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The Effects of Polychlorinated Biphenyls on Dopaminergic PC12 Cells

presented by

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THE EFFECTS OF POLYCHLORINATED BIPHENYLS ON DOPAMINERGIC PC12 CELLS

By

William Gartley Robertson Angus

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ABSTRACT

THE EFFECTS OF POLYCHLORINATED BIPHENYLS ON DOPAMINERGIC PC12 CELLS

By

William Gartley Robertson Angus

Polychlorinated biphenyls (PCBs) are putative neurotoxicants. The dopaminergic model cell system PC12 cells were used to examine the hypothesis that subchronic exposure of dopaminergic cells to PCBs would result in a decrease in cellular dopamine that resulted from a decrease in the synthesis of dopamine, and manifested itself as a decrease in the evoked release of dopamine. Levels of cellular catechols in cells subchronically exposed to Aroclor 1254 or PCB congeners were determined by high performance liquid chromatography coupled to electrochemical detection (HPLC/ECD), and normalized to DNA content. The release of dopamine from subchronically PCB-exposed PC12 cells in response to 56 mM potassium was determined and normalized to DNA content. Results indicated that subchronic exposure of PC12 cells to Aroclor 1254 or PCB congeners resulted in dose-dependent decreases in cellular dopamine that manifested themselves as a decrease in the release of dopamine in response to depolarization with 56 mM potassium. The data from studies using PCB congeners indicated that the effects of PCBs on amounts of cellular

dopamine and dopamine released was specific for each congener. Gas chromatography coupled to electron capture was used to determine the amounts of PCBs in the cells at media concentrations of PCBs at which decreases in cellular dopamine and evoked release of dopamine were observed. The concentrations of PCBs in the cells increased as levels of dopamine decreased, however, there was a lag time between the association of PCBs with the cells and the decrease in dopamine. Possible mechanisms by which PCBs cause decreases in levels of dopamine were examined by measuring 1) the uptake of ^{3}H-tyrosine, 2) the synthesis of dopamine from ^{3}H-tyrosine, and 3) the ability of supplemental dihydroxyphenylalanine (DOPA) to be converted to dopamine. Aroclor 1254 did not cause a decrease in the uptake of ^{3}H-tyrosine, but did cause a decrease in the conversion of DOPA to dopamine, suggesting that one way in which Aroclor 1254 decreases cellular dopamine is by inhibition of the conversion of DOPA to dopamine by L-aromatic amino acid decarboxylase.

DEDICATION

I dedicate this publication to my wife, Tamera for her support and patience. I also dedicate this work to our unborn child and unborn children everywhere that they may not have to experience the consequences of exposure to environmental contaminants.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ALD DH	Aldehyde dehydrogenase
BDNF	Brain derived neurotrophic factor
BH_4	Tetrahydrobiopterin
COMT	Catechol-O-methyltransferase
DA	Dopamine
DBH	Dopamine β hydroxylase
DNA	Deoxyribonucleic acid
DOPA	L-3,4-dihydroxyphenylalanine
DOPAC	Dihydroxyphenylacetic acid
Ca/CAMPK	Calcium/calmodulin-dependent protein kinase
ECD	Electrochemical detection
EPI	Epinephrine
GC	Gas chromatography
GC/MS	Gas chromatography coupled to mass spectrometry
HPLC	High performance liquid chromatography
LAAAD	L-aromatic amino acid decarboxylase
LNAA	Large neutral amino acids
MAO	Monoamine oxidase
MPP^+	N-methyl-4-phenylpyridinium ion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NE	Norepinephrine
NGF	Nerve growth factor
OCN	Octachloronaphthalalene
OSS	Octyl sodium sulfate
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyl
PKA	Protein kinase A
PKC	Protein kinase C
PNMT	Phenylethanolamine-N-methyltransferase
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
TH	Tyrosine hydroxylase
5-HT	Serotonin
5-HTP	5-Hydroxytryptophan
6-OHDA	6-Hydroxydopamine

CHAPTER 1

INTRODUCTION

Polychlorinated Biphenyls

Polychlorinated biphenyls (PCBs) are chlorinated aromatic hydrocarbons consisting of two six-carbon aromatic rings joined at the 1-position with chlorine atoms attached in various combinations at the other 10 positions (Figure 1.1; for reviews see Safe 1984, 1994, 1990; Safe et al., 1987; Silberhorn et al., 1990; Hutzinger et al., 1974). There are 209 possible combinations of chlorine arrangements called congeners. These PCB congeners can be classified into two groups, coplanar and non-coplanar, based on the orientation of the two phenyl rings.

PCBs used in industry were marketed as mixtures of these congeners. For the U.S. and Great Britain, the Monsanto Company was the major producer of PCB mixtures, which were marketed under the trade name Aroclor. The various Aroclor mixtures were identified by a four digit code whereby 12 represented the code for chlorinated biphenyl. The second two digits, such as 54 or 60, indicated the approximate weight of chlorine in the compound as a percent of total weight. Hence, Aroclor 1254 consists of a mixture of chlorinated biphenyls with about 54% of its weight coming from chlorine atoms (Hutzinger et al., 1974). Further, the percentage of chlorine in Aroclor mixtures indicates the degree of congener chlorination in the mixture. For instance, Aroclor 1254 contains mostly tetra-, penta-, and hexachlorobiphenyls; whereas Aroclor 1260 contains mostly

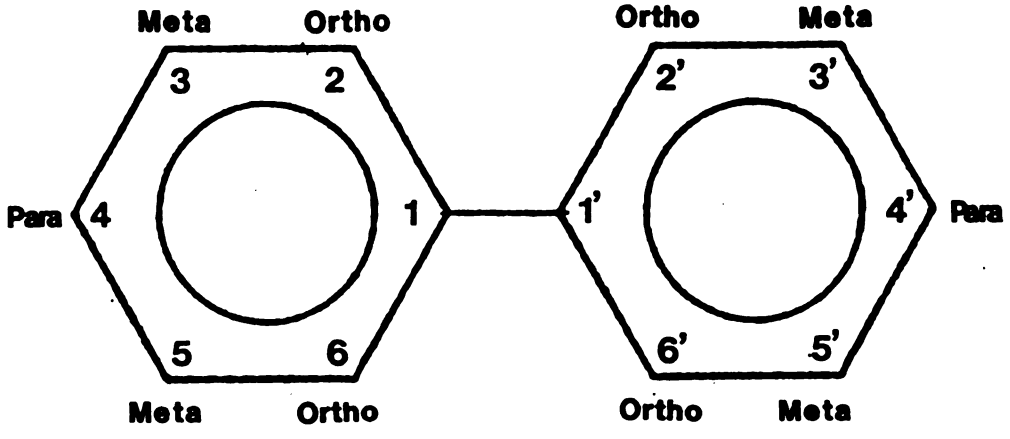


Figure 1.1 Structure of Polychlorinated Biphenyl

highly chlorinated congeners -- penta- through octachlorinated biphenyls (Hutzinger et al., 1974).

PCBs were used industrially until 1977 for such purposes as hydraulic fluids, plasticizers, heat transfer fluids, wax extenders, lubricants, dielectric fluids in capacitors and transformers, and sealants for farm silos. The properties that made these compounds appropriate for the above industrial uses, namely resistance to thermal breakdown, high dielectric current, hydrophobicity, and miscibility with organic solvents (Safe, 1984) have also allowed them to become persistent, bioaccumulative environmental contaminants.

Due to the widespread use of PCBs and ignorance of their toxic effects on biological systems, disposal of these compounds was unregulated and unsupervised during most of the timespan of their use. Consequently, substantial amounts of these compounds are currently circulating and redistributing throughout the environment (Nisbet and Sarofim, 1972; Safe et al., 1987). A geographic area of particular interest, due to large scale PCB contamination, is the Great Lakes. PCBs in sinks and sources in the lithosphere leach into ground water and runoffs, ending up in large rivers, inland lakes, and eventually the Great Lakes. PCBs in the hydrosphere can be taken up and bioaccumulated by fauna within the lake biosphere and then biomagnified up the food chain (Figure 1.2). Death and

Figure 1.2 Environmental Contamination by PCBs

PCBs can enter lakes and rivers through direct contamination, runoff, or atmospheric deposition. Once within the hydrosphere, one route of distribution that PCBs can undergo is to be bioaccumulated and biomagnified up trophic levels to top predatory species, which are in turn consumed by man. Other routes of distribution include removal from active circulation by sedimentation, or recirculation into the atmosphere through evaporation and windborne droplets containing particulates.
Figure taken from Safe et al., 1987.

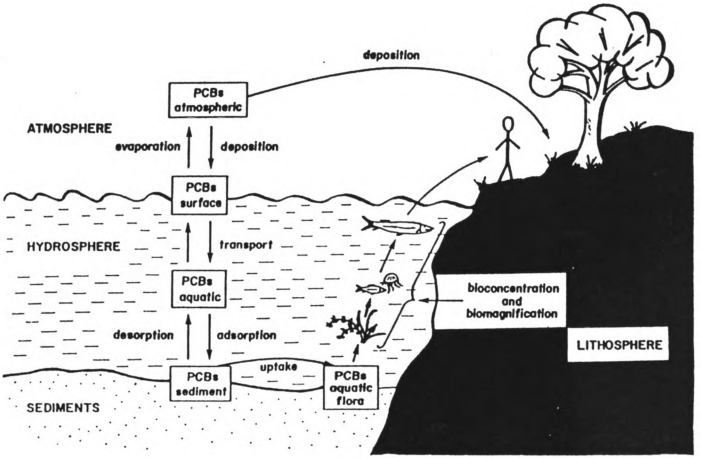

deposition
PCBs atmospheric
ATMOSPHERE
evaporation
deposition
PCBs surface
HYDROSPHERE
transport
PCBs aquatic
desorption
adsorption
uptake
PCBs sediment
PCBs aquatic flora
SEDIMENTS
bioconcentration and biomagnification
LITHOSPHERE

Figure 1.2

decay of aquatic organisms and their predators cause new sinks of PCBs to be formed, some of which become quiescent and result in removal of PCBs from active circulation, others of which are again redistributed throughout the ecosystem. Further, evaporation from contaminated bodies of water (the Great Lakes, for example) and from contaminated terrestrial sinks allows for airborne deposition and redistribution of PCBs into areas not previously contaminated. The net result of this airborne distribution pattern is that there is no place on earth where one can escape being exposed to PCBs (Risebrough and deLappe, 1972; Parkinson and Safe, 1987).

PCBs can gain access to the body through dermal absorption, inhalation, or ingestion. Currently, the most common means of exposure to the general public is through ingestion of foods contaminated with PCBs. This is especially true in the Great Lakes region where game fish such as trout and salmon are reported to have bioaccumulated relatively high levels (Swain, 1983). Once in the body, the more lightly-chlorinated congeners (di- through tetrachlorobiphenyls) are readily metabolized by the liver and excreted. However, highly-chlorinated congeners, those with five or more chlorine atoms, tend to be deposited in adipose tissue and other tissues with high lipid content, and can be highly concentrated in human milk.

The toxicity caused by PCBs is dependent upon age, sex,

and species of the animal, as well as the congeners involved (Safe, 1990). PCBs are not acutely toxic, except in very high doses (Safe, 1984). There is increased toxicity with increased duration of exposure to PCBs. Further, there is a latent period between PCB exposure and onset of the symptoms of toxicity (Parkinson and Safe, 1987). Some symptoms appear after a few days or weeks, while other symptoms become apparent only after months of exposure. In addition to neurotoxic effects, which will be discussed in more detail later, some of the toxic effects of PCBs that have been described in humans and animals are listed below (Parkinson and Safe, 1987):

1. A wasting syndrome, entailing progressive weight loss that unrelated to a decrease in food intake.

2. Skin disorders such as hyperpigmentation and chloracne. Dermal lesions are regarded as some of the most important and characteristic signs of PCB intoxication.

3. Hyperplasia of the epithelial lining of the extrahepatic bile duct, gall bladder, and urinary tract.

4. Lymphoid involution or atrophy of the thymus and spleen that is accompanied by immunosuppression and bone marrow and hematic dyscrasias. Immunotoxic symptoms of PCBs include decreased resistance to host infections, depressed T-cell response to mitogens, and

delayed hypersensitivity.

5. Hepatomegaly and liver damage, which varies with age, sex, and species. Some signs of hepatotoxicity include proliferation of smooth endoplasmic reticulum, focal necrosis of hepatic parenchyma, and increases in cytoplasmic lipid droplets.

6. Porphyria.

7. Endocrine and reproductive dysfunctions such as altered plasma steroid and thyroid hormone levels; menstrual irregularities, increased incidence of spontaneous abortion, hemorrhage and anovulation in females; and testicular atrophy and decreased spermatogenesis in males.

8. Teratogenesis, including cleft palate and kidney malformations.

9. Carcinogenesis, namely hepatocarcinoma.

Studies on the structure-activity relationships of PCBs have shown that coplanar congeners with chlorine substitutions at the *para*-position, or *para*- and *meta*-positions appear to be toxic in the same manner as 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD; Hansen, 1987). Coplanar congeners can interact with the cellular receptor for 2,3,7,8-TCDD, the Ah receptor. The Ah receptor has been shown to mediate the immunotoxic effects of PCBs (Parkinson and Safe, 1987). Most of the other known adverse effects brought about by PCB exposure are also believed to be

mediated through the Ah receptor. PCB congeners with *para*-, or *para*- and *meta*-chlorine substitutions are more likely to have both phenyl rings in the same plane and thus are more apt to fit into the 2,3,7,8-TCDD binding site on the Ah receptor. However, addition of one or more chlorines in the *ortho* position decreases the likelihood that both phenyl rings will remain in the same plane, thus reducing the ability of the PCB congener to bind to the Ah receptor. Interestingly, coplanar congeners are usually only present in very low levels in PCB mixtures, such as those contaminating the environment. Mono- and di*ortho*-substituted analogues, however, are prevalent in these PCB mixtures.

PCBs are differentially metabolized in the human body. Lightly-chlorinated congeners tend to be metabolized more rapidly, usually to mono- or dihydroxylated metabolites that are excreted in the urine and feces (Safe, 1984). Highly-chlorinated congeners are strongly lipophilic and quite resistent to metabolic breakdown.

Neurotoxic Effects of PCBs

Acute exposure of humans to high levels of heat-degraded PCBs has occurred in Japan in 1968 (the Yusho poisoning) and in Taiwan in 1979 (the Yu Cheng poisoning), and chronic exposure to PCBs has been described for workers in industries manufacturing and using PCBs. Children born to Yusho victims demonstrated retarded growth, abnormal

tooth development, hyperpigmentation, small birth size and lower birthweight (Safe, 1987). Yu-Cheng children have been described as being stunted in growth, delayed in developmental milestones, displayed deficits in developmental testing and demonstrated abnormalities in behavioral assessments (Rogan et al., 1988; Guo et al., 1994). The Yu-Cheng children further displayed continuing cognitive deficits at 4 to 5 years of age (Chen et al., 1992). Interestingly, children born to Yu-Cheng mothers up to 6 years after exposure had ceased also displayed cognitive deficits similar to those seen in siblings exposed *in utero* in 1979. A proviso with respect to the data collected on Yusho and Yu-Cheng victims is that the heat-degraded PCBs also contained other halogenated species related to PCBs, such as polychlorinated dibenzofurans.

Furthermore, it was suggested that children born to women consuming PCB-tainted Great Lakes fish also had small birth size and low birthweight (Jacobson et al., 1983, 1984; Fein et al., 1984). The observed effects of PCBs on the offspring of PCB-exposed women demonstrate that PCBs exert deleterious effects on the developing embryo. PCBs can cross the placenta from the maternal to the fetal bloodstream and affect the developing embryo. Milk is another source of infant exposure, as PCBs are concentrated in milk.

A number of studies have been conducted to evaluate the

effects of PCBs on the developing nervous system. These studies included the use of rats, monkeys, human epidemiological surveys, and cell cultures.

Children born to women consuming PCB-contaminated fish to those born to "non-fish eating" women were compared for the purpose of examining cognitive abilities and behavior (Jacobson et al., 1983). Deficits were noted in visual recognition memory as measured by Fagan's test (a measure of infant response to novel visual stimuli) in seven-month old infants exposed *in utero* to PCBs (Jacobson et al., 1985). There was a dose-dependent relationship between poorer scores in Fagan's test and levels of intrauterine exposure to PCB, as determined by PCB levels in the umbilical cord serum. An effect on visual recognition memory in infants exposed to PCB via lactation was not seen. These data suggest that intrauterine exposure to PCBs may have effects on neurological development. These same children were again tested at 4 years of age for PCB-related deficiencies (Jacobson et al., 1990). Intrauterine exposure to PCBs was associated with lower scores in both the verbal memory and numerical (quantitative) memory. Interestingly, PCB exposure via lactation was positively related to memory and verbal scale performance, indicating that there is no adverse effects on verbal and quantitative memory from exposure to PCBs in milk. Thus, it was suggested that intrauterine exposure to PCBs is associated with poor visual

recognition memory, and poor verbal and quantitative abilities.

Another epidemiological study (Rogan et al., 1986) reported that infants exposed to PCBs *in utero* demonstrated hypotonicity and hyporeflexia in a dose-dependent relationship. Furthermore, higher transplacental exposure was associated with lower psychomotor scores at both 6 and 12 months of age (Gladen et al., 1988). There was no relationship between these deficits and nursing exposure, again suggesting that PCB-mediated deficits are due primarily to *in utero* exposure to PCBs. By 2 years of age, these children no longer displayed any deficiencies (Gladen and Rogan, 1991). These data directly contradict the results published by Jacobson et al. (1990), leaving the matter unresolved as to the long-lasting effects of PCBs on young children.

Taken together, the results from the above studies suggest the possibility that intrauterine, transplacental exposure to PCBs does affect human neurological and behavioral development. Therefore, maternal exposure to PCBs does affect the neurological development of humans.

Studies were done with rats to further examine the transfer of PCBs from mother to offspring. Accumulation of ^{14}C-labeled PCB was measured in rat dams and pups given an oral dose of 10 mg/kg Kaneclor 600 (a PCB mixture similar to Aroclor; Takagi et al., 1986). The dams accumulated 44% of

the total dose given, while pups accumulated only 0.003%. Newborn and suckling rat pups' concentrations of PCBs had the following distribution -- liver > heart > skin > muscle > blood > lung > brain. PCB concentrations then decreased upon weaning. Congener accumulation of Aroclor 1254 in rat pups has also been investigated (Shain et al., 1986), and it was determined that the maternal to fetal PCB transfer was not correlated simply with physical and chemical properties of PCBs. Bioaccumulation of congeners in the brain was not correlated with extent of chlorination, suggesting that the number of chlorines did not influence entry into the brain as much as other characteristics of PCB congeners. These investigators suggested that accumulation of congeners is associated with chlorination of the 2 and 4 positions (ie. *ortho* and *para*) of the second phenyl ring, but not with chlorination of the 2 and 5, or 2 and 3 positions (ie. *ortho* and *meta*).

Experiments performed on rats relating ingestion of PCB mixtures to effects on the amounts of brain dopamine (Seegal et al., 1986a, 1991b), suggesting that when a dose of Aroclor 1254 or 1260 equal to 1/10 of the rat LD_{50} was administered by gastric lavage, the levels of dopamine and dihydroxyphenylacetic acid (DOPAC), a dopamine metabolite, were significantly decreased in the caudate region of the brain, but not in the lateral olfactory lobe. When similar doses of Aroclor 1254 were mixed with chow and fed to rats

for 30 days as a chronic exposure, dopamine concentrations in the striatum and lateral olfactory tract were decreased. No decreases in dopamine were seen in other areas of the brain. These data suggest that more brain regions are affected by chronic doses of PCBs than are affected by a single acute dose.

In non-human primates, PCBs also affect behaviors and amounts of dopamine in the brain. After seven months on PCB-containing diets (either 2.5 ppm or 5 ppm Aroclor 1248) infants that were born to a group of rhesus monkeys and survived tended to be hyperactive and did not learn reversal tasks as readily as the control animals, and this altered behavior persisted for two years (Allen et al., 1979). Infants born to these same female monkeys one year after removal from PCB-laced diets showed similar characteristics to those born during the time of the PCB-containing diets, suggesting that PCBs can remain within maternal adipose stores and affect offspring even after direct exposure to PCBs has ceased for a length of time. Work by other investigators (Bowman et al., 1978, 1981; Bowman and Heironimus, 1981) revealed that simian infants born to PCB-exposed mothers had low birthweights and were hyperactive up to 12 months of age; but became hypoactive by 44 months of age. The *in utero* PCB-exposed offspring demonstrated deficits in spatial and color discrimination reversal tasks, and this retarded learning was correlated to the level of

PCB exposure *in utero*. Since the effects of PCBs on learning in animals were observed using complex discrimination tasks and schedule-controlled performance in monkeys, these findings indicate that it is the higher, cognitive levels of CNS function that are disturbed by exposure to PCBs.

Exposure of adult monkeys to Aroclor 1016 (bi- to tetrachlorobiphenyls) resulted in decreases in dopamine in the caudate, putamen, substantia nigra, and hypothalamus (Seegal et al., 1990) that lasted for up to 44 weeks after exposure to PCBs ended (Seegal et al., 1994). Interestingly, three *ortho*-substituted congeners (2,4,4'; 2,2',4,4'; and 2,2',5,5') accounted for most of the PCBs that were accumulated in the brains of these animals (Seegal et al., 1990). Hence, the data suggested that *ortho*-substituted PCBs can gain access to various brain regions and decrease the dopamine content. Again, monkeys were used to determine effects of different PCB mixtures (Aroclors 1016 and 1260) on brain dopamine levels (Seegal et al., 1991a). With Aroclor 1016, decreases in dopamine were again seen in the caudate, putamen, substantia nigra, and hypothalamus, and the same three congeners were seen to accumulate in these brain regions. Yet, when the mixture Aroclor 1260 (mostly pentachlorobiphenyls and larger) was used, only in the caudate, putamen, and hypothalamus were decreases in dopamine levels observed. However, with

Aroclor 1260, the total amount of bioaccumulation of PCBs was much greater than with the 1016. Further, of the congeners making up greater than 5% of total brain PCB residue, most were hexa- or heptachlorinated di-*ortho*-substituted congeners. These data support the notion that *ortho*-substituted congeners are 1) more likely to accumulate in the brain (in agreement with the results of Shain et al. (1991)), and 2) likely to be responsible for changes in brain dopamine levels. Supporting the hypothesis that *ortho*-substituted congeners are the effectors of decreased brain dopamine are studies on the structure-activity relationships of the neurotoxic effects of PCBs in PC12 cells (Shain et al., 1991). Congeners with *ortho*- or *ortho*- and *para*-substitutions were reported to be the most potent in decreasing cellular dopamine. Substitutions in the *meta*-position reportedly enhanced the neurotoxic effects of *ortho*- , but not of *ortho*- and *para*-substituted congeners. Congeners structurally similar to 2,3,7,8-TCDD, such as 3,3'4,4'-tetrachlorobiphenyl and 3,3',4,4',5'-pentachlorobiphenyl, did not cause a decrease in cellular dopamine, suggesting that the Ah receptor does not mediate the effects of PCBs on brain dopamine content. Furthermore, increasing congener chlorination did not correlate with a decrease in potency. Only when one ring of the biphenyl was completely chlorinated, was there a definite decrease in potency, suggesting that the positions of chlorination

rather than the number of chlorines is important in mediating the neurotoxicity of PCBs. Furthermore, the parent PCB congeners, and not their metabolites, were the active mediators in reducing dopamine levels (Shain et al., 1991).

To determine whether the three specific congeners that accumulated in the brains of PCB-treated adult monkeys (2,4,4'; 2,2',4,4'; and 2,2',5,5') were responsible for the decreased amounts of dopamine in specific brain regions, they were applied to rat pheochromocytoma PC12 cells, a model dopaminergic system (Seegal et al., 1990). These congeners caused a decrease in cellular dopamine content. However, application of TCDD-like congeners failed to elicit the decrease, confirming that the Ah receptor is not involved in this toxicity. Aroclor 1254 also produced a dose-dependent decrease in cellular dopamine in PC12 cells in comparison to vehicle-treated control cells (Seegal et al., 1989). These findings suggested that PCBs may affect the dopamine-containing PC12 cells in the same way that they affect the dopaminergic neurons in the brain.

The evidence compiled from human, animal, and cell culture studies suggest that PCB mixtures cross the placenta into the developing embryo, enter neurons or their progenitors, and affect the neurological development of the organism.

PC12 Cells

PC12 cells were isolated by Greene and Tischler (1976) from a solid rat pheochromocytoma of a New England Hospital Deaconess white rat. These small (6-14 μ) round cells with a doubling time of 48 to 96 hours, contain acetylcholine, and various catecholamines (dopamine and norepinephrine) in separate granular chromaffin-like vesicles. The catecholamines are secreted in a calcium-dependent manner in response to depolarization or nicotinic or muscarinic cholinergic agonists (for reviews, see Guroff, 1985; Fujita et al., 1989). PC12 cells contain the synthetic enzymes for catecholamines: tyrosine hydroxylase (TH), L-aromatic amino acid decarboxylase (LAAAD), dopamine beta hydroxylase (DBH), and phenylethanolamine N-methyltransferase (PNMT). Metabolizing enzymes are also present: monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT). Dopamine is the primary catecholamine in PC12 cells.

Addition of nerve growth factor (NGF) to PC12 cells causes a morphological and physiological transformation of the cells into "sympathetic-like neurons". The changes brought about by NGF provide a basis by which the PC12 cell line may be used as a model system to study neuronal differentiation (Guroff, 1985) and effects of neurotoxins (Shafer and Atchison, 1991). NGF is a neurotrophic protein that can be isolated from male mouse submaxillary glands. The 7S form of NGF, contains alpha, beta, and gamma subunits

and has a molecular weight of about 140,000 KDa. The 2.5S form, which has biological activity, is a dimer of noncovalently linked beta subunits with a molecular weight of approximately 26,000 KDa.

When PC12 cells are exposed to NGF, a number of remarkable phenomena occur. These events can be categorized into immediate early (within seconds), delayed early (within minutes to hours), and late events (hours to days). One of the earliest changes seen in PC12 cells exposed to NGF is a ruffling of the membrane, followed by changes in membrane properties that alter the ability of the cells to adhere to plastic substrates. Other early events that occur with NGF administration include the transcription-dependent induction of the protooncogenes c-fos, c-jun, v-src, and H-ras and N-ras. It is believed that the induction of the protooncogene proteins play an important role in later events linked to NGF exposure. Together with protooncogene induction come changes in ion fluxes, alterations in second messenger systems such as changes in the activities of cyclic AMP-dependent protein kinase (PK-A) and protein kinase C (PK-C), activation or increased levels of cytoskeleton-associated proteins such as GAP 43, and cytoskeletal rearrangements. Patterns of protein phosphorylation are also altered.

Later changes in the PC12 cells caused by NGF exposure include the outgrowth of neurites. This change is usually apparent at 24 to 48 hours after NGF addition. Upon

continued exposure to NGF, the neurites become extensive and branched. By 14 days of NGF exposure, PC12 cells have obtained a fully differentiated sympathetic-like neuronal phenotype, demonstrating similar properties to those of a neuron, such as electrical excitability and release of neurotransmitters in response to electrical stimulation or administration of agonists. Removal of NGF from the medium results in retraction of the neurites, however, readdition of the factor causes their immediate reissuance.

NGF binds to two receptor types located on the surface of the PC12 cell, a low- affinity NGF receptor which has a Kd value of approximately 1 x 10^{-9} M, which is not believed to mediate the biological response to NGF; and a high-affinity NGF receptor, with a Kd of approximately 1 x 10^{-11} M, which is considered the biologically relevant form of the receptor. The NGF high-affinity binding protein is the *trk* protooncogene product, $pp140^{trk}$, which has tyrosine kinase activity (Kaplan et al., 1991a,b; Ohmichi et al., 1991; Klein et al., 1991). The $pp140^{trk}$ is distinct from the low-affinity receptor defined by Green and Greene (1986) as demonstrated by immunoprecipitation studies, indicating that the $pp140^{trk}$ is not the low-affinity NGF receptor with an associated protein (for review see Ross, 1991). At the present time, there is great literature debate as to the roles of the high and low affinity receptors in differentiation of neuronal cells.

Dopamine as a Neurotransmitter

The neurotransmitter dopamine appears to be highly integrated in many types of behaviors and in cognition. Dopaminergic projections within the CNS are extensive and occur in, among other regions, the neostriatum, limbic regions, and cortex (Cooper et al., 1991; Graybiel et al., 1994). Dopamine is associated with various behaviors, including: sensorimotor control and learning (substantia nigra, striatum, nucleus accumbens; Cousins et al., 1993; Aosaki et al., 1994), hyperactivity and hypoactivity (mesolimbic, substantia nigra, striatal, nucleus accumbens; Richardson and Tizabi, 1994), motivation and reinforcement (nucleus accumbens; Salamone, 1994), behavioral inhibition (prefrontal cortex), grooming (Francis et al., 1994), aggression (Micsek et al., 1994; Francis et al., 1994), lordosis (striatum; Pednekar and Mascarenhas, 1993), food and water seeking behaviors (caudate and nucleus accumbens; Pal and Thombre, 1993). Dopamine has recently also been implicated in cognition and learning (Graybiel et al., 1994; Crossman, 1987; Albin et al., 1989; Malpaini et al., 1994). Many of the deficits observed in children involved in epidemiological studies on prenatal exposure to PCBs include decreased cognitive and motor functions, suggesting that the deficits in their behaviors may well have a dopaminergic component to them. Additionally, the data obtained on decreased brain dopamine content in prenatally exposed mice

(Agrawal et al., 1981) support the hypothesis that some of the neurological deficits associated with prenatal PCB exposure have at least partial underlying dopaminergic components. However, this is not to suggest that all of the deficits seen due to an *in utero* exposure to PCBs can be attributed solely to a decrease in basal ganglionic dopamine content. Effects of PCBs on other transmitter systems such as acetylcholine and neuropeptides warrant investigation. Further, both prenatal and postnatal exposure to PCBs have been reported to affect endocrine the system (Safe, 1994). The endocrine system plays an important role in the development of the nervous system, and thus some alterations in endocrine function may also play a role in the cognitive and behavioral deficits associated with prenatal PCB exposure. Investigations into this possibility have not yet been reported.

Dopamine Synthesis and Metabolism in PC12 Cells

Changes in dopamine content and synthesis are at the crux of this thesis, therefore, a discussion of them is in order. Figure 1.3 depicts a generic dopaminergic cell. The synthesis of catecholamines involves several reactions. Tyrosine is transported into the cell where the phenyl ring is hydroxylated by TH to form 3,4-dihydroxyphenylalanine (DOPA). The formation of DOPA by TH is the rate-limiting step in the synthesis of catecholamines. DOPA is then decarboxylated by L-aromatic amino acid decarboxylase

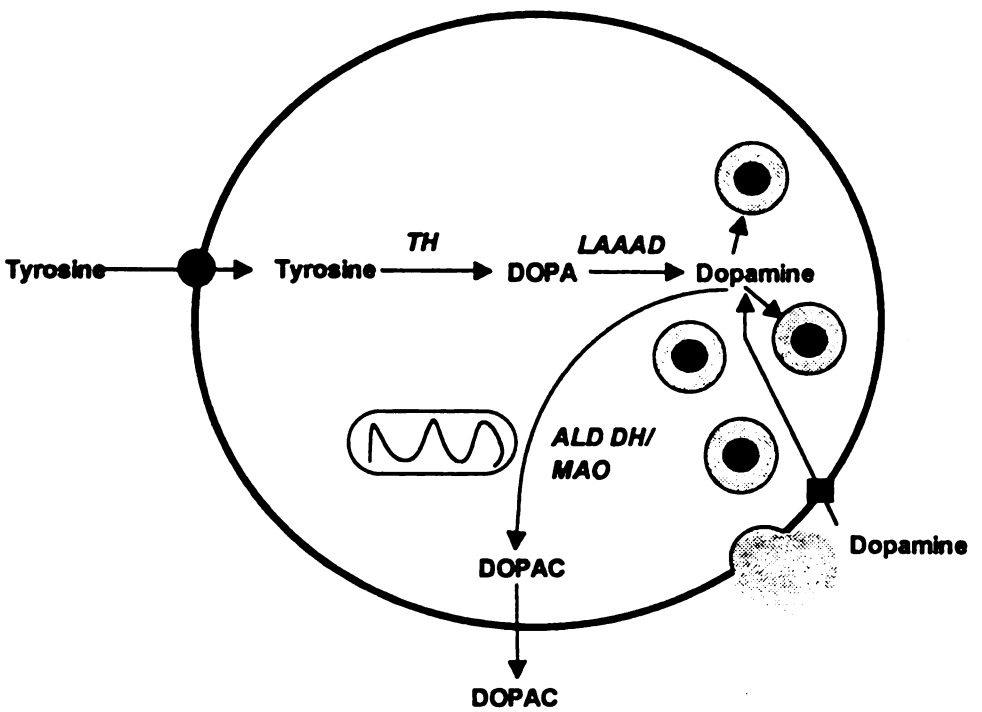


Figure 1.3 Synthesis of dopamine

The amino acid precursor, tyrosine, is actively taken up into the cell where it is hydroxylated by the rate limiting enzyme, tyrosine hydroxylase (TH) to form 3,4-dihydroxyphenylalanine (DOPA). DOPA is decarboxylated by the cytosolic L-aromatic amino acid decarboxylase (LAAAD) to form dopamine. Dopamine may then be packaged into dense core vesicles for release in response to a depolarizing stimulus, or acted upon by an aldehyde reductase (ALD DH) and monoamine oxidase (MAO) associated with the mitochondria to form dihydroxyphenylacetic acid (DOPAC). Each enzyme along the biosynthetic pathway requires a cofactor, for TH, this is tetrahydrobiopterin (BH_4); for LAAAD, the cofactor is pyridoxal phosphate.

(LAAAD) to form dopamine. Dopamine is then taken up into storage vesicles where it may be hydroxylated at the beta position to form norepinephrine by dopamine β hydroxylase (DBH), which is located primarily in the vesicular membrane. Each of the enzymes involved in catecholamine biosynthesis require cofactors. Tyrosine hydroxylase requires tetrahydrobiopterin (BH_4), while LAAAD requires the presence of pyridoxal phosphate. Ascorbate is the cofactor required for DBH activity. In PC12 cells, dopamine is the primary catecholamine since the level of ascorbate is low in these cells (Fujita et al., 1989).

A number of factors can affect the rate of catecholamine synthesis. Cell density can have an effect on TH and DBH levels in PC12 cells (Badoyannis et al., 1991). TH, TH mRNA, and dopamine levels increase when cells are cultured at a high density. However, when PC12 cells are recultured from high to low density, the dopamine, TH, and TH mRNA levels decrease. In contrast, the DBH mRNA level decreases at high density, thereby decreasing the production of norepinephrine.

Data from the work published by Seegal et al. (1989) indicate that the release and degradation of dopamine is not increased in PC12 cells acutely exposed to PCBs, suggesting that the PCB-mediated decrease in dopamine may not be due to decreased synthesis. The levels of DOPA were not reported to be elevated in PC12 cells treated with Aroclor 1254,

suggesting that L-aromatic amino acid decarboxylase is not inhibited by PCB exposure (see Seegal et al., 1989). Inhibition of LAAAD by PCBs would have resulted in DOPA accumulation. The amounts of cellular norepinephrine and dopamine in acutely Aroclor 1254-exposed cells decreased in a parallel fashion, indicating that in the PCB-treated cells, DBH activity was not altered (Seegal et al., 1989). If DBH activity was impaired, dopamine would have increased, due to a slower rate of conversion of dopamine to norepinephrine. Therefore, since the activities of LAAAD and DBH have been reported to be unaffected by exposure to PCB, it appears, based on the reports of Seegal et al., that PCB-mediated reduction in dopamine may be due to inhibition of TH activity. However, results of studies by Seegal et al. omit the possibility that the decrease in levels of cellular dopamine may be due to an alteration in the uptake of tyrosine, the amino acid precursor for dopamine.

In order to be taken up into dopaminergic cells, tyrosine must cross the blood-brain barrier and the neuron plasma membrane. The transport of tyrosine across the blood-brain barrier is active (Guroff et al., 1961) and thought to be facilitated, in part, by a Na^+-independent transporter designated system L (Wiesel et al., 1991). This transporter also transports large neutral amino acids (LNAA). In addition to system L, another Na^+-independent amino acid carrier which prefers aromatic amino acids,

designated system T, may also play a role in controlling the uptake of tyrosine into the brain and nerve cells (Stewart et al., 1989). Several studies using synaptosomes isolated from rat brain as a model have suggested that there are multiple transport routes for tyrosine into neurons. Work by Aragon et al. (1981) suggests that the uptake of tyrosine into an osmotically active space was dependent on a Na^+ gradient, and was stimulated by a membrane potential. Kinetic data suggested two uptake systems with different affinities for tyrosine. Expanding on the findings of Aragon et al. (1981), Diez-Guerra and colleagues (1986, 1989) indicated that L-tyrosine enters the cell through three pathways, each with different K_m values, and each with different roles in the regulation of cellular tyrosine levels. The authors suggested that the transporter with the lowest K_m (6 μM) may play a regulatory role in catecholamine biosynthesis (Diez-Guerra et al., 1986).

Historically, it has been believed that tyrosine hydroxylase activity is the rate-limiting factor in the synthesis of dopamine and other catecholamines. However, this may not always be the case. In the last twenty or so years, numerous researchers, led by Drs. Richard Wurtman and John Fernstrom, have published data suggesting that availability of aromatic amino acid precursors for various neurotransmitters may be rate limiting for synthesis of neurotransmitters, in some situations. This precursor-

dependent limitation to neurotransmitter synthesis has been well established for serotonin (5-HT) and its precursor tryptophan (Carlsson and Lindqvist, 1978, Fernstrom, 1983). For dopamine and its precursor tyrosine, the relationship is more convoluted.

There have been some governing principles put forth by Wurtman (1984) relating the synthesis of neurotransmitters such as serotonin, acetylcholine, and the catecholamines, and the plasma levels of their precursors: "1. The limiting step in the biosynthesis of the transmitter must be catalyzed by a low-affinity enzyme . . . tyrosine hydroxylase -- which at normal substrate concentrations, is unsaturated with the precursor. 2. This enzyme must not be subject to significant end-product feed-back control when the neuron containing it is fired. 3. The amount of the enzyme's substrate (the neurotransmitter precursor) present within the neuron must depend on its concentration in the plasma, either because the nerve terminal is unable to make the precursor ... and obtains it solely by *influx* from the plasma, or because, even though the neuron can synthesize the precursor ... , it tends to lose it by efflux into the plasma at a rate that varies inversely with the precursor's plasma concentration. 4. A mechanism must exist which facilitates the precursor's passage from the bloodstream to the brain, and vice versa (i.e., a blood-brain barrier transport system); moreover, the affinity of

this mechanism for the circulating precursor must ... be relatively *low*. ... 5. Plasma levels of the precursors must, in fact, *change* under normal conditions." Indeed, it has been demonstrated that these principles do apply to the catecholamines and their precursor, tyrosine (Lehnert and Wurtman, 1993; Wurtman, 1984; Fernstrom, 1983; Wurtman et al., 1981; and Wurtman and Fernstrom, 1976).

Traditionally, TH is believed to be saturated with tyrosine, under normal conditions in the brain, and since TH has a slower rate than LAAAD, is the rate limiting step in catecholamine synthesis. Many studies have demonstrated that tyrosine availability can be rate-limiting for catecholamine synthesis, but only under conditions where the neurons in question are rapidly firing, as in normally highly active, catecholaminergic brain regions, or in catecholaminergic neurons responding to a pharmacological challenge. This condition of rapid firing of the neuron results in phosphorylation of TH, thus decreasing the K_m for the cofactor, BH_4, and increasing the K_i for end-product inhibition. This, in effect, causes TH to perform at a maximal rate, thus making tyrosine availability rate limiting (see Fernstrom, 1983; Milner and Wurtman, 1986; Wurtman, 1988; and Lehnert and Wurtman, 1993). However, recent data have suggested that high activity is not necessarily required for tyrosine availability to have effects on catecholamine synthesis and release. Using

microdialysis, *in vivo* studies have shown that tyrosine availability can affect unstimulated catecholamine release in the brain (Lehnert and Wurtman, 1993; Kreutz et al., 1990; and Acworth et al., 1988).

Thus, there are many avenues for polychlorinated biphenyls to disrupt the synthesis of dopamine. For instance, PCBs can alter the uptake of tyrosine through a variety of mechanisms, namely, competitive or non-competitive inhibition of amino acid uptake, or disruption of the uptake protein subsequent to increased fluidization of the membrane. One report (Wurtman et al., 1974) suggested that there is not a linear relationship between cellular tyrosine levels and DOPA formation, that a small decrease in cellular tyrosine can lead to a sizable decrease in DOPA synthesized. Hence, a small disruption of tyrosine uptake by PCBs could lead to a serious decrease in levels of cellular dopamine. Another route for PCBs to cause decreased dopamine synthesis is by affecting the activity of TH. TH is a tetrameric enzyme with a molecular weight of approximately 210-220 KDa with individual subunits having a molecular weight of approximately 62,000 KDa (Markey et al., 1980). Iron and BH_4 are the cofactors needed for TH activity (Wang et al., 1991). TH activity can be inhibited by dopamine through a negative feedback loop. Phosphorylation of the TH enzyme has been reported to cause a decrease in the K_m for BH_4 and an increase in the K_i for

dopamine (Albert et al., 1984; Haycock and Haycock, 1990), resulting in an increase in TH activity. The phosphorylation of TH can be catalyzed by several protein kinases. Agents activating PKA, PKC, and calcium/calmodulin-dependent protein kinase (Ca/CAMPK) increase phosphorylation of TH at Serine 40 (Campbell et al.,1986; Haycock, 1990). Ca/CAMPK phosphorylates an additional site as well (Campbell et al., 1986), which as been reported to be Serine 19 (Haycock, 1990). Vulliet et al. (1989) have also described a proline-directed kinase capable of phosphorylating TH at Serine 8.

Theoretically, PCBs could alter the K_m for BH_4 or alter the K_i for dopamine. Alterations in K_m are likely to come from alterations in the phosphorylation of the enzyme. Phosphorylation by a calcium/calmodulin-dependent protein kinase enhances the activity of tyrosine hydroxylase without altering the kinetic association constants. However, a cAMP-dependent protein kinase may phosphorylate the enzyme, resulting in decreased K_m for BH_4, and increased K_i for dopamine which leaves the enzyme activity dependent on the amount of tyrosine available to be utilized (Lehnert and Wurtman, 1993). It is possible that PCBs may affect the activity of tyrosine hydroxylase by competing either with substrate (tyrosine), BH_4, or dopamine, thereby disrupting the kinetics and regulation of the enzyme. Finally, PCBs may disrupt dopamine synthesis by altering the activity of

LAAAD, which converts DOPA to dopamine. LAAAD is a dimer with a molecular weight of approximately 100 KDa (Coge et al., 1989). LAAAD converts DOPA to dopamine, and also 5-hydroxytryptophan (5-HTP) to serotonin (5-HT). For activity, LAAAD requires the presence of pyridoxal pyrophosphate, B_6, with a K_m of approximately 0.28 μM (Young et al., 1993; Hadjiconstantinou et al., 1993). The V_{max} of LAAAD for DOPA is about 37 nmol/mg protein/20 min, while the K_m for DOPA is around 32 μM (Young et al., 1993; Hadjiconstantinou et al., 1993). Neuronal and non-neuronal isoforms of the enzyme have been reported (Morgan et al., 1986, Krieger et al., 1991), although there appears to be only one gene (Albert et al., 1987), indicating possible tissue-specific alternative splicing of the RNA. However, at this time, there appears to be debate in the literature about how many forms of decarboxylase exist (see Melamed et al., 1980 and Sims et al., 1973, reviewed in Ebadi and Simonneaux, 1991). The activity of LAAAD appears to be regulated acutely by PKA, resulting in a change in V_{max}, without alterations in K_m values (Young et al., 1993). Both D_1 and D_2 receptors have been reported to chronically regulate striatal LAAAD. Antagonists for the dopamine receptors increase the activity of striatal LAAAD, while agonists decrease the activity (Hadjiconstantinou et al., 1993). The increase in activity caused by dopamine receptor antagonists appears to be caused by an increase in V_{max}, and

not alterations in K_m values.

Hence, PCBs could have effects on any of the enzymes involved in dopamine synthesis or on the uptake of tyrosine into cells. Inhibition of tyrosine uptake or dopamine synthesis could result in a decrease in the release of dopamine since it has been shown that newly synthesized catecholamines are preferentially released (Kopin et al., 1968; Besson et al., 1969; Gewirtz and Kopin, 1970). Another deficit in the understanding of the significance of PCBs causing a decrease in the amount of cellular dopamine is information on a functional significance of the decrease. Because cellular levels of dopamine decline, is this enough to manifest an alteration in the release of dopamine? At this time, the effects of polychlorinated biphenyls on the release of dopamine are unknown.

Hypothesis and Aims

Polychlorinated biphenyls have been reported to be associated with various neurological deficits in animals and humans, some of which may have a dopaminergic component. Acute studies using dopaminergic cell culture systems have suggested that PCBs cause decreases in cellular dopamine, and that *ortho* substituted PCB congeners play a major role in the deleterious effects of PCBs on dopaminergic cells. The purpose of these studies was to examine the subchronic effects of polychlorinated biphenyls on the dopaminergic function of PC12 cells. Specifically, the goal was to

examine the hypothesis that polychlorinated biphenyls cause decreases in levels of cellular dopamine in PC12 cells that result from altered synthesis of dopamine, and is manifested as a decrease in the release of dopamine in response to a depolarizing stimulus. The scope of the research included several specific aims:

- Determine the subchronic effects of Aroclor 1254 and selected PCB congeners on cellular dopamine content, using undifferentiated and differentiating PC12 cells as a model system.
- Determine whether the neurotrophic factor, nerve growth factor can protect dopaminergic cells from damage caused by subchronic exposure to Aroclor 1254, using PC12 cells as a model system.
- Determine whether subchronic exposure of PC12 cells to Aroclor 1254 and selected PCB congeners results in altered release of dopamine in response to stimulation with 56 mM potassium, and whether the PCB-mediated decreases in cellular dopamine contributed to any alterations observed.
- Determine the amounts of Aroclor 1254 or PCB congeners associated with PC12 cells after a subchronic exposure, and whether the amounts of PCBs associated with the cells relate to the effects seen in the cells after subchronic exposure to PCBs.
- Examine possible mechanisms to explain the decreases in amounts of cellular dopamine and release of dopamine from

PC12 cells following subchronic exposure to Aroclor 1254. Targets for investigation include 1) the effects of Aroclor 1254 on the uptake of tyrosine, 2) the effects of Aroclor 1254 on the rates of conversion of ^{3}H-tyrosine to ^{3}H-DOPA and ^{3}H-dopamine, and 3) the effects of Aroclor 1254 on LAAAD, using supplementation of cell growth medium with DOPA and examining the cellular dopamine content.

CHAPTER 2

MATERIALS AND METHODS

Materials

Nerve growth factor (NGF) was isolated from male mouse submaxillary glands by the procedure of Bocchini and Angeletti (1969). Stock solutions of Aroclor 1254 (courtesy of Dr. J. Quensen, Michigan State University) were prepared using dimethyl sulfoxide (DMSO) as a solvent. Stock solutions of Aroclor 1254 were diluted into the cell culture medium to achieve the desired concentrations. The final DMSO concentration in the culture medium was at most 0.1% (v/v). Polychlorinated biphenyl congeners [2,2'-dichlorobiphenyl (PCB 04); 3,3'-dichlorobiphenyl (PCB 11); 4,4'-dichlorobiphenyl (PCB 15); 2,4,4'-trichlorobiphenyl (PCB 28); 2,2',4,4'-tetrachlorobiphenyl (PCB 47); 2,2',5,5'-tetrachlorobiphenyl (PCB 52); 2,3,4,4',5-pentachlorobiphenyl (PCB 114); 2,3',4,4',5-pentachlorobiphenyl (PCB 118); 3,3',4,4',5-pentachlorobiphenyl (PCB 126); 2,2',3,3',4,4'-hexachlorobiphenyl (PCB 128); and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153)] were obtained from AccuStandard, Inc. (New Haven, CT; see Appendix I for PCB congener structures). Stocks of congeners were prepared in DMSO, then diluted into the culture medium to achieve the desired medium concentration. The final DMSO concentration in the medium was at most 0.5% (v/v). The reagents L-3,4-dihydroxyphenylalanine (L-DOPA), 3-hydroxytyramine (dopamine, DA), L-3,4-dihydroxyphenylethanolamine (norepinephrine, NE), and 3,4-dihydroxyphenylacetic acid

(DOPAC) were purchased from the Sigma Chemical Co. (St. Louis, MO) and standard solutions were prepared in 0.1 M HCl at a concentration of 10 ng/ml. Hoechst 33258 (bis-benzamide) was also purchased from the Sigma Chemical Co. (St. Louis, MO). Octyl sodium sulfate (OSS) was obtained from Eastman Kodak Co. (Rochester, NY). (Methyl-^{3}H)thymidine and (methyl-^{3}H)-choline were obtained from Amersham Corp. (Arlington Heights, IL) or New England Nuclear (Boston, MA), while L-(ring 3,5-^{3}H)-tyrosine, L-^{14}C-leucine and L-^{14}C-arginine were obtained from New England Nuclear. All other chemicals were of reagent or high performance liquid chromatography (HPLC) grade.

Maintenance of PC12 Cell Stock Cultures

PC12 cells were grown in monolayer form in growth medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 6% fetal bovine serum, 6% horse serum, 100 μg/ml of streptomycin, 100 units/ml of penicillin, and 25 mM HEPES at pH 7.4. Cells were incubated at 37°C in a humidified atmosphere containing 6% CO_2, and subcultured at least once per week.

Preparation of PC12 Cells for Determination of Cellular Catechols

To examine the effects of PCBs on amounts of dopamine in PC12 cells treated simultaneously with or without NGF, cells were seeded on polylysine-coated 6-well plates in cell culture medium with or without Aroclor 1254, in the presence

and absence of NGF. Cells exposed only to 0.1 % (v/v) DMSO in the presence and absence of NGF were used as controls. DMSO alone did not alter levels of cellular dopamine. After treatment, cells at an approximate density of 2.1 x 10^4 cells/cm^2 were harvested following incubation in 1.0 ml of phosphate buffered saline (PBS) containing 0.05% trypsin and 0.53 mM EDTA for 10 minutes at room temperature. The PBS consisted of 138 mM NaCl, 2.7 mM KCl, 7.3 mM KH_2PO_4, and 8 mM Na_2HPO_4. An additional 2 ml of ice-cold PBS was added and the cells were subsequently collected. Each well was then washed with an additional 2 ml of ice-cold PBS. After trituration of the cells, 1 ml of each cell suspension was removed for determination of DNA content. The remainder of each sample was centrifuged at 5000 g x min at 4°C. Subsequently, each pellet was resuspended in 0.5 ml of 0.2 M $HClO_4$ containing 0.263 mM EGTA and incubated on ice for 30 min. The samples were again centrifuged at 5000 g x min and the supernatants were stored at -20°C until assayed for amounts of cellular catechols.

To examine the effect of Aroclor 1254 on amounts of dopamine in NGF-pretreated PC12 cells, cells were seeded in polylysine-coated 6-well plates in DMEM with or without NGF and cultured for various periods of time in the absence of PCBs. Every 4 days, fresh DMEM with or without NGF was added. After the appropriate incubation period without PCBs, 25 μg/ml Aroclor 1254 was then added. After 2 days of

exposure to Aroclor 1254, the cells were then harvested and prepared for DNA and HPLC analysis as described above.

The effects of various PCB congeners on amounts of catechols in PC12 cells was investigated by seeding cells and harvesting them as described above. After three days of exposure, cells at an approximate density of 2.1×10^4 cells/cm^2 were harvested and prepared for DNA determination and HPLC analysis of cell extracts as described above.

Determination of DNA Content

Determination of cellular DNA content was performed according to the method of Labracca and Paigen (1980). Briefly, the samples were centrifuged at 5000 g x min, the supernatant aspirated, and the pellet resuspended in 650 μl of an assay buffer consisting of 2.0 M NaCl, 0.05 M Na_2HPO_4, and 2.0 mM EDTA at pH 7.4. The samples were sonicated and 50 to 300 μl aliquots were incubated with 1.2 ml of Hoescht 33258 and 0 to 250 μl of assay buffer. The final volume of the incubation was 1.5 ml. Relative fluorescence was measured with a spectrophotofluorometer using excitation and emission wavelengths of 356 and 448 nm, respectively. The DNA content in samples was quantitated from the relative fluorescence values, using calf thymus DNA as a standard.

Measurement of Dopamine, Precursors and Metabolites

Amounts of the catechols DOPA, DOPAC, and dopamine in cell extracts were measured using HPLC coupled with electrochemical detection, based on a modification of the

method of Seegal et al. (1986). Catechols were eluted through a C-18 reverse phase column at a rate of 0.9 to 1.0 ml/min with a mobile phase consisting of 2.6 mM NaH_2PO_4, 0.05 to 0.064% OSS, 0.1 mM EDTA, 2.5% triethylamine, and 20 to 22.5% methanol at pH 3.3. The catechols were then oxidized on a glassy carbon electrode at a potential of 0.775 V, versus a Ag/AgCl reference electrode, and the resulting signals were quantitated as peak height. The minimal sensitivity of the system for determining amounts of catechols was 25 pg. Peak heights of standards containing 1 ng of catechols were used to quantitate catechols in each sample. Following quantitation of levels of catechols in the cell extracts, values were calculated as pg/ng DNA and reported as percent of control value in the absence of PCBs.

Measurement of Neurite Outgrowth

Induction of neuronal differentiation in PC12 cells was accomplished by incubation of the cells with 100 ng/ml of NGF. To determine the effects of Aroclor 1254 on neurite outgrowth, photographs of random fields of cells were taken. Photographic images were digitized and analyzed using the BIOQUANT computer system (R & M BIOMETRICS, INC.) to determine percent of cells with neurites, number of neurites per cell, and length of neurites. Neurites were defined as processes issuing from the cell that were at least one cell diameter in length.

Measurement of DNA and Protein Synthesis

To determine the effects of Aroclor 1254 on DNA synthesis, cells were synchronized by culturing the cells for 72 hours in a low serum-containing medium consisting of DMEM with 5 ng/ml selenous acid, 5 μg/ml insulin, 5 μg/ml transferrin, 0.075% bovine serum albumin, and 2% horse serum. Cells were then reintroduced to the normal serum-containing DMEM, plated onto polylysine-coated 12-well plates, and treated with or without Aroclor 1254. After 3 days, cells were exposed to 0.5 μCi/ml of (methyl-^{3}H)thymidine for 6 hours. Cells were harvested by the method of Madhukar et al. (1989). Briefly, the cells were washed with PBS containing 0.5 mM $MgCl_2$ and 0.9 mM $CaCl_2$. The cells were then incubated with 0.5 ml of 10% trichloroacetic acid for 10 minutes on ice, and then washed sequentially with 70% and 95% ethanol. Solubilization of cells was accomplished by incubation in 0.5 ml of 1 N NaOH. The base in the samples was neutralized with 0.5 ml of 1 N HCl, and the radioactivity in each sample was determined by scintillation counting. Results were reported as dpm/ng DNA.

To determine the effects of Aroclor 1254 on protein synthesis in PC12 cells, a modification of the method of Madhukar et al. (1989) was used. Cells were synchronized as previously described, then plated onto polylysine-coated 12-well plates and exposed to Aroclor 1254 for 48 hours.

Subsequently, ^{14}C-leucine was added in 100 µl PBS to obtain a final concentration of 1 µCi/ml. One day later, cells were harvested as described above for measurement of the (methyl-^{3}H)thymidine incorporation.

Measurement of Lactate Dehydrogenase

PC12 cells in 6-well polylysine coated plates were incubated with 0.1% DMSO or 25 µg/ml Aroclor 1254 for 3 days. A 2 ml volume of media was removed for determination of the media content of lactate dehydrogenase (LDH). The cells remaining on the plates were lysed using 2 ml of 0.1% Triton X-100. After a brief sonication of both cells and media, the samples were centrifuged in a tabletop centrifuge for 10 minutes. A 40 µl aliquot of each cell and media sample was added to triplicate wells in a 96-well plate. A 200 µl aliquot of the LDH reagent consisting of 1.27 mM pyruvate, 233 µM disodium β-nicotinamide adenine dinucleotide (reduced form), and 46.8 mM potassium phosphate buffer at pH 7.5 was added to each well. Immediately after addition of the LDH reagent, the absorbance at 340 nm was monitored for 3 min for each sample using a 96-well plate reader. Media LDH was expressed as a percent of total LDH.

Measurement of Release of Dopamine

Spontaneous and evoked release of dopamine from PC12 cells were determined using a modification of the methods of Melega and Howard (1984). Following incubation of the cells with either DMSO or various media concentrations of PCBs for

3 days, the culture medium was removed and the PC12 cells were rapidly washed with, then incubated with a low potassium-containing buffer consisting of 125 mM NaCl, 25 mM HEPES, 5.6 mM glucose, 4.8 mM KCl, 1.2 mM $MgCl_2$, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, and 0.5 mM pargyline at pH 7.3. The cells were incubated in this buffer solution for 5 min at 37°C. Subsequently, the buffer was collected to determine the spontaneous release of dopamine. The cells were then incubated for 5 minutes in a high potassium-containing buffer in which the KCl concentration was raised to 56 mM, and the NaCl concentration was reduced to 73.8 mM. The buffer was collected and used to determine the evoked release of dopamine. The cells were then harvested from the plates as described below, and the cell extracts and the release buffers were analyzed for catechol content.

Cells were harvested by incubating them in 1.0 ml of PBS containing 0.05% trypsin and 0.53 mM EDTA for 10 minutes at room temperature. An additional 3 ml of ice-cold PBS was added and the cells were subsequently collected. After trituration of the cells, 1 ml of each cell suspension was removed for determination of DNA content. The remainder of each sample was centrifuged at 1000 g for 5 min at 4°C. Each pellet was subsequently resuspended in 0.5 ml of 0.2 M $HClO_4$ containing 0.263 mM EGTA and incubated on ice for 1 hour. The samples were again centrifuged at 1000 g for 5 min. The resulting supernatants were stored at -20°C until

assayed for amounts of catechols.

To determine the fraction of dopamine released from the cells, the ng of dopamine released by the cells in response to stimulation by 56 mM K^+ was divided by the total amount of dopamine, in ng, in the sample. This calculation was performed for each replicate in each experiment and the results analyzed for statistical significance.

Measurement of the Uptake of Tyrosine, Choline, and Amino Acids

PC12 cells were cultured in 25 cm^2 polylysine-coated tissue culture flasks with various concentrations of Aroclor 1254 or a PCB congener for 3 days. The medium was aspirated away and the cells were collected and washed with PBS. Following centrifugation of the cells at 5000 g x min and aspiration of the PBS wash, the cells were resuspended in PBS containing 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, and 25 mM glucose (PBS•Ca•Mg). PC12 cells from each treatment group in 400 μl of PBS•Ca•Mg were added to duplicate 12 x 75 mm plastic tubes and incubated for 10 min with 10 μl of 10 mM pargyline at 37° C. After preincubation with pargyline, 40 μl of PBS•Ca•Mg and 50 μl of ^{3}H-tyrosine (3 μCi) were added to each tube, mixed and incubated for 40 min at 37° C. Following incubation, the uptake of ^{3}H-tyrosine was halted by addition of 3 ml of PBS•Ca•Mg to each tube. The contents of the tubes were filtered through Whatman GF/B filters. The filters were rinsed 2 to 3 times with 3 ml of PBS•Ca•Mg.

The amount of radioactivity associated with the cells was determined by scintillation spectroscopy. The radioactivity in each sample was normalized to the DNA content, and the data were reported as a percent of the values for DMSO-treated samples. The same procedures were used to determine the uptake of L-^{14}C-leucine, L-^{14}C-arginine, and ^{3}H-methyl-choline.

Supplementation of PCB-treated cells with DOPA

Cells were cultured either with or without PCBs in the medium for 3 days. Six hours prior to harvest of the cells, various concentrations of L-DOPA were added to the culture medium as a 100-fold concentrate in PBS. The cells were subsequently harvested and the amounts of cellular catechols and DNA were determined. Following quantitation of catechols, values were calculated as pg/ng DNA and reported as percent of the DMSO-treated control for the corresponding level of DOPA in the medium.

Measurement of the Conversion of ^{3}H-tyrosine to ^{3}H-Dopamine

Cells were grown in 150 cm^{2} tissue culture flasks in the presence of either 0.1% DMSO or 25 μg/ml Aroclor 1254 for 3 days. Media was aspirated away and the cells were dislodged in 10 ml of PBS. Samples were centrifuged at 1000 g for 5 min and the supernatant aspirated away. Cells were resuspended in serum- and antibiotic-free DMEM. For incubation, 50 μl of radiolabled tyrosine (20 μCi) was added to 450 μl aliquots of cells. Samples used for a 0-time

point were kept on ice, as were triplicate 450 μl aliquots of cells used for DNA determination. Samples were vortexed and incubated at 37°C for the appropriate amount of time. Following incubation, cells were prepared for determination of catechols as described earlier.

The analysis of catechols was done as described earlier in the "Methods", but the effluent was collected using a fraction collector. The mobile phase was altered such that the methanol content was 16%, which increased the run time and spread out the peaks for better fraction collection. The amount of radioactivity in each fraction was determined using scintillation spectroscopy. Using the chromatograph from each sample produced by the HPLC run, the fractions were related to peaks of catechols. The amounts of radiolabeled catechols were reported in dpm per peak.

Preparation of PC12 Cells for Determination of Cellular Concentrations of PCBs

Cells were cultured in 150 cm^2 polylysine-coated flasks for 3 days in the presence of various media concentrations of either Aroclor 1254 or a PCB congener. Flasks containing only PCB-treated medium were also included for use in determining the background amounts of non-cell-associated PCBs that were carried along in the harvest process. The concentrations of PCBs chosen reflected the IC_{50} values of the PCBs for causing a decrease in amounts of cellular dopamine, plus a concentration higher and lower than the

IC_{50}. The exact volume of stock PCB in DMSO added to each flask was also added to a glass vial to be diluted in hexane and used to determine the total amount of PCB present in each flask at each concentration for each PCB congener or Aroclor 1254. Cells and media were collected for determination of PCB content. Media was decanted into glass vials, and 10 ml of PBS was added to each flask to wash the cells. The PBS was then decanted into a second set of glass vials. To dislodge the cells from the flasks, cells were incubated with 8 ml of PBS containing 0.05% trypsin and 0.53 mM EDTA for 10 min at room temperature, then the flasks were struck to free the cells. Subsequently, 8 ml of PBS was added and the cells decanted into a third set of glass vials. From the third set of vials, two 500 μl aliquots were removed for determination of DNA content, and for counting of the cells. Finally, the flasks were washed with 10 ml of hexane to remove any PCBs bound to the container. This wash was decanted into a fourth set of glass vials. The samples containing 500 μl of cells that were to be used for cell counting were centrifuged at 15,400 g for 3 min, the supernatant was aspirated, and 200 μl of PBS was added. The cells were resuspended in PBS and counted on a hemacytometer.

Extraction and Determination of PCBs in PC12 Cells by Gas Chromatographic Analysis

The contents of the vials containing the cells suspended in PBS and the media combined with the non-hexane wash were acidified by addition of 0.2 ml of concentrated sulfuric acid. A volume of hexane containing 1.6 parts per million octachloronaphthalalene (OCN) as an internal standard for gas chromatographic analysis was added. The volume of hexane containing OCN was equal to the final volume of the PCB solution after extraction which was used for GC analysis. The PCBs and OCN were extracted three times using 5 ml of a hexane-acetone mixture as an extraction solvent. Emulsions formed by the vigorous agitation of the extraction process were broken by addition of acetone in a dropwise fashion until the emulsion dissipated. The organic layer was removed using a pasteur pipette and then cleaned by passage through a copper-florisil column. The hexane was evaporated off and the extracted PCB residue was dissolved in hexane, the same volume as was added when hexane with OCN was added at the beginning of the extraction. Samples were analyzed on a Hewlett-Packard gas chromatograph using a capillary column, as described previously (Quensen, et al. 1988, 1990). Amounts of PCB in each sample were determined using either Aroclor 1254 or a mixture of appropriate congeners as a standard. Generally, 95 to 98% of the PCB added to the cell

culture system was recovered between the medium and the cells.

Levels of PCBs in the media and hexane washes were also determined as described above. Results were reported as total μg per sample. The amounts of PCBs, in μg, were divided by the volume of cells in each sample. The volume of cells for each sample was determined by determining the average diameter of PC12 cells (30 μm) and calculating the volume of a sphere ($^4/_3 \pi r^2$), then multiplying the volume of one cell by the total number of cells in the sample. This method of determining cell volume was not exact, due to the fact that the cells were adhered to a flat surface, hence the volume is an estimate of the volume of the cells, and may overstate the actual volume of the cells in each sample. The concentrations of PCBs in the cells were expressed either in μg/ml or μM. The percent of total PCBs associated with the cells was calculated by dividing the amount of PCBs in the cellular fraction by the total PCBs in the sample.

Preparation of PC12 Cells for Determination of Cellular Concentrations of Biphenyl

PC12 cells were exposed to various media concentrations of biphenyl for 3 days. After exposure, media was decanted into a set of glass vials, and the cells were washed with 10 ml of PBS. The PBS wash was decanted into a second set of glass vials. Five ml of PBS containing 0.05% trypsin and

0.53 mM EDTA was added to the cells for 10 min at room temperature. Cells were dislodged and another 5 ml of PBS was added to the cells. Cells were decanted into a third set of glass vials. Two 500 μl aliquots were removed from the third set of vials for determination of DNA content and for cell counting by hemocytometry. The 150 cm^2 flasks were washed with 5 ml of hexane to remove biphenyl adherent to the container, and this wash was decanted into a fourth set of glass vials. All vials were stored at 4°C until assayed for biphenyl content.

Extraction and Determination of Biphenyl in PC12 Cells

The glass vials containing cells were decanted into large glass tubes. The vials were washed with 2 ml of hexane and the wash was transferred to the tubes containing the cells. The biphenyl in the cells was extracted into the hexane by shaking for 15 min. Emulsions were broken by centrifugation of the samples at 1000 g for 5 min. Aliquots of the hexane extract were transferred to autosample vials and amounts of biphenyl were determined by gas chromatography coupled to mass spectrometry (GC/MS). Samples were injected onto a 15 meter x 0.25 mm column (J&W DB5-MS) for separation before analysis by mass spectrometry (Finnigan TSQ-70). Appendix 2 contains more information on the run conditions for this procedure.

The vials containing the media and PBS wash were combined for each sample and extracted with 3 ml of hexane

by shaking for 15 min. Two ml of a saturated NaCl solution in water were added to each sample to decrease the formation of emulsions. Following extraction, samples were sonicated for 1 min, then centrifuged at 1,800 g for 10 min. Aliquots of hexane extract were analyzed for biphenyl as described above. Meanwhile, aliquots of flask hexane wash were not extracted but were analyzed for the content of biphenyl as described above.

The amount of biphenyl in each sample, in ng, was divided by the volume of cells from each sample, which was calculated as described on page 50. The concentrations of biphenyl in the cells were expressed in nM. The percent of total biphenyl associated with the cells was calculated by dividing the ng in the cellular fraction by the total amount present in the sample.

Determination of Cell Viability by Trypan Blue Exclusion

An aliquot of cells was suspended in PBS. A 200 µl aliquot of 0.4% trypan blue was added to 800 µl of PBS and mixed. A 500 µl aliquot of cells and a 500 µl aliquot of the trypan blue were mixed and allowed to stand for at least 5 min. Cells were counted by hemocytometry and the number of cells staining dark blue (inviable) were calculated as a percentage of total cells counted (viable and inviable).

Statistical Analysis

Since the data were often expressed nonparametrically as ratios, the Kruskall-Wallis k-Sample test was used to

determine if any values in a given experiment differed from control. The Wilcoxon-Mann-Whitney test was used to determine which specific treatments differed. A level of $p < 0.05$ was used to determine significance in both the Kruskall-Wallis and the Wilcoxon-Mann-Whitney tests.

CHAPTER 3

EFFECTS OF POLYCHLORINATED BIPHENYLS ON CELLULAR DOPAMINE IN PC12 CELLS

INTRODUCTION

Over the past 15 years, studies involving the effects of PCBs on living organisms have suggested that, in addition to the dioxin-like effects of PCBs on non-neuronal tissues (Safe, 1984, 1990), PCBs may also affect nervous tissue. Studies involving human infants exposed to PCBs *in utero* have suggested that PCBs affect visual recognition memory and other mental and neurological capabilities (Jacobson et al., 1985, 1990; Gladen and Rogan, 1991; Rogan and Gladen, 1992). Observations of altered behaviors in animals exposed to PCBs *in utero* have also been documented, such as a spinning syndrome in mice (Chou et al., 1979; Tilson et al., 1990), hyperactivity and impaired learning abilities in monkeys (Bowman et al., 1978; Schantz et al., 1991; Levin et al., 1988), and impaired active avoidance learning and impaired visual discrimination task memory in rats (Overmann et al., 1987; Pantaleoni et al., 1988; Lilienthal and Winneke, 1991). Decreases in amounts of brain dopamine and dopamine receptors have been reported in mice exposed to PCBs *in utero* (Agrawal et al., 1981), and decreased amounts of dopamine have been documented in the brains of PCB-exposed rats and monkeys (Seegal et al., 1985, 1986, 1990, 1991a, b), suggesting that decreases in dopamine may underlie some of the PCB-mediated alterations in behavior.

To better understand the acute effects of Aroclor 1254 on dopaminergic cells, Seegal et al., (1989, 1990) employed

a commonly used model to study the dopaminergic system, the rat PC12 pheochromocytoma cell line. Exposure of PC12 cells to various media concentrations of Aroclor 1254 for 6 hours caused decreased dopamine levels in quiescent undifferentiated PC12 cells. The acute effects of PCB congeners on amounts of cellular dopamine in PC12 cells have also been examined to try to determine the structure activity relationships between PCBs and decreases in cellular dopamine. The effects of 6 hours of exposure of PC12 cells to various PCB congeners were reported by Shain et al. (1991), and the data suggest that PCB congeners with di-*ortho* substitutions are the most efficacious at decreasing cellular dopamine. Further, the data suggest that co-planar, non-*ortho* substituted PCB congeners are ineffective at decreasing cellular dopamine in PC12 cells.

However, these studies did not address the subchronic or chronic effects of PCBs on exponentially dividing cells or differentiating neuronal cells. The current studies used exponentially dividing cells, as would be found in a developing organism *in vivo*. Further, there are no known reports on the effects of exposure of dopaminergic cells to PCB congeners for periods of longer than 6 hours. Cells in an actively growing early embryonic organism are generally undergoing exponential growth until they differentiate. *In utero*, undifferentiated and differentiating dopaminergic neurons in the developing embryo and fetus may be exposed to

low levels of PCBs from the maternal bloodstream (Levin et al., 1988; Allen and Barsotti, 1976) on at least a subchronic, if not a chronic basis.

Hence, a study of the effects of subchronic exposure of undifferentiated and differentiating dopaminergic PC12 cells to Aroclor 1254 was performed to understand further the effects of Aroclor 1254 on developing dopaminergic cells. NGF-treated PC12 cells are a commonly used model system of differentiating neurons (Guroff, 1985; Fujita et al., 1989), since the dopaminergic PC12 cells neuronally differentiate in response to NGF over a period of two weeks (Fujita et al., 1989). After two weeks of exposure to NGF, PC12 cells display a fully differentiated, sympathetic-like neuronal phenotype. Thus, some of the studies presented in this chapter were done with PC12 cells that were subchronically exposed to Aroclor 1254 and treated concomitantly with or without NGF to examine the subchronic effects of Aroclor 1254 on undifferentiated and differentiating dopaminergic cells. Further studies were done to examine the effects of Aroclor 1254 on amounts of cellular dopamine following pretreatment of PC12 cells with NGF for various periods of time prior to exposure to PCBs. These studies were done to determine whether NGF can partially protect PC12 cells from Aroclor 1254-induced decreases in cellular dopamine. PC12 cells were also used to examine the effects of a subchronic, 3 day exposure to various media concentrations of numerous

PCB congeners on cellular catecholamine levels in dopaminergic cells.

RESULTS

Aroclor 1254:

Exposure of PC12 cells to PCBs was not without toxic effects on the cells. At 100 μg/ml of Aroclor 1254, greater than 85% cell death occurred, while at 50 μg/ml, greater than 70% cell death occurred, as determined by trypan blue exclusion. The control DNA, dopamine, DOPA, and DOPAC levels, as determined in the presence of 0.1% dimethylsulfoxide (DMSO) were not significantly altered from the amounts observed in untreated cells, and the amounts of catechols and DNA in the DMSO-exposed cells were within 10% of the amounts determined in naive cells. The average 3 day cultured sample control or DMSO-treated sample contained between 20 and 50 ng of dopamine.

Aroclor 1254 caused a large, dose-dependent decrease in cellular dopamine and DOPAC in undifferentiated, non-NGF-treated PC12 cells after 3 days of exposure. Concurrently, the amounts of cellular DOPA increased in a dose-dependent manner (Figure 3.1). Exposure of PC12 cells to Aroclor 1254, at total media concentrations of up to 100 μg/ml for 1 to 7 days was associated with dose-dependent decreases in cellular dopamine (Figure 3.2). Between 1 and 3 days of exposure to Aroclor 1254, the dose-response curves depicting both dopamine and DNA shifted significantly to the left

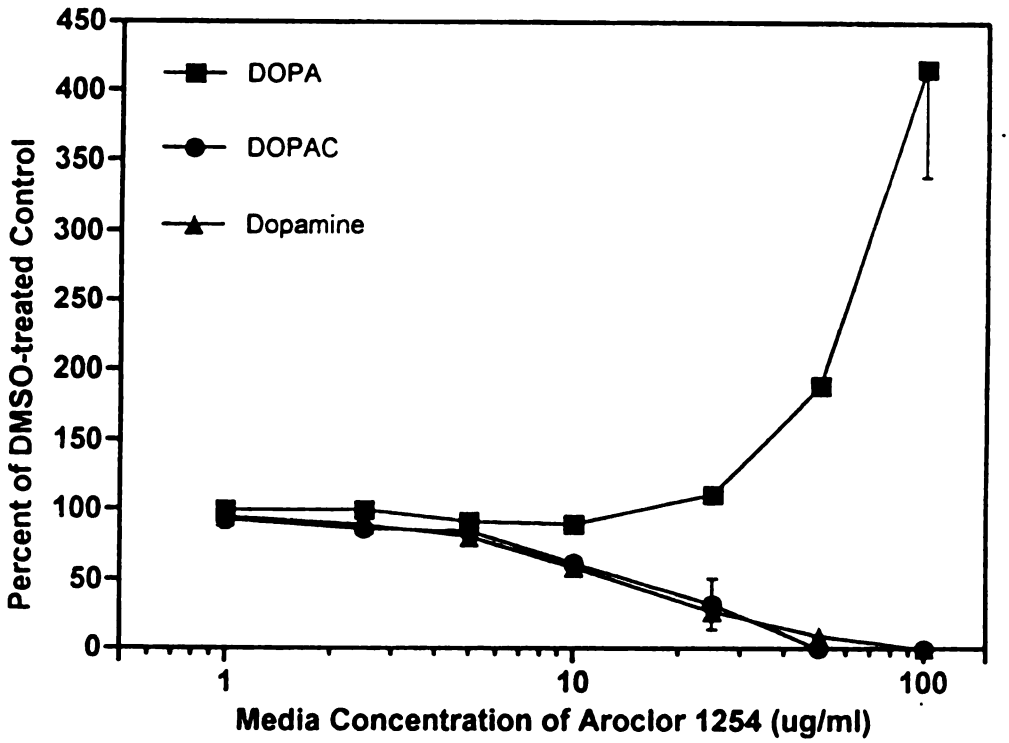


Figure 3.1 Effects of Subchronic Exposure to Aroclor 1254 on Cellular Catechols

PC12 cells were exposed to various media concentrations of Aroclor 1254 for 3 days. Cells were harvested and levels of cellular DOPA, DOPAC, and dopamine were determined as described in Chapter 2, "Materials and Methods". Each point represents the mean ± S.E.M. for 3 separate experiments. When error bars are not noticeable, they are smaller than the corresponding symbol.

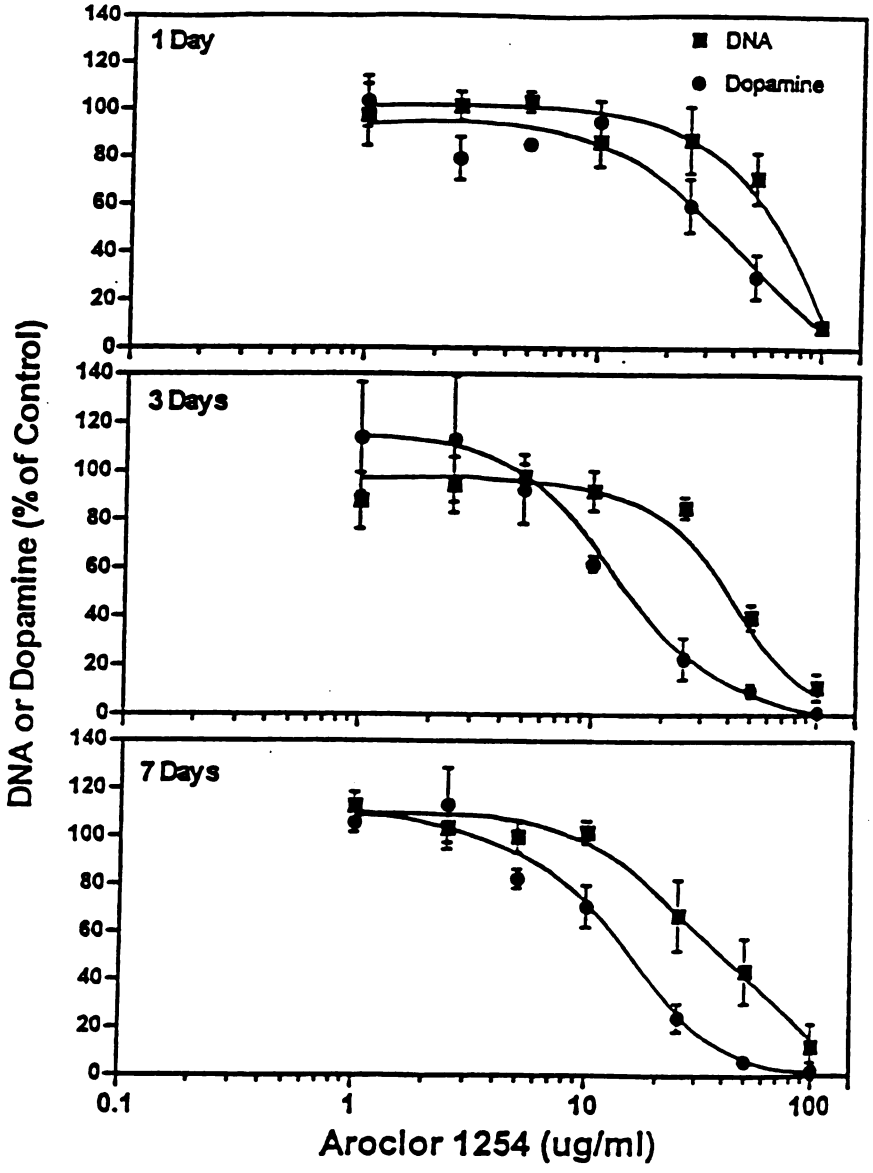


Figure 3.2 Subchronic Exposure of Undifferentiated PC12 Cells to Aroclor 1254

PC12 cells were exposed to various total media concentrations of Aroclor 1254 for 1 (top), 3 (center), or 7 (bottom) days. Cells were harvested and the amounts of DNA and cellular dopamine were determined as described in Chapter 2, "Materials and Methods". Each point represents the mean ± S.E.M. for 3 to 5 separate experiments.

indicating that amounts of cellular dopamine decreased significantly at lower media Aroclor 1254 concentrations, and thus the cells were sensitive to lower media PCB concentrations. For cellular dopamine, the IC_{50} values were 36.3 ± 5.2 μg/ml and 18.4 ± 4.4 μg/ml after 1 and 3 days of exposure to Aroclor 1254, respectively. The IC_{50} values for DNA content were 78.2 ± 9.7 μg/ml and 44.2 ± 5.3 μg/ml after 1 and 3 days of exposure to Aroclor 1254, respectively. Increasing the time of exposure to Aroclor 1254 for longer than 3 days did not result in further shifts in the curves.

To examine the effects of exposure of Aroclor 1254 on rates of DNA synthesis, the incorporation of (methyl-^{3}H)thymidine into DNA was examined following 3 days of exposure to various concentrations of Aroclor 1254 (Table 3.1). There were no statistically significant alterations in DNA synthesis at or below 25 μg/ml media concentration of Aroclor 1254 at 3 days of exposure. Studies examining the incorporation of ^{14}C-leucine into PC12 cells previously exposed to Aroclor 1254 for a total of 3 days demonstrated a decrease in cellular protein synthesis only at the highest concentration of Aroclor 1254 tested (Table 3.1). Release of lactate dehydrogenase (LDH), a large cytosolic enzyme used as an indicator of plasma membrane integrity, into the cell culture medium was also unchanged from control as a result of exposure to a 25 μg/ml media concentration of Aroclor 1254 or less for 3 days, however, the LDH levels in

TABLE 3.1 Effect of Aroclor 1254 on DNA and Protein Synthesis

Aroclor 1254 (μg/ml)	(Methyl-^{3}H)-Thymidine Incorporation (% DMSO Control)	^{14}C-Leucine Incorporation (% DMSO Control)
5	96 ± 3	120 ± 13
10	123 ± 24	134 ± 21
25	102 ± 5	135 ± 12
50	15 ± 4 *	68 ± 14 *

PC12 cells were exposed to various concentrations of Aroclor 1254 for 3 days. The incorporation of (Methyl-^{3}H)-thymidine and ^{14}C-leucine was determined as described in Chapter 2, "Materials and Methods". Data represent the mean ± S.E.M. for 3 separate experiments. * $P \leq 0.05$, significantly different from control levels.

the media at 50 μg/ml Aroclor 1254 increased from approximately 35% to about 70% of total LDH (Figure 3.3).

To examine the effect of Aroclor 1254 on NGF-stimulated differentiating cells, PC12 cells were simultaneously exposed to Aroclor 1254 and NGF. The dose response curves for Aroclor 1254-mediated decreases in cellular dopamine and DNA content (Figure 3.4) were similar to the curves obtained from cells in the absence of NGF (Figure 3.2). For dopamine, the IC_{50} values were 43.8 ± 6.7 μg/ml and 22.6 ± 4.0 μg/ml for 1 and 3 days of exposure to Aroclor 1254, respectively. The IC_{50} values for DNA were 84.1 ± 1.3 and 54.9 ± 4.6 μg/ml for 1 and 3 days of exposure, respectively.

To examine the effects of Aroclor 1254 on PC12 cells that were already differentiating, PC12 cells were cultured with or without NGF for various periods of time before being exposed to Aroclor 1254 at a media concentration of 25 μg/ml for 2 days. As shown in Figure 3.5, there was no significant difference in the Aroclor 1254-mediated decrease in cellular dopamine in cells pretreated without or with NGF for up to 3 days. At 7 and 14 days, the cells pretreated with NGF demonstrated an attenuation of the decrease in cellular dopamine associated with exposure to 25 μg/ml Aroclor 1254 (Figure 3.5).

The similarity in the decreases in cellular dopamine and DNA content associated with Aroclor 1254 exposure in cells simultaneously treated with and without NGF (Figures

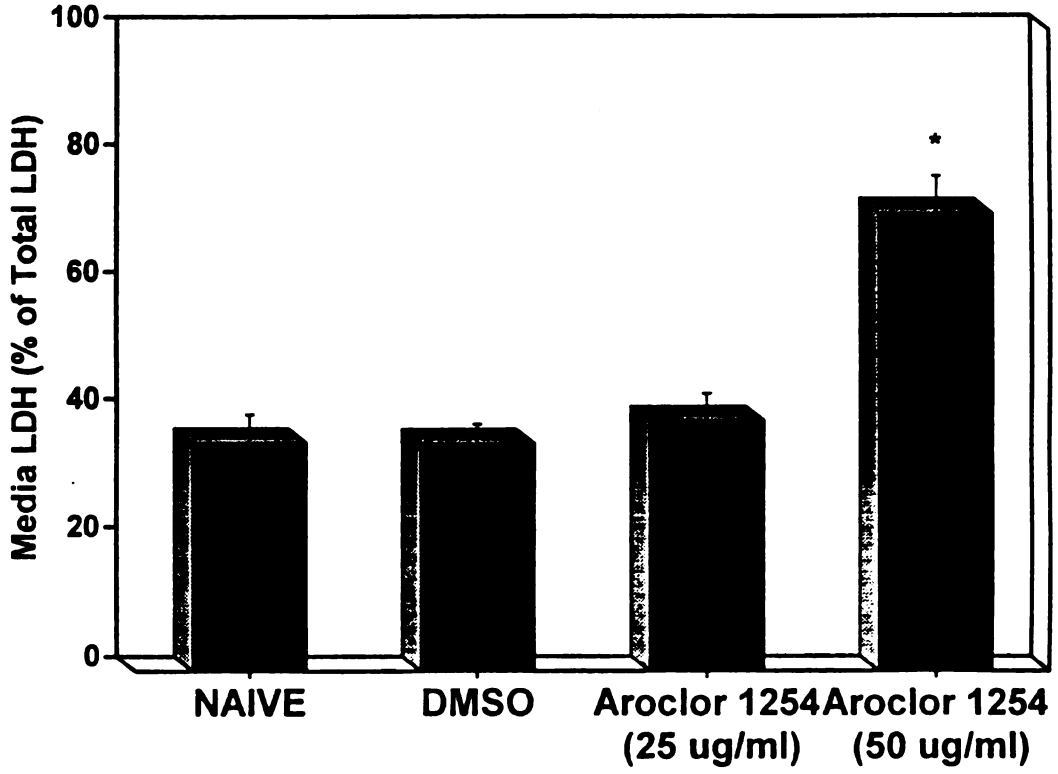


Figure 3.3 Media Lactate Dehydrogenase Levels Following Subchronic Exposure to Aroclor 1254

Media levels of lactate dehydrogenase were determined for naive PC12 cells or cells exposed to DMSO or 25 or 50 μg/ml media concentration of Aroclor 1254 as described in Chapter 2, "Materials and Methods". Values represent the mean ± S.E.M. for 3 separate experiments.

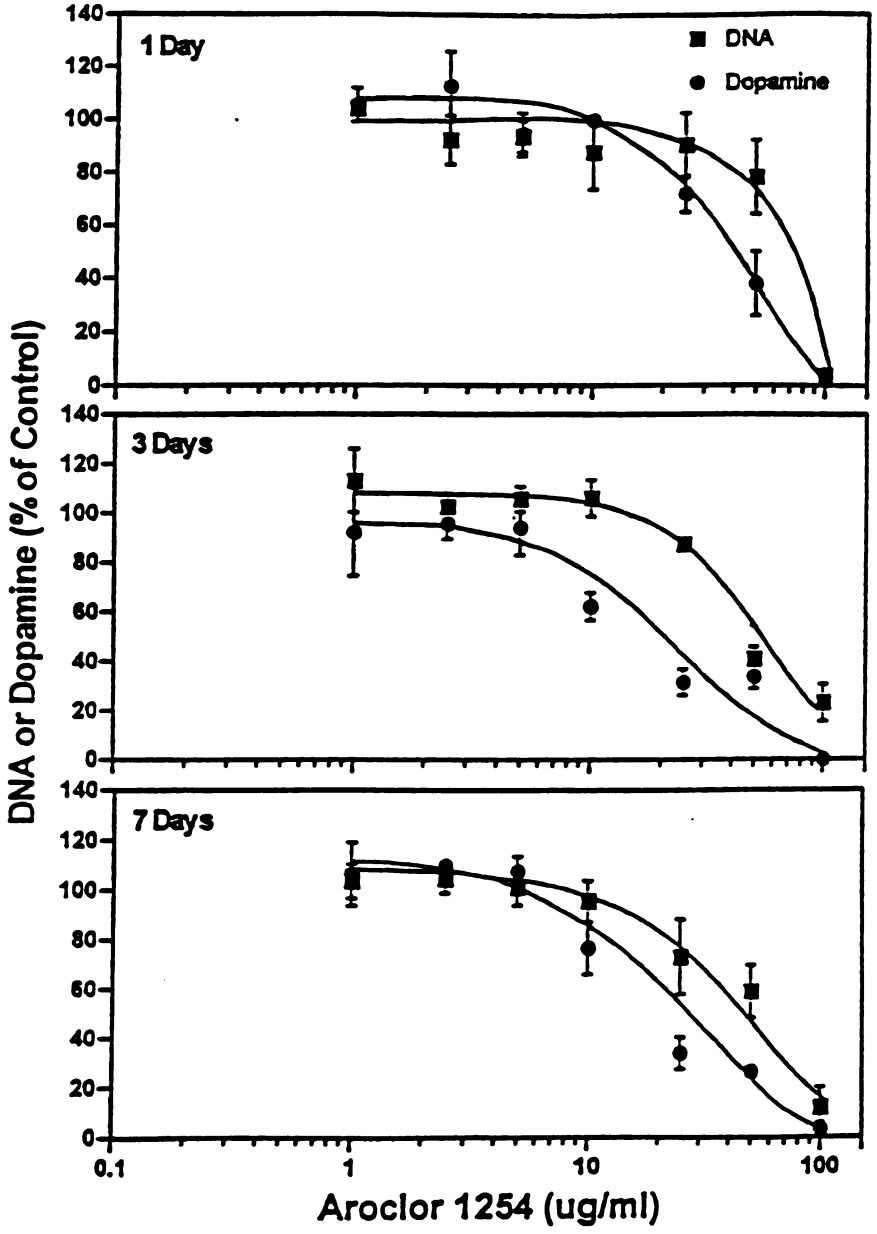


Figure 3.4 Subchronic Exposure of NGF-stimulated Differentiating PC12 Cells to Aroclor 1254

PC12 cells were simultaneously exposed to various total media concentrations of Aroclor 1254 and 100 ng/ml NGF for 1 (top), 3 (center), or 7 (bottom) days. Cells were harvested and the amounts of DNA and cellular dopamine were determined as described in Chapter 2, "Materials and Methods". Each point represents the mean ± S.E.M. for 3 to 5 separate experiments.

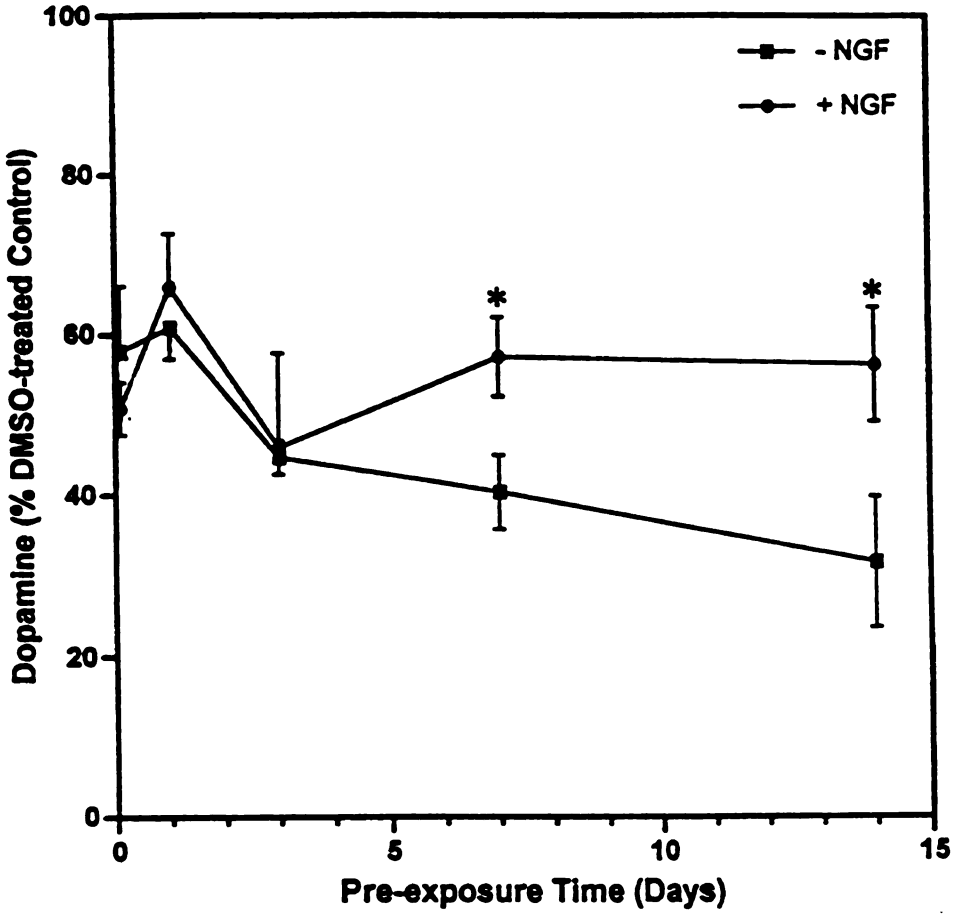


Figure 3.5 Effect of NGF Pretreatment on Aroclor 1254-mediated Decreases in Cellular Dopamine

PC12 cells were preincubated without (■) or with (○) NGF (100 ng/ml) for different periods of time prior to exposure to 25 μg/ml total media concentration of Aroclor 1254 for 2 days. Cells were harvested and the amounts of dopamine and DNA were determined as described in Chapter 2, "Materials and Methods". Each point represents the mean ± S.E.M. for 3 to 4 separate experiments normalized to control values for each timepoint. * $p \leq 0.05$ compared to the corresponding PCB-treated, NGF-untreated cells for each treatment time.

3.2 and 3.4) did not mean that PC12 cells did not respond to NGF in the presence of Aroclor 1254. Exposure of the cells to either DMSO or Aroclor 1254 alone did not induce neurite outgrowth in the PC12 cells (Figure 3.6). However, concurrent exposure of PC12 cells to Aroclor 1254 and NGF produced a dose-dependent enhancement in neurite elongation (Figure 3.7). The length of neurites was markedly increased in the presence of Aroclor 1254 as compared to that seen in cells treated with NGF alone. With increasing time of exposure to Aroclor 1254 and NGF, the effect of Aroclor 1254 became more apparent. Although neurite length was increased with exposure to PCBs, the percentage of cells bearing neurites and the number of neurites per cell did not change (Table 3.2).

PCB Congeners: Exposure of PC12 cells to 0.1 to 0.5 % DMSO did not affect levels of catechols in the cells, nor did it alter the number of cells, as indicated by the amount of DNA present in the plate.

Treatment of PC12 cells with PCB congeners for 3 days resulted in varying changes in amounts of cellular DOPA, DOPAC, and dopamine (Figure 3.8). Amounts of DOPA were elevated in a dose-dependent fashion in response to exposure to 2,2'-dichlorobiphenyl; 2,2',4,4'-tetrachlorobiphenyl; 2,2',5,5'-tetrachlorobiphenyl, and 2,2',4,4',5,5'-hexachlorobiphenyl. In general, cellular DOPAC declined in a dose-dependent manner for all congeners that also caused

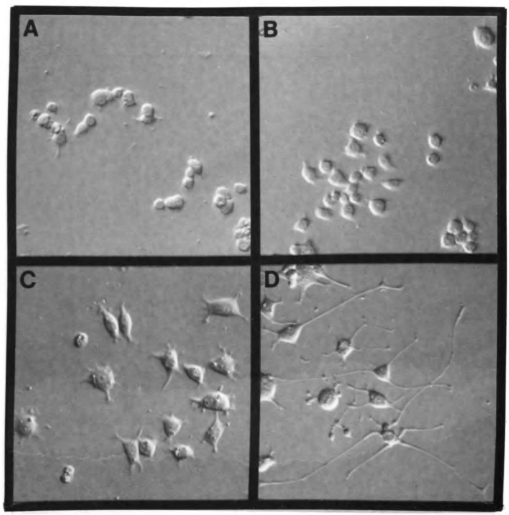


Figure 3.6 Effect of Aroclor 1254 on NGF-induced Neurite Outgrowth
PC12 cells were incubated for 48 hours with A) 0.1% DMSO, B) 25 μg/ml total media concentration of Aroclor 1254, C) 0.1% DMSO and 100 ng/ml NGF, and D) 25 μg/ml Aroclor 1254 and 100 ng/ml NGF. Photographs shown are representative of 3 to 4 experiments.

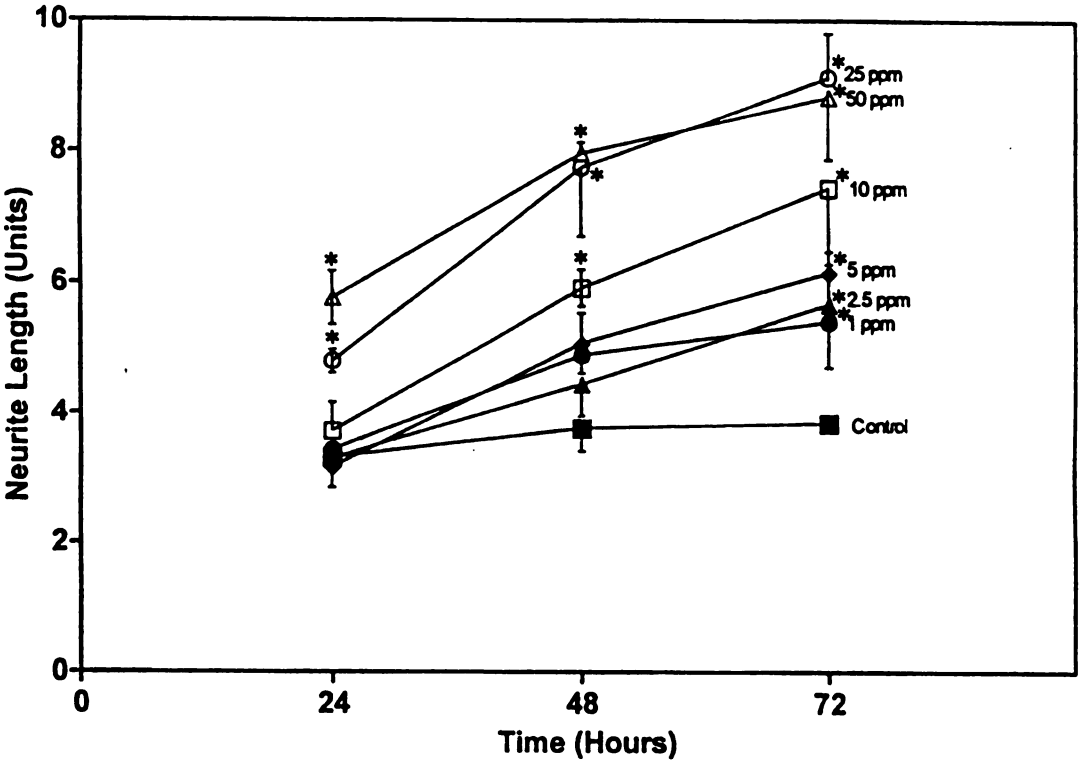


Figure 3.7 Effect of Aroclor 1254 on NGF-induced Neurite Elongation.

Photographs were taken of PC12 cells exposed to various media concentrations of Aroclor 1254 in the presence of NGF for increasing periods of time. Images were analyzed using the BIOQUANT system as described in Chapter 2, "Materials and Methods". Each point represents the mean ± S.E.M. for 3 to 4 separate experiments for each timepoint and Aroclor 1254 concentration. * $p \leq 0.05$ compared to NGF-treated control cells. The term "Units" on the Y-axis refers to arbitrary computer-generated units.

Table 3.2 Percent of PC12 Cells Expressing Neurites and Number of Neurites per Cell Following Subchronic Exposure to Aroclor 1254 and NGF

PC12 cells were exposed concomitantly to 100 ng/ml NGF and various media concentrations of Aroclor 1254. Photographs of random fields were taken and the number of neurites on each cell were determined using the BIOQUANT system. The average number of neurites per cell and the percentage of cells bearing neurites were calculated. Values represent the mean ± S.E.M. for 3 to 4 separate experiments.

Table 3.2 Percent of PC12 Cells Expressing Neurites and Number of Neurites per Cell Following Subchronic Exposure to Aroclor 1254 and NGF

Media Aroclor 1254 Conc. (μg/ml)	24 Hours		48 Hours		72 Hours	
	% with	No. Per Cell	% with	No. Per Cell	% with	No. Per Cell
Naive	1.2 ± 0.6	0.3 ± 0.3	1.8 ± 1.8	0.3 ± 0.3	0.2 ± 0.2	0.5 ± 0.5
NGF	27.3 ± 8.2	1.6 ± 0.1	41.8 ± 1.5	1.6 ± 0.2	41.0 ± 19.7	1.6 ± 0.3
DMSO+NGF	23.2 ± 6.3	1.7 ± 0.2	37.2 ± 5.1	1.7 ± 0.1	31.2 ± 5.6	1.6 ± 0.4
1.0+NGF	36.7 ± 4.1	1.4 ± 0.1	39.5 ± 10.4	1.6 ± 0.1	34.5 ± 6.7	1.6 ± 0.2
2.5+NGF	33.9 ± 3.4	1.5 ± 0.1	43.2 ± 4.0	1.6 ± 0.2	37.9 ± 5.7	1.5 ± 0.1
5.0+NGF	30.7 ± 7.5	1.4 ± 0.1	42.4 ± 2.8	1.4 ± 0.1	43.3 ± 8.7	1.7 ± 0.1
10.0+NGF	40.4 ± 5.9	1.3 ± 0.1	37.8 ± 1.5	1.7 ± 0.1	44.0 ± 5.4	1.8 ± 0.2
25.0+NGF	43.3 ± 11.6	1.4 ± 0.1	45.8 ± 3.6	1.7 ± 0.1	44.7 ± 2.1	1.8 ± 0.2
50.0+NGF	42.5 ± 7.3	1.3 ± 0.1	47.4 ± 6.0	1.4 ± 0.2	36.4 ± 4.4	1.6 ± 0.2

Figure 3.8 Subchronic Exposure of PC12 Cells to 11 PCB Congeners

Cells were exposed for 3 days to various media concentrations of 11 PCB congeners:
PCB 04 (2,2'-dichlorobiphenyl)
PCB 11 (3,3'-dichlorobiphenyl)
PCB 15 (4,4'-dichlorobiphenyl)
PCB 28 (2,4,4'-trichlorobiphenyl)
PCB 47 (2,2',4,4'-tetrachlorobiphenyl)
PCB 52 (2,2',5,5'-tetrachlorobiphenyl)
PCB 114 (2,3,4,4',5-pentachlorobiphenyl)
PCB 118 (2,3',4,4',5-pentachlorobiphenyl)
PCB 126 (3,3',4,4',5-pentachlorobiphenyl)
PCB 128 (2,2',3,3',4,4'-hexachlorobiphenyl)
PCB 153 (2,2',4,4',5,5'-hexachlorobiphenyl).
Cells were harvested and cellular content of DNA, DOPA, DOPAC, and dopamine were determined as described in Chapter 2, "Materials and Methods". Each point represents the mean ± S.E.M. for 3 to 10 separate experiments.

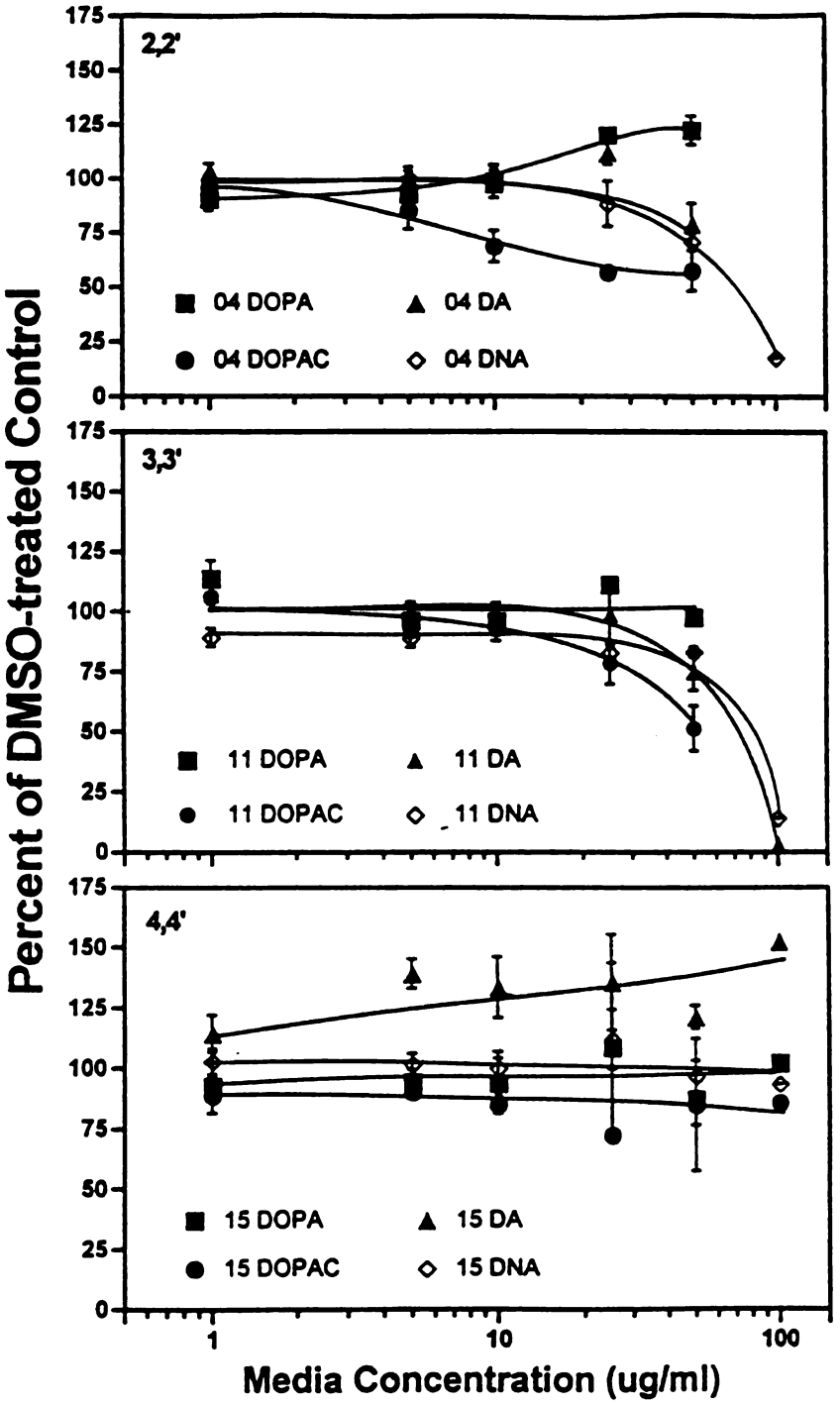

2,2'
3,3'
4,4'
04 DOPA
04 DA
04 DOPAC
04 DNA
11 DOPA
11 DA
11 DOPAC
11 DNA
15 DOPA
15 DA
15 DOPAC
15 DNA
Percent of DMSO-treated Control
Media Concentration (ug/ml)
175
150
125
100
75
50
25
0
1
10
100

Figure 3.8

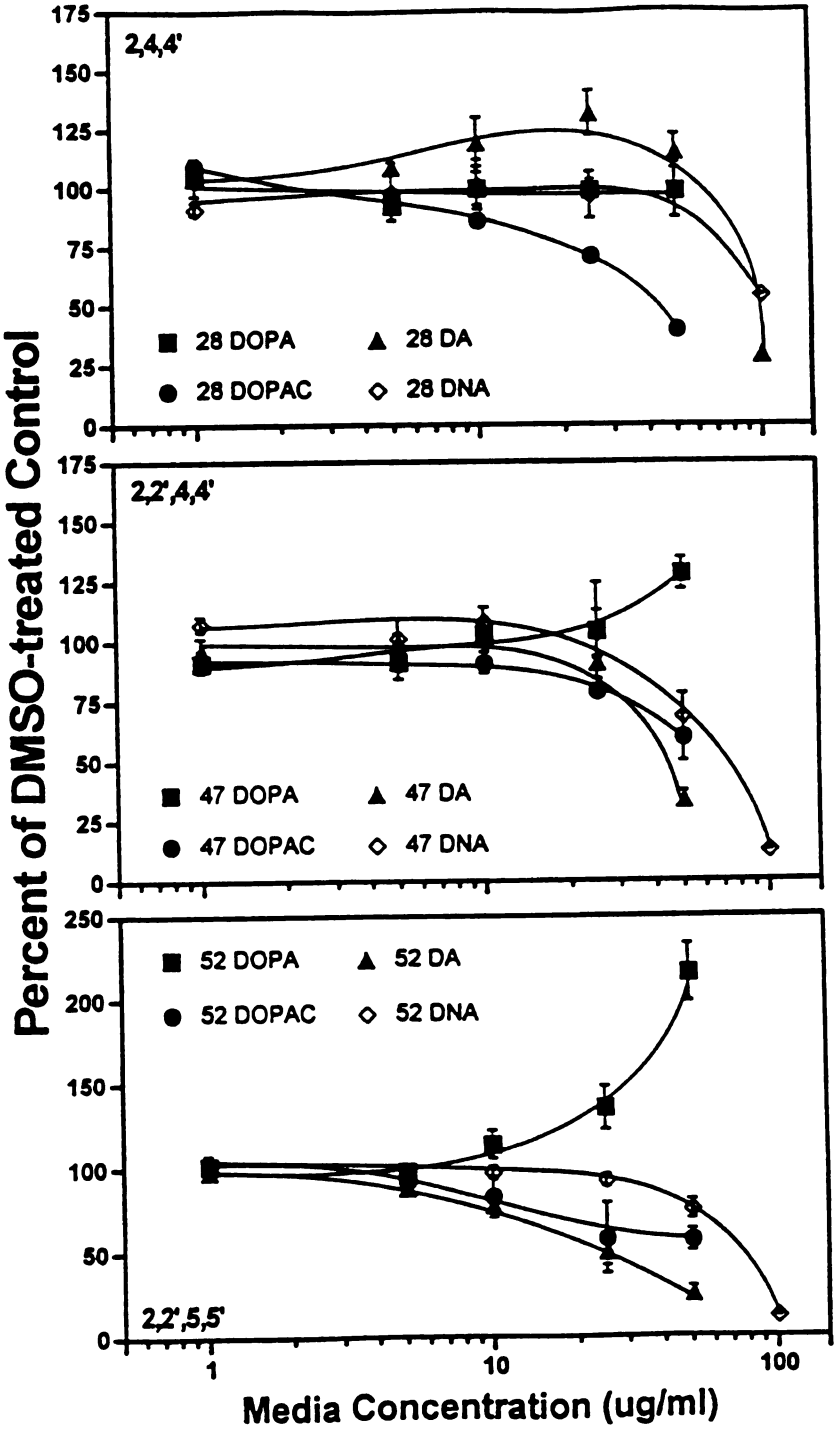


Figure 3.8 Continued

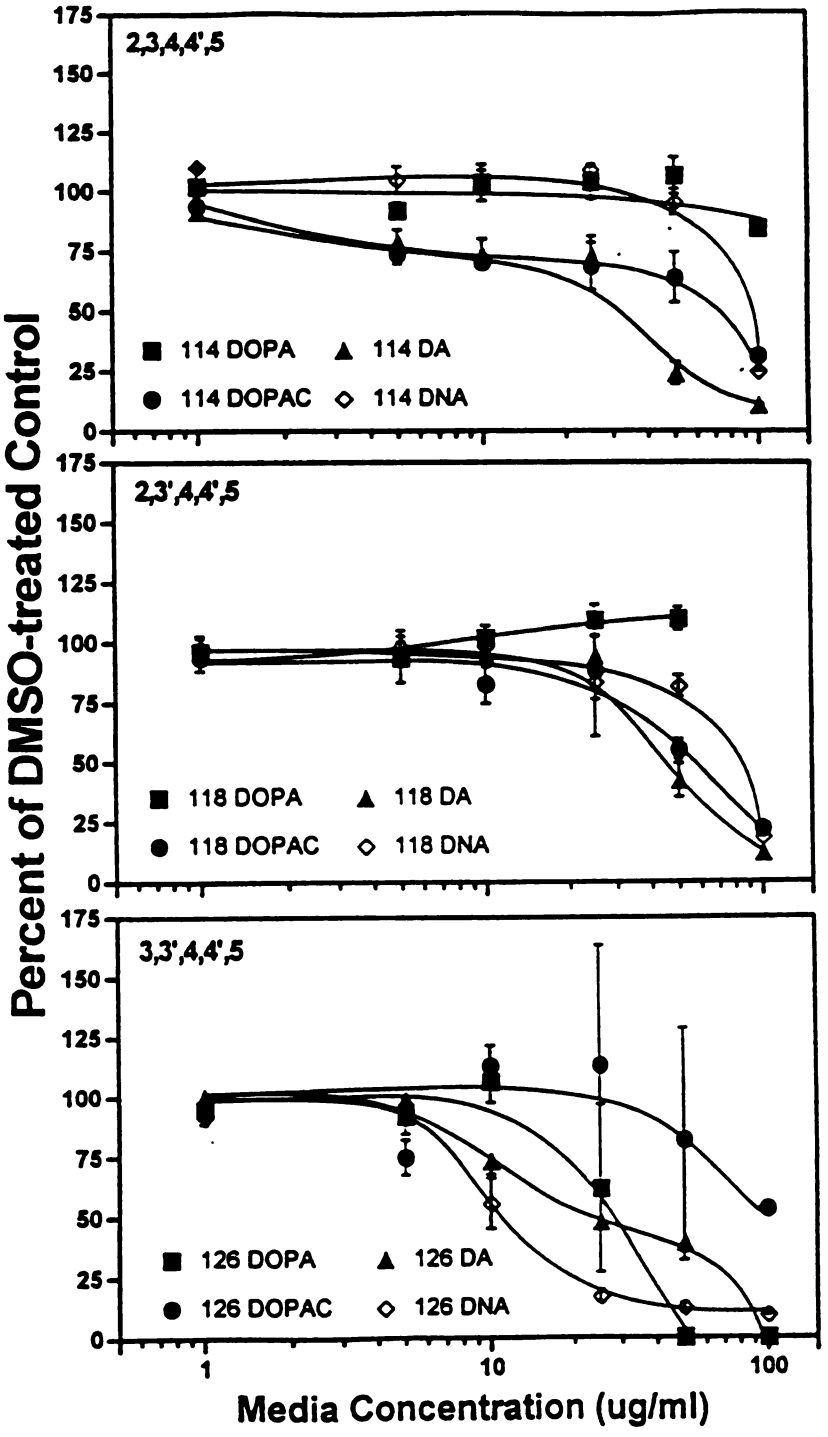


Figure 3.8 Continued

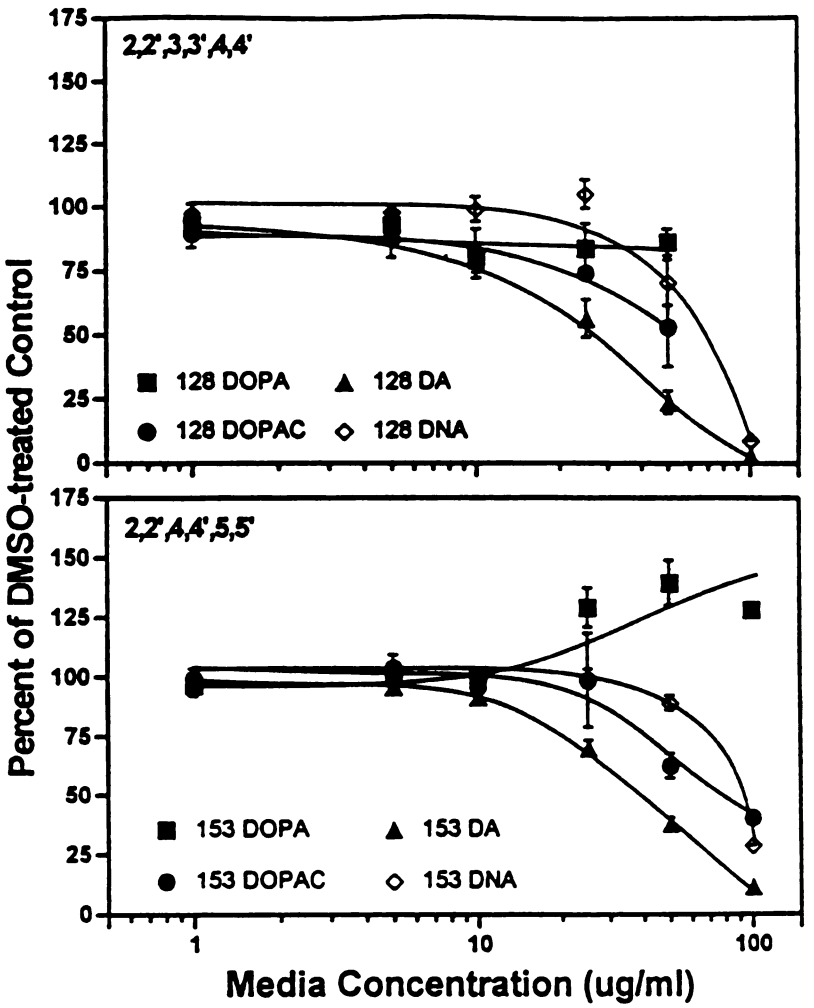


Figure 3.8 Continued

a decrease in dopamine. Cellular dopamine decreased with exposure to 2,2',4,4'-tetrachlorobiphenyl; 2,2',5,5'-tetrachlorobiphenyl; 2,3,4,4',5-pentachlorobiphenyl; 2,3',4,4',5-pentachlorobiphenyl; 3,3',4,4',5-pentachlorobiphenyl; 2,2',3,3',4,4'-hexachlorobiphenyl; and 2,2',4,4',5,5'-hexachlorobiphenyl. Only 4,4'-dichlorobiphenyl did not cause a dose-dependent decrease in cellular dopamine. For the coplanar congener 3,3',4,4',5-pentachlorobiphenyl, the dose-dependent decrease in DNA content occurred at media PCB concentrations identical to those at which decreases in amounts of cellular catechols were observed.

The media PCB concentration IC_{50} values were calculated for the effects of PCB congeners and Aroclor 1254 on amounts of cellular dopamine (Table 3.3). Aroclor 1254 had the lowest IC_{50} for decreasing cellular dopamine and 2,2',4,4',5,5'-hexachlorobiphenyl had the highest. The effects of a 6 hour exposure of quiescent cells to 2,2'-dichlorobiphenyl were examined using the method of Seegal et al. (1989) to try to explain differences between our results with the congener, and those of Seegal pertaining to cellular amounts of catechols. The results indicated that cellular dopamine, DOPA and DOPAC were unaffected by exposure of the cells to 2,2'-dichlorobiphenyl until the media concentrations reached 100 μg/ml, where a modest decrease occurred (Figure 3.9).

Table 3.3 Effect of Aroclor 1254 and Various PCB Congeners on Levels of Cellular Dopamine in PC12 Cells

Congener	n	Cellular Dopamine IC_{50} ± S.E.M. (μg/ml)
Aroclor 1254	7	18.4 ± 4.4
2,2'	4	n.d.
2,2',5,5'	4	31.8 ± 3.0
2,3',4,4',5	4	38.1 ± 2.8
3,3',4,4',5	4	27.6 ± 1.6
2,2',3,3',4,4'	4	34.4 ± 2.8
2,2',4,4',5,5'	5	41.8 ± 1.8

PC12 cells were exposed to various media concentrations of Aroclor 1254 or selected PCB congeners for 3 days. The determination of cellular dopamine was performed as described in Chapter 2, "Materials and Methods". **n.d.** not determinable since amounts of cellular dopamine never decreased below 50%. Data represent the mean ± S.E.M. IC_{50} values determined from 4 to 7 separate experiments for each congener.

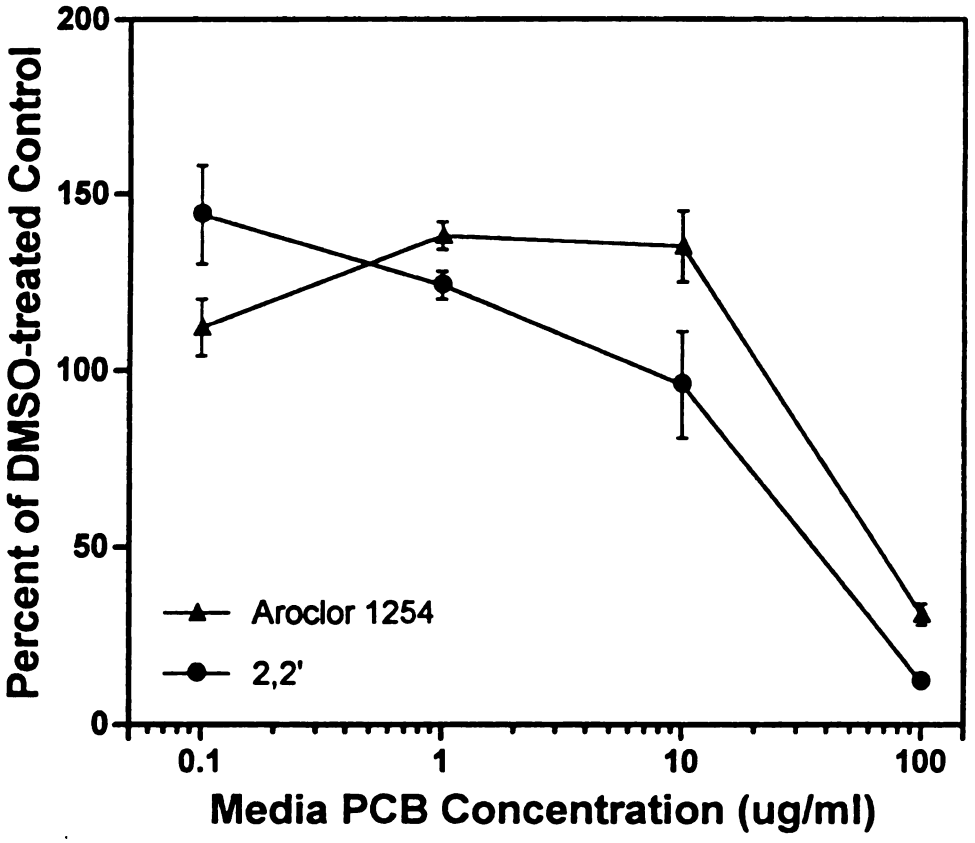


Figure 3.9 Cellular Dopamine Content Following Acute Exposure to Aroclor 1254 or 2,2'-Dichlorobiphenyl

PC12 cells were exposed for 6 hours to Aroclor 1254 or 2,2'-dichlorobiphenyl. Cellular dopamine content was determined as described in Chapter 2, "Materials and Methods". Amounts of cellular dopamine were normalized to protein levels as determined by incorporation of ^{14}C-leucine as described by Seegal et al., 1989. Values represent the mean ± S.E.M. for 4 separate measurements.

DISCUSSION

Aroclor 1254: Exposure of cells to Aroclor 1254 for 6 hours has been reported to be sufficient to attain a maximal decrease of approximately 70% in cellular dopamine at a media concentration of 100 μg/ml of Aroclor 1254 (Seegal et al., 1989). In the present studies, 100 μg/ml of Aroclor 1254 was lethal to the cells by 24 hours, suggesting that this concentration of PCBs elicits a cytotoxic effect. To further determine the cytotoxicity of lower concentrations of Aroclor 1254 at 3 days of exposure in PC12 cells, total media concentrations up to and including 50 μg/ml of the PCB mixture were further examined. The results of studies on the incorporation of (methyl-^{3}H)-thymidine as a measure of DNA synthesis, incorporation of ^{14}C-leucine as a measure of protein synthesis, and leakage of LDH into the culture medium as a measure of membrane integrity all suggested that at concentrations of Aroclor 1254 up to and including 25 μg/ml, there was no generalized cytotoxicity at 3 days. At a 50 μg/ml media concentration of Aroclor 1254, where DNA content began to decrease, decreases in ^{3}H-thymidine and ^{14}C-leucine incorporation indicated that the cells were beginning to show signs of cytotoxicity suggesting that at 50 μg/ml or greater, Aroclor 1254-mediated decreases in cellular dopamine may be at least partially attributable to general cytotoxicity. However, at media concentrations of Aroclor 1254 lower than 50 μg/ml, cytotoxicity cannot

account for the PCB-induced decrease in the amounts of cellular dopamine and thus may be due to decreases in dopamine synthesis. Furthermore, these data support the use of DNA content measurements as indicators of general cytotoxicity.

In undifferentiated, non-NGF-treated cells, the PCB mixture Aroclor 1254 caused dose-dependent decreases in cellular dopamine and its major metabolite, DOPAC. Amounts of cellular DOPA, however, were elevated, compared to control cells. These results suggest that Aroclor 1254 may affect the enzyme converting DOPA to dopamine, LAAAD. An examination of the possible mechanisms for the effects of Aroclor 1254 on cellular dopamine will be addressed in Chapter 6. Amounts of DOPAC in PC12 cells exposed to Aroclor 1254 decreased in a dose-dependent manner, along with amounts of cellular dopamine. Whether the decreased cellular amounts of DOPAC were due to a direct effect of Aroclor 1254 on the enzyme converting dopamine to DOPAC, monoamine oxidase (MAO), or if the decreases in DOPAC solely reflects a diminished amount of dopamine available to be metabolized is not known at the present. However, if MAO were inhibited, and increase in dopamine would be expected. More study into the mechanism for the dose-dependent decreases in cellular DOPAC will be required to elucidate this issue.

In undifferentiated, or non-NGF-exposed PC12 cells, the

dose-response curves depicting both cellular dopamine and DNA content were shifted to the left with longer exposure, up to 3 days. There was no significant shift in the dose-response curves between 3 and 7 days, however, suggesting that the maximal ability of Aroclor 1254 to decrease cellular dopamine levels was attained within 3 days.

PC12 cells in the process of differentiating responded to Aroclor 1254 in the same manner as that observed with undifferentiated cells. Differentiating PC12 cells, those exposed simultaneously to NGF and Aroclor 1254, also demonstrated shifts to the left in the dopamine and DNA dose-response curves between 1 and 3 days of exposure. The shifts were of the same magnitude as those seen with undifferentiated PC12 cells. Thus the data suggest that concomitant exposure of PC12 cells to NGF and Aroclor 1254 did not result in any increase or decrease in the sensitivity of these differentiating dopaminergic cells to decreases in cellular dopamine or DNA content as a result of exposure to Aroclor 1254, when compared to undifferentiated PC12 cells. Further, the data suggest that differentiating dopaminergic PC12 cells exposed to PCBs beginning in the early stages of differentiation may be as susceptible to PCB-mediated decreases in cellular dopamine as are undifferentiated cells.

However, exposure of PC12 cells to NGF for 7 or 14 days prior to exposure to Aroclor 1254 resulted in partial

protection from the decreases in cellular dopamine caused by Aroclor 1254. Attenuation of the effects of other neurotoxic compounds such as 6-hydroxydopamine (6-OHDA), MPTP, and N-methyl-4-phenylpyridinium ion (MPP^+) by neurotrophic factors have previously been shown. Preincubation with brain-derived neurotrophic factor (BDNF) or NGF was shown by Spina et al. (1992) to offer a degree of protection from the neurotoxic effects of both 6-OHDA and MPP^+. In SH-SY5Y human neuroblastoma cells, 24 hours of pretreatment with either BDNF or NGF prior to exposure to 6-OHDA or MPP^+ resulted in significant protection from these neurotoxicants. In primary nigral dopaminergic cell cultures, 6 days or 4 days of pretreatment with BDNF offered partial protection from the neurotoxic effects of 6-OHDA or MPP^+, respectively. Further, Hyman et al. (1991) have demonstrated that a 24 hour preincubation of dopaminergic embryonic rat ventral mesencephalic cells with BDNF resulted in partial protection from the neurotoxic effects of MPTP. It is apparent that neurotrophic factors can play an important role in attenuating the ability of toxic compounds to damage dopaminergic cells. In the case of PCBs, the mechanisms by which NGF offers partial protection from the Aroclor 1254-mediated decrease in cellular dopamine are unclear and merit further investigation.

There appear to be multiple effects of Aroclor 1254 on PC12 cells. For instance, effects on neurite elongation and

effects on cellular dopamine, may or may not be related since the dose-response curves for these effects are different. It is unclear why the simultaneous treatment of PC12 cells with NGF did not afford partial protection from the effects of Aroclor 1254, but pretreatment with NGF did partially protect the cells. Possibly, NGF induced an event during the first 7 days of differentiation that allowed the cells to be partially resistent to the dopamine-depleting effects of Aroclor 1254. This event, however, was apparently blocked or altered in the presence of Aroclor 1254, since concurrent administration of NGF and Aroclor 1254 resulted in similar decreases in dopamine as were seen in cells treated only with Aroclor 1254. This suggests that cells in early differentiation are more susceptible to the effects of PCBs than more differentiated cells are, consequently, embryos in the earliest stages of development may be the most sensitive.

Aroclor 1254 did not block the NGF-induced morphological differentiation of PC12 cells. With concomitant exposure to NGF, Aroclor 1254 caused a dose-dependent increase in neurite elongation in PC12 cells without altering the percentage of cells bearing neurites or the number of neurites per cell, suggesting that Aroclor 1254 enhanced NGF-stimulated elongation of neurites. It is possible that the mechanism underlying this enhanced neurite elongation may be related to ion flux and homeostasis,

especially that of Ca^{++}, since Ca^{++} is involved in the regulation of neurite elongation (Lankford and Letourneau, 1989). Alterations in Ca^{++} flux and homeostasis have been reported in cerebellar granule cells upon exposure to PCB congeners (Kodavanti et al., 1993a). Furthermore, PCB congener-related alterations in phosphoinositide signalling and protein kinase C (PKC) translocation have also been reported (Kodavanti et al., 1993b). PKC has been implicated in cell growth and also plays a role in neuritogenesis, hence, the effects of PCBs on neurite elongation in PC12 cells may also involve PKC. Other, non-calcium, non-PKC mediated mechanisms may also be involved. Further work will be required to determine the mechanism by which PCBs enhance neurite elongation. While it is clear that concomitant exposure of PC12 cells to NGF and Aroclor 1254 results in enhanced neurite elongation, it is unknown whether these neurites form synapses with other cells, or if synapses formed are operable. Hence, more research will be required to determine the physiological consequences of the PCB-mediated enhanced neurite elongation in PC12 cells.

PCB Congeners: Of the 11 PCB congeners examined, those with at least 4 chlorine substitutions generally caused a dose-dependent decrease in cellular dopamine in PC12 cells. Amounts of cellular DOPA were increased by 2 di-*ortho* tetrachlorobiphenyls (2,2',4,4'-tetrachlorobiphenyl and 2,2',5,5'-tetrachlorobiphenyl), and 1 di-*ortho*

hexachlorobiphenyl (2,2',4,4',5,5'-hexachlorobiphenyl). All three of these congeners can be found in Aroclor 1254 (Hutzinger et al., 1974), and two of these congeners (2,2',4,4'-tetrachlorobiphenyl and 2,2',5,5'-tetrachlorobiphenyl) have been found to be concentrated in the brains of adult monkeys who had ingested PCBs (Seegal et al. 1990, 1991). These animals demonstrated a decrease in amounts of brain dopamine that was apparent up to 44 weeks following cessation of exposure to PCBs (Seegal et al, 1994). The increase in cellular DOPA suggests that these congeners may, like Aroclor 1254, decrease the activity of LAAAD. In addition to their effects on cellular dopamine and DOPA, the more highly substituted PCB congeners caused a general decrease in the cellular DOPAC. As with Aroclor 1254, the mechanism(s) for this decrease are not known, but may result from a decrease in available substrate, and/or may also be due to direct effects of PCBs on MAO.

The PCB congeners with 2 or 3 chlorine substitutions tested failed to decrease the amount of cellular dopamine in PC12 cells until media levels of PCBs reached a cytotoxic concentration of 100 μg/ml. Amounts of cellular DOPA were unaffected by these more lightly chlorinated congeners, however, amounts of cellular DOPAC were decreased by 2,2'-dichlorobiphenyl; 3,3'-dichlorobiphenyl; and 2,4,4'-trichlorobiphenyl. These 3 congeners decreased DOPAC without a concomitant or prior decrease in the amount of

cellular dopamine. These results suggest the possibility that these congeners may affect MAO directly, since the levels of substrate appeared to be unaffected by these PCBs. Hypothetically, if these lightly substituted PCB congeners were directly affecting MAO, the amounts of cellular dopamine would be expected to increase. However, it is possible that inhibition of TH by the end product, dopamine, may have compensated for the expected increase in the amount of cellular dopamine by causing less DOPA to be formed. The only congener tested that was wholly without effect on levels of catecholamines in PC12 cells was 4,4'-dichlorobiphenyl. The reasons for this are unknown.

The PCB congener 2,2'-dichlorobiphenyl has been reported to be the most potent PCB congener for decreasing cellular dopamine in acutely exposed PC12 cells (Shain et al., 1991). In our system, 2,2'-dichlorobiphenyl failed to cause any changes in cellular catechols, except for a decrease in the metabolite DOPAC. The reasons for discrepancy are unknown, but it may be that a difference in the clone of PC12 cells utilized was sufficient to cause such a discrepancy. Even when the methods used by Seegal et al. (1989) were duplicated using our PC12 cells, 2,2'-dichlorobiphenyl failed to affect the amount of cellular dopamine in our PC12 cells.

The PCB congener 3,3',4,4',5-pentachlorobiphenyl, has been reported to act through the Ah-receptor to exert its

effects (Safe 1984). In PC12 cells, 3,3',4,4',5-pentachlorobiphenyl appeared to be toxic, decreasing in DNA content (used as an indicator of cytotoxicity) beginning at 10 μg/ml media concentration. Indeed, 3,3',4,4',5-pentachlorobiphenyl caused decreases in cellular dopamine, DOPA, and DOPAC that concurred with decreased DNA content, suggesting that with this congener, cytotoxicity may be the cause of the decreased cellular catechols in PC12 cells.

For a number of selected congeners (2,2'-dichlorobiphenyl; 2,2',4,4'-tetrachlorobiphenyl; 2,2',5,5'-tetrachlorobiphenyl; 2,3,4,4',5-pentachlorobiphenyl; 2,3',4,4',5-pentachlorobiphenyl; 2,2',3,3',4,4'-hexachlorobiphenyl; and 2,2',4,4',5,5'-hexachlorobiphenyl) and Aroclor 1254, IC_{50} values were determined based on the media concentrations of PCBs at which the amount of cellular dopamine decreased by 50%. 2,2'-dichlorobiphenyl was the exception, since cellular dopamine never fell below 50% at media concentrations up to 50 μg/ml. The congeners 2,2',5,5'-tetrachlorobiphenyl; 2,3',4,4',5-pentachlorobiphenyl; 2,2',3,3',4,4'-hexachlorobiphenyl; and 2,2',4,4',5,5'-hexachlorobiphenyl each comprise a large percentage of Aroclor 1254 (4.2%, 9.5%, 1.5%, and 6.1%; Hansen, 1987). By examining these congeners, it was hoped that one or some of the components of Aroclor 1254 that caused the decrease in cellular catechols observed in PC12 cells may become more apparent. Aroclor 1254 was the most

potent of the PCBs examined for decreasing cellular dopamine in PC12 cells. Surprisingly, for the individual congeners for which IC_{50} values could be calculated, the values were similar to each other, and were about twice the value for Aroclor 1254. However, the PCB congeners may differentially associate with the cells, and thereby have different potencies to reduce cellular dopamine. This possibility will be investigated in Chapter 5 of this dissertation.

These data on the IC_{50} values for congeners and Aroclor 1254 also raised a question as to whether PCB congeners in a mixture, such as Aroclor 1254, interact with each other to enhance each others' effects. The effects of 3 congeners on dopamine levels in PC12 cells has been previously examined (Seegal et al., 1990). The data suggested that a mixture of the congeners was more effective at decreasing cellular dopamine than any one of the component congeners alone. These data indicate that more work will be needed to examine possible interactions of individual PCBs within a mixture of congeners, and how possible congener interactions may affect dopaminergic cells.

In the current studies, increasing the *ortho* substitution from non-*ortho* to di-*ortho* appeared to lead to a slightly enhanced ability of a PCB congener to decrease cellular dopamine without a concurrent decrease in DNA content, supporting the findings of Shain et al., 1991. The congener 2,2'-dichlorobiphenyl appeared to be an exception

in our system, even though 2,2'-dichlorobiphenyl has been reported to be the most potent congener for decreasing cellular dopamine in PC12 cells (Shain et al., 1991). Data presented earlier indicated that exposure of the cells to the non-*ortho* substituted congener 3,3'4,4',5-pentachlorobiphenyl was toxic to the cells and decreased cellular dopamine could be related to decreases in DNA content. However, with exposure to increasing media concentrations of the mono-*ortho* substituted congener 2,3',4,4',5-pentachlorobiphenyl and the di-*ortho* substituted congener 2,2',3,3',4,4'-hexachlorobiphenyl, the amounts of cellular dopamine began to decline at concentrations lower than those at which decreases in DNA content were observed. This suggests that these last two congeners may have effects on the biosynthetic pathway for dopamine that is not due to cell death. Furthermore, a comparison of the dose-response data from 3,3',4,4',5-pentachlorobiphenyl; 2,3',4,4',5-pentachlorobiphenyl and 2,2',3,3',4,4'-hexachlorobiphenyl suggest that di-*ortho* substituted congeners are more efficacious at decreasing cellular dopamine in PC12 cells than mono-*ortho* substituted congeners; which in turn appear to be more efficacious at decreasing cellular dopamine than non-*ortho* substituted congeners at non-cytotoxic media concentrations. These findings support the conclusions drawn by Shain et al. (1991) that *ortho* substituted congeners may be primary effectors in the ability of PCBs to

decrease cellular dopamine.

However, Shain et al. (1991) suggested that substitution in the *meta* positions decrease the efficacy of a congener to decrease cellular dopamine, especially if the congener were substituted at the *para* position. Congeners with *ortho, para* and minimal *meta* patterns of substitution were reported to be very effective at decreasing cellular dopamine. Data from our studies, however, suggests that chlorine substitution in the *meta*-5 positions may be important in predicting the ability of a congener to decrease cellular dopamine, possibly via decreasing dopamine synthesis. For instance, 3,3'-dichlorobiphenyl (which could be called 5,5'-dichlorobiphenyl) failed to decrease amounts of cellular dopamine in PC12 cells; however 2,2',5,5'-tetrachlorobiphenyl was the most potent congener for decreasing the amounts of cellular dopamine. Furthermore, increasing the *ortho* substitution of 4,4'-dichlorobiphenyl to 2,4,4'-trichlorobiphenyl to 2,2'4,4'-tetrachlorobiphenyl, does not enhance the ability of the congener to cause a decrease in cellular dopamine content. Therefore, these data suggest that, while *ortho* substitution may be required, it is not in itself sufficient for a PCB congener to cause a decrease in cellular dopamine.

Hence, PCBs, either as a mixture (Aroclor 1254) or as individual congeners, generally appear to decrease the amount of cellular dopamine in PC12 cells. Some PCB

congeners and Aroclor 1254 also affected cellular DOPA and DOPAC. The mechanism(s) explaining these phenomena are unknown, but may include alterations in tyrosine uptake, TH activity, or LAAAD activity. Furthermore, increasing the *ortho* substitution of the congener appeared to enhance its ability to decrease cellular dopamine; and the substitution of the *meta*-5 position may also be an important determinant in predicting the ability of a congener to cause a decrease in cellular dopamine. Concurrent exposure of PC12 cells to PCBs and NGF did not alter the PCB-mediated decrease observed in cellular dopamine, however, pretreatment of PC12 cells with NGF prior to exposure to PCBs appeared to protect against the cellular dopamine decreases. Furthermore, Aroclor 1254 enhanced the elongation of neurites of cells exposed concomitantly to NGF, indicating that PCBs have a multitude of effects on these cells.

CHAPTER 4

EFFECTS OF POLYCHLORINATED BIPHENYLS ON RELEASE OF DOPAMINE FROM PC12 CELLS

INTRODUCTION

Using a model dopaminergic cell system, the PC12 rat pheochromocytoma cell line, data have been obtained demonstrating that both acute and subchronic exposure of dopaminergic cells to PCBs, either as mixtures or as individual congeners, can result in a dose-dependent decrease in cellular dopamine content (Seegal et al., 1989, 1990; Angus and Contreras, 1994; Chapter 3). To our knowledge, no studies have yet investigated the relationship between the PCB-mediated decrease in cellular dopamine and possible changes in the amount of dopamine released from the cells. Thus, cells were exposed to various media concentrations of Aroclor 1254 or six individual PCB congeners [2,2' dichlorobiphenyl (PCB 04); 2,2',5,5' tetrachlorobiphenyl (PCB 52); 2,3',4,4',5 pentachlorobiphenyl (PCB 118); 3,3',4,4',5 pentachlorobiphenyl (PCB 126); 2,2',3,3',4,4' hexachlorobiphenyl (PCB 128); and 2,2',4,4',5,5' hexachlorobiphenyl (PCB 153)] to determine their effects on the amounts of dopamine released spontaneously or in response to stimulation of the cells with 56 mM K^+. Four of these congeners, 2,2',5,5' tetrachlorobiphenyl, 2,2',3,3',4,4' hexachlorobiphenyl, 2,3',4,4',5 pentachlorobiphenyl and 2,2',4,4',5,5' hexachlorobiphenyl, are found in relatively great abundance in Aroclor 1254, comprising 4.2%, 1.5%, 9.5%, and 6.1% of the mixture,

respectively (Hansen, 1987). The congener 3,3',4,4',5 pentachlorobiphenyl is a coplanar PCB congener known to interact with the Ah-receptor; thus it acts through a similar mechanism as 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD), and is representative of the dioxin-like class of PCBs. The congener 2,2' dichlorobiphenyl was tested because it has been reported to be the most potent congener in decreasing cellular dopamine in PC12 cells (Shain et al., 1991) although my previous results disagree (Chapter 3).

Results

PC12 cells displayed both a spontaneous and K^+-evoked release of dopamine. The amount of evoked released dopamine increased over time for cells exposed to media concentrations of 25 μg/ml Aroclor 1254 (Figure 4.1). An exposure time of 5 min was chosen for use in these studies. Exposure of the cells to 0.1 to 0.5% of the vehicle, dimethylsulfoxide (DMSO), had no effects on either the amount of spontaneous or K^+-evoked release of dopamine from PC12 cells, the amount of cellular dopamine, or the DNA content (data not shown). For all of the aforementioned parameters, the intraexperimental variability between naive and DMSO-treated cells was at most 10%.

Treatment of PC12 cells for 3 days with various media concentrations of Aroclor 1254 led to a dose-dependent decrease in both spontaneous and K^+-evoked release of

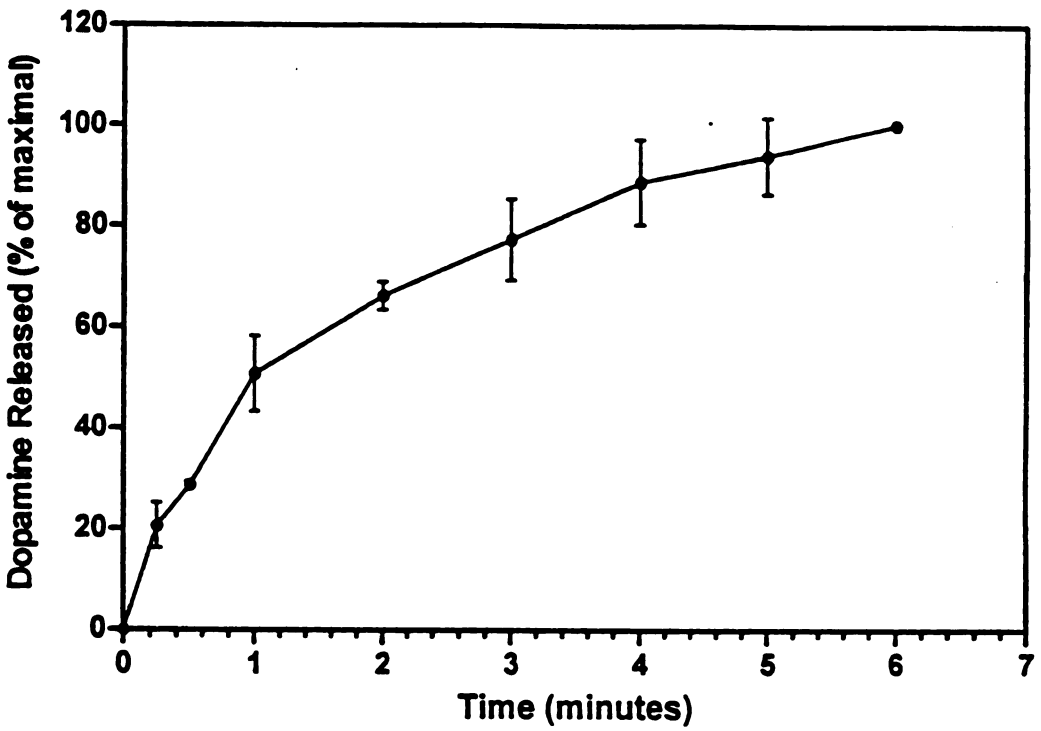


Figure 4.1 Dopamine Released by PC12 Cells in Response to 56 mM K^+

PC12 cells were stimulated to release dopamine by exposure to a HEPES buffer containing 56 mM K^+. The buffer was collected and analyzed for amounts of dopamine as described in Chapter 2, "Materials and Methods". Each point represents the mean ± S.E.M. for 3 separate experiments.

dopamine (Figure 4.2). The spontaneous and K^+-evoked release of dopamine from the cells decreased significantly at 10 μg/ml media concentration of Aroclor 1254, which did not cause decreases in DNA content (Figure 4.2). Further, the dose-response curves depicting the decreased release of dopamine and cellular dopamine caused by Aroclor 1254 appear to coincide (Figure 4.2). Spontaneous release of dopamine began to decline at a media Aroclor 1254 concentration of 10 μg/ml, but then remained relatively steady at an approximate 30% decrease.

To examine the specificity of the effects of PCBs on cellular dopamine and amounts of dopamine released, PC12 cells were exposed for 3 days to various media concentrations of six different PCB congeners. The effects of the different PCB congeners on the release of dopamine from PC12 cells were variable, as follows:

2,2' dichlorobiphenyl caused no decrease in cellular dopamine, spontaneous or evoked release of dopamine at media concentrations up to and including 50 μg/ml (Figure 4.3). At media concentrations greater than 50 μg/ml, there were decreases in released dopamine and cellular dopamine, coincident with a decrease in DNA content.

2,2',5,5' tetrachlorobiphenyl demonstrated the ability to cause similar dose-dependent decreases in the amounts of cellular dopamine and evoked release of dopamine at media concentrations up to and including 100 μg/ml (Figure 4.3).

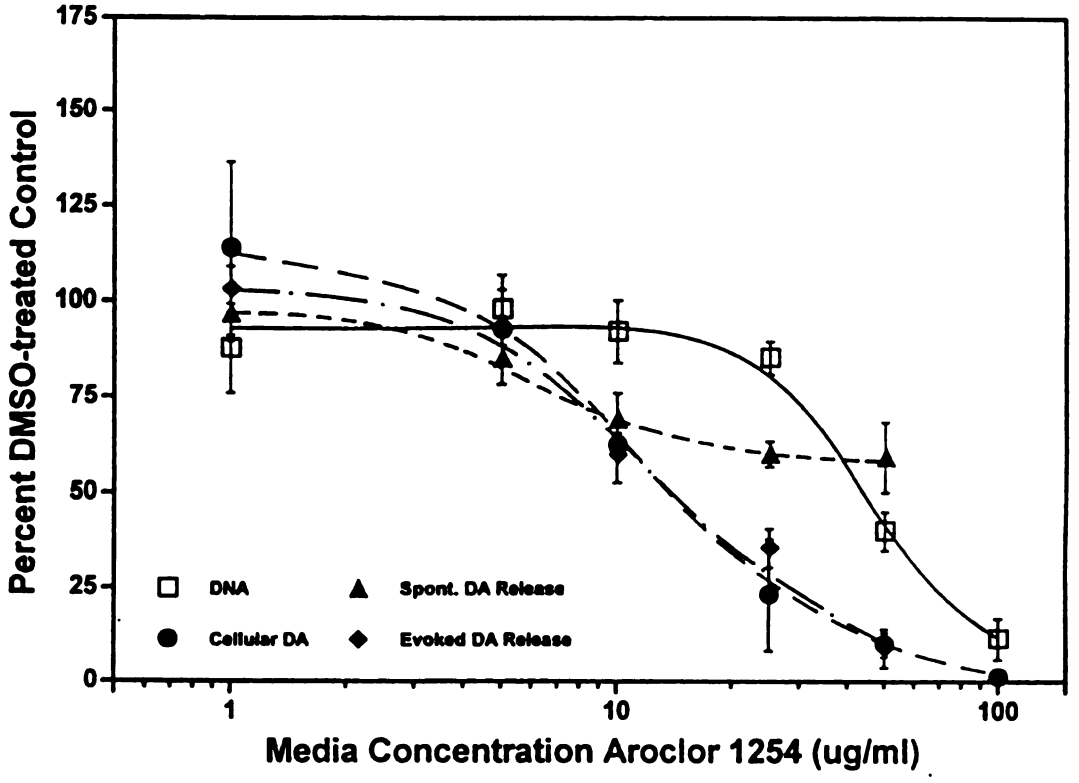


Figure 4.2 Effects of Exposure to Aroclor 1254 on Release of Dopamine From PC12 Cells

PC12 cells were exposed to various concentrations of Aroclor 1254 in the culture medium for 3 days. The release of dopamine, and the cellular dopamine and DNA contents were determined as described in Chapter 2, "Materials and Methods". Each point represents the mean ± S.E.M. for 3 to 7 separate experiments.

The spontaneous release of dopamine decreased at a media concentration of 10 μg/ml, and remained steady at a decrease of about 70%. Only at media concentrations greater than 50 μg/ml was there a decrease in the DNA content (Figure 4.3).

2,2',4,4',5,5' hexachlorobiphenyl decreased cellular dopamine and the amount of dopamine released in response to a stimulation by 56 mM K^+ at media concentrations up to and including 100 μg/ml (Figure 4.3). As was seen with Aroclor 1254, the spontaneous release of dopamine decreased by about 30% at 10 μg/ml media concentration of this PCB congener. Amounts of DNA began to decline at media PCB concentrations greater than 50 μg/ml.

3,3'4,4',5 pentachlorobiphenyl caused DNA content and cellular dopamine to decline significantly at media concentrations of 10 μg/ml. The amount of evoked released dopamine declined between 10 and 25 μg/ml (Figure 4.3). Amounts of dopamine spontaneously released were elevated. Visual observation of the cells treated with 3,3'4,4',5 pentachlorobiphenyl indicated that this PCB congener caused blebbing and vacuole formation on or in the cells.

2,3',4,4',5 pentachlorobiphenyl did not affect cellular dopamine or the amount of evoked dopamine released until media concentrations exceeded 25 μg/ml (Figure 4.3). The amount of spontaneously released dopamine was unaffected at media PCB concentrations up to and including 50 μg/ml.

Figure 4.3 Effects of PCB Congeners on Release of Dopamine From PC12 Cells

PC12 cells were exposed to various concentrations of PCB congeners in the culture medium for 3 days. The spontaneous and evoked release of dopamine, the cellular dopamine and DNA contents were determined as described in Chapter 2, "Materials and Methods". Each point represents the mean ± S.E.M. for 3 to 4 separate experiments.

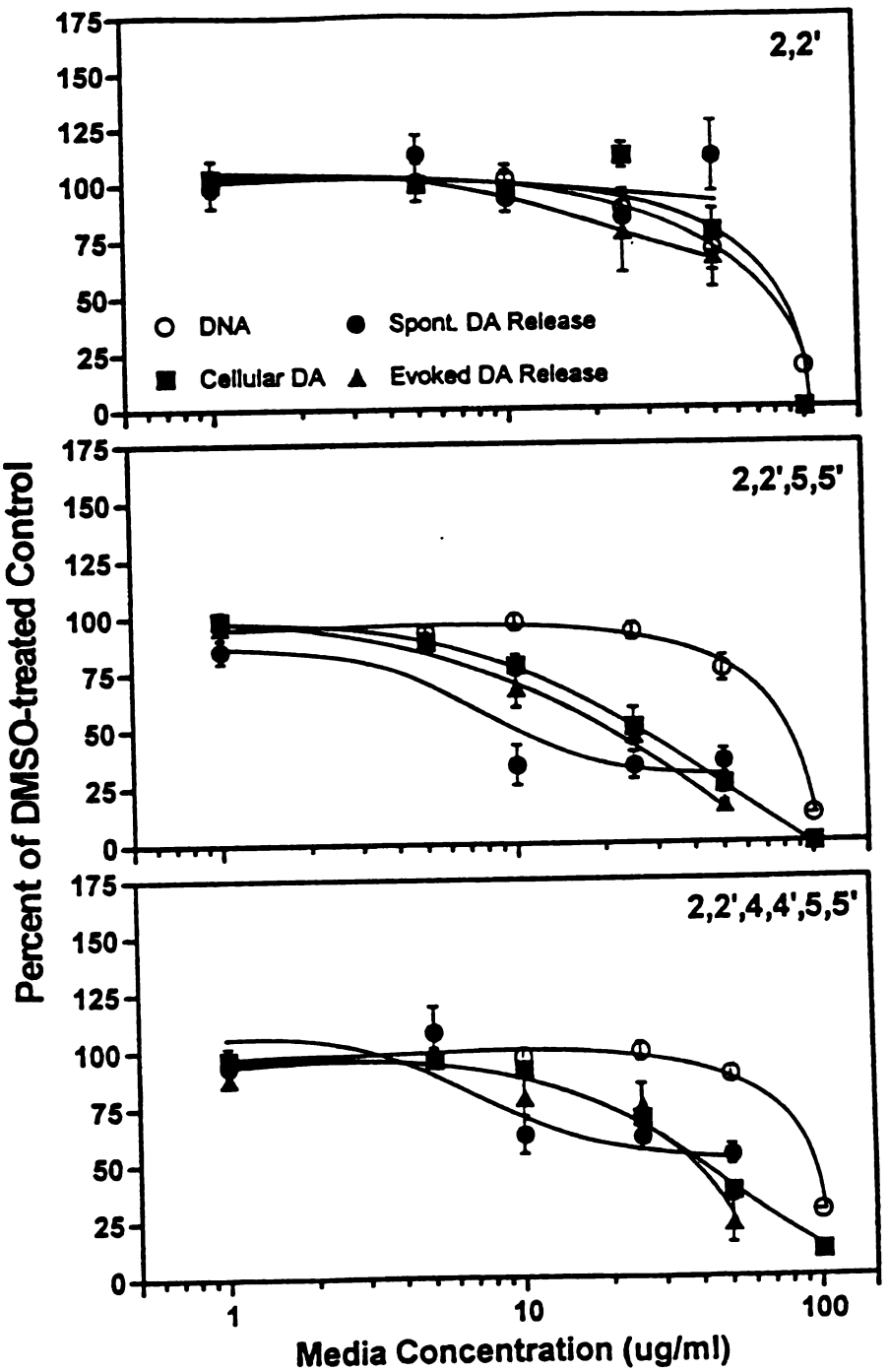

2,2'
2,2',5,5'
2,2',4,4',5,5'
DNA
Spont. DA Release
Cellular DA
Evoked DA Release
Percent of DMSO-treated Control
Media Concentration (ug/ml)
175
150
125
100
75
50
25
0
1
10
100

Figure 4.3

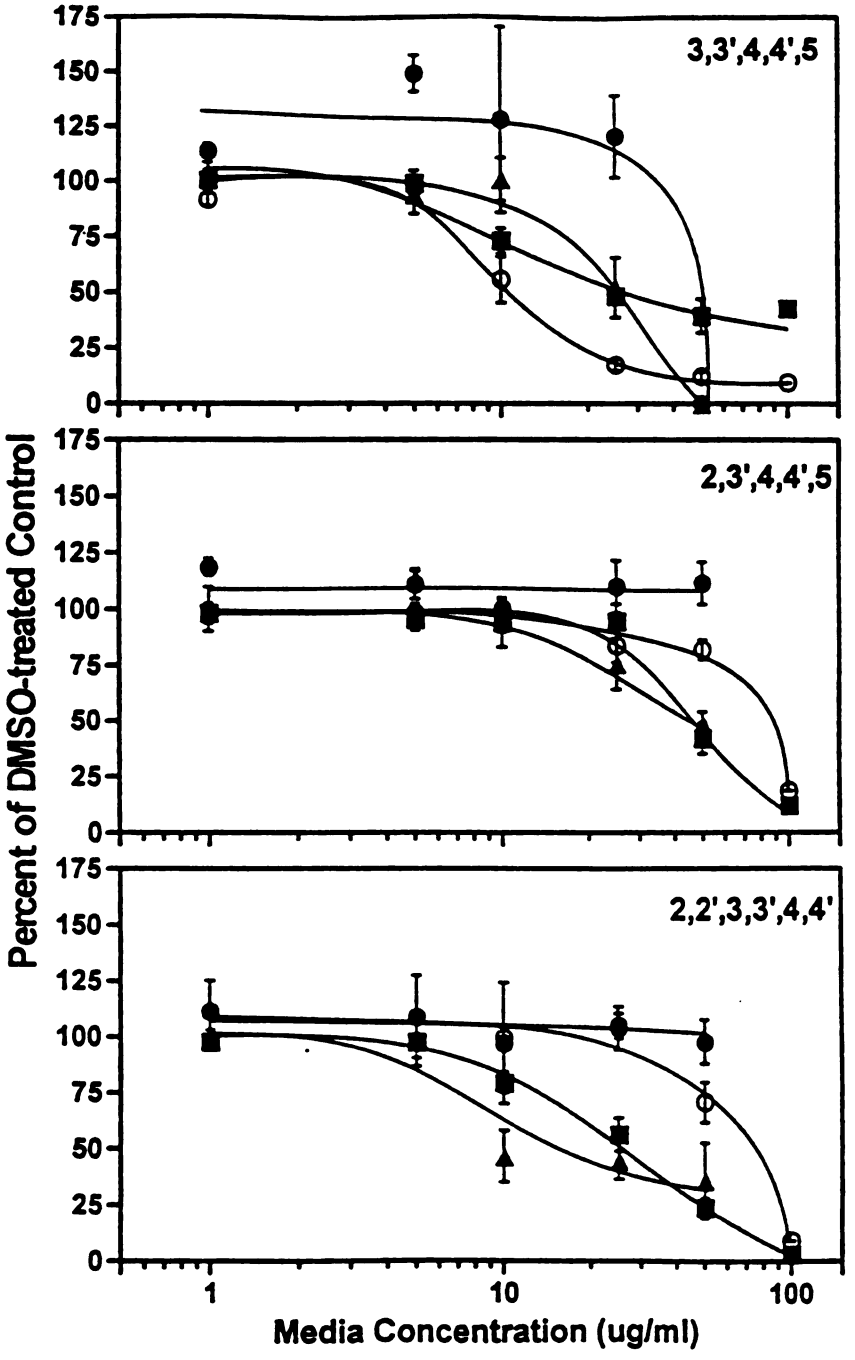


Figure 4.3 Continued

2,2',3,3',4,4' hexachlorobiphenyl caused dose-dependent decreases in cellular dopamine and the amount of dopamine released in response to a stimulation by 56 mM K^+ (Figure 4.3) at media congener concentrations of up to and including 100 μg/ml. Spontaneous release of dopamine was unaffected by this PCB congener. DNA content decreased at media concentrations of greater than 50 μg/ml.

The fraction of total dopamine released upon stimulation of PC12 cells with 56 mM K^+ did not change significantly with increasing media concentration of Aroclor 1254 (Figure 4.4). The fraction of total dopamine released in response to K^+ for each of the individual PCB congeners examined also did not change significantly with increasing media concentrations of PCBs (Figure 4.4).

Discussion

In general, polychlorinated biphenyls caused a significant decrease in the evoked release of dopamine from PC12 cells. This decrease in the evoked release of dopamine followed a similar concentration-response relationship as the decrease in cellular dopamine content caused by PCBs. These observations suggest that the decrease in the release of dopamine may be related to the decrease in cellular dopamine. PCBs have been suggested to cause a decrease in dopamine synthesis (Seegal et al., 1989, 1990; Angus and Contreras, 1994). Since it has been reported that newly synthesized catecholamines are preferentially released in

Figure 4.4 Fractional Release of Dopamine From PC12 Cells

The amount of dopamine, in ng, released by the cells when stimulated with 56 mM K^+ was divided by the total amount of dopamine, in ng, from each sample. Each bar represents the mean ± S.E.M. for 4 separate experiments.

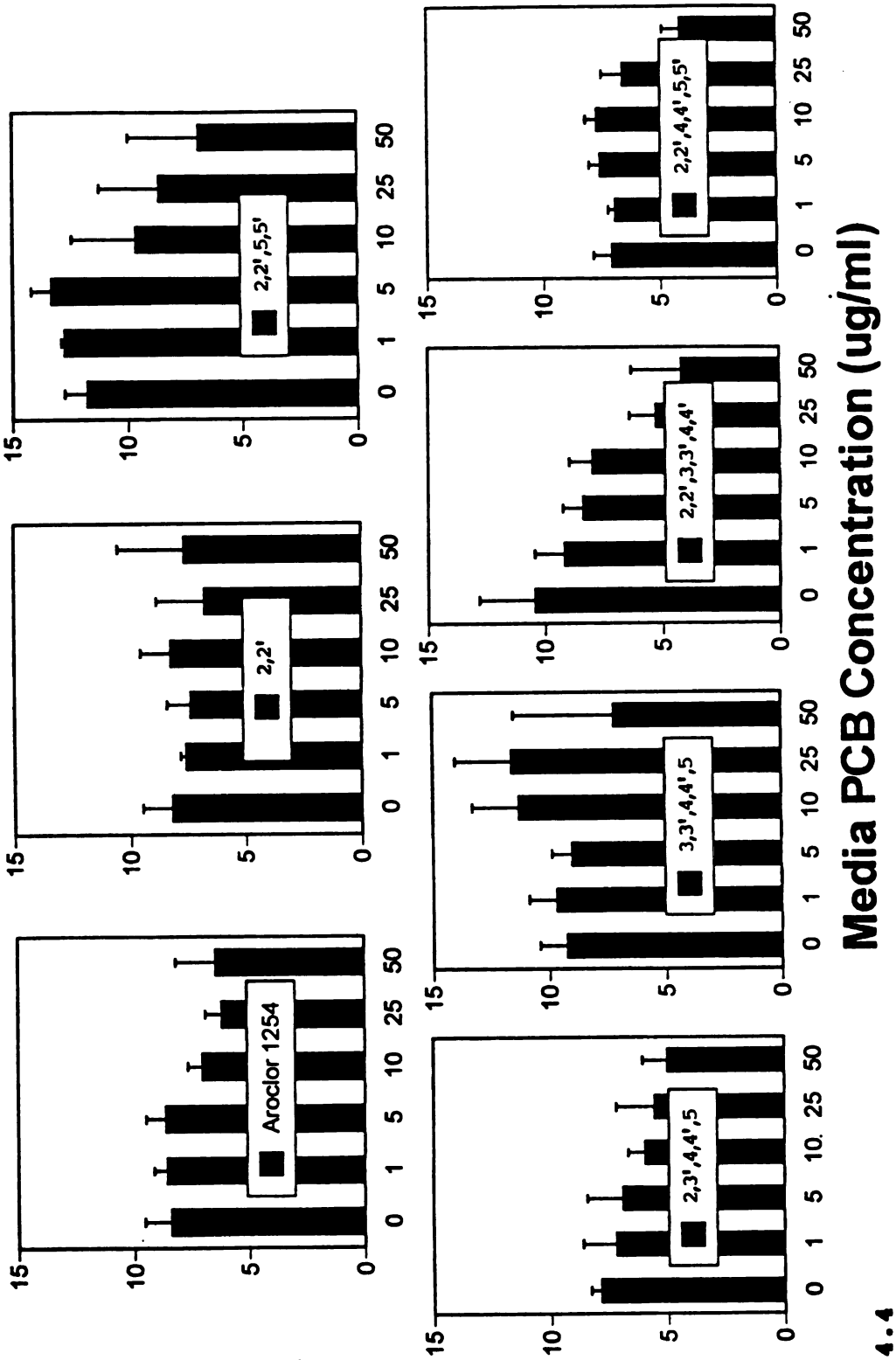

Percent of Total Dopamine
Media PCB Concentration (ug/ml)
Aroclor 1254
2,2'
2,2',5,5'
2,3',4,4',5
3,3',4,4',5
2,2',3,3',4,4'
2,2',4,4',5,5'
0
5
10
15
0 1 5 10 25 50

Figure 4.4

response to a depolarizing stimulus (Kopin et al., 1968; Besson et al., 1969; Gewirtz and Kopin, 1970), a decrease in dopamine synthesis could contribute to the observed decrease in the evoked release of dopamine from PCB-treated cells.

The spontaneous release of dopamine from PC12 cells decreased after exposure to Aroclor 1254, 2,2',5,5'-tetrachlorobiphenyl and 2,2'4,4'5,5'-hexachlorobiphenyl at media concentrations of PCBs that did not decrease DNA, but did decrease the amount of cellular dopamine. For these three PCBs, decreased dopamine synthesis could lead to decreased spontaneous release of dopamine. Data from these studies suggest that the *meta*-5 positions may be important in determining the propensity of a congener to decrease spontaneous release of dopamine, since the 5-positions of both of the congeners decreasing spontaneous release of dopamine are chlorine substituted. By inference, this pattern of substitution may thus be important in predicting a congener's potential to cause a decrease in the amount of cellular dopamine, possibly via decreasing dopamine synthesis, since dopamine may be spontaneously released from cells by diffusion across the membrane from the cytoplasm, prior to being packaged into vesicles or metabolized into dihydroxyphenylacetic acid (DOPAC). Also, a 2,2'-and-5,5' substitution pattern appears to be common in both of the congeners causing a decrease in the spontaneous release of dopamine from PC12 cells. These two congeners, 2,2',5,5'-

tetrachlorobiphenyl and 2,2',4,4',5,5'-hexachlorobiphenyl, make up a significant component of Aroclor 1254 (4.2% and 6.1%, respectively; Hansen, 1987), and thus, may partially account for the action of Aroclor 1254 on spontaneous release of dopamine.

In general, increasing the *ortho* substitution from non- to di-*ortho* appeared to lead to a slightly enhanced ability of a PCB congener to decrease both the evoked release of dopamine, and cellular dopamine content. The congener 2,2' dichlorobiphenyl was an exception in our system. Upon exposure of the cells to the non-*ortho* substituted congener, 3,3'4,4',5 pentachlorobiphenyl, amounts of DNA and evoked dopamine were substantially decreased at low media concentrations. This suggested that the decreases in DA release due to this congener were related more to the congener's cytotoxicity than to effects of the congener on the synthetic pathway for dopamine *per se*. Yet, exposure to increasing media concentrations of the mono-*ortho* substituted congener 2,3',4,4',5 pentachlorobiphenyl and the di-*ortho* substituted congener 2,2',3,3',4,4' hexachlorobiphenyl caused a decrease in evoked dopamine release to decline at media concentrations of the congeners that were less than those needed to decrease DNA content. These results suggest that 1) these two *ortho* substituted congeners (2,3',4,4',5-pentachlorobiphenyl and 2,2',3,3',4,4'-hexachlorobiphenyl) may have effects on the

biosynthetic pathway for dopamine that is not due to cell death, and 2) even though the degree of chlorination is the same between 3,3',4,4',5- and 2,3',4,4',5-pentachlorobiphenyl, the degree of *ortho* substitution contributed to the neurotoxicity and cytotoxicity of each congener. These data further suggest that, while *ortho* substitution may be required, it is not sufficient for observed effects on the evoked or spontaneous release of dopamine from PC12 cells in the absence of cytotoxicity. This supposition is supported by the observation that 2,2'-dichlorobiphenyl is a di-*ortho* substituted congener that does not decrease evoked release of dopamine from PC12 cells. These findings are further supported by the conclusions drawn by Shain et al. (1991) that *ortho* substituted congeners may be primary effectors in the ability of PCBs to decrease amounts of cellular dopamine (see Discussion of Chapter 3). However, Shain et al. (1991) did not investigate the effects of PCB congeners on the release of dopamine from PC12 cells.

An interesting observation is that none of the PCB congeners tested, except for 3,3',4,4',5 pentachlorobiphenyl (due to its cytotoxicity), were more potent than Aroclor 1254 in decreasing evoked release of dopamine or cellular dopamine content. This observation might encourage speculation about cooperative, permissive, or synergistic effects of PCB congeners on this dopaminergic system. It

has been suggested (Seegal et al., 1990) that a mixture of PCB congeners was more effective at decreasing the amount of cellular dopamine than any one of the component congeners alone. More work will be needed to determine potential interactions of individual congeners within a mixture of PCBS.

The exact mechanism(s) defining how polychlorinated biphenyls alter evoked DA release are unclear. Sites of action by PCBs could include 1) the enzymes involved in the synthesis of dopamine, 2) the packaging of dopamine into vesicles for release, and/or 3) the mechanisms involved in the release of dopamine form the cells. Obviously, if the synthesis of dopamine is decreased, either through effects of PCBs on biosynthetic enzymes or by decreasing the uptake of the precursor amino acid tyrosine, the amount of newly synthesized dopamine available for release will be reduced and subsequently, the amount of dopamine released in response to a stimulus will be reduced. The results of the present studies demonstrate that the fraction of dopamine released as a percentage of total available cellular dopamine is not affected by increasing media concentrations of PCBs. These findings suggest that the packaging of the dopamine into vesicles is not affected by PCBs, since an inhibition in the packaging of dopamine would have resulted in a decrease in the fraction of dopamine released. These data further suggest that decreases in evoked release of

dopamine may be directly related to decreases in the synthesis of dopamine, rather than to alterations in the mechanisms involved in the release of dopamine. If PCBs were stimulating or inhibiting the process of release of dopamine, the fraction of dopamine released in response to K^+ would either increase or decrease compared to control cells.

Polychlorinated biphenyls decrease the amounts of cellular dopamine and dopamine released in response to a depolarizing stimulus in PC12 cells. In the brain, dopamine is an important neurotransmitter in the numerous cognitive, behavioral, and emotional functions. Dopamine is also important in the regulation of motor functions, as demonstrated by a decrease in dopamine content in the substantia nigra of persons with Parkinson's disease. Together, this information suggests the possibility that an exposure of neuronal dopaminergic tissue to PCBs *in utero* may alter the distribution and kinetics of dopamine, potentially leading to cognitive, behavioral, emotional, and/or motor functions of the developing organism. This hypothesis is supported by studies involving the effects of *in utero* exposure of animals and humans to PCBs (Jacobson, et al, 1990, Bowman et al., 1981, Schantz et al., 1991). A decrease in the release of dopamine from dopaminergic neurons may not be great enough to result in an overt demonstration of cognitive, emotional or behavioral

deficits, or a Parkinsonian-like symptomology. However, deficits may occur under conditions of stress, such as learning new tasks or testing. In previous studies (Angus and Contreras, 1994), 25 μg/ml of Aroclor 1254 caused about a 90% decrease in cellular dopamine content at 3 days. Furthermore, there was an approximate 80% decrease in the amount of dopamine released from cells exposed to the same media concentrations of Aroclor 1254. Decreases of this magnitude in PC12 cells raise the question of the functional significance of an 80-90% reduction in cellular dopamine. Does a decrease of this magnitude translate into a physiological deficit? Insight into this question can be gained through studies using the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in monkeys. Monkeys treated subchronically with low doses of MPTP and displaying no gross motor deficits or overt Parkinsonian symptoms were shown by Taylor et al. (1990a,b) to have persistent deficits in acquisition and performance of tasks requiring complex cognition. Subtle deficits in motor coordination were also noted. Further, HVA (a dopamine metabolite) was reduced by approximately 65% in the cerebra-spinal fluid during the time period when these animals were tested for the complex cognitive tasks. Similarly, monkeys exposed chronically to MPTP that were motor asymptomatic for Parkinsonism displayed deficits in complex cognition tasks (Schneider and Kovelowski, 1990; Schneider, 1990).

Alterations in behaviors, such as increased irritability and decreased attentiveness, were also noted. Subtle motor deficits were observed in the MPTP-treated monkeys which were especially apparent during the testing of the complex cognition. Some of these subtle motor deficits included difficulty in reaching for and manipulating food rewards during the testing procedures, which indicated effects on fine motor coordination. Dopamine content in certain brain regions of these motor asymptomatic, MPTP-treated monkeys was significantly decreased. The amount of dopamine in the striatal was decreased 84-98%, while dopamine levels in the putamen were decreased some 80-90%. Therefore, in answer the question of functional significance for a 70-90% decrease in cellular dopamine levels, theoretically, there could be subtle deficits in cognition and motor coordination with dopamine decreases of this magnitude. In fact, the work of Jacobson et al. (1985, 1990), Rogan and Gladen (1992) and Gladen and Rogan (1991), cited in the introduction suggest that, in humans, there are indeed deficits in cognitive processes associated with PCB exposure.

Deficits in cognitive, behavioral, emotional, and motor abilities have been described in animal models in which MPTP has decreased the amount of dopamine in the brain, and such deficits have also been described in humans exposed to PCBs *in utero*. Exposure of dopaminergic PC12 cells to PCBs

resulted in a decrease in cellular dopamine to extents consistent with decreases observed in the brains of animals exposed to the dopaminergic neurotoxin MPTP. PCBs also decreased the evoked release of dopamine from PC12 cells that appears to be directly related to decreased cellular dopamine content. Together, the data suggest that polychlorinated biphenyls are agents capable of altering the function of dopaminergic cells. This altered function may underlie some of the PCB-mediated behavioral and cognitive deficits observed in children exposed *in utero* to PCBs. The current studies suggest that a decrease in the amount of cellular dopamine caused by a subchronic exposure to PCBs can manifest itself as a decrease in the evoked release of dopamine in dopaminergic PC12 cells. Further research needs to be done to examine mechanisms by which PCBs can decrease cellular dopamine and decrease evoked release of dopamine. Possible mechanisms by which PCBs act to cause the putative alterations in levels of cellular dopamine will be discussed in Chapter 6.

CHAPTER 5

ASSOCIATION OF POLYCHLORINATED BIPHENYLS WITH PC12 CELLS

Introduction

Polychlorinated biphenyls are putative neurotoxins, especially to dopaminergic cells, either upon acute or subchronic exposure (Seegal et al., 1989, 1990; Shain et al., 1991; Angus and Contreras, 1994). Data from previous chapters from this dissertation have demonstrated that both PCB mixtures and congeners decrease cellular dopamine, which functionally manifests itself as a decrease in the release of dopamine in response to a depolarizing stimulus. Although the evidence indicates that PCBs alter dopamine levels and synthesis, it is unclear at what cellular concentrations of PCBs these effects are seen.

Only one study has attempted to quantitate the amount of PCBs that are associated with cells when PCB-mediated decreases in cellular dopamine are observed (Shain et al., 1991). The weaknesses of that study were that 1) the procedure used in the study analyzed for PCB content in cell remnants previously extracted for determination of cellular catechols, therefore the amount of PCBs measured may have been underestimated; and 2) the study involved an acute exposure of PC12 cells to Aroclor 1254. Extraction of whole, subchronically PCB-exposed cells may provide a better indication of the cellular concentration of PCBs at a given media concentration that results in decreased cellular dopamine in the subchronically exposed cells. Thus, in the current studies, PC12 cells were subchronically exposed for

three days to either DMSO or Aroclor 1254 or PCB congeners. The PCBs were then extracted from the whole cells and amounts of PCBs in the cells were quantitated using gas chromatography coupled to electron capture, as described in Chapter 2.

To support a contention that the effects on cellular dopamine seen with exposure of PC12 cells to PCBs were not due just to exposure to an aromatic hydrocarbon, cellular concentrations of and effects of unchlorinated biphenyl were also determined in biphenyl-treated cells. Cellular biphenyl concentrations were determined in subchronically exposed PC12 cells using gas chromatography coupled to mass spectroscopy. Results of these studies suggested that PCBs were more effective at decreasing cellular dopamine than biphenyl; and that this is likely the case because the cellular PCB concentrations were greater than the cellular concentrations of the unchlorinated biphenyl. Further, a study involving PCB congeners suggested that the association of PCB congeners with the cells appears to vary with degree of chlorination and pattern of substitution, however, no discernable structure-activity relationship could be identified.

Results

A timecourse depicting the amounts of Aroclor 1254 contained in PC12 cells after various periods of exposure, indicated that the amount of Aroclor 1254 associated with

the cells increased over time (Figure 5.1). However the maximal concentrations of PCBs were associated with the cells at 1 day of incubation, and the cellular concentrations decreased in the days following to 7 days, which was the last time examined (Figure 5.1). Interestingly at 6 hours of exposure to Aroclor 1254, the amounts of Aroclor 1254 in the cells was similar to the amounts determined in the cells after 3 days of exposure.

Studies examining the partition of 25 μg/ml Aroclor 1254 in serum-free culture medium indicated that less than 1% was in the medium proper; however, if 12% serum was added to the medium, greater than 90% of the PCBs were found to be associated with the medium. Hence, serum in the media appeared to be a source for PCB binding in this test system. The data depicting the amounts of PCBs in the cells in relation to the amounts of PCBs in the media indicated that beyond a certain media concentration of PCBs, the amounts of PCBs in the cells increased at a greater rate than that seen at lower media concentrations of PCBs (Figure 5.2). In other words, the serum in the medium acted as a buffer for PC12 cells against the accumulation of Aroclor 1254.

A comparison of the effects of Aroclor 1254 on cellular dopamine and amount of cellular Aroclor 1254 at a given media concentration of Aroclor 1254 indicated that the cellular concentrations of PCBs increased as amounts of cellular dopamine decreased (Figure 5.3). Media

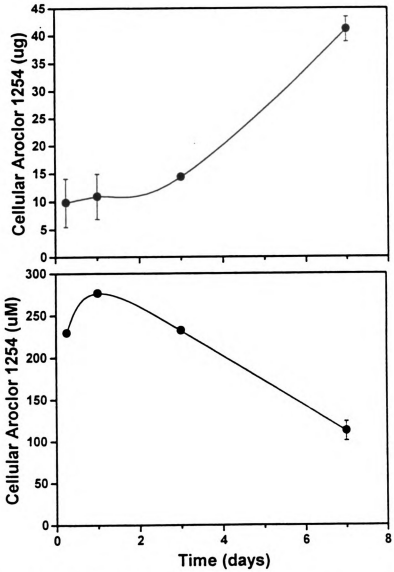


Figure 5.1 Cellular Association of Aroclor 1254 With PC12 Cells Over Time

PC12 cells were exposed to a media concentration of 35 μg/ml Aroclor 1254 for various amounts of time. Amounts of Aroclor 1254 in each sample (top) and cellular Aroclor 1254 concentrations (bottom) were determined as described in Chapter 2, "Materials and Methods". Each point represents the mean ± S.D. of 2 measurements.

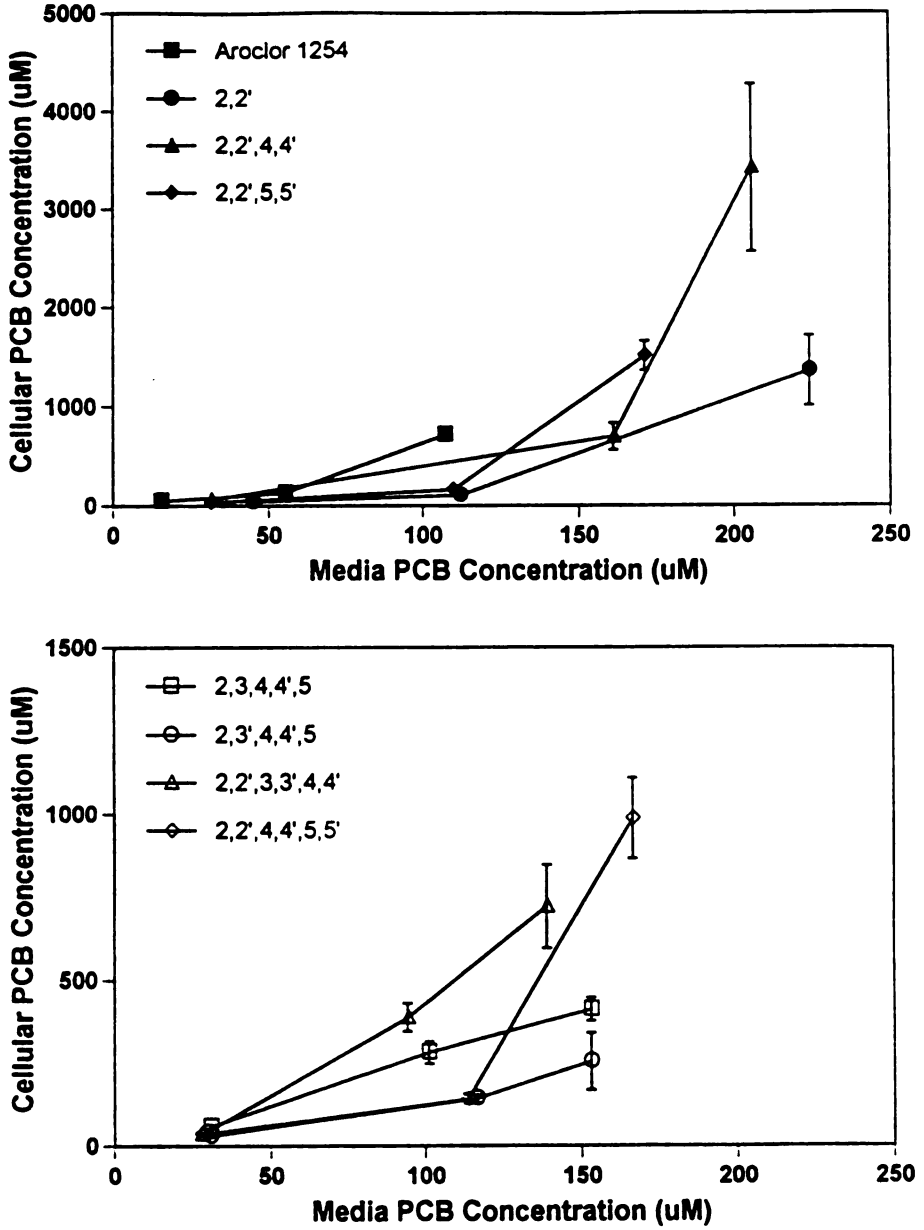


Figure 5.2 Association of Aroclor 1254 and PCB Congeners With PC12 Cells

PC12 cells were exposed to various media concentrations of Aroclor 1254 or 7 PCB congeners for 3 days. Cellular concentrations of PCBs were determined as described in Chapter2, "Materials and Methods". Points represent the means ± S.E.M. for 3 separate measurements. The molecular weight of Aroclor 1254 used for calculations of molarity was 327 and was taken from Hutzinger et al., 1974.

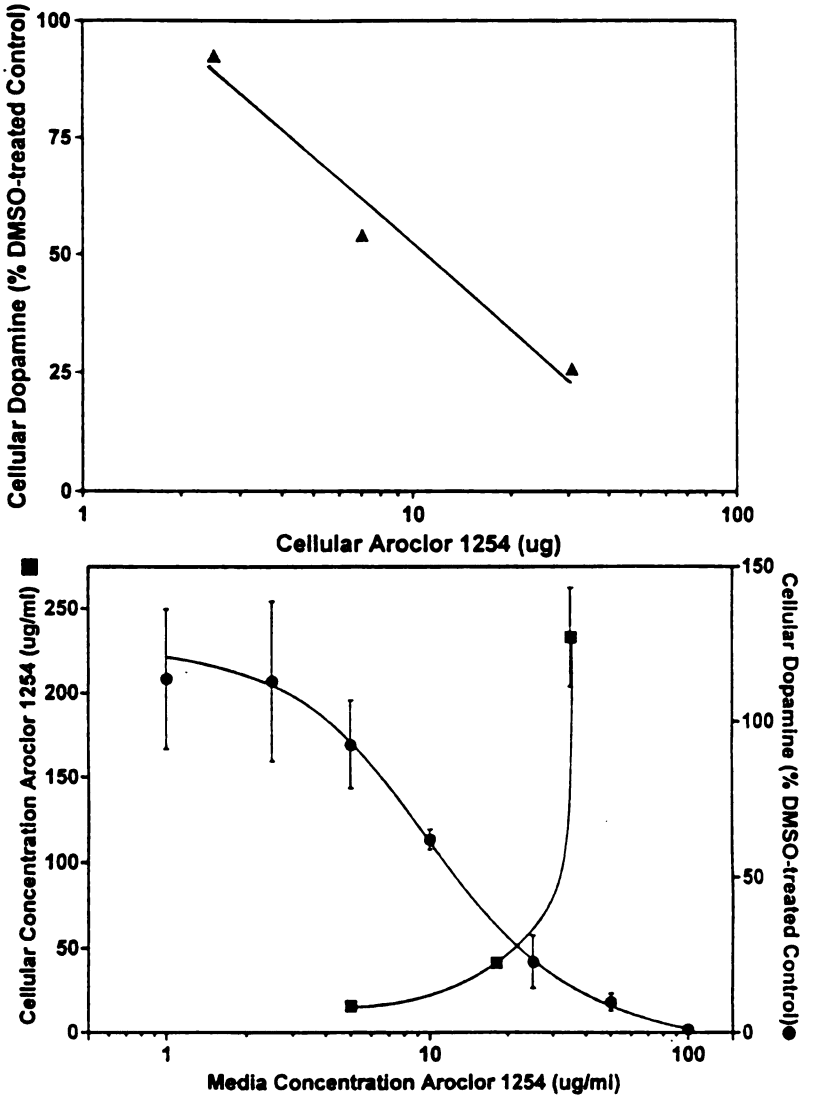


Figure 5.3 Relation of Cellular Dopamine to Cellular Aroclor 1254 Concentration in PC12 Cells

Data from effects of Aroclor 1254 on cellular dopamine content and from the cellular concentration of Aroclor 1254 was graphed onto one figure to visualize the relationship between the two processes. Top: Cellular dopamine and cellular Aroclor 1254 concentrations have an inverse relationship as media concentrations of Aroclor 1254 increase. Bottom: The decreases in cellular dopamine are directly related to increasing cellular Aroclor 1254 concentrations.

concentrations used for the determination of the cellular amounts of Aroclor 1254 were chosen based on the IC_{50} values for Aroclor 1254 that caused a decrease in levels of cellular dopamine (see Chapter 3), with one media concentration above the IC_{50} and one media concentration below the IC_{50}. The percent of total PCB added to the media that was associated with the cells was determined, as well as the cellular concentration of PCBs (Table 5.1). Generally, about 1 to 3% of the total Aroclor 1254 added to the culture medium was associated with the cells. Further, PC12 cells appeared to concentrate the Aroclor 1254 from the medium. A media concentration of Aroclor 1254 of 25 μg/ml (which was used in the timecourse studies) represented a molar concentration of 76.5 μM. Yet, the cellular concentrations of Aroclor 1254 was around 230 to 280 μM in the timecourse studies.

To determine if the effects on cellular dopamine observed with Aroclor 1254 could be duplicated with a non-chlorinated aromatic hydrocarbon, PC12 cells were exposed to various media concentrations of unchlorinated biphenyl. The concentrations of media biphenyl were chosen based on the molar concentrations of PCBs previously examined. This was done to compare the effects of the different compounds over the same range of media concentrations. Subchronic exposure of PC12 cells to polychlorinated biphenyls (Aroclor 1254) for 3 days was significantly more effective AT decreasing

Table 5.1 Cellular Concentrations of Aroclor 1254 and PCB in PC12 Cells

The amounts of cellular Aroclor 1254 or individual PCB congeners in subchronically treated PC12 cells was determined as described in Chapter 2, "Materials and Methods". Values represent the mean ± S.E.M. for 3 determinations.

Table 5.1 Aroclor 1254 and PCB Congeners Associated With PC12 Cells

Media Conc.in μg/ml (μM)	% of total PCB associated with cells	Cellular conc. (μg/ml)	Cellular conc. (μM)
PCB 04			
10 (44.8)	1.65 ± 0.83	8.54 ± 3.64	38.0 ± 16.0
25 (112)	1.15 ± 0.25	18.34 ± 8.15	103.4 ± 0.4
50 (224)	1.92 ± 0.46	304.7 ± 135.91	1,363.7 ± 609.5
PCB 47			
10 (31.3)	2.75 ± 0.23	18.0 ± 3.74	61.7 ± 12.9
47 (161)	2.94 ± 0.2	203.62 ± 68.42	697.7 ± 234.4
60 (205.6)	3.20 ± 1.2	997.37 ± 435	3,417 ± 1,491
PCB 52			
10 (31.3)	1.53 ± 0.31	9.47 ± 1.56	32.4 ± 5.36
32 (109.6)	2.94 ± 0.35	47.65 ± 6.42	163.2 ± 22.0
50 (171)	3.61 ± 0.28	441.13 ± 74.59	1,511.4 ± 255.6
PCB 114			
10 (30.6)	2.75 ± 0.31	19.57 ± 3.44	60.0 ± 10.6
33 (101.1)	2.94 ± 0.80	77.92 ± 19.05	238.8 ± 58.4
50 (153)	2.44 ± 0.05	134.85 ± 19.74	413.3 ± 60.5

Table 5.1 Aroclor 1254 and PCB Congeners Associated With PC12 Cells (Continued)

Media Conc. in μg/ml (μM)	% of total PCB associated with cells	Cellular conc. (μg/ml)	Cellular conc. (μM)
PCB 118			
10 (30.6)	2.84 ± 0.93	13.27 ± 6.36	29.6 ± 4.9
38 (116.5)	2.40 ± 0.87	46.91 ± 7.11	143.8 ± 21.8
50 (153)	1.69 ± 0.20	105.72 ± 43.62	256.9 ± 149.8
PCB 128			
10 (27.7)	2.14 ± 0.38	12.77 ± 2.33	35.4 ± 6.43
34 (94.2)	5.01 ± 0.18	140.36 ± 26.32	389.0 ± 72.9
50 (139)	1.98 ± 0.16	399.9 ± 248.52	720.7 ± 216.7
PCB 153			
10 (27.7)	2.13 ± 0.20	12.99 ± 2.20	36.0 ± 6.1
41 (113.6)	1.57 ± 0.39	51.79 ± 9.43	143.5 ± 26.2
60 (166.3)	3.52 ± 0.04	355.86 ± 76.69	986.4 ± 121.6
Aroclor 1254			
5 (15.3)	2.47 ± 0.06	15.70 ± 0.86	48.0 ± 2.7
18 (55.1)	2.00 ± 0.20	41.54 ± 0.89	127.0 ± 2.7
35 (107.0)	4.40 ± 0.70	232.88 ± 29.34	712.1 ± 89.7

cellular dopamine in PC12 cells than 3 days of subchronic exposure to equimolar media concentrations of biphenyl (Figure 5.4). The amounts of PCBs determined to be associated with subchronically treated cells was on the order of two to three magnitudes greater than biphenyl for exposure to equimolar media concentrations -- the cellular concentrations of biphenyl was in the nM range, as compared to the μM range for PCBs (Tables 5.1 and 5.2). Less than 1% of the total biphenyl added to the culture medium was found in the cells (Table 5.2).

The relationship between subchronic exposure of PC12 cells to individual PCB congeners and the cellular concentrations of those congeners at media concentrations which decreased cellular dopamine was also examined. The media concentrations of individual congeners examined included the IC_{50} concentration, 10 μg/ml, and either 50 or 60 μg/ml (60 μg/ml was used for congeners whose IC_{50} concentrations were 40 μg/ml or greater). The cellular concentrations of PCB congeners, reported either as μg/ml or as μM, (Table 5.1) changed with differing amount of congener chlorination. Higher concentrations of lightly substituted congeners such as 2,2'-dichlorobiphenyl, or the tetrachlorobiphenyls were associated with the cells, as compared to penta- or hexachlorobiphenyls (Table 5.1). However, the cellular concentrations of PCB congeners did not vary significantly among congeners with the same number

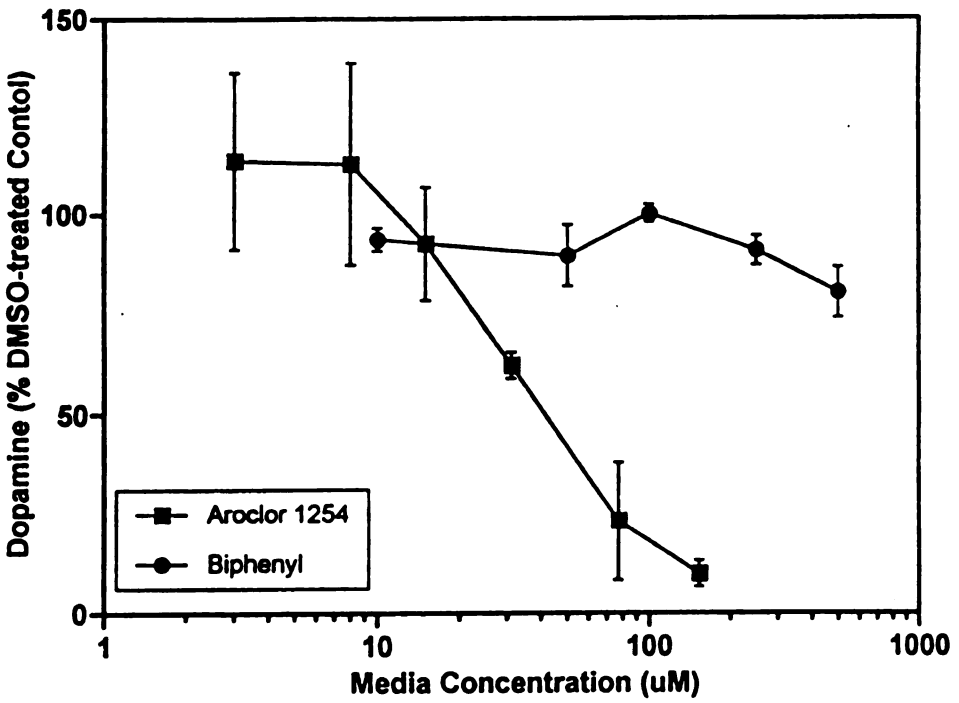


Figure 5.4 Effects of Aroclor 1254 and Biphenyl on Cellular Dopamine

PC12 cells were exposed to various media concentrations of either Aroclor 1254 or biphenyl for 3 days. The amounts of cellular dopamine were determined as described in Chapter 2, "Materials and Methods". Each point represents the mean ± S.E.M. for 3 to 5 separate experiments.

Table 5.2 Biphenyl Associated With PC12 Cells

Media Conc. of Biphenyl (μM)	% of Total Biphenyl Assoc. with Cells	Cellular Conc. of Biphenyl (ng/ml)	Cellular Conc. of Biphenyl (nM)
50	0.061 ± 0.001	773.33 ± 74.91	5.02 ± 0.49
100	0.177 ± 0.021	6,348.22 ± 218.84	41.20 ± 1.41
500	0.158 ± 0.011	56,971.78 ± 9,867.84	369.0 ± 64.05

PC12 cells were subchronically exposed to various media concentrations of unchlorinated biphenyl. Amounts of cellular biphenyl were determined as described in Chapter 2, "Materials and Methods". Values represent the mean ± S.E.M. for 3 measurements.

of chlorine substitutions, but not the same patterns of substitution (for example 2,2',3,3',4,4'-hexachlorobiphenyl and 2,2',4,4',5,5'-hexachlorobiphenyl; Figure 5.2; Table 5.1). Furthermore, there was an inverse relationship between amounts of cellular dopamine and cellular concentrations of PCB congeners for each congener examined (data not shown), as previously described for Aroclor 1254 above.

Discussion

A question that needed to be addressed in the research for this dissertation concerned the effects of PCBs on dopaminergic cells -- are the decreases in cellular dopamine after acute or subchronic exposure of PC12 cells to PCBs due specifically to PCBs or can other non-halogenated aromatic hydrocarbons, such as biphenyl, cause similar effects on cellular dopamine at similar molar concentrations in the culture medium? Results suggest that, at a given media concentration, Aroclor 1254 was greater than 2 orders of magnitude more potent at causing a decrease in cellular dopamine in PC12 cells than unchlorinated biphenyl. Therefore, these data suggest that a degree of halogenation is necessary for the aromatic hydrocarbon to cause a decrease on cellular dopamine in PC12 cells.

A determination of the cellular concentrations of PCBs and biphenyl in subchronically exposed PC12 cells indicated that, over the same range of molar concentrations in the

media, cellular concentrations of PCBs were 10 to 1000 times greater than cellular concentrations of unchlorinated biphenyl. Thus, the apparent greater potency of Aroclor 1254 to decrease cellular dopamine compared to unchlorinated biphenyl may be due to an increase in the ability of Aroclor 1254 to become associated with the cells as compared to biphenyl. PCBs have been described as being more lipophilic with increasing chlorination, and, since the molecular volume of chlorinated biphenyl is greater than the molecular volume of unchlorinated biphenyl, these factors may provide at least a partial explanation for the higher cellular concentrations of Aroclor 1254 with the cells compared to unchlorinated biphenyl.

A timecourse examining how rapidly cellular concentrations of Aroclor 1254 increased in PC12 cells demonstrated that by 6 hours, the cellular concentrations of PCBs were maximal. Even though the total amounts of Aroclor 1254 associated with the cells increased over time, the cellular concentrations of Aroclor 1254 decreased by 7 days. This decrease cellular PCB concentration may be due to a limited availability of Aroclor 1254 in the medium due to its binding to serum proteins, coupled with an exponentially dividing cell population, however more studies will be needed to examine the kinetics of PCB binding and dissociation in serum-containing media to be able to address this issue. PCBs bind to serum proteins, especially albumin

(Mohammed et al., 1990; Borlakoglu et al., 1990a,b, 1993; Busbee et al., 1985), thus the amount of PCBs available for interaction with the cells may be limited; an increase in cell number could therefore, under these conditions, lead to a decreased cellular concentration of PCBs. In Chapter 3, the data indicated that the dose-response curves for cellular dopamine shifted significantly to the left between 1 and 3 days of exposure to Aroclor 1254 indicating an increased sensitivity of cellular dopamine content to lower media concentrations of Aroclor 1254 (Figure 3.2). The results of the current experiments demonstrate that the cellular concentrations of PCBs are actually the same at 3 days and at 6 hours. These observations suggest that the effects of Aroclor 1254 on cellular dopamine and evoked release of dopamine from PC12 cells are not immediate and require a lag time to manifest themselves. The cellular events that occur during the lag between PCB exposure and decrease in cellular dopamine are unknown, but may involve alterations in second messengers, phosphorylation of enzymes, or transcription or translation of DNA or RNA. Further studies will be needed to determine the sequelae of events occurring between the time of PCB exposure and the manifestation of the decrease in cellular dopamine content in PC12 cells.

To examine the relationship between increasing cellular concentrations of Aroclor 1254 and decreasing amounts of

cellular dopamine, the amount of cellular dopamine and cellular concentrations of Aroclor 1254 were plotted against the molar concentration of Aroclor 1254 in the culture medium. Results indicated that the cellular Aroclor 1254 concentration was inversely related to the amount of cellular dopamine.

Studies examining the cellular concentrations of individual PCB congeners provided interesting results, as the cellular concentrations of PCBs of the different PCB congeners examined varied. The di- and tetrachlorobiphenyls accumulated to the highest concentrations in PC12 cells, while the penta- and hexachlorobiphenyls had lower cellular PCB concentrations. Congeners with the same degree of chlorine substitution, but different patterns of chlorine substitution appeared not to differ greatly with respect to cellular PCB concentrations. It was discussed in Chapters 3 and 4 that PCB congeners differ slightly in their ability to decrease cellular dopamine and decrease the evoked release of dopamine from PC12 cells. One factor that may play a role in the ability of an individual PCB congener to decrease dopamine is the ability of the different individual congeners to accumulate in the cells to different extents. The amount of a congener's chlorination appears to relate to how greatly it associates with the cells, for instance, di- and tetrachlorobiphenyls accumulate in cells to higher concentrations than penta- and hexachlorobiphenyls do.

However, as was reported by Shain et al. (1986), these data did not distinguish a structure-activity relationship describing the most effective patterns of chlorine substitution for the prediction of how congeners accumulate in cells.

Another observation made during the current studies related to the concentrations of PCBs in the cell culture medium versus the cellular concentrations of PCBs. For the PCB mixture Aroclor 1254, 25 μg/ml media concentration translated to a concentration of 76.5 μM. However, in the timecourse experiments, for example, the cellular concentrations of Aroclor 1254 were on the order of 150 to 280 μM. These data strongly suggest that PC12 cells concentrate PCBs out of the culture medium. This notion is supported by studies examining the distribution of PCBs in the body tissues of humans (Luotamo, 1991) and animals (Takagi et al., 1986). Generally, the amounts of PCBs in the blood are a fraction of the levels seen in stationary tissues such as brain or adipose, suggesting that these tissues take up and concentrate PCBs out of the blood.

The current studies suggest that the decreases in cellular dopamine associated with PCBs are not caused by unchlorinated aromatic hydrocarbons, at least not over the range of media concentrations examined in the current studies. This may be so because of the differences in the molecular volume, which impacts the lipophilicity, between

halogenated and non-halogenated aromatic compounds. Furthermore, PC12 cells are able to concentrate PCBs out of the culture medium. Finally, the extent to which cellular dopamine content is reduced is dependent on the cellular concentrations of PCBs, however, there is a lag time between the association of PCBs with the cells and the manifestation of the decrease in cellular dopamine.

CHAPTER 6

MECHANISMS BY WHICH POLYCHLORINATED BIPHENYLS DECREASE CELLULAR DOPAMINE IN PC12 CELLS

INTRODUCTION

The toxic effects of PCBs have been studied in numerous organ systems in a variety of species, including man. Recent research has suggested that PCBs have detrimental effects on the nervous system, and particularly the dopaminergic portion of the nervous system. Studies by Seegal et al. (1989) and Angus and Contreras (1994) have demonstrated that PCB mixtures, such as Aroclor 1254, can cause a dose-dependent decrease in cellular dopamine in PC12 rat pheochromocytoma cells, a dopaminergic model system. However, the mechanism(s) by which PCBs cause this decrease is unknown. Previous studies (Chapter 4) have demonstrated that PCBs decrease the spontaneous and evoked release of dopamine from PC12 cells; therefore, the decreases in cellular dopamine caused by exposure to PCBs is not due to an increase in the release of dopamine by the cells. Furthermore, the decreases in cellular dopamine are associated with a decrease in cellular DOPAC. Thus, PCBs do not cause a decrease in cellular dopamine by increasing the metabolism of dopamine. Hence, PCBs probably effect a decrease in cellular dopamine content through a decrease in the synthesis of dopamine. The current studies were undertaken to examine the effects of PCBs on several steps in the biosynthetic pathway for dopamine. The results indicate that PCBs do not affect the uptake of tyrosine, the amino acid precursor for dopamine, but do decrease the

conversion of DOPA to dopamine.

RESULTS

The uptake of ^{3}H-tyrosine proved to be linear with respect to time through 90 min (Figure 6.1). The uptake of L-^{14}C-leucine, L-^{14}C-arginine, and ^{3}H-methyl-choline was also linear with respect to time (data not shown). A timepoint of 40 min was chosen for the subsequent uptake studies since it was in the linear portion of the uptake curves. The results of the experiments examining the uptake of radiolabeled amino acids and choline into PC12 cells demonstrated that media concentrations of PCBs below 50 μg/ml did not affect the uptake of these substances (Figure 6.2). At high 50 μg/ml media concentrations of Aroclor 1254, there was a decrease in the uptake of amino acids concomitant with a decrease in the DNA content in the sample, which was used as an indicator of cytotoxicity.

PCB congeners also did not affect the uptake of ^{3}H-tyrosine below media concentrations of 50 μg/ml (Figure 6.3). At media concentrations of 50 μg/ml, the uptake of tyrosine decreased with 2,2',5,5'-tetrachlorobiphenyl and 2,2',3,3',4,4'-hexachlorobiphenyl. The congener 2,3',4,4',5-pentachlorobiphenyl did not significantly increase the uptake of ^{3}H-tyrosine, while the congener 2,2',4,4',5,5'-hexachlorobiphenyl had no effect on the uptake of ^{3}H-tyrosine at any media concentration examined.

Exposure of PC12 cells to Aroclor 1254 resulted in an

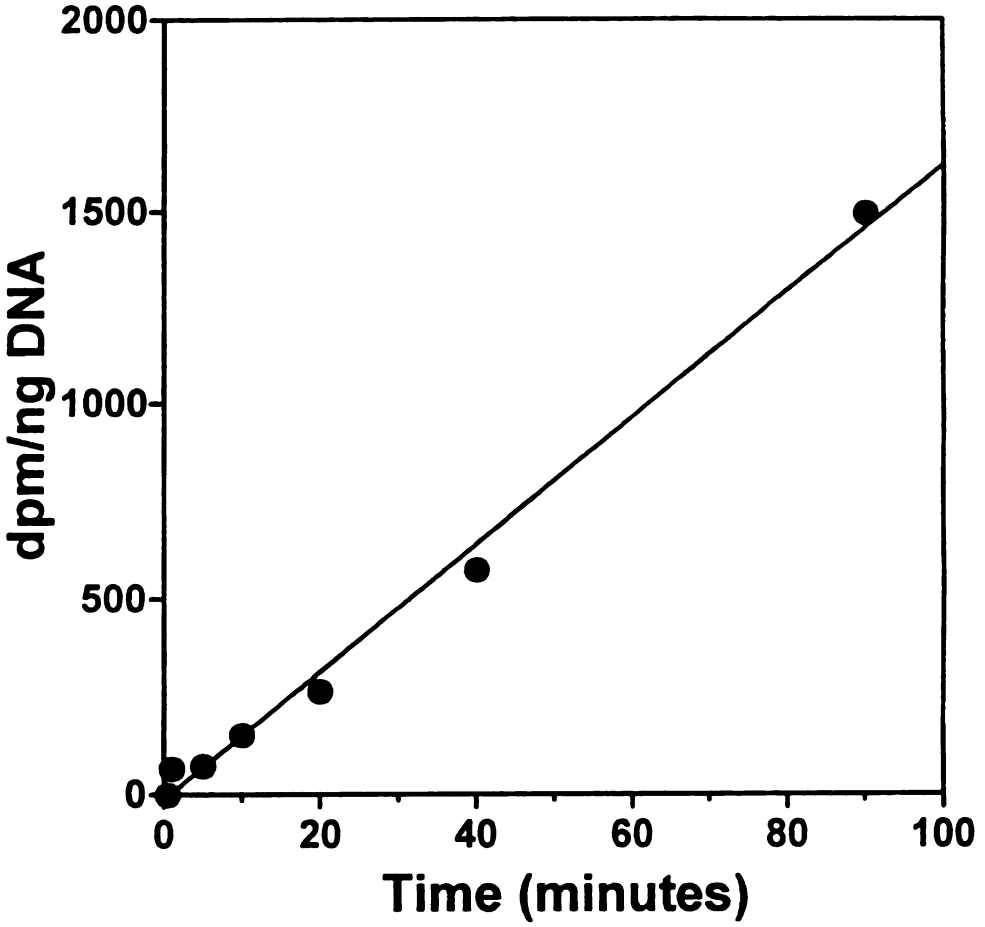


Figure 6.1 Uptake of ^{3}H-tyrosine Into PC12 Cells

PC12 cells were exposed to 3 μCi of ^{3}H-tyrosine for 0, 2.5, 5, 10, 20, 40, or 90 min. The uptake of tyrosine was determined as described in Chapter 2, "Materials and Methods". A representative graph is shown.

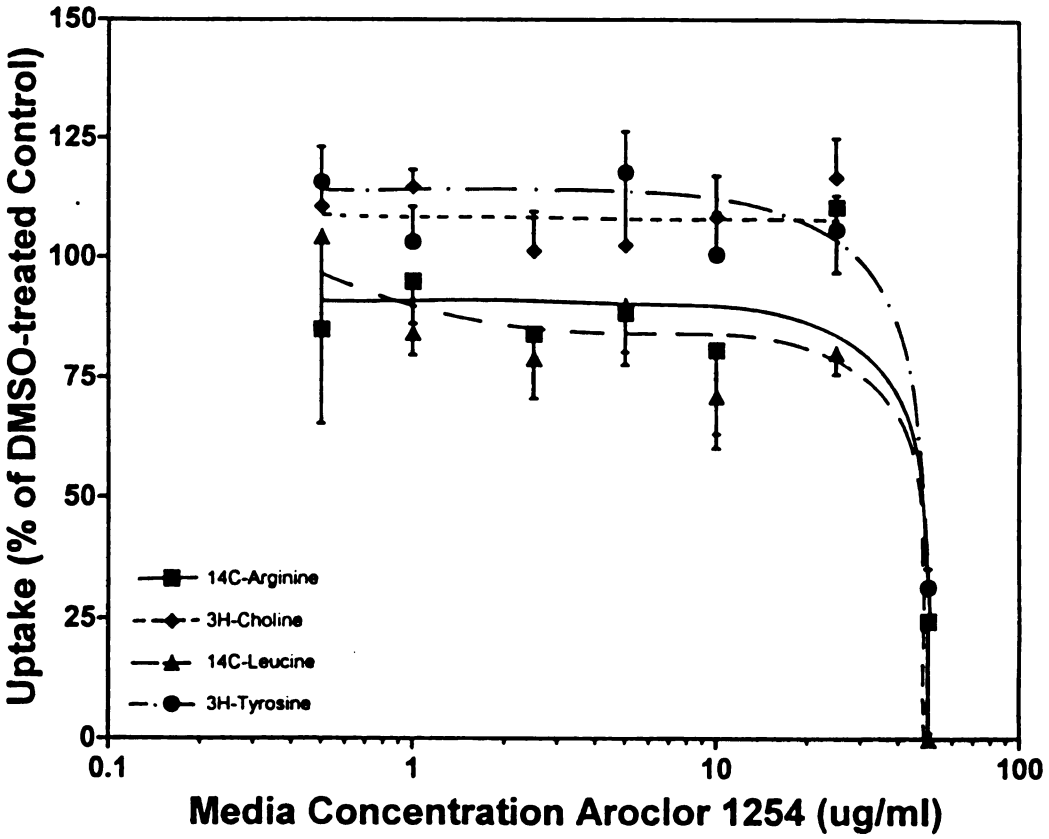


Figure 6.2 Uptake of Amino Acids or Choline by PC12 Cells Exposed to Aroclor 1254

PC12 cells were exposed to various media concentrations of Aroclor 1254 for 3 days. The uptake of ^{14}C-arginine, ^{14}C-leucine, ^{3}H-tyrosine, and ^{3}H-choline were determined as described in Chapter 2, "Materials and Methods". Each point represents the mean ± S.E.M. for 3 to 8 separate experiments.

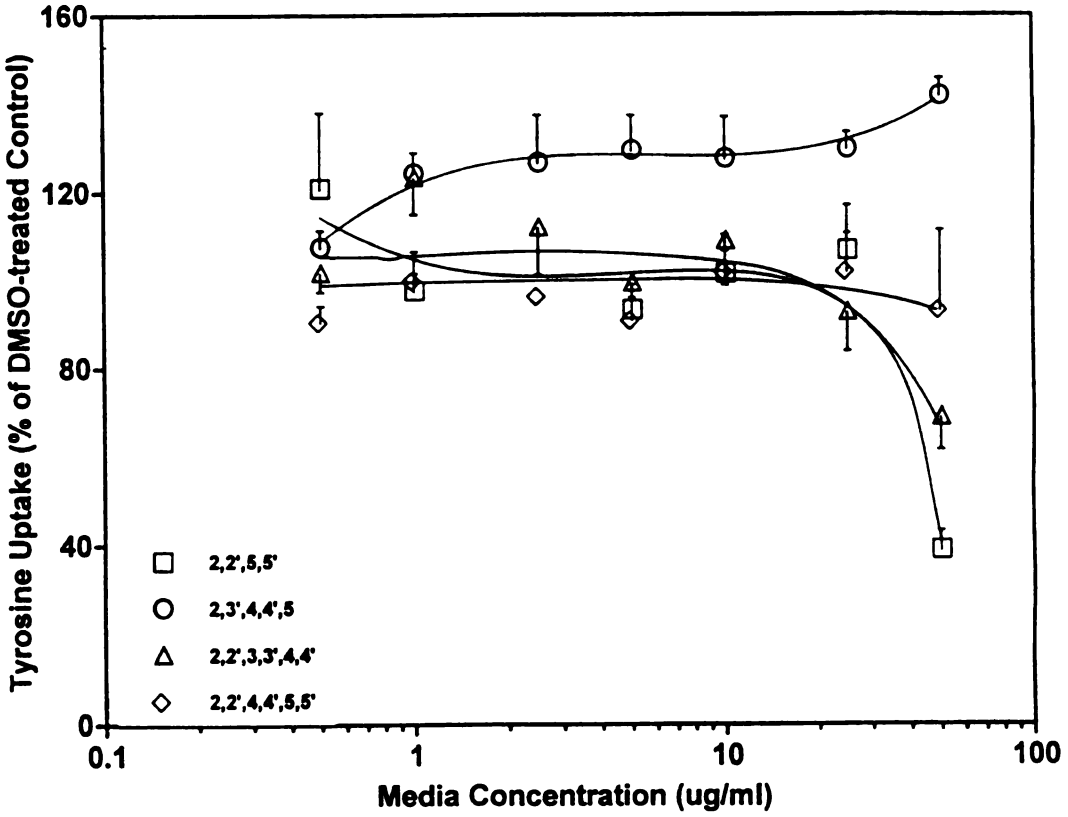


Figure 6.3 Uptake of ^{3}H-tyrosine Into PC12 Cells Exposed to PCB Congeners

PC12 cells were exposed to various media concentrations of different PCB congeners for 3 days. The uptake of ^{3}H-tyrosine was determined as described in Chapter 2, "Materials and Methods". Each point represents the mean ± S.E.M. for 3 to 4 separate experiments.

increase in the cellular DOPA as the amount of cellular dopamine declined (Figure 6.4). This increase in cellular DOPA suggested that LAAAD was being inhibited by exposure to PCBs. However, the effects of PCBs on the activity of tyrosine hydroxylase were also unknown. To examine the effects of Aroclor 1254 on the activity of TH and LAAAD, the conversion of ^{3}H-tyrosine to ^{3}H-DOPA and ^{3}H-dopamine was measured. PC12 cells treated for 3 days with 25 μg/ml Aroclor 1254 demonstrated an increase in the synthesis of ^{3}H-DOPA from ^{3}H-tyrosine after 120 min. Exposure to 25 μg/ml Aroclor 1254 for 3 days also caused a decrease in the synthesis of ^{3}H-dopamine, that was apparent at both 30 and 120 min (Figure 6.5).

To further investigate the effects of Aroclor 1254 on LAAAD, various concentrations of DOPA were added to the culture medium of cells treated for 3 days with or without 25 μg/ml Aroclor 1254. The exposure to DOPA occurred for 6 hours following the exposure to 25 μg/ml Aroclor 1254. With increasing concentrations of DOPA in the culture medium, the cellular DOPA content increased to the same extent in both PCB-treated and DMSO control cells (Figure 6.6). However, even though the content of cellular DOPA was similar in the control and experimental cells, the amount of cellular dopamine in PCB-treated cells was still decreased approximately 75% compared to the amount in vehicle-treated control cells (Figure 6.6).

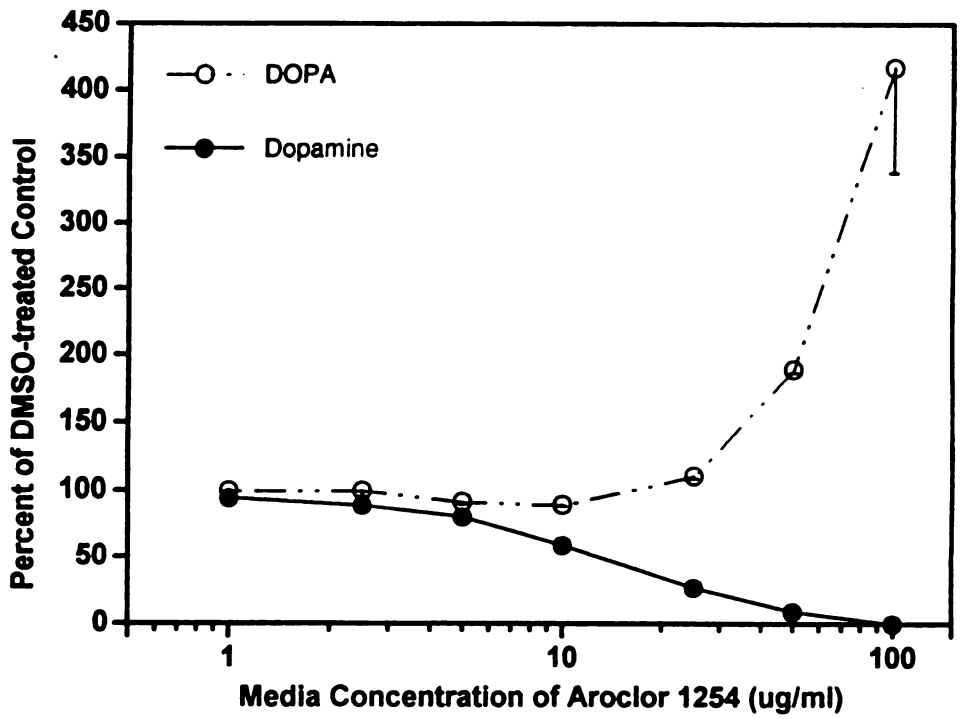


Figure 6.4 Effects of Subchronic Exposure to Aroclor 1254 on Cellular Catechols

PC12 cells were exposed to various media concentrations of Aroclor 1254 for 3 days. Cells were harvested and amounts of cellular DOPA, DOPAC, and dopamine were determined as described in Chapter 2, "Materials and Methods". Each point represents the mean ± S.E.M. for 3 separate experiments.

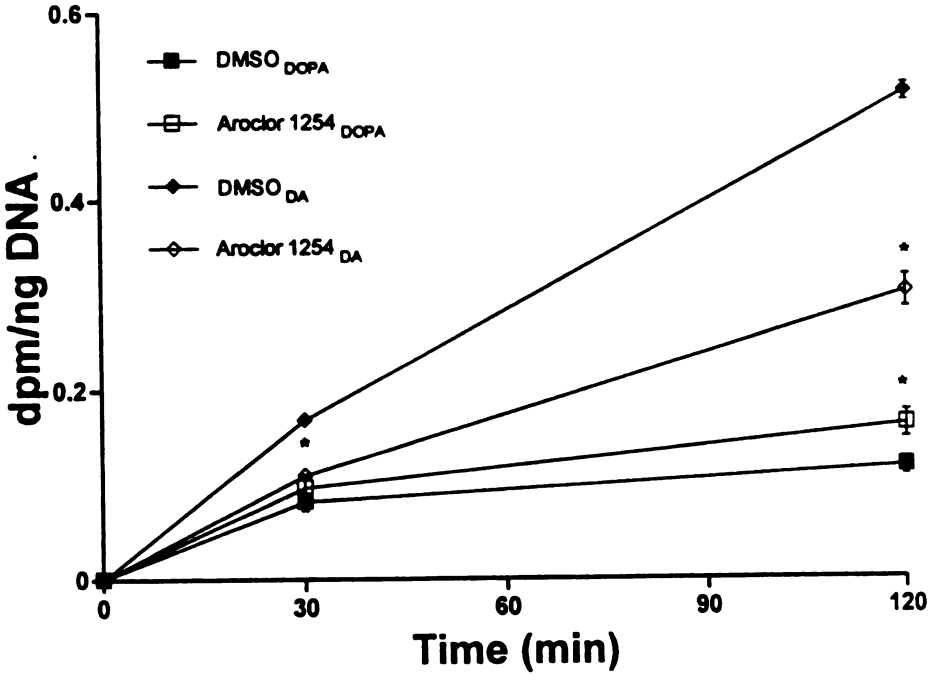


Figure 6.5 Synthesis of ^{3}H-DOPA and ^{3}H-Dopamine From ^{3}H-tyrosine

PC12 cells were exposed to media concentrations of 25 μg/ml Aroclor 1254 for 3 days. The conversion of ^{3}H-tyrosine to ^{3}H-DOPA and ^{3}H-dopamine was determined as described in Chapter 2, "Materials and Methods". Each point represents the mean ± S.E.M. for 6 separate measurements.

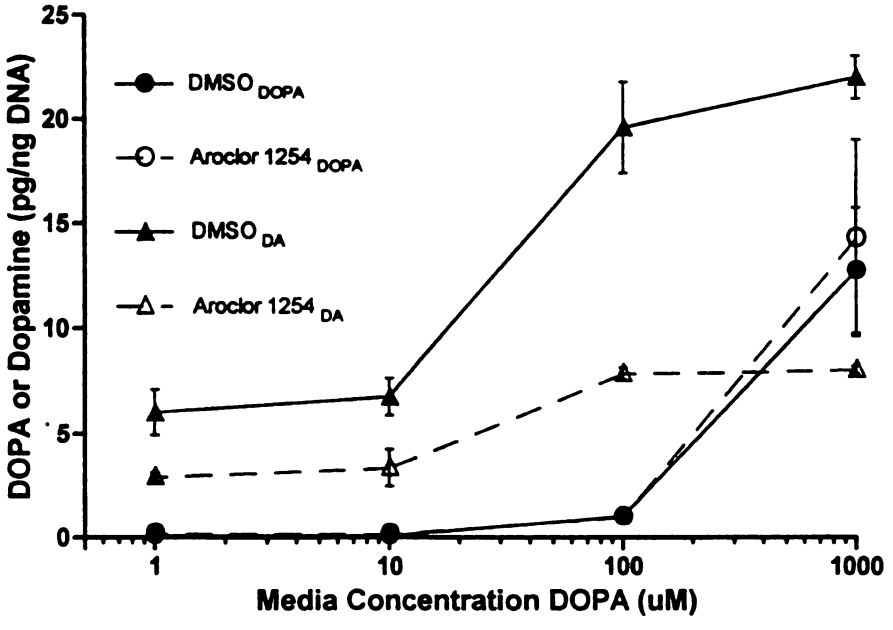


Figure 6.6 Effects of DOPA Supplementation on DOPA and Dopamine in Aroclor 1254-exposed Cells

Cells were incubated for 6 hours with various media DOPA concentrations following subchronic exposure to Aroclor 1254. Amounts of cellular DOPA and dopamine were determined as described in Chapter 2, "Materials and Methods". Each point represents the mean ± S.E.M. for 3 to 5 separate experiments, and are representative of cells treated with or without 25 μg/ml of Aroclor 1254.

Various PCB congeners also decreased the activity of LAAAD. Cells were exposed for 3 days to 25 μg/ml of the PCB congeners 2,2',4,4'-tetrachlorobiphenyl; 2,2',5,5'-tetrachlorobiphenyl; 2,2',3,3',4,4'-hexachlorobiphenyl; 2,2',4,4',5,5'-hexachlorobiphenyl; or DMSO, followed by 6 hours of exposure to various concentrations of DOPA in the medium. In both PCB-exposed and vehicle-treated control cells, cellular DOPA content increased to the same extent (Figures 6.7 and 6.8). However, even though cellular DOPA content in the PCB-treated and control cells was comparable, there was still a decrease of about 50% in cellular dopamine in the congener-treated cells compared to the controls.

Discussion

Subchronic exposure of PC12 cells to Aroclor 1254 did not affect the uptake of ^{3}H-tyrosine into PC12 cells at non-cytotoxic media concentrations of PCBs. The uptake of ^{3}H-tyrosine into PC12 cells decreased at 50 μg/ml of Aroclor 1254 after 3 days of exposure, but the decreases in cellular dopamine content caused by Aroclor 1254 began at 10 μg/ml after 3 days of exposure (see Chapter 3, Figure 3.2); therefore a decrease in the uptake of ^{3}H-tyrosine into the cells could not account for a decrease in cellular dopamine content caused by Aroclor 1254. These data suggest that exposure of the cells to Aroclor 1254 does not result in a decrease in cellular dopamine by causing a decrease in the uptake of amino acids or other precursors by the cells.

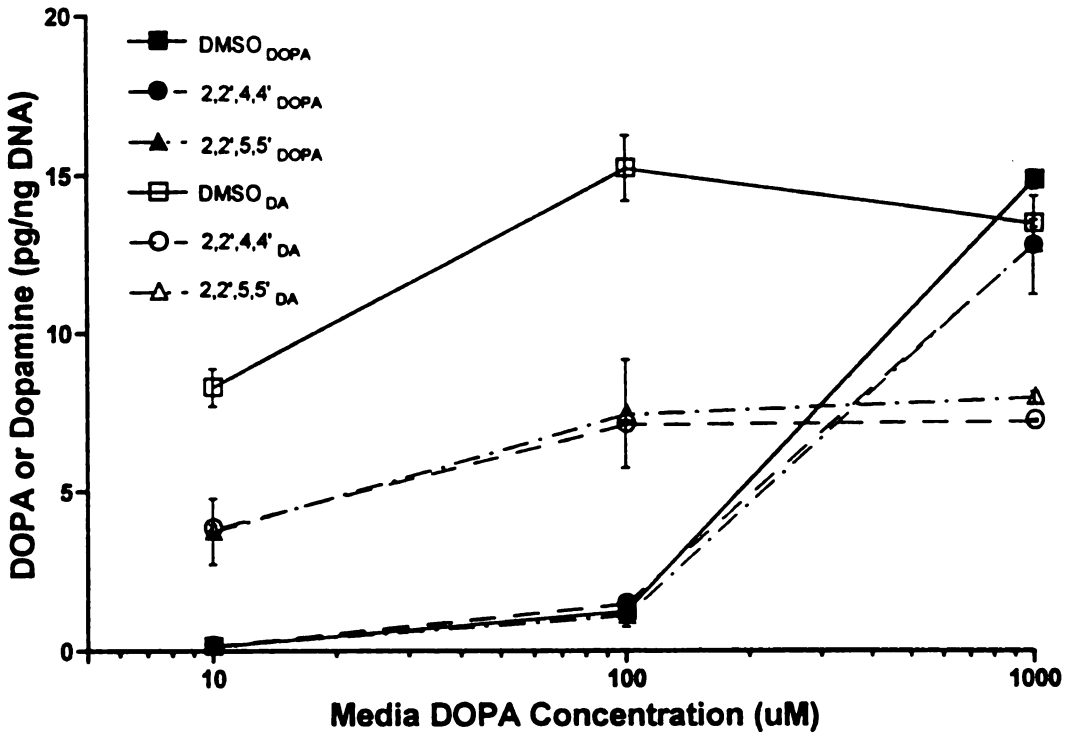


Figure 6.7 Effects of DOPA Supplementation on Cellular Dopamine and DOPA in Tetrachlorobiphenyl-exposed Cells

PC12 cells were exposed to supplemental DOPA in the media for 6 hours following subchronic exposure to tetrachlorobiphenyl. The cellular DOPA and dopamine contents were determined as described in Chapter 2, "Materials and Methods". Each point represents the mean ± S.D. for 3 separate measurements, and are representative of cells treated with or without 25 μg/ml of either 2,2',4,4'- or 2,2',5,5'-tetrachlorobiphenyl.

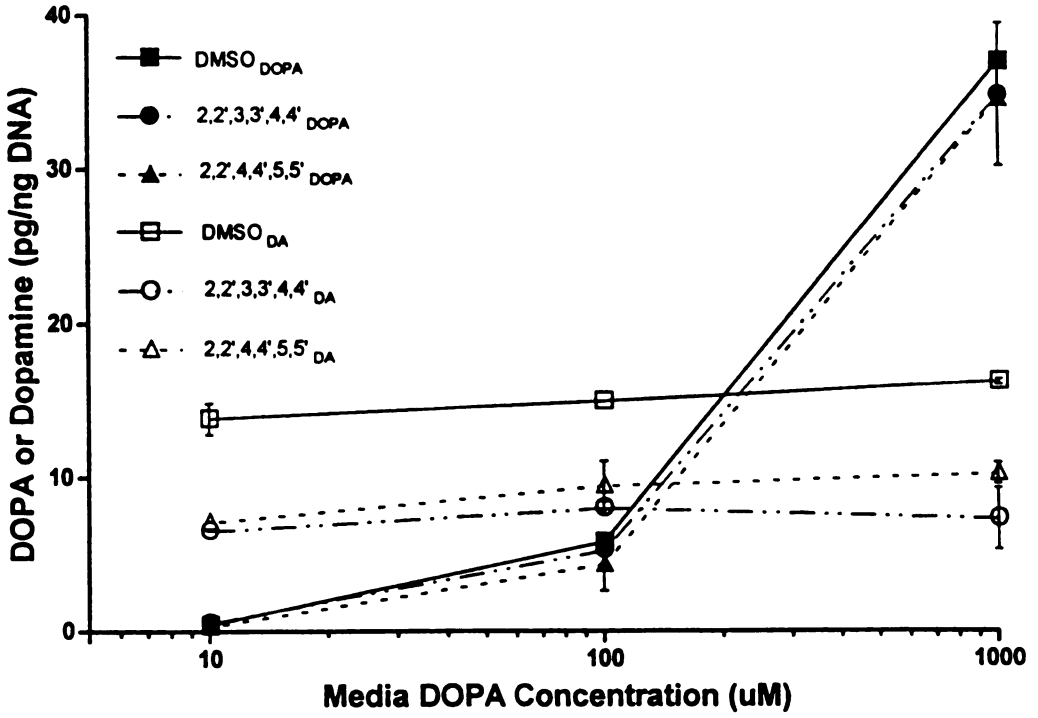


Figure 6.8 Effects of DOPA Supplementation on Cellular Dopamine and DOPA in Hexachlorobiphenyl-exposed Cells

PC12 cells were exposed to supplemental DOPA in the media for 6 hours following subchronic of exposure to hexachlorobiphenyls. The amounts of cellular DOPA and dopamine were determined as described in Chapter 2, "Materials and Methods". Each point represents the mean ± S.D. for 3 measurements, and are representative of cells treated with or without 25 μg/ml of 2,2',3,3',4,4'- or 2,2',4,4',5,5'-hexachlorobiphenyl.

Additionally, the PCB congeners 2,2',5,5'-tetrachlorobiphenyl; 2,3',4,4',5-pentachlorobiphenyl; 2,2',3,3',4,4'-hexachlorobiphenyl; or 2,2',4,4',5,5'-hexachlorobiphenyl did not significantly decrease or increase the uptake of ^{3}H-tyrosine into PC12 cells at media concentrations below 50 μg/ml. As previously mentioned, these four congeners are present in relatively large percentages in the PCB mixture Aroclor 1254 (Chapter 3). Cellular dopamine content began to decrease around media concentrations of 10 to 25 μg/ml of these congeners (Chapter 3, Figure 3.7); but the uptake of ^{3}H-tyrosine by these congeners was not effected until media concentrations reached 50 μg/ml. Therefore, decreases in the uptake of ^{3}H-tyrosine into the PC12 cells caused by these congeners cannot account for the congeners' abilities to decrease cellular dopamine.

Tyrosine and leucine are both taken up, though not exclusively, through a membrane transport system designated system L (Christensen, 1989; Stewart et al., 1989; Diez-Guerra and Gimmenez, 1989; Diez-Guerra et al., 1986), therefore, they act as controls for the effects of PCBs on the uptake of each other. Arginine was chosen to represent a charged amino acid, while choline was used to determine if the uptake of the precursor for the other neurotransmitter produced by PC12 cells (acetylcholine) was affected. However, exposure to Aroclor 1254 did not alter the uptake

of ^{14}C-leucine, ^{14}C-arginine, or ^{3}H-methyl choline into PC12 cells. Thus, PCBs do not appear to have effects on transport systems for amino acids or choline, suggesting that PCBs do not have a generalized effect on plasma membrane proteins.

Subchronic exposure to Aroclor 1254 caused a dose-dependent increase in cellular DOPA content, suggesting that LAAAD may be inhibited. Thus, conversion of ^{3}H-tyrosine to ^{3}H-DOPA and ^{3}H-dopamine was used to examine the effects of subchronic exposure to Aroclor 1254 on the synthetic enzymes for dopamine in PC12 cells. Results indicated that the amounts of newly synthesized DOPA in PCB-treated cells increased significantly over amounts in control cells, while the amounts of newly synthesized dopamine were decreased compared to controls. These data indicate that Aroclor 1254 decreased the activity of LAAAD. The increase in newly synthesized DOPA in the PCB-treated cells likely reflected an accumulation of DOPA due to decreased LAAAD activity. However, these findings do not completely rule out an additional effect of PCBs on TH. A recent report (Seegal and Shain, 1994) suggests that TH activity is inhibited by PCBs in NE115 neuroblastoma cells. In order to determine the effects of Aroclor 1254 on TH, its kinetic parameters, and cofactors, more work will be required.

In order to further investigate the effects of Aroclor 1254 on the activity of LAAAD, cell culture media was

supplemented with various concentrations of DOPA for 6 hours after subchronic exposure to Aroclor 1254, and this treatment did not restore cellular dopamine content in the PCB-treated cells to control levels, suggesting that Aroclor 1254 inhibited LAAAD in a manner that cannot be overcome by increasing cellular DOPA content.

The effects of selected PCB congeners on the activity of LAAAD were also investigated. Subchronic exposure of PC12 cells to the congeners 2,2'5,5'-tetrachlorobiphenyl and 2,2',4,4',5,5'-hexachlorobiphenyl caused an increase in cellular DOPA content and a decrease in cellular dopamine, similar to the results seen with subchronic exposure of PC12 cells to Aroclor 1254. Yet, subchronic exposure to the congeners 2,2',4,4'-tetrachlorobiphenyl and 2,2',3,3',4,4'-tetrachlorobiphenyl did not cause an increase in cellular DOPA content, even though they did cause decreases in cellular dopamine (Chapter 3, Figure 3.7). Hence, the PCB congeners were paired -- 2,2'4,4'- with 2,2'5,5'-tetrachlorobiphenyl, and 2,2',3,3',4,4'- with 2,2',4,4',5,5'-hexachlorobiphenyl -- and amounts of cellular DOPA and dopamine were determined following a 3 day subchronic exposure to 25 μg/ml of each congener, and 6 hours of DOPA supplementation in the media prior to cell harvest. The expected results were to see 2,2',5,5'-tetrachlorobiphenyl and 2,2',4,4',5,5'-hexachlorobiphenyl decrease LAAAD activity, since they increased cellular DOPA

content, but to see no effects on LAAAD activity by the other two congeners, since they do not cause an increase in cellular DOPA. The actual results of these studies indicated that for all four congeners, cellular DOPA content increased to the same extent in PCB-treated and vehicle-treated control cells, reflecting increased concentrations of DOPA in the media. However, cellular dopamine was decreased compared to controls for all four congeners, suggesting that each congener had decreased the activity of LAAAD. These results suggest that a decrease in the activity of LAAAD may be only one mechanism by which PCBs can decrease cellular dopamine. The observations that the congeners 2,2',4,4'-tetrachlorobiphenyl and 2,2',3,3',4,4'-hexachlorobiphenyl decrease cellular dopamine, decrease activity of LAAAD, but do not increase cellular DOPA content, suggest that these two congeners may also be affecting TH as well as LAAAD. A decrease in TH activity might prevent an apparent accumulation of DOPA in the cells secondary to decreased LAAAD activity. More studies will be required to determine if this indeed is true.

L-aromatic amino acid decarboxylase is a dimer with a molecular weight of approximately 100 KDa (Coge et al., 1989). For activity, LAAAD requires the presence of pyridoxal pyrophosphate, B_6, with a K_m of approximately 0.28 μM (Young et al., 1993; Hadjiconstantinou et al., 1993). The V_{max} of LAAAD for DOPA is about 37 nmol/mg protein/20

min, while the K_m for DOPA is around 32 μM (Young et al., 1993; Hadjiconstantinou et al., 1993). Neuronal and non-neuronal isoforms of the enzyme have been reported (Morgan et al., 1986, Krieger et al., 1991), although there appears to be only one gene (Albert et al., 1987). The activity of LAAAD appears to be regulated acutely by PKA, resulting in an increase in V_{max}, without alterations in K_m values (Young et al., 1993). Chronically, striatal LAAAD has been reported to be regulated through dopamine receptors, both D_1 and D_2. Antagonists for the dopamine receptors increase the activity of striatal LAAAD, while agonists decrease the activity (Hadjiconstantinou et al., 1993; Rossetti et al., 1990). The increase in activity caused by dopamine receptor antagonists appears to be caused by an increase in V_{max}, and not alterations in K_m values. Thus, the activity of LAAAD appears to be regulated and this regulation can result in the altered synthesis of dopamine.

To confirm the effects of PCBs on LAAAD, *in vitro* studies using the isolated enzyme need to be done. PCBs might alter the cellular level of the cofactor, pyridoxal pyrophosphate, reduce the binding of the cofactor to LAAAD, or alter the activity or amount of the enzyme. PCBs have been suggested to affect second messenger systems (Kodavanti et al., 1993a,b) and could thereby be altering the activity of LAAAD (see Introduction for a discussion of LAAAD regulation). More work needs to be done to determine the

mechanism for the PCB-mediated decrease in LAAAD activity.

Polychlorinated biphenyls appear to inhibit the activity of LAAAD in PC12 cells as one mechanism by which they cause a decrease in cellular dopamine. PCBs do not appear to alter the uptake of amino acids or choline, nor do they appear, from the data obtained in these experiments, to overtly alter the activity of TH. Work will have to be done on *in vitro* enzyme preparations in order to better understand the effects of PCBs directly on the enzymes for dopamine synthesis.

CHAPTER 7

SUMMARY

Polychlorinated biphenyls are putatively neurotoxic to dopaminergic cells. Studies involving animals and humans (see Chapter 1) have described cognitive and behavioral deficits in individuals exposed to PCBs *in utero*. In humans, *in utero* exposure to PCBs has been implicated in impaired visual recognition memory, deficits in verbal and numeric manipulatory abilities, decreased psychomotor skills, and hypotonia and hyporeflexia (Fein et al., 1984, Jacobson et al., 1985, 1984, 1990; Gladen and Rogan, 1991; Rogan and Gladen, 1992; Chen et al., 1992). Prenatal exposure of animals to PCBs is also associated with defects, such as learning disabilities, hyperactivity, decreased muscular strength, and a behavior described as a "spinning syndrome" (Chou et al., 1979; Bowman et al., 1979, 1981; Agrawal et al., 1981; Tilson et al., 1990). These PCB-mediated deficits in learning and behavior may be due, in part, to altered dopaminergic function in the brain, as a decrease in brain dopamine content and number of dopamine receptors has been reported in mice prenatally exposed to PCBs (Agrawal et al., 1981). At the cellular level, direct acute (Seegal et al., 1989) exposure of dopaminergic cells to PCBs also results in decreased cellular dopamine. Thus, the data indicate that a decrease in cellular levels of dopamine may potentially play a role in the cognitive and behavioral deficits observed in humans and animals following prenatal exposure to PCBs.

The results from studies by Seegal et al. (1989) indicated that acute, 6 hour exposure of dopaminergic PC12 cells to Aroclor 1254 caused a dose-dependent decrease in cellular dopamine content. However, in a prenatal exposure situation, the exposure of developing dopaminergic cells to PCBs would be on a subchronic to chronic basis. Therefore, studies were needed to examine the subchronic exposure of dopaminergic cells to PCBs.

The current studies were undertaken to examine the hypothesis that polychlorinated biphenyls decrease cellular dopamine in PC12 cells, which results from a decreased dopamine synthesis and, which manifests itself as a decreased release of dopamine in response to a depolarizing stimulus. To investigate this hypothesis, five specific aims were addressed. Each is listed, along with a brief summary of the results below. Following a review of the specific aims and the brief summary of their results, a more comprehensive discussion will bring together aspects of the results of this dissertation as they pertain first to Aroclor 1254, then to individual PCB congeners.

Review of Aims

Aim 1 **Determine the subchronic effects of Aroclor 1254 and select PCB congeners on cellular dopamine content, using undifferentiated and differentiating PC12 cells as a model system.** Subchronic 3-day exposure to Aroclor 1254 decreased cellular dopamine content in a dose-dependent fashion in

both undifferentiated and differentiating PC12 cells. Amounts of cellular dopamine decreased at lesser medium concentrations of Aroclor 1254 with longer times of exposure, as indicated by a shift to the left in the dose-response curves for cellular dopamine between 1 and 3 days of exposure. In both undifferentiated and differentiating cells, the maximal effect of Aroclor 1254 on cellular dopamine content was observed at 3 days of exposure.

Cellular dopamine content was decreased in a dose-dependent manner upon subchronic exposure to the following PCB congeners: 2,2',4,4'-tetrachlorobiphenyl; 2,2',5,5'-tetrachlorobiphenyl; 2,3,4,4',5-pentachlorobiphenyl; 2,3',4,4',5-pentachlorobiphenyl; 2,2',3,3,'4,4'-hexachlorobiphenyl; and 2,2',4,4',5,5'-hexachlorobiphenyl. The coplanar congener 3,3',4,4',5-pentachlorobiphenyl was toxic to the cells. The lightly substituted congeners 2,2'-dichlorobiphenyl' 3,3'-dichlorobiphenyl; 4,4'-dichlorobiphenyl; and 2,4,4'-trichlorobiphenyl were ineffectual at decreasing cellular dopamine content in the PC12 cells.

Aim 2 **Determine whether the neurotrophic factor, NGF can protect dopaminergic cells from damage caused by subchronic exposure to Aroclor 1254, using PC12 cells as a model system.** A preincubation of the cells with NGF for 0 to 3 days prior to subchronic exposure to Aroclor 1254 did not result in any protection from the dopamine-decreasing

effects of Aroclor 1254. However, preincubation of the cells with NGF for 7 or 14 days resulted in an attenuation of the decrease in cellular dopamine content caused by subchronic exposure to Aroclor 1254.

Aim 3 **Determine whether subchronic exposure to PC12 cells to Aroclor 1254 and selected PCB congeners results in altered release of dopamine in response to stimulation with 56 mM potassium, and whether the PCB-mediated decreases in cellular dopamine contribute to any alterations observed.** Subchronic exposure of PC12 cells to Aroclor 1254 resulted in dose-dependent decreases in the evoked release of dopamine. The dose-response curves for evoked dopamine release and cellular dopamine content appeared to coincide, indicating a possible cause and effect relationship. The fraction of evoked dopamine released from the cells was unchanged with increasing PCB concentrations, indicating that Aroclor 1254 did not affect the packaging of dopamine into vesicles or the mechanisms for the release of dopamine. The PCB congener 2,2'-dichlorobiphenyl failed to decrease evoked release of dopamine. Meanwhile, the decreased release of dopamine caused by the congener 3,3',4,4',5-pentachlorobiphenyl was attributable to the congener's toxicity. The congeners 2,2',5,5'-tetrachlorobiphenyl; 2,3',4,4',5-pentachlorobiphenyl; 2,2',3,3',4,4'-hexachlorobiphenyl; and 2,2',4,4',5,5'-hexachlorobiphenyl all caused dose-dependent decreases in the evoked release of

dopamine. For all four congeners, the dose-response curves for evoked release of dopamine closely followed the decrease in cellular dopamine content, again indicating a possible cause and effect relationship. The fraction of evoked dopamine released by the cells following subchronic exposure to any one of the six congeners examined did not change with increasing amounts of PCB in the media. These results indicate that the packaging and release mechanisms for dopamine were not affected by these PCB congeners and suggested that the decrease in amount of cellular dopamine was responsible for the decrease in dopamine release.

Aim 4 **Determine the concentrations of Aroclor 1254 or PCB congeners in the cells following a subchronic exposure, and whether the cellular PCB concentrations are related to the effects observed on cellular dopamine content and evoked release.** A timecourse examining the rate of association of Aroclor 1254 with PC12 cells indicated that the PCBs are associated with the cells after 6 hours of exposure. However, effects of Aroclor 1254 on cellular dopamine content are not optimal until 3 days of exposure, thus there is a lag time between maximal association of Aroclor 1254 with the cells and a maximal manifestation of the decrease in cellular dopamine content. PC12 cells also concentrated Aroclor 1254 from the culture medium. An examination of the relationship between cellular Aroclor 1254 concentration and cellular dopamine content indicated an inverse relationship,

indicating a direct effect of Aroclor 1254 on cellular dopamine.

PCB congeners also demonstrated an inverse relationship between cellular PCB concentration and cellular dopamine content. PCB congeners were concentrated by the cells from the medium, however, they accumulated to different extents within the cells. There appeared to be no discernable structure-activity relationship to describe the accumulation of PCB congeners into the cells.

Aim 5 **Examine possible mechanisms to explain the decrease in amounts of cellular dopamine and evoked release of dopamine from PC12 cells following subchronic exposure to Aroclor 1254.** It was observed that subchronic exposure to Aroclor 1254 resulted in a dose-dependent increase in cellular DOPA content, suggesting that LAAAD was being inhibited. However, to rule out the possibility that the uptake of tyrosine was also being inhibited by subchronic exposure to Aroclor 1254, it was determined that the uptake of ^{3}H-tyrosine was not affected at media Aroclor 1254 concentrations at which decreased cellular dopamine content was observed. An examination of the synthesis of ^{3}H-DOPA and ^{3}H-dopamine from ^{3}H-tyrosine indicated that DOPA synthesis was elevated following subchronic exposure to Aroclor 1254, while synthesis of dopamine was substantially decreased. These results indicated an inhibition of LAAAD. Furthermore, following supplementation of culture medium

with DOPA, Aroclor 1254-exposed cells were able to take up DOPA to achieve a cellular content equivalent to control cells, however, they were unable to convert the DOPA to dopamine to the same extent as in control cells, further indicating that one mechanism by which Aroclor 1254 may decrease cellular dopamine content is by inhibition of LAAAD.

However, not all PCB congeners increased cellular DOPA content. The congeners 2,2',5,5'-tetrachlorobiphenyl and 2,2',4,4',5,5'-hexachlorobiphenyl did increase cellular DOPA, and were experimentally paired with the congeners 2,2',4,4'-tetrachlorobiphenyl and 2,2',3,3',4,4'-hexachlorobiphenyl, congeners which did not increase cellular DOPA. These congener pairings were made to test the hypothesis that inhibition of LAAAD was a major mechanism causing the decrease in cellular dopamine content. Theoretically, only 2,2',5,5'-tetrachlorobiphenyl and 2,2',4,4',5,5'-hexachlorobiphenyl should have caused an inhibited conversion of supplemented DOPA to dopamine, since they increased cellular DOPA levels. Surprisingly, cellular dopamine content in the cells exposed to any one of the 4 congeners were decreased, even though the cellular DOPA contents of the PCB-treated cells matched the cellular DOPA contents of the control cells. These data indicate that inhibition of LAAAD is only one mechanism by which PCBs can decrease cellular dopamine.

Discussion

Aroclor 1254:

Subchronic exposure of undifferentiated or NGF-stimulated differentiating dopaminergic PC12 cells to the PCB mixture Aroclor 1254 resulted in dose-dependent decreases in cellular dopamine (Chapter 3). Yet, if PC12 cells were pretreated with NGF for at least 7 days prior to exposure to Aroclor 1254, the decrease in levels of cellular dopamine was partially attenuated. These data suggest that cells early in the process of differentiation are equally susceptible to the dopamine-decreasing effects of Aroclor 1254 as undifferentiated cells, however cells that receive the insult after differentiation had begun were less susceptible to the effects of Aroclor 1254 on cellular dopamine.

The decreases in cellular dopamine content did manifest themselves as a decrease in the evoked release of dopamine (Chapter 4). Further, the fraction of dopamine released from the cells in response to a depolarizing stimulus did not change with increasing media concentrations of Aroclor 1254, suggesting that 1) the mechanisms for the packaging of dopamine into vesicles was not inhibited, otherwise the fraction released would have decreased; 2) the mechanisms for the release of dopamine from PC12 cells were neither stimulated nor inhibited, or else a corresponding increase or decrease in the fraction of dopamine released would have

been observed; and 3) the decreased evoked release of dopamine was caused by a decrease in the amount of cellular dopamine available for packaging.

A decrease in the levels of cellular dopamine could, by decreasing the evoked release of dopamine, lead to learning and behavioral, as well as physical deficits. As discussed in Chapter 4, a substantial decrease (approximately 85 to 90%) in the brain dopamine content were observed in primates treated subchronically with very low doses of MPTP. These monkeys remained motor asymptomatic until challenged to perform a task requiring fine motor coordination skills. Further, these normal-appearing and behaving animals displayed cognitive and behavioral defects when challenged to perform tasks involving cognition and motor coordination. It is therefore possible that brain dopamine content can be significantly compromised without an overt manifestation of cognitive, behavioral, or motor deficits. However, under conditions involving stress, such as testing, the deficits may reveal themselves.

The PCB mixture Aroclor 1254 decreased amounts of cellular dopamine and evoked release of dopamine at media concentrations between 10 and 50 μg/ml. Therefore, in a culture dish containing 2 ml of media, the total amount of Aroclor 1254 present was 20 to 100 μg. However, the culture medium that the cells were grown in contained 12% serum, and PCBs have been reported to bind to serum proteins,

especially albumin (Mohammed et al., 1990; Borlakoglu et al., 1990a,b). Hence, the cellular concentrations of Aroclor 1254 under the experimental conditions used for the current studies was unknown. The concentrations of Aroclor 1254 in cells cultured in a 12% serum-containing medium was determined using GC/MS, and the amount of cellular Aroclor 1254 was normalized to the volume of the cells. The data suggested that 1) only about 2% of total Aroclor 1254 added to the culture medium became associated with the cells; and 2) dopaminergic cells concentrate PCBs from the cell culture medium. A time course examining the rate of association of Aroclor 1254 with PC12 cells indicated that a nearly maximal concentration of PCBs became associated with the cells by six hours; however, data from Chapter 3 demonstrated that the maximal effect of Aroclor 1254 on levels of cellular dopamine lagged behind at three days of exposure. Moreover, results from the current studies indicated that the cellular concentrations of Aroclor 1254 and the amount of cellular dopamine were inversely related (Chapter 5). Together, the results from Chapter 5 suggest that Aroclor 1254 had a direct effect on cellular dopamine in PC12 cells that resulted, after a lag time, from exposure to Aroclor 1254. The mechanisms and pathways involved in the lag will require further investigation.

Aroclor 1254 did not appear to decrease cellular dopamine content by 1) increasing metabolism of dopamine, as

a decrease in cellular DOPAC was observed as levels of cellular dopamine decreased; or 2) increasing the release of dopamine, since release decreased as amounts of cellular dopamine decreased. Elimination of Aroclor 1254 action on metabolism and release of dopamine oriented the focus of the studies on the synthetic pathway for dopamine. Aroclor 1254 could therefore potentially affect LAAAD, TH, or the uptake of tyrosine into the PC12 cells. Following subchronic exposure of PC12 cells to Aroclor 1254, the uptake of ^{3}H-tyrosine into the cells did not decrease until media concentrations of Aroclor 1254 reached 50 μg/ml; however levels of cellular dopamine began to decrease at 10 μg/ml. Therefore, a decrease in the uptake of tyrosine could not account for the decrease in the levels of cellular dopamine. Studies examining the synthesis of ^{3}H-DOPA and ^{3}H-dopamine from ^{3}H-tyrosine indicated that Aroclor 1254 increased the synthesis of ^{3}H-DOPA and a decreased the synthesis of ^{3}H-dopamine; hence Aroclor 1254 appeared to decrease the activity of LAAAD to cause decreases in cellular dopamine content. The synthesis of ^{3}H-DOPA was not reduced in Aroclor 1254-treated cells, which may indicate that Aroclor 1254 did not inhibit TH activity. However, the lack of an effect of Aroclor 1254 on the activity of TH still needs to be directly confirmed with *in vitro* studies involving the enzyme.

To further examine the effects of Aroclor 1254 on the

activity of LAAAD, the culture medium of subchronically exposed cells was supplemented with DOPA. Cellular DOPA content increased with increasing amounts of DOPA in the medium in both Aroclor 1254-treated and control cells. However, cellular dopamine content in the Aroclor 1254-treated cells was still significantly lower than in control cells. This effect of PCBs on LAAAD to cause a decrease in the synthesis of dopamine is probably one mechanism by which PCBs cause a decrease in dopamine levels. Thus, further *in vitro* studies on isolated TH and LAAAD themselves will be required to confirm the effects of PCBs on the enzymes involved in dopamine synthesis.

PCB Congeners:

The interactions of Aroclor 1254 and individual PCB congeners with PC12 cells resulted in a variety of effects on amounts of cellular dopamine, DOPAC, and DOPA; and on the release of dopamine. Furthermore, the ability of a PCB congener to become associated with cells differed.

A subchronic exposure of PC12 cells to 2,2'-dichlorobiphenyl; 3,3'-dichlorobiphenyl; and 4,4'-dichlorobiphenyl generally failed to have significant effects on cellular dopamine or the evoked release of dopamine at media concentrations of the congeners that did not cause a general cytotoxicity. Very high cellular concentrations of the congener 2,2'-dichlorobiphenyl (1,363.7 μM at 50 μg/ml) were observed, even though this

congener failed to elicit significant effects on cellular dopamine content or evoked release of dopamine until cytotoxic media concentrations of 100 μg/ml were reached. These data suggest that the amount of PCB congener associated with the cells is not predictive of the ability of the congener to decrease cellular dopamine.

Meanwhile, 2,2',4,4'- and 2,2',5,5'-tetrachlorobiphenyl differed greatly in their abilities to alter cellular dopamine and accumulated in the cells. The congener 2,2',4,4'-tetrachlorobiphenyl had no significant effects on cellular DOPA content, and decreased amounts of cellular dopamine and DOPAC at media concentrations of 50 μg/ml. Yet, the congener 2,2',5,5'-tetrachlorobiphenyl greatly increased the level of cellular DOPA, while significantly decreasing levels of cellular dopamine and DOPAC at media concentrations as low as 25 μg/ml. While the 2,2',5,5'-tetrachlorobiphenyl, but not the 2,2',4,4'-tetrachlorobiphenyl did cause an increase in cellular DOPA, both congeners appeared to decrease LAAAD activity, as supplementation of cell culture medium with DOPA failed to restore the synthesis of dopamine to control levels. Interestingly, these two congeners, 2,2',4,4'-tetrachlorobiphenyl and 2,2',5,5'-tetrachlorobiphenyl, accumulated in the cells to different concentrations, 61.7 ± 12.9 and 32.4 ± 5.4 at 10 μg/ml media concentrations, respectively; and these differences could not be attributed

to any physical or chemical property differences between them. These findings further support the contention made earlier that association of congener with the cells is not necessarily predictive of the ability of the congener to cause a decrease in cellular dopamine; that is to say that a larger cellular concentration of PCBs does not necessarily translate into a greater decrease in cellular dopamine content.

The three pentachlorobiphenyls examined, 2,3,4,4',5-; 2,3',4,4',5-; and 3,3',4,4',5-pentachlorobiphenyl, presented an interesting situation. The congener tested representative of the dioxin-like, coplanar PCB congeners, 3,3',4,4',5-pentachlorobiphenyl, decreased cellular dopamine, DOPAC, and DOPA together with DNA, suggesting that this congener elicited its effects through general cytotoxicity. The effects of 3,3',4,4',5-pentachlorobiphenyl on the evoked release of dopamine, and a determination of its cellular concentration was not performed in these studies since its effects were attributed to cytotoxicity. The congeners 2,3,4,4',5- and 2,3',4,4',5-pentachlorobiphenyl both decreased levels of cellular dopamine and DOPAC at concentrations that did not cause a decrease in DNA content. These congeners did not alter levels of cellular DOPA. Of the two congeners, the cellular concentration of 2,3,4,4',5-pentachlorobiphenyl was slightly greater than the cellular concentration of 2,3',4,4',5-

pentachlorobiphenyl. Further, a difference in the abilities to decrease levels of cellular catechols was also seen between the hexachlorobiphenyls examined, 2,2',3,3',4,4'-hexachlorobiphenyl and 2,2',4,4',5,5'-hexachlorobiphenyl, even though no difference was observed in the cellular concentrations of these congeners. The congener 2,2',4,4',5,5'-hexachlorobiphenyl increased levels of cellular DOPA while decreasing levels of cellular dopamine and DOPAC. The congener 2,2',3,3',4,4'-hexachlorobiphenyl decreased levels of cellular dopamine and DOPAC, but did not increase the cellular DOPA content. However, as will be discussed later, DOPA supplementation of the culture medium did not return cellular dopamine content to that of controls in cells treated with either congener. Both of these congeners decreased the evoked release of dopamine from the PC12 cells.

The association of PCB congeners with the PC12 cells did not appear to follow any structure-activity relationship. Patterns of substitution and extent of chlorination did not relate to the ability of the congeners to accumulate in the cells. For example, the di- and tetrachlorobiphenyls accumulated in the cells to the largest molar concentrations, followed by the hexachlorobiphenyls and then the pentachlorobiphenyls. PCBs have been reported to become more hydrophobic as chlorination increases; hence, it would be expected that the hexachlorobiphenyls would

become associated with the cells to the greatest extent. Further, as mentioned above, congeners with the same number of chlorine substitutions did not become accumulate in the cells to the same concentrations. These observations support the findings of Shain et al. (1986) that the association of a congener with PC12 cells is not due to a generalizable pattern of chlorination of the congener, the extent of congener chlorination, or the physical properties of the congener (such as molecular volume); but rather association with cells depends on the structure of the congener itself.

Selected congeners were used to determine the effects of individual PCBs on the uptake of tyrosine into the PC12 cells. The congeners 2,2',5,5'-tetrachlorobiphenyl; 2,3',4,4',5-pentachlorobiphenyl; 2,2',3,3',4,4'-hexachlorobiphenyl; and 2,2',4,4',5,5'-hexachlorobiphenyl, all of which constitute large percentages of Aroclor 1254, failed to significantly increase or decrease the uptake of ^{3}H-tyrosine into PC12 cells at non-cytotoxic media concentrations. As with Aroclor 1254, the uptake of tyrosine decreased at 50 μg/ml, while levels of cellular dopamine declined beginning at around 10 μg/ml media PCB congener concentration. These data suggested that individual PCB congeners do not decrease levels of cellular dopamine by decreasing the uptake of the precursor amino acid for dopamine synthesis, tyrosine.

Furthermore, studies involving supplementation of culture media with DOPA indicated that both of the tetrachlorobiphenyls (2,2',4,4'- and 2,2',5,5'-tetrachlorobiphenyl) and both of the hexachlorobiphenyls (2,2',3,3',4,4'- and 2,2',4,4',5,5'-hexachlorobiphenyl) examined appeared to inhibit LAAAD, even though only one member of each pair tested (2,2',5,5'-tetrachlorobiphenyl and 2,2',4,4',5,5'-hexachlorobiphenyl, respectively) increased levels of cellular DOPA. The two congeners that increased levels of cellular DOPA were expected to demonstrate an inhibition in LAAAD activity, while the other two congeners were not. These results suggested that the inhibition of LAAAD is only one mechanism by which PCB congeners can decrease in cellular dopamine content. It is possible that TH activity may be compromised, in combination with the activity of LAAAD or not, by the congeners that decreased cellular dopamine content and did not increase that of cellular DOPA. Hence, further work will be needed to determine other mechanisms by which PCB congeners can cause decreases in cellular dopamine.

Caveats:

PC12 cells are commonly used as model dopaminergic cell systems (Guroff, 1985; Fujita et al., 1989). These cells are, however, limited models. As with neural dopaminergic cells, PC12 cells synthesize and store catecholamines (Greene and Rein, 1977a,b). They also release

catecholamines upon stimulation by depolarization or following drug stimulation (Schubert et al., 1980), and contain specific uptake systems for the catecholamines (Greene and Rein, 1977a,b). Most neuronal cells have feedback inhibition of catecholamine synthesis and release (Cooper et al., 1991), which may occur in PC12 cells, although the issue is currently unclear (Courtney et al., 1991; Althaus et al., 1991). Both types of cells appear to have tyrosine hydroxylase as the rate limiting enzyme for catecholamine biosynthesis, however, PC12 cells can synthesize only small quantities of norepinephrine because of a lack of the cofactor for dopamine beta hydroxylase, ascorbate. Supplementation of PC12 cells with ascorbate has been successful in restoring the activity of DBH and increasing NE in the cells (Greene and Rein, 1979). Due to the lack of ascorbate and the pursuant decreased NE content, epinephrine is not easily detected in these cells. However, EPI and its synthetic enzyme phenylethanolamine-N-methyltransferase (PMNT) have been determined to be present in PC12 cells (Byrd et al., 1986). Furthermore, PC12 cells have been used as a model system for the investigation of the regulation and activity of TH (Haycock, 1990). Another difference between PC12 cells and neuronal catecholaminergic cells is that LAAAD cannot be inhibited in the PC12 cells by the decarboxylase inhibitor NSD 1015 (WGR Angus, unpublished results). Furthermore, PC12 cells are an immortalized cell

line derived from a pheochromocytoma, a tumor of adrenal cortical origin, whereas neuronal cells are of central nervous system origin. Neuronal catecholaminergic cells require neurotrophic factors, such as NGF or BDNF for survival. PC12 cells, on the other hand do not require these factors for survival, but do require NGF for differentiation. Upon differentiation with NGF, PC12 cells, like neuronal cells, are electrically excitable and can form synapses with other cell types. Moreover, one report (Knudson and Poland, 1980) has suggested that cultured cells and cell lines are not as sensitive to the effects of dioxins and related compounds as are cells *in vivo*. Therefore, while PC12 cells are used as models for catecholaminergic cells, they do have some distinct differences from true neuronal cells.

PCBs have been suggested to affect humans in a variety of ways (see Introduction for a discussion and references). Determination of the direct effects of PCBs on humans is difficult due to confounding factors, such as, for example, co-exposure to other potentially toxic compounds, uncertainty about exact amounts of exposure, routes of distribution of PCBs to various tissues, and motility of humans between regions of greater and lesser contamination. Some of these confounding factors can be limited by the use of laboratory animals. Under laboratory conditions, the availability of PCBs can be monitored, as well as amounts of

exposure, limitations to co-exposure to other toxins, and mobility of the animals to areas of differing contamination. However, neither in animal or human studies, can the direct mechanistic effects of PCBs on certain cell types be examined. Cell culture systems are advantageous for these investigations because a large quantity of tissues can be generated and the environment and exposures of the cells can be directly controlled by the investigators. Further, tissue culture systems are easier to manipulate for mechanistic studies and lend themselves to analysis more easily than do animals or humans. However, as discussed above, cells in culture, such as PC12 cells, are not always accurate models for normal cells. Thus, for both cell culture systems and animal models, extrapolating the results back to either an *in vivo* situation or to humans, respectively, is uncertain at best. Human investigations, animal model systems, and cell culture systems all have distinct advantages and disadvantages, but used in a coordinated investigation into a toxicological problem, can prove invaluable for determining the actions of a toxic compound.

In the current studies, PCBs were introduced into cell culture medium containing serum and cells. The availability of the PCBs to the cells was in saturating excess, which is not likely the situation in vivo. As previously mentioned, only about 0.1% of total PCBs administered to pigeons is

found to be associated with the serum (Borlakoglu et al., 1993). Only about 1% of total PCBs were found to be associated with brain tissue. While these data support the hypothesis that neuronal cells can concentrate PCBs from blood, they do not specify what regions of the brain contain what concentration of PCBs. Moreover, larger percentages of total PCBs administered were found associated with non-neural tissues such as liver and adipose, indicating that the availability of PCBs to the brain are low initially, and when the kinetics and dynamics of exchange of PCBs between tissues and blood serum are factored in, one can only speculate about how closely the current studies mimic the events of neural exposure to PCBs in vivo. Nonetheless, the current studies did, for the first time, relate the effects of PCBs on cellular dopamine content, cellular concentrations of PCBs, and the relationship in time between exposure of cells to PCBs and a manifestation of an event.

In conclusion, polychlorinated biphenyls can cause decreases in levels of cellular dopamine in dopaminergic PC12 cells. The decreased levels of cellular dopamine can manifest themselves as a decrease in the evoked release of dopamine in response to a depolarizing stimulus. For Aroclor 1254 and PCB congeners that decrease cellular dopamine, the extent of the decrease reflected an increased cellular concentration of Aroclor 1254 or congener. However, the accumulation of the various PCB congeners in

the cells does not appear to have any determinable structure-activity relationship. The effects of PCBs on cellular levels of dopamine are direct, but require a lag time before manifestation. Furthermore, one mechanism by which PCBs may cause decreased cellular dopamine is through inhibition of LAAAD, however, this does not appear to be the only mechanism by which PCBs may affect dopaminergic cells. More research will be required to further the understanding of the effects of PCBs on dopaminergic cells.

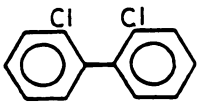
APPENDICES

APPENDIX 1

Table A.1. Structure and Nomenclature of Congeners Used

PCB Congener Name	IUPAC Name for PCB Congener
2,2'-Dichlorobiphenyl	PCB 04
3,3'-Dichlorobiphenyl	PCB 11
4,4'-Dichlorobiphenyl	PCB 15
2,4,4'-Trichlorobiphenyl	PCB 28
2,2',4,4'-Tetrachlorobiphenyl	PCB 47
2,2',5,5'-Tetrachlorobiphenyl	PCB 52
2,3,4,4',5-Pentachlorobiphenyl	PCB 114
2,3',4,4',5-Pentachlorobiphenyl	PCB 118
3,3',4,4',5-Pentachlorobiphenyl	PCB 126
2,2',3,3',4,4'-Hexachlorobiphenyl	PCB 128
2,2',4,4',5,5'-Hexachlorobiphenyl	PCB 153

STRUCTURES



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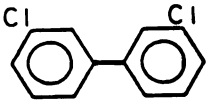

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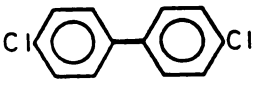

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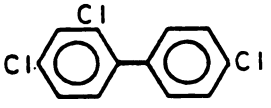

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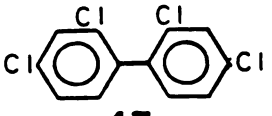

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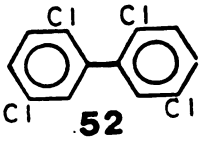

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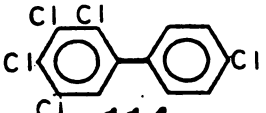

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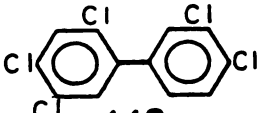

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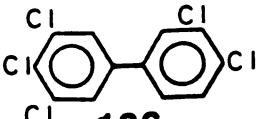

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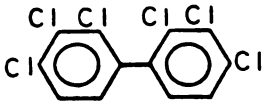

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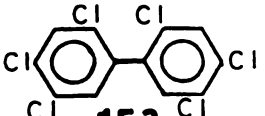

153

APPENDIX 2

GC/MS PARAMETERS

TSQ-70 OPERATING CONDITIONS
for Biphenyl project project

Finnigan,TSQ-70

Mass spectrometer conditions:

electron impact	70 EV
electron current	300 UA
conversion dynode	-10 KV
electron multiplier	1200 V
eletrometer gain	10e8 V
electronic zero	-45 V
source temperature	200 C
manifold temperature	70 C
Quad 3	
forepump pressure	31 MTORR
manafold pressure	6.0 x 10e-7 M TORR

Gas chromatagraph condition: (Varian 3400 GC)

splitless injection	
column head pressure	5 PSI
maximum column temp.	180 C
Injector temp.	210 C
transfer line temp.	325 C

see attached for column oven temperature program

Gc column:

J&W DB5-MS
15 meter
0.25 mmeter
0.25 micron coating
part # 122-5012
serial # 0231722A

Analysis Procedures:

ICL procedure	rob
Autosampler	Headman 1ul inj
Run time	7.5 minutes

Figure A.1 Operating Parameters For Gas Chromatograph and Mass Spectrometer

The operating conditions for the gas chromatograph and mass spectrometer were compiled by Pat Rumler at the Animal Health Diagnostics Laboratories at Michigan State University. These conditions were used for the analysis of biphenyl content in PC12 cells.

APPENDIX 3

DNA Content Versus Cell Number

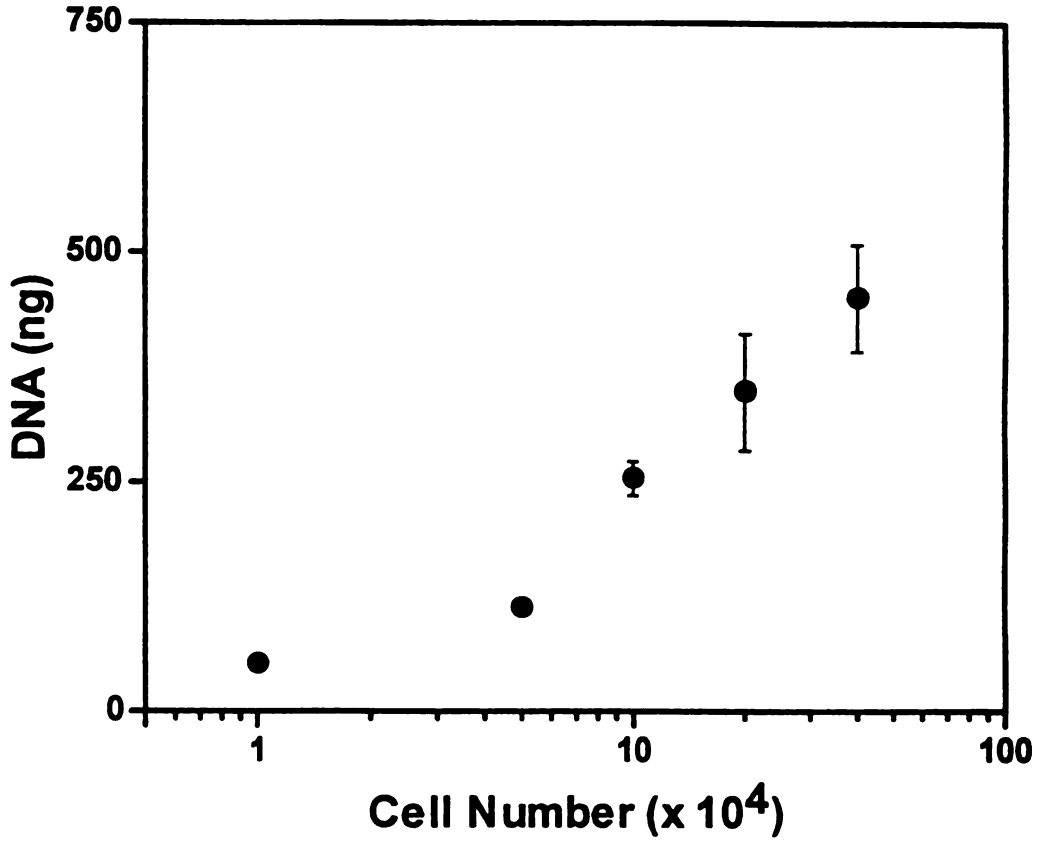

Figure A.2 Cellular DNA Content as a Reflection of Cell Number

Various numbers of PC12 cells were analyzed for DNA content as described in Chapter 2, "Materials and Methods". The average ng of DNA ± S.E.M. was plotted for each number of cells examined. Values represent the results of 3 experiments.

BIBLIOGRAPHY

BIBLIOGRAPHY

Acworth IN, During MJ, and Wurtman RJ. Tyrosine: Effects on catecholamine release. Brain Res Bull 1988; 21:473-477

Agrawal AK, Tilson HA, and Bondy SC. 3,4,3',4'-Tetrachlorobiphenyl given to mice prenatally produces long-term decreases in striatal dopamine and receptor binding sites in the caudate nucleus. *Toxicol Lett* 1981; 7:417-424

Albert KA, Helmer-Matyjek E, Nairn AC, Muller TH, Haycock JW, Greene LA, Goldstein M, and Greengard P. Calcium/phospholipid-dependent protein kinase (protein kinase C) phosphorylates and activates tyrosine hydroxylase. *Proc Natl Acad Sci, USA* 1984; 81:7713-7717

Allen JR and Barsotti DA. The effects of transplacental and mammary movement of PCBs on infant rhesus monkeys. *Toxicology* 1976; 6:331-340

Allen JR, Barsotti DA, Lambrecht LK, and VanMiller JP. Representative effects of halogenated aromatic hydrocarbons on nonhuman primates. *Ann NY Acad Sci* 1979; 419-425

Angus WGR and Contreras ML. The effects of Aroclor 1254 on undifferentiated and NGF-stimulated differentiating PC12 cells. *Neurotoxicology* 1994; 15(4):in press

Aosaki T, Graybiel AM, and Kimura M. Effect of the nigrostriatal dopamine system on acquired neural responses in the striatum of behaving monkeys. *Science* 1994; 265(5170):412-415

Aragon MC, Gimenez C, Mayor, Jr. F, Marvizon JG, and Valdivieso F. Tyrosine transport by membrane vesicles isolated from rat brain. *Biochim Biophys Acta* 1981; 646:465-470

Badoyannis HC, Sharma SC, and Sabban EL. The differential effects of cell density and NGF on the expression of tyrosine hydroxylase and dopamine beta-hydroxylase in PC12 cells. *Molec Brain Res* 1991; 11:79-87.

Besson MJ, Cheramy A, Feltz P, and Glowinski J. Release of newly synthesized dopamine from dopamine-containing terminals in the striatum of the rat. *Proc Natl Acad Sci, U.S.A.* 1969; 62:741-748

Bocchini B and Angeletti PU. The nerve growth factor: Purification as a 30,000-molecular weight protein. *Proc Natl Acad Sci, USA* 1969; 64:787-794

Borlakoglu JT, Wilkins JPG, and Dils RR. Distribution and elimination *in vivo* of polychlorinated biphenyl (PCB) isomers and congeners in the pigeon. *Xenobiotica* 1991; 21(4):433-445

Borlakoglu JT, Welch VA, Wilkins JPG, and Dils RR. Transport and cellular uptake of polychlorinated biphenyls (PCBs) -- I: Association of individual PCB isomers and congeners with plasma lipoproteins and proteins in the pigeon. *Biochem Pharmacol* 1990a; 40(2):256-272

Borlakoglu JT, Welch VA, Wilkins JPG, and Dils RR. Transport and cellular uptake of polychlorinated biphenyls (PCBs) -- II: Changes *in vivo* in plasma lipoproteins and proteins of pigeons in response to PCBs, and a proposed model for the transport and cellular uptake of PCBs. *Biochem Pharmacol* 1990b; 40(2):273-281

Bowman RE and Heironimus MP. Hypoactivity in adolescent monkeys perinatally exposed to PCBs and hyperactive as juveniles. *Neurobehav Toxicol Teratol* 1981a; 3:15-18

Bowman RE, Heironimus MP, and Allen JR. Correlation of PCB body burden with behavioral toxicology in monkeys. *Pharmacol Biochem Behav* 1978; 9:49-56

Bowman RE, Heironimus MP, and Barsotti DA. Locomotor hyperactivity in PCB exposed rhesus monkeys. *Neurotoxicology* 1981b; 2:251-268

Busbee DL, Yoo J-SH, Norman JO, and Joe CO. Polychlorinated biphenyl uptake and transport by lymph and plasma components. *Proc Soc Exp Biol Med* 1985; 179:116-122

Bush B, Snow J, and Koblintz R. Polychlorobiphenyl (PCB) congeners, *p,p'*-DDE, and hexachlorobenzene in maternal and fetal cord blood from mothers in upstate New York. *Arch Environ Contamin Toxicol* 1984; 13:517-527.

Byrd JC, Hadjiconstantinou M, and Cavalla D. Epinephrine synthesis in the PC12 pheochromocytoma cell line. *Eur J Pharmacol* 1986; 127:139-142

Campbell DG, Hardie DG, and Vulliet PR. Identification of four phosphorylation sites in the N-terminal region of tyrosine hydroxylase. *J Biol Chem* 1990; 261(33):10489-10492

Carlsson A and Lindqvist M. Dependence of 5-HT and catecholamine synthesis on concentrations of precursor amino-acids in rat brain. *Naunyn-Schmiedeberg's Arch Pharmacol* 1978; 303:157-164

Chen Y-C J, M.D.,MPH, Guo Y-L, M.D., Ph.D., Hsu C-C, M.D., Ph.D., and Rogan WJ, M.D. Cognitive development of Yu-Cheng ('Oil Disease') children prenatally exposed to heat-degraded PCBs. *JAMA* 1992; 268:3213-3218

Chou SM, Mike T, Payne WM, and Davis GJ. Neuropathology of "spinning syndrome" induced by prenatal intoxication with PCB in mice. *Ann NY Acad Sci* 1979; 320:373-396

Chu I, Villeneuve DC, Valli VE, Ritter L, Norstrom RJ, Ryan JJ, and Becking GC. Toxicological response and its reversibility in rats fed Lake Ontario or Pacific coho salmon for 13 weeks. *J Environ Sci Health [B].* 1984; 19:713-732

Christensen HA. Distinguishing amino acid transport systems of a given cell or tissue. *Meth Enzymol* 1989; 173:576-616

Coge F, Krieger-Poullet M, Guillemot J-C, Ferrara P, Gros F, and Thibault J. Purification et sequencage partiel de la L-DOPA decarboxylase de pheochromocytome de rat. *C R Acad Sci Paris* 1989; t. 309, serie 3:587-592

Cooper JR, Bloom FE, and Roth RH. The Biochemical Basis of Neuropharmacology, Sixth Edition. Oxford University Press, Inc., Cary, N.C. 1991; pp. 285-337

Cousins MS, Sokolowski JD, and Salamone JD. Different effects of nucleus accumbens and ventrolateral striatal dopamine depletions on instrumental response selection in the rat. *Pharmacol Biochem Behav* 1993; 46(4):943-951

Diez-Guerra FJ and Gimenez C. Na^+ dependence of tyrosine transport across the synaptosomal membrane reflects changes in the morphology of synaptosomes. *J Neurochem* 1989; 52:1366-1374

Diez-Guerra FJ, Mayor, Jr. F, and Gimenez C. Tyrosine transport into isolated rat brain synaptosomes. Ionic dependence and kinetic studies. *Biochim Biophys Acta* 1986; 857:209-216

Ebadi M and Simonneaux V. Ambivalence on the multiplicity of mammalian aromatic-L-amino acid decarboxylase. *Adv Exp Med Biol* 1991; 294:115-125

Eisenreich SJ and Johnson TC. PCBs in the Great Lakes: Sources, Sinks, Burdens, In: PCBs: Human and Environmental Hazards (D'Itri, F.M. and Kamrin, M.A., eds.), Butterworth Publishers, Boston, 1983; pp. 49-75

Eveleth DD, Gietz RD, Spencer CA, Nargang FE, Hodgetts RB, and Marsh JL. Sequence and structure of the dopa decarboxylase gene of *Drosophila*: Evidence for novel RNA splicing variants. *EMBO J* 1986; 5(12):2663-2672

Fein GG, Jacobson JL, Jacobson SW, Schwartz PM, and Dowler JK. Prenatal exposure to polychlorinated biphenyls: Effects on birth size and gestational age. *J Pediatr* 1984; 105:315-320

Fernstrom JD. Role of precursor availability in control of monoamine biosynthesis in brain. *Physiol Rev* 1983; 63(2):484-546

Francis PC, Carlson KH, Owen NV, and Adams ER. Preclinical toxicology studies with the new dopamine agonist pergolide. Acute, subchronic, and chronic evaluations. *Arzneimittelforschung* 1994; 44(3):278-284

Fujita K, Lazarovici P, and Guroff G. Regulation of the differentiation of PC12 rat pheochromocytoma cells. *Env Health Perspect* 1989; 80:127-142

Gewirtz GP and Kopin IJ. Effect of intermittent nerve stimulation on norepinephrine synthesis and mobilization in the perfused cat spleen. *J Pharmacol Exp Ther* 1970; 175(2):514-520

Gladen BC and Rogan WJ. Effects of perinatal polychlorinated biphenyls and dichlorodiphenyl dichloroethene on later development. *J Pediatr* 1991; 119:58-63

Gladen BC, Rogan WJ, Hardy P, Thullen J, Tinglestad J, and Tully M. Development after exposure to polychlorinated biphenyls and dichlorodiphenyl dichloroethene transplacentally and through human milk. *J Pediatr* 1988; 56:991-995

Green SH and Greene LA. A single Mr~103,000 ^{125}I-beta-nerve growth factor-affinity-labeled species represents both the low and high-affinity forms of the nerve growth factor receptor. *J Biol Chem* 1986; 261:15316-15326

Greene LA and Rein G. Short-term regulation of catecholamine biosynthesis in a nerve growth factor responsive clonal line of rat pheochromocytoma cells. *J Neurochem* 1979; 30:549-555.

Greene LA and Rein G. Release, storage and uptake of catecholamines by a clonal line of nerve growth factor responsive pheochromocytoma cells. *Brain Res* 1977a; 129:247-263

Greene LA and Rein G. Release of (^{3}H)-norepinephrine from a clonal line of pheochromocytoma cell (PC12) by nicotinic cholinergic stimulation. *Brain Res* 1977b; 138:521-528

Greene LA and Tischler AS. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc Natl Acad Sci, USA* 1976; 73(7):2424-2428

Gou YL, Lin CJ, Yao WJ, Ryan JJ, and Hsu CC. Musculoskeletal changes in children prenatally exposed to polychlorinated biphenyls and related compounds (Yu-Cheng children). *J Toxicol Environ Health* 1994; 41:83-93

Graybiel AM, Aosaki T, Flaherty AW, and Kimura M. The basal ganglia and adaptive motor control. *Science* 1994; 265:1826-1831

Guroff G. PC12 cells as a model of neuronal differentiation, In: Cell Culture in the Neurosciences, (Bottenstein, J.E. and Sato, G., eds.), Plenum Publishing Corp., 1985

Guroff G, King W, and Udenfriend S. The uptake of tyrosine by rat brain *in vitro*. *J Biol Chem* 1961; 236(6):1773-1777

Hadjiconstantinou M, Wemlinger TA, Sylvia CP, Hubble JP, and Neff NH. Aromatic-L-amino acid decarboxylase activity of mouse striatum is modulated via dopamine receptors. *J Neurochem* 1993; 60:2175-2180

Hansen LG. Environmental toxicology of polychlorinated biphenyls, In: Polychlorinated Biphenyls (PCBs): Mammalian and Environmental Toxicology (Safe, S., ed.), Springer-Verlag, New York, 1987; pp. 1-14

Harada M. Intrauterine poisonings: Clinical and epidemiological studies of the problem. *Bull Inst Const Med (Kumamoto Univ)* 1976; 25:1-60

Haycock JW. Phosphorylation of tyrosine hydroxylase in situ at serine 8, 19, 31, and 40. *J Biol Chem* 1990; 265(20):11682-11690

Haycock JW and Haycock DG. Tyrosine hydroxylase in rat brain dopaminergic nerve terminals. *J Biol Chem* 1990; 266(9):5650-5657

Hempstead BL, Martin-Zanca D, Kaplan DR, Parda LF, and Chao MV. High affinity NGF binding requires coexpression of the *trk* proto-oncogene and low affinity NGF receptor. *Nature* 1991; 350:678-683

Hertzler DR. Neurotoxic behavioral effects of Lake Ontario salmon diets in rats. *Neurotoxicol Teratol* 1990; 12:139-143

Hutzinger O, Safe S, and Zitko V. *The Chemistry of PCBs.* Boca Raton, CRC Press Inc., Fla, 1974

Hyman C, Hofer M, Barde Y-A, Juhasz M, Yancopoulos GD, Sqinto SP, and Landsay RM. BDNF is a neurotropic factor for dopaminergic neurons of the substantia nigra. *Nature* 1991; 350:230-232

Jacobson JL, Fein GG, Jacobson SW, Schwartz PM, and Dowler JK. The transfer of polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs) across the human placenta and into maternal milk. *Am J Public Health* 1984; 74:378-379

Jacobson JL, Jacobson SW, and Humphrey HEB. Effects of in utero exposure to polychlorinated biphenyls and related contaminants on cognitive functioning in young children. *J Pediatr* 1990; 116:38-45

Jacobson SW, Fein GG, Jacobson JL, Schwartz PM, and Dowler JK. The effect of interuterine PCB exposure on visual recognition memory. *Child Development* 1985; 56:853-860

Jacobson SW, Jacobson JL, Schwartz PM, and Fein GG. Intrauterine exposure of human newborns to PCBs: Measures of exposure, In: PCBs: Human and Environmental Hazards (D'Itri, F.M. and Kamrin, M.A., eds.), Butterworth Publishers, Boston, 1983; pp. 311-343

Kaplan DR, Hempstead BL, Martin-Zanca D, Chao MV, and Parda LF. The *trk* proto-oncogene product: A signal transducing receptor for nerve growth factor. *Science* 1991a; 252:554-557

Kaplan DR, Martin-Zanca D, and Parda LF. Tyrosine phosphorylation and tyrosine kinase activity of the *trk* proto-oncogene product induced by NGF. *Nature* 1991b; 350:158-160

Klein R, Jing S, Nanduri KV, O'Rourke E, and Barbacid M. The *trk* proto-oncogene encodes a receptor for nerve growth factor. *Cell* 1991; 65:189-197

Knutson JC and Poland A. 2,3,7,8-Tetrachlorodibenzo-p-dioxin: Failure to demonstrate toxicity in twenty-three cultured cell types. *Toxicol Appl Pharmacol* 1980; 54:377-383

Kodavanti PRS, Shafer TJ, Ward TR, Mundy WR, Freudenrich T, Harry GJ, and Tilson HA. Differential effects of polychlorinated biphenyl congeners on phosphoinositide hydrolysis and protein kinase C translocation in rat cerebellar cells. *Soc Neurosci Abs* 1993b; 19(1):751

Kodavanti PRS, Shin D, Tilson HA, and Harry GJ. Comparative effects of two polychlorinated biphenyl congeners on calcium homeostasis in rat cerebellar granule cells. *Toxicol Appl Pharmacol* 1993a; 123:97-106

Kopin, I.J.; Breese, G.R.; Krauss, K.R.; and Weise, V.K. Selective release of newly synthesized norepinephrine from the cat spleen during sympathetic nerve stimulation. *J Pharmacol Exp Ther* 1968; 161(2):271-278

Kreutz M, Acworth IN, Lehnert H, and Wurtman RJ. Systemic administration of thyrotropin-releasing hormone enhances striatal dopamine release *in vivo*. *Brain Res* 1990; 536:347-352

Krieger M, Coge F, Gros F, and Thibault J. Different mRNAs code for dopa decarboxylase in tissues of neuronal and nonneuronal origin. *Proc Natl Acad Sci, USA* 1991; 88:2161-2165

Labracca C and Paigen K. A simple, rapid. and sensitive DNA assay procedure. *Analytical Biochem* 1980; 102:344-352

Lankford KL and Letourneau PC. Evidence that calcium may control neurite outgrowth by regulating the stability of actin filaments. *J Cell Biol* 1989; 109:1229-1243

Lehnert H and Wurtman RJ. Amino acid control of neurotransmitter synthesis and release: Physiological and clinical implications. *Psychother Psychosom.* 1993; 60:18-32

Levin ED, Schantz SL, and Bowman RE. Delayed spatial alteration deficits resulting from perinatal PCB exposure in monkeys. *Arch Toxicol* 1988; 62:267-273

Lilienthal H, Neuf M, Munoz C, and Winneke G. Behavioral effects of pre- and postnatal exposure to a mixture of low chlorinated PCBs in rats. *Fund Appl Toxicol* 1990; 15:457-467

Lilienthal H and Winneke G. Sensitive periods for behavioral toxicity of polychlorinated biphenyls: Determination by cross-fostering in rats. *Fund Appl Toxicol* 1991; 17:368-375

Luotamo M. Congener specific assessment of human exposure to polychlorinated biphenyls. *Chemosphere* 1991; 11-12:1685-1698

Madhukar BV, Oh SY, Chang CC, Wade M, and Trosko JE. Altered regulation of intercellular communication by epidermal growth factor, transforming growth factor-β, and peptide hormones in normal human keratinocytes. *Carcinogenesis* 1989; 10:13-20

Markey KA, Kondo S, Shenkman L, and Goldstein M. Purification and characterization of tyrosine hydroxylase from a clonal pheochromocytoma cell line. *Mol Pharmacol* 1980; 17:79-85

Melamed E, Hefti F, and Wurtman RJ. L-3,4-Dihydroxyphenylalanine and L-5-hydroxytryptophan decarboxylase activities in rat striatum: Effect of selective destruction of dopaminergic or serotoninergic input. *J Neurochem* 1980; 34(6):1753-1756

Malapani C, Pillon B, Dubois B and Agid Y. Impaired simultaneous cognitive task performance in Parkinson's disease: a dopamine-related dysfunction. *Neurology* 1994; 44(2):319-326

Melega, WP and Howard, BD. Biochemical evidence that vesicles are the source of the acetylcholine released from stimulated PC12 cells. *Proc Natl Acad Sci, USA* 1984; 81:6535-6538

Miczek KA, Weerts E, Haney M, and Tidey J. Neurobiological mechanisms controlling aggression: Preclinical developments for pharmacotherapeutic interventions. *Neurosci Biobehav Rev* 1994; 18(1):97-110

Milner JD and Wurtman RJ. Catecholamine synthesis: Physiological coupling to precursor supply. *Biochem Pharmacol* 1986; 35(6):875-881

Mohammed A, Eklund A, Ostlund-Lindqvist A-M, and Slanina P. Distribution of toxaphene, DDT, and PCB among lipoprotein fractions in rat and human plasma. *Arch Toxicol* 1990; 64:567-571

Morgan BA, Johnson WA, and Hirsh J. Regulated splicing produces different forms of dopa decarboxylase in the central nervous system and hypoderm of *Drosophila melanogaster*. *EMBO J* 1986; 5(12):3335-3342

Nisbet ITC and Sarofim AF. Rates and Routes of Transport of PCBs in the environment. *Env Health Perspect* 1972; April:21-38

Ohmichi M, Decker SJ, Pang L, and Saltiel AR. Nerve growth factor binds to the 140 kd *trk* proto-oncogene product and stimulates its association with the *src* homology domain of phospholipase C 1. *Biochem Biophys Res Comm* 1991; 179(1):217-223

Overmann SR, Kostas J, Wilson LR, Shain W, and Bush B. Neurobehavioral and somatic effects of perinatal PCB exposure in rats. *Environ Res* 1987; 44:56-70

Pal GK and Thombre DP. Modulation of feeding and drinking by dopamine in caudate and accumbens nuclei in rats. *Indian J Exp Biol* 1993; 31(9):750-754

Pantaleoni GC, Fanini D, Sponta AM, Palumbo G, Giorgi R, and Adams PM. Effects of maternal exposure to polychlorobiphenyls (PCBs) on F1 generation behavior in the rat. *Fund Appl Toxicol* 1988; 11:440-449

Parkinson A and Safe S. Mammalian biologic and toxic effects of PCBs, In: Polychlorinated Biphenyls (PCBs): Mammalian and Environmental Toxicology, Springer-Verlag, New York, 1987; pp. 49-76

Pednekar JR and Mascarenhas JF. Role of striatal dopamine in the lordosis behaviour. *Indian J Physiol Pharmacol* 1993; 37(4):333-336

Quensen JF, Boyd SA, and Tiedje JM. Dechlorination of four commercial polychlorinated biphenyl mixtures (Aroclors) by anaerobic microorganisms from sediments. *Appl Env Microbiol* 1990; 56(8):2360-2369

Quensen, III JF, Tiedje JM, and Boyd SA. Reductive dechlorination of polychlorinated biphenyls by anaerobic microorganisms from sediments. *Science* 1988; 242:752-754

Richardson SA and Tizabi Z. Hyperactivity in the offspring of nicotine-treated rats: Role of the mesolimbic and nigrostriatal dopaminergic pathways. *Pharmacol Biochem Behav* 1994; 47(2):331-337

Risebrough RW and deLappe B. Accumulation of polychlorinated biphenyls in ecosystems. *Env Health Perspect* April, 1972, pp. 39-45

Rogan WJ and Gladen BC. Neurotoxicology of PCBs and related compounds. *Neurotoxicology* 1992; 13:27-36

Rogan WJ, Gladen BC, Hung KL, Koong S-L, Shia L-Y, Taylor JS, Wu Y-C, Yang D, Ragan NB, and Hsu C-C. Congenital poisoning by polychlorinated biphenyls and their contaminants in Taiwan. *Science* 1988; 241:334-336

Rogan WJ, Gladen BC, McKinney JD, Carreras N, Hardy P, Thullen J, Tinglestad J, and Tully M. Neonatal effects of transplacental exposure to PCBs and DDE. *J Pediatr* 1986; 109:335-341

Ross AH. Identification of tyrosine kinase *Trk* as a nerve growth factor receptor. *Cell Reg* 1991; 2:685-690

Rossetti ZL, Silvia CP, Krajnc D, Neff NH, and Hadjiconstantinou M. Aromatic-L-amino acid decarboxylase is modulated by D_1 dopamine receptors in rat retina. *J Neurochem* 1990; 54:787-791

Safe S Polychlorinated biphenyls (PCBs): Environmental impact, biochemical and toxic responses, and implications for risk assessment. *Crit Rev Toxicol* 1994; 24(2):87-149

Safe S. Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: Environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). *CRC Crit Rev Toxicol* 1990; 21(1):51-83

Safe S. PCBs and human health, In: Polychlorinated Biphenyls (PCBs): Mammalian and Environmental Toxicology, Springer-Verlag, New York, 1987; pp. 133-146

Safe S. Polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs): Biochemistry, toxicology, and mechanism of action. *CRC Crit Rev Toxicol* 1984; 13(4):319-395

Safe S, Safe L, and Mullin M. Polychlorinated biphenyls: Environmental occurrence and analysis. In: *Polychlorinated Biphenyls (PCBs): Mammalian and Environmental Toxicology.* New York, Springer-Verlag, 1987, pp. 1-14

Salamone JD. The involvement of nucleus accumbens dopamine in appetitive and aversive motivation. *Behav Brain Res* 1994; 61(2):117-133

Saltiel AR and Decker SJ. Diversity in cellular signals for nerve growth factor and insulin: Variations on a common theme. *Molec Cell Endocrin* 1991; 81:C197-C202

Scally MC, Ulus I, and Wurtman RJ. Brain tyrosine level controls striatal dopamine synthesis in haloperidol-treated rats. *J Neural Transm* 1977; 41:1-6

Schantz SL, Levin ED, and Bowman RE. Long-term neurobehavioral effects of perinatal polychlorinated biphenyl (PCB) exposure in monkeys. *Environ Toxicol Chem* 1991; 10:747-756

Schneider JS. Chronic exposure to low doses of MPTP. II. Neurochemical and pathological consequences in cognitive-impaired, motor asymptomatic monkeys. *Brain Res* 1990; 534:25-36

Schneider JS and Kovelowski, II CJ. Chronic exposure to low doses of MPTP. I. Cognitive deficits in motor asymptomatic monkeys. *Brain Res* 1990; 519:122-128

Schubert D, LaCorbiere M, Klier FG, and Steinbach JH. The modulation of neurotransmitter sytnthesis by steroid hormones and insulin. *Brain Res* 1980; 190:67-79

Seegal RF, Brosch KO, and Bush B. Polychlorinated biphenyls produce regional alterations of dopamine metabolism in rat brain. *Toxicol Lett* 1986a; 30:197-202

Seegal RF, Brosch KO, and Bush B. High-performance liquid chromatography of biogenic amines and metabolites in brain, cerebrospinal fluid, urine and plasma. *J Chromatography* 1986b; 377:131-144

Seegal RF, Brosch KO, Bush B, Ritz M, and Shain W. Effects of Aroclor 1254 on dopamine and norepinephrine concentrations in pheochromocytoma (PC12) cells. *Neurotoxicology* 1989; 10:757-764

Seegal RF, Bush B, and Brosch KO. Polychlorinated biphenyls induce regional changes in brain norepinephrine concentrations in adult rats. *Neurotoxicology* 1985; 6:13-24

Seegal RF, Bush B, and Brosch KO. Comparison on effects of Aroclors 1016 and 1260 on non-human primate catecholamine function. *Toxicology* 1991a; 66:145-163

Seegal RF, Bush B, and Brosch KO. Sub-chronic exposure of the adult rat to Aroclor 1254 yields regionally-specific changes in central dopaminergic function. *Neurotoxicology* 1991b; 12:55-66

Seegal RF, Bush B, and Brosch KO. Decreases in dopamine concentrations in adult, non-human primate brain persist following removal from polychlorinated biphenyls. *Toxicology* 1994; 86:71-87

Seegal RF, Bush B, and Shain W. Lightly chlorinated ortho-substituted PCB congeners decrease dopamine in non-human primate brain and in tissue culture. *Toxicol Appl Pharmacol* 1990; 106:1-9

Seegal RF and Shain W. Polychlorinated biphenyls reduce tyrosine hydroxylase activity in rat brain. *Soc Neurosci Abs* 1994; 20(2):1650

Shafer TJ and Atchison WD. Transmitter, ion channel and receptor properties of pheochromocytoma (PC12) cells: A model for neurotoxicological studies. *Neurotoxicology* 1991; 12:473-492

Shain, W; Bush, B; and Seegal, RF. Neurotoxicity of polychlorinated biphenyls: Structure-activity relationship of individual congeners. *Toxicol Appl Pharmacol* 1991; 111:33-42

Shain W, Overmann SR, Wilson LR, Kostas J, and Bush B. A congener analysis of polychlorinated biphenyls accumulating in rat pups after perinatal exposure. *Arch Env Contam Toxicol* 1986; 15:687-707

Shiota K. Postnatal behavioral effects of prenatal treatment with PCBs (Polychlorinated Biphenyls) in rats. *Okajimas Fol anat jap* 1976; 53:105-114

Silberhorn EM, Glauert HP, and Robertson LW. Carcinogenicity of polyhalogenated biphenyls: PCBs and PBBs. *Crit Rev Toxicol* 1990; 20(6):439-469

Sims KL, Davis GA, and Bloom FE. Activities of 3,4-dihydroxy-L-phenylalanine and 5-hydroxy-L-tryptophan decarboxylases in rat brain: Assay characteristics and distribution. *J Neurochem* 1973; 20:449-464

Spina MB, Sqinto SP, Miller J, Lindsay RM, and Hyman C. Brain-derived neurotrophic factor protects dopamine neurons against 6-hydroxydopamine and N-methyl-4-phenylpyridinium ion toxicity: Involvement of the glutathione system. *J Neurochem* 1992; 59:99-106

Stewart BH, Collarini EJ, Pisoni RL and Christensen HN. Separate and shared lysosomal transport of branched and aromatic dipolar amino acids. *Biochim Biophys Acta* 1989; 987:145-153

Swain WR. An overview of the scientific basis for concern with polychlorinated biphenyls in the Great Lakes, In: PCBs: Human and Environmental Hazards (D'Itri, F.M. and Kamrin, M.A., eds), Butterworth Publishers, Boston, 1983; pp. 11-48

Takagi Y, Abdura S, Hashimoto, K, and Kitaura, T. Transfer and distribution of accumulated (^{14}C)polychlorinated biphenyls from maternal to fetal and suckling rats. *Arch Environ Contamin Toxicol* 1986; 15:709-715

Taylor JR, Elsworth JD, Roth RH, Sladek, Jr JR, and Redmond, Jr DE. Cognitive and motor deficits in the acquisition of an object retrieval/detour task in MPTP-treated monkeys. *Brain* 1990a; 113:617-637

Taylor JR, Roth RH, Sladek, Jr JR, and Redmond, Jr DE. Cognitive and motor deficits in the performance of an object retrieval task with barrier-detour in monkeys (*Cercopithecus aethiops sabaeus*) treated with MPTP: Long-term performance and effect of transparency of the barrier. *Behav Neurosci* 1990b; 104(4):564-576

Tilson HA, Jacobson JL, and Rogan WJ. Polychlorinated biphenyls and the developing nervous system: Cross-species comparisons. *Neurotoxicol Teratol* 1990; 12:239-248

Vulliet PR, Hall FL, Mitchell JP, and Hardie DG. Identification of a novel proline-directed serine/threonine protein kinase in rat pheochromocytoma. *J Biol Chem* 1989; 264(27):16292-16298

Wang Y, Citron BA, Ribeiro P, and Kaufman S. High-level expression of rat PC12 tyrosine hydroxylase cDNA in *Escherichia coli*: Purification and characterization of the cloned enzyme. *Proc Natl Acad Sci, USA* 1991; 88:8779-8783

Wiesel FA, Blomqvist G, Halldin C, Sjogren I, Bjerkenstedt L, Venizelos N, and Hagenfeldt L. The transport of tyrosine into human brain as determined with L-[1-^{11}C] tyrosine and PET. *J Nucl Med* 1991; 32:2043-2049

Wurtman RJ. Amino acid control of neurotransmitter synthesis and release: Physiological and clinical implications. *Psychother Psychosom* 1993; 60:18-32

Wurtman RJ. Effects of their nutrient precursors on the synthesis and release of serotonin, the catecholamines, and acetylcholine: Implications for behavioral disorders. *Clin Neuropharmacol* 11, Suppl 1: S187-S193, 1988

Wurtman RJ and Fernstrom JD. Control of brain neurotransmitter synthesis by precursor availability and nutritional state. *Biochem Pharmacol* 1976; 25:1691-1696

Wurtman RJ, Hefti F, and Melamed E. Precursor control of neurotransmitter synthesis. *Pharmacol Rev* 1981; 32(4):315-335

Wurtman RJ, Larin F, Mostafapour S, and Fernstrom JD. Brain catechol synthesis control by brain tyrosine concentrations. *Science* 1974; 185:183-184

Young EA, Neff NH, and Hadjiconstantinou M. Evidence for cyclic AMP-mediated increase of aromatic-L-amino acid decarboxylase activity in the striatum and midbrain. *J Neurochem* 1993; 60:2331-2333

Zhu MY, Juorio AV, Paterson IA, and Boulton AA. Regulation of aromatic-L-amino acid decarboxylase by dopamine receptors in the rat brain. *J Neurochem* 1992; 58:636-641