogenesis and lipolysis.

We evaluated the effects of DEX exposure of neonatal rats in two treatment periods, PN 1-3 and 7-9, bracketing stages of development in the rat that are equivalent to those in second- to early-third trimester human fetuses, the stage in which glucocorticoid use is recommended for preterm infants [64]; we focused on doses within the therapeutic

range (0.2 or 0.8 mg/kg) as well as a much lower dose (0.05 mg/kg) likely to be more representative of stress-related glucocorticoid actions. The three-day regimens were chosen to correspond to multiple glucocorticoid courses, as used in approximately 85% of all cases of preterm delivery [41]. We then evaluated the impact on the AC signaling cascade in adulthood (PN75), focusing on each individual step in the pathway (Figure 7); the studies were modeled after our earlier work on OPs [13,113]. In addition to assessing the effects on basal AC activity, we evaluated the response to βARs and glucagon receptors, both of which stimulate AC through via activation of the stimulatory G-protein, G_s. We also determined the effect of fluoride, which evokes maximal activation of both G_s and the corresponding inhibitory protein, G_i. We then measured the maximal activation of AC itself by forskolin, which acts directly on the enzyme by binding to the catalytic core [80]. Finally, we measured ligand binding for βARs and for the inhibitory m_2-muscarinic acetylcholine receptors (m_2AChRs).

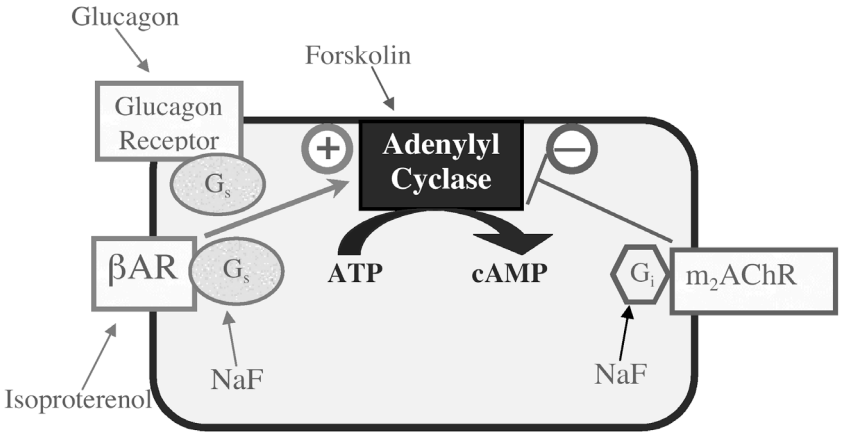

Figure 7: Mechanisms controlling AC activity, showing probes for each step in the pathway: isoproterenol for the βAR, glucagon for the glucagon receptor, NaF for the G-proteins and forskolin for AC itself. Both βARs and glucagon receptors enhance AC activity through the stimulatory G-protein, G_s, whereas m_2AChRs inhibit AC through mediation of the inhibitory protein, G_i.

MATERIALS AND METHODS

Animal treatments. All experiments were carried out humanely and with due regard for alleviation of suffering, with protocols approved by the Institutional Animal Care and Use Committee and in accordance with all federal and state guidelines. Timed-pregnant Sprague-Dawley rats were housed in breeding cages, with a 12-hr light/dark cycle and free access to food and water. On the day of birth, all pups were randomized and redistributed to the dams with a litter size of 10 to maintain a standard nutritional status. Pups received daily subcutaneous injections of DEX phosphate (0.05, 0.2, or 0.8 mg/kg) on PN1–3 or PN7–9, whereas controls received equivalent volumes (1 ml/kg) of isotonic saline vehicle. On each day of treatment and at intervals of several days thereafter, pups were re-randomized within their respective treatment groups and in addition, dams were rotated among litters to distribute any maternal caretaking differences randomly across litters and treatment groups. Offspring were weaned on PN21. On PN75, one male and one female from each of the finally-assigned litters were decapitated and the heart and one liver lobe were dissected, blotted, frozen in liquid nitrogen and maintained at -45° C.

Assays. Tissues were thawed and homogenized (Polytron; Brinkmann Instruments, Westbury, NY) in 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 then sedimented at 40,000 × *g* for 15 min. The pellets were washed twice and then resuspended in 250 mM sucrose, 2 mM $MgCl_2$ and 50 mM Tris. For determinations of AC activity, aliquots of

the 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 $MgCl_2$, 10 μM GTP, 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. The enzymatic reaction was stopped by heating and sedimentation, and the supernatant solution was then assayed for cAMP using commercial radioimmunoassay or immunoassay kits; the two types of kits gave equivalent results. In addition to assessing basal AC activity, we evaluated responses to 100 μM isoproterenol, 3 μM glucagon, 10 mM NaF and 100 μM forskolin. These concentrations produce maximal responses to each stimulant as assessed in earlier studies [13,204,206].

For the ligand binding determinations, there were technical limitations imposed by the large number of membrane preparations that had to be examined. The overall strategy was to determine binding at a single, subsaturating ligand concentration to enable the detection of changes that originate either in altered K_d or B_{max}. To evaluate βAR binding, aliquots of the same membrane preparation were incubated with 67 pM [^{125}I]-iodopindolol in 145 mM NaCl, 2 mM $MgCl_2$, 1 mM sodium ascorbate, 20 mM Tris (pH 7.5), for 20 min at room temperature; samples were evaluated with and without 100 μM isoproterenol to displace specific binding. Incubations were stopped by addition of 3 ml ice-cold buffer, and the labeled membranes were trapped by rapid vacuum filtration onto glass fiber filters, which were washed with additional buffer and counted by liquid scintillation spectrometry. For cardiac m_2AChR binding, the membrane suspension was reconstituted in 10 mM sodium-potassium phosphate buffer (pH 7.4) and incubated with

1 nM [^{3}H] AFDX384, with or without 1 μM atropine to displace specific binding; determinations were not done in the liver, since this tissue is sparse in m_2AChRs and lacks sufficient AC response to m_2AChR agonists.

Data analysis. Data are presented as means and standard errors obtained from 6 animals in each treatment group for each sex and treatment regimen. To establish treatment differences, a global analysis of variance (ANOVA; data log transformed because of heterogeneous variance across tissues and AC stimulants) was first conducted for all variables: the *in vivo* treatment groups (control vs. DEX doses), sex, tissue and the stimulant condition under which the measurement was made (basal AC, isoproterenol-stimulated AC, glucagon-stimulated AC, fluoride-stimulated AC, forskolin-stimulated AC); the latter was considered to be a repeated measure because the same membrane preparation was used for each of the multiple assay conditions. As justified by significant interactions of treatment with the other variables, data were then subdivided to permit testing of individual treatments and AC stimulant responses 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 DEX 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 [173]. The criterion for interaction terms was not used to assign significance to the effects but rather to identify interactive variables requiring subdivision for lower-order tests of main effects of DEX,

the variable of chief interest. Where treatment effects were not interactive with other variables, we report only the main treatment effects without performing lower-order analyses of individual values.

To enable ready visualization of treatment effects across different tissues, treatment regimens and stimulants, the results are given as the percent change from control values, but statistical procedures were always conducted on the original data. For reference, the corresponding control values are detailed in Table 1. For the liver, there were two control cohorts (those receiving vehicle injections on PN1-3 and PN7-9) and the values shown in Table 1 were normalized and combined across the two groups; however, the effects of DEX were compared only to the appropriately matched control cohort.

Materials. Animals were purchased from Charles River (Raleigh, NC). [^{125}I]Iodopindolol (specific activity, 2200 Ci/mmol) and [^{3}H]AFDX384 (115 Ci/mmol) both came from PerkinElmer Life Sciences (Boston, MA), and cAMP radioimmunoassay and enzyme immunoassay kits were purchased from GE Healthcare Biosciences (Piscataway, NJ). All other chemicals were bought from Sigma Chemical Company (St. Louis, MO).

RESULTS

In control rats (Table 1), both liver and heart AC activities showed robust responses to stimulants ($p < 0.0001$ for the main effect of each stimulant compared to basal activity).

Table 1: Adenylyl Cyclase Activities and Receptor Binding in Controls

	Liver		Heart	
	Male	Female	Male	Female
Basal AC[a]	4.1 ± 0.1	4.1 ± 0.1	6.4 ± 0.3	7.7 ± 0.2*
Isoproterenol-Stimulated AC[a]	6.3 ± 0.3	6.7 ± 0.3	27 ± 1	35 ± 2*
Glucagon-Stimulated AC[a]	43 ± 2	45 ± 2	14 ± 1	20 ± 1*
NaF-Stimulated AC[a]	22 ± 1	24 ± 1	48 ± 3	49 ± 1
Forskolin-Stimulated AC[a]	121 ± 6	123 ± 6	205 ± 11	246 ± 8*
βAR Binding[b]	2.8 ± 0.1	3.6 ± 0.1*	6.2 ± 0.2	6.0 ± 0.2
m_2AChR Binding[b]	—	—	119 ± 2	141 ± 9*
Tissue weight	6.2 ± 0.3[c]	3.9 ± 0.1[c]*	1.22 ± 0.03[c]	0.81 ± 0.03[c]*

Body weights (grams): male 453 ± 7; female 280 ± 6*
[a]pmol/min per mg protein
[b]fmol/mg protein
[c]grams; liver weight is for one lobe only
***significant difference between males and females**

However, differences in the overall pattern of AC activity between liver and heart, reflected disparities in the relative effects of the various AC stimulants. In the liver, glucagon produced a much larger response than did isoproterenol, whereas the opposite was true for the heart; this reflects the relatively greater physiologic importance of glucagon signals in the liver as compared to βAR signals in the heart. Similarly, in the liver, glucagon produced a greater stimulatory response than did fluoride, reflecting the mixed involvement of both stimulation (G_s-related) and inhibition (G_i-related) for the latter agent; in the heart, isoproterenol produced a smaller signal than did fluoride.

Superimposed on these basic patterns, the heart showed generally higher AC activities and m_2AChR binding in females as compared to males, whereas the liver showed sex-related differences only for βAR binding.

At the lowest dose, neonatal DEX treatment for either the PN1-3 or PN7-9 regimen did not have significant effects on body weights at PN75 but DEX did evoke statistically significant reductions at the two higher doses with either regimen (Figure 8).

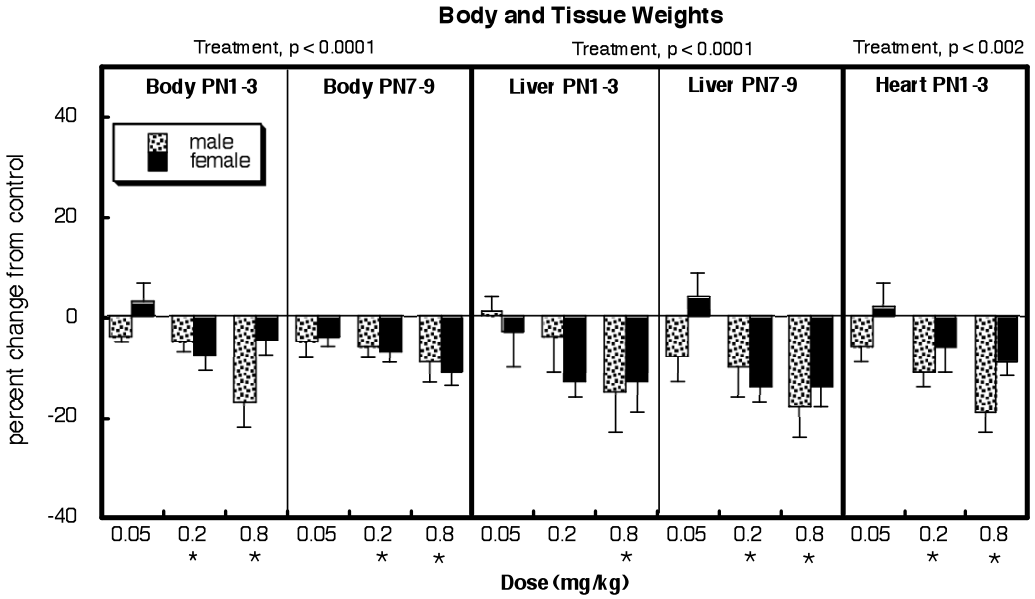

Figure 8: Effects of neonatal DEX treatment on body and tissue weights. Data represent means and standard errors obtained from 6 animals in each group, presented as the percent change from the corresponding control values (Table 1). ANOVA appears at the top and asterisks denote values that differ from the control.

Liver weights were taken from a single lobe, making sure to take the same lobe from each animal. In general, liver weights were reduced with the same pattern as body weights, as were heart weights.

Because we made assessments for two treatment regimens (PN1-3, PN7-9) in the liver but only one regimen (PN1-3) in the heart, we initially performed two multivariate

ANOVAs to evaluate the effects of DEX treatment. In the liver, the global test indicated a significant main effect of DEX treatment reflecting overall increases in AC activity ($p < 0.0002$) that depended upon regimen and sex (treatment × regimen × sex, $p < 0.02$). Accordingly, we separated the data by regimen (PN1-3 and PN7-9) and sex, and then performed lower-order tests to reexamine the results for treatment effects and interactions with the remaining variable of AC stimulant. In the second multivariate ANOVA, we assessed effects on liver and heart AC for the PN1-3 regimen and identified a main treatment effect of DEX ($p < 0.0001$) that was interactive with tissue ($p < 0.0001$) and sex ($p < 0.002$); accordingly, we separated the values for the heart and liver, and for males and females for the lower-order tests.

Liver AC. In the liver, PN1-3 DEX treatment elicited a significant overall increase in AC activity in both males (Figure 9A) and females (Figure 9B), without distinction among the various AC stimulants (no treatment × stimulant interaction). Importantly, both sexes showed significant effects even at the lowest dose (0.05 mg/kg), which did not cause any growth restriction. However, there was a sex difference in that males showed significant increases at each of the three different DEX doses whereas females showed a nonmonotonic dose-effect relationship, with no significant increases at the highest dose.

Sex disparities became more evident when the treatment window was shifted later, to PN7-9. In males (Figure 9C), we still saw significant overall upregulation of AC activity (main treatment effect, $p < 0.02$) whereas now, there were no significant effects in females (Figure 9D).

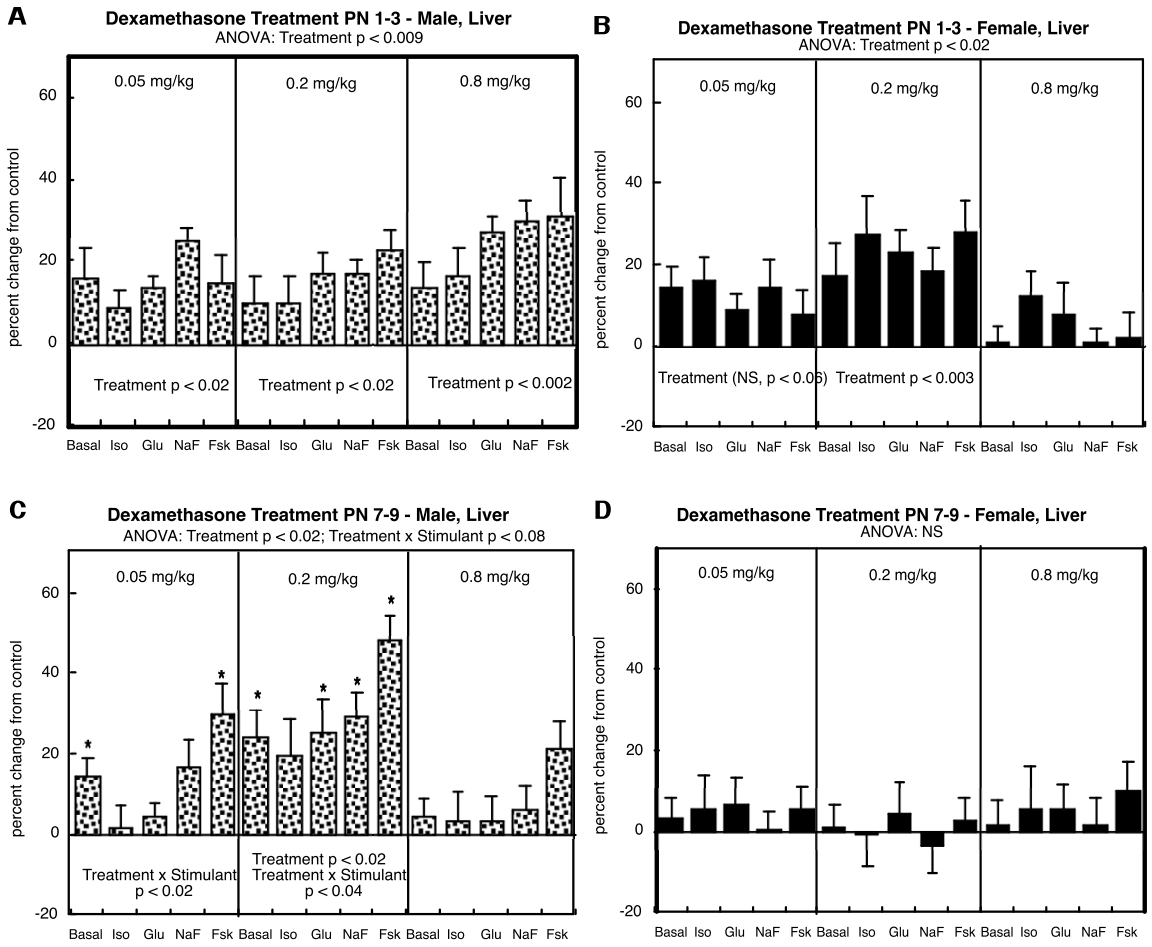

Figure 9: Liver AC activity in animals given DEX on PN1-3 (A,B) or PN7-9 (C,D). Data represent means and standard errors obtained from 6 animals in each group, presented as the percent change from the corresponding control values (Table 1). ANOVA appears at the top of each panel and lower-order tests are shown within the panels. Where there was a significant treatment × stimulant interaction, asterisks denote specific responses that differ from the control. Abbreviations: Iso, isoproterenol; Glu, glucagon; NaF, sodium fluoride; Fsk, forskolin; NS, not significant.

For the males, the dose-effect relationship and stimulant response pattern differed from that obtained with the PN1-3 regimen. Exposure on PN7-9 produced significant increases in AC responses even at the lower dose, but the dose-effect relationship was nonmonotonic, showing loss of effect at the highest dose. Furthermore, DEX augmented the response to forskolin significantly more than for the other stimulants (significant treatment × stimulant interactions, Figure 9C). In the group given 0.05 mg/kg DEX,

there was no significant increase for isoproterenol-stimulated AC or glucagon-stimulated AC, and the response to fluoride showed a small, nonsignificant effect. Raising the dose to 0.2 mg/kg did produce significant increases for glucagon- and fluoride-stimulated AC but the response to forskolin was still enhanced to a greater extent.

Heart AC. To determine the selectivity of the effects of DEX on AC signaling, we compared the effects to those seen in the heart, focusing on PN1-3, the regimen which evoked increases in both sexes. Males exposed to DEX again displayed significant overall increases in AC activity (Figure 10A). However, the dose-effect relationship was distinctly different from that seen in the liver, with a large increase obtained at the lowest dose and a progressive loss of effect as the dose was raised. Further, the response to glucagon showed the greatest increase, a pattern not seen in the liver. In females, the differences between heart and liver were even more stark (Figure 10B). There were no significant effects at 0.05 mg/kg, and increasing the dose to 0.2 and then 0.8 mg/kg produced deficits in AC responses, at first involving glucagon and then encompassing all the AC stimulants, with the largest effect still exerted toward the glucagon response.

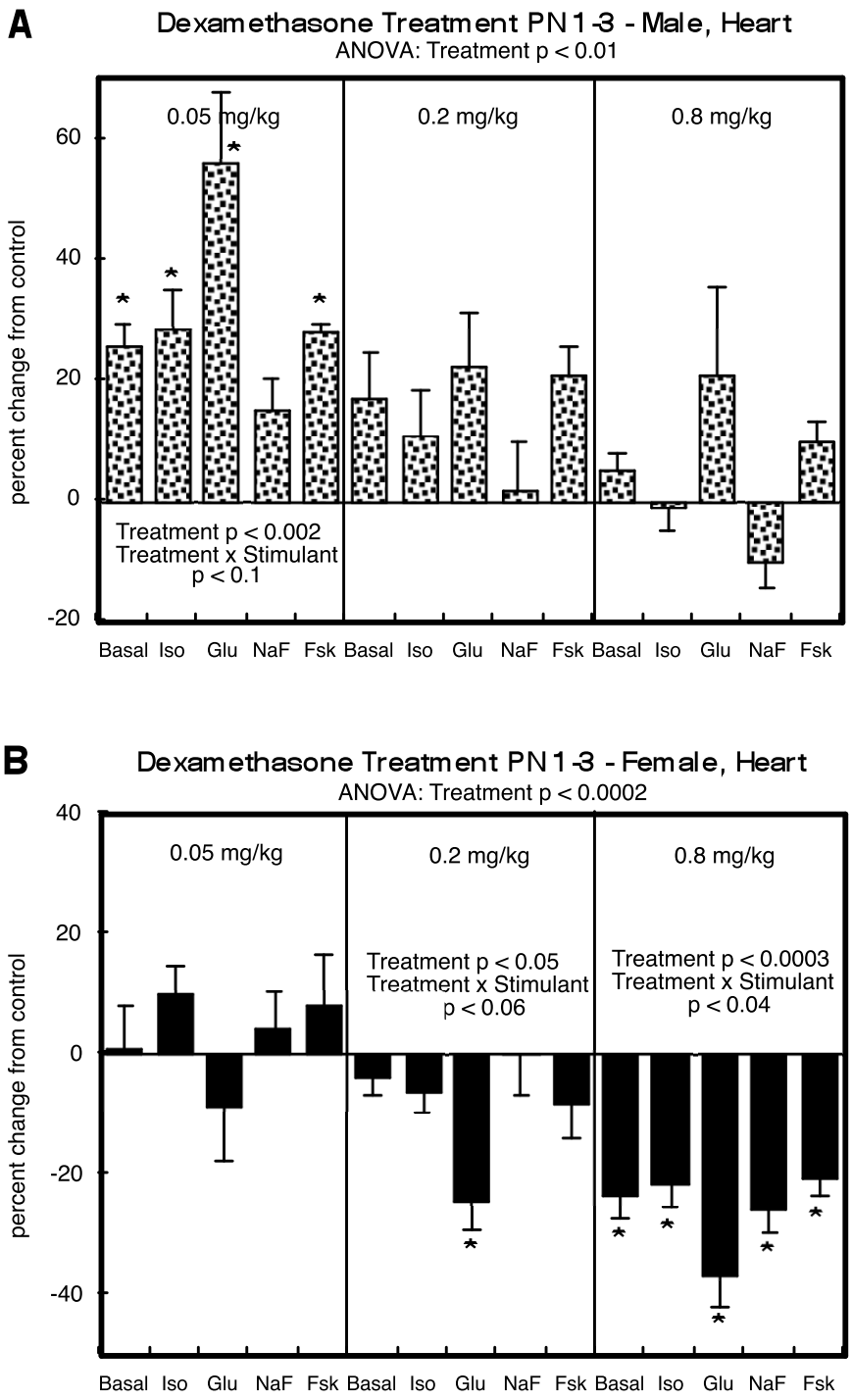

Figure 10: Heart AC activity in animals given DEX on PN1-3. Data represent means and standard errors obtained from 6 animals in each group, presented as the percent change from the corresponding control values (Table 1). ANOVA appears at the top of each panel and lower-order tests are shown within the panels. Where there was a significant treatment × stimulant interaction, asterisks denote specific responses that differ from the control. Abbreviations: Iso, isoproterenol; Glu, glucagon; NaF, sodium fluoride; Fsk, forskolin; NS, not significant.

Receptor binding (Figure 11). For liver βARs, multivariate ANOVA identified a weak treatment × regimen ($p < 0.09$); therefore, we separated the values for both treatment regimens and examined lower-order main treatment effects and interactions of treatment with other variables, but no significant differences were seen after the subdivision. There was a trend toward elevated βAR values for females with PN1-3 DEX exposure, an effect that did not correspond to any selective increase in the AC response to isoproterenol. In the heart, there was significant upregulation of βARs and downregulation of m_2AChRs.

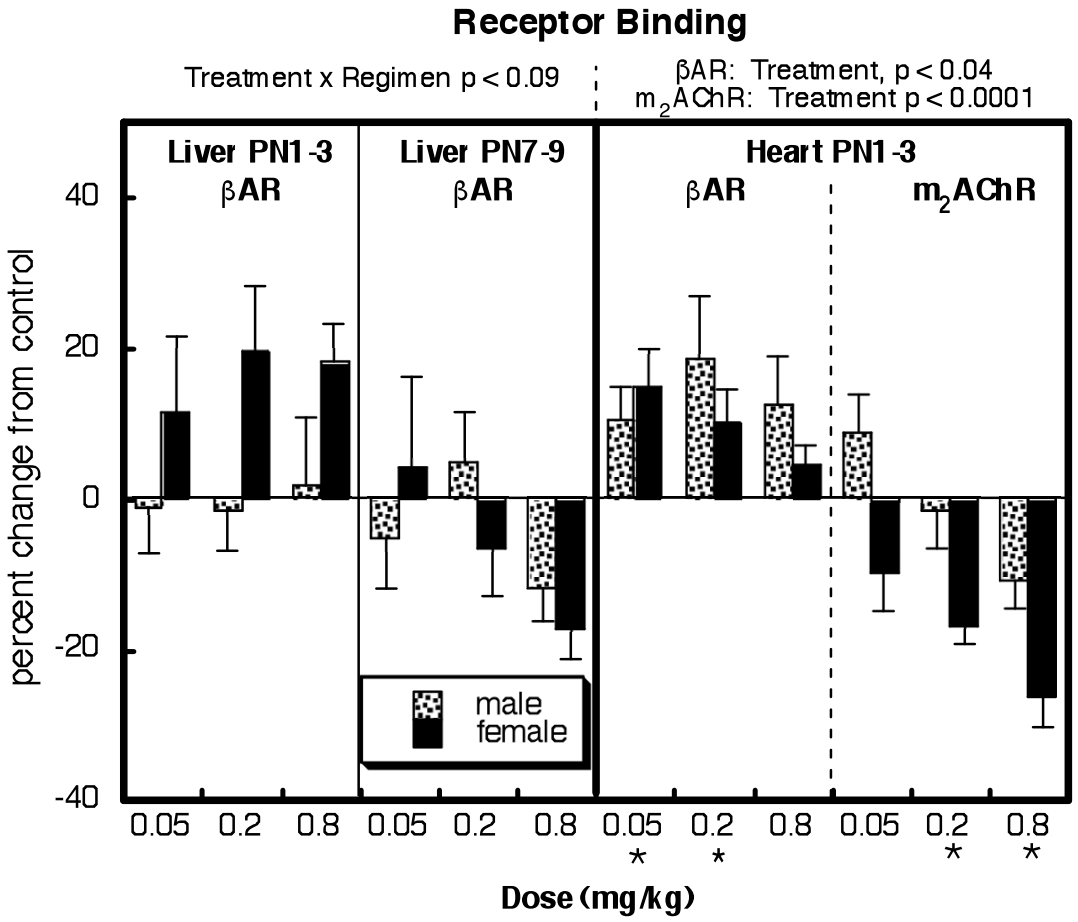

Figure 11: Effects of neonatal DEX treatment on liver βAR binding and on heart βARs and m_2AChRs. Data represent means and standard errors obtained from 6 animals in each group, presented as the percent change from the corresponding control values (Table 1). ANOVA appears at the top and asterisks denote values that differ from the control.

DISCUSSION

There are four main findings of this study. First, DEX treatment in the developmental phases corresponding to those in which glucocorticoids are used in preterm infants, elicits global increases in hepatic AC cell signaling. Second, robust effects were seen even at 0.05 mg/kg, a dose well below those used therapeutically, and likely to be more representative of the actions of endogenous glucocorticoids released in stress. Third, the effects involved AC gain-of-function, thus producing heterologous sensitization, in which the responses to disparate inputs all showed the same augmented effect. Fourth, there were distinct sex differences as well as disparities in the effects on liver vs. heart, indicative of selective actions rather than ubiquitous increases in AC signaling.

The results for the hepatic effects of DEX treatment on PN1-3 provide the clearest example of heterologous sensitization of the AC pathway. At each of the three different doses, we found significant increases in AC regardless of whether the activity was assessed in the basal state, in response to receptor stimulants or G-protein activation, or with direct AC activation by forskolin. The parallel changes point to gain-of-function of AC itself as the underlying mechanism. Accordingly, there is an augmented response to any upstream signal, including the responses to activation of βARs or glucagon receptors, which together provide major inputs to glucose and lipid metabolism; further emphasizing the point that sensitization involves AC itself, we did not observe any significant βAR upregulation that would signify a selective, upstream effect at the receptor level. The results thus resemble those seen in our earlier work with

developmental exposure to OPs [13,113]; in that case, we found that this change in hepatic cellular function leads to prediabetes [93,150], the same outcome as found for AC polymorphisms in humans [119]. Given the known connection between early-life glucocorticoid exposure and later emergence of similar metabolic disorders [43,74,120,121,199], our results thus provide one of the likely contributory mechanisms that connect DEX exposure to adult disease outcomes.

Superimposed on the heterologous activation of AC signaling, DEX treatment on PN1-3 showed selectivity for both sex and tissue. Although females, like males, showed hepatic AC sensitization, the dose-response curve was nonmonotonic, with loss of effect at the highest dose. A similar nonmonotonic relationship between dose and effect was seen in the heart. In males, cardiac AC was maximally sensitized by the lowest dose of DEX and then showed a progressive loss of effect as the dose was raised; females showed a dose-dependent *reduction* in AC instead of the increase that had been seen in the liver. Since high doses of DEX are known to disrupt neuroendocrine function and to produce general somatic deficits, it is likely that the loss of the promotional effect on AC signaling involves mechanisms other than a direct influence on the signaling cascade [29,57,58,63,65,101,103,104,106,108,194,195]. Nevertheless, just as for the metabolic outcomes related to the effects on hepatic cell signaling, the functional consequences for cardiac structure and function are known: outright cell loss [168], alterations in the expression of cardiac contractile proteins [26] and impaired heart rate control [75,96]. As seen here, the greater negative impact on the glucagon response may indicate a further deficit in cardiac glucose utilization [12]. Superimposed on these effects, DEX exposure

impairs the development of cardiac sympathetic projections, which would augment any deficits in AC signaling downstream from βAR input [148]. Viewed in this light, the small βAR upregulation and m_2AChR downregulation found here may represent partial compensation to offset the effects of the downstream changes in signaling; it would be worthwhile to pursue the balance of physiological responses (heart rate, contractility) to sympathetic and parasympathetic input to determine how these cellular changes influence physiological responses.

Shifting the DEX exposure to PN7-9 again produced heterologous hepatic AC sensitization but with notable differences from the effects of the PN1-3 regimen. In males, the dose-response curve became nonmonotonic, with loss of effect at the highest DEX dose; this is compatible with the greater deficits in somatic growth with the PN7-9 treatment, which would then be expected to offset the direct effects of DEX on signaling as shown above. However, in females, there was no significant effect at any of the doses; this points instead to the closing of the critical developmental period in which DEX evokes lasting changes in AC signaling. A similar conclusion was reached in our earlier work with neurodevelopmental indices [86,88,152]. Thus, the most sensitive period for reprogramming of cell signaling evoked by DEX exposure corresponds to the developmental phase in which glucocorticoids are most likely to be used in preterm labor [64]. The later treatment also showed selective effects on the responses to AC stimulants that were not seen with the PN1-3 regimen, characterized by smaller effects on the responses to receptor stimulants than on the direct AC response to forskolin. This indicates reductions in the efficiency of receptor coupling to cAMP generation; future

studies should address the issue of whether these targeted deficits are of functional significance over and above the heterologous effects exerted at the level of AC itself.

Our findings thus extend the Barker Hypothesis, which originally related prenatal stress and associated growth restriction to subsequent development of cardiovascular disease and diabetes [21]. Specifically, our finding that glucocorticoid exposures below the threshold for somatic growth impairment nevertheless lead to cell signaling changes that underlie metabolic and cardiac dysfunction points to the greater relative importance of stress as distinct from growth impairment in adverse outcomes. In turn, this means that a wider variety of prenatal stressors and chemical exposures that are insufficient to elicit growth impairment, may nevertheless contribute to the worldwide increase in the incidence of diabetes. Finally, our results indicate that the common use of glucocorticoids in preterm labor may ultimately contribute to these outcomes through AC signaling alterations similar to those of DZN and PRT, as we shall see in the next chapter.

Chapter 3: Neonatal Organophosphate Exposure Alters the Developmental Trajectory of Cell Signaling Cascades Controlling Metabolism: Differential Effects of Diazinon and Parathion in Liver, Heart and Cerebellum

In the previous chapter our findings show that, in contrast to growth restriction, the glucocorticoids associated with stress or the therapy of preterm labor are more sensitive and more important contributors to the cellular abnormalities underlying subsequent metabolic and cardiovascular dysfunction. These findings agree with recent results that suggest that environmental contaminants, drugs, and chemicals can produce outcomes similar to the original Barker Hypothesis without growth restriction. In this chapter, we will investigate how exposures to common pesticides might provide a route to later-emerging disorders, in particular focusing on OPs, which represent 50% of worldwide insecticide use [32].

Since cell signaling is targeted by OP mechanisms other than their shared property as cholinesterase inhibitors, there is no reason to presuppose that all OPs will act in the same way. Accordingly, the current chapter compares whether the divers outcomes of early-life exposure to different OPs are associated with differences in their long-term effects on AC signaling. We evaluated function at each step in the cascade in liver and heart (refer to Figure 7) after neonatal exposure to DZN and PRT over periods ranging from early adolescence through young adulthood and late adulthood. Further, we measured ligand binding for βARs and for the inhibitory m_2AChRs. The effects of each OP on the trajectory of development of hepatic and cardiac AC signaling were compared to the those in the cerebellum, a brain region that shares the same, high degree of AC

response to βAR stimulation [170].

MATERIALS AND METHODS

Animal treatments. Animal housing, care, litter size, and randomization was identical to that of Chapter 2. Because of their poor water solubility, DZN and PRT were dissolved in dimethylsulfoxide to provide consistent absorption [196] and were injected subcutaneously in a volume of 1 ml/kg once daily on PN1–4. Control animals received equivalent injections of dimethylsulfoxide vehicle, which does not itself produce developmental toxicity [176,196]. At the specified ages, one male and one female from each final litter assignment were decapitated and the heart, one liver lobe and cerebellum were dissected, blotted, frozen in liquid nitrogen and maintained at -45° C. Other tissues and brain regions were reserved for future studies.

The dosing paradigms were chosen to achieve a toxicodynamic match between DZN and PRT [154,169], and similarly, to match earlier studies using CPF [175]. For each OP, we chose doses straddling the threshold for barely-detectable cholinesterase inhibition (5-20% inhibition), well below the 70% inhibition required for signs of cholinergic hyperstimulation [36]: 0.5 and 2 mg/kg/day for DZN; 0.1 and 0.2 mg/kg/day for PRT. Assessments were carried out in adolescence (PN30), early adulthood (PN60), and full adulthood (PN100).

Assays. All procedures used in this study were presented in detail in Chapter 2. Additionally, it is important to note that we did not assess m_2AChR binding in the cerebellum because of the sparsity of the receptors in this tissue. Furthermore, we did not evaluate the effects of glucagon in the cerebellum, a tissue in which this metabolic

hormone has no known biologic role.

Data analysis. Handling of data and the presentation of results are presented in detail in Chapter 2. For reference, the corresponding control values are shown in Table 2; because there were two control cohorts (controls for the DZN study, controls for the PRT study, each comprising 6 males and 6 females for each age point), the values were normalized and presented as a single set. However, statistical comparisons of the effects of DZN and PRT were made only with the appropriately matched control cohort.

Materials. All materials purchased were the same as Chapter 2 except DZN and PRT were obtained from Chem Service (West Chester, PA).

Table 2: Adenylyl Cyclase Activities and Receptor Binding in Controls

	PN30		PN60		PN100	
	Male	Female	Male	Female	Male	Female
Liver						
Basal AC[a]	3.1 ± 0.1	3.1 ± 0.1	4.0 ± 0.1	4.1 ± 0.2	3.2 ± 0.1	3.0 ± 0.1
Isoproterenol-Stimulated AC[a]	4.8 ± 0.2	5.8 ± 0.2*	5.8 ± 0.1	6.3 ± 0.3	4.5 ± 0.2	4.6 ± 0.1
Glucagon-Stimulated AC[a]	29 ± 1	28 ± 1	34 ± 1	31 ± 1*	30 ± 1	27 ± 1*
NaF-Stimulated AC[a]	16.9 ± 0.5	16.9 ± 0.6	22.0 ± 1.1	21.5 ± 0.7	19.3 ± 0.4	18.8 ± 0.5
Forskolin-Stimulated AC[a]	58 ± 4	68 ± 2	77 ± 3	69 ± 2	73 ± 3	67 ± 3
βAR Binding[b]	3.2 ± 0.1	3.5 ± 0.2	3.6 ± 0.2	4.3 ± 0.2	2.7 ± 0.1	3.2 ± 0.1*
Heart						
Basal AC[a]	46 ± 1	44 ± 2	28 ± 1	29 ± 1	20 ± 1	23 ± 1*
Isoproterenol-Stimulated AC[a]	95 ± 2	95 ± 3	62 ± 2	67 ± 3	46 ± 2	54 ± 2*
Glucagon-Stimulated AC[a]	68 ± 2	68 ± 3	50 ± 2	50 ± 2	34 ± 2	40 ± 1*
NaF-Stimulated AC[a]	129 ± 3	127 ± 5	89 ± 2	93 ± 4	78 ± 3	83 ± 3
Forskolin-Stimulated AC[a]	803 ± 7	772 ± 22	646 ± 17	693 ± 24	554 ± 17	585 ± 16
βAR Binding[b]	11.1 ± 0.3	10.3 ± 0.3	8.0 ± 0.2	6.9 ± 0.3*	7.6 ± 0.4	7.3 ± 0.2
m_2AChR Binding[b]	183 ± 4	182 ± 6	161 ± 7	152 ± 7	170 ± 6	188 ± 6*
Cerebellum						
Basal AC[a]	—	—	—	—	166 ± 5	174 ± 4
Isoproterenol-Stimulated AC[a]	—	—	—	—	220 ± 7	208 ± 5
NaF-Stimulated AC[a]	—	—	—	—	212 ± 5	194 ± 5*
Forskolin-Stimulated AC[a]	—	—	—	—	1138 ± 45	1202 ± 60
βAR Binding[b]	—	—	—	—	22.5 ± 0.4	22.4 ± 0.4

[a]pmol / min / mg protein
[b]fmol / mg protein
***significant difference between males and females**

RESULTS

In control rats (Table 2), liver and heart AC activities both showed robust responses to stimulants ($p < 0.0001$ for the main effect of each stimulant compared to basal activity) but the relative response of each stimulant differed among tissues and ages, and between sexes: tissue × stimulant, $p < 0.0001$; sex × stimulant, $p < 0.0005$; age × stimulant, $p < 0.0001$; tissue × age × stimulant, $p < 0.0001$; age × sex × stimulant, $p < 0.06$; tissue × age × sex × measure, $p < 0.007$. Superimposed on the differences in response to stimulants, the overall temporal pattern of AC activity differed between liver and heart (tissue × age, $p < 0.0001$; tissue × age × sex, $p < 0.005$). As shown earlier [118], liver AC declines sharply in the immediate postnatal period, whereas heart AC peaks in early adolescence and then declines; accordingly, here we saw an overall decrease in heart AC from adolescence to adulthood ($p < 0.0001$ for the main effect of age), whereas liver AC showed a slight rise between PN30 and PN60 and a subsequent minor decline by PN100 ($p < 0.0001$ for the main effect of age). Both tissues showed age- and sex-related differences in AC activity and/or stimulant responses, necessitating a point-by-point comparison of sex differences for each measure: liver, $p < 0.06$ for age × sex, $p < 0.003$ for sex × stimulant, $p < 0.0001$ for age × stimulant, $p < 0.007$ for age × sex × stimulant; heart, $p < 0.08$ for age × sex, $p < 0.0001$ for age × stimulant. Nevertheless, the individual sex differences were only sporadic, with the exception of the heart on PN100, where females showed significantly higher AC values than males (main effect of sex, $p < 0.03$; Table 2). The major response differences between liver and heart reflected disparities in the relative effects of the various AC stimulants. In the liver, glucagon

produced a much larger response than did isoproterenol, whereas the opposite was true for the heart; this reflects the relatively greater physiologic importance of glucagon signals in the liver as compared to βAR signals in the heart. Similarly, in the liver, glucagon produced a greater stimulatory response than did fluoride, reflecting the mixed involvement of both stimulation (G_s-related) and inhibition (G_i-related) for the latter agent; in the heart, isoproterenol produced a smaller signal than did fluoride. Finally, in the cerebellum, we again saw robust stimulatory responses to isoproterenol, fluoride and forskolin (all at $p < 0.0001$) but without any sex differences (no main effect of sex or sex × stimulant interaction; Table 2).

Neonatal OP exposure elicited small, but statistically significant effects on body weight (main treatment effect, $p < 0.004$) and heart weight ($p < 0.0001$); liver weights could not be compared because we dissected only a single lobe. The effects on body and heart weight depended on which OP was given, as evidenced by significant interactions of treatment × [DZN vs. PRT]: $p < 0.02$ body weight, $p < 0.005$ for heart weight. DZN produced a significant overall reduction in body weight at either 0.5 or 2 mg/kg, amounting to net deficits of 5% ($p < 0.003$) and 6% ($p < 0.0006$), respectively, consistent with earlier findings [138]; for heart weight, the reductions were slightly larger, 8-9% ($p < 0.0001$ for either dose). In contrast, for PRT we did not find any statistically significant effects on body or heart weights in the animals used for this study; however, these animals were part of a much larger cohort that was used for additional studies [94,153], and in the overall group, PRT caused a small (2-3%) but significant elevation in body weight at the low dose in males, and reductions of about 4% at either dose in females.

Global ANOVA for AC measurements indicated a significant main effect of OP treatment reflecting overall increases in activity ($p < 0.0001$; OP-treated > control) that differed between liver and heart (treatment × tissue, $p < 0.0001$) and between DZN and PRT (treatment × [DZN vs. PRT], $p < 0.04$), as well as displaying more complex interactions: $p < 0.004$ for treatment × [DZN vs. PRT] × age, $p < 0.02$ treatment × [DZN vs. PRT] × age × tissue, $p < 0.0001$ for treatment × AC stimulant, $p < 0.0001$ for treatment × tissue × AC stimulant. Accordingly for presentation, we separated the data into the individual tissues (liver, heart, cerebellum) and treatments (DZN, PRT), and then performed lower-order tests to reexamine the results for treatment effects and interactions.

Liver AC. In the liver, neonatal OP exposure elicited a significant main treatment effect ($p < 0.0001$) that differed between the two OPs and among ages (treatment × [DZN vs. PRT], $p < 0.08$; treatment × [DZN vs. PRT] × age, $p < 0.0001$) and also showed selectivity among the various AC stimulants (treatment × AC stimulant, $p < 0.0001$). Accordingly, we separated the results for DZN and PRT for each age point, and then reexamined the treatment effects.

DZN exposure elicited a significant main treatment effect ($p < 0.0001$), reflecting an overall gain-of-function, with dependence on age (treatment × age, $p < 0.08$) and AC stimulant (treatment × stimulant, $p < 0.005$). On PN30 there was a trend toward overall increases in AC activity in males that did not by itself achieve statistical significance (Figure 12A); however, as noted below, the same pattern was seen in the heart and the overall effect across both tissues was significant. By young adulthood (PN60), the main

effect of DZN treatment became significant for both sexes ($p < 0.001$), reflecting a robust ($p < 0.0003$) enhancement of activity in the animals exposed to 2 mg/kg (Figure 12B). Superimposed on this general increase, there were selectively greater effects on stimulants acting through G_s-coupled receptors (isoproterenol, glucagon) than on the response to fluoride, which stimulates both G_s and G_i (treatment × stimulant, $p < 0.02$); the response to forskolin was also increased to a greater extent than that to fluoride. By PN100 (Figure 12C), both the low and high doses of DZN elicited significant increases in AC ($p < 0.0001$ for all treatments; $p < 0.001$ for 0.5 mg/kg vs. control; $p < 0.0001$ for 2 mg/kg vs. control). Like the PN60 values, the fluoride response was affected to a lesser extent than the receptor stimulants or forskolin ($p < 0.002$ for treatment × stimulant across the two age points).

For hepatic effects of PRT exposure, the global ANOVA indicated a significant main treatment effect ($p < 0.0009$) that depended on age (treatment × age, $p < 0.003$), AC stimulant (treatment × stimulant, $p < 0.0001$) and sex (treatment × stimulant × sex, $p < 0.06$). Most notably, the temporal pattern of effects was completely distinct from that of DZN. On PN30 (Figure 12D), neonatal PRT exposure elicited significant upregulation of AC activity at both 0.1 mg/kg and 0.2 mg/kg ($p < 0.0003$ for the main effect of both treatments; $p < 0.02$ for the low dose vs. control; $p < 0.0001$ for the high dose vs. control). Superimposed on the overall increase, there were selectively greater effects of isoproterenol and forskolin as compared to glucagon or fluoride at the lower dose ($p < 0.002$ for treatment × stimulant); the same pattern was seen at the higher dose ($p < 0.005$ for treatment × stimulant), although the greater overall effect increased the response

sufficiently to achieve statistical significance for all the individual measures. On PN60, the point at which DZN elicited even greater increases in hepatic AC activity, PRT instead showed a loss of effect (Figure 12E) and the same was true in full adulthood, by PN100 (Figure 12F); to make certain that later-emerging changes were not occurring with PRT exposure, we performed an additional set of determinations at 5 months of age and again found no persistent effects on AC.

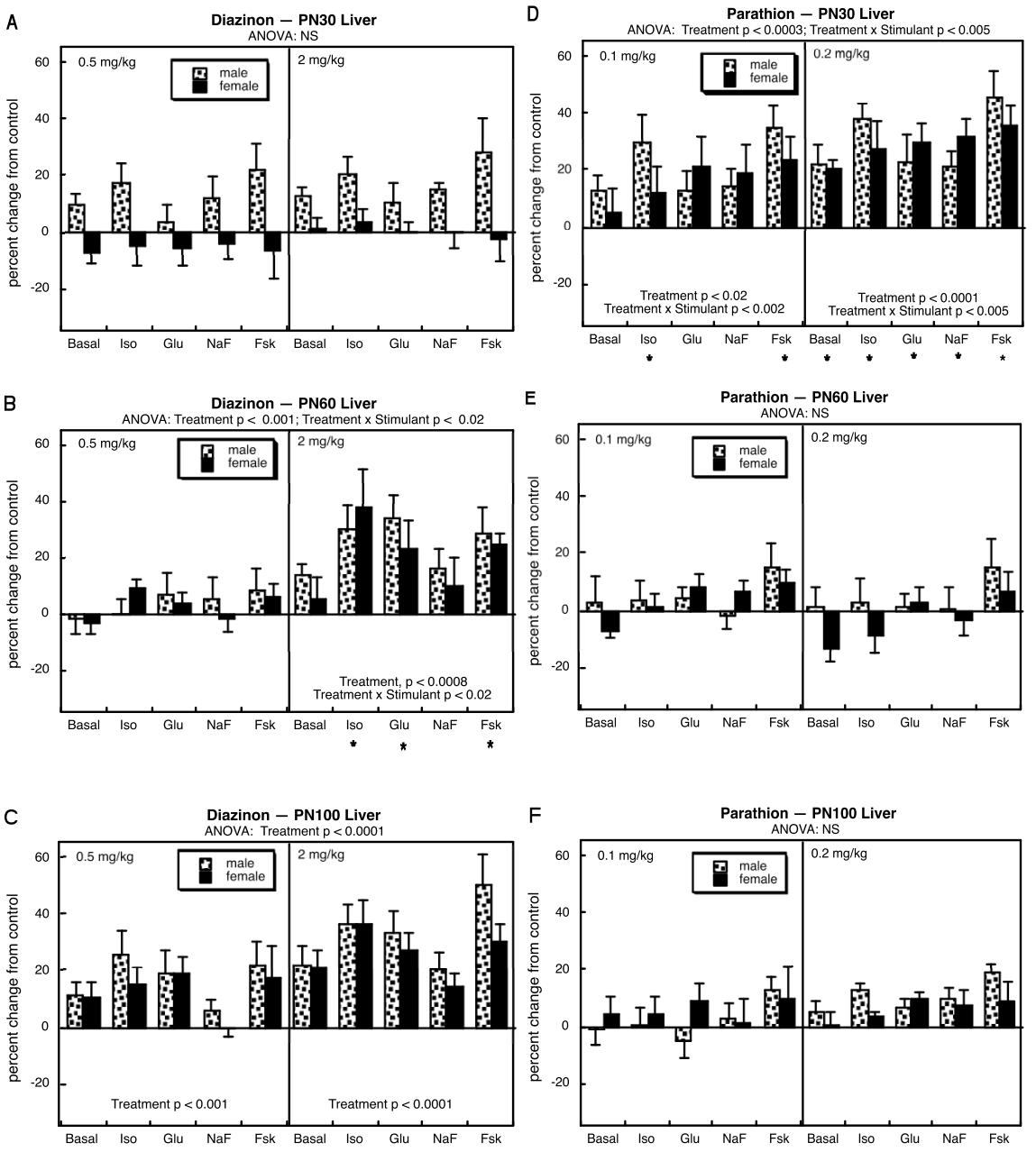

Figure 12: Effects of neonatal exposure to DZN (A,B,C) or PRT (D,E,F) on liver AC activity on PN30 (A,D), PN60 (B,E) and PN100 (C,F). Data represent means and standard errors obtained from 6 animals in each group, presented as the percent change from the corresponding control values (Table 2). ANOVA incorporating the factors of treatment, sex and AC stimulant appears at the top of each panel and lower-order tests are shown within the panels. Where there was a significant treatment × stimulant interaction, asterisks denote specific responses that differ from the control animals; separate tests for males and females were not carried out because of the absence of treatment × sex interactions. Abbreviations: Iso, isoproterenol; Glu, glucagon; Fsk, forskolin; NS, not significant.

Heart AC. In the heart, the effects of neonatal toxicant exposure were less statistically robust than for the liver, but we nevertheless found differences between the two OPs (treatment × [DZN vs. PRT], $p < 0.07$), as well as treatment interactions with age and AC stimulant (treatment × age × AC stimulant, $p < 0.04$). Again, values were separated into the individual treatments and ages for comparisons of lower-order treatment effects. On PN30 (Figure 13A), DZN treatment enhanced AC activity in a sex- and stimulant-selective manner ($p < 0.06$ for treatment × sex, $p < 0.05$ for treatment × stimulant). At the lower dose, there were small overall effects that achieved statistical significance for 2 of the 10 parameters. More robust effects were evident at the higher dose, reflecting a net overall increase in males ($p < 0.04$); in females, the responses to isoproterenol and glucagon were decreased by DZN exposure whereas the response to fluoride was unchanged and that to forskolin was increased. The augmented responses seen in males were similar in direction and magnitude to those noted for the liver at the same age (Figure 12A); ANOVA incorporating both tissues confirmed a main effect of DZN treatment ($p < 0.008$) without tissue selectivity (no treatment × tissue interaction). By PN60, we could no longer detect any significant effects of DZN on heart AC activity (Figure 13B), and although a slight enhancement reappeared by PN100, this did not achieve statistical significance (Figure 13C).

In contrast to the effect of DZN on heart AC, we did not detect significant effects of PRT on the same parameters (Figure 13D,E,F). Again, we performed a follow-up study at 5 months of age to determine if alterations might emerge even later on, but did not find any significant treatment effects.

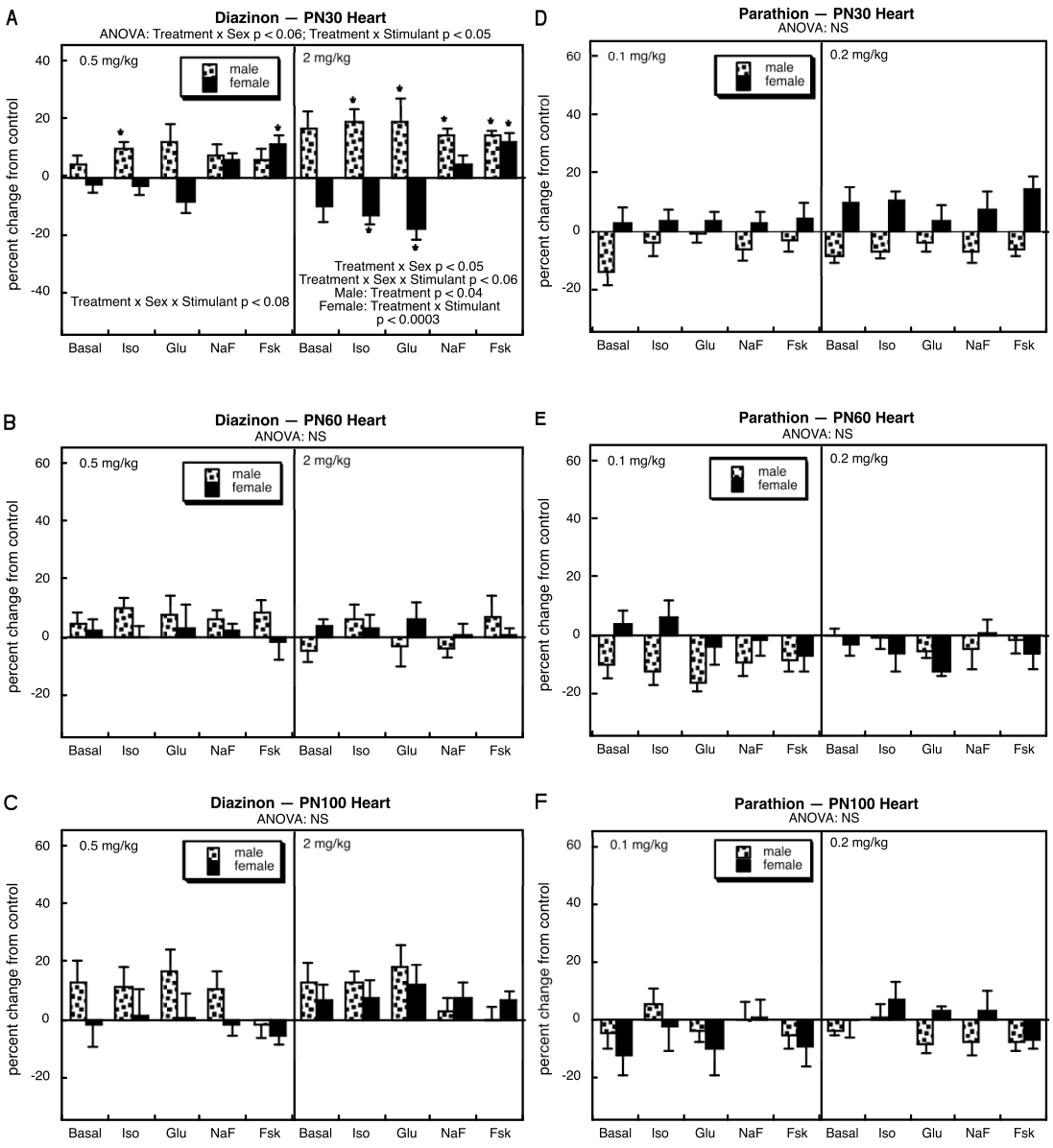

Figure 13: Effects of neonatal exposure to DZN (A,B,C) or PRT (D,E,F) on heart AC activity on PN30 (A,D), PN60 (B,E) and PN100 (C,F). Data represent means and standard errors obtained from 6 animals in each group, presented as the percent change from the corresponding control values (Table 2). ANOVA incorporating the factors of treatment, sex and AC stimulant appears at the top of each panel and lower-order tests are shown within the panels. Where there was a significant treatment × stimulant interaction, asterisks denote specific responses that differ from the control animals. Abbreviations: Iso, isoproterenol; Glu, glucagon; Fsk, forskolin; NS, not significant.

Receptors. For βARs, global ANOVA identified interactions of treatment × age ($p < 0.004$), treatment × sex ($p < 0.05$), treatment × tissue ($p < 0.09$), treatment × [DZN vs. PRT] × sex ($p < 0.01$), treatment × [DZN vs. PRT] × tissue ($p < 0.1$) and treatment × [DZN vs. PRT] × tissue × age ($p < 0.08$). Again, we separated the values for the DZN and PRT treatments and examined lower-order main treatment effects and interactions of treatment with other variables. For DZN (Figure 14A), there were treatment interactions with sex ($p < 0.05$) and tissue ($p < 0.09$). Separation of the values by tissue did not reveal any individually significant treatment effects. However, separation by sex confirmed an overall, significant reduction in βAR binding in females exposed to the lower dose of DZN; the magnitude of this effect was <10%, far smaller than the 30-40% changes seen for the AC response to βAR stimulation, and were in the opposite direction (decrease for βARs, increase for AC response). Neonatal PRT treatment also evoked significant changes in βAR binding that depended on age and tissue (Figure 14B). Separate analyses for each tissue indicated an age-dependent effect in the liver (treatment × age, $p < 0.03$), reflecting an increase on PN30 and a decrease on PN60, restricted to the high dose group. In the heart, βAR binding was decreased selectively in males ($p < 0.002$ in the 0.1 mg/kg group, $p < 0.02$ in the 0.2 mg/kg group).

We evaluated m_2AChR binding only in the heart, since these receptors are sparse in the liver and cerebellum. The global ANOVA identified a significant treatment × sex interaction ($p < 0.006$) and subdivision of the results found a significant treatment effect in females ($p < 0.03$). Nevertheless, the net effect was quite small and the only determinations that achieved statistical significance were those for 0.5 mg/kg DZN

(Figure 14C).

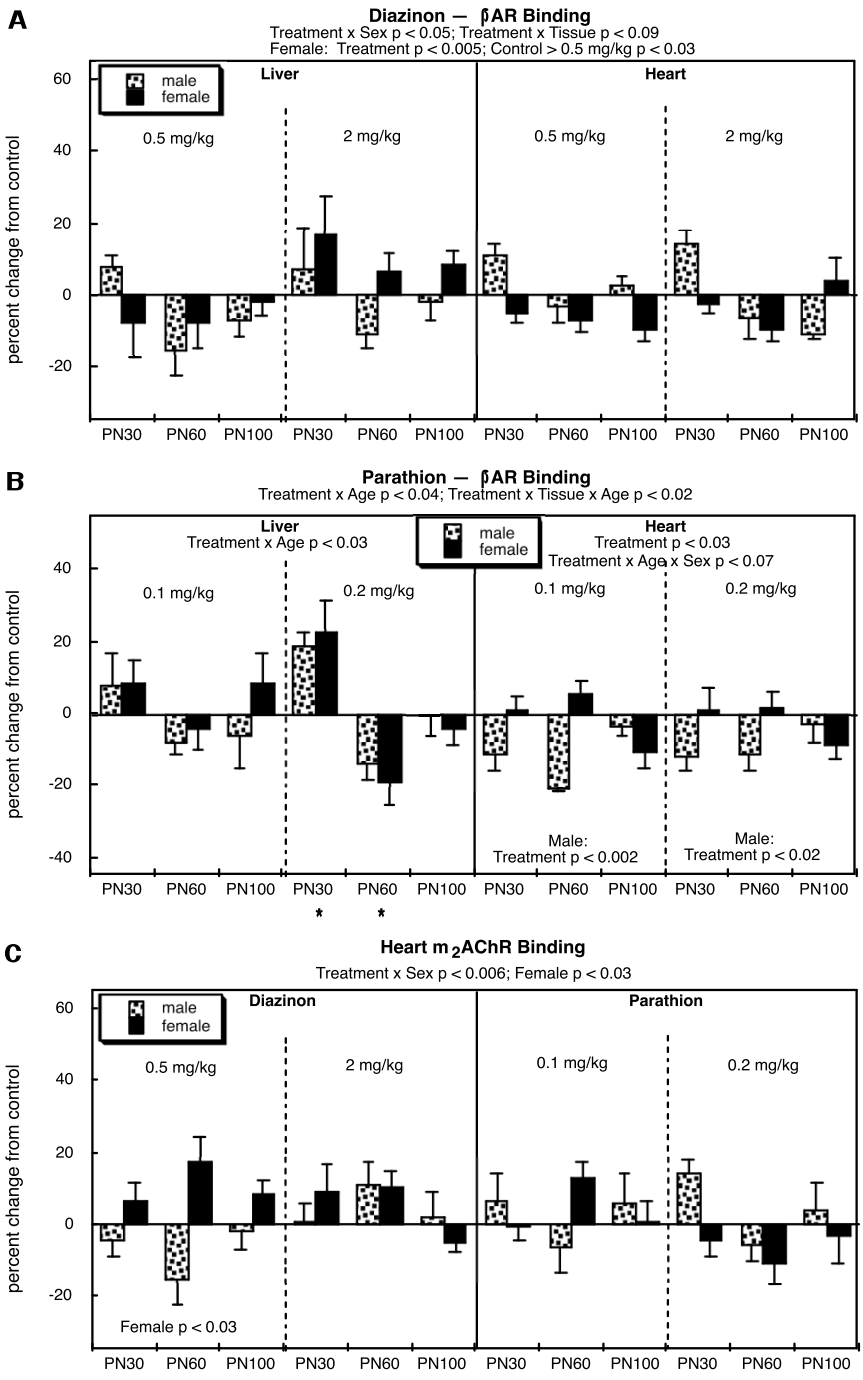

Figure 14: Effects of neonatal DZN or PRT exposure on liver and heart receptors: (A) βAR binding in DZN-exposed animals, (B) βAR binding in PRT-exposed animals, (C) cardiac m_2AChR binding. Data represent means and standard errors obtained from 6 animals in each group, presented as the percent change from the corresponding control values (Table 2). ANOVA incorporating the factors of treatment, sex, age and tissue appears at the top of each panel and lower-order tests are shown within the panels. Asterisks in (B), where there was a treatment × age interaction, show ages for which the PRT group differs from the control.

Cerebellum. Global ANOVA identified treatment effects that differed between DZN and PRT (treatment × [DZN vs. PRT], $p < 0.006$) and between males and females (treatment × sex, $p < 0.06$). Neonatal exposure to the lower dose of DZN elicited significant increases in AC activity that were selective for males ($p < 0.007$) but the higher dose instead produced small, but significant ($p < 0.04$) decrements (Figure 15); the magnitude of the effects was substantially smaller than that seen in the liver at the same age (PN100). The low dose of PRT did not elicit significant alterations in cerebellar AC parameters but the high dose produced an overall increase ($p < 0.05$) without sex selectivity. None of the treatments had a significant effect on cerebellar βAR binding.

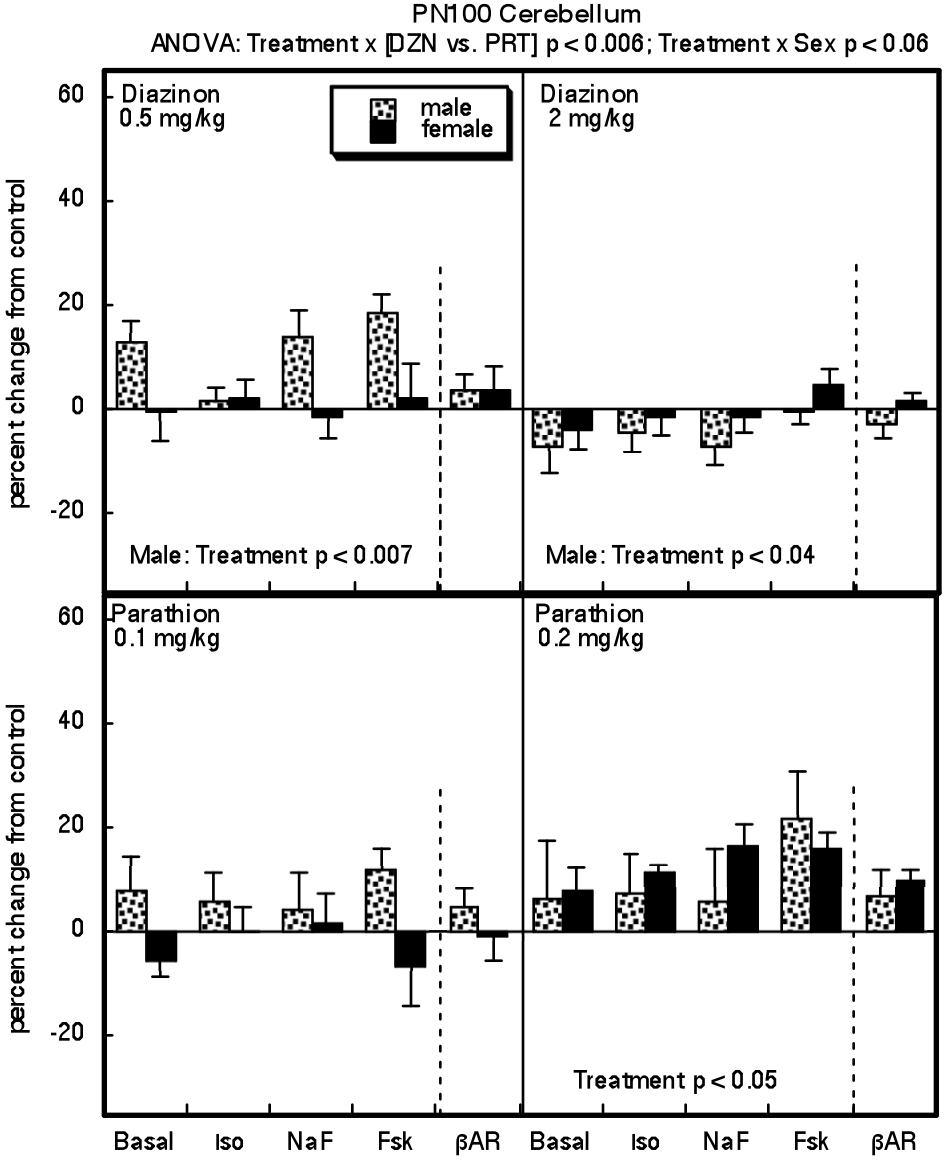

Figure 15: Effects of neonatal DZN or PRT exposure on the cerebellum, evaluated at PN100. Data represent means and standard errors obtained from 6 animals in each group, presented as the percent change from the corresponding control values (Table 2). ANOVA incorporating the factors of treatment, [DZN vs. PRT], sex and AC stimulant appears at the top of each panel and lower-order tests are shown within the panels. Abbreviations: Iso, isoproterenol; Fsk, forskolin.

DISCUSSION

Results of this study reinforce the concept that the developmental effects of OPs extend beyond the nervous system. Neonatal exposures to DZN and PRT altered the developmental trajectory of AC-mediated cell signaling in peripheral tissues, and in the case of the liver, to a greater extent than in the brain. Although in general, OP exposure elicited a net AC gain-of-function, the effects differed among the various tissues, effectively ruling out a global effect on expression of receptors, G-proteins or AC, and instead pointing to selectively greater effects on specific aspects of hepatic function, as will be discussed below. This conclusion was further reinforced by the fact that DZN and PRT exposures evoked different trajectories, despite the fact that the doses of each OP were chosen to produce equivalent degrees of cholinesterase inhibition [169]: the effects of DZN intensified with age, whereas those of PRT waned. Thus, although OPs elicit acute systemic toxicity through their shared ability to inhibit cholinesterase [114,130], lower-level OP exposures, spanning the threshold for anticholinesterase actions, nevertheless disrupt the development of cell-signaling cascades that control cell differentiation and other critical functions [68,145-147]. Indeed, the comparative differences in outcomes seen here between DZN and PRT, and previously for CPF [113], all point to actions independent of cholinesterase inhibition; in turn, because the effects on cholinesterase are produced by the oxon metabolites of each of the OPs, our results point to the native compounds, or perhaps other metabolites as the agents that target the development of cell signaling.

In our earlier work with CPF, we found persistent, global increases in all

measures of hepatic AC signaling, an effect restricted to males [113]; this connotes heterologous sensitization of the pathway, wherein upregulation of AC activity itself (enhanced forskolin response) produces an augmented response to stimulants acting on G_s-coupled receptors (isoproterenol, glucagon), as well as to direct activation of G-proteins (fluoride). Equally important, the CPF effects displayed a critical period of sensitivity, indicating that these are specifically *developmental* actions [113]. As found here, neonatal DZN exposure produced a similar heterologous effect that appeared in adolescence and young adulthood at the higher dose but that also became significant at the lower dose by full adulthood; notably, although the effect emerged first in males, it eventually encompassed both sexes, thus differing in outcome from the sex-specific effects seen for CPF [113]. Superimposed on heterologous sensitization, we also found alterations in specific elements of the signaling cascade. If the upregulation of AC itself were the only effect of DZN exposure, then all pathway stimulants should show the same degree of enhancement. Instead, on both PN60 and PN100, the response to fluoride was augmented to a significantly smaller extent than for forskolin or either of the receptor stimulants. Fluoride differs from isoproterenol and glucagon in that it also activates G_i, and consequently, our findings point to an increase in expression and/or function of the inhibitory G-protein after neonatal DZN exposure; again this differs from the effects of CPF, which produces uniform enhancement of responses as expected from activation of AC itself [113]. Effects on G_i will also produce heterologous alterations in AC signaling, since they affect the response to any receptor acting through this G-protein. Thus, all the effects we noted were heterologous, involving either AC itself or the G-proteins, rather

than reflecting homologous effects on the expression of the neurotransmitter receptors or their specific coupling to the control of AC; indeed, none of the small effects seen for receptor binding could account for the robust augmentation of AC responses.

In contrast to DZN, neonatal PRT exposure produced a much larger initial sensitization of hepatic AC in adolescence, with significant increases at either dose and in both males and females. Again, the effects were primarily heterologous as evidenced by the augmented forskolin response but there were additional pathway effects evident at the lower dose, reflecting smaller increments for glucagon and fluoride than for isoproterenol and forskolin. In turn, these imply homologous desensitization for glucagon and heterologous increases in G_i-mediated inhibition, respectively; the effect of PRT on the glucagon response was not shared by DZN, again pointing out specific differences related to each individual OP. Most notably, though, whereas the effects of DZN intensified over time, those of PRT waned, so that by young adulthood and thereafter, there were no longer any significant differences. Thus, the two OPs differ completely in their effects on the developmental *trajectory* of hepatic AC signaling, with large effects in adolescence for PRT but not DZN, supplanted by the opposite pattern in adulthood.

In addition to selective effects for DZN as compared to PRT, we also found substantial tissue-related differences, with the liver affected far more than either the heart or the cerebellum. Neonatal DZN exposure elicited significant changes in cardiac AC only on PN30, reflecting increases in signaling responses for males and decreases for females; PRT had no significant effects on cardiac AC whatsoever. In the cerebellum, the lower dose of DZN produced small but significant upregulation in males that was

reversed at the higher dose, whereas PRT produced significant increases at the higher dose; none of these was comparable in magnitude to the large changes seen in the liver. For the heart (but not the cerebellum), there were significant changes in receptor binding without apparent connection to the effects on AC signaling, again reinforcing the importance of heterologous sensitization downstream from the receptors as the primary site of regulatory disruption.

The clear implication is that neonatal OP exposure is likely to affect hepatic responses to a greater extent than those in the heart or in the central nervous system, and it is therefore critical to examine how the cellular changes seen here might then contribute to alterations in tissue function. In the liver, where βARs and glucagon receptors are linked through AC to enhanced gluconeogenesis and lipolysis, heterologous sensitization of AC signaling leads to corresponding metabolic abnormalities. Earlier work with CPF established the presence of hyperlipidemia, but serum glucose levels were maintained within normal limits [150]; however, glucose homeostasis was maintained only by compensatory hypersecretion of insulin, thus producing a metabolic profile akin to prediabetes. Critical to the proposed mechanistic connections, the sex-selectivity of the metabolic effects (males) exactly matched that for the heterologous sensitization of hepatic AC signaling [113]. More recently, evidence of prediabetes after neonatal PRT exposure has been found [94]. In this case, there was no corresponding increase in serum insulin, and as a result, the animals displayed a frank prediabetic profile, characterized by hyperglycemia and impaired glucose and lipid utilization [94]. It is thus important to note that the effects of PRT on hepatic AC signaling seen here were restricted to

adolescence, and unlike those of CPF, did not persist into adulthood. Accordingly, for PRT, either the AC changes are unrelated to the metabolic disorders, or alternatively, the effects in adolescence may be sufficient to reprogram metabolism so that defects emerge later, despite the subsequent normalization of signaling parameters. Obviously, future work will need to dissect the temporal emergence of prediabetes after neonatal PRT exposure in order to distinguish these two possibilities. However, current data point toward the latter interpretation, since PRT-exposed animals show a switch from enhanced to suppressed weight gain coinciding with the time course for the disappearance of the effects on AC signaling [94]. Although detailed metabolic studies have not been done for DZN, based on the results for hepatic AC signaling, we would expect to see bigger metabolic defects than for PRT, consistent with the greater weight loss seen here and in earlier reports [94,138]; unlike CPF, we would further predict that DZN will target metabolic function in both males and females, since the cellular effects were not sex-selective. Finally, there are human studies showing a connection of diabetes to long-term OP exposure [116,140] and of a link between gain-of-function AC gene polymorphisms and diabetes susceptibility [119]. Our results thus provide a mechanistic underpinning for these population studies.

There are similar implications for the significant, albeit lesser effects of DZN and PRT exposure on AC signaling in the heart and brain. Transgenic animals that produce AC hyperstimulation through overexpression of βARs or G_s show development of cardiomyopathies and abnormal heart rate regulation [60,81,188]. Alterations in m_2AChR expression, which we detected for PRT, also have corresponding effects on

cardiac function and the response to autonomic input [90]. Importantly, the fact that DZN reduced heart weight significantly and to a greater extent than body weight indicates the need to pursue potential consequences for cardiac function, which have been much less studied than hepatic function. Indeed, the prediabetic changes seen after neonatal OP exposure are themselves likely to contribute to further cardiovascular morbidities. In the cerebellum, we found a nonmonotonic effect of DZN, with enhanced AC signaling at the low dose that disappeared or was reversed at the high dose; this likely represents the positive neurotrophic effect of acetylcholine produced by a small degree of cholinesterase inhibition, offsetting the direct effects of low exposure [183]. In contrast, PRT produced upregulation with a typical, monotonic dose-effect relationship. Most importantly, the disparate dose-effect patterns of effects on AC signaling for both DZN and PRT correspond to the differences in behavioral findings between the two agents [183,184].

Our results support the view that developmental exposure to OPs targets the trajectory of AC signaling in peripheral tissues, thus extending their actions outside the nervous system. The effects on hepatic and cardiac AC appear to be unrelated to the shared mechanism for systemic OP toxicity, cholinesterase inhibition [114], with the consequences that: (a) the effects on signaling occur with nonsymptomatic exposures that are below the threshold for detectable anticholinesterase actions, and (b) effects differ among OPs at exposures that produce similar effects on cholinesterase. Further, the liver appears to be especially sensitive to persistent disruption of AC signaling, involving heterologous sensitization of the entire pathway via induction of AC activity. In turn, this

provides a likely mechanism for the metabolic consequences of neonatal OP exposure identified in earlier studies, indicative of a prediabetic state [93,150]. Indeed, heterologous sensitization is likely to have widespread consequences in general because it affects all humoral signals that operate through cAMP, rather than involving any single input. Our findings thus extend the Barker Hypothesis, which originally related prenatal growth restriction to subsequent development of cardiovascular disease and diabetes [21], to include otherwise nonsymptomatic chemical exposures that may produce similar outcomes without the precondition of fetal/neonatal growth restriction. Our findings point out the need to explore the possibility that developmental exposure to common chemical contaminants contribute to the explosive worldwide increase in diabetes and obesity.

Chapter 4: Neonatal Parathion Exposure and Interactions with a High-Fat Diet in Adulthood: Adenylyl Cyclase-Mediated Cell Signaling in Heart, Liver and Cerebellum

The findings of Chapter 3 indicate that OPs alter the trajectory of hepatic cell signaling in a manner consistent with the observed emergence of prediabetes-like metabolic dysfunction, and with a greater effect than in heart or brain. Specifically, neonatal PRT evoked later upregulation of the hepatic AC pathway in adolescence but the effect waned by young adulthood; nevertheless metabolic changes resembling prediabetes persisted. These effects occured at low exposures and were unrelated to cholinesterase inhibition. Therefore, various OPs differ in their net impact on the peripheral AC signaling, and in a manner divergent from their effects on neurobehavioral outcomes. In this chapter, we explored whether the additional metabolic stress imposed by consuming a high-fat diet in adulthood could unmask persistent effects of neonatal PRT exposure on AC signaling, an approach modeled after our earlier studies on metabolic effects of OPs [93,94,138]. As before, we focused on PRT treatment regimens straddling the threshold for cholinesterase inhibition [154] and then in adulthood, we switched half the animals to a high-fat diet that more than doubles serum β-hydroxybutyrate concentrations [94]. To assess the impact on AC signaling, we evaluated function at each step in the cascade. Further, we measured ligand binding for βARs and for the inhibitory m_2AChRs. We conducted our studies in peripheral tissues (heart, liver) and compared the effects to those seen in a brain region (cerebellum) that shows a similar AC response to βAR activation. Finally, we also assessed βAR binding in a skeletal muscle (gastrocnemius) to determine whether this prominent site of energy

utilization was affected by PRT or the high-fat diet, separately or together.

MATERIALS AND METHODS

Animal treatments and diet. Animal housing, care, litter size, and randomization was identical to those presented in Chapter 2. PRT treatments were the same as those presented in Chapter 3. Each group was defined as a specific neonatal treatment, dietary condition and sex. After weaning, animals were separated by sex and housed in groups according to standard guidelines.

Beginning at 15 weeks of age, half the rats were switched to a high-fat diet (OpenSource D12330), providing 58% of total calories as fat; 93% of the fat is hydrogenated coconut oil. The remaining rats continued on the standard diet, which provides 13.5% of total calories as fat; with this diet, 27% of the fat is saturated. Although the high-fat diet contains 37% more calories per gram, we found that animals on this diet reduce their food intake by approximately the same proportion [94], so that the total dietary intake is isocaloric; nevertheless, animals gain excess weight when fed a diet with a higher fat content [94]. During the 24^{th} postnatal week, animals from each of the finally-assigned litters were decapitated and the heart, one liver lobe (the same lobe from each animal), cerebellum and gastrocnemius muscle were dissected, blotted, frozen in liquid nitrogen and maintained at -45° C.

Assays. The procedures used in this study were presented in detail in Chapter 2. Additionally, it is important to note that we did not assess m_2AChR binding in the gastrocnemius muscle because of the sparsity of the receptors in this tissue. We conducted preliminary studies of AC activity in the gastrocnemius muscle and found

extremely variable results; accordingly, these determinations were not carried out in the present work.

Data analysis. Handling of data and the presentation of results are presented in detail in Chapter 2. Only analysis that varies from the previous chapters follows: To ensure that treatment and diet effects could be compared across all groups, all assays were conducted simultaneously on all samples for a given tissue and sex, but technical limitations dictated that each tissue and sex had to be performed in divided runs. Accordingly, the control values for tissue vs. tissue or for males vs. females should not be compared directly, since each tissue was assayed separately, as was each sex. However, treatment and diet effects and their interactions with tissue and sex can be interpreted, since these depend solely on the internal comparison to the matched control groups that were run together. Because of the large range of AC activities for different tissues and stimulants, the scales for each graph were adjusted to give an equivalent visual presentation of the treatment- and diet-related differences, irrespective of the differences in absolute values.

Materials. All materials utilized were purchased from the same companies detailed in the previous chapters except for the following: LabDiet 5001 came from PMI Nutrition (St. Louis, MO) and the OpenSource D12330 diet came from Research Diets Inc. (New Brunswick, NJ).

RESULTS

Body and tissue weights. Global ANOVA incorporating all variables (treatment, diet, sex) and the five dependent measures (body weight, heart weight, liver lobe weight,

cerebellum weight, gastrocnemius weight) identified significant main effects of diet ($p < 0.0001$), sex ($p < 0.0001$) and tissue ($p < 0.0001$), along with interactions among these three variables: $p < 0.0004$ for diet × sex, $p < 0.0001$ for diet × tissue, $p < 0.0001$ for sex × tissue, and $p < 0.02$ for diet × sex × tissue. Since there was no main treatment effect of PRT or interaction of treatment with the other variables, we separated the values for the individual tissues and still obtained a diet × sex interaction for each peripheral measure ($p < 0.04$ for body weight, $p < 0.02$ for heart, $p < 0.009$ for liver, $p < 0.02$ for gastrocnemius) but not for the cerebellum. For body weights, the main effect of diet was significant in both males ($p < 0.0003$) and females ($p < 0.0001$), with the sex difference reflecting a greater effect of the high-fat diet in females, as reported earlier [94]. Similarly, the high-fat diet evoked significant weight increases of 15-20% in heart ($p < 0.0002$), liver ($p < 0.003$) and gastrocnemius ($p < 0.0001$) in females but not males.

The weight results represent the values from the animals used in the current study. Earlier, we reported on the longitudinal effects of neonatal PRT treatments and dietary manipulations on body weights in a much larger group from the same cohort of animals [94], which revealed further effects not evident from the smaller group. PRT had no initial effect on body weight during or immediately after the exposure period but after weaning, the PRT group displayed a small (2-3%), significant elevation in weight at the low dose in males, and reductions of about 4% at either dose in females. Regardless of the neonatal treatment, the high fat diet increased body weights by about 10% in males and 30% in females, with the low dose of PRT augmenting the effect of the high-fat diet on weight gain.

Adenylyl cyclase. The global ANOVA (factors of treatment, diet, sex, tissue; dependent measures for each AC stimulant condition) indicated interactions of sex × measure ($p < 0.0001$), tissue × measure ($p < 0.0001$), diet × tissue × measure ($p < 0.02$), sex × tissue × measure ($p < 0.0001$), and diet × tissue × measure ($p < 0.02$). Accordingly, we separated the values for the individual tissues and then performed lower-order analyses. The global test had to omit the response to glucagon, since this stimulant was not tested in the cerebellum; this measure was reinstated for the separate analyses of heart and liver, which then uncovered significant treatment effects of PRT. Additionally, the repeated measures analysis of the different AC stimulants confirmed significant responses to each of the agents ($p < 0.0001$ collectively as well as individually in each tissue). However, the stimulant responses differed among the three tissues (stimulant × tissue interaction, $p < 0.0001$). In the heart, the rank order was forskolin > fluoride > isoproterenol > glucagon > basal AC, pointing out the predominant role of βAR input as compared to glucagon. In the liver, the sequence was forskolin > glucagon > fluoride > isoproterenol > basal AC, reflecting the greater physiological importance of glucagon in the metabolic response; indeed, glucagon stimulation in the liver was greater than that of fluoride, which maximally activates both excitatory (G_s-related) and inhibitory (G_i-related) G-protein responses. In the cerebellum, the rank order of responses was forskolin > isoproterenol ≈ fluoride > basal, indicating that βAR stimulation is highly coupled to G-proteins in this brain region.

In the heart, the global ANOVA identified a significant interaction of diet × measure $p < 0.0001$, necessitating a separate consideration for each AC measurement;

however, there were no PRT -related effects, nor was there a significant interaction of PRT × diet or PRT × diet × other variables. The high-fat diet did not have any net effect on basal AC (Figure 16A), isoproterenol-stimulated AC (Figure 16B) or glucagon-stimulated AC (Figure 16C), but evoked a significant decrement in the response to fluoride (Figure 16D) and forskolin (Figure 16E). To illustrate the differences in the main effect of diet across the three treatment groups and both sexes, we collapsed all the interactive variables and determined the average effect (geometric mean) on each AC parameter (Figure 16F). This procedure dilutes any specific decreases by averaging them with smaller effects, so that the absolute magnitude is reduced; nevertheless there was a clear hierarchy of effects reflecting the conclusions reached in the multivariate ANOVA, namely a net decrease evoked by the high-fat diet for the fluoride and forskolin responses.

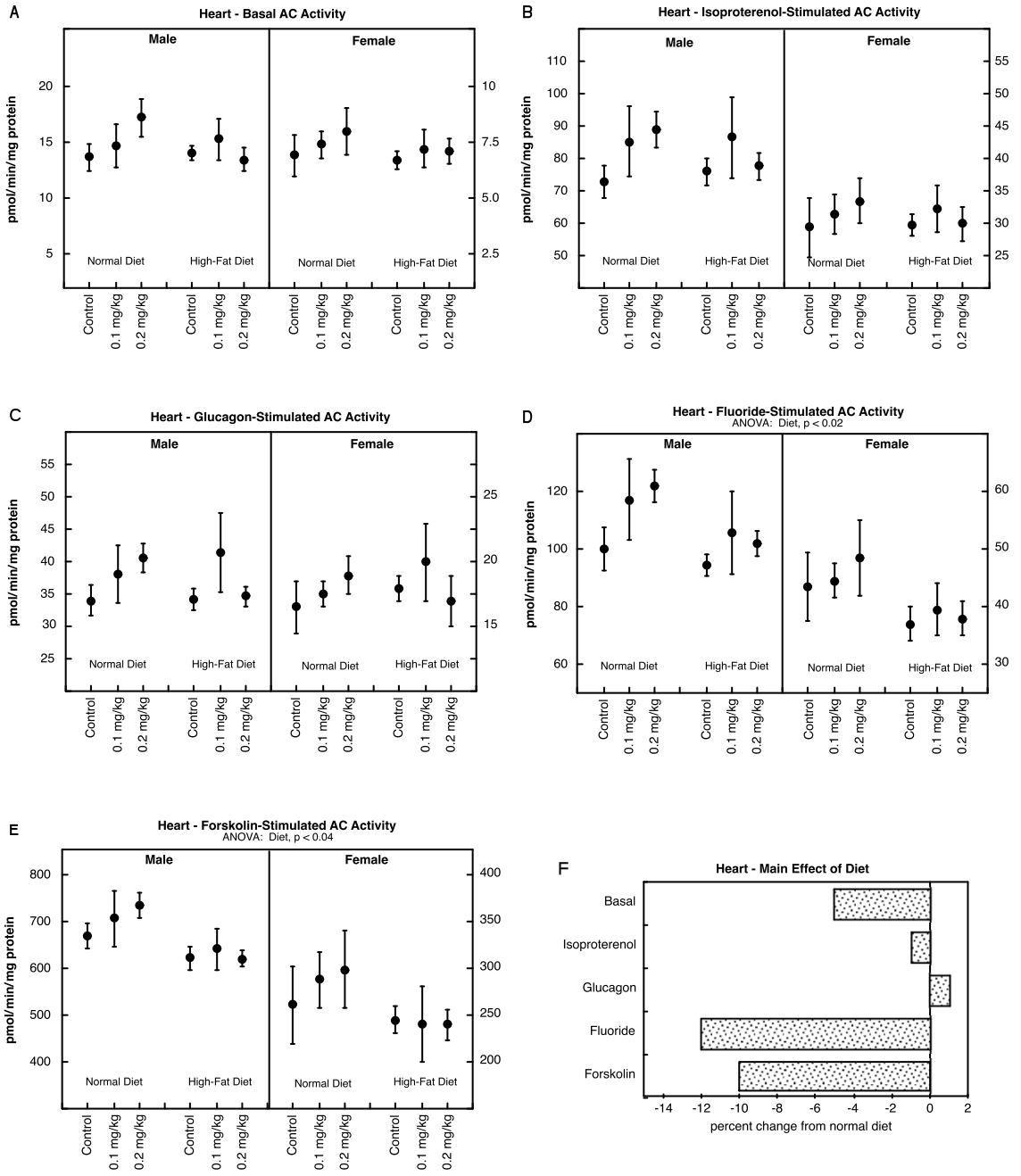

Figure 16: Effects of neonatal PRT exposure and subsequent adult consumption of a high-fat diet on cardiac AC activity: (A) basal AC, (B) isoproterenol-stimulated AC, (C) glucagon-stimulated AC, (D) fluoride-stimulated AC, (E) forskolin-stimulated AC. Data represent means and standard errors obtained from 6 animals in each group. Significant ANOVAs appear at the top of the corresponding panels. Panel (F) shows the simple main effect of diet, collapsed across all the other variables.

In the liver, the global test indicated sex-dependent effects of both PRT treatment and diet: $p < 0.05$ for treatment × sex and $p < 0.05$ for diet × sex × measure. Accordingly separate evaluations of each measure were conducted for males and females. Again, there were no PRT or diet-related effects on basal AC (Figure 17A) or on the AC response to isoproterenol (Figure 17B). However, there were sex-selective effects on the responses to other stimulants that targeted males: the high-fat diet increased the response to glucagon (Figure 17C) and the low-dose PRT treatment suppressed the fluoride response (Figure 17D). Unlike the heart, the liver showed no changes in the forskolin response as a result of dietary manipulation or neonatal PRT exposure, separately or together (Figure 17E). The main effects for the two significant changes are shown in Figure 17F, collapsed across the interactive variables; this clearly shows the selective increase in the glucagon response evoked by high-fat diet in males and the decrease in the fluoride response evoked by the low dose of PRT.

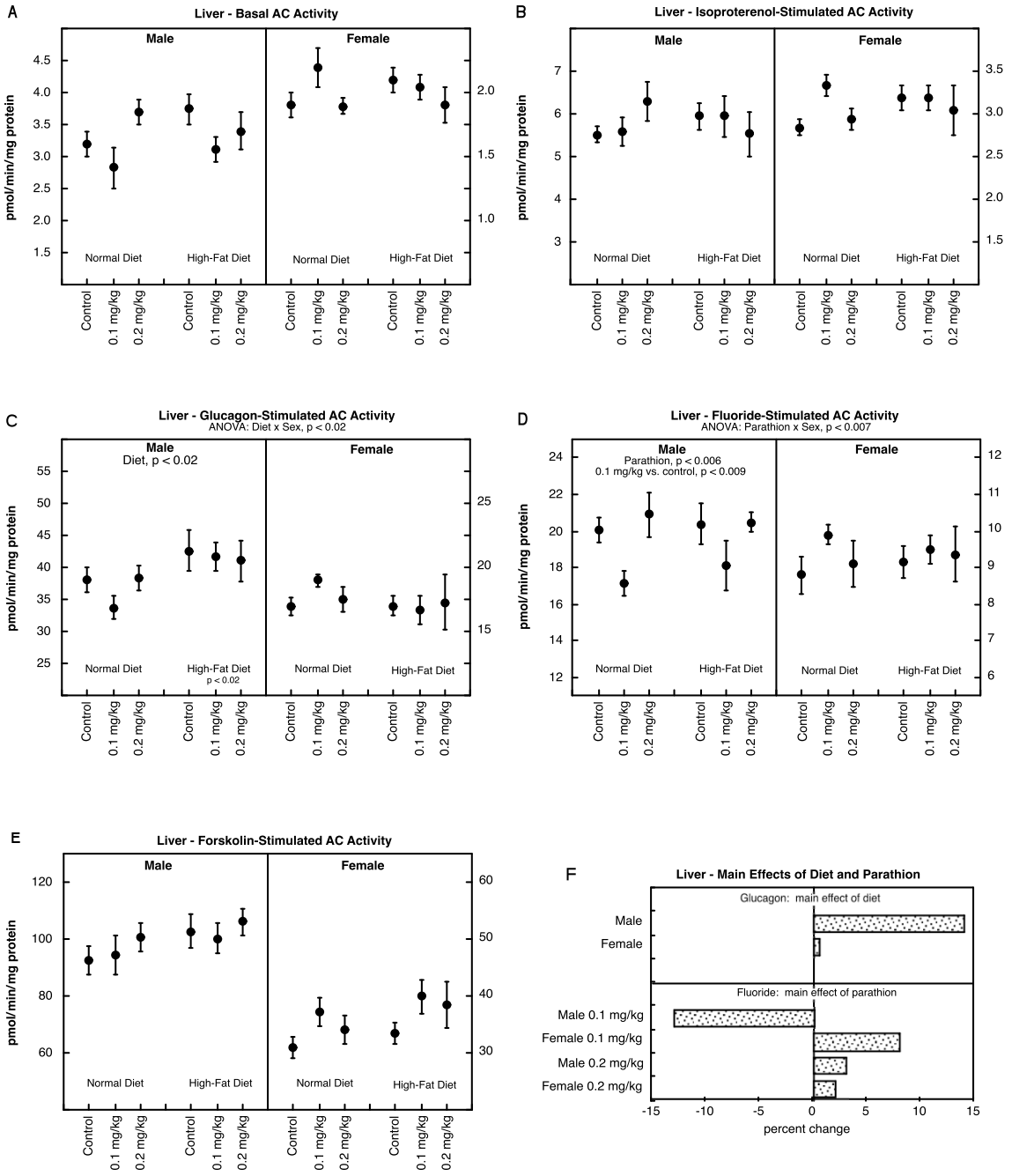

Figure 17: Effects of neonatal PRT exposure and subsequent adult consumption of a high-fat diet on hepatic AC activity: (A) basal AC, (B) isoproterenol-stimulated AC, (C) glucagon-stimulated AC, (D) fluoride-stimulated AC, (E) forskolin-stimulated AC. Data represent means and standard errors obtained from 6 animals in each group. Significant ANOVAs appear at the top of the corresponding panels. and, where justified by the interactions, lower-order tests are shown within panels. Panel (F) shows the simple main effects for the two measures showing significant changes, collapsed across all the other variables.

In contrast to the peripheral tissues, the global ANOVA for the cerebellum revealed nostatistically significant AC signaling effects of either PRT treatment or diet, separately or together, nor any significant interactions of these factors with the other variables (Table 3).

Table 3: Adenylyl Cyclase Activities in the Cerebellum*

Treatment	Diet	Basal	Isoproterenol	Fluoride	Forskolin
Male					
Control	Normal	215 ± 11	252 ± 15	246 ± 15	828 ± 37
	High-Fat	177 ± 19	232 ± 8	258 ± 13	780 ± 32
Parathion 0.1 mg/kg	Normal	201 ± 14	233 ± 13	249 ± 14	809 ± 60
	High-Fat	178 ± 19	235 ± 8	256 ± 11	807 ± 30
Parathion 0.2 mg/kg	Normal	199 ± 15	239 ± 12	242 ± 14	807 ± 40
	High-Fat	200 ± 22	239 ± 6	235 ± 6	772 ± 39
Female					
Control	Normal	192 ± 9	245 ± 10	242 ± 7	901 ± 50
	High-Fat	177 ± 19	216 ± 19	212 ± 14	840 ± 49
Parathion 0.1 mg/kg	Normal	174 ± 22	210 ± 14	235 ± 14	856 ± 69
	High-Fat	178 ± 19	251 ± 28	233 ± 16	930 ± 75
Parathion 0.2 mg/kg	Normal	209 ± 21	278 ± 30	275 ± 18	960 ± 86
	High-Fat	200 ± 22	245 ± 21	232 ± 10	896 ± 58

***pmol/min per mg protein**

Receptor binding. For βAR binding, the global test (PRT treatment, diet, sex, tissue) identified a main effect of PRT ($p < 0.006$) that also depended on sex (treatment × sex, $p < 0.06$) and tissue (treatment × tissue, $p < 0.02$); there was also a tissue-selective effect of diet (diet × tissue, $p < 0.02$). Accordingly, we again separated the tissues for examination of lower-order main effects. In the heart, neonatal PRT exposure evoked a significant reduction in βARs at the lower dose but not at the higher dose (Figure 18A); the high-fat diet by itself also produced a decrement. In contrast, there were no effects of

either PRT or dietary manipulation on βAR binding in the liver (Figure 18B). Like the heart, the cerebellum displayed a significant decrease in receptor binding from the high-fat diet (Figure 18C); although there was a significant interaction of PRT × diet × sex, none of the differences was statistically significant from control values after separation by the interactive variables. Uniquely, the gastrocnemius muscle showed βAR upregulation in response to PRT exposure but was unaffected by the high-fat diet (Figure 18D).

For cardiac m_2AChRs (Figure 18E), we identified a main effect of treatment ($p < 0.03$) that was significant at either dose of PRT ($p < 0.008$ individually vs. control) and that displayed sex-selectivity (treatment × sex, $p < 0.04$). After subdivision of the results, males were significantly affected by PRT ($p < 0.003$ for the main treatment effect, $p < 0.006$ at 0.1 mg/kg, $p < 0.002$ at 0.2 mg/kg), whereas females showed no significant effect. In addition, the high-fat diet by itself caused a significant decrease (main effect of diet, $p < 0.0001$).

Figure 18F shows the main effects of diet and PRT exposure on receptor binding, collapsed across the remaining interactive variables: the diet-induced reduction in βARs in the heart and cerebellum, and in cardiac m_2AChRs; the PRT -induced decrement in heart βAR binding at the low dose and the increase in the gastrocnemius at the higher dose, as well as increases in cardiac m_2AChR binding at either dose.

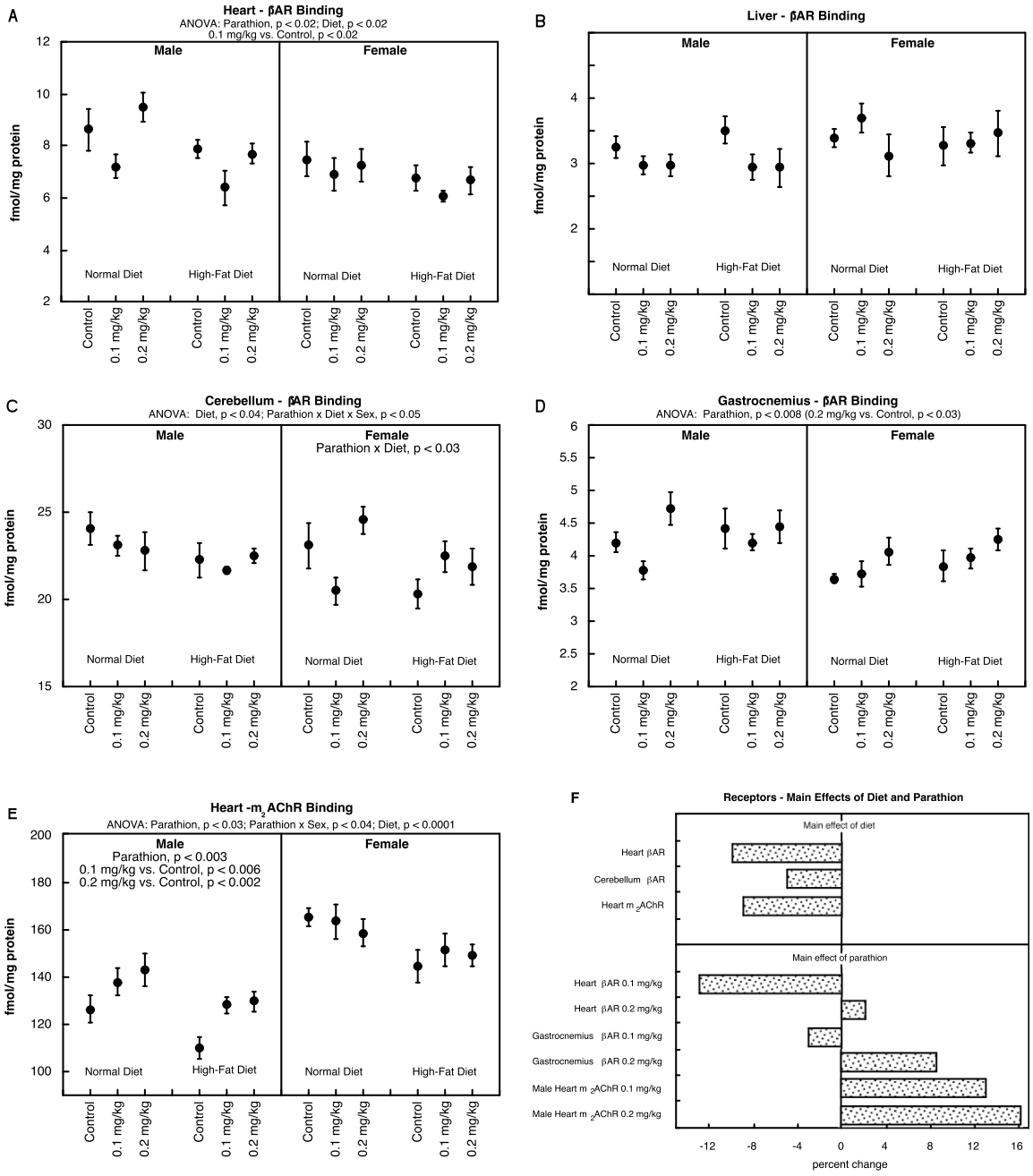

Figure 18: Effects of neonatal PRT exposure and subsequent adult consumption of a high-fat diet on receptor binding: (A) heart βARs, (B) liver βARs, (C) cerebellum βARs, (D) gastrocnemius βARs, (E) heart m_2AChRs. Data represent means and standard errors obtained from 6 animals in each group. Significant ANOVAs appear at the top of the corresponding panels, and, where justified by the interactions, lower-order tests are shown within panels.. Panel (F) shows the simple main effects for the measures showing significant changes, collapsed across all the other variables.

DISCUSSION

In our earlier studies, we found that early-life OP exposure results in sensitized hepatic AC signaling in response to neuronal and hormonal signals that promote gluconeogenesis, glycolysis and lipolysis that are evident later in life [7,113]. The consequent metabolic dysfunction resembles prediabetes, although different OPs vary in their specific pattern of effects [92-94,150]. With CPF, serum glucose is maintained within normal limits, but only because of insulin hypersecretion, and the animals display hyperlipidemia, leptin dysregulation and excessive weight gain [92,150]. PRT produces hyperglycemia, loss of lipid homeostasis and adipose inflammation, all without incurring hyperinsulinemia [93,94]. These differences are also reflected in disparities in OP effects on hepatic and cardiac AC signaling. Whereas sensitization of the pathway is maintained throughout the lifespan with CPF and DZN, the effects of PRT are prominent only through adolescence and then wane in young adulthood [7,113]. Here, we explored whether PRT's effects might emerge even later, in full adulthood, but still did not find the global upregulation of the AC pathway that we saw for CPF and DZN. However, there were focal abnormalities involving G-protein function, exemplified by a decreased hepatic AC response to fluoride; because the effects was not shared by stimulants that activate G_s-coupled receptors (isoproterenol, glucagon), this result implies that PRT exposure enhances the expression or function of the inhibitory G-protein, G_i. Interestingly, the effect was nonmonotonic, since the reduction was significant in the group receiving 0.1 mg/kg PRT but not the group given the higher dose. This is in keeping with the effects on body weight [94] and thus implies that the metabolic

consequences of early-life PRT exposure are distinctly different once the levels exceed the threshold for cholinesterase inhibition and the emergence of systemic toxicity.

Similarly, although there were no significant effects of PRT on heart AC parameters, we did see a reduction in βAR expression with the same, nonmonotonic dose-effect relationship. Since PRT also evoked upregulation of m_2AChRs, which produce opposing physiological effects to the actions of βARs, our result point to the potential for sympathetic/parasympathetic imbalance that could ultimately contribute to cardiovascular disorders. Clearly, future work should address this possibility. In contrast, we found the opposite effect, increased βAR binding, in the gastrocnemius muscle, reinforcing the fact that the changes are related to specific metabolic functions rather than reflecting a global effect on βAR expression; this interpretation is reinforced by the different sensitivity of the skeletal muscle, which required exposure to the high dose in order to show effects (Figure 18D), whereas effects in the heart (Figure 18A) or liver (Figure 18B) were seen at the lower dose of PRT. Since skeletal muscle is a major site for glucose and lipid utilization, the changes seen here are likely to be involved in the general prediabetic profile seen with early-life OP exposure [93,150]. Finally, none of these effects were seen for AC signaling or βAR expression in the cerebellum, pointing again to specific targeting of peripheral organ function, unrelated to the developmental neurotoxicity of OPs.

Despite their differences in effects on AC signaling and metabolic profiles, DZN and PRT both share a common response to a high fat diet, in that animals exposed to either agent gain more weight than do controls [94,138]. Here, we did not observe any

interaction between PRT and the high-fat diet at the level of AC signaling or receptors, implying that the augmented weight gain represents separate actions. It is therefore notable that we found effects of the high-fat diet on signaling that coincide with some of those obtained with OP exposure. In the liver, the high-fat diet enhanced the AC response to glucagon in males without producing a corresponding increase in the response to isoproterenol, fluoride or forskolin. Accordingly, this points to a specific enhancement of glucagon receptor expression or coupling to G_s; the sex-selectivity seen here for signaling effects is paralleled by differences in several metabolic parameters [93,94]. Increased responsiveness to gluconeogenic signals later in life is also a hallmark of neonatal OP exposure [7,113], so the dietary effect seen here represents a endpoint that converges with that of OPs.

The effects of the diet in the heart again point out the targeting of specific organ function as opposed to global changes in cell signaling. Unlike the liver, the high-fat diet reduced the cardiac AC responses to fluoride and forskolin, implying heterologous changes in the expression or function of G-proteins and AC, downstream from the receptors. Notably, though this should have produced a parallel reduction in the responses to receptor stimulants but instead, these were unchanged; in turn, that implies that there are compensatory increases in receptor coupling that maintain the response to isoproterenol and glucagon in the face of downstream impairment. These adaptations are not at the level of receptor expression, since cardiac βARs were decreased, not increased. Although the cerebellum also showed reduced βARs in response to the high-fat diet, there were no corresponding changes in AC signaling, implying that this effect is

functionally unimportant.

In conclusion, unlike CPF or DZN, neonatal PRT exposure does not produce global upregulation of hepatic AC cell signaling that is sustained throughout the life span. Rather, it elicits heterologous sensitization only through adolescence [7]; although this effect then wanes in young adulthood, more subtle changes in hepatic signaling then emerge even later, involving the expression or function of G_i, accompanied by changes in the concentration of βARs in skeletal muscle, which govern glucose and lipid utilization. The disparities among the various OPs in their impact on cell signaling are likely to contribute to the differences in their effects on metabolic function [92-94,150]. Although addition of a high-fat diet did not augment the effect of neonatal PRT exposure on signaling, the diet by itself affected the AC pathway in both liver and heart, indicating that OP exposure produces changes in the same signaling events that are targeted by excessive fat consumption. In that fashion, early OP exposure, separately or in combination with dietary factors, may contribute to the worldwide increase in the incidence of obesity and diabetes.

Nevertheless, it looks like we may have chosen the wrong OP for these studies. Future work may find the investigation of DZN or CPF more productive to look at than PRT. Again, it is important to note that sensitization of the AC pathway is maintained throughout the lifespan with CPF and DZN while the effects of PRT are prominent only through adolescence and then wane in young adulthood [7,113]. Furthermore, our previous studies have shown that early-life CPF exposure results in metabolic dysfunction that resembles prediabetes. With CPF, serum glucose is maintained within

normal limits, but only because of insulin hypersecretion, and the animals display hyperlipidemia, leptin dysregulation and excessive weight gain [92,150]. Additionally, DZN and PRT both share a common response to a high fat diet, in that animals exposed to either agent gain more weight than do controls [94,138].

Chapter 5: Disparate Developmental Neurotoxicants Converge on the Cyclic AMP Signaling Cascade, Revealed by Transcriptional Profiles *In Vitro* and *In Vivo*

In this chapter, we set out to determine if the effects of OPs were direct or indirect, which cannot be distinguished *in vivo*. Therefore our studies were carried out through *in vitro* investigation. We assessed 69 genes, from a preexisting database, that encode AC isoforms and regulators, G-protein α- and β,γ-subunits, protein kinase A subtypes and the phosphodiesterase family. We focused on two OPs, CPF and DZN, an organochlorine pesticide (dieldrin) and a heavy metal (Ni^{2+}). The two non-OP agents were used for contrast and tests of specificity for the OPs. All four toxicants have been well-characterized for effects on neural cell replication and differentiation [156,161-164,167].

For our evaluations, we used PC12 cells, a classical *in vitro* model for neuronal development [182] that has already been validated to reproduce the mechanisms and outcomes found after *in vivo* OP exposures [15,16,39,40,42,52,83,84,98,117,133,134, 155,156,166,167,176,187]. With the addition of nerve growth factor (NGF), PC12 cells begin to differentiate, forming neuritic projections and acquiring electrical excitability and neuronal phenotypes [56,176,182]. We evaluated gene expression patterns with cDNA microarrays, using a planned comparisons approach that focused on the relevant gene families, rather than performing a blanket evaluation of the entire transcriptome [160,164-167]. Planned comparisons are based instead on testing a specific hypothesis that centers around a defined set of genes, and rests on known, validating outcomes from prior work, in this case for the OPs. With examination of the entire genome, verification

via RT-PCR and other techniques is required because the enormous number of comparisons generates many false positive findings (e.g. the >2000 genes that would be false positives if we had considered all 42,000 probes on the array). For our study, the 69 genes we compared generated only 3-4 false positives. We then assessed concordance of the effects of the various agents across gene classes within the cAMP pathway, rather than relying on changes in the expression of individual genes [162,163,167]. Thus, the odds of all these genes being false positives are astronomically small. First, we determined the extent to which CPF, DZN, dieldrin and Ni^{2+} show convergent or divergent actions on gene expression in differentiating cells. Second, we assessed whether these changes involve a critical period by comparing the effects of CPF on undifferentiated PC12 cells versus cells undergoing neurodifferentiation. Finally, we contrasted the concordance of CPF and DZN *in vitro* to the *in vivo* effects in the developing rat brain after neonatal exposure to the same agents, so as to determine the relative contributions of direct neurotoxicant effects on gene expression in isolated cells as compared to those that depend on more complex processes involving the intact brain.

MATERIALS AND METHODS

Cell cultures. Because of the clonal instability of the PC12 cell line [56], the experiments were performed on cells that had undergone fewer than five passages. As described previously [135,176], PC12 cells (American Type Culture Collection, 1721-CRL, obtained from the Duke Comprehensive Cancer Center, Durham, NC) were seeded onto poly-D-lysine-coated plates in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% inactivated horse serum (Sigma Chemical Co., St. Louis, MO), 5% inactivated fetal bovine serum (Sigma), and 50 μg/ml penicillin streptomycin (Invitrogen). Incubations were carried out with 7.5% CO_2 at 37°C, standard conditions for PC12 cells. To initiate neurodifferentiation [83,156,182] twenty-four hours after seeding, the medium was changed to include 50 ng/ml of 2.5 S murine NGF (Invitrogen). Along with the NGF, we added 30 μM of each of the test agents: CPF (Chem Service, West Chester, PA), DZN (Chem Service), dieldrin (Chem Service) or $NiCl_2$ (Sigma). The concentration was chosen from earlier studies that demonstrated adverse effects on differentiation of PC12 cells without outright cytotoxicity [84,133,156,166]. Because of the limited water solubility of the three insecticides, these agents were dissolved in dimethylsulfoxide (final concentration 0.1%), which was also added to the control cultures and to cultures containing $NiCl_2$; this concentration of dimethylsulfoxide has no effect on PC12 cell growth or differentiation [133,135,176]. Cultures were examined 24 and 72 hr after commencing exposure, with 5-8 independent cultures evaluated for each treatment at each time point. Each culture was run on a separate array. We used two time points so as to be able to evaluate changes in gene expression regardless of whether

the mRNA for a given gene has a rapid turnover (and hence can rise rapidly) or a slower turnover that would require a longer period to show corresponding increases or decreases. For CPF, we evaluated the effects both on undifferentiated cells (without addition of NGF) and during differentiation, whereas for the other agents, we studied the effects only during differentiation.

Animal treatments. No additional animal studies were conducted for this work. Instead, we utilized the gene expression data from our previously-published microarray study of the effects of CPF and DZN in neonatal rats [160], with the new addition of concordance comparisons as required for the present study. Details of the animal treatments have been published [160] and therefore will be described only briefly. Neonatal rats received daily injections of CPF or DZN in dimethylsulfoxide vehicle on PN 1-4, whereas control animals received equivalent injections of the vehicle. For both agents, we utilized doses below the threshold for growth retardation and the first signs of systemic toxicity [31,154,196]: 1 mg/kg for CPF and 1 or 2 mg/kg for DZN. The CPF treatment and the higher dose of DZN elicit less than 20% cholinesterase inhibition, well below the 70% threshold necessary for symptoms of cholinergic hyperstimulation [36], whereas the lower dose of DZN produces no measurable inhibition [145,146,169,175,196]. On postnatal day 5 (24 hr after the last dose), one male pup was selected from each of five litters in each treatment group and separate determinations were made for the forebrain and brainstem from each animal.

Microarray determinations. Our earlier studies detailed all the techniques for mRNA isolation, preparation of cDNA, conversion to cRNA incorporating cyanine-3

(reference RNA) or cyanine-5 (sample RNA), verification of RNA purity and quality, hybridization to the microarrays, washing and scanning [160,165,166]. These all involve commercial kits and standardized procedures, and since the current studies were done identically, the techniques will not be described here. The mRNA used for the reference standard was created by pooling aliquots from each of the samples in the study so as to ensure measurable levels of all genes expressed over the background. Array normalizations and error detection were also carried out by standard procedures described previously [160,165,166]. We used Agilent Whole Rat Genome Arrays (Agilent Technologies, Palo Alto, CA), type G4131A for the studies of CPF in undifferentiated and differentiating cells and for the *in vivo* studies, whereas type G4131F was used for the studies of DZN, dieldrin and Ni^{2+} in differentiating cells. The two chips contain exactly the same gene sequences but the latter has a lower detection threshold; however, all the genes reported here passed the quality control filters with both arrays.

For many of the genes, the arrays contain multiple probes and/or replicates of the same probe in different locations on the chip, and these were used to verify the reliability of values and the validity of the measures on the chip. In these cases, to avoid artificially inflating the number of genes utilized for concordance analysis, we limited each gene to a single set of values, selecting those obtained for the probe showing the smallest intragroup variance. The other values for that gene were used only to corroborate direction and magnitude of change. We also validated the readings on the arrays through the use of duplicate arrays for one sample selected from each treatment group [160,165].

Statistical procedures. Because of the requirement to normalize the data across

arrays and within each gene, the absolute values for a given gene are meaningless, so only the relative differences between treatments can be compared. Accordingly, concordance was evaluated using the percentage change from the control values, calculating the linear correlation coefficient between pairs of agents for each class of genes. For reference, the raw values (mean ± SE) for normalized expression for all the genes appear in Table 4 for the evaluations of CPF effects in undifferentiated and differentiating cells. Table 5 shows the values for the effects of DZN, dieldrin and Ni^{2+} in differentiating cells and the corresponding values for the *in vivo* study were published previously [160]. It should be noted that the absolute values cannot be compared for the three sets of determinations because reference mRNAs were constructed from the samples contained in each study, as described above, and thus differed among the studies; nevertheless, the percentage changes from the control value can be compared, since taking the experimental values as a ratio to the corresponding control normalizes the values for the inter-experimental differences. Additional details of the nomenclature and sequence for each gene appeared earlier [160].

In addition to pairwise concordance comparisons of the various agents, we also evaluated groupings of correlations for each class of genes and across classes, using the χ^2 test for combining p-values: $\chi^2 = \Sigma[-2 \ln(p_i)]$, with degrees of freedom corresponding to twice the number of p-values used in the calculation. In the two cases where a correlation coefficient connoted an opposite direction of change from the others, the associated χ^2 value for that comparison was subtracted from the sum instead of being added to it. Significance for all tests was assumed at $p < 0.05$.

Table 4: Effects of chlorpyrifos on gene expression in undifferentiated and differentiating cells

Gene Group	Gene	Treatment	Undifferentiated		Differentiating	
			1d	3d	1d	3d
Adenylyl Cyclase	*adcy2*	Control	1.11 ± 0.07	1.02 ± 0.09	1.46 ± 0.10	0.93 ± 0.03
		CPF	0.87 ± 0.08	1.06 ± 0.25	1.05 ± 0.11	0.96 ± 0.12
	adcy3	Control	1.28 ± 0.15	1.14 ± 0.14	1.15 ± 0.07	0.99 ± 0.08
		CPF	0.93 ± 0.02	0.86 ± 0.03	1.04 ± 0.08	1.07 ± 0.03
	adcy4	Control	1.07 ± 0.20	—	0.88 ± 0.10	—
		CPF	0.84 ± 0.23	—	0.94 ± 0.06	—
	adcy5	Control	0.97 ± 0.09	1.16 ± 0.11	1.05 ± 0.05	0.97 ± 0.04
		CPF	0.99 ± 0.05	1.01 ± 0.05	0.99 ± 0.05	0.95 ± 0.05
	adcy6	Control	1.01 ± 0.02	1.03 ± 0.02	0.92 ± 0.04	1.01 ± 0.02
		CPF	1.03 ± 0.02	1.03 ± 0.03	0.94 ± 0.02	1.08 ± 0.02
	adcy8	Control	0.91 ± 0.13	0.95 ± 0.14	0.95 ± 0.09	1.00 ± 0.23
		CPF	0.94 ± 0.08	1.05 ± 0.06	0.88 ± 0.09	1.08 ± 0.06
	adcy9	Control	0.88 ± 0.06	0.98 ± 0.08	1.00 ± 0.08	1.14 ± 0.09
		CPF	0.94 ± 0.08	0.94 ± 0.04	1.12 ± 0.07	1.15 ± 0.08
	sac	Control	0.50 ± 0.09	1.12 ± 0.35	0.77 ± 0.12	1.25 ± 0.12
		CPF	1.12 ± 0.19	1.03 ± 0.20	1.17 ± 0.26	0.94 ± 0.30
	adcyap1	Control	0.93 ± 0.04	0.98 ± 0.03	0.99 ± 0.04	1.03 ± 0.04
		CPF	0.91 ± 0.05	0.97 ± 0.02	1.01 ± 0.03	1.05 ± 0.02
	adcyap1r1	Control	1.21 ± 0.15	1.16 ± 0.25	0.91 ± 0.10	1.24 ± 0.20
		CPF	1.15 ± 0.14	1.19 ± 0.14	0.96 ± 0.20	1.06 ± 0.08
G-Protein α-Subunits	*gna11*	Control	1.04 ± 0.08	0.95 ± 0.07	0.97 ± 0.03	1.03 ± 0.03
		CPF	0.85 ± 0.11	1.04 ± 0.06	1.07 ± 0.03	1.00 ± 0.08
	gna12	Control	1.07 ± 0.20	1.34 ± 0.27	1.01 ± 0.12	1.30 ± 0.12
		CPF	0.95 ± 0.14	0.98 ± 0.12	0.81 ± 0.03	1.07 ± 0.04
	gna14	Control	0.94 ± 0.06	0.95 ± 0.03	1.13 ± 0.07	1.07 ± 0.03
		CPF	0.90 ± 0.06	0.88 ± 0.06	1.11 ± 0.08	1.10 ± 0.07
	gna15	Control	0.76 ± 0.07	0.86 ± 0.12	0.89 ± 0.11	0.97 ± 0.11
		CPF	1.11 ± 0.13	1.30 ± 0.20	1.28 ± 0.21	1.14 ± 0.13
	gnai1	Control	0.62 ± 0.05	0.63 ± 0.04	1.14 ± 0.09	1.39 ± 0.09
		CPF	0.69 ± 0.03	0.83 ± 0.05	1.38 ± 0.08	1.71 ± 0.09
	gnai2	Control	0.96 ± 0.02	0.94 ± 0.02	1.08 ± 0.03	1.12 ± 0.03
		CPF	0.93 ± 0.02	0.97 ± 0.01	1.11 ± 0.02	1.06 ± 0.03
	gnai3	Control	0.90 ± 0.03	0.92 ± 0.03	1.02 ± 0.02	1.06 ± 0.02
		CPF	0.91 ± 0.03	0.98 ± 0.02	1.01 ± 0.01	1.06 ± 0.02
	gnao	Control	0.98 ± 0.02	0.89 ± 0.02	1.05 ± 0.03	1.39 ± 0.05
		CPF	0.98 ± 0.04	0.90 ± 0.03	1.01 ± 0.05	1.39 ± 0.03
	gnaq	Control	0.86 ± 0.06	0.99 ± 0.06	1.00 ± 0.07	1.10 ± 0.05
		CPF	1.05 ± 0.10	0.85 ± 0.07	1.02 ± 0.06	1.13 ± 0.09
	gnas	Control	0.96 ± 0.02	0.95 ± 0.02	1.07 ± 0.03	2.03 ± 0.08
		CPF	0.98 ± 0.03	0.87 ± 0.03	1.00 ± 0.01	2.28 ± 0.14
	gnat1	Control	0.95 ± 0.07	0.96 ± 0.04	0.90 ± 0.14	1.13 ± 0.21
		CPF	0.93 ± 0.10	1.08 ± 0.20	1.13 ± 0.14	0.98 ± 0.14
	gnat2	Control	0.80 ± 0.13	0.92 ± 0.07	1.28 ± 0.19	1.03 ± 0.03
		CPF	1.19 ± 0.12	1.18 ± 0.23	0.98 ± 0.12	0.99 ± 0.11
	gnat3	Control	0.95 ± 0.06	1.15 ± 0.16	—	0.79 ± 0.08
		CPF	1.13 ± 0.14	0.98 ± 0.07	—	1.07 ± 0.10
	gnaz	Control	0.90 ± 0.01	0.88 ± 0.02	1.07 ± 0.01	1.30 ± 0.01
		CPF	0.93 ± 0.01	0.96 ± 0.03	1.01 ± 0.01	1.21 ± 0.01

Table 4 continued

G-Protein β,γ-Subunits	*gnb1*	Control	0.91 ± 0.01	0.89 ± 0.01	1.16 ± 0.01	1.14 ± 0.02
		CPF	0.90 ± 0.01	0.93 ± 0.01	1.21 ± 0.03	1.14 ± 0.02
	gnb2	Control	0.95 ± 0.03	0.95 ± 0.01	1.07 ± 0.03	1.17 ± 0.03
		CPF	0.90 ± 0.02	0.95 ± 0.01	1.11 ± 0.04	1.11 ± 0.03
	gnb5	Control	1.08 ± 0.03	1.06 ± 0.04	1.02 ± 0.03	0.93 ± 0.02
		CPF	1.03 ± 0.05	1.11 ± 0.04	0.94 ± 0.02	0.91 ± 0.02
	gng3	Control	1.18 ± 0.02	1.21 ± 0.02	0.82 ± 0.03	0.91 ± 0.01
		CPF	1.09 ± 0.01	1.16 ± 0.03	0.79 ± 0.02	0.84 ± 0.02
	gng4	Control	1.10 ± 0.02	1.10 ± 0.01	0.96 ± 0.02	0.99 ± 0.01
		CPF	1.04 ± 0.02	1.09 ± 0.02	0.92 ± 0.01	0.95 ± 0.02
	gng5	Control	0.88 ± 0.01	0.92 ± 0.02	1.07 ± 0.01	1.06 ± 0.03
		CPF	0.94 ± 0.01	0.91 ± 0.02	1.14 ± 0.02	1.25 ± 0.04
	gng7	Control	1.05 ± 0.10	1.36 ± 0.17	0.80 ± 0.03	1.11 ± 0.12
		CPF	0.88 ± 0.07	0.89 ± 0.09	0.98 ± 0.10	1.08 ± 0.10
	gng8	Control	0.93 ± 0.01	1.04 ± 0.02	0.90 ± 0.01	1.38 ± 0.01
		CPF	0.96 ± 0.02	1.06 ± 0.02	0.94 ± 0.01	1.43 ± 0.03
	gng10	Control	1.12 ± 0.03	1.15 ± 0.02	0.92 ± 0.02	0.85 ± 0.02
		CPF	1.08 ± 0.03	1.10 ± 0.02	0.93 ± 0.04	0.84 ± 0.04
	gng11	Control	1.06 ± 0.11	1.08 ± 0.10	1.14 ± 0.19	0.99 ± 0.06
		CPF	1.16 ± 0.06	0.97 ± 0.02	1.05 ± 0.14	1.02 ± 0.08
	gng12	Control	0.86 ± 0.05	0.85 ± 0.05	1.29 ± 0.12	1.46 ± 0.05
		CPF	0.84 ± 0.07	0.90 ± 0.02	1.54 ± 0.03	1.67 ± 0.08
Protein Kinase A	*prkaa1*	Control	1.02 ± 0.08	0.85 ± 0.03	1.48 ± 0.21	1.05 ± 0.07
		CPF	1.16 ± 0.06	0.88 ± 0.05	1.18 ± 0.13	1.38 ± 0.23
	prkaa2	Control	0.93 ± 0.04	0.89 ± 0.03	1.14 ± 0.05	1.15 ± 0.03
		CPF	0.86 ± 0.03	0.87 ± 0.05	0.98 ± 0.04	1.15 ± 0.04
	prkab1	Control	1.08 ± 0.03	1.05 ± 0.03	1.00 ± 0.01	0.84 ± 0.04
		CPF	1.15 ± 0.06	1.08 ± 0.05	0.99 ± 0.02	0.77 ± 0.02
	prkab2	Control	1.35 ± 0.16	1.08 ± 0.03	0.97 ± 0.10	0.86 ± 0.08
		CPF	0.93 ± 0.10	0.98 ± 0.07	0.85 ± 0.06	1.14 ± 0.09
	prkaca	Control	1.09 ± 0.03	0.84 ± 0.05	0.86 ± 0.06	0.94 ± 0.06
		CPF	1.09 ± 0.04	1.04 ± 0.07	0.95 ± 0.06	1.04 ± 0.03
	prkacb	Control	0.99 ± 0.07	1.05 ± 0.05	0.90 ± 0.06	1.09 ± 0.09
		CPF	0.96 ± 0.07	1.03 ± 0.07	0.94 ± 0.10	0.86 ± 0.07
	prkag1	Control	0.95 ± 0.01	0.97 ± 0.02	1.01 ± 0.04	1.00 ± 0.02
		CPF	0.95 ± 0.02	1.03 ± 0.02	1.07 ± 0.02	1.03 ± 0.02
	prkag2	Control	1.14 ± 0.05	1.19 ± 0.05	0.72 ± 0.02	0.90 ± 0.04
		CPF	1.17 ± 0.04	1.31 ± 0.03	0.73 ± 0.03	0.84 ± 0.02
	prkar1a	Control	0.77 ± 0.01	0.82 ± 0.01	1.12 ± 0.03	1.28 ± 0.03
		CPF	0.78 ± 0.02	0.86 ± 0.03	1.18 ± 0.04	1.26 ± 0.02
	prkar2a	Control	1.32 ± 0.10	1.06 ± 0.13	1.21 ± 0.18	1.31 ± 0.17
		CPF	0.94 ± 0.12	0.93 ± 0.05	0.88 ± 0.08	0.92 ± 0.12
	prkar2b	Control	1.11 ± 0.02	1.07 ± 0.02	0.96 ± 0.03	0.95 ± 0.02
		CPF	1.15 ± 0.02	1.06 ± 0.03	0.92 ± 0.02	0.92 ± 0.02

Table 4 continued

Phosphodiesterases	*pde1a*	Control	0.89 ± 0.08	1.02 ± 0.06	0.85 ± 0.09	1.00 ± 0.06
		CPF	0.94 ± 0.09	1.07 ± 0.15	1.25 ± 0.12	1.16 ± 0.07
	pde1b	Control	1.06 ± 0.04	0.97 ± 0.04	1.02 ± 0.03	1.06 ± 0.03
		CPF	0.97 ± 0.01	0.98 ± 0.04	0.99 ± 0.03	1.00 ± 0.02
	pde1c	Control	1.07 ± 0.11	0.85 ± 0.15	0.84 ± 0.15	1.09 ± 0.29
		CPF	0.74 ± 0.07	0.96 ± 0.18	0.85 ± 0.11	1.11 ± 0.17
	pde2a	Control	1.62 ± 0.36	1.20 ± 0.17	1.01 ± 0.13	1.53 ± 0.08
		CPF	0.96 ± 0.08	0.78 ± 0.07	0.81 ± 0.13	0.87 ± 0.10
	pde3a	Control	1.04 ± 0.16	0.95 ± 0.05	1.12 ± 0.05	0.81 ± 0.06
		CPF	1.03 ± 0.14	0.92 ± 0.02	1.05 ± 0.03	0.83 ± 0.06
	pde3b	Control	1.07 ± 0.05	1.13 ± 0.05	1.01 ± 0.02	0.94 ± 0.03
		CPF	1.08 ± 0.03	1.16 ± 0.04	0.87 ± 0.03	0.88 ± 0.02
	pde4a	Control	0.94 ± 0.09	0.91 ± 0.07	1.07 ± 0.09	1.21 ± 0.12
		CPF	0.86 ± 0.08	0.88 ± 0.07	1.10 ± 0.04	1.15 ± 0.09
	pde4b	Control	1.00 ± 0.08	1.17 ± 0.10	0.97 ± 0.10	0.85 ± 0.03
		CPF	1.29 ± 0.07	1.27 ± 0.07	0.86 ± 0.06	0.90 ± 0.04
	pde4c	Control	1.03 ± 0.09	1.06 ± 0.12	0.98 ± 0.09	1.27 ± 0.12
		CPF	1.04 ± 0.09	1.03 ± 0.18	1.06 ± 0.05	0.97 ± 0.06
	pde4d	Control	1.02 ± 0.09	1.13 ± 0.05	0.89 ± 0.11	0.80 ± 0.07
		CPF	1.31 ± 0.04	1.31 ± 0.14	0.80 ± 0.03	0.73 ± 0.03
	pde5a	Control	1.36 ± 0.11	1.06 ± 0.12	1.08 ± 0.13	0.77 ± 0.14
		CPF	1.01 ± 0.05	1.00 ± 0.09	1.00 ± 0.07	0.91 ± 0.03
	pde6a	Control	1.15 ± 0.15	0.81 ± 0.10	1.07 ± 0.14	1.13 ± 0.11
		CPF	0.94 ± 0.12	0.99 ± 0.13	1.14 ± 0.17	1.03 ± 0.13
	pde6b	Control	0.94 ± 0.06	0.87 ± 0.05	0.91 ± 0.12	1.07 ± 0.07
		CPF	1.06 ± 0.07	0.93 ± 0.02	1.03 ± 0.04	1.13 ± 0.06
	pde6c	Control	0.67 ± 0.11	0.71 ± 0.14	0.81 ± 0.18	1.04 ± 0.14
		CPF	0.77 ± 0.17	0.70 ± 0.12	0.97 ± 0.26	1.28 ± 0.20
	pde6d	Control	0.95 ± 0.02	0.85 ± 0.03	1.10 ± 0.03	1.12 ± 0.04
		CPF	0.93 ± 0.02	0.91 ± 0.01	1.15 ± 0.05	1.19 ± 0.05
	pde6h	Control	1.04 ± 0.14	1.00 ± 0.08	1.02 ± 0.10	1.07 ± 0.07
		CPF	1.05 ± 0.11	0.99 ± 0.08	1.03 ± 0.02	1.00 ± 0.09
	pde7a	Control	0.95 ± 0.02	0.95 ± 0.02	1.08 ± 0.03	1.04 ± 0.03
		CPF	0.99 ± 0.02	0.94 ± 0.01	1.08 ± 0.02	1.04 ± 0.03
	pde7b	Control	0.85 ± 0.07	1.13 ± 0.10	1.24 ± 0.14	1.10 ± 0.08
		CPF	1.16 ± 0.13	0.62 ± 0.13	0.97 ± 0.15	0.92 ± 0.11
	pde8a	Control	0.81 ± 0.09	1.35 ± 0.14	0.95 ± 0.06	0.81 ± 0.07
		CPF	1.22 ± 0.08	1.15 ± 0.10	0.93 ± 0.10	0.87 ± 0.11
	pde8b	Control	0.98 ± 0.13	0.79 ± 0.11	1.29 ± 0.29	0.95 ± 0.15
		CPF	1.04 ± 0.16	1.12 ± 0.25	0.63 ± 0.07	2.02 ± 0.40
	pde9a	Control	1.03 ± 0.26	0.85 ± 0.17	1.31 ± 0.19	0.95 ± 0.16
		CPF	0.92 ± 0.18	1.32 ± 0.28	0.98 ± 0.11	0.77 ± 0.14
	pde10a	Control	0.79 ± 0.03	1.06 ± 0.05	0.86 ± 0.06	1.00 ± 0.05
		CPF	1.01 ± 0.09	1.20 ± 0.06	0.92 ± 0.06	0.99 ± 0.08
	pde11a	Control	0.81 ± 0.04	1.41 ± 0.20	1.04 ± 0.08	0.99 ± 0.07
		CPF	1.04 ± 0.12	1.04 ± 0.11	1.00 ± 0.05	0.98 ± 0.09

Data represent means and standard errors of normalized expression ratios. Missing values are those that did not pass the quality control filters.

Table 5: Effects of diazinon, dieldrin and Ni^{2+} on gene expression in differentiating cells

Gene Group	Gene	Time	Control	DZN	Dieldrin	Ni^{2+}
Adenylyl Cyclase	*adcy2*	1 day	0.98 ± 0.09	1.17 ± 0.12	0.92 ± 0.06	0.89 ± 0.07
		3 days	1.08 ± 0.09	0.97 ± 0.08	1.12 ± 0.10	1.07 ± 0.07
	adcy3	1 day	1.00 ± 0.04	0.97 ± 0.03	1.02 ± 0.03	0.92 ± 0.03
		3 days	1.01 ± 0.06	1.09 ± 0.03	0.99 ± 0.03	0.96 ± 0.04
	adcy4	1 day	0.90 ± 0.10	1.09 ± 0.10	1.02 ± 0.09	0.90 ± 0.06
		3 days	1.05 ± 0.11	0.99 ± 0.08	1.08 ± 0.14	1.11 ± 0.09
	adcy5	1 day	1.11 ± 0.06	1.01 ± 0.10	1.09 ± 0.11	0.94 ± 0.10
		3 days	0.96 ± 0.09	0.94 ± 0.06	0.97 ± 0.08	0.89 ± 0.06
	adcy6	1 day	0.97 ± 0.03	0.89 ± 0.03	0.97 ± 0.03	0.84 ± 0.03
		3 days	1.11 ± 0.03	1.12 ± 0.03	1.07 ± 0.03	1.04 ± 0.04
	adcy8	1 day	0.96 ± 0.12	1.12 ± 0.13	1.17 ± 0.22	1.18 ± 0.18
		3 days	1.16 ± 0.13	0.90 ± 0.09	1.20 ± 0.14	0.98 ± 0.06
	adcy9	1 day	0.96 ± 0.01	0.95 ± 0.01	1.00 ± 0.01	0.94 ± 0.01
		3 days	1.14 ± 0.03	1.10 ± 0.01	1.07 ± 0.01	1.07 ± 0.03
	sac	1 day	0.89 ± 0.08	1.06 ± 0.09	0.93 ± 0.09	0.92 ± 0.09
		3 days	1.05 ± 0.07	0.97 ± 0.07	1.05 ± 0.10	0.97 ± 0.05
	adcyap1	1 day	0.98 ± 0.06	1.01 ± 0.04	1.02 ± 0.07	1.07 ± 0.05
		3 days	0.97 ± 0.09	1.01 ± 0.05	1.01 ± 0.09	1.12 ± 0.06
	adcyap1r1	1 day	1.01 ± 0.14	1.11 ± 0.14	1.15 ± 0.15	1.07 ± 0.18
		3 days	1.05 ± 0.13	1.04 ± 0.19	1.02 ± 0.12	0.82 ± 0.07
G-Protein α-Subunits	*gna11*	1 day	1.00 ± 0.01	0.97 ± 0.03	0.98 ± 0.02	0.96 ± 0.05
		3 days	1.06 ± 0.03	1.02 ± 0.03	1.03 ± 0.02	1.01 ± 0.02
	gna12	1 day	0.97 ± 0.02	0.96 ± 0.04	1.00 ± 0.04	0.92 ± 0.04
		3 days	1.01 ± 0.05	1.08 ± 0.02	0.99 ± 0.03	1.02 ± 0.06
	gna14	1 day	1.01 ± 0.01	1.00 ± 0.01	1.04 ± 0.03	0.97 ± 0.02
		3 days	0.97 ± 0.03	1.06 ± 0.03	1.02 ± 0.01	0.95 ± 0.02
	gna15	1 day	0.85 ± 0.14	1.08 ± 0.13	1.17 ± 0.12	1.01 ± 0.10
		3 days	0.96 ± 0.15	0.94 ± 0.08	1.08 ± 0.10	0.97 ± 0.08
	gnai1	1 day	0.82 ± 0.04	1.05 ± 0.03	0.81 ± 0.04	0.73 ± 0.02
		3 days	0.99 ± 0.05	1.31 ± 0.03	1.18 ± 0.05	1.32 ± 0.03
	gnai2	1 day	1.02 ± 0.03	0.98 ± 0.05	1.07 ± 0.03	0.95 ± 0.03
		3 days	1.00 ± 0.04	1.06 ± 0.03	0.95 ± 0.03	1.01 ± 0.03
	gnai3	1 day	1.03 ± 0.01	1.03 ± 0.02	1.00 ± 0.03	0.99 ± 0.02
		3 days	0.99 ± 0.03	1.00 ± 0.02	0.95 ± 0.02	1.00 ± 0.02
	gnao	1 day	0.93 ± 0.02	0.90 ± 0.01	0.92 ± 0.02	0.94 ± 0.02
		3 days	1.34 ± 0.05	1.29 ± 0.03	1.27 ± 0.03	1.19 ± 0.01
	gnaq	1 day	0.99 ± 0.03	0.99 ± 0.05	0.96 ± 0.04	0.97 ± 0.06
		3 days	0.95 ± 0.03	1.05 ± 0.03	0.99 ± 0.05	1.13 ± 0.05
	gnas	1 day	0.67 ± 0.01	0.66 ± 0.01	0.63 ± 0.01	0.73 ± 0.03
		3 days	1.30 ± 0.04	1.32 ± 0.02	1.33 ± 0.02	1.44 ± 0.02
	gnat1	1 day	1.04 ± 0.09	1.07 ± 0.11	1.17 ± 0.12	0.87 ± 0.06
		3 days	1.06 ± 0.10	1.13 ± 0.11	1.01 ± 0.09	1.04 ± 0.08
	gnat2	1 day	1.18 ± 0.14	1.01 ± 0.08	0.96 ± 0.09	0.86 ± 0.06
		3 days	1.03 ± 0.06	0.99 ± 0.07	1.02 ± 0.10	0.97 ± 0.07
	gnat3	1 day	0.90 ± 0.08	1.14 ± 0.12	0.99 ± 0.09	1.00 ± 0.10
		3 days	1.02 ± 0.09	1.10 ± 0.09	1.11 ± 0.10	0.99 ± 0.08
	gnaz	1 day	0.97 ± 0.01	0.99 ± 0.01	0.97 ± 0.01	0.94 ± 0.03
		3 days	1.13 ± 0.03	1.12 ± 0.01	1.07 ± 0.01	0.98 ± 0.01

Table 5 continued

G-Protein β,γ-Subunits	*gnb1*	1 day	1.03 ± 0.01	0.98 ± 0.02	1.02 ± 0.02	0.97 ± 0.01
		3 days	1.03 ± 0.01	0.99 ± 0.02	1.01 ± 0.01	0.92 ± 0.02
	gnb2	1 day	1.05 ± 0.03	0.97 ± 0.02	1.06 ± 0.03	0.96 ± 0.05
		3 days	0.99 ± 0.02	1.02 ± 0.02	1.00 ± 0.02	0.94 ± 0.03
	gnb5	1 day	1.00 ± 0.02	0.99 ± 0.02	0.97 ± 0.03	0.92 ± 0.02
		3 days	1.02 ± 0.04	1.15 ± 0.03	1.02 ± 0.02	1.01 ± 0.03
	gng3	1 day	1.02 ± 0.02	1.00 ± 0.01	0.98 ± 0.02	0.91 ± 0.02
		3 days	1.05 ± 0.03	1.11 ± 0.02	1.02 ± 0.02	0.94 ± 0.02
	gng4	1 day	1.00 ± 0.01	0.90 ± 0.03	0.98 ± 0.01	1.00 ± 0.01
		3 days	1.12 ± 0.03	1.02 ± 0.02	1.02 ± 0.01	0.99 ± 0.01
	gng5	1 day	0.95 ± 0.02	0.99 ± 0.02	0.98 ± 0.02	1.10 ± 0.04
		3 days	0.93 ± 0.02	0.92 ± 0.02	1.14 ± 0.01	1.18 ± 0.03
	gng7	1 day	0.97 ± 0.05	0.91 ± 0.09	1.40 ± 0.21	1.03 ± 0.13
		3 days	1.11 ± 0.11	0.94 ± 0.05	1.01 ± 0.10	1.12 ± 0.16
	gng8	1 day	0.76 ± 0.01	0.77 ± 0.01	0.79 ± 0.01	0.81 ± 0.02
		3 days	1.18 ± 0.02	1.21 ± 0.01	1.29 ± 0.02	1.16 ± 0.01
	gng10	1 day	1.04 ± 0.04	1.01 ± 0.04	0.99 ± 0.06	0.98 ± 0.05
		3 days	1.01 ± 0.04	0.98 ± 0.04	0.95 ± 0.03	0.98 ± 0.05
	gng11	1 day	0.98 ± 0.19	1.15 ± 0.26	0.92 ± 0.10	1.10 ± 0.20
		3 days	1.12 ± 0.14	1.30 ± 0.21	1.12 ± 0.10	1.16 ± 0.19
	gng12	1 day	0.91 ± 0.01	1.00 ± 0.02	0.98 ± 0.03	0.94 ± 0.03
		3 days	0.96 ± 0.02	1.05 ± 0.02	1.07 ± 0.01	1.10 ± 0.02
Protein Kinase A	*prkaa1*	1 day	1.10 ± 0.05	1.03 ± 0.03	1.05 ± 0.03	1.11 ± 0.07
		3 days	0.90 ± 0.02	0.90 ± 0.04	0.90 ± 0.03	0.96 ± 0.04
	prkaa2	1 day	0.94 ± 0.02	0.92 ± 0.02	0.93 ± 0.01	0.96 ± 0.02
		3 days	1.08 ± 0.03	1.06 ± 0.01	1.11 ± 0.01	1.08 ± 0.03
	prkab1	1 day	1.14 ± 0.04	1.06 ± 0.04	1.14 ± 0.03	1.09 ± 0.04
		3 days	0.90 ± 0.02	0.92 ± 0.03	0.88 ± 0.02	0.89 ± 0.03
	prkab2	1 day	0.89 ± 0.05	0.83 ± 0.05	0.84 ± 0.07	0.97 ± 0.05
		3 days	1.05 ± 0.09	1.04 ± 0.04	1.08 ± 0.09	1.19 ± 0.06
	prkaca	1 day	1.03 ± 0.01	0.98 ± 0.02	1.02 ± 0.02	0.98 ± 0.03
		3 days	1.02 ± 0.02	0.99 ± 0.01	0.94 ± 0.02	1.00 ± 0.03
	prkacb	1 day	0.96 ± 0.01	0.90 ± 0.02	0.92 ± 0.02	1.00 ± 0.03
		3 days	1.11 ± 0.03	1.02 ± 0.01	1.06 ± 0.02	1.11 ± 0.02
	prkag1	1 day	1.06 ± 0.04	1.02 ± 0.04	1.11 ± 0.03	0.97 ± 0.02
		3 days	0.93 ± 0.03	1.08 ± 0.03	1.03 ± 0.03	0.98 ± 0.03
	prkag2	1 day	0.91 ± 0.01	0.97 ± 0.02	0.88 ± 0.01	0.88 ± 0.03
		3 days	1.12 ± 0.03	1.19 ± 0.01	1.04 ± 0.01	1.09 ± 0.02
	prkar1a	1 day	1.01 ± 0.01	0.99 ± 0.01	0.99 ± 0.01	1.09 ± 0.03
		3 days	0.97 ± 0.03	0.96 ± 0.02	0.98 ± 0.02	1.06 ± 0.02
	prkar2a	1 day	1.05 ± 0.02	1.01 ± 0.02	1.04 ± 0.02	0.96 ± 0.02
		3 days	1.04 ± 0.04	1.04 ± 0.02	0.99 ± 0.02	0.95 ± 0.02
	prkar2b	1 day	1.02 ± 0.02	0.98 ± 0.02	0.99 ± 0.02	1.04 ± 0.02
		3 days	1.09 ± 0.03	0.95 ± 0.02	1.00 ± 0.03	0.99 ± 0.02

Table 5 continued

Phosphodiesterases	*pde1a*	1 day	1.04 ± 0.09	1.06 ± 0.12	0.98 ± 0.10	0.85 ± 0.09
		3 days	1.25 ± 0.10	1.00 ± 0.11	1.11 ± 0.09	1.01 ± 0.07
	pde1b	1 day	1.07 ± 0.02	0.97 ± 0.07	0.96 ± 0.04	0.97 ± 0.06
		3 days	1.11 ± 0.07	1.05 ± 0.05	0.95 ± 0.04	1.01 ± 0.05
	pde1c	1 day	0.92 ± 0.10	1.10 ± 0.14	0.98 ± 0.11	1.02 ± 0.13
		3 days	1.03 ± 0.11	0.94 ± 0.08	1.14 ± 0.10	1.03 ± 0.07
	pde2a	1 day	0.81 ± 0.08	1.14 ± 0.09	0.94 ± 0.11	0.95 ± 0.08
		3 days	1.05 ± 0.11	0.90 ± 0.08	0.96 ± 0.12	0.95 ± 0.06
	pde3a	1 day	1.13 ± 0.08	1.25 ± 0.06	1.01 ± 0.07	1.11 ± 0.08
		3 days	0.94 ± 0.07	0.80 ± 0.08	0.69 ± 0.07	0.97 ± 0.06
	pde3b	1 day	1.15 ± 0.05	1.07 ± 0.06	1.08 ± 0.05	1.00 ± 0.07
		3 days	1.02 ± 0.09	0.98 ± 0.05	0.94 ± 0.03	0.97 ± 0.05
	pde4a	1 day	0.81 ± 0.04	0.80 ± 0.04	0.74 ± 0.03	0.74 ± 0.07
		3 days	1.27 ± 0.08	1.18 ± 0.04	1.31 ± 0.05	1.26 ± 0.04
	pde4b	1 day	0.97 ± 0.05	1.44 ± 0.08	0.80 ± 0.03	1.18 ± 0.04
		3 days	0.78 ± 0.04	1.04 ± 0.06	0.60 ± 0.02	1.34 ± 0.05
	pde4c	1 day	0.72 ± 0.05	1.05 ± 0.10	0.84 ± 0.04	0.73 ± 0.08
		3 days	1.27 ± 0.09	1.31 ± 0.03	1.21 ± 0.03	0.99 ± 0.04
	pde4d	1 day	1.03 ± 0.02	1.07 ± 0.06	0.88 ± 0.03	1.15 ± 0.04
		3 days	0.93 ± 0.04	0.92 ± 0.03	0.75 ± 0.02	1.20 ± 0.03
	pde5a	1 day	1.03 ± 0.12	1.07 ± 0.09	1.33 ± 0.24	0.93 ± 0.07
		3 days	1.01 ± 0.04	0.83 ± 0.07	1.02 ± 0.04	0.97 ± 0.06
	pde6a	1 day	0.95 ± 0.10	1.02 ± 0.11	1.04 ± 0.17	0.86 ± 0.06
		3 days	1.06 ± 0.10	1.41 ± 0.24	0.99 ± 0.09	0.90 ± 0.10
	pde6b	1 day	0.96 ± 0.08	1.08 ± 0.10	0.89 ± 0.06	0.90 ± 0.06
		3 days	1.05 ± 0.09	0.96 ± 0.08	1.09 ± 0.10	1.04 ± 0.07
	pde6c	1 day	0.95 ± 0.11	1.16 ± 0.09	0.99 ± 0.09	0.96 ± 0.09
		3 days	1.02 ± 0.17	1.06 ± 0.14	1.10 ± 0.08	0.95 ± 0.10
	pde6d	1 day	1.00 ± 0.02	1.06 ± 0.03	1.00 ± 0.03	0.91 ± 0.01
		3 days	0.85 ± 0.04	1.13 ± 0.03	0.99 ± 0.02	1.02 ± 0.01
	pde6h	1 day	0.95 ± 0.07	1.03 ± 0.11	1.08 ± 0.12	0.80 ± 0.06
		3 days	1.34 ± 0.09	1.04 ± 0.11	1.25 ± 0.20	0.99 ± 0.05
	pde7a	1 day	0.98 ± 0.01	1.00 ± 0.01	1.00 ± 0.01	0.95 ± 0.03
		3 days	1.05 ± 0.03	1.01 ± 0.01	1.12 ± 0.02	0.92 ± 0.02
	pde7b	1 day	0.97 ± 0.10	1.08 ± 0.10	1.05 ± 0.11	0.87 ± 0.05
		3 days	1.06 ± 0.10	0.94 ± 0.10	1.10 ± 0.11	0.99 ± 0.06
	pde8a	1 day	1.12 ± 0.03	1.12 ± 0.05	1.03 ± 0.06	1.10 ± 0.06
		3 days	0.77 ± 0.08	0.88 ± 0.03	0.91 ± 0.06	0.93 ± 0.05
	pde8b	1 day	1.02 ± 0.11	1.00 ± 0.11	0.99 ± 0.09	0.92 ± 0.08
		3 days	0.95 ± 0.08	0.91 ± 0.11	1.04 ± 0.10	1.01 ± 0.05
	pde9a	1 day	1.19 ± 0.10	1.04 ± 0.12	1.08 ± 0.06	0.96 ± 0.12
		3 days	1.16 ± 0.10	0.91 ± 0.06	0.93 ± 0.05	0.81 ± 0.04
	pde10a	1 day	1.05 ± 0.03	1.08 ± 0.05	0.90 ± 0.04	1.08 ± 0.06
		3 days	0.98 ± 0.06	0.94 ± 0.03	0.72 ± 0.02	1.26 ± 0.04
	pde11a	1 day	0.98 ± 0.09	1.10 ± 0.10	0.99 ± 0.08	0.88 ± 0.07
		3 days	1.10 ± 0.10	0.99 ± 0.08	1.14 ± 0.11	1.08 ± 0.07

Data represent means and standard errors of normalized expression ratios.

RESULTS

CPF, DZN, dieldrin and Ni^{2+} in differentiating PC12 cells. Despite the fact that CPF and DZN are both OPs, there was only slight, nonsignificant concordance in their effects on AC gene expression in differentiating cells (Figure 19A). Similarly, the effects of CPF showed small, positive correlations with those of dieldrin (Figure 19B) and Ni^{2+} (Figure 19C), as did that for DZN and dieldrin (Figure 19D), with none of the individual effects of sufficient magnitude to achieve statistical significance. However, the relationship between DZN and Ni^{2+} was stronger (Figure 19E), and that of dieldrin and Ni^{2+} strongest (Figure 19F). It was notable that, even for the nonsignificant regression relationships, all the correlations were positive. Accordingly, besides the pairwise comparisons, we established the overall significance of the concordance across all four agents using the χ^2 test for combining p-values; the global relationship for effects on AC gene expression was highly significant ($p < 0.002$, Table 6).

In general, the concordance for effects of CPF, DZN, dieldrin and Ni^{2+} on G-protein α-subunit genes was much higher than for AC. CPF and DZN showed a strong, statistically significant correlation (Figure 20A) and the relationship between CPF and dieldrin was even stronger (Figure 20B). CPF also showed a positive correlation with Ni^{2+} (Figure 20C) but the effect was smaller and nonsignificant. On the other hand, all the remaining pairwise combinations were robust and significant: DZN versus dieldrin (Figure 20D), DZN versus Ni^{2+} (Figure 20E) and dieldrin versus Ni^{2+} (Figure 20F). Reflecting the positive correlations, the overall concordance for effects of the four agents on G-protein α-subunit gene expression was highly significant ($p < 0.0001$, Table 6).

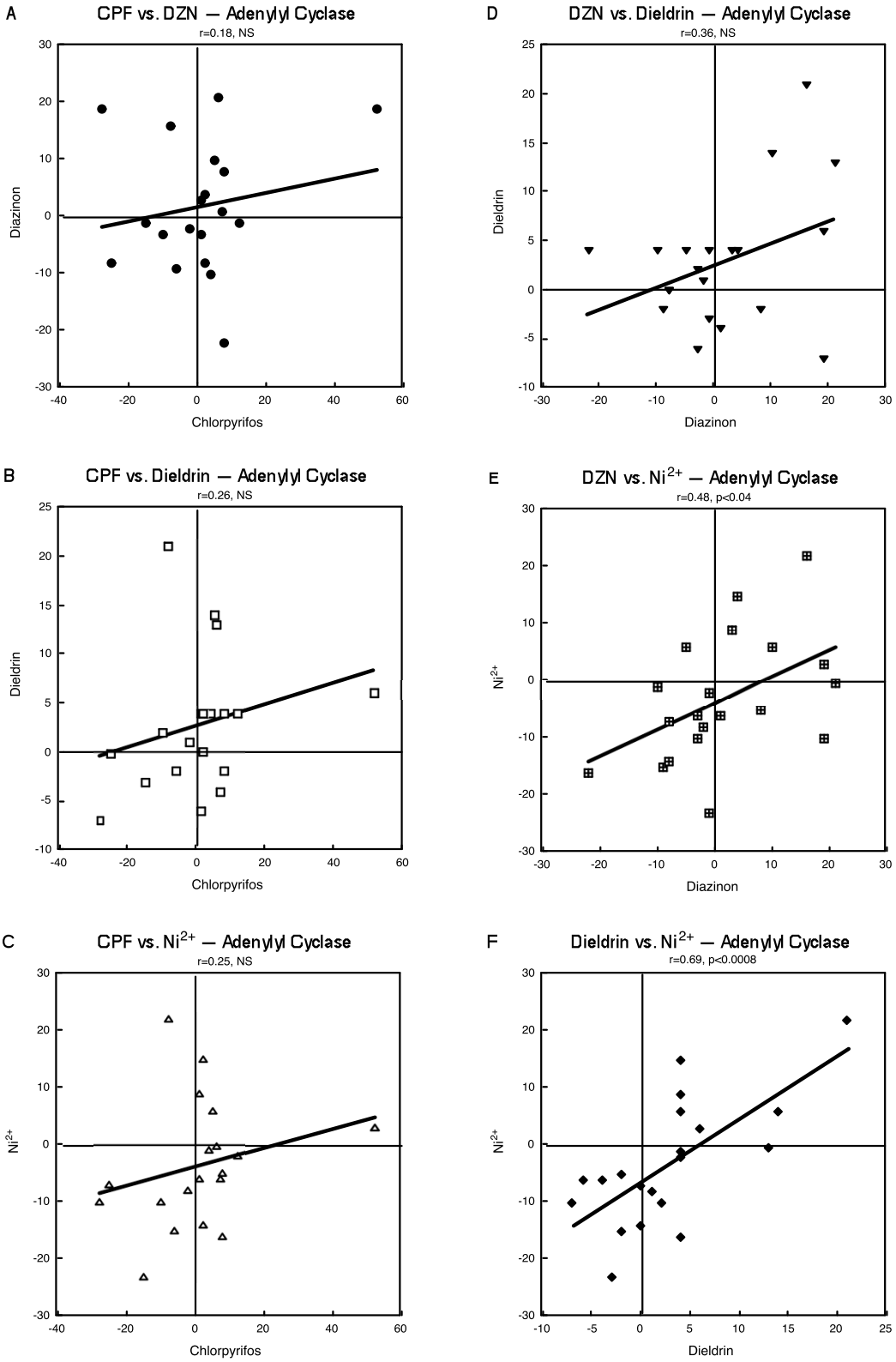

Figure 19: Pairwise correlations of the effects of CPF, DZN, dieldrin and Ni^{2+} on expression

of AC genes, calculated from Tables 4.1 and 4.2 as the percent change from corresponding control values. Linear correlation coefficients are shown at the top of each panel and the line represents the least-squares fit of the data. NS, not significant.

Table 6: Compound comparisons of gene expression effects across gene classes and treatments

	AC	G-protein α-subunits	G-protein β,γ-subunits	Protein kinase A	Phospho-diesterase	Row χ^2, p-value
Differentiating cells						
CPF vs. DZN	0.70	0.014	0.80	0.35	0.89	12, NS
CPF vs. dieldrin	0.30	0.0001	0.0001	0.08	0.26	47, <0.0001
CPF vs. Ni^{2+}	0.18	0.11	0.004	0.03	0.41	31, <0.001
DZN vs. dieldrin	0.11	0.0002	0.81	0.03	0.08	34, < 0.001
DZN versus Ni^{2+}	0.04	0.0003	0.10	0.58	0.0004	44, < 0.0001
dieldrin vs. Ni^{2+}	0.0008	0.002	0.02	0.15	0.33	41, < 0.0001
Column χ^2, p-values	32, < 0.002	77, <0.0001	43, <0.0001	26, <0.02	24, <0.02	205, <0.0001
CPF in undifferentiated vs. differentiating cells	0.03	0.41	0.88	0.14	0.09	18, NS
CPF vs. DZN in vivo	0.0001	0.0001	0.0001	0.0001	0.0001	110, <0.0001

Values shown are the individual p-values for each comparison, along with the corresponding χ^2 test evaluating the effects for each row, and for differentiating cells, each column. NS, not significant.

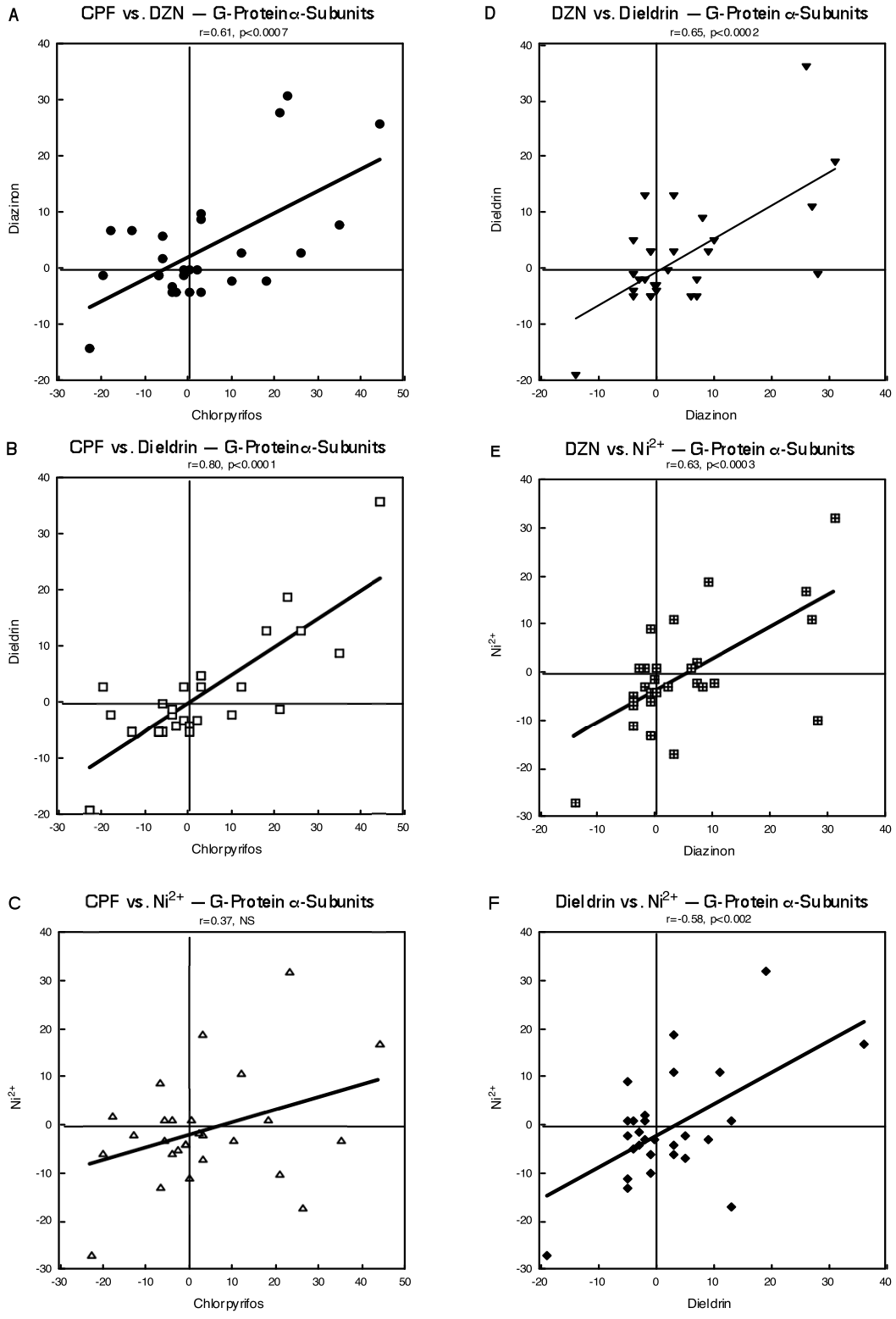
A
CPF vs. DZN — G-Protein α-Subunits
r=0.61, p<0.0007
Diazinon
Chlorpyrifos
D
DZN vs. Dieldrin — G-Protein α-Subunits
r=0.65, p<0.0002
Dieldrin
Diazinon
B
CPF vs. Dieldrin — G-Protein α-Subunits
r=0.80, p<0.0001
Dieldrin
Chlorpyrifos
E
DZN vs. Ni2+ — G-Protein α-Subunits
r=0.63, p<0.0003
Ni2+
Diazinon
C
CPF vs. Ni2+ — G-Protein α-Subunits
r=0.37, NS
Ni2+
Chlorpyrifos
F
Dieldrin vs. Ni2+ — G-Protein α-Subunits
r=-0.58, p<0.002
Ni2+
Dieldrin

Figure 20: Pairwise correlations of the effects of CPF, DZN, dieldrin and Ni^{2+} on expression of G-protein α-subunit genes, calculated from Tables 4.1 and 4.2 as the percent change from corresponding control values. Linear correlation coefficients are shown at the top of each panel and the line represents the least-squares fit of the data. NS, not significant.

The interrelationships of neurotoxicant effects on the genes encoding G-protein β,γ-subunits were still significant but less uniform than for the α-subunits. CPF and DZN did not show any apparent concordance (Figure 21A) but CPF was highly concordant with dieldrin (Figure 21B) and Ni^{2+} (Figure 21C). DZN and dieldrin had unrelated effects (Figure 21D); DZN and Ni^{2+} showed a stronger relationship that still did not achieve statistical significance (Figure 21E). On the other hand, the effects of dieldrin and Ni^{2+} were positively and significantly correlated (Figure 21F). Taken together, the fact that all correlations were in the same direction (positive) reinforced an overall common pattern as established by the χ^2 test ($p < 0.0001$, Table 6) but this clearly reflected the dominating effect of the three individual correlations that were significant.

For the protein kinase A gene family, CPF and DZN again showed a slightly positive but nonsignificant relationship (Figure 22A); the correlation between CPF and dieldrin was better (Figure 22B) and that between CPF and Ni^{2+} even stronger, reaching statistical significance (Figure 22C). A similar, significant relationship was seen for DZN and dieldrin (Figure 22D), whereas the correlations for DZN versus Ni^{2+} (Figure 22E) and dieldrin versus Ni^{2+} (Figure 22F) were positive but not sufficiently large to cross the threshold for statistical significance. The weaker relationship for this class of genes was evident in a less robust outcome for the combined χ^2 test ($p < 0.02$, Table 6). Likewise, the results for the phosphodiesterase gene family showed significant but weaker overall relationships among the neurotoxicants, with positive but nonsignificant

correlations for CPF versus DZN (Figure 23A), CPF versus dieldrin (Figure 23B), CPF versus Ni^{2+} (Figure 23C) and DZN versus dieldrin (Figure 23D). There was more robust, significant concordance between DZN and Ni^{2+} (Figure 23E) but the relationship between dieldrin and Ni^{2+} was slightly discordant (i.e. nonsignificant, negative correlation). Nevertheless, the positive direction for five of the six pairwise comparisons resulted in a statistically significant overall concordance ($p < 0.02$, Table 6).

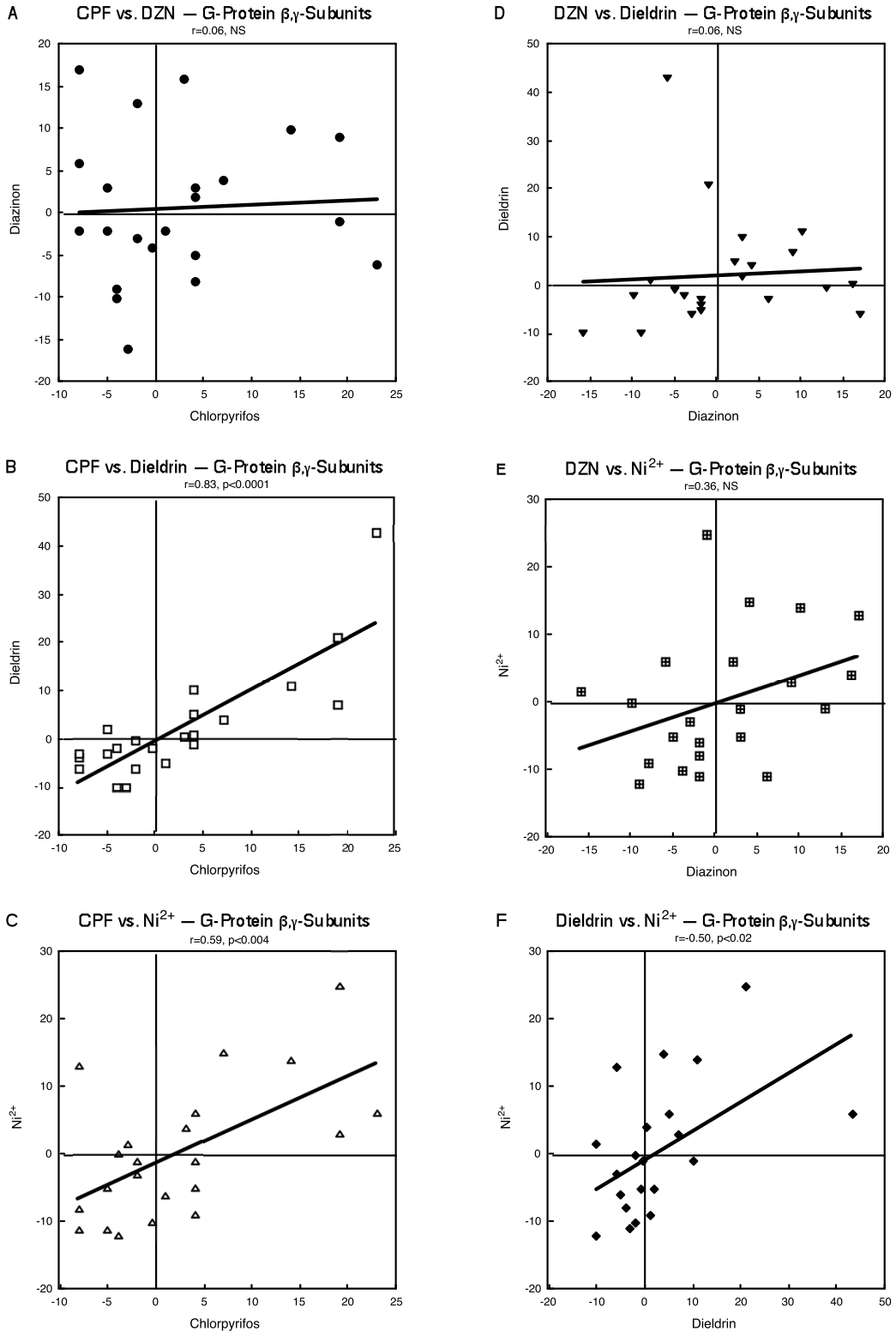

Figure 21: Pairwise correlations of the effects of CPF, DZN, dieldrin and Ni^{2+} on expression of G-protein β,γ-subunit genes, calculated from Tables 4.1 and 4.2 as the percent change from

corresponding control values. Linear correlation coefficients are shown at the top of each panel and the line represents the least-squares fit of the data. NS, not significant.

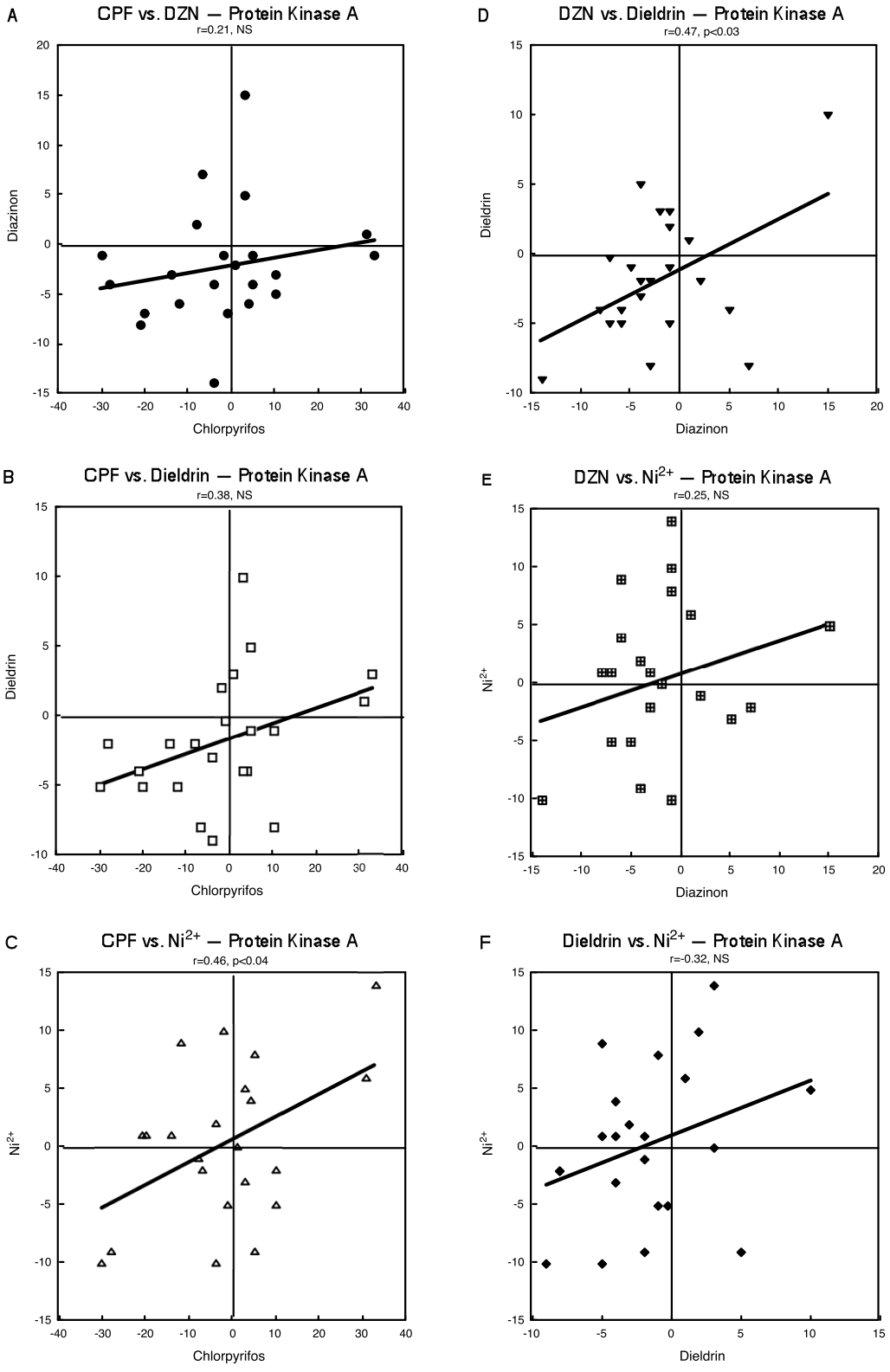

Figure 22: Pairwise correlations of the effects of CPF, DZN, dieldrin and Ni^{2+} on expression

of protein kinase A genes, calculated from Tables 4.1 and 4.2 as the percent change from corresponding control values. Linear correlation coefficients are shown at the top of each panel and the line represents the least-squares fit of the data. NS, not significant.

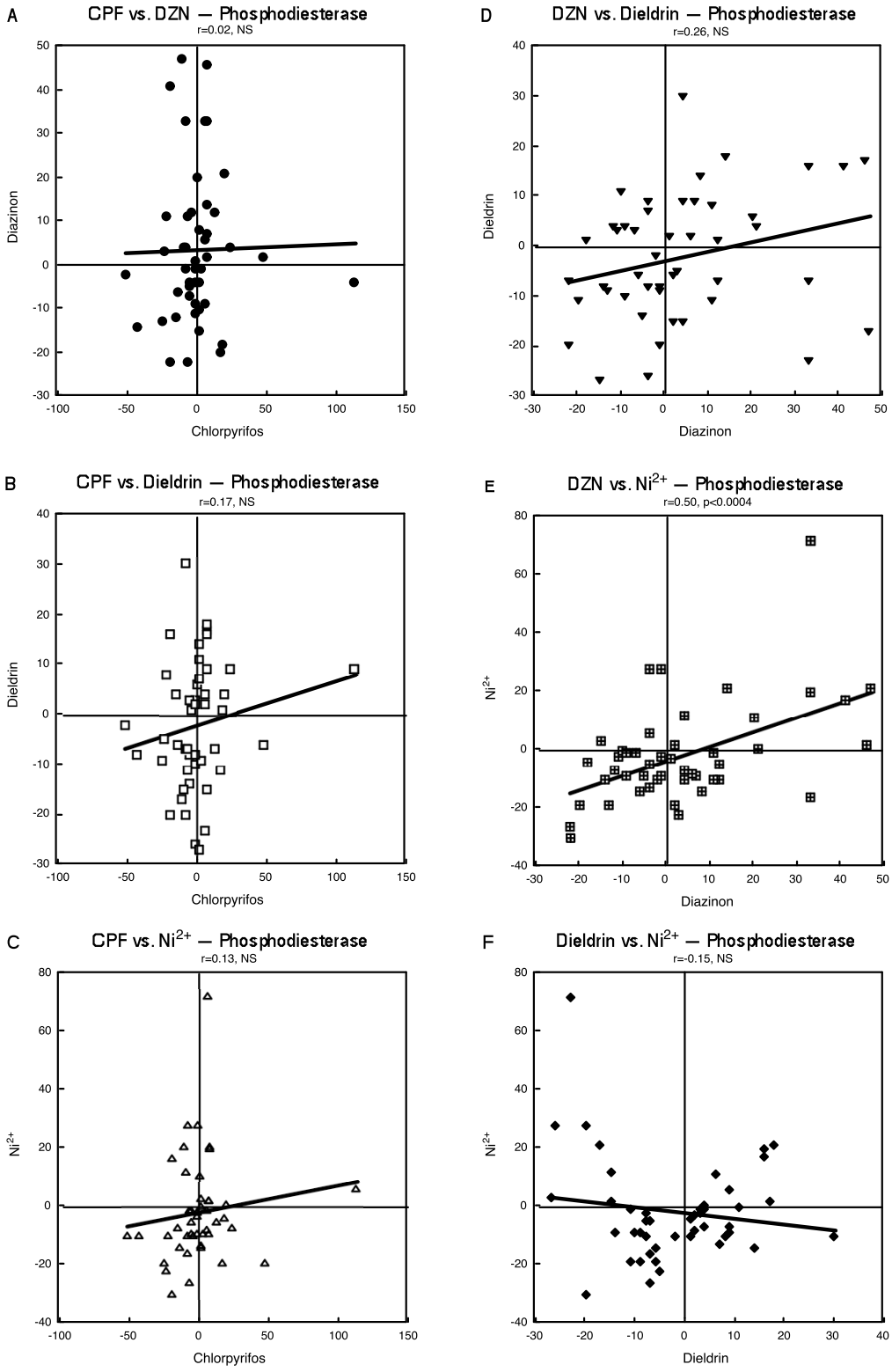

Figure 23: Pairwise correlations of the effects of CPF, DZN, dieldrin and Ni^{2+} on expression of phosphodiesterase genes, calculated from Tables 4.1 and 4.2 as the percent change from

corresponding control values. Linear correlation coefficients are shown at the top of each panel and the line represents the least-squares fit of the data. NS, not significant.

CPF in undifferentiated versus differentiating PC12 cells. The effects of CPF on AC genes correlated significantly between undifferentiated and differentiating cells but the relationship depended completely on a single value (*gnat3* at 1d of exposure), without which there would have been no significant concordance (Figure 24A). Similarly, the effects of CPF on G-protein α-subunits (Figure 24B) and β,γ-subunits (Figure 24C) showed virtually no correlation between undifferentiated and differentiating cells; although the relationships for protein kinase A (Figure 24D) and phosphodiesterase (Figure 24E) were stronger, none of these achieved statistical significance. The overall weakness of the correlation was confirmed by the lack of statistical significance for compound comparisons of all gene families using the χ^2 test (Table 6).

CPF and DZN in vivo. CPF and DZN administration to neonatal rats elicited significantly concordant transcriptional effects for all five gene families in forebrain and brainstem, resulting in a highly-significant overall relationship ($p < 0.0001$, Table 6). Regardless of whether the comparison involved AC (Figure 25A), G-protein α-subunits (Figure 25B), G-protein β,γ-subunits (Figure 25C), protein kinase A (Figure 25D), or phosphodiesterase (Figure 25E), the correlations of CPF with either dose of DZN were strongly positive and statistically significant.

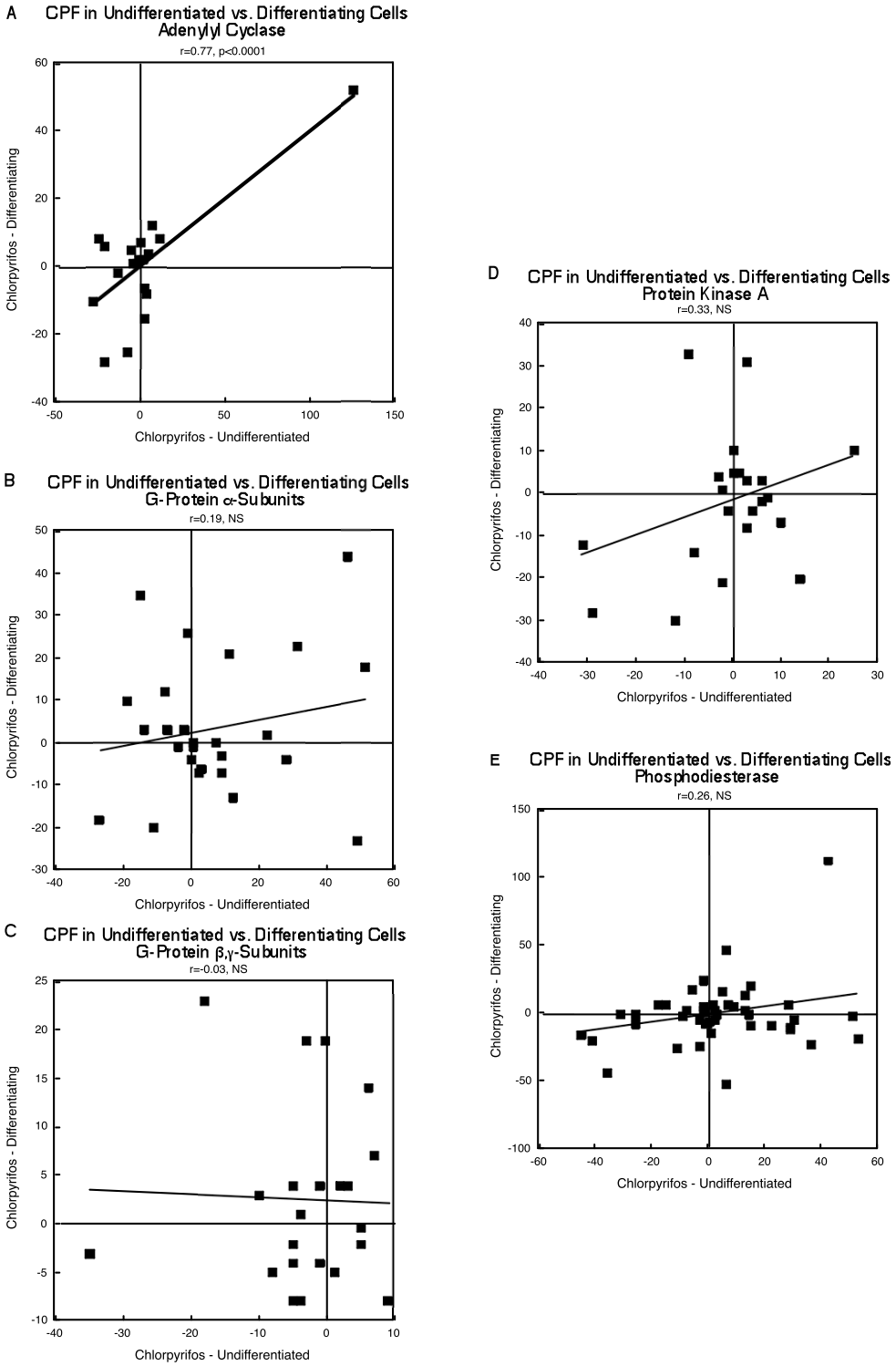

Figure 24: Correlations of the effects of CPF on undifferentiated versus differentiating cells for each the five classes of genes, calculated from Tables 4.1 and 4.2 as the percent change from corresponding control values: AC (A), G-protein α-subunits (B), G-protein β,γ-subunits (C), protein

kinase A (D) and phosphodiesterase (E). Linear correlation coefficients are shown at the top of each panel and the line represents the least-squares fit of the data. NS, not significant.

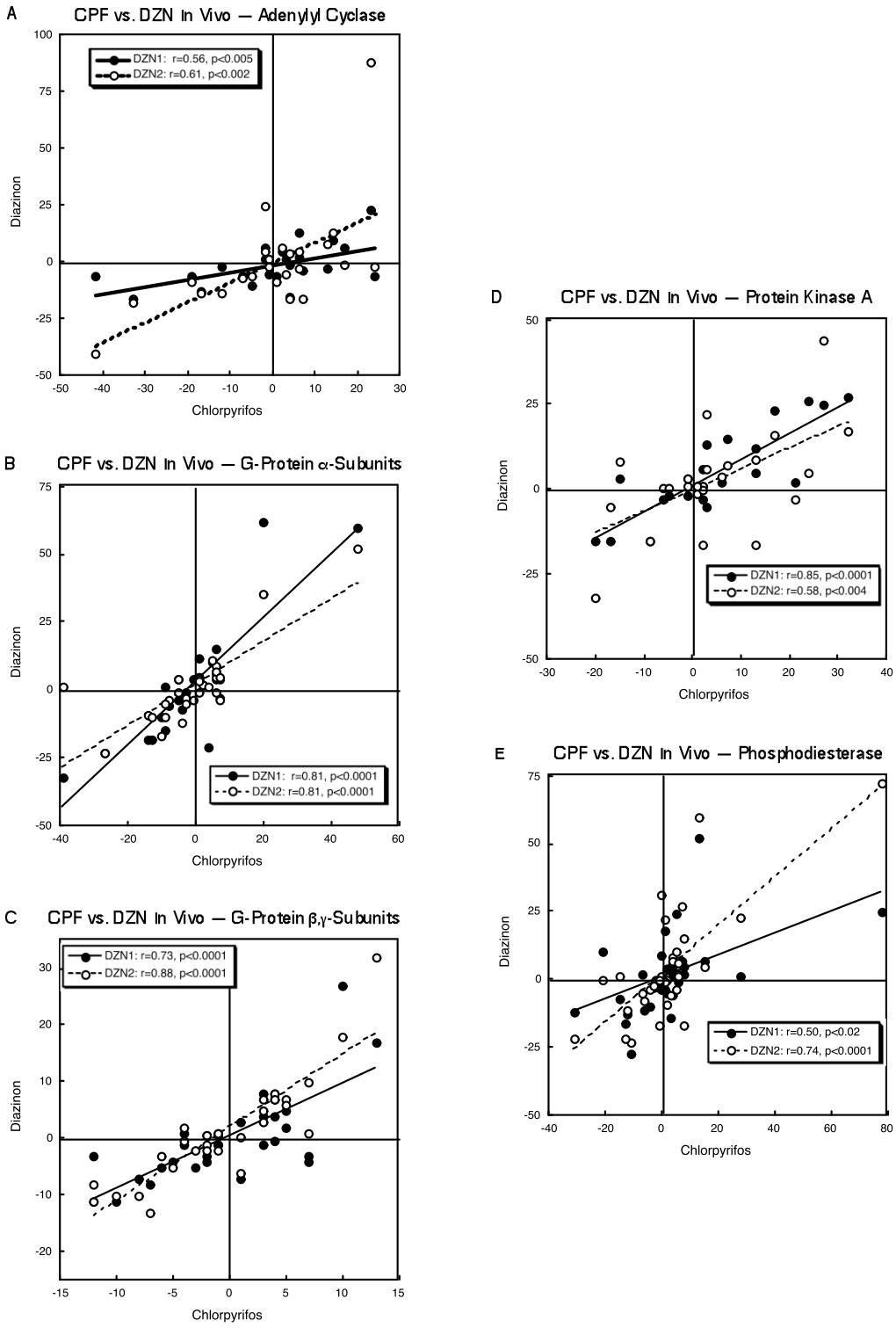

Figure 25: Correlations of the effects of CPF (1 mg/kg) and DZN (1 or 2 mg/kg) on gene expression *in vivo*, evaluated on postnatal day 5 in forebrain and brainstem from neonatal rats given

each agent daily from PN 1-4: AC (A), G-protein α-subunits (B), G-protein β,γ-subunits (C), protein kinase A (D) and phosphodiesterase (E). Linear correlation coefficients are shown within each panel and the line represents the least-squares fit of the data.

DISCUSSION

Our results indicate that otherwise unrelated developmental neurotoxicants nevertheless converge on cell signaling involving cAMP, while at the same time, there are clear dichotomies between OPs, CPF and DZN, that might be expected to be the most similar. Considering the four compounds together, we found strong concordance for combining all the potential pairwise comparisons (χ^2=205, $p < 0.0001$, Table 6), a reflection of the fact that, of the 30 evaluations, 29 showed a positive correlation and only one a negative correlation, a highly, non-random outcome ($p < 0.0001$ compared to a random expectation of 15 positive correlations and 15 negative correlations). Further, these similarities were observed in isolated cells, pointing to direct effects of the agents on transcription of genes involved in the control of cAMP. Embedded within the overall interrelationships, the concordance was strongest for effects on G-proteins, followed by the AC genes, and least for the genes involved in downstream effectors and regulators, protein kinase A and phosphodiesterase (see column χ^2 values, Table 6). Thus, effects on the steps required for the generation of cAMP provide the predominant points of similarity; consistent with this conclusion, for CPF and DZN, we found that *in vivo* exposures produce alterations in cAMP generation in response to stimulants acting at the level of G-proteins and AC itself [7,111,175]. The implication is thus inescapable that OPs, organochlorines and metals may all lead to similar neurodevelopmental outcomes despite the underlying differences in their chemical properties and classification. In turn, the *in vitro* findings can thus guide future *in vivo* examinations to test that prediction.

Superimposed on the general concordance of effects among CPF, DZN, dieldrin

and Ni^{2+}, there were important dichotomies that provide further mechanistic information and predictions about *in vivo* effects. First, the effects of CPF were highly dependent on the differentiation state of the cells, with little relationship evident between gene transcription in undifferentiated cells and those undergoing neurodifferentiation. This is consistent with the existence of critical periods of vulnerability of developing neurons to OPs. Thus, although OP effects can be seen at neurodevelopmental stages ranging from cell replication through the final stages of neurodifferentiation, the period surrounding the transition from replication to differentiation appears to be one of the most sensitive phases [83,145-147,156,161,166,167,176]. In the current study, our evaluations in differentiating cells specifically occupied the initial phases of that transition, highlighting the dissimilarities to the effects on gene expression in the undifferentiated state. The outcome is thus critically dependent upon the stage of neurodifferentiation at which exposure occurs, even for the same agent. Given the important role of cAMP in neurodifferentiation [102,107,177,197], our findings again reinforce the mechanistic relationships between stage-specific effects of neurotoxicants on gene expression controlling cAMP levels, and their ultimate disruption of neurodevelopment.

The second dichotomy appeared in the pairwise comparisons of individual agents, where there was a surprising divergence between CPF and DZN, the two toxicants that might be expected to be the most related, since they are both OPs. In fact, of the six pairings, CPF and DZN were the least alike (row χ^2 comparisons, Table 6). The effects of these two OPs on gene expression involved in the cAMP signaling pathway clearly do not depend on their shared property as cholinesterase inhibitors, the mechanism that

instead contributes to their similarities for systemic toxicity. Indeed, studies of downstream cAMP phosphorylation targets confirm that CPF produces its effects at concentrations well below those required for cholinesterase inhibition [141]. Our findings reinforce earlier conclusions showing that CPF has unique and/or more sensitive actions on signaling and neurotrophic pathways as compared to other OPs [163,165-167]; in contrast, DZN, even when given in the same developmental period as CPF and in pharmacodynamically-equivalent doses, elicits lesser long-term disruption of synaptic function and behavior [9,10,97,138,149,151,158,159,183]. Despite their dissimilarities, however, both CPF and DZN had strong concordance with the effects of the other two agents (row χ^2 comparisons, Table 6). The interrelationships can best be illustrated with schematic Venn diagrams. Although both CPF and DZN have significant overlap with the effects of dieldrin, the two OPs have little concordance with each other (Figure 26A), so that the common relationship to dieldrin involves different genes for each OP; however, CPF, dieldrin and Ni^{2+} all have significant mutual overlap (Figure 26B) and similar relationships pertain to DZN and the other two agents (not shown). The close resemblance of the transcriptional responses to DZN, dieldrin and Ni^{2+} are entirely consistent with their shared effects on neurotrophic responses [167] and downstream neurotoxic endpoints [156,161]. The implication is inescapable: these results predict that OPs such as CPF and DZN, dieldrin (an organochlorine) and Ni^{2+} (a metal) may in fact produce convergent developmental neurotoxicant outcomes, even if the OPs themselves may diverge in part.

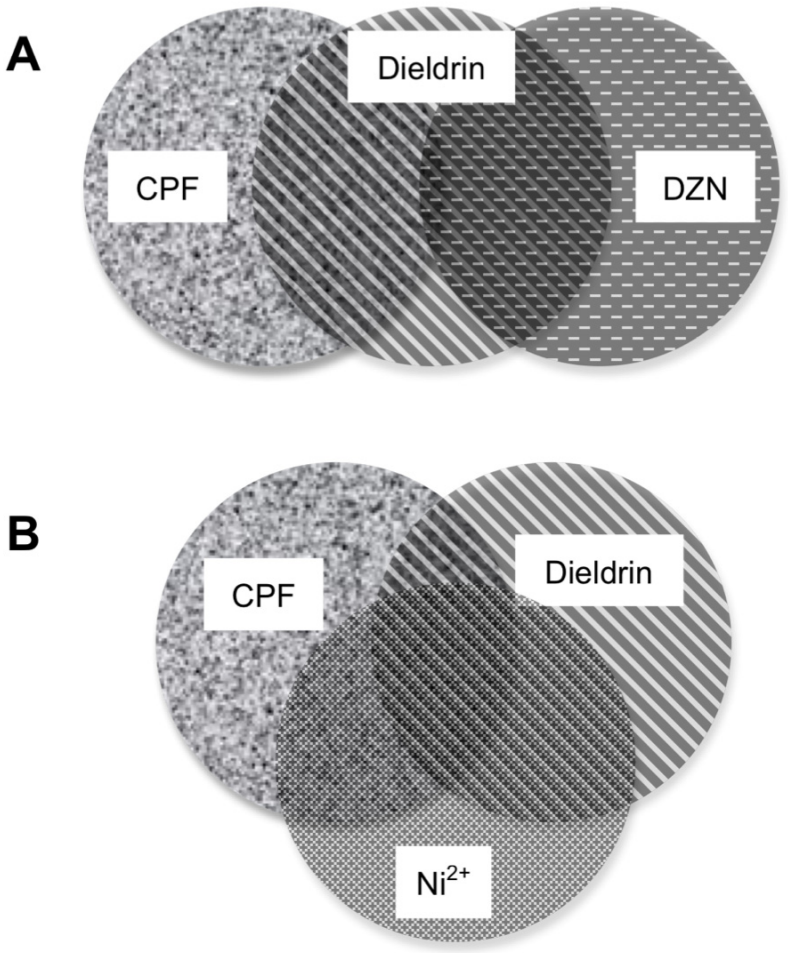

Figure 26: Venn diagram illustrating the relationships among CPF, DZN, dieldrin and Ni^{2+} for their global effects on the five classes of genes as calculated in Table 6. (A) CPF and dieldrin have strong concordance, as do DZN and dieldrin, but the effects of CPF and DZN are only weakly related because the gene effects shared between CPF and dieldrin differ from those involved in the correlation of DZN and dieldrin. (B) CPF, dieldrin and Ni^{2+} all share common patterns of effects on gene expression.

Although *in vitro* test systems enable the detection of direct effects of neurotoxicants on gene transcription and differentiation endpoints, they are inherently limited in that they cannot evaluate effects that depend on interactions between neurons and glia, architectural assembly of the brain, or any of the myriad, more complex

interactions that occur in the intact organism. Here, we found strong dichotomies between CPF and DZN in their effects on gene transcription related to the cAMP signaling pathway in PC12 cells and we have already highlighted how those differences are reflected in divergent outcomes. Nevertheless, CPF and DZN also have a number of similarities in their developmental neurotoxicant effects, particularly as they pertain to targeting of specific brain regions and neurotransmitter systems and their associated behaviors, as well as shared actions on formation of neurites and on proliferation of glial cells [133,138,145-147,149,156,158,164,169,183]. Accordingly, in addition to the *in vitro* studies conducted here, we performed a comparable concordance evaluation using gene expression data from our earlier work on the effects of CPF and DZN *in vivo*, in neonatal rats; we identified much stronger concordance involving highly significant correlations for each class of genes and across all classes together (Table 6). The strong *in vivo* relationship can be seen by comparing concordance of all the cAMP pathway genes in a single correlation: whereas there was no significant overall correlation for the *in vitro* evaluations (Figure 27A), the same comparisons were highly concordant for *in vivo* exposures (Figure 27B). Thus, in the intact brain, CPF and DZN do actually produce similar effects on the cAMP-related genes but the common outcome reflects indirect actions requiring the intact brain, rather than depending upon direct effects on differentiating neural cells, as seen with the PC12 cell model. Clearly, although *in vitro* models permit identification of direct effects of neurotoxicants on neurodifferentiation, a full picture of similarities and differences of neurotoxicant effects directed toward a given pathway requires additional examinations of the effects in the intact organism. It would

thus be worthwhile to pursue similar examinations of the effects of dieldrin and Ni^{2+}.

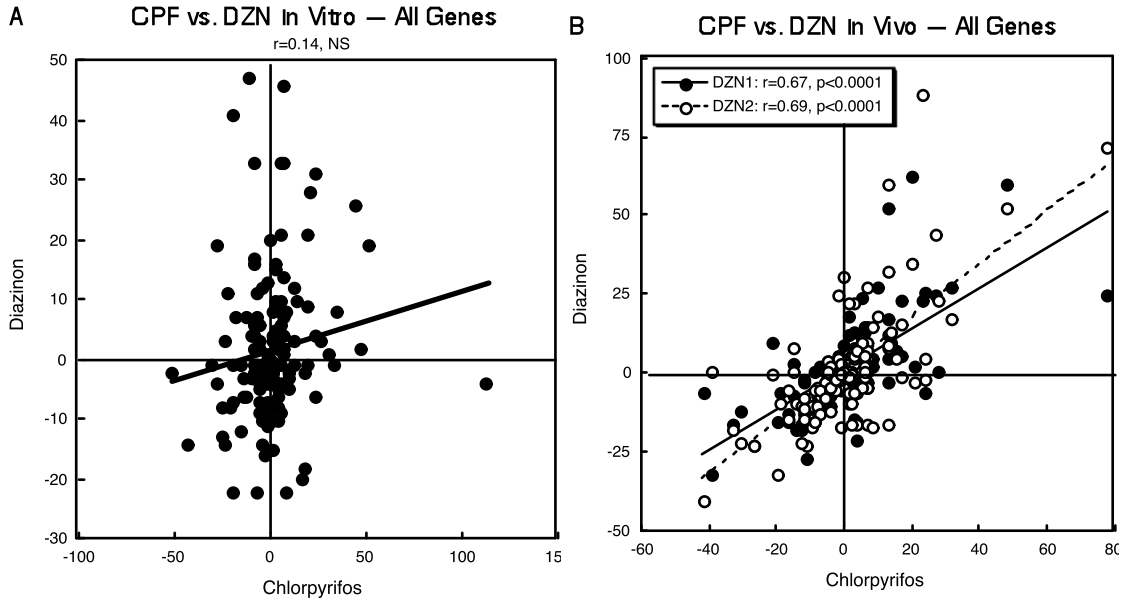

Figure 27: Comparison of *in vitro* (A) and *in vivo* (B) correlations of gene expression values combined from all five classes. Linear correlation coefficients are shown at the top of each panel and the line represents the least-squares fit of the data. NS, not significant.

Similarly, a key limitation of the microarray approach is that the measured effects occur at the mRNA level, which does not necessarily entail a corresponding change at the level of protein expression or cell function. Therefore, cell culture studies in Chapter 6 were utilized to evaluate the extent to which functional changes correspond to the effects of each toxicant on gene expression.

In this study, we used planned comparisons of specific pathways targeted by the neurotoxicants and analyzed the data through the determination of shared properties (i.e. a standard "principal components" approach); the rationale for this has appeared previously [160,165,166] but is worth repeating here. Planned comparisons and pathway analysis are distinct from the use of microarrays to find a handful of genes that are

affected the most, within the global examination of the tens of thousands of genes present on the microarrays. Planned comparisons are based instead on testing a specific hypothesis that centers around a defined set of genes, and rests on known, validating outcomes from prior work, in this case for the OPs. With examination of the entire genome, verification via RT-PCR and other techniques is required because the enormous number of comparisons generates many false positive findings (e.g. the >2000 genes that would be false positives if we had considered all 42,000 probes on the array). For our study, we compared only 69 genes that would generate only 3-4 false positives, and for interpretation, we relied on the overall pattern of multiple gene changes for each agent, as well as effects that were repeated across different treatments and/or different times, rather than changes in any one gene. The odds of all those genes being false positives are astronomically small. However, even for individual genes, there were multiple probes and multiple spots on a given array (see Materials and Methods), so the changes cannot be "chance." Unlike many array studies, where a single mRNA set combined from multiple samples might be evaluated, we evaluated separate samples for each treatment condition, so again it is inconceivable that one could statistically produce these outcomes by accident. However, it is important to point out that our interpretation of the overall pattern of multiple gene changes, rather than changes in any one gene, provides the possible risk of missing strong relationships between individual genes. Indeed, one of the key points of this study is to demonstrate that a planned comparisons approach may provide a superior strategy for the use of microarray data, provided that the relevant target pathways are known in advance.

In conclusion, our findings bolster the increasing evidence that the various OPs differ in their underlying mechanisms of developmental neurotoxicity, over and above their shared property as cholinesterase inhibitors, in this case involving distinct outcomes at the levels of the genes encoding the critical proteins of the cAMP signaling pathway. In addition, we found unexpected concordance in the effects of unrelated neurotoxicants, dieldrin and Ni^{2+}, on the same gene families, indicating that different classes of compounds can nonetheless converge on common final pathways. Finally, the results obtained here illustrate how a combined use of a cell culture system, an animal model, and microarrays can guide future studies toward specific endpoints that can distinguish similarities and disparities in the effects of diverse developmental neurotoxicants.

Chapter 6: Organophosphate Exposure During a Critical Developmental Stage Reprograms Adenylyl Cyclase Signaling in PC12 Cells

In Chapters 3 and 4, we showed how early life exposures to CPF, DZN or PRT all evoke lasting effects on components of the AC cascade [6,111,113,175]. Notably, these effects were found to extend outside the central nervous system. Indeed, neonatal OP exposures produce subsequent gain-of-function of hepatic AC signaling that contributes to the emergence of metabolic dysregulation akin to prediabetes [7,13,93,113,150].

These findings raise the possibility that, during a critical developmental period, OP exposures directly reprogram the functioning of the AC signaling pathway, a hypothesis that would be difficult to evaluate *in vivo*, given the myriad systemic changes elicited when these agents are given to animals. With this in mind, experiments in Chapter 5 utilized planned comparison gene expression (mRNA) analysis to assess AC isoforms and regulators. Importantly, these studies at the transcriptional level, showed a relative lack of concordance between undifferentiated and differentiating cells in the impact of CPF on the mRNAs encoding AC isoforms and G-proteins [6].

Here, in Chapter 6, we then investigated how mRNA findings tie into function at the level of protein. We used the same PC12 cell model to examine the effects of different OPs on AC signaling under conditions spanning different developmental stages from cell replication through early and later stages of differentiation. We had three specific objectives: first, to determine if OPs affect AC signaling during a discrete stage of cell development; second, to establish whether the effects persist so long as OP

exposure continues or rather whether effects extend beyond the exposure period; and third, to evaluate whether the effects on AC signaling are separable from effects on general aspects of cell growth. Here, we compared the effects of CPF, DZN and PRT on AC signaling in the undifferentiated state, at the initiation of differentiation, and after a more prolonged period of differentiation. Our AC assessments focused on measures that evaluate pathway function at sequential steps: basal enzymatic activity, the response to global stimulation of G-proteins by fluoride, and the responses to two direct AC stimulants, forskolin and Mn^{2+}. Because the two stimulants act at different loci on the AC molecule, the preferential effects for one versus the other defines shifts in the expression and catalytic activities of different AC isoforms [13,204,206]. We then compared the effects on AC signaling to those on cell growth, focusing on measures of cell number and neurite formation. Each neural cell contains a single nucleus, so that measuring the DNA content evaluates the number of cells [198], whereas the expansion of the membrane surface area that accompanies the formation of neurites during neurodifferentiation leads to an increase in the membrane protein/DNA ratio [4,82,156,176].

MATERIALS AND METHODS

Cell cultures. Because of the clonal instability of the PC12 cell line [56], the experiments were performed on cells that had undergone fewer than five passages and all studies were repeated several times with different batches of cells. As described previously [39,135,176], 3×10^6 PC12 cells (American Type Culture Collection, 1721-CRL; Duke Comprehensive Cancer Center, Durham, NC) were seeded onto 100 mm poly-D-lysine-coated plates in RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% horse serum (Sigma), 5% fetal bovine serum (Sigma), and 50 μg/ml penicillin streptomycin (Invitrogen, Carlsbad, CA); cells were incubated with 5% CO_2 at 37°C. All cultures were evaluated 7 days after plating to ensure a comparable basis for measurement regardless of treatment time or differentiation state. The medium was changed 24 hours after plating and at intervals of 48 hours thereafter. For studies in the undifferentiated state, OPs were introduced after 5 days in culture, 50 μM CPF, DZN or PRT (Chem Service, West Chester, PA), and cells were then examined after 2 days of exposure. Because of their poor water solubility, the toxicants were dissolved in dimethylsulfoxide (Sigma), achieving a final concentration of 0.1% in the culture medium. The control cultures also included the vehicle, which has no effect on replication or differentiation of PC12 cells [133,135,176].

For studies in differentiating cells with 2 days of exposure to the OPs, the cells were cultured in the undifferentiated state for 5 days, as already described. At that point, the medium was changed to include 50 ng/ml of 2.5 S murine NGF (Invitrogen) along with the test agents and culturing continued for the ensuing 2 days. For treatment effects

after 6 days of exposure, NGF and test agents were added 24 hours after plating, with subsequent media changes at 2 day intervals, including replacement of the test agents. In some experiments, toxicant exposure was continued throughout the 6 day period, whereas in others, exposure was limited to the initial 2 day period, followed by 4 days in culture without the toxicants. We chose the 50 μM test concentration because it elicits robust oxidative stress, inhibition of DNA synthesis and interference with cell acquisition, without producing outright cytotoxicity [40,42,83,133-135,156,176]. Each culture was examined under a microscope to verify the outgrowth of neurites after NGF treatment.

DNA and protein assays. For determinations of DNA content and membrane protein, the medium was aspirated and the culture was rinsed with a buffer consisting of 154 mM NaCl and 10 mM sodium phosphate (pH 7.4). Cells were harvested in ice-cold buffer, homogenized (Polytron, Brinkmann Instruments, Westbury, NY) and an aliquot was withdrawn for measurements of DNA [186]. The cell membrane fraction was prepared by sedimentation at 40,000 × *g* for 15 min. The pellets were washed twice and then resuspended in 250 mM sucrose, 2 mM $MgCl_2$, and 50 mM Tris and aliquots were withdrawn for determination of membrane protein [172] and for AC assays.

Adenylyl cyclase activity. The assay procedures and stimulant concentration profiles have been described in detail previously [13,110,155,204]. Aliquots of the 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 $MgCl_2$, 10 μM GTP, 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 (all reagents from Sigma). The enzymatic reaction was stopped by heating and sedimentation, and the supernatant solution was then assayed for cAMP using commercial immunoassay kits (GE Healthcare Biosciences, Piscataway, NJ). In addition to assessing basal AC activity, we evaluated responses to 10 mM NaF, 100 μM forskolin and 10 mM $MnCl_2$ (all reagents from Sigma). These concentrations produce maximal responses to each stimulant as assessed in earlier studies [13,204,206]. Activities were determined as the amount of cAMP formed per minute per mg of membrane protein.

Data analysis. Handling of data and the presentation of results are presented in detail in Chapter 2. Only analysis that varies from the previous chapters follows: All studies were performed in multiple batches of cells, with several independent cultures for each treatment in each batch. For reference, the normalized control values are shown in Figure 28; however, statistically significant differences for each study were computed by comparing treated groups only to the contemporaneous control group.

RESULTS

In control cells, NGF treatment elicited the expected switch from cell replication to neurodifferentiation, as evidenced by significantly lower numbers of cells (Figure 28A) and greater membrane surface area (Figure 28B) as compared to undifferentiated cells cultured for the same amount of time. NGF also elicited a significant overall reduction in AC activities relative to membrane protein ($p < 0.0001$ for the main effect of NGF), with selective effects on the responses to the various AC stimulants ($p < 0.0001$ for the interaction of NGF × stimulant measure). For basal activity (Figure 28C), the net reduction was completed within 2 days of NGF treatment and showed no further

reduction by the 6 day time point. In contrast, the response to fluoride showed a progressive loss over time (Figure 28D). The decline in responses to forskolin (Figure 28E) and Mn^{2+} (Figure 28F) resembled that of basal activity, with a complete effect evident after 2 days of NGF; further, with the onset of differentiation, there was a small but significant decline in the Mn^{2+}/forskolin response ratio (Figure 28G). The patterns for basal AC and the forskolin and Mn^{2+}-mediated responses were thus entirely distinct from the changes in cell number and membrane protein/DNA ratio, which showed distinct progression between 2 and 6 days of NGF exposure. Differentiation also altered the relative response to each of the stimulants. For fluoride, the increase over basal activity was 8-fold in undifferentiated cells, rising to 13-fold after NGF treatment; for forskolin the increase was from 25-fold to nearly 40-fold, and for Mn^{2+} the values were 12-fold and 18-fold, respectively.

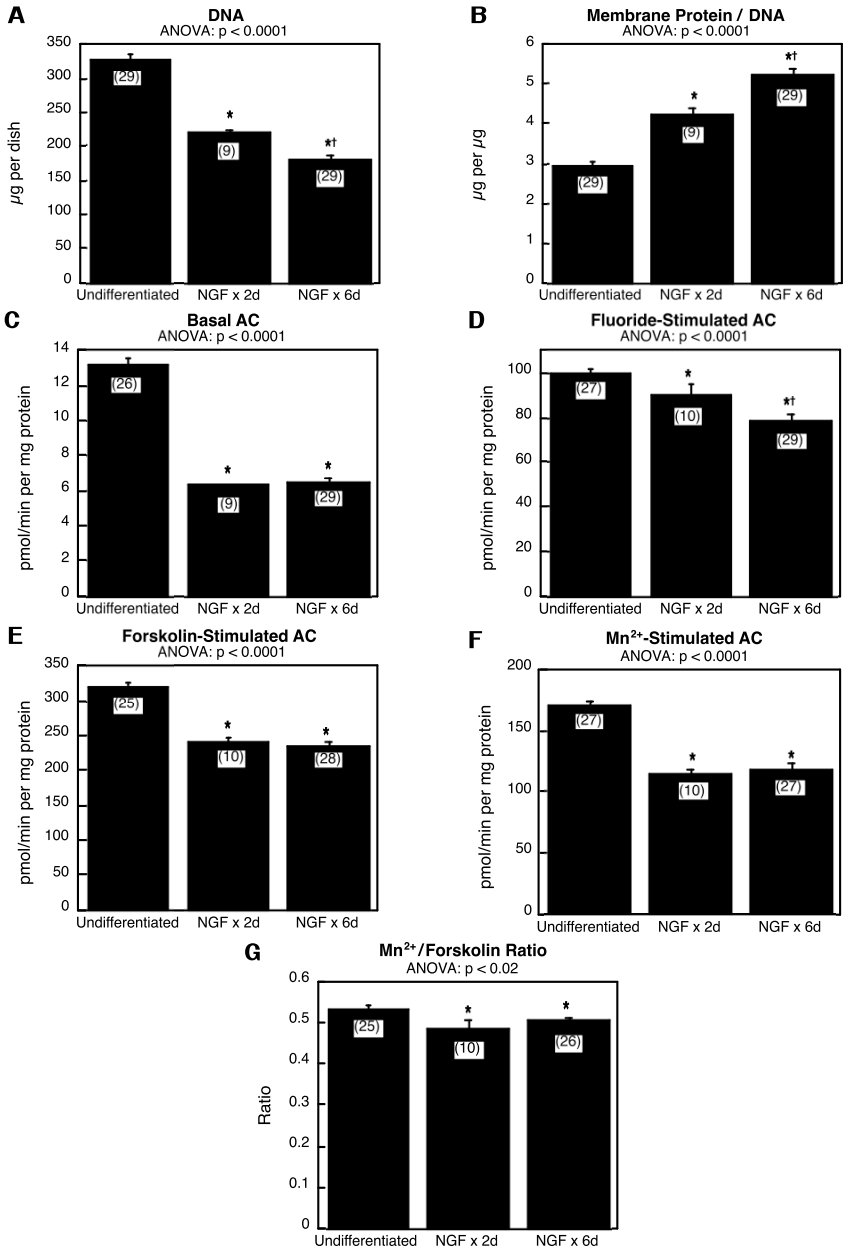

Figure 28: Effects of NGF on parameters of cell growth and AC signaling: (A) DNA, (B) membrane protein/DNA ratio, (C) basal AC, (D) fluoride-stimulated AC, (E) forskolin-stimulated AC, (F) Mn^{2+}-stimulated AC, and (G) Mn^{2+}/forskolin ratio. Cells were cultured for a total of 7 days. NGF was introduced after either 5 days in culture (2 days of NGF treatment, NGF × 2d) or after 1 day in culture (6 days of NGF treatment, NGF × 6d). Data represent means and standard errors of the number of determinations shown in parentheses. ANOVA appears above each panel; asterisks denote values for differentiating cells that differ from the undifferentiated state and daggers denote differences between 2 days and 6 days of NGF exposure. The values shown here were normalized and pooled from the control groups across all experiments; however OP treatment effects in the remaining figures were assessed against only the matched contemporaneous controls for each study.

In undifferentiated cells, a 2 day exposure to CPF or PRT had little or no effect on DNA content but DZN produced a significant, albeit small, decrement (Figure 29A).

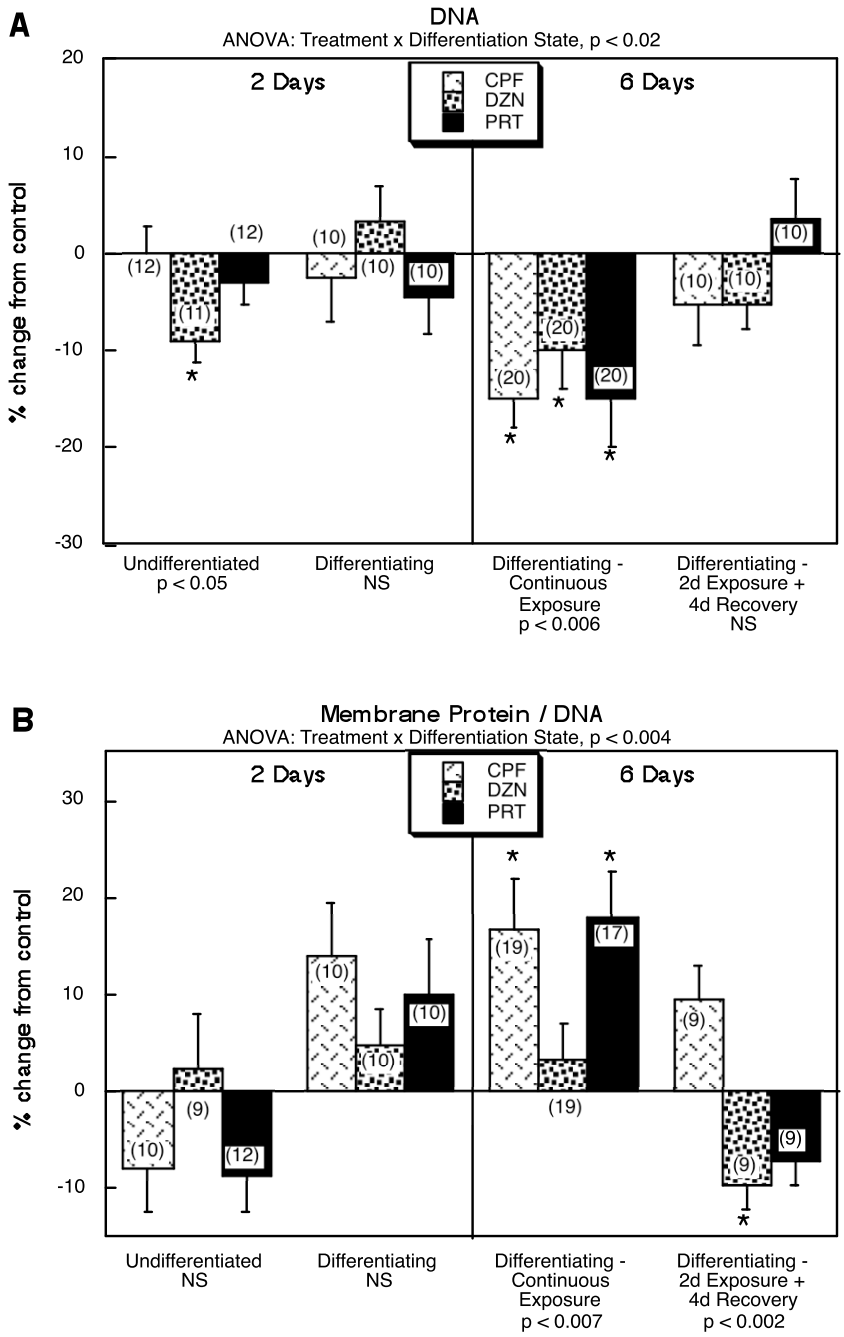

Figure 29: Effects of OP exposure on cell growth parameters: (A) DNA, (B) membrane protein/DNA ratio. Data represent means and standard errors of the number of determinations

shown in parentheses, given as the percentage change from control values. ANOVA appears above each panel and lower-order tests for each differentiation state appear at the bottom. Asterisks denote individual values that differ significantly from the corresponding control. Abbreviation: NS, not significant.

In differentiating cells, the 2 day OP treatment had no discernible effect on DNA but extending the exposure to 6 days produced a significant decline with all three agents. In contrast, when OP exposure was limited to the first 2 days of differentiation, followed by a 4 day recovery period, there were no DNA deficits. For the membrane protein/DNA ratio, undifferentiated cells showed no significant effects after a 2 day OP exposure (Figure 29B). Differentiating cells showed a trend toward increases at 2 days that became statistically significant for CPF and PRT after 6 days. Again, limiting the exposure of differentiating cells to the first 2 days, followed by a 4 day recovery period, completely obtunded the increases and instead, there was a slight but significant decline seen for DZN.

Exposure of undifferentiated cells to the three OPs for a period of 2 days did not have any statistically significant overall effects on AC signaling parameters (Figure 30A) but in differentiating cells there was a robust suppression of activity regardless of stimulant condition, an effect that was statistically significant overall as well as individually for CPF, DZN and PRT (Figure 30B). With continued exposure of differentiating cells for 6 days, there was a complete reversal of the inhibitory effect of the OPs on AC signaling parameters (Figure 30C). Indeed, the response to forskolin became significantly elevated for all three agents, and similar but nonsignificant trends were present for fluoride and Mn^{2+}; the nonsignificant increases for the latter two were statistically indistinguishable from the significant increase in the forskolin response.

Importantly, the same effect was noted in cells that had been exposed for only the first 2 days of differentiation and that were then carried out for an additional 4 days in the absence of the OPs. The OP exposure in differentiating cells also reduced the

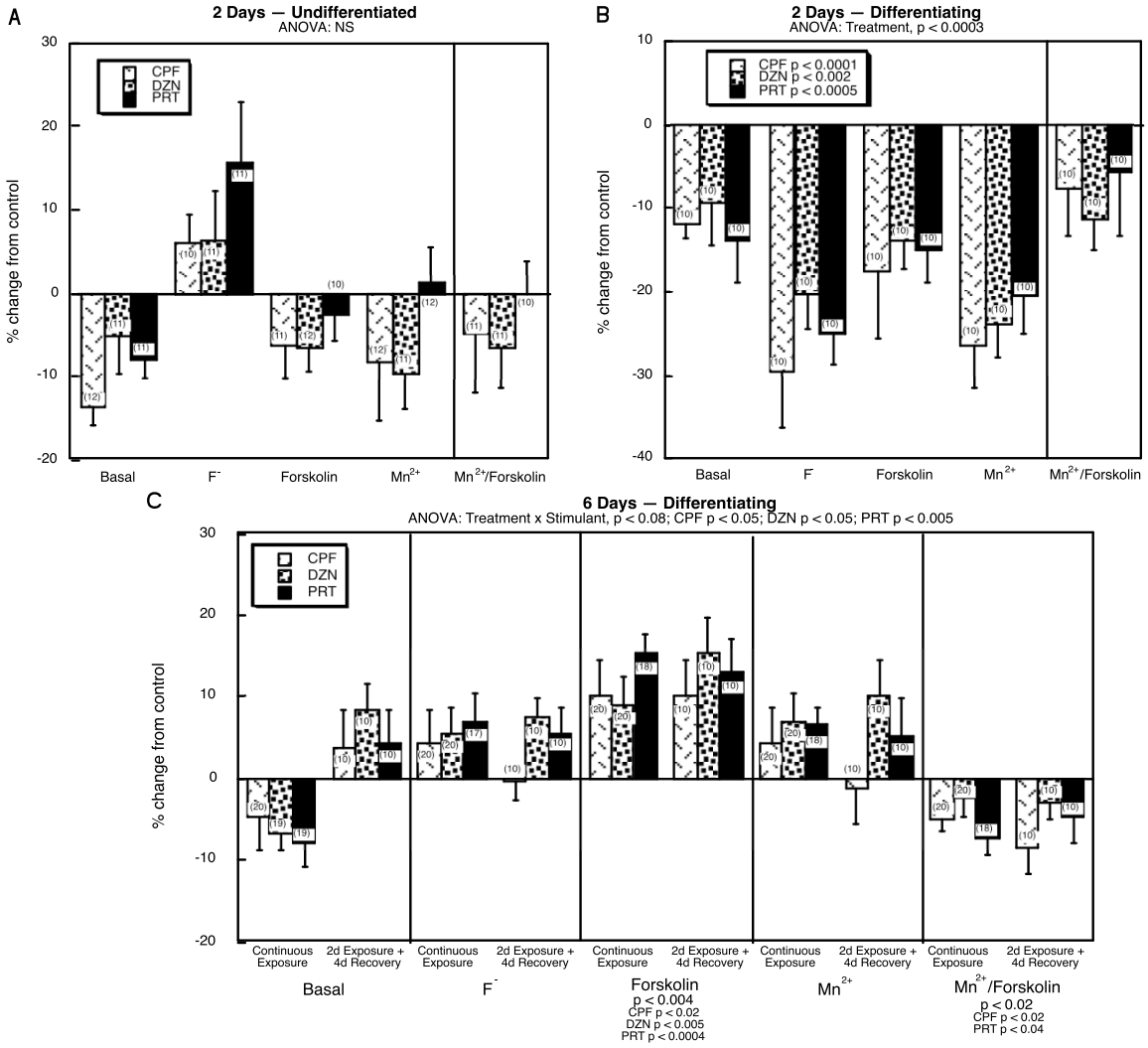
A
2 Days — Undifferentiated
ANOVA: NS
CPF
DZN
PRT
% change from control
Basal
F-
Forskolin
Mn2+
Mn2+/Forskolin
B
2 Days — Differentiating
ANOVA: Treatment, p < 0.0003
CPF p < 0.0001
DZN p < 0.002
PRT p < 0.0005
C
6 Days — Differentiating
ANOVA: Treatment x Stimulant, p < 0.08; CPF p < 0.05; DZN p < 0.05; PRT p < 0.005
Continuous Exposure
2d Exposure + 4d Recovery
Forskolin
p < 0.004
CPF p < 0.02
DZN p < 0.005
PRT p < 0.0004
Mn2+/Forskolin
p < 0.02
CPF p < 0.02
PRT p < 0.04
D
Concordance for Stimulant Responses at 6 Days
r = 0.90, p < 0.0001
2d Exposure + 4d Recovery
6d Continuous Exposure

Figure 30: Effects of OP exposure on AC activities (figure on preceding page): (A) undifferentiated cells with a 2 day exposure, (B) differentiating cells with a 2 day exposure, (C) differentiating cells with a 6 day continuous exposure or with a 2 day exposure followed by a 4 day recovery period. Data represent means and standard errors of the number of determinations shown in parentheses, given as the percentage change from control values. A global ANOVA incorporating all variables and dependent measures in a single test identified a main treatment effect ($p < 0.02$) and interactions of treatment × differentiation state ($p < 0.0007$) and treatment × state × stimulant ($p < 0.03$), necessitating subdivision into the individual differentiation states (A,B,C). Accordingly, separate ANOVAs for each differentiation state appear above the panels. Lower order tests were not carried out for (A) and (B) because of the absence of treatment interactions with other variables; in (B), the main effects for each OP are shown within the legend box. In (C), the treatment × stimulant interaction necessitated separate examinations for each stimulant condition and the corresponding ANOVAs are shown below the panel; main effects for each OP are shown without conducting separate tests for each exposure regimen because of the absence of a treatment × regimen interaction. Panel (D) shows the concordance (least squares fit and linear correlation coefficient) between OP effects on stimulant responses (fluoride, forskolin, Mn^{2+}, Mn^{2+}/forskolin) for differentiating cells with 6 days of continuous OP exposure vs. 2 days of exposure + 4 days of recovery. Abbreviation: NS, not significant.

Mn^{2+}/forskolin response ratio. The effect on the ratio was statistically significant for either of the 6 day regimens but not for the 2 day exposure; however, the effect at 2 days was in the same direction as at 6 days and was not statistically distinguishable from the significant reduction at the later time points, and in fact, a comparison of all three regimens for differentiating cells identified only a main treatment effect ($p < 0.04$) without a treatment × regimen interaction.

To reinforce the similarities in the patterns of OP effects on AC stimulant responses seen with 6 days of continuous exposure and with 2 days of exposure + 4 days of recovery, we replotted the data to examine the correlation between the two regimens and found high concordance (Figure 30D); thus, the similar outcomes did not depend solely on the significant difference in the forskolin response but rather was reflected in the overall pattern for all the AC stimulants.

DISCUSSION

The results obtained in this study indicate that OP exposure reprograms the AC signaling pathway during a discrete developmental stage at the commencement of neurodifferentiation, with effects that continue to emerge after OP exposure is discontinued, actions that are distinct from the impact on cell growth. In control cells, we noted that the first two days after the start of NGF-induced neurodifferentiation had a critical effect on AC activity profiles. Although there was a drop in total activity, stimulants acting at the level of G-proteins or AC itself then elicited larger proportional increases over basal activity than in the undifferentiated state. Further, differentiation produced a drop in the Mn^{2+}/forskolin response ratio, indicative of a shift in AC isoform

expression [205]. Over the ensuing four days, most of the effects of NGF on AC signaling leveled off, whereas those on cell growth parameters (DNA, membrane protein/DNA) showed a clear progression over time. This suggested to us that the initial stages of neurodifferentiation might indeed be especially sensitive for disruption of AC signaling by OPs and we designed our studies to test that hypothesis.

If OPs act on AC signaling during a specific stage of neurodifferentiation, we would expect to see a distinct difference in the effects on undifferentiated PC12 cells as compared to those exposed to NGF. After a 2 day exposure to CPF, DZN or PRT, there were no significant changes in AC signaling in the undifferentiated state but there was a robust, global decrement in the differentiating cells. This resembles our earlier finding of overall decreases in AC activities in differentiating PC12 cells with a more prolonged exposure to lower concentrations of CPF [155]. More importantly, though, the same effects are seen in the developing brain after CPF exposure in neonatal rats [175], reinforcing the view that the effects of OPs on AC signaling are mediated directly on the differentiating neurons themselves. These conclusions are bolstered by studies at the transcriptional level, which show a relative lack of concordance between undifferentiated and differentiating cells in the impact of CPF on the mRNAs encoding AC isoforms and G-proteins [6].

Given the OP-induced reductions in AC signaling in differentiating cells at the 2-day point, we were surprised to find that continuing the exposure to 6 days resulted not only in disappearance of the deficits, but actual increases in responses; again, in contrast to the findings for AC, the more general indices of cell numbers and neurite formation

showed progressively greater effects of the OPs at 6 days than at 2 days. The findings thus indicate either that the cells adapt to the continued presence of the OPs, or alternatively, that exposure during the critical first 2 days of neurodifferentiation reprograms the function of the pathway. To distinguish between these two possibilities, we exposed the cells to the OPs for the first 2 days and then discontinued exposure for the ensuing 4 days. If the biphasic response represents an adaptation to continuous OP exposure, then at the end of the 4 day recovery period, we would expect to see either a small residual deficit or restoration of normal AC activities. If on the other hand, the elevations seen after 6 days of continuous exposure represented a reprogramming elicited by exposure in the first 2 days, then the pattern at the end of the recovery period should be indistinguishable from that seen with continuous exposure. In fact, the latter turned out to be true: the entire pattern of initial AC deficits, subsequent elevations and a decrement in the Mn^{2+}/forskolin ratio was recapitulated with just an initial 2 day exposure conducted at the onset of neurodifferentiation. Indeed, these findings with an *in vitro* model reproduce exactly the pattern seen for exposure of neonatal rats to OPs, namely initial deficits in AC signaling, followed by persistent upregulation and supersensitivity emerging well after the end of OP exposure [7,111]; indeed the *in vivo* outcomes are similar for AC signaling in peripheral tissues [7,113], reinforcing the concept of a direct effect of early OP exposure that reprograms the function of this signal transduction pathway, unrelated to cytotoxicity or generalized actions on cell growth.

Separable from their impact on AC signaling, the OPs showed selective effects on indices of cell growth that are of additional interest. Given their shared antimitotic and

proapoptotic properties [147,156,160,202,203], it was not surprising that all three OPs elicited a decline in the number of cells after 6 days of exposure, as evidenced by a deficit in the DNA content. Unlike the AC effects, the cell loss required the continuous presence of the OPs over the 6 day span: when we limited exposure to the first 2 days and then allowed 4 days for recovery, cell loss was no longer evident. This points out that the sensitivity of the AC pathway to reprogramming by OP exposure is far more sensitive than are general effects on cell acquisition or loss. The membrane protein/DNA ratio displayed a dichotomy, with a significant increase evident for CPF and PRT but not DZN. These findings support earlier conclusions about differential effects of the various OPs on the formation of neuritic projections. CPF blunts the development of long axons while favoring the creation of short dendritic branches [76]. In contrast, DZN shows greater overall inhibitory effects on neurite outgrowth, whereas PRT is more like CPF [14,154]. Again, though, these growth-related effects of CPF and PRT were dependent on continuous exposure over the 6 day span; interestingly, the 2 day exposure appeared to augment the inhibitory effects of DZN, an unexpected finding that should be followed up with structural measures.

In conclusion, results of this study show that OP exposure during early neurodifferentiation reprograms the development of the AC signaling cascade, with initial deficits replaced by subsequent upregulation resulting in a net gain-of-function. The effects in PC12 cells mimic those seen after OP exposure in neonatal rats both in the brain and peripheral tissues, effects that are thus likely to contribute to neurobehavioral deficits and metabolic dysfunction. Accordingly, the lasting impact of early-life OP

exposure on cell signaling cascades is likely to represent one of the most critical noncholinesterase targets for these common pesticides.

Chapter 7: Conclusions and Implications

The results obtained in this investigation point out four main findings. First, early-life CPF, DZN and PRT exposure produces lasting gain-of-function of the AC pathway in the liver, likely contributing to metabolic dysfunction. Second, CPF, DZN and PRT elicit distinct sex differences as well as disparities in their effects on tissue, indicative of selective actions rather than ubiquitous changes in AC signaling. Third, all OPs do not elicit identical effects, reflecting underlying mechanisms that do not depend on their shared property as cholinesterase inhibitors. Fourth, there are major differences in (direct vs. indirect) programming of the AC signaling pathway at the transcriptional and translational levels.

Although OPs are likely to disrupt metabolism and cardiovascular function at many different levels, one common feature is their ability to produce lasting changes in AC signaling, notably involving a net gain of pathway function, an effect noted for CPF, DZN and PRT [7,113]. In Chapter 3, we saw that neonatal exposures to DZN and PRT altered the developmental trajectory of AC-mediated cell signaling in the heart and liver but the liver appeared to be especially sensitive to persistent gain-of-function via induction of AC itself. The presence of heterologous sensitization of the entire pathway means that any signal operating through AC will show hyperresponsiveness. In turn, these findings provide strong evidence of a likely mechanism for the metabolic consequences of neonatal OP exposure that have been identified in earlier studies, indicative of a prediabetic state [93,150].

There is also a known connection between early-life glucocorticoid exposure and later emergence of similar metabolic disorders as stated in Chapter 3 [43,74,120,121,199]. With this in mind, we used the DEX experiments in Chapter 2 to determine whether changes in AC signaling influence the susceptibility to metabolic/cardiovascular dysfunction in the same manner as our OP experiments in Chapter 3. The results for the hepatic effects of DEX treatment on PN1-3 also provided a clear example of heterologous sensitization of the AC pathway. Here, at each of the three different doses, we found significant increases in AC regardless of whether the activity was assessed in the basal state, in response to receptor stimulants or G-protein activation, or with direct AC activation by forskolin. Again, the parallel changes point to gain-of-function of AC cell signaling. Accordingly, there is an augmented response to activation of βARs or glucagon receptors, which together provide major inputs to glucose and lipid metabolism, and point to possible metabolic dysfunction outcomes.

The results of Chapters 2 and 3 are consistent with earlier work with developmental exposure to CPF [13,113] and with the functional consequence of prediabetes [93,150], the same outcome as found for AC polymorphisms in humans [119]. Therefore, our results reveal that OP exposures that are devoid of signs of intoxication and that are insufficient to elicit growth impairment, still may lead to lasting alterations in hepatic cell signaling that are likely to contribute to metabolic dysfunction.

We have also learned that the early-life OP exposure regimens we implemented showed distinct selectivity for both sex and tissue. These findings clearly indicate selective actions rather than ubiquitous changes in AC signaling. In Chapter 3, the

effects on the liver were more substantial than those on the heart, which displayed only transient effects of DZN on AC function in adolescence, and no significant effects of PRT. Furthermore, the hepatic effects were greater in magnitude than those in the cerebellum, a brain region that shares similar AC cascade elements. It is also important to note that earlier work with CPF showed persistent, global increases in all measures of hepatic AC signaling, an effect restricted to males [113]. As found here, neonatal DZN exposure produced a similar effect that appeared in adolescence and young adulthood at the higher dose but that also became significant at the lower dose by full adulthood. Notably, although the effect emerged first in males, it eventually encompassed both sexes, thus differing in outcome from the sex-specific effects seen for CPF [113]. In contrast to DZN, neonatal PRT exposure produced a much larger initial sensitization of hepatic AC in adolescence, with significant increases at both doses and in both males and females. The clear implication is that neonatal OP exposure is likely to affect hepatic responses to a greater extent than those in the heart or in the central nervous system, and it is therefore critical to examine how the cellular changes seen here might then contribute to alterations in tissue function. In the liver, where βARs and glucagon receptors are linked through AC to enhanced gluconeogenesis and lipolysis, heterologous sensitization of AC signaling may lead to corresponding metabolic abnormalities.

The specific mechanisms underlying the targeting of males or females have not been thoroughly investigated. Similar to the present findings, many other developmental disruptors also produce sexually dimorphic changes in cardiovascular and/or metabolic outcomes in males and females, both in animal studies and in humans [5,85,121]. For

example, DEX exposure of rats *in utero* has been shown to produce post-glucose hyperglycemia and hyperinsulinemia in males in adulthood, while in females it produces hypertension. Notably, the OPs investigated in this study have been found to be weakly estrogenic [11,190], but certainly, secondary endocrine effects are certainly plausible [70]. Clearly sex differences need to be taken into account in future evaluations of similar outcomes from developmental OP exposure.

This study also has implications for the significant, although lesser effects of DZN and PRT exposure on AC signaling in the heart. Transgenic animals that produce AC hyperstimulation through overexpression of βARs or G_s show development of cardiomyopathies and abnormal heart rate regulation [60,81,188]. In Chapter 3, alterations in m_2AChR expression, which we detected for PRT, also have corresponding effects on cardiac function and the response to autonomic input [90]. Importantly, the fact that DZN reduced heart weight significantly and to a greater extent than body weight indicates the need to pursue potential consequences for cardiac function, which have been much less studied than hepatic function. Indeed, the prediabetic changes seen after neonatal OP exposure are themselves likely to contribute to further cardiovascular morbidities.

Although the systemic toxicity and signs of OP intoxication reflect their shared ability to inhibit cholinesterase [114,130], recent data—including data from this study—indicate that lower-level OP exposures which are below the threshold for anticholinesterase actions, can disrupt cell-signaling cascades that control cell differentiation and other critical regulatory functions [68,145-147]. Therefore, since cell

signaling is targeted by OP mechanisms other than their shared property as cholinesterase inhibitors, there is no reason to assume that all OPs will act in the same way. In recent studies, it is important to note that consequent metabolic dysfunction resembles prediabetes while different OPs vary in their specific pattern of effects [92-94,150]. For example, with CPF, serum glucose is maintained within normal limits, but only because of insulin hypersecretion, and the animals display hyperlipidemia, leptin dysregulation and excessive weight gain [92,150]. However, PRT produces hyperglycemia, loss of lipid homeostasis and adipose inflammation, all without incurring hyperinsulinemia [93,94]. These differences are also reflected in disparities in OP effects on hepatic and cardiac AC signaling. Whereas sensitization of the pathway is maintained throughout the lifespan with CPF and DZN, we have shown that the effects of PRT are prominent only through adolescence and then wane in young adulthood [7,113]. In Chapter 4, we explored whether PRT's effects might emerge even later, in full adulthood, but still did not find the global upregulation of the AC pathway that we saw for CPF and DZN. However, PRT exposure elicits nonmonotonic effects in keeping with the effects on body weight [94], and thus implies that the metabolic consequences of early-life PRT exposure are distinctly different once the levels exceed the threshold for cholinesterase inhibition and the emergence of systemic toxicity.

The addition of a high-fat diet in adulthood did not unmask persistent effects of neonatal PRT exposure on AC signaling. The diet by itself affected the AC pathway in both liver and heart. The convergent effects of PRT and elevated dietary fat on the same signaling cascade may thus elicit greater net hepatic and cardiac dysfunction when these

two challenges are combined. In that fashion, early OP exposure, separately or in combination with dietary factors, may contribute to the worldwide increase in the incidence of obesity and diabetes. Nevertheless, it looks like we may have chosen the wrong OP for these studies. Future work may find the investigation of DZN or CPF more productive to look at than PRT. Again, it is important to note that sensitization of the AC pathway is maintained throughout the lifespan with CPF and DZN while the effects of PRT are prominent only through adolescence and then wane in young adulthood [7,113]. Furthermore, our previous studies have shown that early-life CPF exposure results in metabolic dysfunction that resembles prediabetes. Additionally, DZN and PRT share a common response to a high fat diet, in that animals exposed to either agent gain more weight than do controls [94,138].

The study of direct and indirect programming, at the transcriptional and translational levels, proved to be of extreme importance. We hypothesized that our results for OP effects on AC signaling could reflect either direct effects on the expression or function of proteins in the AC pathway, or could result through indirect effects mediated through any of the myriad changes evoked in whole animal physiology by the early-life OP exposure. In Chapter 5 we first set out to determine the extent to which related and unrelated developmental neurotoxicants alter cAMP signaling through direct effects on activation or repression of genes encoding the key elements of the pathway. We used PC12 cells because they are a well-characterized neurodevelopmental model [182] that reproduces many of the key mechanisms and features of the adverse effects of OPs *in vivo* [15,16,39,40,42,52,83,98,117,133,134,145-147,176,187,201]. Furthermore,

with the addition of NGF, PC12 cells begin to differentiate, forming neuritic projections and acquiring electrical excitability and neuronal phenotypes [56,176,182]. Notably, there are problems with hepatic cell lines and their ability to dedifferentiate. This characteristic of hepatic cell lines would not provide an opportunity for our experiments to follow a cell differentiation time course—which proved to be important in this study. Here, PC12 cells work because there are lasting effects of CPF, DZN, and PRT in the central nervous system.

CPF and DZN were surprisingly the least alike, whereas there was strong concordance of dieldrin and Ni^{2+} with each other and with each individual OP. Interestingly, the effects of CPF differed substantially depending on whether cells were undifferentiated or differentiating. This finding points to unique features of CPF in its effects on the AC pathway and is consistent with the existence of critical periods of vulnerability of developing neurons to OPs. Thus, although OP effects can be seen at neurodevelopmental stages ranging from cell replication through the final stages of neurodifferentiation, the period surrounding the transition from replication to differentiation appears to be one of the most sensitive phases [83,145-147,156,161,166,167,176]. To resolve the disparities between CPF and DZN, we performed analyses in rat brain regions after *in vivo* neonatal exposures; unlike the *in vitro* results, there was strong concordance. The strong *in vivo* relationship can be seen by comparing the concordance of all the cAMP pathway genes in a single correlation: whereas there was no significant overall correlation for the *in vitro* evaluations, the same comparisons were highly concordant for *in vivo* exposures. Thus, in the intact brain, CPF

and DZN do actually produce similar effects on the cAMP-related genes, but the common outcome reflects indirect actions requiring the intact brain, rather than depending upon direct effects on differentiating neural cells, as seen with the PC12 cell model. Clearly, although *in vitro* models permit identification of direct effects of neurotoxicants on neurodifferentiation, a full picture of the similarities and differences of neurotoxicant effects directed toward a given pathway will require additional study of the effects in the intact organism.

Our results for DZN, dieldrin and divalent nickel show that unrelated developmental neurotoxicants can nevertheless produce similar outcomes by targeting cell signaling pathways involved in neurodifferentiation during a critical developmental period of vulnerability. Therefore, toxicants may all lead to similar neurodevelopmental outcomes despite the underlying differences in chemical properties and classification. Importantly, toxicant effects on signaling pathways are a new concept in developmental toxicity and could allow for new groupings of toxicants. For example, one pathway that has received recent attention is the protein kinase C cascade. Protein kinase C is a cell-signaling cascade that integrates the actions of neurotrophic factors involved in brain development. Our lab compared two OP insecticides with an organochlorine and a metal for the similarities and differences in their effects on gene expression encoding subtypes of protein kinase C and their modulators. Evaluations were performed in PC12 cells, with each agent introduced for 24 or 72 hours. We found that the protein kinase C cascade contributes to the cholinesterase-independent developmental neurotoxicant actions of CPF and its differences from DZN. In addition, the effects of DZN, dieldrin

and Ni^{2+} showed basic similarities despite the fact that three come from different classes of toxicants. Therefore, this pathway has helped identify mechanistic convergence between otherwise unrelated toxicants that provides predictions about common neurodevelopmental outcomes [163]. Nevertheless, a full evaluation of the concordance between different toxicants requires not just evaluations of *in vitro* systems that detect direct effects (as done here), but also *in vivo* systems that allow for more complex interactions that converge on the same pathway.

A key limitation of the microarray approach is that the measured effects occur at the mRNA level, which does not necessarily entail a corresponding change at the level of protein expression or cell function. Therefore, the disparities between CPF and DZN that were seen in Chapter 5 may have been more apparent than real. For example, the OPs might have still shown the same affect at the protein/function level even if their effects on transcription differ. With this in mind, we then investigated how the mRNA (transcriptional) findings of Chapter 5 might tie into function at the level of protein (translation) in Chapter 6. The findings of Chapter 6 revealed that OP exposures during early neurodifferentiation reprogram the development of the AC signaling cascade, with initial deficits replaced by subsequent upregulation resulting in a net gain-of-function. The effects in PC12 cells mimic those seen after OP exposure in neonatal rats both in the brain [175] and peripheral tissues [7,113], effects that are thus likely to contribute to neurobehavioral deficits as well as metabolic dysfunction. Interestingly, the results of Chapter 6 showed that *in vitro* cell-culture testing of CPF, DZN, and PRT yielded corresponding changes at the level of protein expression or cellular function of the cAMP

cascade. These results differed from those of our *in vitro* mRNA studies. Our mRNA work showed that CPF and DZN were unrelated in altering the cAMP signaling through direct effects on activation or repression of genes encoding the key elements of the pathway. Therefore, the aforementioned findings point to post-transcriptional events as perhaps more important. Overall, the results of Chapters 5 and 6 highlight the importance of comparing *in vitro* and *in vivo* findings that can guide future examinations to test a hypothesis. Equally important, we have shown the need for combined use of cell culture systems, animal models, and microarrays to guide future studies toward specific endpoints.

There are some additional studies that could be included with this investigation, and furthermore, would provide answers to questions that were discovered as the study progressed. This study would have benefitted from additional experiments that included PRT in our gene expression study. It would be interesting to see if PRT elicits the same *in vitro* and *in vivo* effects that we saw between CPF and DZN at the level of mRNA. Furthermore, we would also learn how PRTs gene expression ties into function at the protein level. These additional data would provide a more complete picture of how all three OPs affect the AC pathway and likely contribute to metabolic dysfunction. In addition, in Chapter 3, the effects of PRT on hepatic AC signaling were restricted to adolescence, and unlike those of CPF, did not persist into adulthood. Interestingly, both OPs still elicit metabolic changes related to prediabetes. Thus, for PRT, either the AC changes are unrelated to metabolic disorders, or alternatively, the effects in adolescence may be sufficient to reprogram metabolism so that defects will emerge later, despite

subsequent normalization of signaling parameters. In this case, future work will need to dissect the temporal emergence of prediabetes after neonatal PRT exposure. For example, we would gain a further understanding of the temporal emergence of prediabetes from neonatal PRT exposure with prediabetic marker testing at adolescence (PN30), young adulthood (PN60), and adulthood (PN100). This study could include markers that have proven to be beneficial in our lab's assessment of metabolic function, such as, serum glucose, serum insulin, cholesterol, β-hydroxybutyrate, nonesterified fatty acids, triglycerides, and glycated hemoglobin. Again, we also proved that the addition of a high-fat diet in adulthood does not unmask persistent effects of neonatal PRT exposure on AC signaling, however, the question remains: How does early-life exposure to PRT affect AC signaling with the introduction of a high-fat diet when the dietary change is begun in juvenile stages as opposed to adulthood? This regimen would reflect a more realistic scenario for human dietary preference. Therefore, it is evident that early-life exposure to PRT in conjunction with high-fat implementation commencing at weaning may change weight and/or metabolic responses differently from that of an adult. This may lead to exacerbated weight gains and possibly a metabolic profile that surpasses prediabetes and reflects the more drastic consequence of diabetes. This type of scenario may prove to be pivotal for providing a deeper understanding of glucose and lipid metabolism-related variables that change in metabolism for these particular physiologic and pathophysiologic circumstances.

In conclusion, the results of this study show that early-life OP exposure produces lasting AC pathway gain-of-function in the liver. Importantly, these findings indicate

that OPs alter the trajectory of hepatic cell signaling in a manner consistent with the observed emergence of prediabetes-like metabolic dysfunction, and with a greater effect than in heart or brain. Because the effects occur at low exposures and are unrelated to cholinesterase inhibition, the various OPs differ in their net impact on peripheral AC signaling, and in a manner divergent from their effects on neurobehavioral outcomes. Our findings thus extend the Barker Hypothesis, which originally related prenatal growth restriction to the subsequent development of cardiovascular disease and diabetes [21]; we now include otherwise nonsymptomatic chemical exposures that may produce similar outcomes without the precondition of fetal/neonatal growth restriction. These findings point out the need to explore the possibility that developmental exposure to common chemical contaminants contribute to the explosive worldwide increase in diabetes and obesity. The standard view of OPs as developmental toxicants that specifically target the nervous system may thus require a paradigm change.

References

[1] S.M. Abdel-Halim, A. Guenifi, A. Khan, O. Larsson, P.O. Berggren, C.G. Ostenson, S. Efendic, Impaired coupling of glucose signal to the exocytotic machinery in diabetic GK rats: a defect ameliorated by cAMP, Diabetes 45 (1996) 934-40.

[2] M. Abdollahi, M. Donyavi, S. Pournourmohammadi, M. Saadat, Hyperglycemia associated with increased hepatic glycogen phosphorylase and phosphoenolpyruvate carboxykinase in rats following subchronic exposure to malathion, Comparative Biochemistry and Physiology Toxicology and Pharmacology 137 (2004) 343-347.

[3] M.B. Abou-Donia, Organophosphorus ester-induced chronic neurotoxicity, Archives of Environmental Health 58 (2003) 484-497.

[4] Y. Abreu-Villaça, F.J. Seidler, D. Qiao, T.A. Slotkin, Modeling the developmental neurotoxicity of nicotine in vitro: cell acquisition, growth and viability in PC12 cells, Developmental Brain Research 154 (2005) 239-246.

[5] L.S. Adair, T.J. Cole, Rapid child growth raises blood pressure in adolescent boys who were thin at birth, Hypertension 41 (2003) 451–456.

[6] A.A. Adigun, F.J. Seidler, T.A. Slotkin, Disparate developmental neurotoxicants converge on the cyclic AMP signaling cascade, revealed by transcriptional profiles in vitro and in vivo, Brain Res 1316 (2010) 1-16.

[7] A.A. Adigun, N. Wrench, F.J. Seidler, T.A. Slotkin, Neonatal organophosphorus pesticide exposure alters the developmental trajectory of cell signaling cascades controlling metabolism: differential effects of diazinon and parathion, Environmental Health Perspectives 118 (2010) 210-215.

[8] A. Al Mamun, D.A. Lawlor, R. Alati, M.J. O'Callaghan, G.M. Williams, J.M. Najman, Does maternal smoking during pregnancy have a direct effect on future offspring obesity? Evidence from a prospective birth cohort study, American Journal of Epidemiology 164 (2006) 317-325.

[9] J.E. Aldridge, E.D. Levin, F.J. Seidler, T.A. Slotkin, Developmental exposure of rats to chlorpyrifos leads to behavioral alterations in adulthood, involving serotonergic mechanisms and resembling animal models of depression, Environmental Health Perspectives 113 (2005) 527-531.

[10] J.E. Aldridge, F.J. Seidler, T.A. Slotkin, Developmental exposure to chlorpyrifos elicits sex-selective alterations of serotonergic synaptic function in adulthood: critical periods and regional selectivity for effects on the serotonin transporter,

receptor subtypes, and cell signaling, Environmental Health Perspectives 112 (2004) 148-155.

[11] H.R. Andersen, A.M. Vinggaard, T.H. Rasmussen, I.M. Gjermandsen, E.C. Bonefeld-Jorgensen, Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity *in vitro*, Toxicology and Applied Pharmacology 179 (2002) 1-12.

[12] N. Anousis, R.A. Carvalho, P. Zhao, C.R. Malloy, A.D. Sherry, Compartmentation of glycolysis and glycogenolysis in the perfused rat heart, NMR Biomed (2004) 51-59.

[13] J.T. Auman, F.J. Seidler, T.A. Slotkin, Neonatal chlorpyrifos exposure targets multiple proteins governing the hepatic adenylyl cyclase signaling cascade: implications for neurotoxicity, Developmental Brain Research 121 (2000) 19-27.

[14] J.C. Axelrad, C.V. Howard, W.G. McLean, The effects of acute pesticide exposure on neuroblastoma cells chronically exposed to diazinon, Toxicology 185 (2003) 67-78.

[15] D. Bagchi, M. Bagchi, E.A. Hassoun, S.J. Stohs, In vitro and in vivo generation of reactive oxygen species, DNA damage and lactate dehydrogenase leakage by selected pesticides, Toxicology 104 (1995) 129-140.

[16] D. Bagchi, G. Bhattacharya, S.J. Stohs, In vitro and in vivo induction of heat shock (stress) protein (Hsp) gene expression by selected pesticides, Toxicology 112 (1996) 57-68.

[17] P.L. Ballard, Glucocorticoids and differentiation, Monogr Endocrinol 12 (1979) 493-515.

[18] D.J. Barker, The fetal and infant origins of adult disease, BMJ 301 (1990) 1111.

[19] D.J. Barker, The fetal origins of adult hypertension, J Hypertens Suppl 10 (1992) S39-44.

[20] D.J. Barker, J.G. Eriksson, T. Forsen, C. Osmond, Fetal origins of adult disease: strength of effects and biological basis, Int J Epidemiol 31 (2002) 1235-9.

[21] D.J.P. Barker, The developmental origins of adult disease, European Journal of Epidemiology 18 (2003) 733-736.

[22] D.J.P. Barker, In utero programming of chronic disease, Clinical Science 95 (1998) 115-128.

[23] S. Barone, K.P. Das, T.L. Lassiter, L.D. White, Vulnerable processes of nervous system development: a review of markers and methods, Neurotoxicology 21 (2000) 15-36.

[24] N. Begin-Heick, Liver beta-adrenergic receptors, G proteins, and adenylyl cyclase activity in obesity-diabetes syndromes, Am J Physiol 266 (1994) C1664-72.

[25] N.R. Bhat, G. Shanker, R.A. Pieringer, Cell proliferation in growing cultures of dissociated embryonic mouse brain: macromolecule and ornithine decarboxylase synthesis and regulation by hormones and drugs, Journal of Neuroscience Research 10 (1983) 221-230.

[26] X. Bian, M.M. Briggs, F.H. Schachat, F.J. Seidler, T.A. Slotkin, Glucocorticoids accelerate the ontogenetic transition of cardiac ventricular myosin heavy-chain isoform expression in the rat: promotion by prenatal exposure to a low dose of dexamethasone, Journal of Developmental Physiology 18 (1992) 35-42.

[27] X. Bian, F.J. Seidler, T.A. Slotkin, Fetal dexamethasone exposure interferes with establishment of cardiac noradrenergic innervation and sympathetic activity, Teratology 47 (1993) 109-117.

[28] X.-P. Bian, F.J. Seidler, T.A. Slotkin, Promotional role for glucocorticoids in the development of intracellular signalling: enhanced cardiac and renal adenylate cyclase reactivity to β-adrenergic and non-adrenergic stimuli after low-dose fetal dexamethasone exposure, Journal of Developmental Physiology 17 (1992) 289-297.

[29] M.C. Bohn, Glucocorticoid induced teratologies of the nervous system, in: J. Yanai (Ed.), Neurobehavioral Teratology, Elsevier, Amsterdam, 1984, pp. 365-387.

[30] J. Buck, M.L. Sinclair, L. Schapal, M.J. Cann, L.R. Levin, Cytosolic adenylyl cyclase defines a unique signaling molecule in mammals, Proc Natl Acad Sci U S A 96 (1999) 79-84.

[31] C.G. Campbell, F.J. Seidler, T.A. Slotkin, Chlorpyrifos interferes with cell development in rat brain regions, Brain Research Bulletin 43 (1997) 179-189.

[32] J.E. Casida, G.B. Quistad, Organophosphate toxicology: safety aspects of nonacetylcholinesterase secondary targets, Chem Res Toxicol 17 (2004) 983-998.

[33] G. Celsi, A. Kistner, A.C. Eklof, S. Ceccatelli, R. Aizman, S.H. Jacobson, Inhibition of renal growth by prenatal dexamethasone and the programming of blood pressure in the offspring., Journal of the American Society of Nephrology 8 (1997) A1360-A1360.

[34] A. Chen, M.L. Pennell, M.A. Klebanoff, W.J. Rogan, M.P. Longnecker, Maternal smoking during pregnancy in relation to child overweight: follow-up to age 8 years, International Journal of Epidemiology 35 (2006) 121-30.

[35] W.C. Claycomb, Biochemical aspects of cardiac muscle differentiation, Journal of Biological Chemistry 251 (1976) 6082-6089.

[36] D.J. Clegg, M. van Gemert, Determination of the reference dose for chlorpyrifos: proceedings of an expert panel, Journal of Toxicology & Environmental Health Part A 2 (1999) 211-255.

[37] T.J. Cole, J.A. Blendy, A.P. Monaghan, K. Krieglstein, W. Schmid, A. Aguzzi, G. Fantuzzi, E. Hummler, K. Unsicker, G. Schutz, Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation, Genes Dev 9 (1995) 1608-21.

[38] T.J. Cole, J.A. Blendy, A.P. Monaghan, W. Schmid, A. Aguzzi, G. Schutz, Molecular genetic analysis of glucocorticoid signaling during mouse development, Steroids 60 (1995) 93-6.

[39] T.L. Crumpton, F.J. Seidler, T.A. Slotkin, Developmental neurotoxicity of chlorpyrifos in vivo and in vitro: effects on nuclear transcription factor involved in cell replication and differentiation, Brain Res 857 (2000) 87-98.

[40] T.L. Crumpton, F.J. Seidler, T.A. Slotkin, Is oxidative stress involved in the developmental neurotoxicity of chlorpyrifos?, Developmental Brain Research 121 (2000) 189-195.

[41] O. Dammann, S.G. Matthews, Repeated antenatal glucocorticoid exposure and the developing brain, Pediatric Research 50 (2001) 563-564.

[42] K.P. Das, S. Barone, Neuronal differentiation in PC12 cells is inhibited by chlorpyrifos and its metabolites: is acetylcholinesterase inhibition the site of action?, Toxicology and Applied Pharmacology 160 (1999) 217-230.

[43] M.J. De Blasio, M. Dodic, A.J. Jefferies, K.M. Moritz, E.M. Wintour, J.A. Owens, Maternal exposure to dexamethasone or cortisol in early pregnancy differentially alters insulin secretion and glucose homeostasis in adult male sheep offspring, American Journal of Physiology - Heart & Circulatory Physiology 293 (2007) E75-E82.

[44] A. De Peyster, W.O. Willis, C.A. Molgaard, T.M. MacKendrick, C. Walker, Cholinesterase and self-reported pesticide exposure among pregnant women, Archives of Environmental Health 48 (1993) 348-352.

[45] M. Dodic, T. Abouantoun, A. O'Connor, E.M. Wintour, K.M. Moritz, Programming effects of short prenatal exposure to dexamethasone in sheep, Hypertension 40 (2002) 729-34.

[46] M. Dodic, V. Hantzis, J. Duncan, S. Rees, I. Koukoulas, K. Johnson, E.M. Wintour, K. Moritz, Programming effects of short prenatal exposure to cortisol, FASEB J 16 (2002) 1017-26.

[47] A.J. Drake, J.I. Tang, M.J. Nyirenda, Mechanisms underlying the role of glucocorticoids in the early life programming of adult disease, Clin Sci (Lond) 113 (2007) 219-32.

[48] A.J. Drake, J.I. Tang, M.J. Nyirenda, Mechanisms underlying the role of glucocorticoids in the early life programming of adult disease, Clin Sci 113 (2007) 219-232.

[49] D.J. Echobiochon, Toxic Effects of Pesticides, Casarett and Doull's Toxicology, 5 Edition, McGraw-Hill, New York 1996, pp. 643-698.

[50] M. Ehrich, L. Correll, B. Veronesi, Acetylcholinesterase and neuropathy target esterase inhibitions in neuroblastoma cells to distinguish organophosphorus compounds causing acute and delayed neurotoxicity, Fundam Appl Toxicol 38 (1997) 55-63.

[51] M.A. Elobeid, D.B. Allison, Putative environmental-endocrine disruptors and obesity: a review, Curr Opin Endocrinol Diabetes Obes 15 (2008) 403-8.

[52] J. Flaskos, W.G. McLean, A.J. Hargreaves, The toxicity of organophosphate compounds towards cultured PC12 cells, Toxicology Letters 70 (1994) 71-76.

[53] K.M. Flegal, M.D. Carroll, R.J. Kuczmarski, C.L. Johnson, Overweight and obesity in the United States: prevalence and trends, 1960-1994, Int J Obes Relat Metab Disord 22 (1998) 39-47.

[54] A.L. Fowden, Endocrine regulation of fetal growth, Reprod Fertil Dev 7 (1995) 351-63.

[55] T.M. Frayling, N.J. Timpson, M.N. Weedon, E. Zeggini, R.M. Freathy, C.M. Lindgren, J.R.B. Perry, K.S. Elliott, H. Lango, N.W. Rayner and others, A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity, Science 316 (2007) 889-894.

[56] K. Fujita, P. Lazarovici, G. Guroff, Regulation of the differentiation of PC12 pheochromocytoma cells, Environmental Health Perspectives 80 (1989) 127-142.

[57] K. Fuxe, A. Cintra, G. Chadi, J.Å. Gustafsson, L.F. Agnati, Central glucocorticoid receptors and neuronal plasticity, Meth Neurosci 22 (1994) 372-382.

[58] K. Fuxe, R. Diaz, A. Cintra, M. Bhatnagar, B. Tinner, J.A. Gustafsson, S.O. Ogren, L.F. Agnati, On the role of glucocorticoid receptors in brain plasticity, Cell Mol Neurobiol 16 (1996) 239-258.

[59] M. Gallo, N. Lawryk, Organic Phosphorous Pesticides, in: W.J.H.a.E.R. Laws (Ed.), Handbook of Pesticide Toxicology, Academic Press, San Diego, 1991, pp. 917-1123.

[60] X.M. Gao, A. Agrotis, D.J. Autelitano, E. Percy, E.A. Woodcock, G.L. Jennings, A.M. Dart, X.J. Du, Sex hormones and cardiomyopathic phenotype induced by cardiac β_2-adrenergic receptor overexpression, Endocrinology 144 (2003) 4097-4105.

[61] Y.J. Gao, A.C. Holloway, Z.H. Zeng, G.E. Lim, J.J. Petrik, W.G. Foster, R.M. Lee, Prenatal exposure to nicotine causes postnatal obesity and altered perivascular adipose tissue function, Obesity Res 13 (2005) 687-692.

[62] S.J. Garcia, F.J. Seidler, T.L. Crumpton, T.A. Slotkin, Does the developmental neurotoxicity of chlorpyrifos involve glial targets? Macromolecule synthesis, adenylyl cyclase signaling, nuclear transcription factors, and formation of reactive oxygen in C6 glioma cells, Brain Res 891 (2001) 54-68.

[63] G.M. Gilad, V.H. Gilad, Y. Eliyayev, J.M. Rabey, Developmental regulation of the brain polyamine-stress-response, International Journal of Developmental Neuroscience 16 (1998) 271-278.

[64] L.C. Gilstrap, R. Christensen, W.H. Clewell, M.E. D'Alton, E.C. Davidson, M.B. Escobedo, D.K. Gjerdingen, J. Goddard-Finegold, R.L. Goldenberg, D.A. Grimes and others, Effect of corticosteroids for fetal maturation on perinatal outcomes, Journal of the American Medical Association 273 (1995) 413-418.

[65] E. Gould, P. Tanapat, B.S. McEwen, Activation of the type 2 adrenal steroid receptor can rescue granule cells from death during development, Developmental Brain Research 101 (1997) 265-268.

[66] T.J. Gould, P.C. Bickford, Age-related deficits in the cerebellar *beta* adrenergic signal transduction cascade in Fischer 344 rats, Journal of Pharmacology and Experimental Therapeutics 281 (1997) 965-971.

[67] A. Guidotti, Adenosine 3',5'-monophosphate concentrations and isoproterenol-induced synthesis of deoxyribonucleic acid in mouse parotid gland, Molecular Pharmacology 8 (1972) 521-530.

[68] R.C. Gupta, Brain regional heterogeneity and toxicological mechanisms of organophosphates and carbamates, Toxicology Mechanisms and Methods 14 (2004) 103-143.

[69] S. Gurunathan, M. Robson, N. Freeman, B. Buckley, A. Roy, R. Meyer, J. Bukowski, P.J. Lioy, Accumulation of chlorpyrifos on residential surfaces and toys accessible to children, Environmental Health Perspectives 106 (1998) 9-16.

[70] M. Guven, F. Bayram, K. Unluhizarci, F. Kelestimur, Endocrine changes in patients with acute organophosphate poisoning, Human and Experimental Toxicology 18 (1999) 598-601.

[71] C.N. Hales, D.J.P. Barker, Type-2 (on-Insulin-Dependent) Diabetes-Mellitus - the Thrifty Phenotype Hypothesis, Diabetologia 35 (1992) 595-601.

[72] J. Hanoune, N. Defer, Regulation and role of adenylyl cyclase isoforms, Annu Rev Pharmacol Toxicol 41 (2001) 145-74.

[73] J. Hanoune, Y. Pouille, E. Tzavara, T. Shen, L. Lipskaya, N. Miyamoto, Y. Suzuki, N. Defer, Adenylyl cyclases: structure, regulation and function in an enzyme superfamily, Mol Cell Endocrinol 128 (1997) 179-94.

[74] M.J. Holness, M.C. Sugden, Dexamethasone during late gestation exacerbates peripheral insulin resistance and selectively targets glucose-sensitive functions in β cell and liver, Endocrinology 142 (2001) 3742-3748.

[75] Q.-C. Hou, T.A. Slotkin, Effects of prenatal dexamethasone or terbutaline exposure on development of neural and intrinsic control of heart rate, Pediatric Research 26 (1989) 554-557.

[76] A.S. Howard, R. Bucelli, D.A. Jett, D. Bruun, D.R. Yang, Chlorpyrifos exerts opposing effects on axonal and dendritic growth in primary neuronal cultures, Toxicology and Applied Pharmacology 207 (2005) 112-124.

[77] M.D. Howard, C.N. Pope, *In vitro* effects of chlorpyrifos, parathion, methyl parathion and their oxons on cardiac muscarinic receptor binding in neonatal and adult rats, Toxicology 170 (2002) 1-10.

[78] A. Hultgårdh-Nilsson, V. Querol-Ferrer, B. Jonzon, U. Krondahl, J. Nilsson, Cyclic AMP, early response gene expression, and DNA synthesis in rat smooth muscle cells, Exp Cell Res 214 (1994) 297-302.

[79] D.L. Hunter, T.L. Lassiter, S.M. Chanda, S. Barone, S. Padilla, Pharmacokinetics of chlorpyrifos and its metabolites in maternal and fetal brain and liver tissue following gestational exposure, Toxicologist 42 (1998) 157-158.

[80] J.H. Hurley, Structure, mechanism, and regulation of mammalian adenylyl cyclase, Journal of Biological Chemistry 274 (1999) 7599-7602.

[81] M. Iwase, S.P. Bishop, M. Uechi, D.E. Vatner, R.P. Shannon, R.K. Kudej, D.C. Wight, T.E. Wagner, Y. Ishikawa, C.J. Homcy and others, Adverse effects of chronic endogenous sympathetic drive induced by cardiac $G_{s\alpha}$ overexpression, Circ Res 78 (1996) 517-524.

[82] R.R. Jameson, F.J. Seidler, D. Qiao, T.A. Slotkin, Adverse neurodevelopmental effects of dexamethasone modeled in PC12 cells: identifying the critical stages and concentration thresholds for the targeting of cell acquisition, differentiation and viability, Neuropsychopharmacology 31 (2006) 1647-1658.

[83] R.R. Jameson, F.J. Seidler, D. Qiao, T.A. Slotkin, Chlorpyrifos affects phenotypic outcomes in a model of mammalian neurodevelopment: critical stages targeting differentiation in PC12 cells, Environmental Health Perspectives 114 (2006) 667-672.

[84] R.R. Jameson, F.J. Seidler, T.A. Slotkin, Nonenzymatic functions of acetylcholinesterase splice variants in the developmental neurotoxicity of organophosphates: chlorpyrifos, chlorpyrifos oxon and diazinon, Environmental Health Perspectives 115 (2007) 65-70.

[85] I.Y. Khan, P.D. Taylor, V. Dekou, P.T. Seed, L. Lakasing, D. Graham, A.F. Dominiczak, M.A. Hanson, L. Poston, Gender-linked hypertension in offspring of lard-fed pregnant rats, Hypertension 41 (2003) 168–175.

[86] M.L. Kreider, J.E. Aldridge, M.M. Cousins, C.A. Oliver, F.J. Seidler, T.A. Slotkin, Disruption of rat forebrain development by glucocorticoids: critical perinatal periods for effects on neural cell acquisition and on cell signaling cascades mediating noradrenergic and cholinergic neurotransmitter/neurotrophic responses, Neuropsychopharmacology 30 (2005) 1841-1855.

[87] M.L. Kreider, E.D. Levin, F.J. Seidler, T.A. Slotkin, Gestational dexamethasone treatment elicits sex-dependent alterations in locomotor activity, reward-based memory and hippocampal cholinergic function in adolescent and adult rats, Neuropsychopharmacology 30 (2005) 1617-1623.

[88] M.L. Kreider, C.A. Tate, M.M. Cousins, C.A. Oliver, F.J. Seidler, T.A. Slotkin, Lasting effects of developmental dexamethasone treatment on neural cell number and size, synaptic activity and cell signaling: critical periods of vulnerability, dose-effect relationships, regional targets and sex selectivity, Neuropsychopharmacology 31 (2006) 12-35.

[89] S.L. Krein, M.M. Funnell, J.D. Piette, Economics of diabetes mellitus, Nurs Clin North Am 41 (2006) 499-511, v-vi.

[90] C. LaCroix, J. Freeling, A. Giles, J. Wess, Y.-F. Li, Deficiency of M2 muscarinic acetylcholine receptors increases susceptibility of ventricular function to chronic adrenergic stress, American Journal of Physiology - Heart & Circulatory Physiology 294 (2008) H810-H820.

[91] S.C. Langley-Evans, G.J. Phillips, R. Benediktsson, D.S. Gardner, C.R. Edwards, A.A. Jackson, J.R. Seckl, Protein intake in pregnancy, placental glucocorticoid metabolism and the programming of hypertension in the rat, Placenta 17 (1996) 169-72.

[92] T.L. Lassiter, S. Brimijoin, Rats gain excess weight after developmental exposure to the organophosphorothionate pesticide, chlorpyrifos, Neurotoxicology and Teratology 30 (2008) 125-130.

[93] T.L. Lassiter, I.T. Ryde, E.D. Levin, F.J. Seidler, T.A. Slotkin, Neonatal exposure to parathion alters lipid metabolism in adulthood: interactions with dietary fat intake and implications for neurodevelopmental deficits, Brain Research Bulletin 81 (2010) 85-91.

[94] T.L. Lassiter, I.T. Ryde, E.A. MacKillop, K.K. Brown, E.D. Levin, F.J. Seidler, T.A. Slotkin, Exposure of neonatal rats to parathion elicits sex-selective reprogramming of metabolism and alters the response to a high-fat diet in adulthood, Environmental Health Perspectives 116 (2008) 1456-1462.

[95] C. Lau, J.M. Rogers, Embryonic and fetal programming of physiological disorders in adulthood, Birth Defects Res C Embryo Today 72 (2004) 300-12.

[96] C. Lau, T.A. Slotkin, Maturation of sympathetic neurotransmission in the rat heart. VII. Supression of sympathetic responses by dexamethasone, Journal of Pharmacology and Experimental Therapeutics 216 (1981) 6-11.

[97] E.D. Levin, N. Addy, N.C. Christopher, F.J. Seidler, T.A. Slotkin, Persistent behavioral consequences of neonatal chlorpyrifos exposure in rats, Developmental Brain Research 130 (2001) 83-89.

[98] W.W. Li, J.E. Casida, Organophosphorus neuropathy target esterase inhibitors selectively block outgrowth of neurite-like and cell processes in cultured cells, Toxicology Letters 98 (1998) 139-146.

[99] J. Liu, C.N. Pope, Comparative presynaptic neurochemical changes in rat striatum following exposure to chlorpyrifos or parathion, J Toxicol Environ Health A 53 (1998) 531-44.

[100] A.D. Lopez, C.D. Mathers, M. Ezzati, D.T. Jamison, C.J. Murray, Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data, Lancet 367 (2006) 1747-57.

[101] S. Maccari, M. Darnaudery, S. Morley-Fletcher, A.R. Zuena, C. Cinque, O. Van Reeth, Prenatal stress and long-term consequences: implications of glucocorticoid hormones, Neuroscience and Biobehavioral Reviews 27 (2003) 119-127.

[102] M.D. Mark, D.R. Storm, Coupling of epidermal growth factor (EGF) with the antiproliferative activity of cAMP induces neuronal differentiation, Journal of Biological Chemistry 272 (1997) 17238-17244.

[103] S.G. Matthews, Antenatal glucocorticoids and programming of the developing CNS, Pediatric Research 47 (2000) 291-300.

[104] S.G. Matthews, D. Owen, S. Banjanin, M.H. Andrews, Glucocorticoids, hypothalamo-pituitary-adrenal (HPA) development, and life after birth, Endocr Res 28 (2002) 709-718.

[105] L. McCabe, D. Marash, A. Li, S.G. Matthews, Repeated antenatal glucocorticoid treatment decreases hypothalamic corticotropin releasing hormone mRNA but not corticosteroid receptor mRNA expression in the fetal guinea-pig brain, J Neuroendocrinol 13 (2001) 425-31.

[106] B.S. McEwen, Steroid hormones: effect on brain development and function, Hormone Res 37 (1992) 1-10.

[107] M.F. McManus, L.C. Chen, I. Vallejo, M. Vallejo, Astroglial differentiation of cortical precursor cells triggered by activation of the cAMP-dependent signaling pathway, Journal of Neuroscience 19 (1999) 9004-9015.

[108] M.J. Meaney, J. Diorio, D. Francis, J. Widdowson, P. LaPlante, C. Caldji, S. Sharma, J.R. Seckl, P.M. Plotsky, Early environmental regulation of forebrain glucocorticoid receptor gene expression: implications for adrenocortical responses to stress, Dev Neurosci 18 (1996) 49-72.

[109] W.J. Meggs, K.L. Brewer, Weight gain associated with chronic exposure to chlorpyrifos in rats, J Med Toxicol 3 (2007) 89-93.

[110] A. Meyer, F.J. Seidler, J.E. Aldridge, T.A. Slotkin, Developmental exposure to terbutaline alters cell signaling in mature rat brain regions and augments the effects of subsequent neonatal exposure to the organophosphorus insecticide, chlorpyrifos, Toxicology and Applied Pharmacology 203 (2005) 154-166.

[111] A. Meyer, F.J. Seidler, J.E. Aldridge, C.A. Tate, M.M. Cousins, T.A. Slotkin, 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 (2004) 295-301.

[112] A. Meyer, F.J. Seidler, M.M. Cousins, T.A. Slotkin, Developmental neurotoxicity elicited by gestational exposure to chlorpyrifos: when is adenylyl cyclase a target?, Environmental Health Perspectives 111 (2003) 1871-1876.

[113] A. Meyer, F.J. Seidler, T.A. Slotkin, Developmental effects of chlorpyrifos extend beyond neurotoxicity: critical periods for immediate and delayed-onset effects on cardiac and hepatic cell signaling, Environmental Health Perspectives 112 (2004) 170-178.

[114] B.E. Mileson, J.E. Chambers, W.L. Chen, W. Dettbarn, M. Ehrich, A.T. Eldefrawi, D.W. Gaylor, K. Hamernik, E. Hodgson, A.G. Karczmar and others, Common mechanism of toxicity: a case study of organophosphorus pesticides, Toxicological Sciences 41 (1998) 8-20.

[115] M.P. Montgomery, F. Kamel, T.M. Saldana, M.C. Alavanja, D.P. Sandler, Incident diabetes and pesticide exposure among licensed pesticide applicators: Agricultural Health Study, 1993-2003, Am J Epidemiol 167 (2008) 1235-46.

[116] D.P. Morgan, L.I. Lin, H.H. Saikaly, Morbidity and mortality in workers occupationally exposed to pesticides, Arch Environ Contam Toxicol 9 (1980) 349-382.

[117] K. Nagata, C.S. Huang, J.H. Song, T. Narahashi, Direct actions of anticholinesterases on the neuronal nicotinic acetylcholine receptor channels, Brain Res 769 (1997) 211-218.

[118] H.A. Navarro, E.M. Kudlacz, T.A. Slotkin, Control of adenylate cyclase activity in developing rat heart and liver: effects of prenatal exposure to terbutaline or dexamethasone, Biology of the Neonate 60 (1991) 127-136.

[119] S. Nordman, A. Abulaiti, A. Hilding, E.C. Långberg, K. Humphreys, C.G. Östenson, S. Efendic, H.F. Gu, Genetic variation of the adenylyl cyclase 3 (AC3) locus and its influence on type 2 diabetes and obesity susceptibility in Swedish men, International Journal of Obesity 32 (2008) 407-412.

[120] K. O'Brien, H. Sekimoto, C. Boney, M. Malee, K. O'Brien, H. Sekimoto, C. Boney, M. Malee, Effect of fetal dexamethasone exposure on the development of adult insulin sensitivity in a rat model, Journal of Maternal-Fetal & Neonatal Medicine 21 (2008) 623-628.

[121] D. O'Regan, C.J. Kenyon, J.R. Seckl, M.C. Holmes, Glucocorticoid exposure in late gestation in the rat permanently programs gender-specific differences in adult cardiovascular and metabolic physiology, American Journal of Physiology - Heart & Circulatory Physiology 287 (2004) E863-E870.

[122] C.L. Ogden, M.D. Carroll, L.R. Curtin, M.A. McDowell, C.J. Tabak, K.M. Flegal, Prevalence of overweight and obesity in the United States, 1999-2004, JAMA 295 (2006) 1549-1555.

[123] K. Olivier, J. Liu, C. Pope, Inhibition of forskolin-stimulated cAMP formation *in vitro* by paraoxon and chlorpyrifos oxon in cortical slices from neonatal, juvenile, and adult rats, Journal of Biochemical and Molecular Toxicology 15 (2001) 263-269.

[124] P. Onkamo, S. Vaananen, M. Karvonen, J. Tuomilehto, Worldwide increase in incidence of Type I diabetes--the analysis of the data on published incidence trends, Diabetologia 42 (1999) 1395-1403.

[125] L.A. Ortiz, A. Quan, A. Weinberg, M. Baum, Effect of prenatal dexamethasone on rat renal development, Kidney International 59 (2001) 1663-1669.

[126] L.A. Ortiz, A. Quan, F. Zarzar, A. Weinberg, M. Baum, Prenatal dexamethasone programs hypertension and renal injury in the rat, Hypertension 41 (2003) 328-334.

[127] E.M. Ostrea, V. Morales, E. Ngoumgna, R. Prescilla, E. Tan, E. Hernandez, G.B. Ramirez, H.L. Cifra, M.L. Manlapaz, Prevalence of fetal exposure to environmental toxins as determined by meconium analysis, Neurotoxicology 23 (2002) 329-339.

[128] T.B. Patel, Z. Du, S. Pierre, L. Cartin, K. Scholich, Molecular biological approaches to unravel adenylyl cyclase signaling and function, Gene 269 (2001) 13-25.

[129] T. Pfeuffer, H. Metzger, 7-O-Hemisuccinyl-Deacetyl Forskolin Sepharose - a Novel Affinity Support for Purification of Adenylate-Cyclase, Febs Letters 146 (1982) 369-375.

[130] C.N. Pope, Organophosphorus pesticides: do they all have the same mechanism of toxicity?, Journal of Toxicology & Environmental Health Part A 2 (1999) 161-181.

[131] C. Power, B.J. Jefferis, Fetal environment and subsequent obesity: a study of maternal smoking, Int J Epidemiol 31 (2002) 413-419.

[132] D. Qiao, F.J. Seidler, S. Padilla, T.A. Slotkin, Developmental neurotoxicity of chlorpyrifos: What is the vulnerable period?, Environmental Health Perspectives 110 (2002) 1097-1103.

[133] D. Qiao, F.J. Seidler, T.A. Slotkin, Developmental neurotoxicity of chlorpyrifos modeled *in vitro*: comparative effects of metabolites and other cholinesterase inhibitors on DNA synthesis in PC12 and C6 cells, Environmental Health Perspectives 109 (2001) 909-913.

[134] D. Qiao, F.J. Seidler, T.A. Slotkin, Oxidative mechanisms contributing to the developmental neurotoxicity of nicotine and chlorpyrifos, Toxicology and Applied Pharmacology 206 (2005) 17-26.

[135] D. Qiao, F.J. Seidler, J.D. Violin, T.A. Slotkin, Nicotine is a developmental neurotoxicant and neuroprotectant: stage-selective inhibition of DNA synthesis coincident with shielding from effects of chlorpyrifos, Developmental Brain Research 147 (2003) 183-190.

[136] S. Rashid, G.F. Lewis, The mechanisms of differential glucocorticoid and mineralocorticoid action in the brain and peripheral tissues, Clin Biochem 38 (2005) 401-9.

[137] V.A. Rauh, R. Garfinkel, R. Perera, H. Andrews, L. Hoepner, D. Barr, D. Whitehead, D. Tang, R.M. Whyatt, Impact of prenatal chlorpyrifos exposure on neurodevelopment in the first 3 years of life among inner-city children, Pediatrics 118 (2006) 1845-1859.

[138] C.S. Roegge, O.A. Timofeeva, F.J. Seidler, T.A. Slotkin, E.D. Levin, Developmental diazinon neurotoxicity in rats: later effects on emotional response, Brain Research Bulletin 75 (2008) 166–172.

[139] D.S. Rohlman, T.A. Arcury, S.A. Quandt, M. Lasarev, J. Rothlein, R. Travers, A. Tamulinas, J. Scherer, J. Early, A. Marin and others, Neurobehavioral performance in preschool children from agricultural and non-agricultural communities in Oregon and North Carolina, Neurotoxicology 26 (2005) 589-598.

[140] T.M. Saldana, O. Basso, J.A. Hoppin, D.D. Baird, C. Knott, A. Blair, M.C.R. Alavanja, D.P. Sandler, Pesticide exposure and self-reported gestational diabetes mellitus in the Agricultural Health Study, Diabetes Care 30 (2007) 529-534.

[141] R.A. Schuh, P.J. Lein, R.A. Beckles, D.A. Jett, Noncholinesterase mechanisms of chlorpyrifos neurotoxicity: altered phosphorylation of Ca^{2+}/cAMP response element binding protein in cultured neurons, Toxicology and Applied Pharmacology 182 (2002) 176-185.

[142] K.B. Seamon, W. Padgett, J.W. Daly, Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells, Proc Natl Acad Sci U S A 78 (1981) 3363-7.

[143] J.R. Seckl, Glucocorticoid programming of the fetus: adult phenotypes and molecular mechanisms, Molecular & Cellular Endocrinology 185 (2001) 61-71.

[144] R.R. Singh, L.A. Cullen-McEwen, M.M. Kett, W.M. Boon, J. Dowling, J.F. Bertram, K.M. Moritz, Prenatal corticosterone exposure results in altered AT1/AT2, nephron deficit and hypertension in the rat offspring, J Physiol 579 (2007) 503-13.

[145] T.A. Slotkin, Cholinergic systems in brain development and disruption by neurotoxicants: nicotine, environmental tobacco smoke, organophosphates, Toxicology and Applied Pharmacology 198 (2004) 132-151.

[146] T.A. Slotkin, Developmental cholinotoxicants: nicotine and chlorpyrifos, Environmental Health Perspectives 107(suppl 1) (1999) 71-80.

[147] T.A. Slotkin, Developmental neurotoxicity of organophosphates: a case study of chlorpyrifos, in: R.C. Gupta (Ed.), Toxicity of Organophosphate and Carbamate Pesticides, Elsevier Academic Press, San Diego, 2005, pp. 293-314.

[148] T.A. Slotkin, G. Barnes, C. Lau, F.J. Seidler, P. Trepanier, S.J. Weigel, W.L. Whitmore, Development of polyamine and biogenic amine systems in brains and hearts of neonatal rats given dexamethasone: role of biochemical alterations in cellular maturation for producing deficits in ontogeny of neurotransmitter levels, uptake, storage and turnover, Journal of Pharmacology and Experimental Therapeutics 221 (1982) 686-693.

[149] T.A. Slotkin, B.E. Bodwell, E.D. Levin, F.J. Seidler, Neonatal exposure to low doses of diazinon: long-term effects on neural cell development and acetylcholine systems, Environmental Health Perspectives 116 (2008) 340-348.

[150] T.A. Slotkin, K.K. Brown, F.J. Seidler, Developmental exposure of rats to chlorpyrifos elicits sex-selective hyperlipidemia and hyperinsulinemia in adulthood, Environmental Health Perspectives 113 (2005) 1291-1294.

[151] T.A. Slotkin, M.M. Cousins, C.A. Tate, F.J. Seidler, Persistent cholinergic presynaptic deficits after neonatal chlorpyrifos exposure, Brain Res 902 (2001) 229-243.

[152] T.A. Slotkin, M.L. Kreider, C.A. Tate, F.J. Seidler, Critical prenatal and postnatal periods for persistent effects of dexamethasone on serotonergic and dopaminergic systems, Neuropsychopharmacology 31 (2006) 904-911.

[153] T.A. Slotkin, T.L. Lassiter, I.T. Ryde, N. Wrench, E.D. Levin, F.J. Seidler, Consumption of a high-fat diet in adulthood ameliorates the effects of neonatal parathion exposure on acetylcholine systems in rat brain regions, Environmental Health Perspectives 117 (2009) 916-922.

[154] T.A. Slotkin, E.D. Levin, F.J. Seidler, Comparative developmental neurotoxicity of organophosphate insecticides: effects on brain development are separable from systemic toxicity, Environmental Health Perspectives 114 (2006) 746-751.

[155] T.A. Slotkin, E.A. MacKillop, I.T. Ryde, F.J. Seidler, Ameliorating the developmental neurotoxicity of chlorpyrifos: a mechanisms-based approach in PC12 cells, Environmental Health Perspectives 115 (2007) 1306-1313.

[156] T.A. Slotkin, E.A. MacKillop, I.T. Ryde, C.A. Tate, F.J. Seidler, Screening for developmental neurotoxicity using PC12 cells: comparisons of organophosphates with a carbamate, an organochlorine and divalent nickel, Environmental Health Perspectives 115 (2007) 93-101.

[157] T.A. Slotkin, C.A. Oliver, F.J. Seidler, Critical periods for the role of oxidative stress in the developmental neurotoxicity of chlorpyrifos and terbutaline, alone or in combination, Developmental Brain Research 157 (2005) 172-180.

[158] T.A. Slotkin, I.T. Ryde, E.D. Levin, F.J. Seidler, Developmental neurotoxicity of low-dose diazinon exposure of neonatal rats: effects on serotonin systems in adolescence and adulthood, Brain Research Bulletin 75 (2008) 640-647.

[159] T.A. Slotkin, F.J. Seidler, The alterations in CNS serotonergic mechanisms caused by neonatal chlorpyrifos exposure are permanent, Developmental Brain Research 158 (2005) 115-119.

[160] T.A. Slotkin, F.J. Seidler, Comparative developmental neurotoxicity of organophosphates in vivo: transcriptional responses of pathways for brain cell development, cell signaling, cytotoxicity and neurotransmitter systems, Brain Research Bulletin 72 (2007) 232-274.

[161] T.A. Slotkin, F.J. Seidler, Developmental neurotoxicants target neurodifferentiation into the serotonin phenotype: chlorpyrifos, diazinon, dieldrin and divalent nickel, Toxicology and Applied Pharmacology 233 (2008) 211-219.

[162] T.A. Slotkin, F.J. Seidler, Oxidative and excitatory mechanisms of developmental neurotoxicity: transcriptional profiles for chlorpyrifos, diazinon, dieldrin and divalent nickel in PC12 cells, Environmental Health Perspectives 117 (2009) 587-596.

[163] T.A. Slotkin, F.J. Seidler, Protein kinase C is a target for diverse developmental neurotoxicants: transcriptional responses to chlorpyrifos, diazinon, dieldrin and divalent nickel in PC12 cells, Brain Res 1263 (2009) 23-32.

[164] T.A. Slotkin, F.J. Seidler, Transcriptional profiles reveal similarities and differences in the effects of developmental neurotoxicants on differentiation into neurotransmitter phenotypes in PC12 cells, Brain Research Bulletin 78 (2009) 211-225.

[165] T.A. Slotkin, F.J. Seidler, F. Fumagalli, Exposure to organophosphates reduces the expression of neurotrophic factors in neonatal rat brain regions: similarities and differences in the effects of chlorpyrifos and diazinon on the fibroblast growth factor superfamily, Environmental Health Perspectives 115 (2007) 909-916.

[166] T.A. Slotkin, F.J. Seidler, F. Fumagalli, Targeting of neurotrophic factors, their receptors, and signaling pathways in the developmental neurotoxicity of organophosphates in vivo and in vitro, Brain Research Bulletin 76 (2008) 424-438.

[167] T.A. Slotkin, F.J. Seidler, F. Fumagalli, Unrelated developmental neurotoxicants elicit similar transcriptional profiles for effects on neurotrophic factors and their receptors in an in vitro model, Neurotoxicology and Teratology 32 (2010) 42-51.

[168] T.A. Slotkin, F.J. Seidler, R.J. Kavlock, J.V. Bartolome, Fetal dexamethasone exposure impairs cellular development in neonatal rat heart and kidney: effects on DNA and protein in whole tissues, Teratology 43 (1991) 301-306.

[169] T.A. Slotkin, C.A. Tate, I.T. Ryde, E.D. Levin, F.J. Seidler, Organophosphate insecticides target the serotonergic system in developing rat brain regions: disparate effects of diazinon and parathion at doses spanning the threshold for cholinesterase inhibition, Environmental Health Perspectives 114 (2006) 1542-1546.

[170] T.A. Slotkin, J. Zhang, E.C. McCook, F.J. Seidler, Glucocorticoid-targeting of the adenylyl cyclase signaling pathway in the cerebellum of young vs. aged rats, Brain Res 800 (1998) 236-244.

[171] M.J. Smit, D. Verzijl, R. Iyengar, Identity of adenylyl cyclase isoform determines the rate of cell cycle progression in NIH 3T3 cells, Proc Natl Acad Sci U S A 95 (1998) 15084-9.

[172] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal Biochem 150 (1985) 76-85.

[173] G.W. Snedecor, W.G. Cochran, Statistical Methods, Editoin Edition, Iowa State University Press, Ames, Iowa, 1967.

[174] E. Somm, V.M. Schwitzgebel, A. Toulotte, C.R. Cederroth, C. Combescure, S. Nef, M.L. Aubert, P.S. Huppi, Perinatal exposure to bisphenol a alters early adipogenesis in the rat, Environ Health Perspect 117 (2009) 1549-55.

[175] X. Song, F.J. Seidler, J.L. Saleh, J. Zhang, S. Padilla, T.A. Slotkin, Cellular mechanisms for developmental toxicity of chlorpyrifos: targeting the adenylyl cyclase signaling cascade, Toxicology and Applied Pharmacology 145 (1997) 158-174.

[176] X. Song, J.D. Violin, F.J. Seidler, T.A. Slotkin, Modeling the developmental neurotoxicity of chlorpyrifos *in vitro*: macromolecule synthesis in PC12 cells, Toxicology and Applied Pharmacology 151 (1998) 182-191.

[177] E.K. Stachowiak, X. Fang, J. Myers, S. Dunham, M.K. Stachowiak, cAMP-Induced differentiation of human neuronal progenitor cells is mediated by nuclear fibroblast growth factor receptor-1 (FGFR1), Journal of Neurochemistry 84 (2003) 1296-1312.

[178] C.J. Stein, G.A. Colditz, The epidemic of obesity, Journal of Clinical Endocrinology & Metabolism 89 (2004) 2522-2525.

[179] K. Sun, K. Yang, J.R. Challis, Differential expression of 11 beta-hydroxysteroid dehydrogenase types 1 and 2 in human placenta and fetal membranes, J Clin Endocrinol Metab 82 (1997) 300-5.

[180] R.K. Sunahara, C.W. Dessauer, A.G. Gilman, Complexity and diversity of mammalian adenylyl cyclases, Annu Rev Pharmacol Toxicol 36 (1996) 461-80.

[181] R.K. Sunahara, R. Taussig, Isoforms of mammalian adenylyl cyclase: multiplicities of signaling, Mol Interv 2 (2002) 168-84.

[182] K.K. Teng, L.A. Greene, Cultured PC12 cells: a model for neuronal function and differentiation, in: J.E. Celis (Ed.), Cell Biology: A Laboratory Handbook, Academic Press, San Diego, 1994, pp. 218-224.

[183] O.A. Timofeeva, C.S. Roegge, F.J. Seidler, T.A. Slotkin, E.D. Levin, Persistent cognitive alterations in rats after early postnatal exposure to low doses of the organophosphate pesticide, diazinon, Neurotoxicology and Teratology 30 (2008) 38-45.

[184] O.A. Timofeeva, D. Sanders, K. Seemann, L. Yang, D. Hermanson, S. Regenbogen, S. Agoos, A. Kallepalli, A. Rastogi, D. Braddy and others, Persistent behavioral alterations in rats neonatally exposed to low doses of the

organophosphate pesticide, parathion, Brain Research Bulletin 77 (2008) 404-411.

[185] A.M. Toschke, A.G. Ehlin, R. von Kries, A. Ekbom, S.M. Montgomery, Maternal smoking during pregnancy and appetite control in offspring, Journal of Perinatal Medicine 31 (2003) 251-256.

[186] J.A. Trauth, F.J. Seidler, T.A. Slotkin, An animal model of adolescent nicotine exposure: effects on gene expression and macromolecular constituents in rat brain regions, Brain Res 867 (2000) 29-39.

[187] S.M. Tuler, A.A. Hazen, J.M. Bowen, Release and metabolism of dopamine in a clonal line of pheochromocytoma (PC12) cells exposed to fenthion, Fund Appl Toxicol 13 (1989) 484-492.

[188] M. Uechi, K. Asai, M. Osaka, A. Smith, N. Sato, T.E. Wagner, Y. Ishikawa, H. Hayakawa, D.E. Vatner, R.P. Shannon and others, Depressed heart rate variability and arterial baroreflex in conscious transgenic mice with overexpression of cardiac $G_{s\alpha}$, Circ Res 82 (1998) 416-423.

[189] R. Van Wijk, W.D. Wicks, M.M. Bevers, J. Van Rijn, Rapid arrest of DNA synthesis by N6,O2'-dibutyryl cyclic adenosine 3',5'-monophosphate in cultured hepatoma cells, Cancer Res 33 (1973) 1331-1338.

[190] A.M. Vinggaard, C. Hnida, V. Breinholt, J.C. Larsen, Screening of selected pesticides for inhibition of CYP19 aromatase activity in vitro, Toxicol in Vitro 14 (2000) 227-234.

[191] R. von Kries, A.M. Toschke, B. Koletzko, W. Slikker, Jr., Maternal smoking during pregnancy and childhood obesity, American Journal of Epidemiology 156 (2002) 954-961.

[192] Y. Wang, Q. Zhang, Are American children and adolescents of low socioeconomic status at increased risk of obesity? Changes in the association between overweight and family income between 1971 and 2002, Am J Clin Nutr 84 (2006) 707-16.

[193] B. Weinhold, Environmental cardiology: getting to the heart of the matter, Environ Health Perspect 112 (2004) A880-7.

[194] M. Weinstock, Alterations induced by gestational stress in brain morphology and behaviour of the offspring, Prog Neurobiol 65 (2001) 427-451.

[195] L.A.M. Welberg, J.R. Seckl, Prenatal stress, glucocorticoids and the programming of the brain, Journal of Neuroendocrinology 13 (2001) 113-128.

[196] K.D. Whitney, F.J. Seidler, T.A. Slotkin, Developmental neurotoxicity of chlorpyrifos: cellular mechanisms, Toxicology and Applied Pharmacology 134 (1995) 53-62.

[197] N.G. Williams, H.Y. Zhong, K.P. Minneman, Differential coupling of α_1-, α_2-, and β-adrenergic receptors to mitogen-activated protein kinase pathways and differentiation in transfected PC12 cells, Journal of Biological Chemistry 273 (1998) 24624-24632.

[198] M. Winick, A. Noble, Quantitative changes in DNA, RNA and protein during prenatal and postnatal growth in the rat, Dev Biol 12 (1965) 451-466.

[199] C.S. Wyrwoll, P.J. Mark, T.A. Mori, B.J. Waddell, Developmental programming of adult hyperinsulinemia, increased proinflammatory cytokine production, and altered skeletal muscle expression of SLC2A4 (GLUT4) and uncoupling protein 3, Journal of Endocrinology 198 (2008) 571-579.

[200] K.R. Yamamoto, Steroid receptor regulated transcription of specific genes and gene networks, Annu Rev Genet 19 (1985) 209-52.

[201] J. Yanai, O. Vatury, T.A. Slotkin, Cell signaling as a target and underlying mechanism for neurobehavioral teratogenesis, Annals of the New York Academy of Sciences 965 (2002) 473-478.

[202] M. Yousefpour, F. Bahrami, B. Shahsavan Behboodi, A. Khoshbaten, A. Asgari, Paraoxon-induced ultrastructural growth changes of rat cultured hippocampal cells in neurobasal/B27, Toxicology 217 (2006) 221-227.

[203] F. Yu, Z. Wang, B. Ju, Y. Wang, J. Wang, D. Bai, Apoptotic effect of organophosphorus insecticide chlorpyrifos on mouse retina in vivo via oxidative stress and protection of combination of vitamins C and E, Exp Toxicol Pathol 59 (2008) 415-423.

[204] J.L. Zeiders, F.J. Seidler, G. Iaccarino, W.J. Koch, T.A. Slotkin, Ontogeny of cardiac β-adrenoceptor desensitization mechanisms: agonist treatment enhances receptor/G-protein transduction rather than eliciting uncoupling, J Mol Cell Cardiol 31 (1999) 413-423.

[205] J.L. Zeiders, F.J. Seidler, T.A. Slotkin, Agonist-induced sensitization of β-adrenoceptor signaling in neonatal rat heart: expression and catalytic activity of adenylyl cyclase, Journal of Pharmacology and Experimental Therapeutics 291 (1999) 503-510.

[206] J.L. Zeiders, F.J. Seidler, T.A. Slotkin, Ontogeny of regulatory mechanisms for β-adrenoceptor control of rat cardiac adenylyl cyclase: targeting of G-proteins and the cyclase catalytic subunit, J Mol Cell Cardiol 29 (1997) 603-615.

[207] H.S. Zhang, J. Liu, C.N. Pope, Age-related effects of chlorpyrifos on muscarinic receptor-mediated signaling in rat cortex, Archives of Toxicology 75 (2002) 676-684.

[208] P. Zimmet, K.G. Alberti, J. Shaw, Global and societal implications of the diabetes epidemic, Nature 414 (2001) 782-787.

Biography

NAME: Abayomi Alexander Adigun

DATE OF BIRTH &

BIRTH PLACE: March 12, 1979; Webster, Texas

EDUCATION: B.A., Cornell University, May 2001, Psychology, Cum laude

Ph.D., Duke University, Expected May 2010,

Pharmacology and Cancer Biology,

Certification in Toxicology

HONORS & AWARDS:

Society of Toxicology Annual Meeting --Student Platform Session Speaker, 2008

Steele Endowment Fund, 2008

Hammes Teaching Award committee member & chairman, 2007-Present

Medical Department Tuition Scholarship, 2006-Present

Nicholas School of Environment Graduate School Award, 2006-Present

Toxicology Award, 2006-Present

The National Dean's List, 2002

Thesis Honors, Psychology Cum laude graduate, 2001

Publications

A. A. Adigun, Ian T. Ryde, Frederic J. Seidler and Theodore A. Slotkin. Organophosphate Exposure During a Critical Developmental Stage Reprograms Adenylyl Cyclase Signaling in PC12 Cells. *Brain Research*, December 2009, Accepted for publication March 2010, *In Press.*

A. A. Adigun, Nicola Wrench, Frederic J. Seidler and Theodore A. Slotkin. Neonatal Organophosphate Exposure Alters the Developmental Trajectory of Cell Signaling Cascades Controlling Metabolism: Differential Effects of Diazinon and Parathion. *Environmental Health Perspectives,* 2010 Feb;118(2):210-5.

A. A. Adigun, Nicola Wrench, Edward D. Levin, Frederic J. Seidler, and Theodore A. Slotkin. Neonatal Parathion Exposure and Interactions with a High-Fat Diet in Adulthood: Adenylyl Cyclase-Mediated Cell Signaling in Heart, Liver and Cerebellum. *Brain Research Bulletin,* 2010 Apr 5;81(6):605-12. Epub 2010 Jan 14.

A. A. Adigun, Frederic J. Seidler and Theodore A. Slotkin. Disparate Developmental Neurotoxicants Converge on the Cyclic AMP Signaling Cascade, Revealed by Transcriptional Profiles *In Vitro* and *In Vivo*. *Brain Research*, 2010 Feb 26;1316:1-16. Epub 2009 Dec 21.

A. A. Adigun, Nicola Wrench, Frederic J. Seidler and Theodore A. Slotkin. Neonatal Dexamethasone Treatment Leads to Alterations in Cell Signaling Cascades Controlling Hepatic and Cardiac Function in Adulthood. *Neurotoxicology and Teratology*, 2009 Oct 20. [Epub ahead of print]