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EXPOSURE OF NERVOUS SYSTEM CELLS TO POLYCHLORINATED
BIPHENYLS (PCBs), RESULTS IN ALTERATIONS OF
NEUROTROPHIC FACTOR EXPRESSION

by

Gordon H. Gurley

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Dr. John Spitsburgen, Advisor

Western Michigan University
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EXPOSURE OF NERVOUS SYSTEM CELLS TO POLYCHLORINATED
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Gordon H. Gurley, Ph.D.

Western Michigan University, 2006

Toxic insult by PCBs results in learning and memory deficits in humans. Alterations in expression of neurotrophic factors (NF) and/or their receptors have been linked to changes in learning and memory. How PCBs affect cognition is not known. We suggest that PCBs affect cognition by altering NF expression.

We exposed cultured C6 rat glioblastoma cells (an astrocyte cell-line) to medium containing PCB (Aroclor 1254 (10ppm)). Control cells were treated with dimethyl sulfoxide (DMSO) or regular medium. Enzyme-linked immunosorbent assays (ELISA) were used to determine glial cell-line derived neurotrophic factor (GDNF) and nerve growth factor (NGF) concentrations in all samples. We also assayed for intracellular NF from cell lysates. Real-time RT-PCR was used to determine GDNF mRNA levels and pharmacological assays were utilized to investigate the role of protein kinase C (PKC) in the signaling pathway by which PCBs may exert their effect on GDNF gene expression. In addition we subjected rats to dietary exposure of PCBs and lead and assayed their brain tissue for NF protein content.

Results show that PCBs increased both synthesis and release of GDNF and increased secretion of NGF in glial cells. We determined that PCBs use the PKC signaling pathway in their effect on GDNF expression in glial cells. Rats exposed to PCBs had reduced NF protein in their brains and there was a negative correlation of brain GDNF protein levels to blood lead levels (BLLs) in the lead exposed animals.

Our data show that exposure of nervous system cells to toxins such as PCBs and lead, alters NF expression and hence offers a basis whereby these neurotoxins may alter neural plasticity leading to compromised cognition.

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Gordon H. Gurley

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CHAPTER I

GLOBAL INTRODUCTION

The objective of this research was to investigate how neurotrophic factor expression is affected by neurotoxic insults. We studied the effects of two widespread environmental toxins, Polychlorinated Biphenyls and Lead (Pb) on the expression of nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF). Numerous studies support the idea of a critical association of altered NF with deficiencies in neural functions, including learning and memory. It is also well established that toxins like PCBs affect learning and memory in animals as well as humans. This study therefore, was significantly driven by the suspicion that toxins like PCBs and lead may mediate some of their adverse effects on learning and memory by altering NF expression.

Our *in vitro* studies utilized the C6 glial cell-line. The *in-vivo* studies were performed in rats. The following sections therefore, offer general and in some cases, brief backgrounds on: NFs, glial cells, PCBs and lead.

Historical Overview of Neurotrophic Factors

NFs are a group of proteins which are able to promote differentiation, and survival of both central nervous system and peripheral neurons. Generally these are small soluble proteins with molecular weights ranging from 13 to 24 Kilodaltons and are often active as homodimers (Lipton and Kalil 1995)

The first NF to be discovered was NGF, discovered in the late 1940s. It belongs to a subset of NFs called neurotrophins which include Neurotrophin 3, Brain Derived Neurotrophic Factor, and Neurotrophin 4/5 (NT-3, BDNF, NT-4/5) (Heerssen and Segal 2002, Levi-Montalcini 1987). Rita Levi-Montalcini, an Italian biologist and Stanley Cohen an American biochemist shared the 1986 Nobel Prize in Physiology or Medicine largely for their discovery of, and work on, NGF.

The serendipitous discovery of NGF has its history in work done in the lab of Viktor Hamburger. Elmer Bueker, a former student of Hamburger, reported the result of an experiment involving the grafting of mouse sarcoma 180 into the body wall of 3-day old chick embryos. Histological observation of the embryos fixed 3- 5 days later, revealed that sensory fibers emerging from adjacent dorsal root ganglia had gained access into the tumor. The experimenter concluded that there was some unique histochemical property of the fast growing mouse sarcoma which offered a favorable field for the growth of sensory fibers. In addition, the sarcoma tissue environment induced enlargement of the exposed ganglion. This led to continuous *in-vivo* and *in-vitro* investigation by Hamburger and Levi-Montalcini using in addition to mouse sarcoma 180 another, sarcoma 37. Not only did this unknown

tumor factor, humeral factor—as they then called it, induce sensory fiber growth but sympathetic as well. Sympathetic fibers tended to grow in large bundles. The factor also caused dense innervation of blood vessels with fibers protruding into the lumen and in some cases to the extent of obstructing blood flow. In experiments where the tumors were inserted next to embryonic ganglion, but separated by a membrane, the trophic effect was still produced, indicating that the factor was dissolvable and diffusible. Sensory ganglia in the presence of the growth promoting extract caused halos of nerve fiber outgrowth *in vitro*.

Later Cohen stumbled upon an even more potent source of this growth factor. He had isolated from the two tumors a nucleoprotein fraction containing the nerve growth promoting activity (Cohen et al. 1954). He used snake venom, a source of phosphodiesterase, to study the mechanism by which the extracted nucleoprotein worked. During the classical halo experiments, by pure chance he discovered—not only did the venom seem to enhance the nerve growth promoting activity of the tumor fractions, but even minute quantities of the venom itself possessed the ability. From the snake venom Cohen isolated and purified a non-dialyzable, heat-labile substance with the nerve growth promoting propensity that he termed nerve growth factor (NGF). Microgram quantities of this identified protein (of approximate molecular weight 20,000) mimicked the effects of the two mouse sarcomas on sensory and sympathetic ganglia and fibers. The researchers continue their search for NGF in several non-sarcoma tissue types. Cohen reasoned that the salivary gland of mouse, the homologue body part with the source of snake venom, should be a good source of

the factor. His intuition paid off with dividend. From the sub-mandibular gland of male mouse, Cohen identified and purified salivary NGF, a protein molecule with molecular weight of 44,000. Salivary gland turned out to be a greater source of NGF than venom (Cohen et al. 1954). The primary structure of male mouse sub-maxillary NGF was determined in 1971 as a result of its successful sequencing (Bradshaw 1978). This led to the identification of its cDNA and subsequent cloning from various animal sources including human. The clones were highly homologous to the mouse NGF (Scott et al. 1983, Ullrich et al. 1983)

The discovery of NGF opened up the “flood-gates” for the search and identification of other such factors. The discovery of other members of the neurotrophin family was quickly forthcoming. Three of them, BDNF, NT-3, and NT-4 also called NT-5, were characterized in mammals. BDNF was first purified from pig brain (Barde et al. 1982) and its gene was later cloned by Liebrock and colleagues (Liebrock et al. 1989; in Lindsay et al. 1996). By comparing conserved sequences of NGF and BDNF, clone homologues were obtained and used to identify NT-3, and NT-4/5 (Lindsay 1996). These four—NGF, BDNF, NT-3 and NT-4/5 are derived from a common ancestral gene, are similar in sequence and structure and therefore were collectively named neurotrophins (Huang and Reichard 2001). Two additional neurotrophins, NT-6 and NT-7 are related to NGF but unlike the afore-mentioned neurotrophins, whose genes have been found also in reptiles, amphibians and mammals, NT-6 and NT-7 have been found only in fish (Hallböök et al. 1998). The discovery and cloning of NT-6 from the teleost fish *Xiphophorus* (Gotz et al. 1994),

and NT-7 from zebra fish (Nilsson et al. 1998), was reported in 1994 and 1998 respectively.

If the quote; “The discovery of molecules with therapeutic value often occurs by chance rather than by purposeful experimentation” is true in the case of NGF the first neurotrophic factor to be discovered, it is also true for a more recent NF that is not a member of the neurotrophin family, that being GDNF. Discovered in 1993 as a result of the suggestion that glia secrete factors that influence growth and differentiation of specific neurons, GDNF was the first member identified of a new family of NFs (Bohn 1998). GDNF is also reported to be a distant member of the transforming growth factor- β (TGF- β) superfamily of proteins (Yajima et al. 1997). Just prior to the discovery of GDNF, Schubert and colleagues performed a study aimed at identifying cell-specific proteins in the nervous system. The study utilized a technique called two- dimensional electrophoresis, to compare expressed proteins by various glial and neuronal cell-lines. The result was astonishing. The gel patterns of intracellular proteins of the cell lines were almost identical, whereas those of the secreted proteins were remarkably different. This lead to the idea of investigating secreted protein fraction of a cell line as a good source for the identification of cell-specific proteins. This approach seemingly had the potential for revealing novel molecules with possible trophic effects on different cell types. Jurgen Engele, a postdoc at the time in Bohn’s lab, pursued the concept despite the unavailability of the now advanced molecular tools. He cultured dopaminergic (DA) neurons, some glial tumor cell lines and pheochromocytoma cells (PC12), a neuron cell line. Using

burdensome bioassays, he screened for protein fractions that promoted varied trophic activities of the DA neurons. These growth references included the following: neuronal survival, degree of neurite outgrowth, and extent of high-affinity DA uptake. He found that all the glial cell lines secreted proteins that had effects on DA growth activity but the PC12 cells did not (Bohn 1998). Utilizing these bioassays, Lin and co-workers (Lin et al. 1993) isolated from one of the glial cell lines, B49, a glycoprotein of 134 amino acids with intense DA neurotrophic propensity, called GDNF. GDNF was found to have greater potency than any previously discovered DA neurotrophic factors (Bohn 1998).

In December of 1996, Kotzbauer and colleagues reported their discovery of yet another NF, a member of the GDNF family called Neurturin (NTN), also a distant member of the (TGF)- β superfamily (Kotzbauer et al. 1996). While investigating whether factors other than NGF supported sympathetic neuronal survival in culture, the researchers discovered that conditioned medium of Chinese hamster ovary cells contained long-term survival of superior cervical ganglion (SCG) sympathetic neurons activity in culture. This SCG survival-promoting factor was thermo-stable and trypsin sensitive. This molecule had the attributes of a peptide but it was not affected by neutralizing antibodies against NGF and based on its chemical properties it was distinguishable from any other known neurotrophins. Yet, it promoted the survival of 100% of neurons supported by NGF. Sequencing of this molecule revealed it to be a new factor whose sequence was then used to clone mouse complementary DNA (cDNA), subsequently yielding mature protein upon proteolytic cleavage of the

pro-protein. It was found that mature NTN of humans exhibits 91% homology with that of mouse NTN and that its amino-acid sequence shares approximately 42% similarity with mature GDNF (Kotzbauer et al. 1996).

In February of 1998, Persephin another member of the GDNF family was identified. Persephin did not bind receptors known to bind GDNF and NTN, so it was suggested that persephin utilizes additional or different receptors to GDNF and NTN. Persephin however, is about 40% identical to the mature regions of GDNF and NTN (Milbrandt et al. 1998).

The discovery of a forth member of the GDNF family called Artemin was reported just 9 months subsequent to the report of Persephin (Baloh et al. 1998). Artemin is almost identical to another GDNF family member reported to have been cloned and characterized just soon after the reporting of Artemin, called Enovin (Masure et al. 1999). Interestingly, although Enovin is reported by its researchers to be very similar to but different from Artemin, the OMIM gene database lists it to be synonymous to Artemin (OMIM 2005).

All four/ five aforementioned GDNF family members are distantly related to the members of the TGF β family of growth factors (Lin et al., 1993; Buj-Bello et al., 1995; Hoyer et., 1998) cited in (Soler et al. 1999). The list of neurotrophic factors has not been exhausted, furthermore, at the rate at which discovery and development in this field has been growing, future identifications of novel NF will be accompanied by very little surprise.

Molecular Structure and Signaling Mechanism of Neurotrophic Factors

Neurotrophins are relatively small homodimeric polypeptides generally of about 120 amino acid residues (Persson and Ibanez 1993). They are closely structurally related. For example, the conserved sequences of the NGF family were studied from varied vertebrates—human, rat, chicken, viper, xenopus, salmon and ray. It was found that BDNF, NT-3 and NT-4/5 all share about 50% sequence identity (Hallböök et al. 1991). The neurotrophins generally interact with two common families of receptors expressed by the cells they target. One receptor they bind to with high affinity, the Trk family of protein tyrosine kinase receptors. The other receptor type they bind to with low affinity, the p75 neurotrophin receptor (p75^{NTR}). The high affinity NGF receptor subsequently classified as the proto-oncogene Trk, a family of tyrosine kinase protein (Martin-Zanca et al. 1989), was first detected by Hosang and Shooter (1987) while working on cell surface receptor proteins of PC12 cells that responded to NGF. They detected a 130,000 Dalton protein that slowly releases NGF and is internalized at 37°C (Alonzo 1991). Each neurotrophin member can influence specific biological activities by preferential binding to ligand specific receptors of the Trk family. Thus cell types such as sympathetic neurons, nerve fibers of the dorsal root ganglion of the spine, and cholinergic neurons of the basal forebrain all express the TrkA receptor which specifically binds NGF (Fagan et al. 1996). BDNF and NT-4/5 preferentially bind TrkB (Klein et al. 1989), which is extensively expressed in both the peripheral and central nervous system, in cells such as spinal motor neurons and nodose ganglion sensory neurons (Meakin and Shooter 1992). NT-

3 interacts with TrkC which is expressed by large sensory and motor neurons of the spine and noradrenergic neurons of the locus coeruleus (Barbacid 1994, Numan et al. 1998). NT-3 to some extent is promiscuous and will also bind, albeit with less affinity, both TrkA and TrkB (Barbacid 1994, Rydén and Ibáñez 1996).

The low affinity neurotrophin receptor, p75, was first identified as a membrane protein that can bind NGF (Radeke et al. 1987), but it was later shown to be capable of binding all of the neurotrophins (Rodriguez-Tabar et al. 1992, Ryden et al. 1995). It was originally suggested that p75^{NTR} functioned as a positive regulator of Trk, probably based on evidence of lost nociceptive and thermosensitive sensory neurons in p75 knockout mice (Lee et al., 1992 Bamji et al. 1998). It was later shown however that p75^{NTR} can signal on its own and has so-called binding death domains that trigger the cell death pathway of neuronal as well as glial cells (Bamji et al. 1998, Murray et al. 2004). In general neurotrophin receptors act as sensors that relay various extracellular and intracellular signals in the cells they target. These signaling activities account for the unique effects neurotrophins have on behavior and higher-order activities (Chao et al. 1992). A model for the NGF family receptor activation is illustrated by the following figure 1.1

.

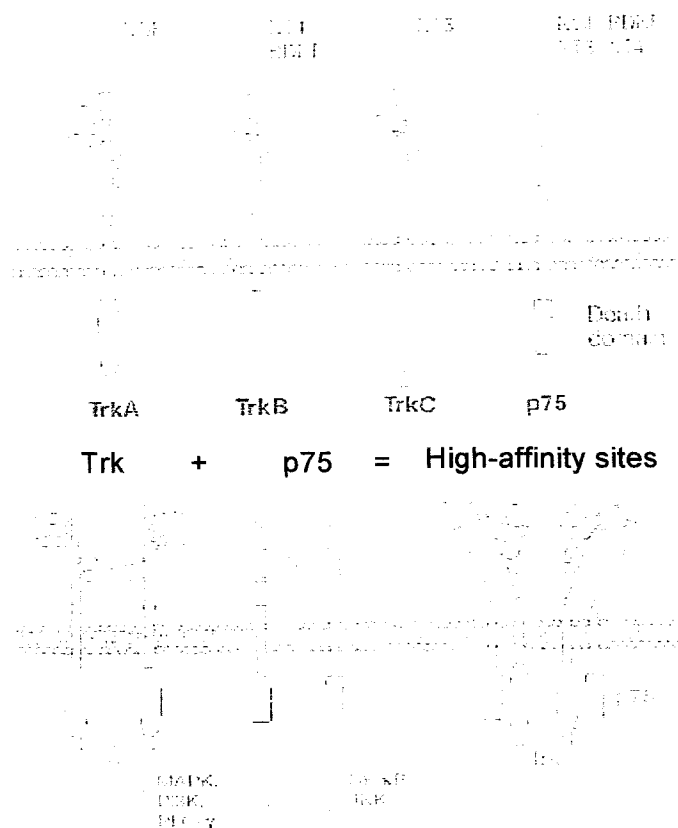


Figure 1.1. Model of Trk and p75 Receptor Activation. Neurotrophin binding results in dimerization of each receptor. Neurotrophins bind selectively to specific Trk receptors, whereas all neurotrophins bind p75. TRk receptors contain extracellular immunoglobulin G (IgG) domains for ligand binding and a catalytic tyrosine kinase sequence in the intracellular domain. Each receptor activates several signal transduction pathways. The extracellular portion of p75 contains four cysteine-rich repeats, and the intracellular part contains a death domain. Neurotrophin binding to p75 receptor mediates survival, cell migration and myelination through several signaling pathways. Interaction between Trk and p75 receptors can lead to changes in binding affinity for neurotrophins.

Figure and legend taken from (Chao, 2003)

The GDNF family of neurotrophic factors, induce their effects through multicomponent receptors consisting of a family of receptors called the Ret receptor kinases, in combination with glycosylphosphatidyl inositol (GPI)-linked receptors called GFR α s. Like the Neurotrophins and their receptors, these proteins, though they share close sequence homology, each signals through their specific receptor. In the case of these proteins however, each of these specific receptors then interacts with a common receptor, the RET receptor tyrosine kinase (Remy et al. 2001). RET activates several intracellular signalling cascades, which regulate cell survival, differentiation, proliferation, migration, chemotaxis, branching morphogenesis, neurite outgrowth and synaptic plasticity (Sariola and Saarma 2003). Figure 1.2 illustrates the basic signaling pathways of this family of NFs:

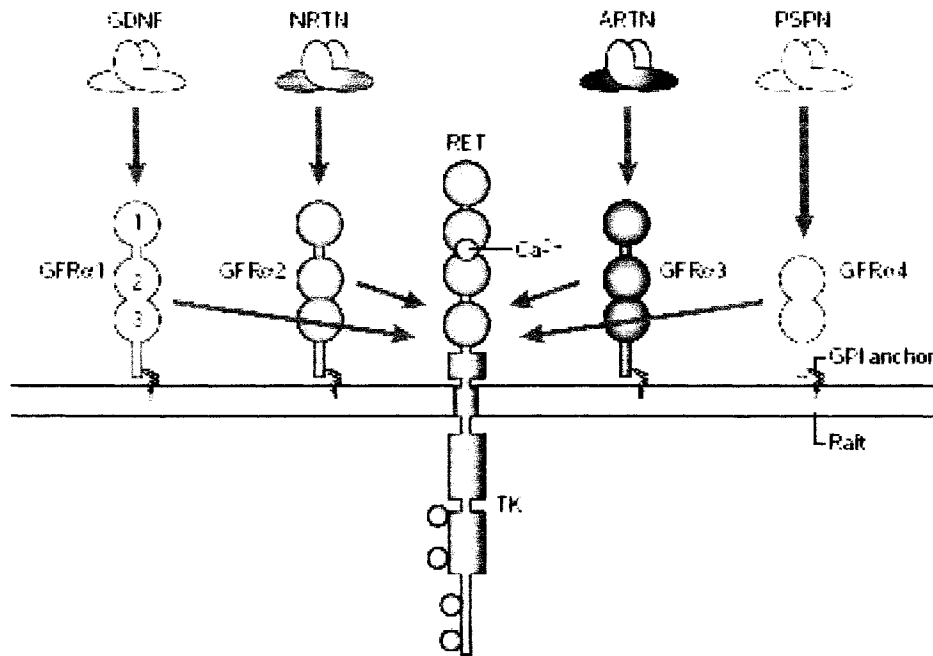


Figure 1.2. GDNF-Family Ligands and Receptor Interactions. Homodimeric glial cell line-derived neurotrophic factor (GDNF)-family ligands (GFLs) activate RET tyrosine kinase (TK) by first binding their cognate GDNF-family receptor- α (GFR α) receptors. Arrows indicate the preferred ligand-receptor interactions that are known to occur physiologically in vivo. GFR α proteins are attached to the plasma membrane by a glycosyl phosphatidylinositol (GPI) anchor and are predicted to have three globular cysteine-rich domains (1,2,3) (except for GFR α 4, which has only two), joined together by less conserved adaptor sequences¹. GFLs bind mainly to the second domain of GFR α receptors, which is also crucial for RET binding. Although the extracellular domain of RET interacts with all four GFL GFR α complexes, the regions of RET that are involved in these interactions have not been delineated. Binding of Ca^{2+} ions to one of the four extracellular cadherin-like domains of RET is required for its activation by GFLs⁸⁷. Four tyrosine residues in the RET intracellular part (Tyr905, Tyr1015, Tyr1062 and Tyr1096; red balls) serve as docking sites for different adaptors. One of them (Tyr1096) is in the carboxy-terminal end of the long isoform of RET (grey). Membrane rafts are shown in yellow. ARTN, artemin; NRTN, neurturin; PSPN, persephin.

Figure and legend taken from (Airaksinen and Saarma 2002)

Relationship Among NF, Nervous System Cells and Cognition

Neurotrophic factors are important for the growth of neurons and for regulation of phenotypic expression during development. Several critical aspects of the ontogeny of neurons for example—determination of neuronal population, neurite branching and synaptogenesis, and synaptic plasticity in adults are regulated by NFs (Sariola and Saarma 2003). Most neurons require a sustained supply of these trophic factors throughout their lifetime for their normal maintenance and for recovery from nervous system injury in the adult organism. NFs have been shown to be important regulators of learning and memory. Results of recent studies suggest that blockade of NGF effects in the brain results in impaired learning performance (Van der Zee 1995), while administration of NGF enhances learning performance (Fiore et al. 2002, Lipinski et al. 1995).

Generally, studies on the mechanism of learning and memory, have focused on activities of the hippocampus in animal models such as mice and rats, and the hippocampus is widely accepted to play a vital and pivotal role in memory (Broadbent et al. 2004, Kumaran and Maguire 2005, Wesierska et al. 2005). The trend of studies investigating the effects of NFs on learning and memory therefore, has been on their effects on hippocampal activities. One study reported that their results indicate a role for NGF acting through its TrkA receptor on two proteins called choline acetyl transferase (ChAT) and vesicular acetylcholine transferase (VACHT) in contextual memory consolidation (Woolf et al. 2001). Mice with a disruption in one of their alleles for NGF (*ngf*^{+/-}) was shown to have reduced NGF mRNA and protein in the

hippocampus. This was associated with shrinkage and loss of basal forebrain cholinergic neuron and a decrease of cholinergic innervation of the hippocampus. The changes in these NGF-mutant mice were accompanied with measurable deficits in learning and memory. Administration of NGF effectively corrected the deficits in size of cholinergic neurons and the density of cholinergic innervation of the hippocampus (Chen et al. 1997). Rattiner and colleagues reported that Long-term potentiation (LTP) within the hippocampus and hippocampally-dependent behaviors have been the primary models for examining the role of BDNF in learning and memory. Both morphological and chemical changes to synaptic organization are most likely involved in the process of learning and memory (Rattiner et al. 2005). It has been shown in *in-vitro* and *in-vivo* studies that BDNF modulates hippocampal LTP (Lee et al. 2005, Messaoudi et al. 2002). Several studies also have shown that neurotrophins induce morphological changes in dendritic spines and synaptic connections in brain regions associated with learning and memory (Gaiarsa 2004, Tyler and Pozzo-Miller 2003, Wirth et al. 2003).

Glial cell line-derived neurotrophic factor (GDNF) is noted for its potent neuroprotection of mid-brain dopaminergic (DA) neurons (Horger et al. 1998, Wang et al. 2002, Winkler et al. 1996). GDNF also promotes the re-growth and survival of DA neurons damaged by neurotoxins *in-vitro*. The neuroprotective and neuro-repair properties of GDNF, highlights it as a favored therapeutic candidate for Parkinson's disease. Parkinson's disease results from degeneration of midbrain nigral DA neurons that innervate the striatum (Kirik et al. 2000, Lin 1996, Patel 2004). Other

GDNF family ligands—neurturin, artemin, and persephin all maintain several neuronal populations in the central nervous system. These NFs also support the survival and regulate differentiation of several peripheral nerve cells, which include sympathetic, parasympathetic, sensory and enteric neurons (Sariola and Saarma 2003).

Studies in mice in which GDNF expression is lowered via gene knockout, indicate that low levels of GDNF in the central nervous system are associated with impaired learning performance (Gerlai et al. 2001). In a study investigating the effects of GDNF on progenitor cell proliferation and differentiation in the hippocampus, (a site that supports adult neurogenesis) GDNF significantly increased cell neurogenesis by 78%. The effects of GDNF on the hippocampus are potentially important in memory and learning processes (Chena et al. 2005).

In-vitro Models Utilized

C6 Glial Cells

Glial cells are a source of NFs in the brain. There are 10 – 50 times as many glial cells within the brain as neurons. They make up 90% of brain cells (Miller et al., 2003). Glial cells, referred to simply as glia were discovered in 1891 by Santiago Ramon y Cajal, a Spanish neuroanatomist. They are non-neuronal cells that nourish, support, and myelinate the nervous system and take part in its signal transmission. Glia can be divided into two main groups—microglia and macroglia. Microglia scavenge and engulf central nervous system (CNS) debris, in essence they are

macrophages. Macroglia of the CNS consist of: Astrocytes, which are the most abundant glial cells, Oligodendrocytes, Ependymal cells, and Radial glia. Schwann cells are glia of the peripheral nervous system (PNS). They have a similar function as oligodendrocytes. That is, to provide myelination to axons of neurons. Each of the previously listed types of glia has special functions in the nervous system (Wikipedia Contributors (2005)-a)

Until recently, glial cells have been thought to be important only as integral support cells of the nervous system, but this concept has changed since it has been recently shown that glial cells play a role in synapse formation. Astrocytes in particular are believed to actually ‘instruct’ neurons to make synapses (Miller et al., 2003). Some researchers reported that although neurons can form synapses without glia, they may require glial derived cholesterol to form high numbers of efficient synapses, suggesting glia as a source for synaptogenesis-promoting signals (Mauch et al., 2001; Pfrieger et al., 2002). Glial cells play important roles in the immune system including, inflammatory and cytotoxic responses (Cheeran et al. 2003, Giri et al. 2004, Lee et al. 2000). Glial cells are also integral components of the blood brain barrier (Vries et al. 1997). In fact, previous researchers believed that the blood brain barrier was formed by glial sheets covering the brain capillaries (Dempsey and Wislocki 1955). It was later found to be more accurately a functional barrier of cerebral endothelial plasma membrane (Brightman and Reese 1969).

The C6 glial cell line is an astrocyte cell line derived from rat. Rat C6 cells possess extensive chemical and functional analogy to normal rat astrocytes (Syapin et

al. 2001). This cell line is widely used among the scientific community as a model especially for the study of response to toxic insult to the nervous system.

In-vivo Model

The *in-vivo* aspect of this study utilized as its model rats that were exposed to PCBs at varying doses and duration of exposure. Rodents such as rats and mice have been used extensively as mammalian models for behavioral studies of learning and memory. Different systems have been developed to test and measure levels of cognition. The Morris water maze developed by neuroscientist Richard G. Morris in 1984 is one such study system. This system is used primarily to explore the role of the hippocampus in the formation of spatial memories (Wikipedia contributors (2005)-b). The radial arm maze developed earlier by Olton has been steadily improved and has been used by collaborators in this study. Roegge and colleagues also used the radial arm maze to explore retardation in cognition of rats exposed to PCBs (Roegge et al. 2000).

Polychlorinated Biphenyls (PCB)

PCBs are persistent environmental bio-accumulative, widely distributive, toxic, pollutants. They accumulate in food chains, especially in certain species of fish and seafood collected in contaminated areas. (Altmann et al. 2001, Li and Hansen 1997, Rao 2000). PCBs are polychlorinated hydrocarbons and they occur as both ortho (non co-planar) and non-ortho (co-planar) substituted molecular

configurations. Based on all the chlorination patterns, there are 209 possible congeners. The basic structure from which all possible congeners are obtained is shown in figure 1.3.

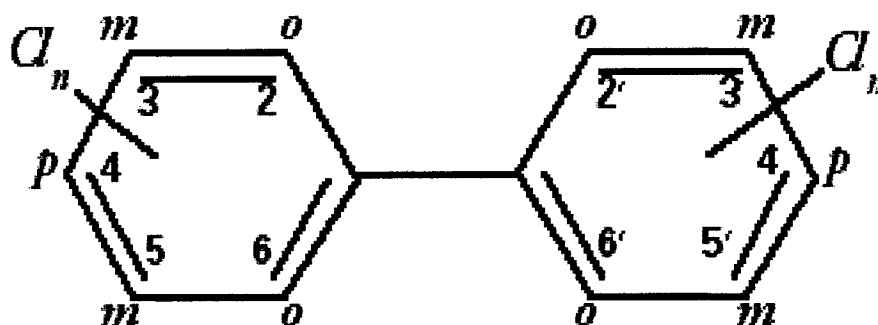


Figure 1.3. The General Structure of PCBs. m, o and p represent chlorine substitutions of the meta, artho and para respectively. (adapted from McKinny et al., 1994)

Commercial mixtures of PCBs called Aroclors, which are made up of several individual congeners, were manufactured in the United States for almost 50 years. They were produced for use as nonflammable dielectrics in electronic parts, lubricants, plasticizers in caulking compounds, paints, adhesives and sealants, vehicles for pesticide application, and pigment suspension agents in carbonless copy paper. (Korrick and Altshul 1998, McKinney and Walter 1994). One of these PCB mixtures, called Aroclor 1254, was the most commonly sold and is the one most commonly found at contaminated sites (Tilson and Kodavanti 1997).

A total of 1,598 hazardous waste sites have been proposed for inclusion on the Environmental Protection Agency's (EPA) list. In at least 500 of these, PCBs have been identified and of these 499 are situated in the United States (HazDat, 2000).

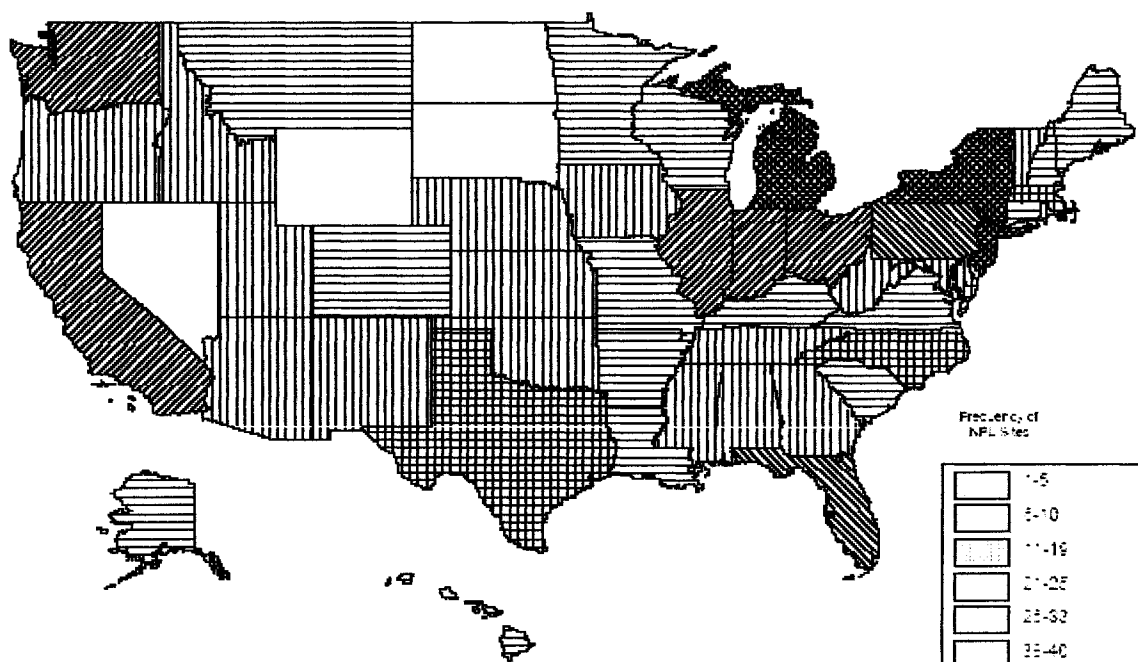


Figure 1.4. The Frequency of Waste Sites with PCB Pollution. Taken from HazDat 2000 in (ATSDR, 2000)

In our immediate vicinity here in Michigan USA, the Kalamazoo River has been identified as a Great Lakes Area-Of-Concern and Superfund site due to historic releases of PCBs from de-inking operations at local paper mills (Beck 1998).

Atmospheric Deposition

Atmospheric deposition is the transfer of pollutants from air to the earth's surface. The atmospheric pathway is significant since it is the pathway by which many toxic substances capable of sustained presence in the environment long after manufacture or use, are widely distributed. PCBs do not easily break down, and so can travel long distances in the air before deposition via rain or snow into bodies of water or on to land. Once the temperature is high enough, these toxic substances can revolatilize back into the air and travel further. This cycling called the *grasshopper effect* can occur numerous times, resulting in pollutants traveling to cooler and cooler environments until temperatures are too low for revolatilization. Atmospheric deposition has been shown to be a significant source of pollutants to the Great Lakes and other water bodies. The grasshopper effect also called global distillation is the reason why high levels of pollutants such as PCBs can be found in the cold Artic, far away from cities and factories (EPA).

Possible Ways of Destroying PCBs

According to the EPA, many items containing PCBs can be appropriately and safely destroyed in hazardous waste incinerators, since PCBs can be destroyed by burning at 2400 °F. Two technologies are currently used for on-site destruction of PCBs. One is by using plasma arc torches, which burn up to 10,000 °F . This method is being used for many soils that are contaminated with large amounts of PCBs. The other process is the use of bacteria—a bioremediation process that is used for rivers

with large sediment concentrations of PCBs. Currently there are over 20 strains of bacteria capable of using biphenyl exclusively as their source of carbon (EPA).

Health Perspective

Even though PCBs have been banned in the USA since 1977 they continue to pose health problems because of their persistent presence in the environment and in the tissue of exposed humans. PCBs are exceedingly toxic and have been shown to cause health problems in humans even at low levels. The highest acceptable concentration in fish—according to the EPA, is two parts per million. Human exposure to PCB can be through inhalation (respiration), digestion, or through the skin (dermal absorption) (EPA). They are lipophilic and are present in the fat of all human populations. The skin and liver are particularly susceptible to PCBs and there is controversy as to their “carcinogenicity”(ability to cause cancer). A major concern has been that of PCBs’ ability to cross the placenta to affect prenatal infants as well as to affect postnatal infants via the milk of exposed mothers (Korrick and Altshul 1998, McKinney and Walter 1994). PCBs are amongst the environmental chemicals capable of endocrine system disruption in animals and humans. The ability of PCBs to mimic natural hormones explains a possible mechanism by which they interfere with endocrine function. The structural shape of these compounds underlies their molecular interactions with biological systems. The coplanar configuration of PCBs phenyl rings and the “laterality” of chlorine atoms are important structural features

governing biological binding affinity and toxic reactivity (McKinney and Walter 1994).

The incidences that are probably most credited for sparking the international concern for and health hazard awareness of PCBs are the mass poisonings called Yusho and Yu-Cheng. These occurred in Japan in 1968 and central Taiwan in 1979, respectively. These incidences were caused separately by ingestion of rice oil contaminated with polychlorinated hydrocarbons including PCBs. Some of the more noticeable symptoms included—pigmentation of nails, skin and mucous membranes, increase eye discharge, acneform eruptions and feeling of weakness. One year after the initial poisoning of the Yu-Cheng victims, blood PCB concentration was 3 – 1156 ppb. Five years after the Yusho incident PCB concentration in blood was 1 – 30 ppb (Masuda 1985). Studies carried out on pregnant mothers with Yusho report that babies from these women were born with PCB unique abnormalities, which the researchers called fetal PCB syndrome (FPS). The manifestations included: Dark brown “CoCa-Cola color” skin pigmentation, gingival hyperplasia, early eruption of the teeth (some actually born with teeth), increased calcification of the skull and low birth weight (Yamashita and Hayashi 1985).

A review article highlighted a study based on the Yu-Cheng children exposed to PCBs in utero and by lactation that reported that these children had poor cognitive development (Aoki 2001). According to Cynthia Washam, a team of researchers from London and Taiwan reported in the July 2002 issue of *The Lancet* (Washam 2003), a weakened ability of males exposed two decades ago to PCB in the Yu-Chen

incident, to father boys. The report indicated that 46% of children born to young men exposed in the oil disaster are males compared to a world average of 51-52%.

Women exposed in the same incident showed no abnormalities in sex ratio of their offspring (Washam, 2003).

In 1989 the World Health Organization reported that contamination of breast milk with PCBs and dioxins is higher in the Netherlands, Belgium, Germany and the United Kingdom than in most other parts of the world (WHO, 1989 in (Schantz et al. 2003)). As a result the Dutch Government launched a longitudinal study known as the Dutch PCB/Dioxin Study to investigate the possible adverse effects of exposure to PCBs and dioxins and growth and development of full-term infants. This study was the first to apply congener-specific analytic techniques. More recent studies have had the advantage of modern analytical methods and as a result congener-specific analysis procedures are widespread and available. These advances in techniques and toxicological knowledge have been instrumental in influencing a renewed risk assessment process. Data from ongoing PCB studies assessing the mediators of neurobehavioral outcomes in children are being published and as a result incriminating evidence for PCB's effects on neurodevelopment is significantly increasing. Studies in Taiwan, Michigan (USA), New York (USA), Holland, Germany, and the Faroe Islands have all reported negative associations between prenatal PCB exposure and measure of cognitive functioning in infancy or childhood (Schantz et al. 2003).

The studies described herein examined the effects of PCBs on the nervous system, with an interest in effects on cognition. The nervous system has been shown to be highly sensitive to the effects of PCBs. Epidemiological studies in humans have demonstrated a variety of nervous system deficits associated with PCB exposure, including alterations in function of the peripheral (Chia and Chu 1984) and central nervous system (Jacobson and Jacobson 1997, Jacobson JL. et al. 1990). Impairments in learning and memory have been documented both in young (Grandjean et al. 2001) and older humans (Schantz et al. 2001) following environmental exposure to PCBs, and in rats exposed to PCBs (Widholm et al. 2001). Developmental exposure to PCBs has been associated with cognitive deficits in children. Experiments with rodents have revealed impairments in learning tasks that involve the hippocampus (Gilbert et al. 2000)

Although PCB exposure has been shown to alter a variety of intracellular signaling processes in neurons, including calcium signaling, protein kinase C signaling and neurotransmitter synthesis (Tilson and Kodavanti 1998), very little is known concerning the mechanism by which PCBs affect learning and memory. The risk to the developing nervous system posed by PCBs is difficult to assess due to a general lack of information on mechanism/s by which PCBs induce neurotoxicity (Howard et al. 2003). Furthermore, very little is known concerning effects of PCBs on neurotrophic factor expression or effects (Angus and Contreras 1994, Angus and Contreras 1995).

At least three mechanisms of action by which PCBs appear to exert their toxicity have been suggested: (1) reversible binding with specific molecular sites of action, such as receptors, enzyme, etc., (2) irreversible covalent interaction between PCB and target molecules—macromolecules such DNA and proteins, and (3) accumulation of highly lipid-soluble, metabolically stable PCBs in lipid-rich tissues or tissue compartments (McKinney and Walker, 1994). Being aware of the many reported studies that established a relationship between NFs and the nervous system, we studied the effects of PCBs on NGF, BDNF and GDNF, utilizing both *in-vitro* and *in-vivo* experiments, in an attempt to suggest an indirect mechanism by which PCBs may cause alterations in the CNS that may lead to compromised cognitive abilities.

Lead

Despite the fact that Pb is one of the oldest (three millennia) known poisons, and that there has been knowledge of its effects on children for over 100 years, its exposure continues to be a major public health problem. As a result investigations of the neurotoxicity of Pb still occupy an area of active research, and there have been recent developments in the study of its basic mechanism of action (Burger and Gochfeld 2004, Lidsky and Schneider 2003)

Unlike PCBs, which are man-synthesized compounds, Pb is a common element of the geosphere, but its ubiquitous presence in the environment in bioavailable forms is largely due to human activities (Rice, 1984, 1996; Bellinger et al., Payton et al., 1998; Laskey et al., 2000 in: (Burger and Gochfeld 2004) For

example, between 1969 and 1971 an ore smelter in El Paso, Texas had discharged 1,116 tons of lead into the atmosphere. Subsequent analysis of soil and 'house-hold-dust' Pb concentrations was conducted in areas close to smelters, and correlative studies of blood lead levels (BLLs) in children and adults were performed. The studies reported that Pb emitted by the smelter and deposited in soil and house-hold dust, appear to be the major source of Pb absorbed by humans (Rosenblum et al. 1973). Since the 1970s lead has been deemed a ubiquitous contaminant with several contributing "human responsible sources" such as Pb based paints and gasoline. Within recent times however decreases in the use of leaded gasoline and lead-based paints in the USA have resulted in reductions of lead exposure and BLLs (ATSDR 1999). Recent studies have lead to a greater awareness of health hazards posed by lead toxicity at much lower BLLs than first expected. In 1971 "undue lead absorption" referred only to BLLs equal to or greater than, 40 µg/dL. But several Subsequent studies reported that BLLs much lower than 40 µg/dL adversely impacted the health of children without causing overt symptoms (Matte and Falk 1997). For example, one study reported lower cognitive test scores and higher teachers' rating of behavioral problems among children with higher dental lead levels but no history of clinically overt lead poisoning (Needleman et al. 1979). Numerous other studies report that in humans and primates, lead exposure at low or medium levels leads to auditory, neurobehavioral, reproductive and systemic effects (Rice 1995) in: (Burger and Gochfeld 2004). Although the relationship between lead exposure and cognitive development has been a lively controversy among certain researchers (Burger and

Gochfeld 2004), many studies agree that even at low doses, lead exposure can produce serious adverse effects on the central nervous system of children and that these effects can last for years.

Background Re-cap and Projection

In this background I provided the general account of the history, origin and distribution of certain environmental toxins, namely PCBs and lead, and their mode of entry into Biological systems. An overview of the risks these pollutants pose and several examples of past, present and future health risks were highlighted. Since there is significant evidence that both of these toxins affect the central nervous system to the extent of causing retardation in learning and memory, the present study was oriented towards trying to understand possible relationship between these toxins and other factors that promote, modify and maintain the integrity of the nervous system. These factors are neurotrophic factors and hence an overview of their discovery, classification, structure and biological functions were given. The objective of this study was to investigate the effects of the studied toxins on the expression of these neurotrophic factors and how such changes may allow room for speculations on how toxins such as PCBs may indirectly affect cognition by their direct effect on neurotrophic factors. An overview of the models—in-vitro and in-vivo used to facilitate the investigations was given.

Following will be: a list of the statistical methods used in this study; chapter II which present the in-vivo investigations and findings relating to PCB exposure;

chapter III which presents the in-vivo investigations and findings relating to lead exposure; chapter IV which presents the in-vitro experimentations and findings and finally chapter V which provides a global discussion as well as offer projections and suggestions of further studies.

Statistical Methods

A one-way ANOVA was used to test differences from controls and each ANOVA was followed by a post-hoc test to determine where differences lie among treatment groups. A two-way ANOVA was used to compare treatment levels and duration followed by Student-Newman-Keuls method of Pairwise Multiple Comparison. A linear regression was used, followed by Pearson Product Moment Correlation, to test whether blood lead levels (BLLs) in exposed rats were predictors of neurotrophic factor protein levels in their brains. Data are expressed as mean + or \pm standard error of the mean--“*” indicates statistical significant difference at $P < 0.05$ and “**” indicates statistical significant difference at $P < 0.001$. Other symbols of specific meanings are displayed and defined on individual figures.

CHAPTER II

THE EFFECTS OF POLYCHLORINATED BIPHENYLS (PCBs) ON NEUROTROPHIC FACTOR (NF) EXPRESSION IN RAT BRAIN AND POSSIBLE IMPLICATIONS IN COGNITIVE DEFICIENCY

Introduction

The aim of the study presented in this chapter, was to determine whether exposure to PCBs (A1254) Alters neurotrophic factor protein expression in the brains of rats. The study also aimed at determining if A1254 induced alterations in NF expression may be correlated to impaired performance in radial arm maze (RAM) tests for spatial learning and memory.

PCBs

PCBs are polychlorinated hydrocarbons. Commercial mixtures of PCB congeners, Aroclors, were manufactured in the United States for almost 50 years for use as nonflammable dielectrics in electronic parts; in several products including: lubricants, paints, and sealants; as vehicles for pesticide application, and as pigment suspension agents in carbonless copy paper (Korrick and Altshul 1998, McKinney and Walter 1994).

The nervous system has been shown to be highly sensitive to the effects of PCBs. Epidemiological studies in humans have demonstrated a variety of nervous system deficits associated with PCB exposure, including alterations in function of the peripheral (Chia and Chu 1984), and central nervous system (Jacobson and Jacobson 1997, Jacobson JL. et al. 1990). Impairments in learning and memory have been

documented both in young (Grandjean et al. 2001) and adult humans (Schantz et al. 2001) following environmental exposure to PCBs, and in rats exposed to PCBs (Widholm et al. 2001). Developmental exposure to PCBs has been associated with cognitive deficits in children.

At least three types of basic mechanisms of action by which PCBs appear to exert their toxicity has been suggested: (1) reversible binding with specific molecular sites of action such as receptors, enzymes, etc., (2) irreversible covalent interaction between PCB and target molecules, macromolecules such as DNA and proteins, and (3) accumulation of highly lipid-soluble, metabolically stable PCBs in lipid-rich tissues or tissue compartments (McKinney and Walker, 1994). Very little is known however, concerning the mechanism by which PCBs affect learning and memory. Little is also known about the effects of PCBs on NF expression or effects (Angus and Contreras 1994, Angus and Contreras 1995)

Neurotrophic Factors

NFs are integral and inevitable agents of the development and maintenance of the nervous system. Several critical aspects of the ontogeny of neurons for example—determination of neuronal population, neurite branching and synaptogenesis, and synaptic plasticity in adults are regulated by NF (Sariola and Saarma 2003). Most neurons require a sustained supply of these trophic factors throughout their lifetime, for their normal maintenance and for recovery from nervous system injury in the adult organism.

Because of the roles NFs play in synaptic plasticity (Elliott et al. 2001, Tyler et al. 2002), it is assumable that agents affecting their homeostasis may also inadvertently affect cognition. Many studies suggest that synaptic plasticity plays a fundamental role in learning and memory (Abbott and Nelson, Johnston et al. 2003, Rampon and Tsien 2000). “A change in the transmission efficacy at the synapse (synaptic plasticity) has been considered to be the cause of memory”(Okano et al. 2000). Other studies demonstrate significant deterioration in spatial learning due to disruption of mechanisms involved in synaptic plasticity (Saura et al. 2004, Sun et al. 2002).

NFs have been shown to be important regulators of learning and memory. Results of previous studies suggest that blockade of nerve growth factor (NGF) effects in the brain results in impaired learning performance (Van der Zee 1995), Mice with a disruption in one allele for NGF (*ngf*^{+/-}) were shown to have reduced NGF mRNA and protein in the hippocampus. These changes were accompanied by measurable deficits in learning and memory (Chen et al. 1997). Several studies also have shown that neurotrophins induce morphological changes in dendritic spines and synaptic connections in brain regions associated with learning and memory (Gaiarsa 2004, Tyler and Pozzo-Miller 2003, Wirth et al. 2003).

GDNF is noted for its potent neuroprotection of mid-brain dopaminergic (DA) neurons (Horger et al. 1998, Wang et al. 2002, Winkler et al. 1996). GDNF also promotes the regrowth and survival of DA neurons damaged by neurotoxins *in-vitro*. Studies in mice in which GDNF expression is lowered via gene knockout, indicate

that low levels of GDNF in the central nervous system is associated with impaired learning performance (Gerlai et al. 2001).

Hypothesis: PCBs may affect learning and memory by altering the expression of neurotrophic factors

The previous overview highlighted a few of the many studies that implicate PCBs as cognitive altering compounds. It also presented other studies that established a relationship between altered NFs and reduced ability in learning and memory. This study questioned therefore, whether a possible means by which PCBs affect cognition is by altering NF expression. We therefore investigated the effects of Aroclor 1254, a commercial mixture of PCBs, on NF protein levels in brains of rats which had undergone learning and memory test (behavioral studies by Paris-Larson, 2004) following treatment with PCBs.

Methods and Materials

A1254 Treatment of Rats for NF Analysis (Experiment 1)

Fischer Rats (7 weeks old) were allowed to feed ad-libitum for 7 days (short term) or 84 days (long term) on a diet containing 10 ppm or 50 ppm Aroclor 1254 (Lot 124-191-B, obtained from Accustandard) or the control diet which was free of PCBs.

To prepare the PCB treated diet, 2.0 kg of Lab Diet 400 rat chow + 500ml ethanol were added to a 1 gallon Nalgene jar. 20.0 ml of 1mg/ml or 2.0 ml of 10 mg/ml (for 10 ppm dose) or 100 ml of 1 mg/ml or 10.0 ml of 10 mg/ml (for 50 ppm)

Aroclor 1254 stock solution was added. The mixture was shaken or rolled to allow initial vigorous mixing. This was followed by four hours of further mixing of which in the first hour each batch was shaken and turned every 15 minutes and then every 30 minutes for the remaining 3 hours. The food was then spread out in foil pans to dry in hoods. Usually at least 48 hours of drying was needed for satisfactory evaporation of solvents. Control food was mixed and dried the same way. Final food A1254 content was determined by GC analysis of batch samples.

A1254 Treatment of Rats for behavioral Analysis (Experiment 2)

Eighteen male Fischer 144 rats (Charles River, Portage MI) were obtained at 21 days of age and delivered to Haenicke Hall animal colony, Western Michigan University. Western Michigan University animal colony was maintained at constant 20-22° C and 20-24% humidity under a 12 hour light/dark cycle. The animals were housed singly in standard plastic hanging cages with free access to food and water.

Treatment

To prepare the Aroclor 1254 treated diet, 500 ml ethanol was mixed with 2.0 ml or 10 ml of 10mg/ml A1254 stock solution (for 10ppm or 50ppm dose). This solution was then poured over 2.0 kg rat chow (Purina). The rat chow/ Aroclor mixture was placed on a rotary shaker for four hours and manually shaken every 15 minutes for the first hour, every half hour thereafter. The food was then placed in a single layer in aluminum pans to dry for three days to allow complete evaporation of

the ethanol. The Aroclor remains in the food and is spread evenly throughout. Each dose was made in individual batches of food. The control food was also soaked in ethanol and dried, to control for any unknown differences in smell or texture. The animals were allowed to feed freely on the treated food for 28 days. On the 28th day all food was replaced with standard Purina rat chow and free access to food was continued for one week, at which time food was restricted to achieve 85% of free feeding body weight.

Behavioral Study Procedure (*All behavioral studies done by Paris-Larson—student of Lisa Baker PhD. Dept. Psychology Western Michigan University*)

Habituation

A small handful of Fruitloops® were placed at the end of each arm of the radial arm maze. The animals were placed in pairs in the center of the maze and allowed to roam throughout the maze, with free access to the Fruitloops®. Each pair was left in the maze for ten minutes. This procedure was repeated for three consecutive days.

Acquisition Phase

Training began on the day following the last habituation session. One Fruitloop® was placed at the end of each arm of the radial arm maze. Each animal was placed individually in the maze and allowed to explore. Each animal was

removed after all eight cereal pieces were eaten or 16 arms were entered. Training sessions for each rat, occurred once a day for 28 days or until each group achieved a mean of 80% accuracy for two consecutive days. An error was considered an entry into a previously entered arm. Six measures were recorded or calculated during acquisition; total time to complete the maze, latency to first bite of food, total number of arm entries, repeat arm entries, number of entries made until first error, and number of correct entries out of the first eight entries.

Delay Win-Shift Phase

In the second phase a delay win-shift procedure was utilized. A Fruitloop® was placed at the end of six randomly chosen, arms, while the other two arms were blocked from entry by an adhesive index card. The blocked arms were randomly chosen prior to each session for every rat. Each animal was removed from the maze when all six Fruitloops® were consumed or 16 arms were entered. Three delays, 20 minutes, 2 hours, and 6 hours were established. After the given delay, each animal was placed back in the maze and the previously blocked arms were now baited while the other six arms were not baited. Each delay was tested for three consecutive days (sessions). Each animal was removed from the maze when either both Fruitloops® were eaten or 16 arms were entered. In this task, 100% accuracy was defined as one entry into each of the two previously unbaited arms.

All data were recorded by two human observers and each session was videotaped for further data collection. All entries were recorded in order of entry and

each entry that resulted in food consumption was circled. The total number of arms entered, repeated arm entries, number of entries until an error occurred, and the number of correct entries out of the first eight were all recorded during or calculated after each session. A stopwatch was used to record the latency, time until the first Fruitloop® was eaten, and the total session time (Paris-Larson 2004).

Brain Collecting Procedure

The following procedure for obtaining rat brains for analysis was applied to all experiments involving PCB exposed rats. The animals were sacrificed by CO₂ euthanization and the brain from each animal was removed after decapitation with a guillotine. Care was taken to remove and freeze the brains within 5 min post sacrifice. Upon excision, the two cerebral hemispheres were immediately dropped into tubes containing 2- methyl butane cooled on dry ice. Upon freezing, excess 2- methyl butane was decanted and tubes were stored at – 80 °C. Half of a brain from each animal was homogenized and processed for neurotrophic factor content, while the complementary halves were cryostat sectioned for immunohistochemistry.

Brain processing for ELISA

Each hemisphere processed for NF quantitative analysis was weighed and then crushed in a polythene envelope by hammering on an aluminum plate cooled with dry ice. The resultant powder was put into a glass homogenizer and homogenized in 4ml of processing buffer (See appendix for buffer recipe), on wet ice. The homogenate

was transferred to centrifuge tubes and spun at 2400 rpm, for 30 min, at 4 °C. The supernatant was collected and stored at -20 °C.

Enzyme-linked Immunosorbant Assay (ELISA)

ELISA was used to determine GDNF, NGF and BDNF protein content in the supernatant obtained from each treated group. For NGF and GDNF analysis, anti-NGF or GDNF, diluted in PBS, was added to 96 well plates, which were sealed and allowed to incubate overnight. PBS was also used to wash plates between treatments. Plates were blocked with 1% BSA for 1 hour and incubated for 2 hours with protein standards and samples. Anti-NGF or anti-GDNF conjugated to biotin was diluted in Tris Buffered Saline (see appendix for recipe) containing 0.1% BSA and 0.3% Tween-20 and was added to plates for two hours of incubation. Streptavidin HRP (*Pierce*) was added to plates followed by a 20 min incubation period. After washing off unbound HRP, the color reagent (tetramethyl benzidine dissolved in phosphate citrate buffer) was added. At full color development, the reaction was stopped with 0.1 M phosphoric acid and plates were scanned at 450 nm wavelength using a microplate scanning spectrophotometer (*Bio-Tek Instruments*). The average absorbance from each column of samples assayed in quadruplicates was calculated and NF concentration was extrapolated from a log standard curve. For BDNF we used ELISA kits from (Promega Corporation) and followed the manufacturer's protocol. All NF protein values were normalized to brain weight.

Results

Effects of PCB on NF Levels in Rat Brains

Short Term (7 days exposure)

We determined the levels of NGF and GDNF protein in the brains of rats exposed to PCB in their diets. Twelve animals (4 rats per treatment group) were exposed for 7 days to 0ppm, 10ppm and 50ppm, respectively, and then sacrificed. One cerebral hemisphere from each animal was processed and NF protein content was determined by ELISA. The results showed significant reduction of both NGF and GDNF protein content in the brains from animals subjected to acute PCB exposure compared to that in controls (Figure 2.1a and 2. 1b.).

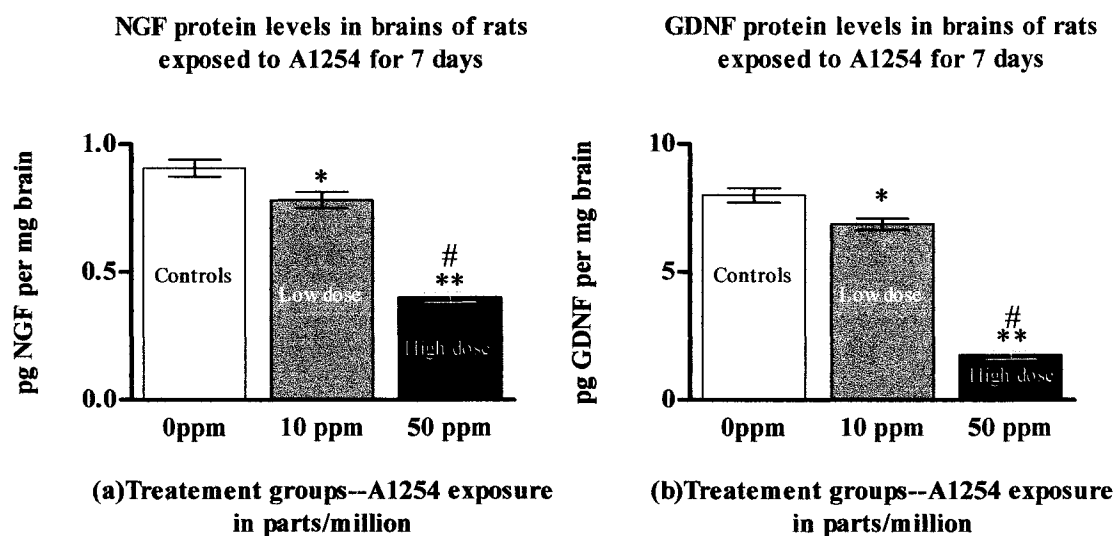


Figure 2.1. NGF and GDNF Protein Levels in Brains of Rats Exposed for 7 Days to 0, 10, or 50 ppm A1254 in Their Diets. The brains were removed and processed for determination of neurotrophic factor protein. The results showed that the A1254 exposed animals had significantly lower levels of both NGF and GDNF in their brains than control animals (*). The higher dose group had lower levels of NGF and GDNF than lower dose group (#). Statistical analysis used: 1-way ANOVA followed by Dunnette post hoc. * = $P < 0.05$, ** = $P < 0.001$.

Long Term (84 days exposure)

Rats were treated in the same manner as the short term exposed rats, except they were exposed for 3 months. The NF protein content of the brain homogenate from these animals was determined by ELISA, as previously described. Results showed that both A1254 exposed groups of rats had significantly less GDNF in their brains compared to control rats. Long term treatment of rats with A1254 had no effect on NGF protein levels, figure 2.2a and 2.2b.

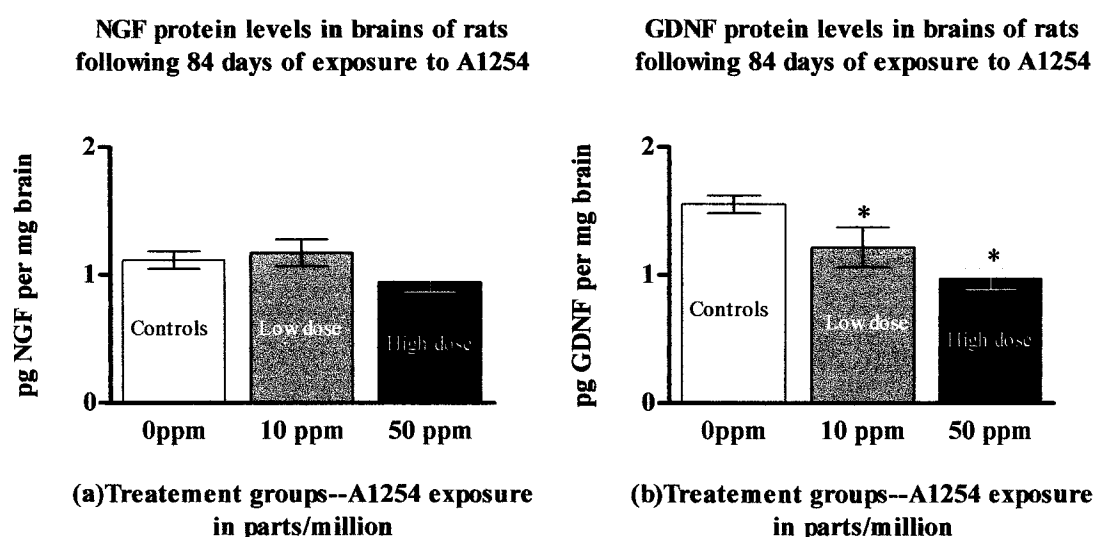


Figure 2.2. NGF and GDNF Protein Levels in Brains of Rats Exposed for 84 Days to 0, 10, or 50 ppm A1254 in Their Diets. The brains were removed and processed for determination of neurotrophic factor protein content. The results showed that the A1254 exposed rats had significantly lower levels of GDNF but no changes in NGF in their brains. Statistical analysis used: 1-way ANOVA followed by post hoc. * = significantly different from controls. $P < 0.05$.

Comparison of Short Term to Long Term A1254 Exposure

A comparison of the effect of A1254 on NF protein expression in the brains of rats exposed for 7 days or 84 days, showed a difference in response based on the period of exposure, especially for the 50ppm exposed groups. The differences between the means of the control groups and the treated groups for 7-days exposed animals was significantly greater than that of the 84-day exposed animals, as illustrated by figures 2.3a and b.

Percent of Control NF Protein Levels in Rat Brains Following 7 and 84 Days of Exposure to A1254

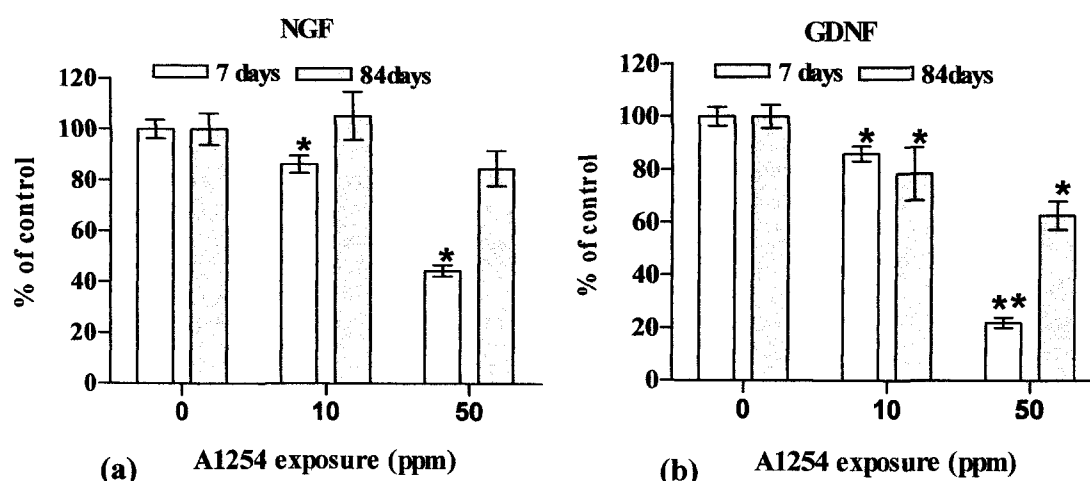
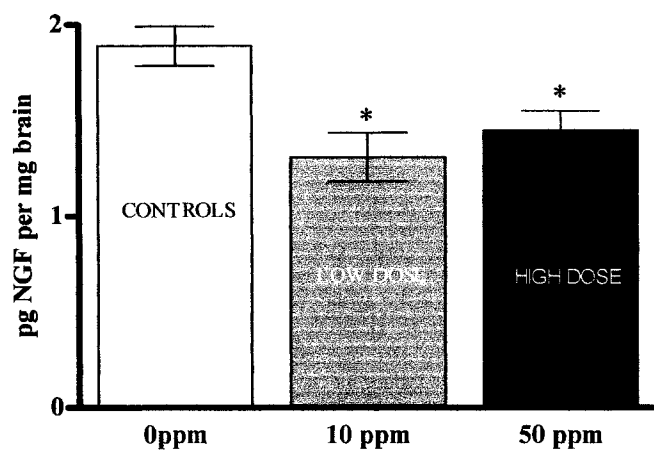


Figure 2.3. Changes in NGF and GDNF Protein Content in Brains of Rats Treated with A1254 for 7 Days (Short-Term), VS 84 Days (Long-Term). Results show a decrease in the effect of A1254 (% change from control) on NGF and GDNF protein levels at the 50ppm exposure level. A similar trend is observed, for NGF low dose but not GDNF low dose. Statistical procedure: **two-way ANOVA** followed by **Student-Newman-Keuls** post hoc.

Result on NFs from Behavioral Groups

NF protein content was also measured in brains of rats which had undergone behavioral tests following A1254 exposure. Rats were given A1254 in their diet for 28 days followed by a recovery period of 7 days before behavioral studies commenced. They were subjected to the radial arm maze test to determine the effect of PCBs on learning and memory—an exercise lasting 5 weeks, resulting in a total of 6 weeks recovery from A1254 exposure. At the conclusion of the behavioral experiments, the rats were euthanized and their brains were excised for determination of NF content.

The results indicated that the A1254 exposed groups of animals had significantly lower levels of NGF, BDNF and GDNF protein in their brains, compared to non-exposed animals (Figures: 2.4, 2.5, and 2.6). There were no dose-dependant differences in NGF and GDNF protein content. The BDNF results however, showed a dose response difference where the 10ppm A1254 caused a significant decrease in BDNF protein while the 50ppm exposure level did not. Moreover, the 50ppm A1254 exposed group of rats had significantly higher BDNF protein in their brains compared to the 10ppm A1254 exposed rats (figure 2.5).



Test groups--A1254 exposure in parts/million

Figure 2.4. NGF Protein Levels in Brains of Rats Exposed to A1254 for 28 Days. Rats were exposed for 28 days to 0, 10, or 50ppm A1254 in their diets, followed by 6 weeks of A1254-free diet. Animals were then euthanized and their brains were processed for determination of NGF protein content. The results showed that the A1254 exposed rats had significantly lower levels of NGF protein in their brains. There was no significant difference between high and low dosage groups. Statistical analysis used: 1-way ANOVA followed by a post hoc. * represents $P < 0.05$, $N = 6$.

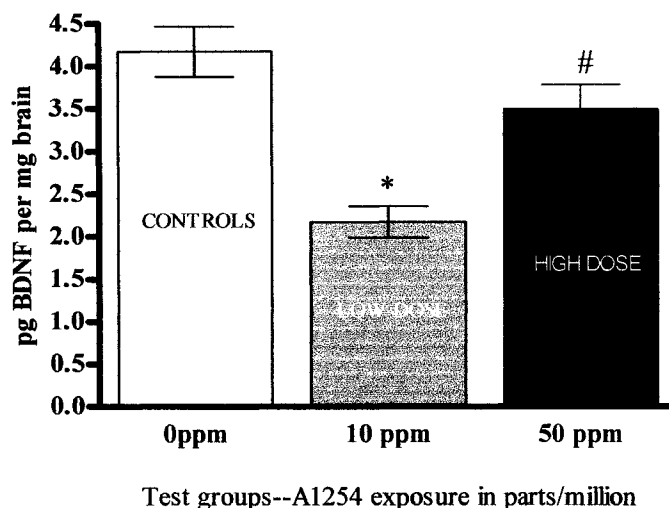


Figure 2.5. BDNF Protein Levels in Brains of Rats Exposed to A1254 for 28 Days. Rats were exposed for 28 days to 0, 10, or 50ppm A1254 followed by 6 weeks of A1254-free diet. Animals were euthanized and their brains were processed for determination of NGF protein content. The results showed that the 10ppm A1254 exposed rats had significantly lower levels of BDNF protein in their brains. The 50ppm exposed group had higher levels of BDNF than the 10ppm exposed group but was not different to controls. Statistical analysis used: 1-way ANOVA followed by post hoc. $P < 0.05$, * = sig. difference from controls and # = sig. difference between exposed groups. $N = 6$.

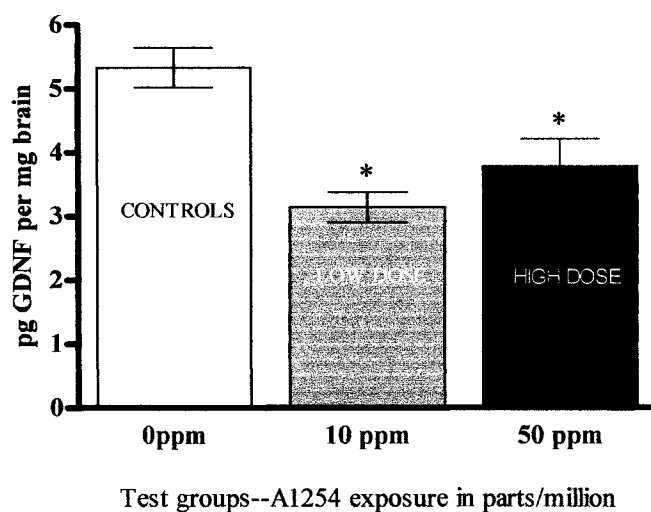


Figure 2.6. GDNF Protein Levels in Brains of Rats Exposed to A1254 for 28 Days. Rats were exposed for 28 days to 0, 10, or 50ppm A1254 in their diets followed by 6 weeks of A1254-free diet. Animals were then euthanized and their brains were processed for determination of GDNF protein content. The results showed that the A1254 exposed rats had significantly lower levels of GDNF protein in their brains. There was no significant difference between high and low dosage groups. Statistical analysis used: 1-way ANOVA followed by a post hoc. * represents $P < 0.05$, $N = 6$.

Observation of Age Related Effects on Expression of GDNF Protein in the Brains of Rats

Comparative observations were made of the control rats from the 7-days A1254 exposed (short term) 28-days A1254 exposed (intermediate), and 84-days A1254 exposed (long term) animals. The observations revealed a natural significant reduction in GDNF as the animals aged. These data are shown in (Figure 2.7).

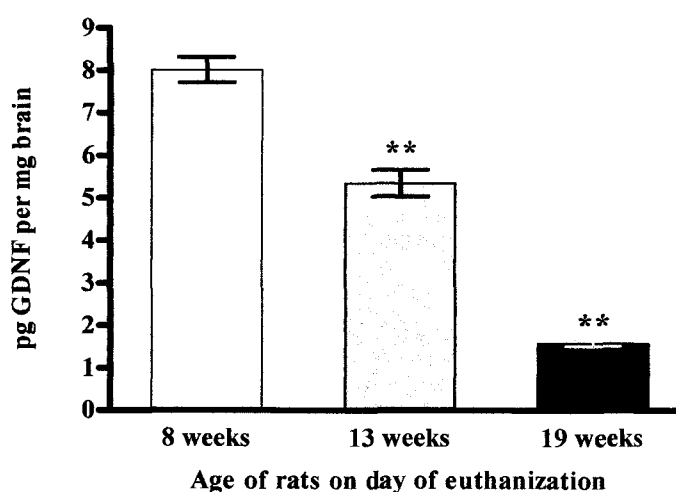


Figure 2.7. Relationship of Brain GDNF Protein Content with Age. GDNF protein content in brains of control rats is plotted against age, for three specific age groups. Results show that there is a reduction of brain GDNF protein content with increase age. Four 8 week, six 13 week and four 19 week old rats were tested for GDNF protein content of their brains. The protein data was obtained by ELISA from one brain hemisphere of each animal.
Statistics: 1- way ANOVA followed by post-hoc testing.

A 2-way ANOVA with age and treatment as variables, followed by Student-Newman-Keuls post hoc was also performed for all animals. Increase in age was negatively correlated with GDNF protein levels for all groups: $P < 0.01$, $N = 42$.

Results from Studies on Effects of Aroclor 1254 on Spatial Learning and Memory

Prior to the removal of whole brains from 18 Fisher 344 rats, they were tested for the effects of dietary exposure to Aroclor 1254 on spatial learning and memory. The results indicated that animals treated with 50ppm A1254 exhibited significantly more errors during the acquisition phase than those treated with 10ppm or control animals. In addition, the control group of rats tended to exhibit greater evidence of learning and better reference memory. The deduction was made based on observation (During the Delay Win-Shift tests) of subjects making less mean number of errors or repeated entries from one delay to another (Paris-Larson, 2004). The results suggest that dietary exposure of rats to A1254, caused impairments in their performances on the RAM tests.

Discussion

In this study there has been considerable evidence provided suggesting that PCBs affected the overall level of the NF proteins, in the brains of exposed rats. The A1254-induced NF protein reductions were seen following short-term exposure (7 days), intermediate exposure (28 days), as well as a long-term exposure (84 days). In addition, it appeared that A1254-induced NF protein reduction in the brains of the animals that underwent behavioral studies may be correlated to impaired performances in the RAM exercises. An important observation is that the A1254 effect on the NF protein levels, in the brains of the rats that underwent the RAM test, was relatively long-lived. Considering that even after 6 weeks of recovery (taken off

the A1254 diet), there were still observable reductions in NGF, BDNF and GDNF protein levels in their brains. This may be due to PCB's ability to remain in body tissue, especially adipose, for long periods of time even extending into years. The brain is among the organs found to have the highest PCB concentrations in exposed individuals (Argonne National Laboratory 2005).

It was observed that the level of GDNF protein in the brains of rats we tested naturally declined with age (figure 2.7). The difference between the protein content measured from the adolescent (2 months old) rats and that from the mature (5 months old) rats represented an 80% reduction. Despite this age related decline in GDNF protein level in rat brains, there was significant reduction in rats exposed to A1254 relative to the controls at all ages for which tests were carried out in this study. Based on these results it appears that A1254 significantly exaggerated the natural decline in GDNF protein in the brains of exposed rats, by the time of maturity. We suggest that such induced reduction may have negative consequences on brain processes that depend on natural levels of GDNF protein in the brain.

In comparing results after 7 days of exposure to a prolonged duration of 84 days, we observed a stronger effect in animals we tested after the shorter exposure period. It seems that although the effect on NF levels in some cases was still significant after 84 days of exposure, that there had been some ablation with time, especially for the high dose group. This we determined by comparing the percent difference in NF protein levels (NGF and GDNF) from control of 7-days A1254 exposed rats with the percent difference obtained from the 84-days A1254 exposed

animals and their controls (figure 2.3). Moreover, the significant difference in NGF protein level seen with the shorter exposure time disappeared by the end of 84 days of A1254 exposure. When animals are exposed to non-lethal dosages of a toxin, the reaction to that toxin on initial introduction may be different than that after prolonged exposure (Klein 1996). Factors such as accumulative effects or induction of intrinsic adaptive mechanisms may be contributors to the changes in response observed. It was Dr. H.F. Smyth, Jr., a toxicologist, who first coined the term "sufficient challenge" based on his observations of toxin-exposed laboratory animals. He discovered that animals to which he fed toxins actually got healthier at a certain point before showing signs of toxicity (Ottoboni 1991).

Ultimately we wanted to determine whether animals that showed altered NFs in their brains as a consequence of PCB exposure, would also exhibit signs of compromised cognition. We compared the results obtained from the behavioral test (RAM) involving the 28-days A1254 exposed animals with the NF results obtained from brains taken from these animals. Interestingly, it was found that A1254 exposed animals shown to have reduced levels of NFs, also exhibited poorer performances on the spatial learning and memory exercises, compared to controls. Low performance on the RAM test by A1254 exposed rats is supported by a study that reported male rats being exposed to A1254 during lactation, exhibited impaired RAM performance at adulthood (Roegge et al. 2000).

The NF analysis of the rats used in the RAM test showed no significant difference in NF protein levels between lower and higher A1254 dosage groups; and

in the case of BDNF, the 50ppm exposed group even had significantly higher levels than the group exposed to 10ppm. Since these animals had 6 weeks of recovery from A1254 exposure before their brains were analyzed for NF protein content, it may suggest that six weeks of recovery allowed for improvement in NF protein levels in the animals that received the higher A1254 dose.

The behavioral investigator reported that the 50ppm A1254 group of animals made significantly more mistakes than the 10ppm exposed group (Paris-Larson, 2004). This does not necessarily contradict the biological outcome seen for example, in the BDNF results shown in figure 2.5. It may indicate that there is no correlation between NF protein and behavior problems for BDNF or it may just be recovery at the NF protein level for BDNF. If in fact, there is a recovery effect for NF expression, it is reasonable to deduce that the greater the insult the greater the recovery, temporary though it may be. If we are assuming however, that extrinsic induced alterations of NFs in turn affect their influences on neuronal structural changes involved in cognition, then a temporary recovery at the level of NF expression, may not parallel a concomitant recovery at the level of cognition.

This study is intended to stimulate thinking along the presented line of arguments. It is difficult to elucidate *in vivo*; the sequence of molecular events involving introduction of PCBs at the level of brain tissue and the subsequent involvement of NFs and neuronal functions leading to compromised cognitive abilities. The work done here is just skimming the surface of potential discoveries. But all successful endings must have a beginning and this study is an important

beginning. From the biological and behavioral observations of these studies, we set out a speculative model represented by the following cartoon, for an indirect effect of PCBs on learning and memory.

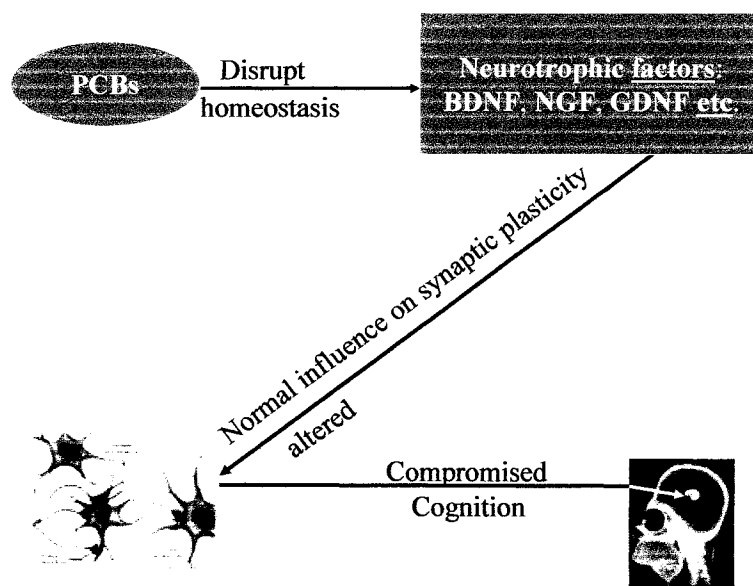


Figure 2.8. Illustrative Model of a Possible Sequence Leading to an Indirect Effect of PCBs on Learning and Memory

OVERVIEW OF CHAPTER II AND LINK TO CHAPTER III

In the previous chapter, the investigations and results of effects of PCBs (A1254) on NF protein expression in the brains of exposed rats were presented. It was found that A1254 caused reduction in NF protein levels in brains of exposed rats. The effects of the higher A1254 dose (50ppm) on NGF and GDNF tended to be more intense with the short term exposure (7 days) than after 84 days of exposure. Rats exposed to A1254 for 28 days and then subjected to the RAM test following 5 weeks of recovery during which they were trained, showed poorer performance than controls. Analysis of brains from animals in the RAM study showed significant reduction in brain NGF, BDNF and GDNF protein.

The aim of the studies in this chapter was to compare the *in-vivo* response (on the level of brain NF expression), to lead exposure with that of A1254 exposure. Here, the effects of lead (Pb) a neurotoxin known to affect learning and memory, was examined.

The association of Pb exposure with decline in cognitive function especially in children but also in adults has been widely studied and documented. One study reported that progressive and ongoing decline in cognitive function, particularly functions such as learning and memory; is associated with adult lead exposure long after exposure ceases (Schwartz et al. 2000). Because Pb has similar effects on cognition as PCBs, and because we are suggesting a role for NFs in activities of PCBs, we were curious as to whether NFs were targets of Pb as well.

CHAPTER III

THE EFFECTS OF LEAD (PB) ON NEUTROPHIC FACTOR EXPRESSION IN THE BRAINS OF EXPOSED RATS—*HOW DOES IT COMPARE TO THAT OF PCBs?*

Introduction

Lead

Lead is a common element of the geosphere, but its ubiquitous presence in the environment in bioavailable forms is largely due to human activities (Rice 1995).

Between 1969 and 1971 an ore smelter in El Paso, Texas had discharged 1,116 tons of lead into the atmosphere. Studies conducted close to these smelters, reported that lead emitted and deposited in soil and house-hold dust, appear to be the major source of lead absorbed by humans (Rosenblum et al. 1973). Lead based paints and gasoline had been “human responsible sources” of Pb since the 1970s. Within recent times however decreases in the use of leaded gasoline and lead-based paints in the USA have resulted in reductions of lead exposure and blood lead levels (BLLs) (ATSDR 1999). BLLs are used as an index of exposure and hence health risks.

Recent studies have lead to a greater awareness of health hazards posed by lead toxicity at much lower BLLs than first expected. In 1971 “undue lead absorption” referred only to BLLs equal to or greater than, 40 µg/dL. But several subsequent studies reported how BLLs much lower than 40 µg/dL adversely impacted the health of children without causing overt symptoms (Matte and Falk 1997). Although lead poisoning can be the result of one high dose, the more common occurrence is due to prolonged exposure to small amounts. There is discrepancy

among researchers and doctors as to what BLL is safe in humans (Hess 2004). One study showed that declines in IQ can be seen in children with blood Pb concentrations below 10 µg/dL (Canfield et al. 2003). The following table: 3.1 from (Hess, 2004) shows results of recent studies linking potential health problems, to measured BLLs in exposed individuals.

Blood lead level	Possible effects
Below 10 µg/dL	<ul style="list-style-type: none"> • May result in small declines in intelligence
10 µg/dL or above	<ul style="list-style-type: none"> • Can impair the learning, growth, and development of young children
15–20 µg/dL	<ul style="list-style-type: none"> • Can harm the body's ability to transport oxygen • May reduce the amount of vitamin D in the body (necessary for the proper formation of bones and teeth)
Up to 25 µg/dL	<ul style="list-style-type: none"> • May affect intelligence (lower IQ) • May slow reaction time
Up to 70 µg/dL	<ul style="list-style-type: none"> • May cause any of the conditions listed above
Above 70 µg/dL	<p>May cause:</p> <ul style="list-style-type: none"> • Further damage to the brain and nervous system • <u>Anemia</u>, which is a decrease in the amount of oxygen-carrying substance in red blood cells • Stomach pains • Kidney damage • <u>Lead encephalopathy</u>, in which extremely high levels of lead cause the brain to swell, increasing pressure within the skull

Table 3.1. Health Risks Associated with Blood Lead Levels (BLLs). Obtained from (Hess, 2004)

Although the relationship between lead exposure and cognitive development has been a lively controversy among certain researchers (Burger and Gochfeld 2004), many studies agree that even at low doses, lead exposure can produce serious adverse effects on the central nervous system of children and that these effects can last for years.

In chapter II we presented our findings on effects of PCBs on levels of different NF in the brains of exposed rats. Results of these studies indicated that PCBs caused reduction in NGF, BDNF and GDNF in the brains of these rats. We speculated that such alterations of NF expression may cause altered neural processes and functions leading to compromised cognitive ability.

Since lead has also been shown to be neurotoxic, and to be associated with cognitive impairment in exposed children, we were curious about the possibility that NFs may be general targets for neurotoxins that affect cognition. The focus of this study therefore was to determine whether NF protein expression is altered in the brains of rats exposed to lead in their diet.

Methods and Materials

Lead Exposure

Six weeks post-weaning male Fischer 344 rats were exposed to 0 ppm, 50 ppm or 500 ppm of Pb^{2+} , in the form of lead acetate, in their drinking water for 30 or 90 days. The control animals were given distilled drinking water. Prior to treatment, all rat diet and drinking water were analyzed by ICP-MS for lead content. Rats were

housed at the Western Michigan University Animal Facility. The animals were treated according to the principles outlined in the NIH Guide for the Care and Use of Laboratory Animals. After each exposure period, rats were euthanized with CO₂; eight rats per treatment from the 30 days exposed group and 5 per treatment from the 90 days exposed group. Following euthanization, the animals were decapitated using a rat guillotine and their brains were removed within 3-4 minutes. Upon excision, the brains were hemisected and immediately dropped into 2-butyl butane cooled on dry ice. Excess fluid was decanted and the brains were stored at -80 degrees until processing for NF content.

Brain Processing

Each hemisphere to be used for NF quantitative analysis was weighed and then crushed in a polythene envelope by hammering on an aluminum plate cooled with dry ice. The resultant powder was put into a glass homogenizer and homogenized in 4-5 ml sample processing buffer (See appendix for buffer recipe) on wet ice to minimize protein degradation. Homogenate was transferred to centrifuge tubes and spun in an ultra centrifuge at 2400 rpm for 40 min at 4 °C. The supernatant was collected and stored at -20 °C.

Enzyme Linked Immunosorbent Assay (ELISA)

We followed the procedure outlined in the method section of chapter II for the ELISA of NGF and GDNF. Ninety-six well plates were used and each sample was

pipette in quadruplicate into columns of wells. A serial dilution ranging from 0 – 1000 pm/ml of NGF and GDNF proteins (obtained from *and R&D*) was prepared as a standard curve. Following color development, plates were read at 450 nm in a plate reader. Average values for samples were calculated and NF concentrations were extrapolated from the log standard curve.

Determination of Lead Exposure—Blood Lead Level (BLL)

Rats were exposed to lead acetate 0ppm 50ppm or 500ppm in their drinking water. The BLL of each exposed rat was determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

Results

30 Days Lead Exposure Results

The NGF and GDNF protein contents of the brains of the lead treated rats were determined by ELISA. A 1-way ANOVA was performed for comparison between groups. Results showed no significant difference between groups for NGF or GDNF protein. The data is given in the following table 3.2:

Treatment	NF in pg/mg brain	Mean	N	SE of mean
Control (0ppm Pb)	NGF	4.58	8	0.68
Low dose (50ppm Pb)	NGF	5.53	7	0.71
High dose (500 ppm Pb)	NGF	4.89	8	0.35
Control (0ppm Pb)	GDNF	4.52	8	0.93
Low dose (50ppm Pb)	GDNF	4.77	7	0.72
High dose (500 ppm Pb)	GDNF	3.52	8	0.36

Table 3.2. NF Protein Content in Brains of Rats Exposed to Lead for 30 Days

The BLLs of each of the 15 lead exposed animals were plotted against corresponding NF levels, obtained by ELISA. A linear regression was performed in order to determine if BLL was a reliable predictor of brain NF level. Results showed no significant relationship between BLLs and brain NGF expression (figure 3.1). There was however a significant correlation between BLLs and brain GDNF expression, with higher BLLs being associated with lower GDNF levels (figure 3.2). Control rats, which had insignificant BLLs, showed no relationship between BLLs and brain GDNF expression. The inclusion of the data from control animals in the linear regression did not change the outcome. These findings suggest that lead exposure may affect GDNF expression in the brains of exposed animals.

90 Days Lead Exposure Results

Data from the results of this experiment was analyzed as previously mentioned for the 30 days Pb treatment experiment. Results showed no significant difference between groups for NGF or GDNF protein. The data is given in the following table:

Treatment	NF in pg/mg brain	Mean	N	SE of mean
Control (0ppm Pb)	NGF	3.23	5	0.22
Low dose (50ppm Pb)	NGF	2.49	5	0.42
High dose (500 ppm Pb)	NGF	2.91	5	0.27
Control (0ppm Pb)	GDNF	4.20	5	1.63
Low dose (50ppm Pb)	GDNF	3.49	5	0.98
High dose (500 ppm Pb)	GDNF	3.57	5	0.64

Table 3.3. NF Protein Content in Brains of Rats Exposed to Lead for 90 Days

Regression analysis was also performed in order to determine if BLL was a reliable predictor of brain NGF level. Results showed no significant relationship between BLLs and NGF or GDNF protein levels in the brains of the animals treated with lead for 90 days. **(Data not shown)**

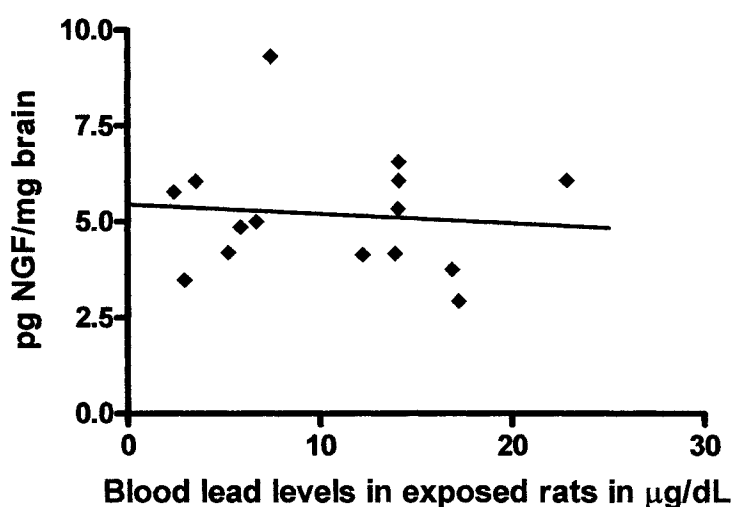


Figure 3.1. The Relationship between Blood Lead Levels and NGF Protein Expression in Brains of Rats Exposed to Lead in Their Drinking Water for 30 Days. The figure shows the BLLs from 15 exposed rats. Seven rats were exposed to 50ppm and 8 were exposed to 500ppm lead, respectively, in the form of lead acetate in drinking water. There was no significant relationship between BBLs and brain NGF expression. Statistical method: Linear regression, N = 15.

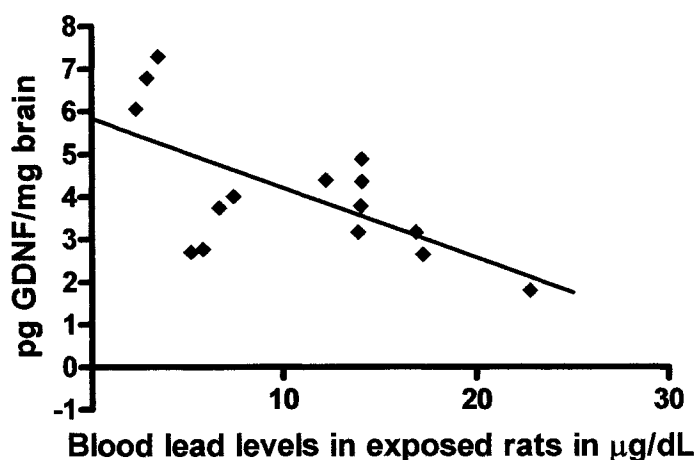


Figure 3.2. The Relationship between Blood Lead Levels and GDNF Protein Expression in Brains of Rats Exposed to Lead in Their Drinking Water for 30 Days. The figure shows the BLLs from 15 exposed rats: Seven rats exposed to 50ppm and 8 were exposed to 500ppm of lead in the form of lead acetate in drinking water. The data show a significant negative relationship between BLLs and brain GDNF expression. Statistical method: Linear regression followed by Pearson Product Moment Correlation: correlation coefficient = - 0.638, P = 0.0105, N = 15.

Discussion

The objective of this study was to investigate the effect of Pb, a known neurotoxin, on brain NF levels. We chose Pb because of the similarity in its effect on neuronal functions with those of PCBs, especially in children.

There was no difference in NF protein content in the brains of rats treated with 50ppm or 500ppm Pb compared to that in control rats. We believe this was due to the high variability in actual lead consumption, since the animals drank the lead treated water *ad libitum*. Comparing BLLs to brain GDNF levels on an individual basis however, showed that there was a significant negative relationship between BLLs and brain GDNF levels in exposed animals. The results for NGF under identical experimental procedure showed no correlation between BLLs and NGF protein content. We do not know any *in-vivo* mechanism by which either of these neurotoxins, PCBs or Pb, exert their effects on NF expression, yet the effect is similar. Exposure to lead seems to be associated with significant alteration in the expression of the neurotrophic factor GDNF. Interestingly, a recent study found that lead exposed rats in an impoverished environment had significantly decreased NF gene expression in the hippocampus (Schneider *et al.*, 2001 in: (Lidsky and Schneider 2003). We found decreased GDNF protein levels in brains of lead exposed rats.

We have been able to demonstrate that two different neurotoxins (PCBs and lead) which have been associated with adverse effects in neuronal functions also caused reductions in brain NFs. The importance of NFs to the nervous system and learning and memory, has been previously discussed in chapter 3. The relationship

between compromised cognition and significant interference of the homeostasis of NFs has been clearly established and documented by several studies. The mechanisms by which PCBs and lead affect NFs may be quite different, the fact that they do however, suggests that neurotoxins capable of affecting learning and memory may do so, in part, by interfering with the homeostasis of NFs thereby altering the influence these NFs have on processes like, synaptogenesis and neuroplasticity.

OVERVIEW OF CHAPTER III AND LINK TO CHAPTER IV

The previous studies investigated the effects of PCBs and lead (Two known neurotoxins) on the expression of NFs in brains of exposed animals. It was shown that PCBs altered the expression of NGF, BDNF and GDNF in the brains of rats that were exposed to A1254 in their diets. It was also shown that BLLs correlated with reduced GDNF protein levels in the brains of rats exposed to lead acetate in their drinking water.

Complementary *in-vitro* studies are very useful because they allow for greater manipulation and ease of determination of the treatment environment. The following manuscript (chapter IV), presents the *in-vitro* aspects of this dissertation. Presented are morphological and physiological responses to A1254 exposure of glial cells in culture.

The model of study was a glioma cell line called C6. These were exposed to 10ppm A1254 in culture for specific exposure periods. Medium from these cultures were assayed to determine levels of secreted NF protein. Total mRNA was extracted from control and A1254 exposed cells and analyzed by RT-PCR to determine effects on GDNF gene regulation. A possible signal pathway by which A1254 exerts its influence was also determined.

CHAPTER IV

EFFECTS OF POLYCHLORINATED BIPHENYLS (PCBs) ON EXPRESSION OF NEUROTROPHIC FACTORS IN C6 GLIAL CELLS IN CULTURE

Introduction

PCBs are persistent environmental toxic pollutants which accumulate in food chains in contaminated areas (ATSDR 1999, ATSDR 2000, Li and Hansen 1997). Commercial mixtures of PCBs called Aroclors, which are made up of several individual congeners, were manufactured in the United States for almost 50 years. They were produced for use as nonflammable dielectrics in electronic parts, lubricants, plasticizers in caulking compounds, paints, adhesives and sealants, vehicles for pesticide application, and pigment suspension agents in carbonless copy paper. (Korrick and Altshul 1998, McKinney and Walter 1994). One of these PCB mixtures, called Aroclor 1254 (A1254) was the most commonly sold and is the one most commonly found at contaminated sites. (Tilson and Kodavanti 1997)

PCBs are exceedingly toxic and have been shown to cause health problems in humans even at low levels. Human exposure to PCB can be through inhalation (respiration), digestion, or through the skin (dermal absorption) (EPA). They are lipophilic and are present in the fat of all human populations. A major concern has been that of prenatal exposure (trans-placenta) and postnatal exposure (from breast feeding) (Korrick et al.,1998) Epidemiological studies in humans have demonstrated a variety of nervous system deficits associated with PCB exposure, including

alterations in function of the peripheral nervous system (Chia and Chu 1984) and central nervous system (Jacobson and Jacobson 1997, Jacobson JL. et al. 1990). Impairments in learning and memory have been documented both in young (Grandjean et al. 2001) and older humans (Schantz et al. 2001) following environmental exposure to PCBs (Widholm et al. 2001). Developmental exposure to PCBs has been associated with cognitive deficits in children and experiments with rodents have revealed impairments in learning tasks that involve the hippocampus (Gilbert et al. 2000).

Although PCB exposure has been shown to alter a variety of intracellular signaling processes, including calcium signaling, protein kinase C signaling and neurotransmitter synthesis (Tilson and Kodavanti 1998), very little is known concerning the mechanism by which PCBs alter neural functions. The risk to the developing nervous system posed by PCBs is difficult to assess due to a general lack of information on mechanisms by which PCBs induce neurotoxicity (Howard et al. 2003).

The main objective of this study was to determine the effects of PCBs on the expression of an important class of proteins called neurotrophic factors (NFs) that are essential to the development and maintenance of the nervous system. These were studied in glial cells, a nervous system cell type that is known to synthesize them. NFs are important for the growth of neurons and for regulation of phenotypic expression during development. Several critical aspects of the ontogeny of neurons; determination of neuronal population, neurite branching and synaptogenesis, and

synaptic plasticity in adults are regulated by NF (McKay et al. 1999, Sariola and Saarma 2003). Most neurons require a sustained supply of these trophic factors throughout their lifetime for normal maintenance and for recovery from nervous system injury in the adult organism. (Catapano et al. 2001, Mattson et al. 2002)

PCBs have been shown to alter neurotrophic factor in whole animals. One study showed that exposure of *Xenopus laevis* tadpoles to A1254 at a concentration level as low as 300-400 ppb, caused significant decrease in NGF gene expression. Subsequent observable health effects were evident consequences (Jelaso et al. 2003).

We investigated the possible NF altering effect of A1254 in culture glial cells. Glial cells are a possible source of NF in the brain. There are 10 – 50 times as many glial cells within the brain as neurons. They make up 90% of brain cells. Until recently, glial cells have been thought to be important only as integral support cells of the nervous system, but this concept has changed since it has recently been shown that glial cells play a role in synapse formation. Astrocytes in particular are believed to actually ‘instruct’ neurons to make synapses (Miller 2003). It has been reported that although neurons can form synapses without glia, they may require them to form high numbers of efficient synapses, suggesting glia as a source for synaptogenesis-promoting signals (Mauch et al. 2001, Pfrieder 2002). Given these attributes and the fact that glial cells produce NFs, they are important models for preliminary studies involving toxic exposure. Results from this study may offer valuable incites towards unraveling a mechanism by which PCBs interfere with neural processes and functions.

Methods and Materials

Tissue Culture

For routine cell culturing we used a medium consisted of: Delbeco's Modified Eagles's Medium (DMEM) (*Sigma, St. Louis MO*) + 10% fetal bovine serum (FBS) (*Invitrogen*) + antibiotic-antimycotic (*Sigma St. Louis MO*), hitherto referred to as medium. For some experiments in which cultures had to be maintained for long periods in the same medium, the cells were grown in medium supplemented with extra glucose (3.5g/L). All medium or buffer solutions were initialized to pH 7.4.

Initial cultures of C6 glial cells (a rat glial cell-line), were established in sterile 100 mm dishes to provide cells for designed experiments. Cells were seeded at approximately 2×10^3 cells/cm² and incubated in a humidified chamber at a constant 5% CO₂ and 37 °C. When initial cultures proliferated to 60 – 70% confluence the cells were lifted with 10% trypsin/EDTA (*Invitrogen*) (in Calcium and Magnesium-free buffered Tyrodes) and then re-suspended in medium. For experiments, samples of the cell suspension were then used to seed 60mm sterile culture dishes at a cell density of approximately: 2×10^3 cells/cm², and then incubated to proliferate to 100% confluence.

Dose Response Treatment

C6 cells were cultured in medium containing A1254 (1ppm, 10ppm, 25ppm and 50ppm) + DMSO (0.01%, 0.1%, 0.25% and 0.5%) respectively, DMSO at the four latter listed concentrations, or in just medium. The cells were monitored visually with an inverted microscope immediately after treatment and then at 10 min, 30 min, 60 min and 24 hrs or longer post-treatment.

Live/Dead Cells Determination

PCB-treated and control C6 glial cells were incubated in 60mm culture dishes for 72 hours or 5 days. After two washes with warm phosphate buffered saline (PBS), plates were incubated for 30 minutes with 2 mM *Calcein AM* (a dye taken up only by live viable cells) and for 10 – 15 minutes with 4 μ M *ethidium homodimer-1* (EthD-1), a dye that enters only dead cells. Calcium AM is virtually non-fluorescence until it is cleaved by intracellular esterases in living cells to produce an intense uniform green fluorescence. EthD-1 is excluded by living cells but enters damaged membranes of dead cells and upon binding to DNA produces an intense red fluorescence color (*Molecular Probes*). Examination of the stained cells was done with a Nikon microscope utilizing fluorescent filters selected for excitation/emission at wavelengths 495/515 and 495/635 nm. Total live and dead cells were counted from each of 8 random visual fields from 2-3 plates per treatment, and averaged to determine the live-dead ratio.

Culture Treatment and Sampling

For all subsequent experiments, when initial C6 cell cultures attain 100% confluence, all medium was removed and plates were replenished with medium containing 10ppm A1254 + 0.1% DMSO, 0.1% DMSO or normal medium. Each 60 mm plate was loaded with 2 ml of medium. Three plates per treatment were cultured for each of the time points: 6, 24, 48 and 72 hours or 5 days. This configuration was chosen to allow each plate to be sampled only once for consistency of treatment concentration at all times. Medium samples were taken for all but the 5 day time point and stored at -20°C. The 5-day old cultures were collected for PCR analysis. Each experiment was repeated a minimum of 5 times. In a subset of experiments, cells from some time points were harvested to facilitate NF normalization per cell.

Culture and Treatment for Effect-on- Proliferation

C6 cells were cultured in 60mm culture dishes and treated as described previously described, one dish per treatment. All medium was withdrawn and cells were counted at 6 hours and 72 hours, those being the earliest and latest time-points of treated culture medium sampling. Cells were lifted with trypsin as previously described, resuspended and counted using a hemocytometer.

Culture, Treatment and Sampling for Cell Lysate NGF and GDNF Determination

C6 glial cells were grown in medium supplemented with 3.5g/L glucose and treated with 10ppm A1254 + 0.1% DMSO or 0.1% DMSO for 6 hrs and 72 hrs. Cells were trypsinized, washed in Ca^{++} Mg^{++} free Tyrodes and centrifuged at approximately 1200 rpm. The supernatant was removed and pellets were weighed. Cells were then re-suspended in Cell Lysing Buffer consisting of: 10% (Stock 10X prod. # 9803, from *Cell Signaling Technology*) + 10mM Benzethonium chloride, 200mM benzamidine, and 164 $\mu\text{L}/100\text{ml}$ aprotinin (*Sigma*). Cells were allowed to vibrate in lyses buffer for an hour and then were centrifuged at 24,000 rpm for 45 min. The supernatant was analyzed for NF by ELISA. NF concentrations were standardized to corresponding cell (pellets) weights.

Cell Fractionation for Intracellular PCB Binding Assay

A1254 treated C6 glial cells were washed with Ca^{++} Mg^{++} free Tyrodes, and their outer membranes were disrupted by vibration in mild lysate buffer as previously mentioned. Separation of sub-cellular components was done by *differential centrifugation* as follows: The resulting mixture of sub-cellular organelles was placed in a centrifuge and spun at 1000g for 10 min. The resulting pellet would normally consist of intact cells, cell membrane and nuclei. The supernatant was then decanted and re-spun at 10,000g for 20 minutes. Intermediate subcellular particles such as mitochondria and lysosomes would normally be in the sediment (pellet) at this stage. The supernatant was carefully decanted into a storage tube. This supernatant

would at this stage contain the tiniest and lightest particles such as, endoplasmic reticulum fragments, ribosomes and microsomes. The intracellular PCB analysis was performed using a modification of that used by Means (1998).

Enzyme-linked Immunosorbant Assay (ELISA)

Neurotrophic factor concentrations in all sampled mediums and supernatants were determined by ELISA. All NGF and GDNF proteins and antibodies were obtained from *R&D systems*.

Anti-NGF or anti-GDNF mono-clonal antibodies diluted in PBS, were added to 96 well ELISA plates, which were then sealed and allowed to incubate at room temperature overnight, for antibody adherence. Plates were then washed with PBS and blocked with 1% BSA for 1 hour. Blocker was washed from plates and NF protein standards and experimental samples were added and incubated for 2 hours while being gently swirled. The plates were washed (one 1 min. and two 5 min. washes) and a polyclonal antibody (conjugated to biotin), which was diluted in Tris Buffered Saline (TBS) + 0.1% Bovine Serum Albumin (BSA) and 0.3% Tween-20, was added. Plates were allowed to incubate for two hours. After washing, the plates were incubated for 20 min. with streptavidin HRP (*Pierce*). After washing off unbound HRP, the color reagent (tetramethyl benzidine in phosphate citrate buffer) was added. When color developed (generally, color indicator developed fully by 10 minutes of incubation), the reaction was stopped with 0.1 M phosphoric acid. The

plates were then scanned at 450 nm wavelength using a microplate scanning spectrophotometer (*Bio-Tek Instruments*).

For each assay, standards and samples were assayed in quadruplicate. For experiments that assayed samples from different exposure times, the protein values from three plates per treatment for each time point were averaged. NF protein concentrations were determined by interpolation from a log standard protein curve plotted from serial dilutions ranging from 0 – 1000pg/ml diluent (example: culture medium or lysing buffer solution).

Messenger RNA Isolation for RT-PCR

Messenger RNA (mRNA) was isolated from cultured C6 cells (n = 5) using a Poly (A) Pure mRNA isolation kit following the manufacturer's instructions (Ambion, Austin, TX.). Cells from the different treatment groups were collected, centrifuged and washed in Rnase-free PBS. For each culture treated for real-time RT-PCR analysis, 50 ng of yeast mRNA (Clontech, Palo Alto, CA) was added to the homogenate (Jelaso et al., 2003) to serve as an exogenous control/standard. Messenger RNA concentration of each treatment group was determined by measuring the absorbance at A260 using a GeneQuant Pro spectrophotometer (Amersham Pharmacia Biotech, Piscataway, NY) (Jelaso et al., 2003)

Real-Time RT – PCR

We determined GDNF gene expression in C6 cells utilizing the Taqman PCR method, a recently developed technique for the rapid and accurate quantification of gene-specific mRNA. This method takes advantage of the 5' nuclease activity of Taq polymerase to cleave a dual labeled fluorescent probe. There is a linked fluorescent reporter dye to the 5' end of the probe while a fluorescent quencher dye is attached to the 3' end (Jelaso et al., 2003). The gene sequence for Rat GDNF was obtained from GenBank. The probe and primers were purchased from Applied Biosystems and are as follows: Forward Primer (GACTTGGGTTTGGGCTACGAA), Reverse Primer (TCTCGGCCGCTTCACAG), and TaqMan TAMRA Probe (CAAGGAGGAACTGATCTTTCGATATTGTAGCGG).

Each RT-PCR reaction was performed in a 25µl final volume containing 1.25 U/µl of MultiScribe reverse transcriptase, forward and reverse primer, 200nM Taqman probe, 2X Master Mix without UNG (uracil *N*-glycosylase) and an equivalent concentration of mRNA template from each treatment group. All RT-PCR reagents were purchased from Applied Biosystems. All RT-PCR runs contained control reactions lacking template and reverse transcriptase. Four or five point standard curves were generated using serial dilutions of mRNA from untreated cells. All reactions were run in duplicate in 96-well plates. An Applied Biosystems Prism 7700 Sequence Detection System was used for amplification and fluorescence detection. Thermal cycling parameters—48°C, 30 min, 10 min at 95°C, and then 40 cycles of 95°C, for 15 sec, 60°C for 1 min. (Jelaso et al., 2003).

Treatment with PKC Inhibitor

To determine the role of Protein kinase C (PKC) in effects induced by A1254, a PKC inhibitor, 2-[1-(3-Dimethylaminopropyl) indol-3-yl]-3-(indol-3-yl) maleimide (bisindolylmaleimide or bis) (TOCRIS) was applied. PCB treated and control cells received 0.132 μM bis, which targets the α , $\beta 1$ and ϵ PKC isoforms, or 5.8 μM bis that also targets the ζ isoform but will also inhibit other kinases such as PKG and PKA. Con-currently, PCB treated and control cells were cultured free of the PKC inhibitor to serve as bis controls. Exposure time was 24 hours prior to medium sampling.

Results

Observable Effects of Aroclor 1254 on C6 Cells

Treatment with DMSO (0.01% – 0.5%) had no observable effect on cells monitored up to 24 hours. Cells exposed to 10 ppm A1254 + 0.1% DMSO or 20 ppm A1254 + 0.2% DMSO for 24 hours also had normal appearance and continued to proliferate. Treatment with 50 ppm A1254 + 0.5% DMSO however, had pronounced observable effects. Within 20 minutes of treatment, clear vacuoles were observed surrounding nuclei of cells. After 60 minutes there was an increase in the incidence of both clear and dark vacuoles as well as retraction of glial processes. Following 24 hours of treatment, all PCB exposed cells contained numerous clear vacuoles with

cells displaying membrane blebbing. By 72 hours the majority of cells treated with 50 ppm A1254 + 0.5% DMSO had lifted off the substrate.

Results from live/dead analysis of cells using Calcein (for live cell detection) and EthD-1 (for dead) showed, that cells exposed for up to 5 days to a lower dose of A1254, 10 ppm, exhibited no difference in cell mortality compared to controls. More than 95% of cells for all treatments were alive and viable (**Table 4.1**). The following photos show examples of visual fields from which the live/dead ratio was obtained (**Figure 4.1**).



Figure 4.1. Comparison of Effect of 10ppm A1254 and 0.1% DMSO (Vehicle) on Mortality of Glial Cells in Culture. Visual fields show live (green) and dead (red) C6 cells.

Having shown that neither A1254 (10ppm) + 0.1% DMSO nor 0.1% DMSO caused any difference in cell death compared to untreated controls, we proceeded to determine whether A1254 or DMSO at the same previously given dosage had an effect on cell proliferation. Cells were seeded at a constant density of $2 \times 10^3 / \text{cm}^2$ and allowed to grow to 100% confluence. Control and treated cells were incubated for

6 hrs or 3 days to test both acute and chronic effects. Cells were counted and the mean number of cells per plate per treatment were compared (**Table 4.2**). It was determined that neither PCB nor DMSO had a significant effect on cell population when compared to untreated control cells.

Results of Intracellular PCB Binding Analysis

In order to roughly determine the binding activity of A1254 once it had entered the cells, C6 glial cells were subjected to membrane disruption followed by separation of sub-cellular fractions by differential centrifugation. Intracellular PCB analysis was performed according to Means (1998). It was found that while there was insignificant DMSO associated with cell membranes and inclusions (indicating that most was dissolved in the cytosol), significant A1254 was found bound to cell fractions. Most of the A1254 was found in association with small cell-inclusions, which will include ribosomes, ER, microsomes etc. The second largest association was with larger cell inclusions, which will include nuclei and mitochondria. There was also some cell membrane-bound A1254 (**figure 4.2**). These results confirm that PCBs dissolved in DMSO, efficiently cross the cell membrane; and can bind to receptors on the nucleus and other organelles, with little or none remaining in the cytosol.

Effect of Aroclor 1254 on GDNF and NGF in C6 Cells

To test whether PCBs affect NF secretion by cultured glial cells, confluent cultures were exposed to 0.1% DMSO or A1254 (10 ppm) + 0.1% DMSO for 24

hours. Following medium sampling, the cells were counted and NF concentration was calculated per cell. A1254 induced a significant increase in the secretion of both NGF and GDNF by C6 cells in culture: One way ANOVA followed by Dunnettes Post Hoc, $P < 0.01$ (Figure 4.3).

Effect of Aroclor 1254 on GDNF in C6 Cells for Up to 72 Hours of Exposure

To determine the time course of effect on GDNF secretion, cells were treated and samples of conditioned medium were taken at various times following exposure. Cells were exposed to the treatments in medium supplemented with 3.5g/L glucose. GDNF content of conditioned cell culture medium was determined by ELISA. Medium sampled at 6hr from control cultures contained; 7.4 ± 0.1 pg GDNF ml^{-1} . In control cultures the concentration of GDNF decreased by 24 hr and then increased at 48 and 72 hours (Figure 4.4). Neither A1254 nor DMSO had an effect on the pattern of GDNF secretion by control cells. Comparison of GDNF quantities at each time point however, indicated that the A1254 treated cultures had significantly higher concentrations for all time points (One-way ANOVA followed by Dunnette Post HOC, $P < 0.05$) (Figure 4.4).

Aroclor 1254's Effect on Intracellular GDNF and NGF in C6 Glial Cells

To determine whether PCBs exert effects on intracellular stores of NGF and GDNF, cells were exposed to A1254 and content of NGF and GDNF in cell lysate was measured. The results showed that there was no difference in intracellular NGF

or GDNF levels in C6 glial cells treated with A1254, compared to untreated controls (Table 4.3).

RT—PCR Results

To determine whether exposure to PCBs affected GDNF gene expression, GDNF mRNA from treated and control C6 cells was measured utilizing real time RT-PCR. Total mRNA was extracted from cells exposed for 5 days to 0ppm A1254, 10 ppm A1254 + 0.1% DMSO or 0.1% DMSO, in medium supplemented with 3.5g/L glucose. A1254 treated cells expressed 1.149 ± 0.109 gene expression value (GEV) compared to 0.761 ± 0.082 GEV for DMSO treated control cells, a 51% increase (Figure 4.5).

Results of PKC Experiments

To determine whether the effects of A1254 on NF secretion, were exerted via signaling pathways involving PKC, experiments were performed in the presence of the PKC blocker bisindolylmaleimide (bis). Our results show that the effects of A1254 on GDNF secretion by C6 glial cells were abolished when cultured in the presence of the PKC inhibitor (bis) for 24 hours (Figure 4.6).

Discussion

The aim of this study was to investigate whether exposure to PCBs (Aroclor 1254) alters NF expression in C6 glial cells. It was important therefore to determine a physiologically working dosage that was not fatal to C6 glial cells. It had been shown

that certain congeners of PCBs kill cerebellar granule cells within 30 min (Tan et al. 2004). We exposed C6 cells to A1254 at concentrations ranging from 5ppm to 50ppm and found that at 50ppm, the cells showed signs of compromised health within 30 minutes. Cells exposed to A1254 at lower concentrations of 10 ppm or 20 ppm showed no visible signs of compromised health for up to several days after exposure. At 10 ppm, cells proliferated to confluence at the same rate as non-exposed cells and there was no visible difference between treated and untreated cultures. In addition our live/dead analysis at 10 ppm A1254 exposure revealed no difference in mortality of cells, compared to controls. The results showed that 10 ppm A1254 increased GDNF mRNA and protein, as well as NGF protein in cultured C6 cells. The results confirmed that PCB congeners included in A1254 preferentially binds small sub-cellular membranes and organelles such as endoplasmic reticulum (ER) and ribosomes. It was also determined that PCBs may exert their effect on GDNF in C6 cells via signaling pathways involving PKC.

An experimental exposure level of 10 ppm A1254 is not unreasonable when compared to environmental levels. One study showed that composite analysis of commercial whole fish collected from Lake Ontario found PCB levels of up to 5 ppm (Johnson 1997). Interestingly the FDA's previous PCB tolerance level was 5 ppm (Goldsmith 1979). Moreover, one year after the initial poisoning of the Yu-Cheng victims (an incident in 1979 where 2,000 people in Yu-Cheng, Taiwan, were exposed to PCBs from contaminated cooking oil), blood PCB concentrations were up to 1.2 ppm (Masuda 1985). Because PCBs are highly lipophilic, (Korrick et al., 1998) lipid

tissue burdens can be much higher. For example a Hudson River Report states—
 “While it is true that PCB levels have declined over the years, the average PCB concentrations for all species are still 644 ppm lipid-based PCBs (lpcb) in the Upper Hudson and 84 ppm lpcb in the Lower Hudson, with levels as high as 3285 ppm lpcb and 429 ppm lpcb in Upper and Lower Hudson River fish, respectively” (Clearwater, 2001).

Our results indicate that rat C6 glial cells exposed to 10 ppm A1254 secreted significantly higher concentrations of NGF and GDNF in the culture medium. We had hypothesized that PCBs would cause alterations in NF. We initially predicted however, that such alterations may have been a down regulation of expression, based on results reported in animal studies. Yet, the glial cells response to the PCBs’ insult we observed may not be surprising. The roles of glia include support and survival promotion. Studies have shown that microglial-conditioned media promote neuronal survival in culture due to neurotrophic factors they produce (Nagata et al. 1993). GDNF as well as NGF are survival factors for various neurons (Grimm et al. 1998, Zhou 1994). We saw that glial cells produce and secrete GDNF and NGF in the presence of A1254 *in vitro*. It has been shown that glial cells increase their production of NF in response to diffusible molecular signals they receive from the compromised but salvageable neurons they support (Aschner et al. 1999). Our results show similar response in the absence of such neuronal cues *in vitro*. This may be an initial default mode of operation for glial cells when a foreign toxin such as A1254 is sensed, or it may suggest that congeners within the A1254 mixture can mimic certain

neuronal chemical ‘help-cues’. PCBs have been reported to have the ability to directly mimic natural hormones, both as potentially potent and persistent agonists and antagonists (McKinney and Walter 1994).

We observed dynamic swings in the secretion of GDNF from C6 glial cells when they were maintained in the same culture for 72 hours, and medium was sampled at several different time points. As can be seen from the data of figure 4.2, this changing pattern of secretion with time was regardless of treatment. This is not unusual; a recent study reported that GDNF exhibits complex pattern of expression (Schaar et al., 1993 in (Caumont AS et al. 2006) and such expression is subject to tight temporal regulation (Caumont AS et al. 2006). Importantly though, despite the common temporal variation in expression, the A1254 exposed cells exhibited increase GDNF during all time points.

Measurements of intracellular NGF and GDNF from A1254 exposed and vehicle (DMSO) exposed cells showed there was no significant difference between them. This suggests that in order to maintain increased secretion without a significant reduction in intracellular concentration, there may be increased production of NF via gene up-regulation. Up-regulation of the GDNF gene was confirmed by Real Time RT- analysis. There was significant increase in GDNF mRNA in the A1254 exposed cells compared to controls. This further supported our finding of increase secreted GDNF protein in the medium of PCB exposed glial cells.

There is support for PCBs’ ability to regulate transcription by their interaction with aryl hydrocarbon receptor (AhR) (Beck 1998, Song and Freedman 2005).

Interestingly we found that A1254 PCBs was most abundantly associated with C6 cell small sub-cellular organelles such as microsomes, ribosomes and ER. The affinity of certain PCB congeners for small sub-cellular organelles was supported by other studies. One such study reported that PCBs have been demonstrated to mobilize microsomal Ca^{2+} by direct interaction with ryanodine receptors localized within muscle SR and neuronal ER (Wong and Pessah 1996).

We investigated a possible signaling pathway by which PCB may modify GDNF gene transcription. The activation of PKC by PCBs is supported by findings of (Grimm et al. 1998, Kodavanti and Tilson 2000). Interestingly, activation of PKC has been shown to up-regulate GDNF in glioblastoma cells (Grimm et al. 1998, Verity et al. 1998). Results of our study indicate that bisindolymaleimide inhibition of PKC (Coultrap et al. 1999, Martiny-Baron et al. 1993, Toullec et al. 1991) had a profound effect on GDNF production by C6 cells *in-vitro*. A1254's effect on GDNF was abolished in cells treated with the PKC inhibitor. Moreover, GDNF values for the groups exposed to the higher dosage of bis, was significantly lower across treatments compared to the lower dosage and negative groups. This suggests that PKC signaling pathways and/or possibly PKA or PKG are important for controlling GDNF expression in general.

It has been demonstrated in this study that PCBs affect NFs that are important components of the nervous system. Effects included: debilitating structural changes to glial cells *in vitro* at high dosage and significant changes in NF (NGF and GDNF) expression and secretion at lower doses. It is important to note that some of these

changes, namely changes in GDNF, are occurring at the gene level and that a pathway for such gene regulation was demonstrated. The relationship between the effects of PCBs on NFs and consequent effects on neuroplasticity is not known. The findings of this study therefore may have interesting implications when considering that numerous studies emphasize the importance of glial cells and neurotrophic factors to the nervous system, and their possible relationship to neural activities such as learning and memory.

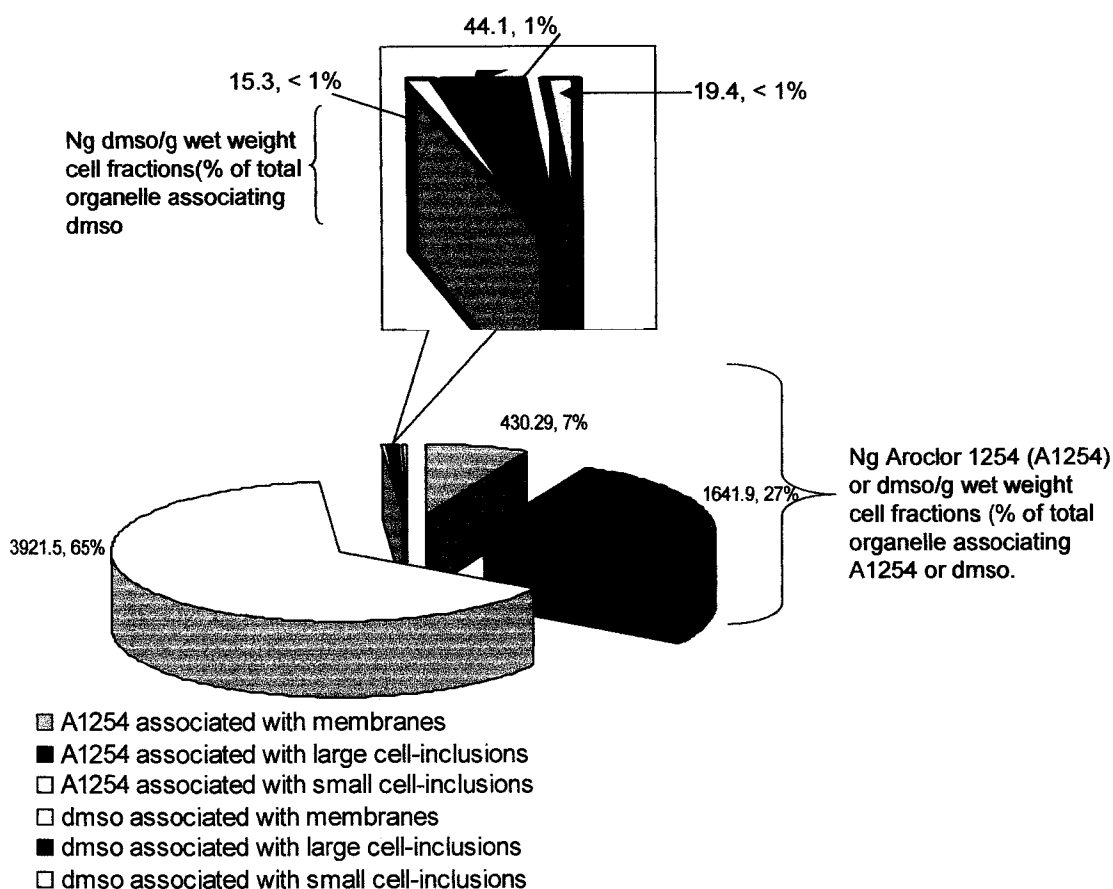


Figure: 4.2. Intracellular Distribution of A1254 and DMSO (Vehicle) in C6 Glial Cells after 24 Hours of Exposure (10ppm A1254 + 0.1% DMSO). The values indicate ng A1234 or DMSO/g wet weight of, and the percent of the total A1254 or DMSO associated with, cell membranes and inclusions. There was insignificant DMSO associated with cell membranes and inclusions, indicating that most DMSO was in the cytosol. A1254 was found bound to cell fractions. Most of the A1254 was bound to small cell-inclusions, which will include ribosomes, ER, and microsomes. The second largest association was with larger cell inclusions, which will include nuclei and mitochondria. These results show an association of PCBs with large organelles which may be consistent with binding to nuclear receptors.

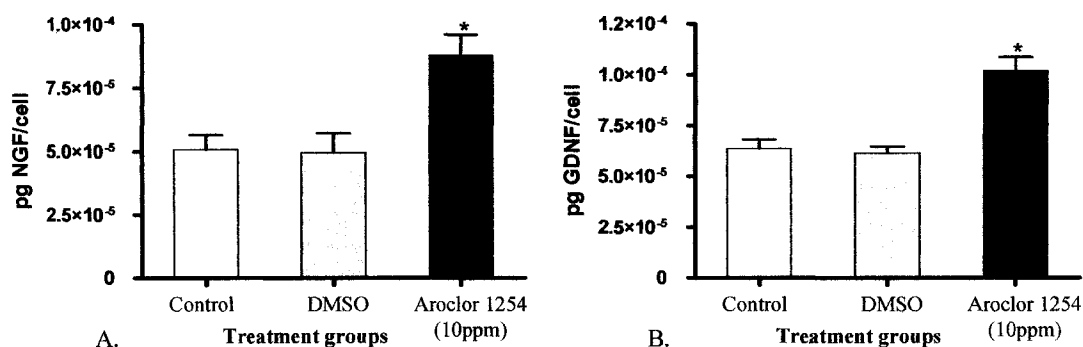


Figure: 4.3. Effects of A1254 on NGF and GDNF Secretion by C6 Glial Cells in Culture. Aroclor 1254 induced a significant increase in the secretion of NGF (A) and GDNF (B) in C6 cells. Cells were exposed to DMSO (0.1%), or DMSO (0.1%) + PCB (10ppm) for 24 hrs. Results were obtained by ELISA. The data shown represent the mean \pm S.E.M of values obtained from 5 experiments. The asterisk * represents significant difference from other groups. One-way ANOVA followed by Dunnette Post Hoc, $P < 0.01$

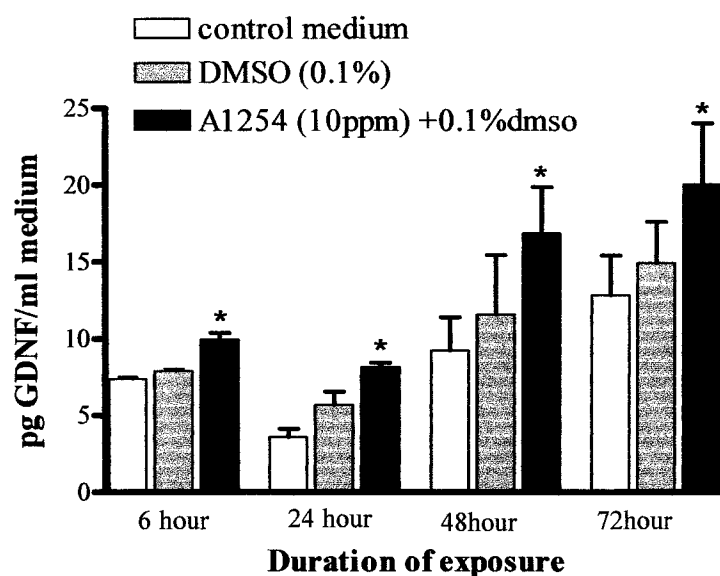


Figure 4.4. Effects of PCB on Secretion of GDNF from C6 Cells Glial Cells in Culture at 4 Different Periods of Exposure. Treatment of C6 glial cells with PCB caused significant increase in GDNF secretion for all time points. Cells cultured in medium supplemented with 3.5g/L glucose, were treated with DMSO (0.1%) or DMSO (0.1%) + PCB (10ppm). Data represents the mean \pm S.E.M. from 5 experiments. * = significant difference from other groups within the respective interval. One way ANOVA followed by Dunnett Post Hoc. $P < 0.050$

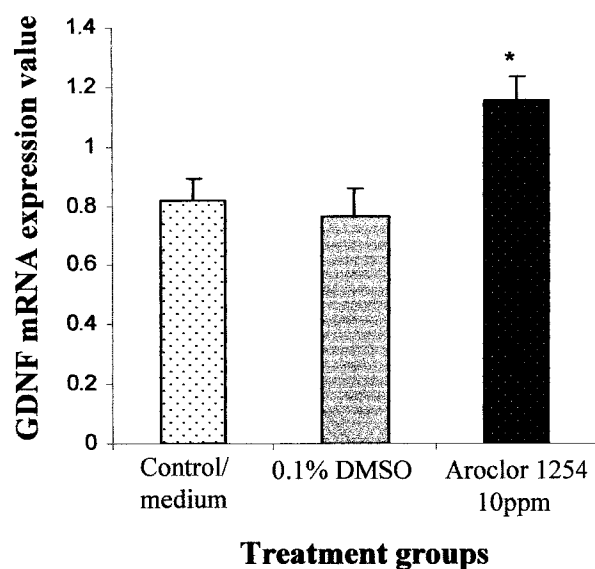


Figure 4.5. The Effect of PCB on GDNF Gene Regulation in Exposed Cultured C6 Glial Cells. Aroclor 1254 significantly increased the expression of GDNF mRNA in exposed C6 glial cells. Data shown represents the mean \pm S.E.M. of values obtained from 4 experiments. One-way ANOVA followed by Post Hoc. $P < 0.05$, * = significantly $>$ all other groups.

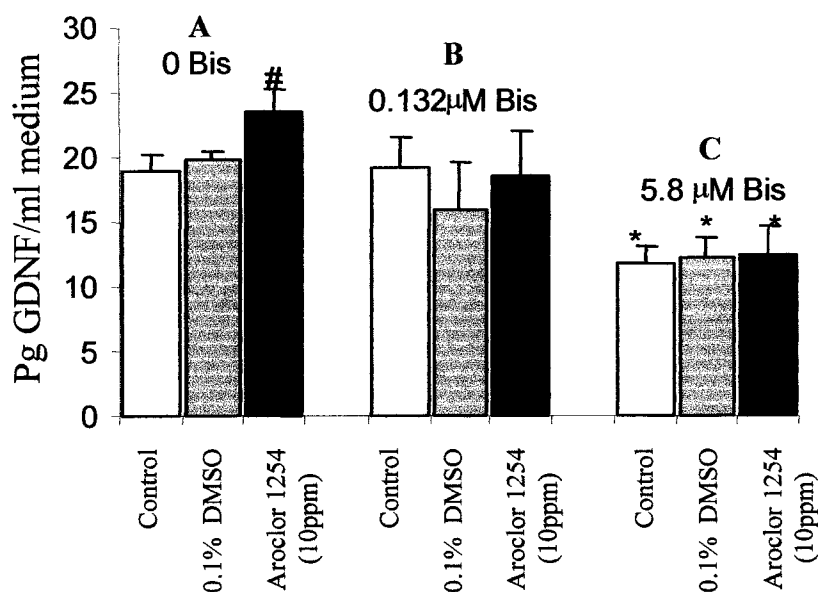


Figure 4.6. The Effect of Inhibited PKC on the Modification of GDNF by A1254 in Cultured C6 Glial Cells. The effect of Aroclor 1254 on GDNF secretion (# group A) was abolished when 0.132 μ M bisindolylmaleimide (bis) was added to the medium in which the cells were cultured for 24 hours (group B). At the much higher dosage--5.8 μ M bis, which at this concentration also blocks PKA and PKG, GDNF levels were significantly lower (* group C) for A 1254-treated as well as controls, compared to group A and B. These results: (1) confirm that PKC and possibly PKA & PKG activation, has strong effects on GDNF, (2) Suggests that A1254 induces its effect via pathways involving PKC. Statistical test: 1-way ANOVA followed by Dunnett Post Hoc, $P < 0.001$. # = sig. diff. within group, * sig. diff. from corresponding treatment in other groups.

SUM OF CELLS COUNTED FROM 8 RANDOM VISUAL FIELDS PER EXPERIMENT									
TREATMENT: Control (medium)				DMSO (0.1%)			Aroclor 1254 (10ppm)		
Experiment	dead	live	% live	dead	live	% live	dead	live	% live
1	18	306	94.444	35	625	94.935	76	1529	95.265
2	37	1178	96.955	24	765	96.958	15	533	97.263
3	11	1475	99.26	22	1698	98.721	10	1257	99.211
Ave. 96.886				Ave. 96.871			Ave. 97.246		

Table 4.1. Comparison of the Live/Dead Ratio of C6 Cells between Treatments.

Results show no significant difference in live/dead ratio between treatments and > 95% live cells were maintained for all groups. Ratio obtained by treatment with Calciem (for live cell detection) and EthD-1 (for dead). Total live and dead cells were counted from each of 8 random visual fields from 2 -3 plates per treatment and averaged.

Treatment	Exposure time	Mean \pm S.E.	N	Treated compared to Control
Aroclor 1254 (10ppm)+ 0.1%DMSO	6 hours	$5 \times 10^5 \pm 2.8 \times 10^4$	3	P = 0.629
	3 days	$1.4 \times 10^6 \pm 3.4 \times 10^5$		P = 0.826
	6 hours	$4.5 \times 10^5 \pm 4.9 \times 10^4$	3	P = 0.475
	3 days	$1.0 \times 10^6 \pm 3.4 \times 10^4$		P = 0.600
CONTROL/ Regular medium	6 hours	$5.9 \times 10^5 \pm 1.2 \times 10^5$	3	
	3 days	$1.3 \times 10^6 \pm 1.2 \times 10^5$		

Table 4.2. Effects of Treatment (10ppm Aroclor or 0.1% DMSO) on Proliferation of C6 Glial Cells. Cell counts indicated no significant difference between treatments for either short (6 hr) or long term (72 hr) exposure time. Cells were seeded at a density of 2.0×10^3 /ml, treated and allowed to grow for 6 or 72 hours. Statistical analysis: 1-way ANOVA and Post Hoc, $P < 0.05$.

	PCB (6hr)	DMSO (6hr)	PCB (72hr)	DMSO (72 hr)
GDNF:	$0.11 \pm 1.4 \times 10^{-2}$	$0.14 \pm 2.9 \times 10^{-4}$	0.19 ± 0.14	0.24 ± 0.17
NGF	$0.07 \pm 4.3 \times 10^{-3}$	$0.087 \pm 5.4 \times 10^{-3}$	$0.17 \pm 6.4 \times 10^{-2}$	$0.185 \pm 6.8 \times 10^{-2}$

Table 4.3 Effects of Aroclor 1254 on Intracellular Concentrations of NGF and GDNF in C6 Glial Cells in Culture. ELISA was used to determine NGF and GDNF levels, and neither NGF nor GDNF levels were significantly changed by treatment with Aroclor 1254 (10ppm) + 0.1% DMSO, compared to treatment with 0.1% DMSO alone (Student t-test, $P < 0.05$)

CHAPTER V

GLOBAL DISCUSSION

The central drive of the studies of this dissertation were to investigate whether neurotoxins such as PCBs and lead, can be shown to alter the expression of neurotrophic factors in brains of exposed animals and or exposed nervous system cells. These investigations were prompted by the hypothesis--PCBs affect learning and memory by altering neurotrophic factor expression and/or effects. The rationale for this line of thinking was based on the many studies supporting the importance of neurotrophic factors to the genesis, development and maintenance of the nervous system and components thereof. The supporting literature also established the roles these factors play in neuronal modifications believed to be the processes required for learning and memory to occur. It must be understood that except for one collaborative study in which descriptive correlations were made with spatial learning and memory (L&M) data, this primary study did not investigate L&M. The references to, and suggestions about L&M therefore are mostly based on the assumption that it is generally accepted, that extrinsically induced alterations in neurotrophic factors are associated with disturbances in cognitive performance.

Most of the reported associations of neurotrophic factors like NGF and BDNF with deficits in learning and memory, were those of lowered levels, and in some studies the result of NF gene knockouts. It was not surprising therefore, that our *in-vivo* studies of Aroclor 1254 exposed rats revealed, in many cases, significant reduction of NFs in their brains. It must be noted however, that our argument is about

significant alterations in NF protein expression that may in turn affect neuronal processes involved in cognition. That means any significant deviation from normal or control NF protein levels.

The studies described in Chapter 2 examined NF protein levels in brains from rats exposed to A1254 in their diet. It is significant that in most of these experiments the recurring evidence is that A1254 caused reduced NF expression. It is also significant that it was not just one NF, but we demonstrated changes in NGF, BDNF and GDNF.

NF alterations were observed following by both short term and chronic exposure, although duration of exposure was shown to influence response. From observation of control animals, we were able to show that young animals contained significantly higher levels of GDNF in their brains than mature animals. This may be expected since neurological and physiological changes in the brain can be considered to be more dynamic in the growing youngster than at maturity. Given the functions described for GDNF, it may be seen why its higher levels are necessary in younger animals.

Based on our experiments however, both *in-vivo* and *in-vitro*, we observed that expression of NFs in general seemed to be quite dynamic, with significant changes occurring in control as well as treated animals, even within a few hours (as seen *in-vitro*). These expression swings seem to continue even during response to toxic interference. It is very unlikely that these fluctuations are random. On the contrary, it would be expected that in a living system these fluctuations must be under

homeostatic control. Changes induced by extrinsic interference to the normal intrinsic dynamics of the system therefore, would most likely be detrimental.

If a mechanism by which PCBs affect cognitive processes is by indirectly disturbing NF homeostasis, then the results demonstrated in our studies will be consistent with our hypothesis. The interpretation of these outcomes in terms of learning and memory however, would be interesting if we can demonstrate--PCB exposure, NF reduction and cognitive deficit all in the same group of animals. Evidence of this combination of events was demonstrated in our collaborative work with Paris-Larson and her mentor—Baker (Paris-Larson, 2004). In that study, A1254 exposed animals, on an average exhibited higher frequencies of mistakes in spatial learning and memory tests and also had average lower levels of NGF, BDNF and GDNF in their brains.

Armed with the convincing evidence of PCBs' effect on NF expression in rat brains a logical next step was to investigate the response of one or more NF to another known neurotoxin. There are several other environmental pollutants that are known neurotoxins. Much is known about Pb and its detriment to neural processes and functions, including learning and memory impairment; especially in children. Pb was therefore the next chosen candidate of investigation. We became curious about the possibility, that NFs may be general targets for neurotoxins that affect cognition. It may have been just a stroke of good luck or 'instinct' in that the first choice of another neurotoxin turned out to be one exhibiting negative correlation to a NF protein level in rat brains. The effect of Pb on brain NGF levels was inconclusive but

its effect on GDNF was significant. GDNF is integral in the support, protection and maintenance of mid-brain dopaminergic neurons (Barnett et al. 2002, Simon et al. 2001). The finding that Pb affects the expression of GDNF maybe important when considering Parkinson's disease, a neural dysfunction associated with lost or compromised midbrain dopaminergic neurons. (Villadiego et al. 2005).

In the studies on C6 glial cells, we took advantage of the ease of manipulation offered by *in-vitro* systems. Here the results were convincing and totally opposite. C6 glial cells exposed to 10ppm A1254 + 0.1% DMSO showed significant increase in NGF and GDNF expression. Moreover, GDNF mRNA was shown to be upregulated in exposed cells—indicating that the increase in GDNF protein was due to gene upregulation. The fact that PCBs are able to upset the homeostasis of regulatory proteins by gene regulation is significant.

The interesting twist of this story (as it may appear), is that NF expression was increased by A1254 in glial cells *in-vitro* while they were decreased in brains of exposed rats. The apparent contradiction should not be surprising and there are several possible explanations for it. The roles of glia include support and survival promotion. Studies have shown that microglial-conditioned media promote neuronal survival in culture due to neurotrophic factors they produce (Nagata et al., 1993). GDNF as well as NGF are survival factors for various neurons (Grimm et al., 1998; Zhou et al., 1994). It has been shown that glial cells increase their production of NF in response to diffusible molecular signals they receive from the compromised but salvageable neurons they support (Aschner et al., 1999). Our results show similar

response in the absence of such neuronal cues *in vitro*. This may be an initial default mode of operation for glial cells when a foreign toxin such as PCB is sensed, or it may suggest that congeners within the Aroclor 1254 mixture can mimic certain ‘early’ neuronal chemical ‘help-cues’. PCBs have been reported to have the ability to directly mimic natural hormones, both as potentially potent and persistent agonists and antagonists (McKinney et al., 1994).

It is possible that the responses we see in culture are early responses that may change with chronic exposure or change in method of exposure. In-fact, in experiments where A1254 inoculated medium was replaced every 24 hours with fresh inoculated medium (mimicking daily animal exposure), the trend we observed with the constant medium over time, was abolished. The longest duration of exposure in culture was 72 hours, and according to our time-response experiments (chapter IV, figure 4.4), the largest change (increase) in GDNF by cultured A1254 exposed C6 cells was between the 24th, and 48th hour interval. The shortest exposure for the animals whose brains we analyzed for NF was 7 days (168 hours).

The conditions in culture are far from being equal to that in the brains. But even if a similar to *in-vitro* condition in terms of NF expression, existed in the brain during the very early stages of exposure, it may have been reversed by the time of our analysis, due to the overwhelming or exhausting of the NF protective mechanisms. It could even be a time when glial cells switch modes in terms of their secretory products. Researchers suggest that *in vivo*, either of the two glial ‘modes’; neurotoxic, to destroy severely damaged neurons or NF-producing to promote

survival of salvageable ones, is triggered by diffusible molecular signals they receive from the neurons (Aschner et al., 1999).

In reality, the conditions for glia in culture as apposed to that in the brains (*in-vivo*) are too dissimilar for reasonable expectation of similar results in response to a toxin. Apart from myriads of compounds in the *in-vivo* tissue fluids, there are also several tissue and cell types, and these are in constant dynamic changes, as appose to a few isolated cells of a pure culture in an essentially static environment, where secreted and excreted products are accumulative. Furthermore the response we observed *in-vitro* was consistent with accounts reported. One study suggested that once glial cells are isolated and maintained *in vitro*, the cells exist in a state of near-maximal activation, profusely secreting several factors including cytokines and neurotrophins (Aschner et al., 1999).

Referring to the same 7-days A1254 exposure *in-vivo* experiments of chapter 2, the NF response was dose dependent. There was significantly greater effect of 50ppm A1254 compared to 10ppm. We don't know though, how much of the A1254 actually crossed the blood brain barrier and eventually entered brain tissue. The animals were exposed to A1254 in their food. Here again there is room for significant difference in dose response when compared to treatment in cell culture.

A consequence of animal exposure to PCBs in their diet is the potential for the formation of reactive metabolites. For example, it has been shown that PCBs are metabolized to mono and dihydroxylated compounds by cytochromes P450. Dihydroxy-PCBs can potentially be oxidized to the corresponding quinones

(Srinivasan et al. 2001). Metabolites such as quinones create a variety of biological threats *in vivo*—including acute cytotoxicity and immunotoxicity (Bolton et al. 2000). Clearly, PCB metabolites have been implicated in the reduction of endogenously produced proteins such as hormones in rats. It has been reported that several hepatic methylsulfonyl-PCB metabolites reduce thyroid hormone levels in rats (Kato et al. 2003). PCB metabolites like those produce by liver activity may have effects on NF *in-vivo*, these effects would be absent in culture.

There are 9 glial cells for every one neuron and it's worth noting that these two cell types can respond differentially to the same substance. It has been shown that GDNF is differentially regulated in neuroblastoma and glioblastoma cell-lines exposed to the same regulating factor (Verity et al., 1999). The data obtained from whole brains therefore can be the resultant of differential responses from the several tissue subtypes to A1254 and or metabolites.

The departure from normal NF levels was in opposite direction when *in-vivo* to *in-vitro* comparison of PCB effect was made. This does not diminish the prediction outlined in the hypothesis of this study. The common effect was significant disruption of the homeostasis of the NFs that were tested.

Substantial work was done on rat brain sections in this study. Several experiments involving immunochemistry were conducted on cryostat sections cut predominantly through hippocampal regions. The hippocampal region was the region of choice because of its frequent association with compromised learning and memory as consequence of its altered NF expression.

Unfortunately these experiments were not presented in the main study because of difficulties with inconsistent staining for NFs. Preliminary observations however, seem to suggest that A1254 can affect the distribution and level of NGF in rat hippocampal regions. In one experiment, we saw differential staining for NGF protein in brain sections from control animals compared to animals exposed to 50ppm A1254. In the section from a control animal a pattern of specific staining of cells was observed. No specific staining was observed in a similar section from a control section to which no primary NGF antibody was added. Staining of cells in the section from the 50ppm A1254 exposed animals was visibly minimal and the distribution looked random. A summary of Immunohistochemistry procedures and preliminary data are given in the appendix.

We have been able to demonstrate that PCBs significantly alter the homeostasis of neurotrophic factors, both *in-vivo* and *in-vitro*. We also showed that blood lead levels correlated negatively with brain GDNF protein levels in exposed rats. We demonstrated an increase in both GDNF mRNA and protein in A1254 exposed glial cells and also discovered a role for PKC in the signal pathway through which A1254 affected GDNF. We showed down regulation of NGF, BDNF and GDNF in brains of A1254 exposed rats. Although we did not investigate effects on NF mRNA in the experimental brains directly, our GDNF level findings from the lead exposed animals were supported by a recent study which reported reduction of NF mRNA in hippocampus of rats exposed to lead (Schneider et al., 2001 in: (Lidsky and Schneider, 2003). We mentioned numerous studies claiming deficits in learning

and memory as a consequence of altered NF expression in the CNS (especially brain). From our collaborative study, there was evidence of correlation between reduced NF and findings of reduced spatial learning and memory in rats exposed to PCB. Based on all this, we speculate that neurotoxins such as PCBs and lead may affect cognition by their ability to disrupt the homeostasis of NFs important in the process of learning and memory.

Future studies: Further in-vitro studies may include the co-culture of neuronal cells with glial cells (Park et al. 2001). At the time when synaptic connections are made between the two types of cells, the co-culture can then be exposed to the A1254. The rational is to try to mimic to some extent, the in-vivo situation where glia are grown in the presence of neurons. Subsequent analysis of NF levels of the co-cultures can then be compared to the present study's findings. In addition, ICC can be done on the co-culture by using for example, the glial fibrillary acidic protein (GFAP) marker to identify the glia or nestin, a neurofilament protein to distinguish the neuronal cells. Effects on NF receptors of the cultures may then be observed by staining against--for example; GFR α and Trk for GDNF and NGF respectively.

More experiments such as RT-PCR are suggested to determine mRNA changes for other NFs other than GDNF, NGF and BDNF. Further pharmacological experiments can be done to elucidate a more complete pathway by which A1254 may be causing effect on GDNF levels in C6 glial cells. And also to determine if a common pathway is involved for other NFs. It would also be interesting to determine

if the A1254 effect is still present at lower A1254 concentrations, say for example: 5ppm or 1ppm.

We suggest further studies in the in-vivo investigations involving A1254 exposed rats. In this study, the brains on which immunohistochemistry was done were taken from non-perfused animals. This choice was due to the fact that other tissues from the experimental animals were needed by our collaborators. These tissues would have been ruined (for their purposes) by perfusion. Staining for neurotrophic factors in brain sections is inherently difficult due to their very low tissue levels and complications with background staining. Here is a direct quote from a renowned and well established corporation in the business of Biotechnology: ***'The neurotrophins have proved difficult to localize which may be due to masking by, for example, their association with Trk receptors or very low concentrations'*** (CHEMICON INTERNATIONAL). Staining of sections from perfused animals is generally the more common choice presumably because of greater chances of success. The suggestion therefore is for the exposure of animals to A1254 with subsequent perfusion to prefix brain tissue, before immunohistochemistry for NFs. We suggest also that blood PCB burden be assayed for each animal for correlation with brain NF findings.

A greater number of correlative studies where A1254 or Lead exposed animals with low brain NFs (due to the exposure) can be analyzed for learning and memory (using different L&M analysis), may serve to verify or reinforce our findings in further support of our objectives and suggestions.

Appendix A

Preliminary Immunohistochemical Studies on Rat Brain Sections

Using Immunohistochemistry to Determine Effects of Aroclor 1254 on Neurotrophic Factor Expression in the Brains of Exposed Rats

This study was intended to complement the study in chapter 2 that involved the A1254 exposed rats. This was an attempt to determine whether there were localized differences in NF occurrence in regions like the cortex and hippocampus. The idea was to stain the brain sections for NGF, BDNF and GDNF and compare distribution and staining intensity among the various treatments. The following is an outline of the brain sectioning and staining procedure used in this study and a preliminary result obtained.

Rat Brain Sectioning

The complementary (with respect to those used for ELISA) hemispheres of brains were sectioned on a cryostat (*Leica CM 3050*) held at -18 °C. Slices were sectioned at 12 – 16µm for regular light microscopy and 40 – 60µm for fluorescence staining and confocal microscopy. The brains were sectioned in such a way as to obtain slices coronally through the hippocampus region. The sections were allowed to adhere to cold slides (*Fisher Scientific*) prior to fixing.

Immunohistochemistry: (Light microscopy)

Microscope slides with brain sections (14 µm) from each of the two treatment groups and from controls were fixed in ice-cold methanol or acetone for 5-10

minutes. After 3 washes in PBS, staining procedures were followed according to protocols adapted from VECTASTAIN (Vector Laboratories, Inc.) with minor modifications. For GDNF, we used a rabbit anti GDNF polyclonal primary antibody (D-20, from Santa Cruz Biotechnology, Inc). The secondary antibody was a mouse anti-rabbit conjugated to biotin, followed by streptavidin conjugated to alkaline phosphatase. The protocol followed for NGF was the same except that we use a rabbit anti-NGF polyclonal antibody from CHEMICON International, Inc.

Fluorescence

Slides with brain sections (40 μ m) from each of the two treatment groups and from controls were fixed in Zamboni's fixative for 10-15 minutes. The sections were washed in PBS (pH 7.4) and were then allowed to air dry for 1 hour. The slides were then blocked with 3-5% BSA in PBS or 3% serum from the same strain of animal from which the secondary antibodies were obtained, in 1 % BSA-PBS. Without washing, excess blocking liquid was blotted away and except for the negative control slides (which were incubated with blocker instead of 1^o antibody) all other slides were incubated with primary antibody at 4 degrees Celsius for a minimum of 24 hours (on occasions we incubated for up to 4 days). The slides were washed 3 times and then incubated for 1 hour with a fluorescence labeled secondary antibody, an Alexafluor (molecular probes). This final incubation was followed by gentle rinsing and the slides were cover-slipped with 50% glycerol solution and sealed with nail polish or rubber cement. The sections were then viewed using a confocal microscope.

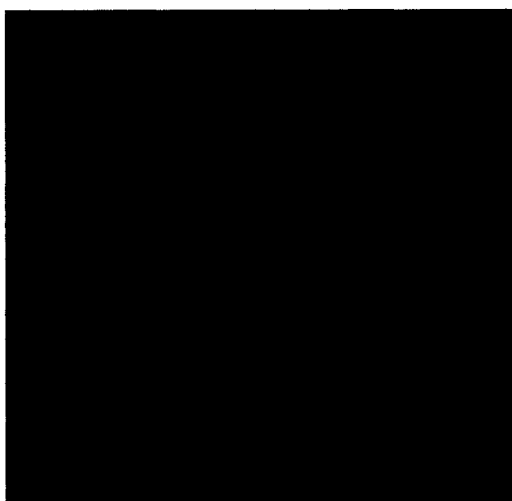
Results

The following photos were obtained from a section of the rat hippocampus bound with anti-NGF monoclonal antibody (R&D). Panels A, B and C display images captured from similar locations in the hippocampal region of the brain. Panel A shows what appears to be specific staining for NGF in cells of the hippocampus. Panel B shows a field taken from hippocampus of a rat exposed to 50ppm A1254. In panel B there appears to be no specific staining for NGF. Panel C shows a section similar to A but to which no primary antibody against NGF was added. These preliminary findings suggest that A1254 exposed rat brains show little positive staining for NGF. Unfortunately, we were unable to repeat these results.

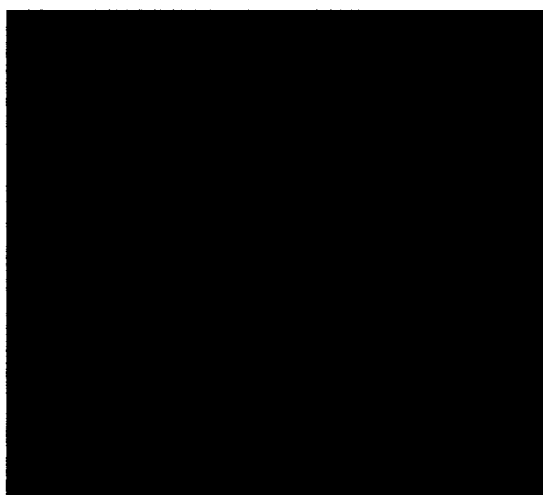


A

Anti-NGF Staining in Brain Section from Control Rats: Software generated 8-bit image @ 20X-- photographed using the Metamorph software. Viewed with a Zeiss microscope fitted with fluorescence filters.

**B**

No Primary (1^o) Control--Anti-NGF Stain in Brain Sections from a Non-Exposed (Negative Control) Rat. Software generated 8-bit image @ 20X-- photographed using the Metamorph software. Viewed with a Zeiss microscope fitted with fluorescence filters.



C

Anti-NGF Stain in Brain Section from a Rat Exposed to A1254 at a Dose of 50ppm. Software generated 8-bit image @ 20X-- photographed using the Metamorph software. Viewed with a Zeiss microscope fitted with fluorescence filters.

Appendix B
Recipes of Solutions Used

(1) Calcium and Magnesium Free Tyrodes:

1. Add 600 ml of cell culture water to a 1 liter beaker.
2. Add 100 ml Stock A.
3. Add 2.2 g/liter of glucose (d-glucose).
4. Add 10 ml HEPES solution (1 M pH=7.4).
5. Add 5 ml of Antibiotic/Antimycotic solution.
6. Bring volume up to approximately 950 ml.
7. Set pH to 7.4.
8. Bring volume up to 1 liter (using volumetric flask).
9. Filter sterilize (0.2 micron filter) into sterile bottle.
10. Store at 4° C.

Stock A Solution.

1. Add the following to a 1000 ml beaker.
 - a) NaCl 75.03 g/liter.
 - b) KCl 2.24 g/liter.
 - c) NaHCO₃ 17.22 g/liter.
- d) Bring to final volume of 1000 ml using cell culture water.
- e) Filter sterilize and store at 4° C.

(2) Phosphate Buffered Saline (PBS).

Final desired volume: 1 liter

1. Initial volume of distilled water 800ml
2. Add NaCl. 9 g
3. Add NaH₂PO₄ (monobasic). 2.4 g
4. Add Na₂HPO₄ (dibasic). 11.4 g
5. Mix well.
7. Adjust pH to 7.4 using NaOH.
8. Adjust to final volume with distilled water.

(3) Sample Processing Buffer (SPB): Wash buffer (PBS + 0.05% tween) + 0.4 M NaCl + 2 mM EDTA, 0.1 mM benzethonium chloride, 2 mM benzamidine, 20 KIU/ml aprotinin, 0.5% BSA.

In wash buffer add 1:100 dilutions of each of the following stock solutions.

- 200 mM EDTA (7.44 g/100 ml WB, pH 8 to dissociate, then set to pH 7.4, store @ 25° C.

- 10 mM benzethonium chloride (0.448 g/100 ml WB, store @ 25° C.

- 200 mM benzamidine (3.13 g/100 ml WB, store @ 25° C.

Then,

Add 1.42 g NaCl per 100 ml wash buffer.

Add 500 mg BSA per 100 ml of sample buffer.

Add aprotinin (164 µl/100 ml sample buffer if using Sigma A-6012).

(4) Zamboni's fixative recipe

To make 100 ml:

- 1 25 ml 8% paraformaldehyde
- 2 0.15 ml of picric acid
- 3 Fill to 100 ml with 0.1 M PB
- 4 pH to 7.3-7.4

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