EFFECTS OF PCBS AND PBDES ON RESPONSE INHIBITION 
AND DOPAMINERGIC SIGNALING

BY

PAUL EUBIG

DISSERTATION

Submitted in partial fulfillment of the requirements 
for the degree of Doctor of Philosophy in VMS – Comparative Biosciences 
in the Graduate College of the 
University of Illinois at Urbana-Champaign, 2013

Urbana, Illinois

Doctoral Committee:

Professor Susan L. Schantz, Chair
Professor Duncan C. Ferguson
Professor Jodi A. Flaws
Associate Professor Joshua M. Gulley
Abstract

Polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) are persistent environmental contaminants that share physical and neurotoxic properties. Developmental PCB exposure has been associated with impulsive behavior in laboratory species and human cohorts, while little is known about whether PBDEs have similar effects. Both PCBs and PBDEs have been shown to perturb dopaminergic neurotransmission. The behavioral and neurochemical effects of PCBs parallel changes seen in children with attention deficit hyperactivity disorder (ADHD). The aims of this research were to further investigate the effects of PCBs and PBDEs on inhibitory control and dopaminergic signaling using a rodent model. The findings were anticipated to provide insight into the role that these environmental contaminants have on mechanisms and manifestations of impulsive behavior and provide insights as to whether exposure to PCBs and/or PBDEs may be a contributing factor in ADHD.

Female Long Evans rats received daily oral doses of either 3 or 6 mg/kg of an environmentally-relevant PCB mixture, 11.4 or 22.8 mg/kg of the commercial PBDE solution DE-71, or corn oil vehicle (i.e., 5 treatment groups) from 4 weeks prior to breeding through weaning at postnatal day (PND) 21. Starting on PND 90, male-female pairs of offspring from each litter were tested on either a task that assesses impulsive action, differential reinforcement of low rates of responding (DRL) task, or a task that assesses impulsive choice, delay discounting (DD). After subjects were trained on either task, they received drug challenges with the D1/D2 receptor antagonist flupenthixol (FLU), amphetamine (AMPH), and then FLU/AMPH together. It was
hypothesized that PCB or PBDE exposure would result in impulsive performance on both tasks, while during the drug trials there would be shifts in the dose-response curves so that performance on both tasks would be improved by AMPH and impaired by FLU to a greater extent than in controls. Tissue punches from 4 brain regions important for DRL and DD performance were collected at PND 90 from littermates of those being behaviorally tested. Western blots were performed on the punches to examine for differences in expression of dopamine transporter (DAT) between treatment groups. It was hypothesized that DAT expression would be reduced with PCB or PBDE exposure.

PCB/PBDE-related effects on performance were not found on either the DRL or DD task, nor did the drug trials differentially alter the primary performance measures of impulsive behavior in treated groups relative to controls. The lack of effects of PCBs on DRL performance is in contrast to previous studies. However, some reasons why positive findings may have been obscured included potential insensitivity of the versions of the tasks used, particularly DD, in eliciting differences in impulsive behavior, and experimental manipulations in diet, contaminant dose, strain of rat or other unrecognized factors. In addition to the behavioral findings, DAT expression did not differ between treated and control subjects. This differs from the findings in previous in vivo and in vitro studies. However, DAT expression was examined 70 days after contaminant exposure ceased in the current study versus immediately after exposure ceased in prior studies. Thus, any possible changes in DAT expression could have resolved by the time brains were examined at PND 90 in the current study. While the current study did not allow insight into the role that PCBs and PBDEs have in impulsive
behavior and dopaminergic signaling, a better understanding of experimental factors that can influence the outcome of *in vivo* and *in vitro* studies was gained.
Dedicated to Tina

You were worth the 40 year wait.
Acknowledgements

There are many people to thank, so please forgive me if I forget anyone. First, my decision to take Sue Schantz’s neurotoxicology course, way back when, was quite fortuitous. Since then Sue has been constantly guiding me to develop my mind and my talents in ways I had not dreamed possible. Thank you, Sue. I will try my utmost to repay your faith in me. I also want to thank my committee members, Duncan Ferguson, Jodi Flaws, and Joshua Gulley. You each have contributed in very different ways to my growth as a scientist.

As for people whose expertise was invaluable in helping me bring this research to fruition, John Widholm is at the top of the list. He taught me the nuts and bolts of making a behavioral task work. My colleagues and friends Helen and Jeff Sable: Helen has been there every step of the way and Jeff got me started on programming. Stan Floresco and Leonard Green both lent their expertise to my drug studies and the delay discounting task. Megan Mahoney was of much assistance with the cryotome, and if not for Liying Gao then I do not think the western blots would have turned out so well.

Thank you to the gang in the lab: Supida Monaikul, Suren Bandara, Steve Neese, and Mindy Howe. Also the ones who escaped: Emily Poon, Brian Powers, and Victor Wang. We took turns testing each other’s rats on weekends and helping with each other’s projects. What a great group!

Last but not least, Tina for her love and unwavering support. Andrew and Will for the Saturdays they spent in my office while I was working. And my parents. They probably did not always know what to do with me, but they always supported my decisions.

This research was funded by NIEHS K08 ES017045 and R01 ES015687.
# Table of Contents

Chapter 1: Background

1. Introduction ................................................................. 1
2. Neurotoxicity of PCBs and PBDEs ................................. 4
3. Response Inhibition and Impulsivity ............................... 7
4. Overview of ADHD ......................................................... 9
5. Impulsive Behavior and ADHD ................................. 11
6. PCBs, PBDEs, and Impulsivity ........................................ 15
7. Similarities in Neurochemical Alterations Seen in ADHD and with PCB and PBDE Exposure ........................................ 19
8. Gaps in Knowledge ...................................................... 26
9. Figure ........................................................................ 29
10. References ................................................................. 30

Chapter 2: Specific Aims .......................................................... 44

Chapter 3: Effects of PCBs and PBDEs on the Differential Reinforcement of Low Rates of Responding Task

1. Introduction ................................................................. 47
2. Methods ....................................................................... 55
3. Results .......................................................................... 68
4. Discussion ....................................................................... 76
5. Conclusions ................................................................. 86
6. Tables and Figures .......................................................... 88
7. References ................................................................. 98

Chapter 4: Effects of PCBs and PBDEs on the Delay Discounting Task

1. Introduction ................................................................. 105
2. Methods ....................................................................... 110
3. Results .......................................................................... 117
4. Discussion ....................................................................... 123
5. Conclusions ................................................................. 131
6. Figures ........................................................................ 132
7. References ................................................................. 145

Chapter 5. Effects of PCBs and PBDEs on Dopamine Transporter Expression in the Brain

1. Introduction ................................................................. 150
2. Methods and Materials .................................................... 152
3. Results .......................................................................... 160
4. Discussion ....................................................................... 160
5. Conclusions ................................................................. 163
6. Figures ........................................................................ 165
7. References ................................................................. 171
Chapter 6: Conclusions.........................................................................................................................173
1. Discussion............................................................................................................................................173
2. Future Studies....................................................................................................................................176
3. References.........................................................................................................................................179

Appendix: Western Blot Procedure........................................................................................................180
Chapter 1: Background

1. Introduction

The neurodevelopment of an individual, which progresses in a predictable manner from conception to adulthood, occurs across 4 main domains: motor, cognitive, speech and language, and social-emotional development (Patel et al. 2010). Abnormalities in normal maturation processes of any of these domains result in neurodevelopmental disabilities. Approximately 1 in 6 children in the United States have been reported as having a neurodevelopmental disability, with the prevalence increasing from 12.84% to 15.04% over the period of 1997-2008 (Boyle et al. 2011). Because the consequences of these disorders often extend throughout the lifespan, neurodevelopmental disabilities carry a high cost to society.

The example of attention deficit hyperactivity disorder (ADHD), which is a common developmental disorder characterized by impulsive behavior, hyperactivity, and inattention (Aguiar et al. 2010), is illustrative of the social cost, not to mention the incalculable personal cost, of developmental disorders. Using data from a large, nationally representative sample of 34,000 adults, it was determined that the subgroup of ADHD individuals in the study had increased prevalence of comorbid psychiatric disorders and significantly higher tendencies to engage in behaviors that reflected poor planning and impulsivity, to suffer more lifetime traumas, and to perceive poorer health and social support and more stress when compared to non-ADHD adults (Bernardi et al. 2012). Furthermore, Pelham et al. (2007) estimated an annual societal cost in the United States of $42.5 billion for ADHD in childhood and adolescence (in 2005 dollars).
This was estimated using a cost of illness approach which included factors such as costs of physical and psychosocial health care, special education, and crime and delinquency.

While it has been long understood that the developing nervous system is especially sensitive to insult from toxicants (Rice & Barone 2000), more recently it has become better understood that developmental chemical exposures not only impact the early life stages (Mendola et al. 2002; Williams & Ross 2007), but also the later life stages (Landrigan et al. 2005). This has led to considerable interest in recent years in examining the intersection of genes and environment early in the life course as a basis for the development of adult health disorders (the Developmental Origins of Health and Disease concept), which has invigorated interest in understanding the role of early environmental exposures in ADHD (Swanson et al. 2009; Barouki et al. 2012).

It has been shown that environmental and genetic factors both have roles in the development of ADHD (Hudziak et al. 2005), with epigenetic processes potentially mediating individual risk for the development of the disorder (Thapar et al. 2007; Mill & Petronis 2008). There are numerous environmental risk factors, including chemical exposures, that have been associated with a diagnosis of ADHD, to varying extents (Banerjee et al. 2007; Pineda et al. 2007; Millchap 2008). However, establishing causality is extremely difficult.

Perhaps a more fruitful approach is to examine the influence of environmental exposures on domains of behavior such as response inhibition or attention that are impaired in ADHD, similar to the approach proposed in Research Domain Criteria project spearheaded by the National Institute of Mental Health (Morris & Cuthbert 2012).
Focusing more on behavioral domains allows for a focus on mechanisms that encompass characteristics found across several disorders. This approach also helps avoid some of the uncertainties associated with investigating clinically diagnosed, heterogeneous disorders such as ADHD (Stefanatos & Baron 2007). This is not to imply that disorders such as ADHD should not be studied, but instead it implies that also studying the individual abnormal behaviors that together comprise a disorder can provide new information about the causes of the disorder.

The study of neurodevelopmental effects of environmental contaminant exposures lends itself to this behavioral domain-oriented approach. For example, a recent publication examined the parallels between the cognitive domains that are affected by lead and polychlorinated biphenyl (PCB) exposures and those affected by ADHD (Eubig et al. 2010). Both similarities and differences between the behavioral domains affected by developmental exposure to these two contaminants and those affected in ADHD were noted (e.g., both affect response inhibition but lead affects attention to a greater extent than PCBs). This allowed a better understanding of the role these contaminants may play as risk factors for ADHD, but it also raised questions about the roles these contaminants may play in other disorders that include impulsive or inattentive behavior in their clinical presentations (e.g., substance abuse).

With this in mind, the current project examined how developmental contaminant exposures may affect different aspects of impulsive behavior. Specifically, two contaminants were examined: PCBs, for which a much greater body of knowledge exists regarding their neurodevelopmental effects, and polybrominated diphenyl ethers (PBDEs), which are similar to PCBs in many ways but for which much less is known
about their neurodevelopmental effects. It is hoped that the findings from this project will provide new insights into how PCBs and PBDEs affect not only impulsive behavior, but also how closely the effects of these compounds on impulsive behavior parallel those seen in ADHD children.

2. Neurotoxicity of PCBs and PBDEs

PCBs are considered a legacy contaminant in that even though production ceased in the United States by 1977, environmental contamination remains ubiquitous and concentrations in air, water, soil, and biota have been declining very slowly because of the chemical stability of PCBs (ATSDR 2000; Beyer & Biziuk 2009). Due to their lipophilic nature and their environmental persistence, PCBs bioaccumulate and then biomagnify up the food chain (Beyer & Biziuk 2009). One of the main sources of PCB exposure in humans is the consumption of fish (Domingo & Bocio 2007). PCB concentrations in individuals who consume fish are significantly higher than levels in non-fish eaters (Humphrey et al. 2000). Transplacental and lactational passage of PCBs from mothers to their offspring occurs (Dekoning & Karmaus 2000), with fish consumption by mothers prior to or during pregnancy significantly increasing PCB concentrations in cord blood and breast milk (Stewart et al. 1999).

PBDEs have seen extensive use as additive flame retardants to plastics and in coatings for textiles (Darnerud et al. 2001). Due to their lipophilic nature and relative environmental persistence, PBDEs are also found in air, water, and sediments, but especially seem to bioaccumulate in aquatic biota (Darnerud et al. 2001; Hale et al. 2003). Until the 2005 phase out of the production of most PBDEs in North America
(Crimmins et al. 2012), concentrations of PBDEs were exponentially increasing both in Great Lakes fish, with a doubling time of 3-4 years (Zhu & Hites 2004), and in humans, with a doubling time of approximately 5 years (Hites 2004), so that human PBDE serum levels surpassed those of PCBs (Sjodin et al. 2004; Schecter et al. 2005). In adults, ingestion of PBDEs through food may be a significant source of exposure, with fish having higher PBDE concentrations than meat or dairy products (Schecter et al. 2004). Transplacental exposure occurs (Schecter et al. 2007), with maternal and cord blood PBDE concentrations being highly correlated and statistically indistinguishable (Mazdai et al. 2003). PBDEs also partition into breast milk (Schecter et al. 2010), with PBDE concentrations in breast milk beginning to surpass those of PCBs (She et al. 2007). This has resulted in nursing infants experiencing the highest daily PBDE exposure of all life stages (Johnson-Restrepo & Kannan 2009). However, PBDEs differ from PCBs in that, after infancy, ingestion of house dust is suspected to the primary exposure route of PBDEs, particularly for toddlers (Jones-Otazo et al. 2005; Johnson-Restrepo & Kannan 2009; Toms et al. 2009).

PCBs and PBDEs share some structural and functional similarities. PCBs consist of 2 phenyl rings with up to 10 chlorine substitutions for a total of 209 possible congeners (ATSDR 2000). Depending on the substitution pattern of the chlorines, the biphenyl rings of individual PCB congeners can either assume a coplanar or a non-coplanar conformation (Safe 1994). Coplanar PCBs are of concern because they can bind aryl hydrocarbon receptor to produce effects such as hepatic enzyme induction and carcinogenicity (Safe 1994). However, coplanar PCBs have limited neurobehavioral effects – it is the non-coplanar congeners that are primarily responsible for behavioral
impairments following developmental exposure (Fischer et al. 1998; Sable & Schantz 2006). PBDEs are comprised of an ether binding 2 phenyl ring constituents that have up to 10 bromine substitutions for a total of 209 possible congeners (Darnerud et al. 2001). The ether linkage, in addition to the larger bromine atoms, forces PBDEs into non-coplanar configurations (Hardy 2002). Accordingly, PBDEs are not active at aryl hydrocarbon receptor (Peters et al. 2006).

Non-coplanar PCBs and PBDEs also affect neurons in similar manners. PCBs and PBDEs have been shown to disturb intracellular calcium homeostasis by inhibiting mitochondrial and endoplasmic reticular calcium sequestration (Kodavanti et al. 1996; Kodavanti & Ward 2005; Dingemans et al. 2010) through their interactions with ryanodine receptors (Wong et al. 1997; Pessah et al. 2006; Kim et al. 2011). PCBs and PBDEs can also stimulate protein kinase C translocation to the cell membrane (Kodavanti et al. 1994; Kodavanti & Ward 2005). Together, these mechanisms can impair normal neuronal growth during development (Kodavanti 2004) as well as contribute to oxidative stress (Fonnum & Mariussen 2009). Non-coplanar PCBs and PBDEs also decrease expression and impair the function of dopamine transporter (DAT) and vesicular monoamine transporter 2 (VMAT2) (Mariussen & Fonnum 2001; Mariussen & Fonnum 2003; Richardson & Miller 2004; Caudle et al. 2006; Bradner et al. 2013), potentially in an additive manner (Dreiem et al. 2010). These changes increase free dopamine in the cytosol, which is readily oxidized and thus contributes to oxidative stress and dopaminergic neuronal damage seen with PCB exposure (Caudle et al. 2006; Lee et al. 2012).
Additionally, both PCBs and PBDEs interfere with normal thyroid hormone functioning through several different mechanisms (Zoeller 2007; Fonnum & Mariussen 2009; Dingemans et al. 2011). Thyroid hormone plays vital roles in many aspects of brain development (Patel et al. 2011), so any chemical that perturbs thyroid hormone signaling may have deleterious effects on neurodevelopment and behavioral functioning (Zoeller et al. 2002). It should be noted that the PCB and PBDE mixtures to be used in the current study have both been shown to decrease circulating thyroid hormone concentrations in neonatal rats (Poon et al. 2011).

Given the similarities in exposure routes, chemical profiles, and neurotoxic effects of PCBs and PBDEs, it should not be a surprise that developmental exposure to either type of contaminant has been associated with detrimental neurobehavioral effects (see Eubig et al. 2010 and Costa & Giordano 2007 for reviews). In examining the laboratory and human epidemiologic studies on the effects of PCBs (Eubig et al. 2010), it is evident that response inhibition is one of the behavioral domains adversely affected by PCBs.

3. Response Inhibition and Impulsivity

The construct of response inhibition describes the inhibition or stopping of actions that occur in response to cues or triggers in the subject’s environment. As situational circumstances or goals change, response inhibition is an important component in stopping a current course of action and reorienting behavior towards an original or new goal (Logan 1994). Response inhibition can occur before an action begins, or it can stop
an action that has already begun. The converse of response inhibition is the inability to inhibit actions, which is commonly termed impulsive behavior or impulsivity.

An oft quoted definition is that “impulsivity encompasses a range of actions which are poorly conceived, prematurely expressed, unduly risky or inappropriate to the situation and that often result in undesirable consequences” (Daruna & Barnes 1993). This broad definition reflects that there are a variety of impulsive behaviors which are interrelated, but which also have their own patterns of behavioral manifestations and underlying neuroanatomical and neurochemical substrates. Several authors have attempted to define and categorize different varieties of impulsivity seen in human and animal subjects (Evenden 1999; Nigg 2000; Dick et al. 2010; Fineberg et al. 2010). Yet the risk of subdividing behavior into too many subcategories is that the ability to see connections between the various types of behavior may be compromised. For this reason, dividing impulsivity into impulsive choice and impulsive action (Winstanley et al. 2010; Broos et al. 2012) is a good point from which to start an investigation. More detailed subcategorizations could then be used to address subsequent experimental questions.

Impulsive behavior is a common problem. A recent survey using data from the National Epidemiologic Survey on Alcohol and Related Conditions concluded that the prevalence of self-reported impulsivity in the adult general population is 16.9% (n=34,653) (Chamorro et al. 2012). Smaller surveys report that impulsivity peaks in childhood and then declines during adolescence and into adulthood (see Steinberg et al. 2008 for a brief review), a trend which was confirmed in the Chamorro et al. (2012) study. The main concern is that impulsive individuals are vulnerable to developing
psychopathologic disorders (Beauchaine et al. 2008). Impulsivity is an important component of attention deficit hyperactivity disorder (ADHD) (Moeller et al. 2001; Adams et al. 2008; Groman et al. 2009), substance and alcohol use disorders (Groman et al. 2009; Dick et al. 2010; Robbins et al. 2012), mood (bipolar) and personality (borderline) disorders (Moeller et al. 2001), and what are termed impulse-control disorders such as pathological gambling, kleptomania, and trichotillomania (obsessive hair pulling) (American Psychiatric Association 2000a).

4. Overview of ADHD

ADHD is a common developmental disorder, with a worldwide prevalence estimated at 5.29% (Polanczyk et al. 2007). However prevalence estimates vary based on criteria for diagnosis, among other factors. For example, the Centers for Disease Control and Prevention (2010) reported a 9.5% prevalence of ADHD among children aged 4-17 years in the United States in 2007 using the relatively loose criteria of parental report of diagnosis.

The current, most commonly used diagnostic criteria for ADHD involve 18 behavioral items divided between an inattentive dimension and a hyperactive/impulsive dimension (American Psychiatric Association, 2000b) (see Figure 1.1, reprinted from Aguiar et al. (2010)). The two dimensions allow distinction between 3 different subtypes of ADHD. A predominately inattentive subtype (ADHD-PI) is diagnosed if at least 6 items from the inattentive dimension are present; a predominately hyperactive/impulsive subtype (ADHD-PH) is diagnosed if at least 6 items from the hyperactive/impulsive dimension are present; and a combined subtype (ADHD-C) is diagnosed if at least 6 items from
each of the two dimensions are present. Additional criteria include that some of the abnormal behaviors must have been present before age 7 and that the behaviors cause significant functional impairments in 2 or more social, academic, or occupational settings.

Classifying patients into different subtypes allows for more individualized treatment, especially in regards to non-pharmacologic therapies. Overall, ADHD is diagnosed approximately 2.5 times more frequently in boys than girls (Polanczyk et al. 2007). ADHD-C is the most frequently reported subtype in both sexes, but ADHD-C is more common in boys and ADHD-PI is more common in girls who are referred for psychiatric evaluation (Biederman et al. 2002).

Up to two-thirds of individuals with ADHD either concurrently exhibit or later develop additional psychiatric disorders, with comorbidity rates ranging up to 50% for internalizing disorders, such as depression, and 90% for externalizing or disruptive disorders (Young 2008; Taurines et al. 2010). Oppositional defiant disorder and conduct disorder are the most commonly diagnosed comorbid disorders (Taurines et al. 2010), with externalizing disorders such as these being more commonly found in ADHD males (Rucklidge 2010). But numerous other conditions including depression, anxiety disorders, substance abuse disorders, and bipolar disorder are also commonly diagnosed (Young 2008; Taurines et al. 2010), with internalizing disorders such as depression and anxiety disorders being more common in ADHD females (Rucklidge 2010).

A particular challenge is that ADHD symptoms are heterogeneous across affected individuals, even within each subtype (Stefanatos & Baron 2007). Although classifying
children by ADHD subtype may be valid for individuals at the time of initial diagnosis, the problem is that individuals do not necessarily remain true to their subtypes over time (Willcutt et al. 2012). This variability between individuals and over time may reflect the influence of different causative pathways in addition to the influence of comorbidities and external factors (Stefanatos & Baron 2007; Swanson et al. 2007; Willcutt et al. 2012). For these reasons, relying upon clinical symptomatology to define ADHD can be important in recognizing the problem and initiating treatment, but it does not provide strong insight into the causative factors that result in the clinical manifestations of ADHD.

5. Impulsive Behavior and ADHD

Several important cognitive functions are impaired in ADHD children, particularly executive functioning, attention, temporal information processing, and response to reinforcement (Nigg & Nikolas 2008). A review by Aguiar et al. (2010) examined the strength of evidence for executive function and attention domains being affected in ADHD based on findings from meta-analyses of behavioral tasks. It was concluded that the strongest evidence was for response inhibition, both verbal and nonverbal working memory, and sustained attention being abnormal in ADHD, although cognitive flexibility, planning and alertness are also abnormal to a lesser extent. This section will focus on the evidence for impaired response inhibition (i.e., impulsive behavior) in ADHD.

Given that behaviors are mediated by distinct neural pathways, using behavioral assessment measures to examine specific neuropsychological functions is an initial step towards gaining further insight into the biological foundations of both normal and
abnormal behavior (Winstanley et al. 2006). Additionally, incorporating behavioral assessment measures allows a more quantitative approach, as opposed to the qualitative information provided by clinical diagnostic scales such as the one in Figure 1.1. Four different behavioral tasks used to evaluate impulsive behavior in ADHD are the stop signal response time (SSRT) task, the continuous performance test (CPT), differential reinforcement of low rates of responding (DRL), and delay discounting.

SSRT and ADHD

The SSRT task requires subjects to make a rapid response to “go” signals (e.g., pressing a button on the left if “<” appears on the video screen and pressing a button on the right if “>” appears). “Stop” signals, which are either an auditory signal (a tone) or a visual signal (a letter on the screen), occur during a limited number of trials (typically 20-25%) in the testing session. The SSRT task allows the assessment of the time required for the subject to inhibit the already initiated response to press a button (i.e., the SSRT) once the stop signal is presented, so one specific aspect of impulsive behavior is being evaluated (Chamberlain & Sahakian 2007). Meta-analyses of the SSRT task show that ADHD is associated with slower inhibition of the ongoing response (increased SSRT), with a moderate effect size for the difference (Cohen’s $d = 0.54-0.63$) (Aguiar et al. 2010). One challenge to these findings arises from recent studies suggesting that longer SSRTs in ADHD children may be due to a more variable and inaccurate response style rather than an inhibitory deficit per se (Alderson et al. 2008; de Zeeuw et al. 2008).

CPT and ADHD
The CPT requires that a subject respond as quickly as possible to a target on the screen while suppressing responses to non-targets. Responding to non-targets (commission errors) is a measure of impulsive action. Meta-analyses of commission errors on the CPT show that a higher rate of errors is associated with ADHD, with a moderate effect size for the difference (Cohen’s $d = 0.51-0.56$) (Aguiar et al. 2010). However, although increased commission errors have been associated with impulsive behavior in ADHD children (as opposed to only being associated with a diagnosis of ADHD) (Egeland & Kovalik-Gran 2010), this has not been demonstrated in all studies (e.g., Epstein et al. 2003). One reason for this variability between studies may be that the error rate can vary depending on the frequency with which the target is presented (Conners et al. 2003), with different studies using different versions of the CPT.

**DRL and ADHD**

DRL assesses impulsive action and the ability to withhold a response over time. Throughout the test session a button or lever is available for the subject to press. During each trial, a press after a pre-determined time interval has elapsed (e.g., 15 sec; DRL 15 sec) results in the delivery of a reinforcer, while a press before the interval has elapsed results in the interval timer resetting. In order to perform well on the task, the subject must realize that it is necessary to withhold pressing for a length of time at the beginning of each trial.

Four studies using DRL to assess ADHD children have been published. Two studies using a DRL 6 sec task, one with a 15 min session length for 6-8 year olds and the other with an 8 min session length for 6-11 year olds, found that hyperactive subjects
had significantly greater number of responses and lower efficiency ratios of reinforced versus total presses (Gordon 1979; McClure & Gordon 1984), which suggests impulse control problems in the hyperactive children. However a similar study (DRL 6 sec for 8 min in 8-13 year olds) did not replicate these findings (Daugherty & Quay 1991). The sample size of each treatment group was smaller in this third study (n = 15 normal, 9 ADHD, 10 conduct disorder/ADHD), as opposed to the 2 studies by Gordon and colleagues (n = 20 normal, 20 ADHD for both), which may have affected the ability to detect significant differences. A more recent study obtained parent and teacher ratings for the presence of characteristic ADHD and oppositional/defiant disorder (ODD) behaviors in 165 6-12 year olds (Avila et al. 2004). The children were tested with a DRL 10 sec task (8 min session length), along with tasks that assessed other aspects of impulsivity. A principal components analysis of the results determined that the DRL efficiency ratio was not significantly correlated with the ADHD behavioral ratings, although it was correlated with ODD ratings. The authors suggest that DRL assesses a different aspect of inhibitory control that is more reflective of delay aversion. This possibility can not be eliminated since impaired response inhibition and delay aversion are both characteristics of ADHD that may vary between individuals (Sonuga-Barke 2005).

Besides its potential useful role in assessing ADHD children, DRL also has been used in children developmentally exposed to environmental contaminants including PCBs (Stewart et al. 2006) and perfluorinated chemicals (Gump et al. 2011), and was sensitive enough to detect an association of exposure with impulsive behavior.
Delay Discounting and ADHD

The delay discounting or delay of gratification paradigm involves making a choice between a smaller reward that is delivered immediately and a larger reward that is delivered after a delay. Typically, subjects choose the larger-delayed reward when the delay is short, but then begin to choose the smaller-immediate reward as the delay length increases (Tesch & Sanfey 2008). Hence they discount the value of the larger reward in order to avoid longer delays. In this context, the decision to choose the smaller-immediate reward is interpreted as impulsive choice, with the paradigm more closely assessing decision-making processes rather than impulsive motor activity (Winstanley et al. 2006).

ADHD children generally choose the smaller-immediate reward more than non-ADHD children (Winstanley et al. 2006; Scheres et al. 2010), with moderate effect sizes reported for individual studies (Cohen’s $d = 0.57-0.71$) (Sonuga-Barke et al. 2008). While the underlying reason that ADHD children favor the smaller-immediate reward may remain a matter of active debate (Sagvolden et al. 2005; Sonuga-Barke et al. 2005), delay discounting has been shown to be able to distinguish a different aspect of impulsivity than is assessed by other tasks, such as the SSRT, in both ADHD and non-ADHD populations (Solanto et al. 2001; Reynolds et al. 2008).

6. PCBs, PBDEs, and Impulsivity

If the role of environmental contaminants in developmental disorders such as ADHD is to be understood, then an important initial step is to examine whether contaminants such as PCBs and PBDEs impact behavioral domains that are affected in ADHD. In this
section, the evidence that PCBs and PBDEs affect response inhibition will be considered.

Studies in rodents, monkeys, and human populations have examined the effects of developmental PCB exposure on response inhibition. Three studies in rats and 1 in monkeys used the DRL task to assess impulsive behavior. While these studies are discussed in detail in Chapter 3, a brief summary follows. Holene et al. (1999) studied the effects of lactational exposure to the non-coplanar congener PCB 153 in female rats. It was found that the PCB-exposed subjects had shorter times between responses and earned fewer rewards than controls. Sable et al. (2009) evaluated the effects of perinatal exposure to an environmentally-relevant PCB mixture (Fox River Mix; Kostyniak et al. 2005) in rats. PCB exposure resulted in smaller efficiency ratios (reinforced:non-reinforced responses), which is in contrast to an earlier study from the same laboratory in which the same perinatal exposure period and DRL testing paradigm were used but PCB-related effects were not found (Sable et al. 2006). The DRL study in monkeys involved exposure to a PCB mixture from birth to 20 weeks of age (Rice 1998). DRL testing at age 4.5 years revealed increased responding, fewer rewards earned, and shorter times between responses in PCB-exposed monkeys. Several other studies of developmental PCB exposure in rats and monkeys have used a related task, the fixed interval task to assess for impaired response inhibition, as reviewed by Eubig et al. (2010). The fixed interval task is similar to DRL, but subjects are not penalized for early responding in the former task. The most common findings in many, but not all, of the fixed interval studies were increased responding and shorter times between responses in PCB-exposed subjects.
The effects of developmental PCB exposure on response inhibition in humans have been examined in 3 prospective cohorts: the Oswego, New York cohort, the Michigan cohort, and the New Bedford, Massachusetts cohort. In the Oswego cohort, participants were tested on a CPT task at ages 4.5, 8, and 9.5 years (Stewart et al. 2003; Stewart et al. 2005). At each age, a higher incidence of commission errors was associated with PCB exposure. The 9.5 year-old participants were also tested on DRL and PCB exposure was associated with increased responding, shorter times between responses, and fewer rewards earned (Stewart et al. 2006). In the Michigan cohort, participants were tested on a CPT at ages 4 and 11 years (Jacobson et al. 1992; Jacobson & Jacobson 2003). A higher incidence of commission errors was associated with PCB exposure at age 11, but not age 4. This association was only seen in children that had been formula fed, indicating that breast feeding had a protective effect despite being a source of PCB exposure postnatally. Participants in the New Bedford cohort were tested on a CPT at age 8, but no association between commission errors and PCB exposure was found (Sagiv et al. 2012). However, the CPT used in this study had a low target presentation rate. This results in a greater likelihood of omission errors and a lesser likelihood of commission errors and, thus, is better at detecting inattention than impulsivity. The same children were assessed for ADHD-like behaviors using the Conners’ Rating Scale for Teachers (Sagiv et al. 2010). Positive associations were found between cord blood levels of PCBs and several subscales of the test, including the DSM-IV Hyperactive-Impulsive subscale.

A limited number of animal studies have examined impulsive behavior subsequent to developmental PBDE exposure in rodents. Driscoll et al. (2009) perinatally exposed rats
to the commercial PBDE solution, DE-71. Exposure then continued post-weaning via the chow through the time the subjects were tested on a version of the 5-choice serial reaction time task. PBDE exposure resulted in increased premature responding, which is considered indicative of impulsive behavior. These results were not replicated when the exposure period was limited to postnatal days (PND) 6 to 12 (Driscoll et al. 2012). In a study by Rice et al. (2009), mice were dosed with congener BDE 209 from PND 2 to 15. Subjects were then either tested on a fixed interval task beginning in young adulthood (PND 90) or at 16 months of age. Only in the older cohort were significant increases in responding seen.

The cognitive effects of developmental PBDE exposure have been examined in prospective human cohorts, although specific tests of impulsive behavior have not been used except for a CPT in one study (Eskenazi et al. 2013). In that study of the CHAMACOS cohort in California, maternal prenatal PBDE levels were not associated with changes in commission errors on the CPT at age 5. The same children were assessed at age 7 for ADHD-like behavior using the Conners’ Rating Scale for Teachers. Higher serum PBDE concentrations in the children at the time of testing were positively associated with higher scores on several subscales, including the Hyperactive-Impulsive subscale, but no association was found with maternal prenatal PBDE levels. Children in the COMPARE cohort in the Netherlands were evaluated at a similar age, 5 to 6 years, using the NEPSY-II neuropsychological battery, and the parents filled out an 18 item ADHD questionnaire (Roze et al. 2009). Levels of individual PBDE congeners from prenatal maternal serum were not associated with poorer scores on the Inattention subscale of the NEPSY-II. Interestingly, there was not an association
for PCB 153 either, but there were positive associations for hydroxylated PCB congeners with the Inattention subscale score. None of the contaminants were associated with the results of the ADHD questionnaire. Children were assessed at a very young age, 2.5 years-old, using the Infant-Toddler Social and Emotional Assessment in a cohort from North Carolina (Hoffman et al. 2012). PBDE concentrations in the mothers' breast milk were associated with a higher score on the Activity/Impulsivity subscale.

Thus, animal studies suggest that PCB exposure has a role in impulsive behavior, and neurobehavioral assessments from human PCB-exposed cohorts corroborate the findings from the animal studies. Animal behavioral studies of PBDE exposure suggest that PBDEs also may have a role in impulsive behavior. However, because only a limited number of PBDE studies address this question, it is difficult to draw conclusions. Although studies of human cohorts suggest that PBDEs may have a role in impulsive behavior, this is based on scores from behavioral rating scales. There is a need for studies in which quantifiable behavioral tasks are used to assess the effects of developmental PBDE exposure on response inhibition.

7. Similarities in Neurochemical Alterations Seen in ADHD and with PCB and PBDE Exposure

Given the similarities in impulsive behavior seen in ADHD and with PCB and, possibly, PBDE exposure, it is important to consider the neurochemical mechanisms that underlie the behavioral effects. Gaining a better understanding of the neurochemical changes involved in PCB and PBDE exposure, and how these changes
relate to ADHD, will be beneficial in elucidating the roles that developmental contaminant exposure could have in the clinical manifestations of ADHD.

**ADHD and the Dopamine Hypothesis**

As already discussed, ADHD is a complex disorder with varying clinical phenotypes. The etiology of ADHD involves a genetic component to a large extent, but it is a disorder involving many genes of small effect, with no gene identified thus far that accounts for more than 3 to 4% of the total variance in ADHD (Smith et al. 2009). Likewise, environmental factors are also acknowledged as having a role in the development of ADHD, with epigenetic processes potentially serving as the interface between genes and environment in mediating risk for the development of ADHD in the individual (Thapar et al. 2007; Mill & Petronis 2008). A current hypothesis about the etiology of ADHD that combines what is known about environmental and genetic factors is the dopamine hypothesis (Swanson et al. 2007; Genro et al. 2010). It is postulated that hypofunctional dopamine signaling is responsible for many of the behavioral deficits seen in ADHD. This is supported by several lines of evidence including animal studies demonstrating the behavioral profile seen with hypodopaminergic functioning, studies of polymorphisms in dopamine-related genes and ADHD risk, imaging studies of dopaminergic functioning in ADHD individuals, and pharmacologic studies of the impact of dopaminergic drugs on ADHD-like behaviors in animal models and ADHD patients.

Mesocortical and mesolimbic dopaminergic modulation of cortical-striatal circuits has been shown to be important for executive functioning, particularly inhibitory control (Sonuga-Barke et al. 2005; Brennan & Arnsten 2008; Dalley et al. 2008). It has been
proposed that hypofunctional mesocortical dopaminergic input to the appropriate
cortical-striatal circuits plays an important role in the deficits in response inhibition and
attention seen in ADHD (Sagvolden et al. 2005). Studies using animal models have
demonstrated the importance of dopaminergic signaling in impulsive behavior (reviewed
by Winstanley et al. 2006). For example, it has been shown that depleting the medial
prefrontal cortex of dopamine increased responding and decreased the time between
responses on a DRL task in rats (Sokolowski & Salamone 1994). Also, although it is
indirect evidence for the dopamine hypothesis, it is interesting that in ADHD patients
similar brain regions which receive dopaminergic innervation are affected.
Neuroimaging studies have found size reductions in areas including the prefrontal
cortex and caudate nucleus of the striatum (Valera et al. 2007; Nigg & Nikolas 2008;
Makris et al. 2009). Functional imaging studies have also demonstrated hypoactivity in
these regions in ADHD patients (Dickstein et al. 2006; Makris et al. 2009).

Candidate gene studies have suggested the involvement of genes involved in
doaminergic neurotransmission in ADHD, with the most consistent findings being for
polymorphisms in the DAT gene ($DAT1$ or $SLC6A3$) and the D4 receptor gene ($DRD4$)
(Gizer et al. 2009; Smith et al. 2009). While the most commonly studied D4 receptor
variant in ADHD has been shown to have a blunted response to dopamine binding
(Biederman & Faraone 2005), the functional significance of DAT variants in ADHD is
less clear and likely context dependent (Madras et al. 2005). Molecular imaging studies
by Volkow et al. have demonstrated decreased striatal D2/3 receptor and DAT binding
in ADHD patients (e.g., Volkow et al. 2009), which suggests that a hypodopaminergic
state exists in ADHD. However, many of the earlier studies showed increased striatal
DAT binding in ADHD patients (Nikolaus et al. 2007; del Campo et al. 2011), which could suggest that ADHD instead involves a hyperdopaminergic state. However, if increased DAT binding is due to primary changes in gene expression of \textit{DAT1}, i.e., overexpression of DAT during neurodevelopment or due to abnormalities of a \textit{DAT1} variant (Krause 2008), then increased DAT expression would result in a hypodopaminergic state because dopamine would be cleared from the synapse more efficiently than usual.

The efficacy of the stimulant medications amphetamine and methylphenidate in treating ADHD is another argument that supports the dopamine hypothesis for ADHD. Stimulants have larger effect sizes than non-stimulants for improvement of ADHD symptoms (Biederman & Faraone 2005). Both medications prevent DAT from clearing dopamine from the synapse, while amphetamine also promotes dopamine release into the synapse, thus increasing synaptic dopamine concentrations (Madras et al. 2005). Lending some support to the argument that a hypodopaminergic state exists in ADHD, Volkow et al. (2007) found that medication-naïve, adult ADHD patients released less dopamine in the caudate when administered methylphenidate than did non-ADHD controls. But concordance of findings in the human studies is elusive. It was demonstrated in medication-naïve, adolescent ADHD patients who were administered methylphenidate that the more impulsive patients experienced a greater release of striatal dopamine than the less impulsive patients (Rosa-Neto et al. 2005). However, given that either too little or too much dopamine release in the prefrontal cortex can impair cognitive functioning (Brennan & Arnsten 2008), the argument follows that if stimulants increase synaptic dopamine levels, then patients in a hypodopaminergic
state are the ones who are more likely to experience improvements in cognitive functioning when administered stimulant medications.

**PCBs, PBDEs, and Dopamine**

PCBs and PBDEs are hypothesized to create a hypodopaminergic state, which could play a role in the cognitive changes seen subsequent to developmental exposure. Both *in vitro* and *in vivo* evidence supports the argument that PCBs reduce dopamine concentrations in the brain and, specifically, in the synapse. Some evidence also exists that PBDEs exert similar effects.

Perinatal exposure to non-coplanar congener PCB 47 has been shown to decrease dopamine concentrations in the frontal cortex and striatum at PND 90 (Seegal et al. 1997). Two studies of prenatal exposure to non-coplanar PCB 153 reported similar findings: either decreased striatal dopamine at PND 21 (Castoldi et al. 2006) or an initial increase in brain dopamine and its metabolites through 6 weeks of age followed by a persistent decrease from 9 weeks to 1 year of age (Honma et al. 2009). However, perinatal exposure to the congener mixtures Aroclor 1016 or Aroclor 1254 (Morse et al. 1996; Zahalka et al. 2001) did not result in significant changes in brain dopamine concentrations. These investigations were carried further by *in vitro* work demonstrating that a 1:1 mixture of Aroclor 1254 and 1260 caused a dose-dependent decrease in dopamine in striatal slices and an increase of dopamine in the surrounding media (Chishti et al. 1996), and that an environmentally relevant PCB mixture (Fox River Mix; Kostyniak et al. 2005) decreased tissue dopamine and increased media dopamine in an organotypic co-culture derived from embryonic rat striatum and ventral mesencephalon
(Lyng et al. 2007). Finally, a study in which in vivo microdialysis of the striatum was used to quantify free (i.e., synaptic) dopamine after Aroclor 1254 exposure revealed that dopamine concentrations were elevated after the first 3 days of exposure, but thereafter dopamine concentrations were decreased as exposure continued beyond 1 week (Seegal et al. 2002). This study also showed that even though changes were occurring in synaptic dopamine levels, striatal tissue levels of dopamine did not differ from controls. Thus, PCB exposure has been shown to induce functionally significant alterations in synaptic dopamine concentrations, regardless of whether tissue concentrations were altered.

Investigations into the mechanisms for dopaminergic changes have shown that tyrosine hydroxylase and L-aromatic amino acid decarboxylase, the 2 enzymes in the synthetic pathway of dopamine from its precursor tyrosine, are affected by PCBs. Choksi et al. (1997) exposed striatal preparations to non-coplanar PCB congeners and found that tyrosine hydroxylase activity was inhibited in the preparations from Sprague-Dawley and, to a lesser extent, Long Evans rats, which suggests that there may be some strain differences in sensitivity to PCBs. In the previously mentioned organotypic co-culture study, it was shown that PCB exposure resulted in decreases in tyrosine hydroxylase and DAT expression (Lyng et al. 2007). In a separate, in vitro study, non-coplanar PCB congeners were shown to increase DOPA and decrease dopamine concentrations in catecholaminergic PC12 cells (Angus et al. 1997). This specifically points to an inhibition of L-aromatic amino acid decarboxylase.

PBDEs have not received as much scrutiny as to their effects on neuronal dopamine content and synthesis. One study involved exposing adult mice to DE-71 for 1 month
(Bradner et al. 2013). Striatal dopamine and metabolites were decreased in PBDE-exposed subjects, but striatal tyrosine hydroxylase expression was unaffected. A separate study exposed mice to 3 different dosages of congener BDE 47 on PND 10 (Gee et al. 2011). Only mice receiving the middle dose (10 mg/kg) demonstrated an increase of dopamine levels in the frontal cortex, but not the striatum, at PND 15, at PND 20, and at 4.5-5 months of age. The differences between these 2 studies suggest that differences in age, dose, and length of PBDE exposure all might differentially mediate changes in dopaminergic signaling.

An area that has generated much interest is the effects of PCBs and PBDEs on the functioning of the dopamine transporters DAT and VMAT2, which clear dopamine from the synapse and cytosol, respectively. This is extensively discussed in Chapter 5, but a brief review follows. Both transporters should function in concert to terminate synaptic signaling and to minimize cytosolic dopamine accumulation. Alterations of synaptic dopamine can impact many different cognitive functions in which dopamine signaling plays important roles (Seamans & Yang 2004). Alterations in cytosolic dopamine can affect neurotransmission but can also, if dopamine is elevated, result in oxidative damage (Guillot & Miller 2009). Several studies have demonstrated that both PCB mixtures and individual non-coplanar congeners reduce the expression and impair the functioning of DAT and VMAT2 (Mariussen & Fonnum 2001; Mariussen et al. 2001; Richardson & Miller 2004; Caudle et al. 2006; Tian et al. 2011). It has been shown that the commercial PBDE mixture, DE-71, also reduces expression and impairs functioning of DAT and VMAT2 (Mariussen & Fonnum 2003; Bradner et al. 2013). Furthermore, it has been demonstrated that the Fox River PCB Mix is 2 to 3 times more potent than
DE-71 at inhibiting DAT uptake of dopamine in a synaptosome model, and that PCBs and PBDEs have an additive effect in this regard (Dreiem et al. 2010).

It is clear that dopaminergic signaling is affected in ADHD and also by PCB and PBDE exposure. There is evidence that a hypodopaminergic state exists in the brain in ADHD, but there are also studies that suggest the opposite. What is emerging from the literature is that DAT expression and other aspects of dopaminergic neurotransmission may vary between ADHD individuals and may also change in response to environmental influences, including pharmacotherapy (del Campo et al. 2011; Swanson et al. 2011). Studies in laboratory species suggest that developmental exposure to PCBs and PBDEs induce a hypodopaminergic state. So it is conceivable that PCBs and PBDEs could contribute to the clinical manifestations of ADHD in individuals through interactions with dopaminergic neurotransmission. However, this direct link would be very challenging to investigate in human populations.

8. Gaps in Knowledge

Neurodevelopmental disorders such as ADHD have their roots in both genetic and environmental factors. Interactions between genes and environment are believed to result in the clinical manifestation of developmental disorders in individual patients. Assessing the contributions of environmental contaminant exposures during neurodevelopment is important for gaining a better understanding of these interactions. In the case of ADHD, affected individuals often have problems with inhibitory control and thus are prone to both performing impulsive actions and making impulsive choices. Furthermore, problems with response inhibition are common across several
developmental disorders, so it may be more fruitful to first investigate the effects of environmental exposures on response inhibition in general, and then to use the findings to inform research on inhibitory control problems seen in individual disorders.

Developmental exposure to PCBs has been shown to cause impulsive action, whereas there has been little inquiry into whether PBDEs, which share several key neurotoxic properties with PCBs, also have similar effects. Whether either type of contaminant affects impulsive choice has not been investigated. It is established that dopamine is an important neuromodulator of response inhibition, with perturbations in synaptic dopamine concentrations resulting in impulsive behavior. There is strong evidence that PCBs affect dopaminergic neurotransmission. A smaller number of studies have addressed whether PBDEs share this effect, but the results so far suggest that they do. Most studies have focused on the effects of PCBs and PBDEs on dopamine in the striatum, which is a brain region that is heavily innervated by dopaminergic neurons. Less attention has been given to whether PCBs and PBDEs affect dopaminergic signaling in other regions such as the prefrontal cortex and nucleus accumbens. These regions, in addition to the striatum, play important roles in the performance of behavioral tasks that are used to assess impulsive behavior.

Investigation of whether PCBs and PBDEs affect impulsive choice is an important step that should be taken in order to better understand the role that developmental exposure to these contaminants plays in disorders such as ADHD in which impulsive choice is an important clinical component. Also, examining the effects of PCBs and PBDEs on dopaminergic signaling in response to pharmacotherapies used to treat disorders such as ADHD, and in regions of the brain that are important for response
inhibition, will lead to a better understanding of how PCBs and PBDEs influence disorders, such as ADHD.
Figure 1.1. Diagnostic Criteria for ADHD\footnote{Reprinted with permission from Aguiar et al. (2010).}

I. At least six behavioral symptoms from list A or list B occur often, have persisted for the preceding 6 months, and are maladaptive and inappropriate given the individual’s developmental level.

A. Inattentive–Disorganized Dimension:
   1. Fails to give close attention to details or makes careless mistakes in schoolwork, work, or other activities
   2. Has difficulty sustaining attention in tasks or play activities
   3. Does not seem to listen when directly spoken to
   4. Fails to follow through on instructions and fails to finish schoolwork, chores, or work duties
   5. Has difficulty organizing tasks and activities
   6. Avoids, dislikes, or is reluctant about engaging in tasks that require sustained mental effort
   7. Loses things necessary for tasks or activities (e.g., toys, school assignments, or tools)
   8. Gets easily distracted by extraneous stimuli
   9. Is forgetful in daily activities

B. Hyperactivity–Impulsivity Dimension:
   1. Fidgets with hands or feet or squirms in seat
   2. Leaves seat in classroom or in other situations in which remaining seated is expected
   3. Runs about or climbs excessively in situations in which it is inappropriate (in adolescents or adults, may be limited to subjective feelings of restlessness)
   4. Has difficulty playing or engaging in leisure activities quietly
   5. Is “on the go” or acts as if “driven by a motor”
   6. Talks excessively
   7. Blurs out answers before questions have been completed
   8. Has difficulty awaiting turn
   9. Interrupts or intrudes on others (e.g., butts into conversations or games)

II. Some symptoms that cause impairment were present before 7 years of age.

III. Some impairment from the symptoms is present in two or more settings.

IV. There is clear evidence of significant impairment in social, school, or work functioning.

V. Symptoms do not happen only during the course of a pervasive developmental disorder, schizophrenia, or other psychotic disorder, and they are not better accounted for by another mental disorder (e.g., mood, anxiety, dissociative, or personality disorder).

Based on criteria I–V, three types of ADHD are identified:

1. Predominantly inattentive (ADHD-PI); if at least six symptoms from list A but not B are present
2. Predominantly hyperactive–impulsive (ADHD-PH); if at least six symptoms from list B but not A are present
3. Combined (ADHD-C); if at least six symptoms from each of the lists, A and B, are present

\footnote{Reprinted with permission from Aguiar et al. (2010).}
10. References


phosphoinositide hydrolysis and protein kinase C translocation in rat cerebellar granule cells. *Brain Res.* 662, 75-82.


Chapter 2: Specific Aims

This research investigated the effects of polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) on inhibitory control using a rodent model. The primary goals were (1) to assess the effects of developmental PCB and PBDE exposure on two distinct aspects of response inhibition, impulsive action and impulsive choice, and (2) to use additional approaches including systemic drug challenges and western blot analyses of dopamine transporter protein expression in specific brain regions to gain a better understanding of the neurochemical mechanisms through which PCBs and PBDEs impair distinct aspect of response inhibition. PCB-exposed animals and children show deficits on several different types of learning tasks that require inhibitory control for their successful execution, but the underlying changes in cognitive processing responsible for these deficits are not well understood. A closer evaluation of specific aspects of inhibitory control impaired by developmental PCB exposure should lead to a better overall understanding of the cognitive deficits associated with this exposure. Additionally, because similar behavioral problems are seen in children with attention deficit hyperactivity disorder (ADHD), these studies may lead to a better understanding of this common childhood disorder. The experiments were performed using a PCB mixture (the Fox River Mix) that models the PCB congener profile in fish consumed by a human population. These studies also evaluated the effects of PBDEs on response inhibition. Worldwide use of PBDEs as flame retardants has resulted in increasing levels in the environment and human tissue. This is of concern given that PBDEs and non-coplanar PCBs have similar molecular
structures and similar intracellular effects, including changes in catecholamine signaling, and, thus, may have similar effects on behavioral function.

The specific aims of this research were to:

1. Use a rodent model (Long Evans rat) to assess the effects of developmental exposure to PCBs and PBDEs on two distinct aspects of response inhibition. Previous studies have reported impaired inhibitory control following developmental PCB exposure, but closer examination of the specific aspects of response inhibition affected by PCBs is lacking. Additionally, recent studies suggest that developmental exposure to PBDEs may also affect inhibitory control. Two behavioral tasks, differential reinforcement of low rates of responding (DRL) and delay discounting (DD), were used to evaluate the effects of PCBs and PBDEs on two aspects of response inhibition, the ability to stop an action before it begins (DRL) and the ability to choose a delayed but larger reward over an immediate but smaller reward (DD). These tasks were also selected to allow better evaluation of the extent to which the deficits seen with PCBs and PBDEs parallel those seen in ADHD. It was hypothesized that PCB- and PBDE-exposed rats would exhibit more pronounced deficits on DRL and DD tasks as compared to controls.

2. Use drug challenges to assess the role of perturbations in dopaminergic neurotransmission in mediating the effects of developmental exposure to PCBs and PBDEs on response inhibition. Reduced dopaminergic neurotransmission has been documented following both PCB and PBDE exposure and is also present in ADHD. Systemically-administered agents that alter dopaminergic synaptic activity were used to further investigate the role of the dopaminergic system in PCB- and PBDE-
induced deficits in response inhibition. It was hypothesized that there would be shifts in the dose-response curves such that performance of PCB- and PBDE-exposed animals on DRL and DD tasks would be more improved by drugs that enhance dopaminergic neurotransmission and would be more disrupted by drugs that reduce dopaminergic neurotransmission as compared to controls.

3. Use western blot analysis to quantify expression of the dopamine transporter in specific regions of the prefrontal cortex and striatum following developmental exposure to PCBs and PBDEs. PCBs and PBDEs have been shown to inhibit synaptic uptake of dopamine via the dopamine transporter. This research expanded the current body of knowledge by quantifying expression of dopamine transporter in 4 specific brain regions: medial prefrontal cortex and orbitofrontal cortex (prefrontal regions) and nucleus accumbens and dorsal striatum (striatal regions). These brain regions are important in the regulation of the DRL and DD tasks. It was hypothesized that expression of dopamine transporter would be reduced in the prefrontal cortex and striatum of rats developmentally exposed to PCBs and PBDEs.

Together these results were anticipated to provide evidence that would help us to understand the role of changes in dopamine signaling in specific regions of the prefrontal cortex and striatum in mediating deficits in specific components of inhibitory control following PCB and PBDE exposure. These studies were also anticipated to provide further insight both into the neurochemical alterations that occur in ADHD and the influence of environmental factors on the clinical manifestations of ADHD.
Chapter 3: Effects of PCBs and PBDEs on the Differential Reinforcement of Low Rates of Responding Task

1. Introduction

The focus of the current project is to examine the effects of developmental exposure to polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) on response inhibition. Impulsive behavior can be divided into two types: impulsive action and impulsive choice (Winstanley et al. 2010; Broos et al. 2012). This chapter will examine the effects of PCBs and PBDEs on impulsive action.

Differential reinforcement of low rates of responding (DRL) is an operant task that assesses impulsive action. In typical DRL tasks, a subject presses a lever to initiate each trial, and then must withhold responding on the lever until a predetermined interval of time (required inter-response time or IRT) passes in order to receive reinforcement for the 2nd lever press. If the lever is pressed before the required IRT elapses, the trial clock is reset, and the subject must wait for another full required IRT to pass before a lever press results in reinforcement. Thus, DRL tasks assess the ability to withhold a response over time.

One advantage of DRL is that it assesses what Evenden (1999a) terms the “execution” stage of carrying out an action without involving overtly aversive punishment (e.g., an electric shock) for an incorrect action, thus avoiding the confounder of anxiety seen in some tests with an aversive component. It is generally accepted that DRL performance allows for assessment of response inhibition, with increases in lever pressing and decreases in reinforcers earned reflecting impulsivity (Evenden 1999b;
Monterosso & Ainslie 1999). Many species have been assessed using the DRL task (see Kramer & Rilling 1970 for an early review), and because homologous brain regions are known to be involved in mediating performance on this task in rodents and humans (Robbins et al. 2012), DRL tasks are well-suited for cross-species comparisons.

The main drawback of DRL is that task performance relies on other various aspects of cognition, including the ability to estimate time, and can be affected by alterations in motivation and attention (Bizot 1998). However this drawback can be said to be true for any behavioral task, so what is most important is to consider these other influences on performance when interpreting study results.

**Neurobiology of DRL Performance**

Anatomic regions important for DRL performance have been investigated, mostly by lesion studies. The roles of the frontal cortex, the dorsal striatum, and the nucleus accumbens have all been investigated in this manner.

Findings from studies where the medial prefrontal cortex (mPFC) was lesioned suggest involvement of this area in DRL performance. However the findings from different studies were not always consistent. Generally, when the more ventral region of the mPFC (prelimbic and infralimbic cortices) was damaged, DRL performance was impaired as indicated by increased frequency of lever pressing and decreased frequency of earning reinforcers in rats (Nonneman et al. 1974; Numan et al. 1975; Rosenklide & Divac 1975; Izaki et al. 2007) and mice (Cho & Jeantet 2010). Yet this did not hold true for all studies (Kolb et al. 1974). When only the dorsal aspect of the mPFC (cingulate cortex) was lesioned, DRL performance was not affected (Neill 1976; Finger
et al. 1987). Findings from studies where the ventrolaterally-located orbitofrontal cortex (OFC) was lesioned are less consistent, with both impairments (Kolb et al. 1974; Neill 1976) and improvements (Nonneman et al. 1974) in DRL performance reported. Given that the authors that did not find effects with mPFC lesions did find effects with OFC lesions, and vice versa, it is possible that differences in DRL methodology between studies may have influenced the findings.

The striatum also appears to have a role in DRL performance, although fewer studies have focused on this region. Lesioning the dorsal striatum in mice (Cho & Jeantet 2010) and the central caudate in rats (Schmaltz & Isaacson 1968) did not have an effect on DRL performance. However the nucleus accumbens (ventral striatum) does appear to have a role in DRL performance, with damage to this region, specifically the core of the nucleus accumbens rather than the shell, impairing successful DRL performance (Reading & Dunnett 1995; Pothuizen et al. 2005).

Thus, both the mPFC and the nucleus accumbens appear to have important roles in DRL performance, while the importance of the OFC and the dorsal striatum is less certain. These regions correspond with the ventromedial PFC and the nucleus accumbens in humans, which are considered important for response inhibition tasks that involve a “waiting” component (Robbins et al. 2012).

It is well established that dopaminergic signaling has an important role in response inhibition and, by extension, in impulsive behavior (Winstanley et al. 2006; Pattij & Vanderschuren 2008; Dalley & Roiser 2012). Evidence for the role of dopaminergic signaling is derived from a variety of studies including systemic injections of dopamine
agonists and antagonists, brain region-specific injections of dopaminergic drugs, and dopamine-specific lesioning studies.

In general, systemic administration of amphetamine (AMPH) increases lever pressing and decreases reinforcers earned in the DRL task in rodents (Robbins & Iversen 1973; Sanger et al. 1974; van Haaren et al. 1986; Wiley et al. 2000; Cheng & Liao 2007; Ferguson et al. 2007). However the effects are biphasic, with higher dosages (≥3.0 mg/kg) typically causing dramatic decreases in lever pressing, likely due to the stereotypies seen with higher AMPH doses (Sanger et al. 1974; van Haaren et al. 1986; Wiley et al. 2000). In accordance with this, treatment with the D1 receptor antagonist SCH 23390 decreased lever pressing, while combining SCH 23390 with AMPH resulted in a cancelling of each individual drug’s effect on lever pressing (Cheng & Liao 2007). The D2 autoreceptor antagonists raclopride (Cheng & Liao 2007) and haloperidol (van Hest 1988) similarly decreased lever pressing, while combining raclopride with AMPH attenuated the effects of either drug alone (Cheng & Liao 2007). Thus, D1 and D2 receptors both have roles in lever pressing on the DRL task, with lever pressing increasing when dopamine binds both receptors, and lever pressing decreasing when binding of dopamine to either receptor alone is antagonized.

The findings from studies focusing on the role of dopamine in specific brain regions in mediating DRL performance are in agreement with the systemic drug studies reviewed above. Infusion of dopamine and AMPH into the ventral anterior striatum in rats increased lever pressing (Neill 1976; Neill & Herndon 1978). In these studies, the infusions were dorsal to the nucleus accumbens. Sokolowski and Salamone (1994) investigated the role of dopamine in the mPFC by injecting 6-hydroxydopamine, which
is selectively neurotoxic for dopaminergic neurons, into the mPFC. They found that DRL lever pressing increased. Although these results seem paradoxical because they mirror those seen with AMPH, the authors speculate that it is possible that the prefrontal cortex inhibits subcortical motor activity. When dopaminergic input to the mPFC is removed, then inhibitory input to behavioral functions such as lever pressing, which is influenced by the striatum, may also be removed, resulting in increased lever pressing.

It is important to recognize that dopamine is not the sole neurotransmitter that modulates response inhibition. Serotonin (5-HT) also plays an important role, with the relative importance of dopamine and serotonin differing with the type of response inhibition and impulsivity (e.g. impulsive action vs. impulsive choice) (Pattij & Vanderschuren 2008; Eagle & Baunez 2010). Administration of a 5-HT$_{1A}$ receptor agonist resulted in decreased DRL lever pressing (Evenden et al. 1995), while 5-HT depletion in the nucleus accumbens, but not the frontal cortex, resulted in increased lever pressing (Fletcher et al. 2009). However results of a study of serotonergic manipulations in combination with AMPH suggest that while 5-HT has a role in DRL performance, the role of dopamine is more prominent (Mele & Caplan 1980).

**Previous Studies Examining the Effects of PCBs and PBDEs on DRL Performance**

Studies of DRL performance subsequent to developmental PCB exposure have demonstrated that PCBs exposure causes less efficient performance in rodents (Holene et al. 1999; Sable et al. 2006; Sable et al. 2009), monkeys (Rice 1998), and humans (Stewart et al. 2006). Studies utilizing the DRL task to examine the effects of PBDE exposure on response inhibition have not been performed. However, Rice et al. (2009)
determined that developmental PBDE exposure in mice resulted in increased lever pressing in the fixed interval (FI) task. In the FI task, there is a predetermined interval of time which must elapse before a lever press results in reinforcement. However, unlike with DRL, premature lever pressing is not penalized.

The effects of lactational exposure to the non-coplanar congener PCB 153 in female rats were examined by Holene et al. (1999). PCB 153 was chosen because it typically comprises a large portion of the sum of PCBs quantified in human biological samples (Longnecker 2003). This study was a follow-up to one in which male rats were exposed to PCB 153 via nursing (Holene et al. 1998). In the earlier study with males, PCB 153 resulted in increased lever pressing on the FI task. The study with females did not find any effects on FI performance, but then the subjects were placed on a conjunctive variable interval 120 sec.-DRL 14 sec. schedule which means that, on average, the DRL component of the schedule was in effect every 120 sec. The PCB-exposed females had shorter IRTs between lever presses and earned significantly less reinforcers than controls.

The effects of perinatal exposure to an environmentally relevant PCB mixture, the Fox River Mix (Kostyniak et al. 2005), on rats of both sexes was examined by Sable et al. (2006). In this study, dams were exposed to 0, 1, 3, or 6 mg/kg/day of the Fox River Mix. Offspring were tested on other behavioral tasks starting at post-natal day (PND) 90, so DRL 15 sec. testing did not begin until approximately PND 240 and then continued for 30 days. Differences between PCB-exposed groups and the control group were not seen. However, the DRL component was followed with an extinction component during which lever pressing was never reinforced. Subjects exposed to 6
mg/kg PCBs lever pressed more frequently than other groups during the extinction component.

In a subsequent study, which also examined developmental methylmercury exposure, PCB-only treatment groups included dams that were exposed to 0, 1, or 3 mg/kg/day of the Fox River Mix (Sable et al. 2009). DRL testing of the offspring began at approximately PND 180. Both the 1 and 3 mg/kg PCB groups had significantly smaller ratios of reinforced:non-reinforced responses. However there was not a significant difference in the number of lever presses or reinforcers delivered between these groups and the control group. The effect of 0.5 and 1 mg/kg AMPH on DRL performance was also evaluated. In controls of both sexes, 0.5 and 1 mg/kg AMPH significantly reduced the ratio of reinforced:non-reinforced lever presses in a dose-dependent manner. In PCB-exposed males (both treatment groups), 1 mg/kg AMPH did not decrease the ratio to the extent that it did in controls, while there was not a difference in the effect of AMPH in females exposed to PCBs versus controls. Thus the higher dose of AMPH was less disruptive to DRL performance in males developmentally exposed to PCBs than it was in control males, but the same was not true for females.

While no studies have examined the effects of PBDEs on DRL performance, in one study Rice et al. (2009) evaluated the effects of daily oral exposure of PBDE congener 209 (BDE 209) from PND 2 to 15 in mice on a FI schedule. No effects on FI performance were seen in subjects that began testing at approximately PND 90. However a separate cohort began testing at 16 months of age. In the older cohort, significant increases in lever pressing were seen in the 20 mg/kg exposure group as compared to controls and the 6 mg/kg exposure group.
In monkeys, performance on DRL was assessed in a cohort of males dosed with a PCB mixture from birth to 20 weeks of age (Rice 1998). The PCB mixture was synthesized to contain 15 PCB congeners typically found in human breast milk in Canada. At 4.5 years of age, the cohort was tested on a DRL 30 sec. schedule (Rice 1998). PCB-exposed subjects had increased responding, earned fewer reinforcements, and had shorter IRTs.

DRL has also been used to assess the consequences of prenatal PCB exposure in children. Stewart et al. (2006) evaluated the performance of 167 9.5-year old children on a DRL 20 sec. schedule during a 1 hour testing session. Both a decreased number of reinforcers earned and shorter IRTs were associated with higher concentrations of PCBs in the umbilical cord blood.

Based on what is known about the effects of PCBs and, to a much lesser extent, PBDEs on response inhibition, it was hypothesized that developmental PCB or PBDE exposure would result in poorer performance on DRL as compared to controls. Experimental measures of performance that could be affected included total lever presses, reinforcers earned, the ratio of reinforced:non-reinforced presses, and post-reinforcement pause.

Based on what is known about the effects of PCBs and PBDEs on dopaminergic signaling, it was further hypothesized that developmental PCB or PBDE exposure would result in a shift in the dose response curve to dopaminergic agents. More specifically, based on in vivo evidence that PCBs result in lower synaptic concentrations of dopamine (Seegal et al. 2002), AMPH administration to PCB- or PBDE-exposed rats was predicted to improve DRL performance at lower doses and impair performance at
higher doses, although to less of an extent than in controls. Also, the D1/D2 receptor antagonist flupenthixol (FLU) was predicted to impair DRL performance to a greater extent in PCB- or PBDE-exposed subjects than in controls.

2. Methods

2.1. Animals and Exposure

Eighty-nine female and 89 male Long Evans rats were purchased from Harlan Laboratories (Indianapolis, IN) in four cohorts spaced 11 weeks apart, on average. Efforts were made to balance the number of subjects in each contaminant treatment group within and across cohorts. The females were approximately 8-10 weeks and the males 10-12 weeks of age upon arrival. The rats were singly housed in polycarbonate shoebox cages with pine bedding in a temperature- and humidity-controlled room (22 °C, 40–55% humidity) on a 12-hour light–dark cycle (lights on at 0830 h). Food pellets (2020X Teklad Global Extruded Rodent Diet, Harlan Laboratories) and tap water were available ad libitum. All subjects were housed in facilities fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). The Institutional Animal Care and Use Committee (IACUC) at the University of Illinois at Urbana-Champaign approved all procedures, which were in accordance with the guidelines of the Public Health Service Policy on Humane Care and Use of Laboratory Animals (National Research Council, 2011).

Females were allowed to adapt to the vivarium for 6 days. They were then assigned to 1 of 5 exposure groups, balanced for body weight, and daily oral exposure to either PCBs or PBDEs exposure was initiated (see Table 3.1 for exposure groups). Exposure
continued for 28 days prior to breeding to establish a maternal burden of PCBs or PBDEs. Exposure then continued during breeding and through pregnancy and lactation until PND 21.

The PCB mixture, termed the Fox River Mix, was formulated to parallel the PCB congener profile found in walleye, a popular fish consumed by sport anglers, in the contaminated Fox River in Wisconsin (Kostyniak et al. 2005). The mixture consisted of a combination of 4 different Aroclor mixtures: 35% Aroclor 1242 (Lot KB 05-415, Monsanto), 35% Aroclor 1248 (Lot F-110, AccuStandards), 15% Aroclor 1254 (Lot KB 05-612, Monsanto), and Aroclor 1260 (Lot 021-020, AccuStandards). A new batch of the Fox River Mix was prepared for these studies using the same protocol described by Kostyniak et al. (2005). The Fox River Mix dosages of 3 and 6 mg/kg/day were chosen based on prior findings in this laboratory of effects on response inhibition as assessed by a DRL task (Sable et al. 2009) and extinction of DRL responding (Sable et al. 2006).

The commercial PBDE mixture, DE-71 (lot 7550OK20A, Great Lakes Chemical Corp.; gift of Kevin Crofton, USEPA), was chosen because its congener profile is similar to that found in fish in the Great Lakes (Manchester-Neesvig et al. 2001). Dreiem et al. (2010) suggested that the Fox River Mix is 2-3 times as potent as DE-71 in affecting dopaminergic signaling in vitro in rats. Given that 11.4 mg/kg DE-71 is isomolar to 6 mg/kg of the Fox River Mix, DE-71 dosages of 11.4 and 22.8 mg/kg were chosen in an effort to have PCB and PBDE dosages that were approximately equipotent to 3 and 6 mg/kg of the Fox River Mix.

For dosing, contaminants were diluted in corn oil (Mazola Corn Oil; ACH Food Companies, Inc.; Cordova, TN) and solutions were pipetted onto ½ of a vanilla wafer
cookie (Nabisco Nilla Wafers; Mondelez International; Deerfield, IL) at an appropriate volume (approximately 0.4 mL/kg body weight) to obtain the target dosages. For each subject, dosing volumes were adjusted daily according to body weight. The control subjects received cookies treated with corn oil alone. The cookies were fed to the females daily at approximately 1100 h. During breeding, the females were separated from the males each day for approximately 2 hours while the cookie was consumed.

The dosing solutions had target concentrations of 7.5 and 15 mg PCB/g, and 28.5 and 57 mg PBDE/g, respectively. After the solutions were prepared, contaminant concentrations in the corn oil solutions were confirmed in the analytical toxicology laboratory of Paul Kostyniak, State University of New York at Buffalo. The results were then used to adjust the volumes of dosing solution given to subjects, if necessary, to attain the target dosages.

2.2. Breeding, Pregnancy, and Weaning

Four weeks after contaminant exposure was initiated, each female was paired with a male in a hanging wire cage for 24 hrs daily, with the exception of the time females were removed for daily contaminant dosing. Food and tap water were available ad libitum. The wire cage allowed for daily assessment of sperm plugs that fell to the paper beneath, with the presence of sperm plugs being evidence that copulation occurred. Females that had evidence of sperm plugs by the 6th morning after pairing began were returned to their home cages for the duration of the experiment. Females that did not have evidence of sperm plugs were re-paired with different males that had produced sperm plugs with other females. Regardless of whether sperm plugs were seen upon
the 2\textsuperscript{nd} pairing, all remaining females were returned to their home cages on the 9\textsuperscript{th} morning.

Dosing was continued throughout pregnancy. Typically dams birthed, cleaned, and nursed their litters overnight, so if a clean litter with milk in their stomachs was present by 1100 h then that day was considered PND 0. If pups were present but had not been cleaned or nursed, a quick count of the number of pups was done and the dam and litter was not disturbed again until the afternoon when the number of pups was recounted. The presence of more pups indicated that parturition had continued and, thus, the next morning was considered PND 0. If no new pups were present, then the current day was considered PND 0. The pups were sexed and examined for gross abnormalities on PND 0. All males and then all females were weighed as 2 separate groups so that average male and female weights could be calculated. Females that did not give birth were retained for a minimum of 23 days past the 9\textsuperscript{th} morning of breeding, and then they were sacrificed and their uteri were examined for implantation sites.

Each litter had to have a minimum of 6 pups birthed by the dam to be included in the study. For litters with more than 8 pups, pups were culled to reduce the litter size to 8 pups on PND 2. If a litter had 6 pups and there was a litter with >8 PND 2 pups available, 1 or 2 pups from the larger litter were cross-fostered to the smaller litter, but only if the donor litter was either in the control treatment group or in a group that was exposed to an equal or lesser dose of the same contaminant. This was done to attempt to equalize the relative amount of contaminant exposure via nursing per pup across litters. A total of 9 pups were cross-fostered from 5 donor litters to 7 recipient litters. One ear of cross-fostered pups was clipped to permit identification so that developmental
data from these pups were excluded from the study. All cross-fostered pups were euthanized on PND 21. On PND 7, PND 14, and PND 21, individual pup weights were recorded for each litter. Beginning on PND 10, pups within each litter were examined for eye opening. The numbers of male and female pups with open eyes were recorded each day until all pups had open eyes.

On PND 21, pups in each litter were ideally allocated as thus: 2 male-female pairs for behavioral testing (one pair for the DRL task; one pair for the delay discounting task, see Chapter 4), 1 pair for western blot analysis of brains on PND 21, and 1 pair for western blot analysis of brains on PND 90. Since litters did not always have equal numbers of each sex, preference was given to forming pairs for behavioral testing. This sometimes resulted in only one sex from a litter being available for PND 21 or PND 90 western blot analysis. All dams and pups allocated for PND 21 analysis were euthanized by overexposure to CO₂ followed by decapitation. The pups' brains were quickly removed, weighed, and then snap frozen in liquid nitrogen. The brains were stored at -80° C until western blot analysis was performed. The dams' livers and the pups' livers and thymis were removed and weighed. The remaining pups were placed in a room with the same ambient and housing conditions described for the dams, except that the 12-hour light–dark cycle was reversed (lights off at 0830 h) for the remainder of the study.

2.3. Behavioral Testing Procedures

Beginning at approximately PND 30, remaining pups of the same sex and same contaminant exposure group were double- or triple-housed. Their ears were punched at
that time to facilitate individual identification. Starting at PND 70, food restriction commenced for pups that were to begin behavioral testing. This was necessary so that food pellets could be used as reinforcement for the behavioral task. Target weights were either 85% of the weight at PND 70 or 350 g for males over 360 g and 250 g for females over 260 g. Food was gradually restricted so that weight loss occurred over 2 weeks. Then enough food was provided to maintain body weight and allow for a maximum of 5 g weight gain each week until maximum weights of 350±25 g for males and 250±15 g for females were attained. Subjects allocated for western blot analysis of PND 90 brains were not food restricted. On PND 90 these subjects were euthanized by overexposure to CO$_2$ followed by decapitation. Brains were collected, frozen, and stored the same as for the PND 21 brains.

Behavioral testing was conducted in 12 automated operant chambers (Med Associates; St. Albans, VT) housed in sound attenuated cubicles, with each ventilated by a fan. Each operant chamber contained 2 retractable response levers located 6 cm above the floor on either side of the centrally located pellet trough. A stimulus cue light was positioned above each response lever. An external pellet dispenser delivered 45 mg AIN-76A purified rodent food pellets (TestDiet; Richmond, IN). An 80 decibel white-noise generator masked extraneous sounds, while a house light positioned at the rear of the chamber provided general illumination. The experimental contingencies were programmed using Medstate Notation programming language (Med Associates; St. Albans, VT).

**Autoshaping (Training Phase 1)**
Daily sessions of operant testing were conducted 6 days per week excluding Sundays. Beginning at PND 90, an autoshaping program (training phase 1) was used to train the subjects to press the response levers. Both levers were extended for the entire session. The right cue light was programmed to illuminate for 15 sec every 3 minutes. If either lever was pressed while the cue light was illuminated, the cue light was extinguished and the response was reinforced. Otherwise, the cue light was extinguished and reinforcement was delivered at the end of the 15 sec period even though a response did not occur. Reinforcement consisted of one food pellet being dispensed and a 90 decibel tone sounding for 40 msec. If either lever was pressed before the 15 sec period began, reinforcement was delivered and the cue light above that lever illuminated for 40 msec. After 10 lever presses were attained by pressing any combination of response levers, delivery of reinforcers became contingent on lever presses so that every lever press was reinforced (i.e., a fixed ratio 1 (FR1) contingency). The house light and white noise remained on for the entire session. Autoshaping sessions terminated after either 60 minutes had elapsed or 100 reinforcers were delivered. Criterion for advancement to the next phase of training was 99 or 100 lever presses for 2 consecutive sessions. Autoshaping was completed in 2.8 sessions on average (range 2 to 4).

**Fixed Ratio Training (Training Phases 2 and 3)**

The next two training phases elicited lever pressing in response to cue light illumination. At the onset of each session for training phase 2, one of the levers was extended at random and the cue light above it was illuminated. Every response was
reinforced, thus continuing the FR1 contingency. After 5 lever presses, the lever retracted and the cue light extinguished while the opposite lever was extended and the opposite cue light illuminated. Active levers and lights alternated after every 5th lever press so the subject would not learn to associate reinforcement with a particular lever (i.e., develop a side bias). White noise was present but the house light was not illuminated throughout this and all subsequent phases. Animals were required to earn 100 reinforcers across three consecutive sessions of phase 2 in order to move to the next training phase. This phase was completed in 3 sessions on average (all subjects completed in 3 days except for one that was mistakenly tested for only 2 days). Training phase 3 followed the same contingencies as phase 2 except that only the right response lever was used, which remained true for all remaining phases of the experiment. Subjects trained on phase 3 for 2 sessions with no criterion for advancement, except 3 subjects were mistakenly trained for 3 days.

**DRL Training (Training Phases 4-6)**

The next 3 phases of training introduced delays during which the subject had to withhold lever pressing in order to receive reinforcement. All trials were initiated by a lever press, and sessions terminated after 90 min elapsed or 200 reinforcers were delivered. During training phase 4, a 5 sec minimum required IRT was required in order to receive reinforcement (DRL 5). If the subject initiated a trial and then pressed the lever before 5 sec elapsed, the clock was reset so that the subject had to wait a minimum of 5 sec before pressing again in order to receive reinforcement. DRL 5 training lasted 2 sessions, except for 3 subjects mistakenly trained for 3 days. Phase 5
required a 10 sec interval to elapse in order for presses to be reinforced (DRL 10). DRL 10 training lasted 2 sessions for all subjects. The required IRT in phase 6 was 15 sec (DRL 15). Subjects remained on this phase for a minimum of 30 daily sessions (range 30 to 34, average 31.8) except for one subject that was mistakenly tested for 29 days. The drug trials commenced for all subjects on the same day.

**Drug Trials (Phase 7)**

The experimental parameters for phase 7 were identical to those in phase 6: DRL 15. AMPH dosages were selected based on those used in similar DRL studies (Sanger et al. 1974; van Haaren et al. 1986; Ferguson et al. 2007). Because studies examining the effects of acute FLU administration on DRL performance could not be located, FLU dosages were selected based on those used in studies of a different task of response inhibition, delay discounting (Cardinal et al. 2000;Floresco et al. 2008). Drugs were prepared each day of administration and then protected from light to prevent photodecomposition. Each drug was administered at 4 different doses, including a saline-only vehicle control which ensured that each subject served as its own control. *cis*-Flupenthixol (FLU) (Sigma-Aldrich; St. Louis, MO) was prepared on injection days by dissolving the drug in 0.9% sterile saline to concentrations of 0 (vehicle), 0.05, 0.125, and 0.25 mg/mL for respective doses of 0, 0.05, 0.125, and 0.25 mg/kg. *d*-Amphetamine sulfate (AMPH) (Sigma-Aldrich; St. Louis, MO) was prepared on injection days by dissolving the drug in 0.9% sterile saline to concentrations of 0 (vehicle), 0.25, 0.5, and 1.0 mg/mL for respective doses of 0, 0.25, 0.5, and 1.0 mg/kg. Intraperitoneal injections of FLU and AMPH were administered 30 and 10 minutes, respectively, before
testing, based on their pharmacokinetic properties (Jorgensen et al. 1969; Kunh and Schanberg 1978).

Injections were administered in 3 successive blocks: FLU, then AMPH, then FLU/AMPH combined (COMBO) (see Figure 3.1). Each dose within a block was administered once to each subject, with the dosing order individually randomized using a balanced Latin Square design. Drugs were administered on Tuesdays and Fridays, with 7 calendar days between each block of injections. Additional saline injections were given both two and one day before the FLU block, one day before the AMPH block, one day before the COMBO block, and one day after the COMBO block. The first additional saline injection was performed to acclimate the subjects to the injection procedure. Results from the subsequent additional saline days were examined to assess whether baseline performance remained stable across the drug trials.

2.4. Data Analysis

All statistical analyses were conducted using SPSS for Windows (version 20.0, SPSS Inc.; Chicago, IL) with statistical significance set at \( p < 0.05 \). For some repeated-measures factors, a sphericity violation was noted. In these instances a Greenhouse-Geisser correction was used to reduce the risk of a Type I error if \( \varepsilon < 0.75 \) and a Huynh-Feldt correction was used if \( \varepsilon \geq 0.75 \) (Rogan et al. 1979). Analyses requiring these corrections are reported using the adjusted degrees of freedom rounded to the nearest integer. When significant main effects or interactions were obtained, post hoc one-way ANOVA followed by Tukey’s least significant difference (LSD) analyses were performed. Data are reported as mean ± SEM.
Reproductive and Developmental Endpoints

Reproductive endpoints (see Table 3.1) included the live birth rate, which is the percent of dams delivering ≥1 live pup. If a dam delivered any live pups then endpoints from her litter were included in analyses of litter size, percent live pups delivered, percent male pups delivered, and percent gestational weight gain. Litter size was the number of live pups delivered on PND 0. Percent live pups delivered was litter size / the number of uterine implantation sites. Percent gestational weight gain was (gestation day 21 weight – gestation day 0 weight) / gestation day 0 weight. In addition, percent lactational weight gain and ratio of liver weight:body weight on PND 21 were measured for the dams with litters with ≥6 pups. Percent lactational weight gain was (highest lactational weight – weight after parturition) / weight after parturition. Dependent measures were analyzed using between-subjects ANOVAs with treatment group as the independent variable.

Developmental endpoints (see Table 3.2) included weights on PND 0 (birth weight), PND 7, PND 14, and PND 21 (weaning weight). Ratios of brain, liver, and thymus to body weight, and day of eye opening (of at least 1 eye), were also examined. Dependent measures were analyzed using between-subjects ANOVAs with treatment group and sex as independent variables.

A total of 66 dams and their litters had data included. Of the 89 dams that began the study, 23 had data excluded for the following reasons: failure to eat daily cookie (1), not pregnant (10), placentation sites present but no live pups delivered (2), <5 pups in litter
(6), poor maternal care and/or cannibalization of pups (4). Seven control dams were among the 23 excluded.

**DRL Dependent Measures**

Sixty male-female pairs completed behavioral testing and were included in the analyses of drug trials. Their allocation to treatment groups is as follows: 12 pairs of control, 13 pairs of 3 mg/kg PCB, 11 pairs of 6 mg/kg PCB, 11 pairs of 11.4 mg/kg PBDE, and 13 pairs of 22.8 mg/kg PBDE. Three pairs of subjects from the 6 mg/kg PCB group were removed from the study: 1 pair because the female died of unknown causes before PND 90, and 2 pairs because 1 member of each pair was injured. The data from one of the latter pairs was included in the analyses of training data but not of drug trials. Three additional litters were not included in the study: 1 litter had all female pups, and 2 litters had only 1 male-female pair which were assigned to a concurrent behavioral study rather than to the DRL study.

Five dependent measures were evaluated. *Total lever presses* were presses that occurred during the IRT phase of DRL, so presses that initiated each trial were excluded. *Reinforcers earned* reflects the number of lever presses that resulted in delivery of reinforcement (i.e., presses that occurred after the required IRT elapsed). The *ratio of reinforced:non-reinforced presses* is the number of presses that were reinforced divided by the number of presses that were premature and thus did not result in reinforcement. *Post-reinforcement pause* spans the time from when the subject made a reinforced lever press to the time when it made the next lever press to begin a new
trial. The latency to collect reinforcement is the length of time the subject took to collect the food pellet after it made a reinforceable press.

**Training**

The dependent measures total lever presses and reinforced:non-reinforced lever presses from the first day of DRL 5, DRL 10, and DRL 15 were analyzed separately using 2 (sex) x 5 (treatment) mixed ANOVAs with sex (nested within litter) as the repeated measures factor (Hughes 1979). Also, total lever presses and reinforced:non-reinforced lever presses from the first 30 days of DRL 15 were averaged across six day blocks to yield 5 blocks of testing. These measures were then separately analyzed using 5 (block) x 2 (sex) x 5 (treatment) mixed ANOVAs with block and sex (nested within litter) as the repeated measures factors.

Performance on all 5 dependent measures (total lever presses, ratio of reinforced:non-reinforced presses, reinforcers earned, post-reinforcement pause, and latency to collect reinforcement) was separately examined for testing block 5 using sex (2) x treatment (5) repeated-measures ANOVAs with sex (nested within litter) as the repeated measures factor. This was done in order to examine baseline performance before drug trials began.

**Drug Trials**

All 5 dependent measures (total lever presses, ratio of reinforced:non-reinforced presses, reinforcers earned, post-reinforcement pause, and latency to collect reinforcement) were analyzed separately for FLU, AMPH, and COMBO trials using 4
(dose) x 2 (sex) x 5 (treatment group) mixed ANOVAs with dose and sex (nested within litter) as the repeated measures factors. For post-reinforcement pause, 1 outlier was noted at 0.05 mg/kg FLU which increased the mean pause time for the same sex and treatment group by 72%. For latency to collect reinforcement, 1 outlier was detected at 0.25 mg/kg FLU and 3 were detected for 0 mg/kg AMPH which increased the mean latencies for the same sex and treatment groups by 44% to 91%. Due to the pronounced effects on performance measures that these 5 outliers had, each of their values was mean replaced with the average performance of other members of the same sex and treatment group.

3. Results

3.1. Reproductive and Developmental Endpoints

No overt clinical signs of toxicosis were noted in the dams. There were no significant differences between treatment groups for the following dam-related measures: live birth rate, litter size, percent live pups, percent male pups, percent gestational weight gain, and dam’s liver weight:body weight ratio (p=0.095 for the last measure). The main effect of treatment for percent lactational weight gain was significant [F(4,61)=3.0, p=0.026]. However post-hoc comparisons between groups did not detect any significant differences between treatment groups. Upon visual inspection of the data, percent lactational weight gain appeared to be less for the 3 mg/kg PCB and 11.4 mg/kg PBDE dams as compared to the control and 6 mg/kg PCB dams. Values for each treatment group are reported in Table 3.1.
No overt clinical signs of toxicosis were noted in the pups. Treatment group significantly affected pup weights at all days measured: birth \( [F(4,140)=8.8, p<0.001] \), PND 7 \( [F(4, 126)=4.7, p=0.002] \), PND 14 \( [F(4,126)=7.2, p<0.001] \), and PND 21 \( [F(4,126)=19.9, p<0.001] \) (see Table 3.2). Post-hoc analysis revealed that at birth, the weights of the 22.8 mg/kg PBDE group were greater than the weights of control and both PCB groups (all \( p<0.05 \)). On PND 7, the 22.8 mg/kg PBDE group no longer weighed more than the control group, but did still weigh more than the 6 mg/kg PCB group \( (p<0.001) \). Although they were not significantly different from controls at birth or PND 7, by PND 14 and 21, the body weights in both PCB groups were significantly less than the control and both PBDE groups (all \( p<0.05 \) at PND 14, all \( p\leq0.005 \) at PND 21). At weaning, the 3 mg/kg PCB group weighed 91% of control and the 6 mg/kg group weighed 85% of control.

The most notable organ weight finding for the pups was the ratio of liver:body weight, which was significantly affected by treatment \( [F(4,103)=124.3, p<0.001] \). Post-hoc analysis revealed that all PCB and PBDE groups had greater liver:body weight ratios than control (all \( p<0.001 \)), and also that both PCB and the 22.8 mg/kg PBDE group had greater liver weight ratios than the 11.4 mg/kg PBDE group (all \( p<0.005 \)).

Thymus:body weight ratio was also significantly affected by treatment \( [F(4,103)=22.3, p<0.001] \). Post-hoc analysis revealed that both PCB groups had smaller thymus:body weight ratios than the control group and both PBDE groups (all \( p\leq0.01 \)). None of the treatment groups differed from control with respect to brain:body weight ratio.
The average day of eye opening was significantly affected by treatment \[F(4,125)=3.6, \ p=0.008\], with *post-hoc* analysis revealing that the 6 mg/kg PCB group opened their eyes 1.3 and 1.2 days earlier than the control and 22.8 mg/kg PBDE groups, respectively (all \(p<0.05\)).

### 3.2. Training

#### Day 1 of DRL 5, DRL 10, DRL 15

Contaminant treatment group was not a significant factor for any of the days examined, nor were there any significant interactions with treatment. Males lever pressed significantly more \[F(1,56)=23.6, \ p<0.001\] (see Figure 3.2), resulting in a significantly smaller ratio of reinforced:non-reinforced lever presses \[F(1,56)=13.9, \ p<0.001\] (not shown) than females on the first session of DRL 5. However sex was not a significant factor for either measure on day 1 of DRL 10 or DRL 15.

#### Training During DRL 15 Phase

Analysis of the data from the DRL 15 training phase did not find a main effect of treatment, but a main effect of blocks resulted from significant decreases in total lever presses \[F(2,108)=88.3, \ p<0.001\] (see Figure 3.3) and increases in reinforced:non-reinforced presses in all groups \[F(3,195)=103.5, \ p<0.001\] (see Figure 3.3). Neither the main effect of sex nor any of the interactions were significant.

Evaluation of all 5 dependent measures (total lever presses, ratio of reinforced:non-reinforced presses, reinforcers earned, post-reinforcement pause, and latency to collect reinforcement) during block 5 did not reveal significant effects of treatment group or sex.
for any of the measures. Analyses were done in order to examine baseline performance after it had stabilized and just before drug trials began. In block 5, there were an average of 299.6 ± 6.6 total lever presses and 121.2 ± 2.6 reinforcers earned per session. The average ratio of reinforced:non-reinforced lever presses was 0.843 ± 0.036. The average post-reinforcement pause was 17.3 ± 0.6 sec while the average latency to collect reinforcement was 0.42 ± 0.01 sec.

3.3. Drug Trials

Total Lever Presses During Drug Trials

While lever pressing during FLU trials was not significantly affected by contaminant treatment group, lever pressing decreased with increasing doses of FLU [F(2,119)=43.9, p<0.001] (see Figure 3.4.A). Post-hoc analysis revealed that the administration of 0.25 mg/kg FLU resulted in significantly less lever pressing than when vehicle or the 2 lower dosages were administered (all p<0.001). Administration of 0.125 mg/kg also resulted in less lever pressing than the vehicle control (p=0.029). Lever pressing decreased to 89% and 64% of control at 0.125 and 0.25 mg/kg, respectively. Neither the main effect of sex nor any of the interactions were significant.

Although treatment group did not have an effect, lever pressing increased with increasing doses of AMPH [F(2,98)=74.5, p<0.001] (see Figure 3.4.B), in contrast to the drug dose effect of FLU. Post-hoc analysis revealed that the administration of 1.0 mg/kg resulted in significantly more lever pressing than when the vehicle control or the 2 lower dosages were administered (all p<0.001). Administration of 0.5 mg/kg also resulted in more lever pressing than the vehicle control (p=0.009). Lever pressing increased to
122% and 185% of control at 0.5 and 1.0 mg/kg, respectively. Neither the main effect of sex nor any of the interactions were significant.

For the drug combinations, treatment group was not significant, but drug dose \([F(3,155)=25.3, p<0.001]\) and sex \([F(1,55)=4.8, p=0.033]\) were significant factors. However, none of the interactions, including the dose x sex interaction, were significant. \textit{Post-hoc} analysis of drug dose revealed that the 0.125/1.0 mg/kg COMBO resulted in significantly more lever pressing than other dosages (all \(p<0.001\)) (see Figure 3.4.C). However \textit{post-hoc} comparison of sex at each dose determined that there were no significant sex differences at any of the individual combination doses.

**Reinforcers Earned During Drug Trials**

The number of reinforcers earned was not affected by contaminant treatment, but it did decrease with increasing doses of FLU \([F(2,100)=74.5, p<0.001]\) (see Figure 3.5.A). Neither the main effect of sex nor any of the interactions were significant. \textit{Post-hoc} analysis revealed that significantly fewer reinforcers were earned after 0.25 mg/kg FLU than with the other 3 doses (all \(p<0.001\)). Additionally, significantly fewer reinforcers were earned with 0.125 mg/kg than with the vehicle control or the 0.05 mg/kg FLU dose (all \(p<0.05\)). The 0.125 and 0.25 mg/kg FLU doses resulted in only 90% or 63%, respectively, of reinforcers earned relative to the vehicle control.

Reinforcers earned were not affected by treatment, but they decreased with increasing doses of AMPH \([F(3,151)=80.4, p<0.001]\) (see Figure 3.5.B), similar to the effect of dose for FLU. Additionally, the main effect of sex was significant \([F(1,55)=6.8, p=0.011]\), but the interaction between sex and dose was not. Females earned less
reinforcers than males. The effect of sex was not seen with FLU or during initial pre-drug testing. Other interactions were not significant. *Post-hoc* analysis revealed that significantly fewer reinforcers were earned after 1.0 mg/kg AMPH than with the other 3 doses (all *p*<0.001). Additionally, significantly fewer reinforcers were earned with 0.5 mg/kg than with control or 0.25 mg/kg AMPH (*p*<0.001). The 0.5 and 1.0 mg/kg AMPH doses resulted in only 87% or 60%, respectively, of reinforcers earned relative to the vehicle control.

For drug combinations, treatment group was not significant, but drug dose 
\[F(3,150)=78.9, \ p<0.001\] and sex 
\[F(1,55)=4.9, \ p=0.031\] were significant factors, with the dose x sex interaction also being significant 
\[F(3,160)=4.5, \ p=0.005\]. Other interactions were not significant. *Post-hoc* analysis revealed that at 0.125/1.0 mg/kg COMBO males earned significantly more reinforcers than females exposed to the same combined FLU/AMPH doses (*p*=0.018), but there were no significant sex differences at any of the other dose combinations (see Figure 3.5.C). Regarding responses to doses within each sex, all 3 COMBO doses resulted in females earning significantly less reinforcers when compared to the vehicle control (all *p*≤0.001) (see Figure 3.5.D). Also females earned significantly less reinforcers at 0.125/1.0 mg/kg than at 0.125/0.5 mg/kg (*p*=0.001). Males receiving the 0.125/1.0 and 0.25/1.0 mg/kg COMBO drug doses earned significantly fewer reinforcers than at 0/0 and 0.125/0.5 mg/kg (all *p*<0.001 except *p*=0.015 for the comparison of 0.125/0.5 to 0.125/1.0) (see Figure 3.5.D).

**Ratio of Reinforced:Non-Reinforced Lever Presses During Drug Trials**
For the FLU trials, contaminant treatment, drug dose, and sex did not significantly affect the ratio of reinforced:non-reinforced presses. There were no significant interactions.

While treatment did not have a significant effect during the AMPH trials, increasing doses of AMPH decreased the ratio of reinforced:non-reinforced presses [F(3,155)=50.3, p<0.001] (see Figure 3.6.A). Neither the main effect of sex nor any of the interactions were significant. *Post-hoc* analysis revealed that the ratios of reinforced:non-reinforced presses at 0.5 and 1.0 mg/kg were each significantly different from the ratios at all other doses, with the ratios at 0.5 and 1.0 mg/kg being 61% and 32% of the vehicle control, (all p<0.005, all p<0.001 respectively).

For combined FLU/AMPH, treatment was not significant, but drug dose was a significant factor [F(3,142)=53.0, p<0.001] (see Figure 3.6.B). Neither the main effect of sex nor any of the interactions were significant. *Post-hoc* analysis revealed that all combination dosages resulted in decreases in the ratio of reinforced:non-reinforced presses compared to vehicle control (all p<0.001). Furthermore the ratios seen with 0.125/1.0 and 0.25/1.0 mg/kg COMBO did not differ from each other but were significantly less than the ratio with 0.125/0.5 mg/kg (all p<0.05).

**Post-Reinforcement Pause During Drug Trials**

While contaminant treatment did not affect post-reinforcement pause, increasing FLU doses increased the pause time [F(2,120)=28.8, p<0.001] (see Figure 3.7). Neither sex nor any interactions were significant. *Post-hoc* analysis revealed that the post-reinforcement pause at a dose of 0.25 mg/kg was significantly greater than the pause
after the vehicle control and 0.05 mg/kg dose (all $p \leq 0.001$), with the pause time increasing 145% from 17.1 sec after vehicle control to 24.8 sec after the 0.25 mg/kg dose.

Treatment had no effect on post-reinforcement pause during the AMPH and COMBO trials. While AMPH dose had no effect on post-reinforcement pause, the main effect of dose for the COMBO trials was significant [$F(2,99) = 4.5$, $p = 0.016$]. However, none of the post-hoc comparisons between the individual doses were significant, although visual inspection of the graph (not shown) suggests that there was a trend for post-reinforcement pause to be longer at the 0.25/1.0 mg/kg COMBO. Neither sex nor any interactions were significant for the AMPH or COMBO trials.

**Average Latency to Collect Reinforcement During Drug Trials**

Contaminant treatment did not have a significant effect on latency to collect reinforcement during any of the drug trials. Increasing FLU doses increased the latency to collect reinforcement [$F(2,101) = 12.2$, $p < 0.001$] (see Figure 3.8). Specifically, the latency was longer at the 0.25 mg/kg dose than at the vehicle control or 0.05 mg/kg FLU doses (all $p \leq 0.001$), increasing 130% from 0.40 sec at control to 0.52 sec at 0.25 mg/kg. AMPH dose alone did not affect the latency, but when both FLU and AMPH were administered together, dose had a significant effect on latency [$F(2,106) = 3.9$, $p = 0.025$] (not shown). Post-hoc analysis revealed that increasing the dose of FLU to 0.25 mg/kg significantly prolonged the latency as compared to 0.125/0.5 mg/kg ($p = 0.047$) and 0.125/1.0 mg/kg ($p = 0.018$) COMBO. Yet none of the combination dosages resulted in a
latency that was significantly different than the control. Sex did not have an effect on the latency during any of the drug trials, nor were any interactions significant.

**Stability of Performance During Drug Trials**

Total lever presses, reinforcers earned, and the ratio of reinforced:non-reinforced lever presses were compared across the 4 saline days that were included before each block of drug injections and at the end of the experiment. Although saline injection day was significant for total lever presses, reinforcers earned, and the ratio of reinforced:non-reinforced lever presses in the repeated-measures analyses (all $F \leq 4.5$, all $p < 0.05$), performance between individual saline injection days did not significantly differ on any of the *post-hoc* comparisons. Visual inspection of the data (not shown) supports that performance did not change over the course of the drug trials.

**4. Discussion**

The doses of PCBs and PBDEs used in this study were sufficient to cause systemic changes including increased liver weights and decreased thymus weights, as well as reductions in pup body weights, at weaning; yet the exposures did not have long term effects on inhibitory control as assessed in the DRL task and did not alter the pattern of response to pharmacologic challenges with the dopaminergic drugs AMPH and FLU on the DRL task. PCB and PBDE treatment did not affect total lever presses, reinforcers earned, the ratio of reinforced:non-reinforced presses, post-reinforcement pause, or the latency to collect reinforcement. The absence of contaminant effects is contrary to the experimental hypotheses. The absence of effects is also contrary to the majority of prior
findings including those of Holene et al. (1999) and Sable et al. (2009) in rats, Rice (1998) in monkeys, and Stewart et al. (2006) in humans, but is consistent with a Sable et al. (2006) study in rats.

**Lack of Contaminant Effects on Behavior at Baseline (Block 5)**

The main goals of this study were to determine whether PCBs or PBDEs cause impulsive performance on DRL or cause differences in dopaminergic-signaling that result in changes in DRL performance that could be detected by drug challenge. However, there were no differences in DRL performance between PCB- or PBDE-exposed groups and controls either before or during challenges.

Because prior studies have shown that PCB exposure impairs DRL task performance (Holene et al. 1999; Sable et al. 2009), it is important to consider the differences between the current study and the prior ones to attempt to understand the results. The study design by Holene et al. (1999) differs the most from the current study. Dams were dosed with 5 mg/kg of one congener, PCB 153, every other day from PND 3 to 13 (6 doses total). Then multiple female pups from each litter underwent behavioral testing, first on a FI-extinction task, then on a conjunctive variable interval 120 sec.-DRL 14 sec. schedule. The latter schedule results in a very low rate of responding because not only must a subject wait at least 14 sec. to lever press to satisfy the DRL component, but reinforcers are only available to be delivered every 120 sec., on average, as dictated by the variable interval component. It was found that the PCB 153-exposed females pressed more frequently during the initial sessions of the task, but that there was no difference with controls by the later sessions of the task.
The current study differs from the PCB 153 study in that a mixture of PCB congeners was evaluated and the subjects were tested solely on a DRL task, with no prior testing experience. The Fox River Mix is comprised of numerous congeners, including PCB 153. Prior studies have demonstrated that individual PCB and PBDE congeners can differ greatly in their effects on intracellular endpoints, especially on calcium sequestration and release, and protein kinase C translocation (Kodavanti & Ward 2005; Fonnum et al. 2006). So the neurobehavioral effects of a PCB mixture would not necessarily be expected to be similar to that of an isolated congener. Another consideration is that the contingencies of the variable interval-DRL schedule likely tax subjects’ response inhibition greater than DRL alone because appropriately timed lever presses that meet the DRL contingency often go unrewarded because of the variable interval contingency. Similar to this, Mele et al. (1986), in a study evaluating perinatal exposure to Aroclor 1248 in monkeys performing a FI task, only elicited increased responding in the PCB-exposed subjects when 25% of the earned reinforcers were randomly omitted. Thus it is possible that the contingencies of the DRL 15 schedule in the current study do not sufficiently tax response inhibition mechanisms to the extent that differences caused by contaminant exposure can be detected.

The findings of Sable et al. (2006) more closely match the current findings. Similar experimental methods were followed in that study, namely a perinatal exposure paradigm in Long Evans rats including daily Fox River Mix dosages of 0, 3, and 6 mg/kg. No significant differences between PCB-exposed subjects and controls were found in the DRL component of the study. Increased lever pressing in PCB-exposed subjects was seen in the extinction component of the study. The current study did not
include an extinction component after the initial training period because drug challenges were administered. A notable difference between studies is that in the 2006 study the subjects did not begin DRL until approximately PND 240, after completing testing on spatial reversal learning and delayed spatial alternation, whereas subjects began testing at PND 90 with no prior testing experience in the current study.

A second study that closely matches the current one in methodology is that by Sable et al. (2009). In that study, there were 1 and 3 mg/kg PCB treatment groups, as compared to 3 and 6 mg/kg groups in the current study. A clear dose response of decreasing ratio of reinforced:non-reinforced presses with increasing PCB dose was seen. Additionally, both groups of PCB-exposed males, but not females, were less sensitive to the disruptive effect of 1 mg/kg AMPH on reinforced:non-reinforced responses. Similar to the 2006 study, the subjects in the 2009 study did not begin DRL until approximately PND 180, after completing testing on spatial reversal learning, delayed spatial alternation, and differential reinforcement of high rates of responding, compared to the current study in which testing began at PND 90 with no prior testing experience.

One possible explanation for the difference in findings is that a DRL 15 sec schedule is not as sensitive to contaminated-related changes as would be ideal. While the ratio of reinforced:non-reinforced responses was significant in the 2009 study, total lever presses and reinforcers delivered, which are the two values used to determine the ratio, did not differ between PCB groups and controls. If this is true, then it is possible that unrecognized factors, such as a greater degree of individual variation between subjects
or the extent of prior testing experience, influenced performance to a great enough extent that any effects due to contaminant exposure were obscured.

Another possibility related to the issue of task sensitivity is whether there are strain differences in DRL performance that may make some strains less suited for detecting differences in performance. If a strain is already relatively impulsive, then it may be harder to detect whether a treatment, be it a contaminant or a drug, makes subjects from that strain more impulsive. The question of whether Long Evans rats may be too impulsive to reliably detect changes that have small to moderate effect sizes is interesting, but it awaits further research before it can be answered.

Another factor that differed between this and the two prior studies was a dietary change made in the current study to reduce exposure to phytoestrogens. In the 2006 and 2009 studies, the subjects were housed with corn cob bedding and fed 8604 diet (Teklad Global Extruded Rodent Diet, Harlan Laboratories). In the current study, the subjects were housed on pine bedding and fed 2020X diet, which is described by the manufacturer as a “minimal” phytoestrogen diet (Harlan Laboratories 2013). Although it seems unlikely, it can not be ruled out that there was a dietary interaction between the 8604 diet (which varies greatly from lot to lot in phytoestrogen content) and the PCBs in the 2009 study which was altered by reducing phytoestrogen exposure in the current study. PCBs (Dickerson & Gore 2007) and PBDEs (Ceccatelli et al. 2006; Hamers et al. 2006) potentially interact with both endogenous estrogen and testosterone. By reducing phytoestrogen exposure in the current study, the balance of endogenous hormones, hormonal xenobiotics, or both in the subjects could have been altered through
mechanisms such as increased biotransformation and clearance (Kirk et al. 2001), resulting in an attenuation of behavioral effects that are mediated hormonally.

The factors which may have reduced the sensitivity of the DRL to detect differences between treated and control subjects may also explain why the findings of the current study were not concordant with previous studies in monkeys (Rice 1998) or humans (Stewart et al. 2006). However, until questions about possible intra-species influences on DRL performance, such as strain differences, are more closely explored, it will remain difficult to make inferences from the findings of the current study to other species.

**Non-Contaminant Effects During Training**

With toxicant-naïve subjects, lever pressing increases on days in which a transition to a longer delay occurs during the DRL task (Pizzo et al. 2009). A prior study of adolescent exposure to Aroclor 1248 revealed that PCB-treated subjects lever pressed on the FI task more frequently than controls on the first day of transitioning to schedules in which a longer period of time elapsed before reinforcement became available (Berger et al. 2001). However, in the current study, PCB or PBDE exposure did not result in a change in frequency of lever pressing relative to controls on these transitional days. Of interest, males lever pressed more frequently than females on the first day of DRL 5, while there were no differences in lever pressing between sexes in the subsequent phases of the experiment. Increased lever pressing by males has been noted in at least one prior study of DRL 15 performance in rats (van Hest et al. 1987). However the
finding of no sex difference in subsequent phases of the task parallels results of DRL studies performed in our lab (Sable et al. 2009) and others (van Haaren et al. 1986).

The finding that total lever presses decreased while the ratio of reinforced:non-reinforced presses increased over the 30 days of DRL 15 training indicates that the subjects learned the experimental contingencies, with their performance becoming increasingly more efficient over the training period. PCB or PBDE exposure did not result in differences in these learning curves, or differences at the end of the training period, that differed from controls.

Non-Contaminant Effects During Drug Trials

AMPH increased lever pressing while FLU decreased lever pressing in DRL 15 in a dose-dependent manner. Additionally, both AMPH and FLU decreased reinforcers earned in a dose-dependent manner. Also, FLU significantly increased the post-reinforcement pause and the latency to collect reinforcement at the highest dosage, 0.25 mg/kg. These findings confirm that dopaminergic signaling influences DRL performance in the absence of contaminant treatment effects.

The finding that AMPH increases DRL lever pressing is consistent with prior studies (Robbins & Iversen 1973; Sanger et al. 1974; van Haaren et al. 1986; Wiley et al. 2000; Cheng & Liao 2007; Ferguson et al. 2007). Several studies have shown that AMPH administration results in a shift to shorter IRTs (Sanger et al. 1974; Wiley et al. 2000; Cheng & Liao 2007). The end result of the IRT shift is that premature responding increases, thus the trial timer is reset more frequently and less reinforcers are earned. The increase in lever pressing was marked enough to greatly reduce the ratio of
reinforced:non-reinforced lever presses. Importantly, AMPH did not alter post-reinforcement pause or the latency to collect reinforcement at the doses used, so the changes can not be attributed to changes in motoric ability or motivation of the subjects.

The effects of FLU on DRL performance have not been reported before, with the exception of a study in which rats were initially trained on DRL, then given 36 weekly injections of a long-lasting depot formulation of FLU, and finally retested on DRL 7 weeks after the last FLU injection (Nielsen 1977). Due to dramatically different study designs, the findings of the 2 studies are difficult to compare, although both found that FLU resulted in decreased lever pressing on a DRL schedule. In the current study, less reinforcers were earned at the higher dosages, although the extent to which lever pressing decreased was not enough to significantly alter the ratios of reinforced:non-reinforced presses. The latency to collect reinforcers was significantly increased at 0.25 mg/kg. This may suggest that the highest dose of FLU affected the subjects’ motivation, but an increase in latency of 0.12 sec above control by itself does not suggest a large motivational deficit. However, a 7.7 sec increase in post-reinforcement pause time above control at 0.25 mg/kg makes it more difficult to discount the argument that motivational and motoric effects are contributing to the impaired performance at the highest FLU dose.

An interesting finding of the COMBO trials is that when AMPH and FLU were administered in a 4:1 dosage ratio, the effects of increased dopamine release by AMPH and D1/D2 receptor antagonism by FLU on lever pressing were balanced so that total lever presses did not differ from control. Increasing the AMPH:FLU ratio to 8:1 resulted
in increased lever pressing, suggesting that FLU is a competitive inhibitor of the effects of dopamine on DRL performance.

Sex effects were seen under 3 conditions in the drug study: females lever pressed more than males during COMBO trials, females earned less reinforcers than males during AMPH trials, and females earned less reinforcers than males at 0.125/1.0 mg/kg COMBO. So in all 3 instances, the performance of females was worse than that of males in drug trials involving AMPH administration. Sable et al. (2009) did not find sex differences in response to AMPH in control subjects, but instead found sex differences in response to AMPH with PCB-exposed subjects, which is different than the current findings. However, it is interesting that in Sable et al.’s study that PCB-exposed females were more sensitive to the effects of AMPH than males. In a separate study that examined sex differences in DRL performance, doses of 0.2, 0.4, and 0.8 mg/kg AMPH all increased lever pressing and decreased reinforcers earned and response efficiency to a greater extent in females than males (van Haaren et al. 1986). Because there were no sex differences during FLU trials, it is more likely that the sex effects during the COMBO trials are due solely to the effect of AMPH. This is particularly evident in the finding that females earned less reinforcers than males at 0.125/1.0 mg/kg COMBO, which is the combination dose at which the FLU dose is smallest relative to the AMPH dose.

**Reproductive and Developmental Findings**

The absence of treatment-related effects in dams parallels the findings of other studies using similar exposure paradigms and dosages of the Fox River Mix (Kostyniak
et al. 2005; Sable et al. 2009; Poon et al. 2011) and DE-71 (Zhou et al. 2002; Poon et al. 2011). The most pronounced differences in developmental endpoints of the offspring were decreased weight gain in PCB groups, increased liver:body weight ratio in PCB and PBDE groups, decreased thymus:body weight ratio in PCB groups, and earlier eye opening in PCB groups. Reductions in weight gain have also been reported in prior PCB studies in our laboratory (Kostyniak et al. 2005; Sable et al. 2009; Poon et al. 2011), in other laboratory species (Safe 1994), and in humans (Schantz et al. 2003), whereas the lack of a difference in weight gain in PBDE subjects replicates what has been seen in prior studies of developmental exposure to DE-71 (Zhou et al. 2002; Poon et al. 2011). Increased liver:body weight ratio in the pups from PCB and PBDE treatment groups match the findings from prior studies of developmental PCB and PBDE exposure in rodent models (Zhou et al. 2002; Kostyniak et al. 2005; Sable et al. 2009; Bondy et al. 2013; Poon et al. 2011) and other laboratory species (Safe 1994). Decreased thymus:body weight ratio in PCB but not PBDE subjects also parallels findings from prior studies in our laboratory (Kostyniak et al. 2005; Sable et al. 2009; Poon et al. 2011) and in other laboratory species (Safe 1994).

Earlier eye opening in the 6 mg/kg PCB group is an interesting finding. PCBs did not affect day of eye opening in previous studies using the Fox River Mix (Sable et al. 2009; Poon et al. 2011) or in some studies of Aroclor 1254 (Overmann et al. 1987; Crofton et al. 2000). However, early eye opening was reported in studies employing higher dosages of Aroclor 1254 (Goldey et al. 1995; Goldey & Crofton 1998). Eye opening is a commonly used physical developmental landmark that is included in developmental
neurotoxicology screening batteries (Henck 2002), with a finding of early eye opening suggesting that a chemical has the ability to affect neurodevelopment.

5. Conclusions

The current study did not detect alterations in DRL performance in subjects developmentally exposed to PCBs or PBDEs, raising the question of whether PCBs and PBDEs truly affect impulsive action. However, as discussed, there are studies across multiple species utilizing DRL tasks that have shown that PCBs result in changes such as increased lever pressing and decreased performance efficiency, which are interpreted as impulsive responding (Rice 1998; Holene et al. 1999; Stewart et al. 2006; Sable et al. 2009).

The question that remains is why did the current study not detect PCB effects? The study by Sable et al. (2009) was almost identical to the current study in methodology and resulted in there being a significant decrease in the ratio of reinforced:non-reinforced presses in PCB groups. However there were not differences in total lever presses or reinforcers earned in that study. This suggests that the DRL task can detect toxicant-induced changes under some circumstances, but that it is possible that subtle factors such as inter-individual variation, strain or dosage differences, and dietary manipulations may hinder the ability for differences to be detected.

Significant inter-individual variation exists in performance of tasks that assess impulsive action (Dellu-Hagedorn et al. 2004) and impulsive choice (Galtress et al. 2012). Methodologies exist for predicting impulsivity in individual subjects, such as locomotor response to cocaine (Stanis et al. 2008) or performance on a screening
behavioral test (Anker et al. 2009). This enables segregating subjects based on impulsive tendency before experimental manipulations are introduced, which allows inter-individual variation to be accounted for in the statistical analysis. However studies which involve perinatal manipulations, such as the current study, do not allow for determination of whether subjects are impulsive prior to the exposure period and, thus, preclude accounting for inter-individual variation in the statistical analysis. Historically, behavioral tests such as DRL have been shown to be useful for assessing the effects of chemical exposures on response inhibition. However, until better methods of accounting for inter-individual variation are devised, the ability of such studies to reliably detect significant effects across studies may be compromised.
6. Tables and Figures

Table 3.1. Reproductive endpoints.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Treatment Group ((n_1,n_2,n_3))\textsuperscript{b}</th>
<th>Live Birth Rate</th>
<th>Litter Size</th>
<th>% Live Pups</th>
<th>% Male Pups</th>
<th>% Gest Weight Gain</th>
<th>% Lact Weight Gain</th>
<th>Liver Weight: BW (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control) (19,15,13)</td>
<td>78.9</td>
<td>10.8±1.1</td>
<td>88.9±3.3</td>
<td>47.6±2.7</td>
<td>41.1±3.0</td>
<td>8.8±1.4</td>
<td>0.0505 ±0.0015</td>
</tr>
<tr>
<td>3 mg/kg FRM (19,17,14)</td>
<td>89.5</td>
<td>8.9±0.8</td>
<td>76.4±6.1</td>
<td>56.8±5.3</td>
<td>44.8±1.9</td>
<td>4.2±0.8</td>
<td>0.0539 ±0.0009</td>
</tr>
<tr>
<td>6 mg/kg FRM (18,16,14)</td>
<td>88.9</td>
<td>10.6±0.6</td>
<td>77.8±6.3</td>
<td>53.7±3.2</td>
<td>42.3±2.5</td>
<td>9.5±2.4</td>
<td>0.0546 ±0.0018</td>
</tr>
<tr>
<td>11.4 mg/kg DE-71 (15,12,12)</td>
<td>80.0</td>
<td>10.2±0.8</td>
<td>89.2±4.6</td>
<td>48.2±5.9</td>
<td>42.4±1.9</td>
<td>3.8±1.0</td>
<td>0.0547 ±0.0016</td>
</tr>
<tr>
<td>22.8 mg/kg DE-71 (18,16,14)</td>
<td>88.9</td>
<td>8.8±0.8</td>
<td>89.5±3.0</td>
<td>53.2±4.4</td>
<td>41.8±2.8</td>
<td>6.6±1.2</td>
<td>0.0551 ±0.0012</td>
</tr>
</tbody>
</table>

BW = body weight; DE-71 = DE-71 commercial PBDE mixture; FRM = Fox River PCB mixture; Gest = gestational; Lact = lactational.

\textsuperscript{a}Data are reported as averages for each treatment group. Mean±SEM reported.

\textsuperscript{b}n_1 = # dams in each treatment group, n_2 = # dams that delivered ≥1 live pup, n_3 = # dams remaining in the study by PND 21.
Table 3.2. Developmental endpoints.*

<table>
<thead>
<tr>
<th>Treatment Group; (n₁, nₛ)</th>
<th>Wt (g)</th>
<th>Organ Wt:BW (g/g)</th>
<th>Eye Opening (PND)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Birth</td>
<td>PND 7</td>
<td>PND 14</td>
</tr>
<tr>
<td>0 (control)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>6.37±0.20</td>
<td>14.85±0.74</td>
<td>29.47±1.03</td>
</tr>
<tr>
<td>Female</td>
<td>6.24±0.27</td>
<td>14.29±0.63</td>
<td>31.44±3.05</td>
</tr>
<tr>
<td>3 mg/kg FRM (15,13)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>C</td>
<td>6.44±0.16</td>
<td>14.23±0.39</td>
</tr>
<tr>
<td>Female</td>
<td>5.98±0.13</td>
<td>13.83±0.40</td>
<td>26.41±0.61</td>
</tr>
<tr>
<td>6 mg/kg FRM (16,14)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>C</td>
<td>5.96±0.16</td>
<td>13.38±0.39</td>
</tr>
<tr>
<td>Female</td>
<td>5.71±0.16</td>
<td>12.84±0.31</td>
<td>25.23±0.42</td>
</tr>
<tr>
<td>11.4 mg/kg DE-71 (12,11)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>C</td>
<td>6.51±0.19</td>
<td>15.58±0.80</td>
</tr>
<tr>
<td>Female</td>
<td>6.23±0.20</td>
<td>14.24±1.40</td>
<td>28.87±0.99</td>
</tr>
<tr>
<td>22.8 mg/kg DE-71 (16,13)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>A</td>
<td>7.06±0.19</td>
<td>16.00±0.56</td>
</tr>
<tr>
<td>Female</td>
<td>6.74±0.21</td>
<td>15.24±0.42</td>
<td>29.01±0.38</td>
</tr>
</tbody>
</table>

BW = body weight; DE-71 = DE-71 commercial PBDE mixture; FRM = Fox River PCB mixture; PND = postnatal day; Wt = weight.

*Data are reported as averages for each treatment group. Means±SEM reported.

*n₁ = # litters in each treatment group at PND 0. A litter had to have ≥6 pups birthed by the dam to be included in the analyses. nₛ = # litters from each treatment group that had subjects advance beyond PND 21 (weaning) to the behavioral study.

Sex had a significant effect on this endpoint. All p<0.05.

A Significantly different from control group; B significantly different from 11.4 mg/kg PBDE group; C significantly different from 22.8 mg/kg PBDE group; all p<0.05.
Figure 3.1. The 3 successive blocks of drug injections including the drug dosages given to each subject within each block. SAL = the relative positions of saline days from which data was used to examine whether baseline performance remained stable across the blocks of drug trials.
Figure 3.2. Comparison of total number of lever presses between sexes on the first session of DRL 5, DRL 10, and DRL 15. Males (M) pressed significantly more frequently than females (F) on day 1 of DRL 5 but not DRL 10 or DRL 15. ***p<0.001.

Figure 3.3. Changes in total lever presses and ratio of reinforced:non-reinforced presses across five 6-day blocks during DRL 15 training phase. As training progressed, total lever presses per session decreased (closed circles) while reinforced:non-reinforced presses increased (open circles).
Figure 3.4. Changes in lever pressing with drug treatment. A. FLU decreased pressing. There was less pressing 0.25 mg/kg than with the other 3 doses. There was also less pressing with 0.125 mg/kg than with control. B. AMPH increased pressing. There was more pressing with 1.0 mg/kg than with the other 3 doses. There was also more pressing with 0.5 mg/kg than with control. *p<0.05, **p<0.01, ***p<0.001.
Figure 3.4 (cont). Changes in lever pressing with drug treatment. C. FLU and AMPH together counteracted each other. When the AMPH dose increased to 1.0 mg/kg, there was significantly more pressing, but this difference was not present when FLU was then increased to 0.25 mg/kg. Sex had a significant effect on lever pressing for COMBO trials, but the interaction between sex x dose was not significant. Females lever pressed more than males when administered FLU/AMPH together. ***p<0.001.
Figure 3.5. Changes in reinforcers earned with drug treatment. A. and B. FLU and AMPH both decreased reinforcers earned. Significantly less reinforcers were earned at the highest 2 doses of each drug as compared to control and the lowest dose, while the highest dose also resulted in significantly less reinforcers than the next highest dose. Sex had a significant effect on reinforcers earned for AMPH, but the interaction between sex x dose was not significant. Females earned less reinforcers than males when administered AMPH. *p<0.05, ***p≤0.001.
Figure 3.5 (cont.). Changes in reinforcers earned with drug treatment. C. Males (M) and females (F) did not differ except at 0.125/1.0 mg/kg COMBO. D. Drug combinations also resulted in significantly less reinforcers at all higher dosages in females and the 2 higher dosages in males. *$p<0.05$, ***$p\leq 0.001$. 
Figure 3.6. Changes in ratio of reinforced:non-reinforced lever presses. A. AMPH caused a decrease in the ratio at 0.5 and 1.0 mg/kg, with both of those doses significantly differing from all other doses. B. All 3 COMBO doses resulted in ratios smaller than control. Also the ratios at the 2 highest COMBO doses were smaller than the ratio at 0.125/0.5 mg/kg COMBO. *p<0.05, **p<0.01, ***p<0.001.
Figure 3.7. FLU increases post-reinforcement pause, but only at the highest dose. ***$p \leq 0.001$.

Figure 3.8. FLU increases the latency to collect reinforcement, but only at the highest dose. ***$p \leq 0.001$. 

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97
7. References


Chapter 4: Effects of PCBs and PBDEs on the Delay Discounting Task

1. Introduction

Examining the effects of developmental exposure to polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) on different aspects of response inhibition is one of the primary goals of the current research. In the previous chapter, the effects of these contaminants on impulsive action were examined. In this chapter the effects of PCBs and PBDEs on impulsive choice will be scrutinized.

Impulsive choice, as assessed through delay discounting (DD) tasks, is one of the better-studied types of impulsivity in laboratory animal and human studies (Winstanley 2011). In DD paradigms, subjects must choose between a smaller reinforcer that is delivered immediately and a larger reinforcer that is delivered after a delay. Subjects typically choose the larger-delayed reward when the delay is short, but then begin to choose the smaller-immediate reward as the delay length increases (Tesch & Sanfey 2008). Hence they discount the value of the larger reward in order to avoid longer delays. In this context, a greater choice of the smaller-immediate reward as compared to controls is considered impulsive. These findings can be graphed as a hyperbolic curve referred to as a discounting curve.

A strength of the DD paradigm is that the relationship between delay and the subjective value of the reward is best described by a hyperbolic function in both humans and animals (Paule et al. 2012), even though the rewards are typically hypothetical in

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1 Some text in this chapter is modified from the manuscript “Sex differences in adult Long Evans rats performing a delay discounting task” authored by Eubig and submitted for publication. The data is original.
human studies and real (i.e., food) in animal studies. Despite this difference in DD methodology between humans and laboratory species, it has been argued that DD studies in the latter have been very beneficial in exploring the underlying mechanisms of impulsive choice (Winstanley et al. 2006a).

Rodent DD models have been important in understanding the neural pathways and neurochemical signaling underlying impulsive choice (Dalley et al. 2008; Pattij & Vanderschuren 2008; Winstanley 2011). Dopaminergic systems, in particular, have been found to be important mediators of DD performance (van Gaalen et al. 2006; Winstanley 2011).

Anatomic regions important for DD performance, including the frontal cortex and nucleus accumbens (NAC), have been investigated by a variety of techniques. One study of the medial prefrontal cortex (mPFC) found that lesioning resulted in flattened discounting curves (Cardinal et al. 2001). This was interpreted to mean that the mPFC has an important role in regulating how the passage of time impacts DD behavior. Other studies have examined the role of the orbitofrontal cortex (OFC) in DD performance. Lesioning the OFC in rats resulted in increased choice of smaller-more immediate rewards (Kheramin et al. 2002; Mobini et al. 2002). In a recent study, electrodes were implanted in the OFC of rats in order to measure firing of individual neurons during DD performance (Roesch et al. 2012). Firing rates were associated with the length of delay to the reward but not the size of the reward, which highlights the importance of the OFC in encoding information about the delay during DD performance.

The role the NAC plays in DD has also been investigated. Lesioning the NAC core resulted in increased choice for the smaller-more immediate reward when the delay to
the reward changed within each experimental session (Cardinal et al. 2001; Bezzina et al. 2007), and resulted in a flattening of the discounting curve when the delay changed between sessions (Acheson et al. 2006). Furthermore, the connectivity between the OFC and the NAC has also been shown to be important in DD performance, with leucotomy of the corpus callosum combined with one-sided lesions of the OFC and NAC resulting in even greater choice for the smaller-more immediate reward than in subjects that were either only lesioned or only callosotomized (Bezzina et al. 2008).

The contributions of striatal regions (i.e., dorsal, ventral) to DD performance have not been scrutinized in animal models. However, several human imaging studies have shown that the ventral striatum is activated during DD performance (McClure et al. 2004; Hariri et al. 2006; Wittman et al. 2010; Sripada et al. 2011; Cho et al. 2012), in addition to the mPFC (McClure et al. 2004; Ballard & Knutson 2009; Sripada et al. 2011; Cho et al. 2012), medial OFC (McClure et al. 2004), and the nucleus accumbens (Ballard & Knutson 2009).

Pharmacologic studies have demonstrated the importance of dopaminergic signaling in DD performance. In general, administration of amphetamine (AMPH), which increases synaptic concentrations of dopamine, increases choice for the larger-delayed reward (Cardinal et al. 2000; van den Bergh et al. 2006; van Gaalen et al. 2006; Floresco et al. 2008). However, the effect is biphasic. AMPH dosages of approximately 1.0 mg/kg or higher increase choice for the smaller-immediate reward (Evenden & Ryan 1996; Cardinal et al. 2000; Slezek & Anderson 2009; Koffarnus et al. 2011). Administration of the D1 receptor antagonist SCH 23390 increased choice of the small-immediate reward (van Gaalen et al. 2006; Koffarnus et al. 2011), while the D2 receptor
antagonist eticlopride did not affect choice (van Gaalen et al. 2006). However when either dopamine receptor antagonist was administered together with 0.5 mg/kg AMPH, both SCH 23390 and eticlopride attenuated the effect of AMPH on choice of the large-delayed reinforcer (van Gaalen et al. 2006). Along these lines, flupenthixol (FLU), a D1/D2 receptor antagonist, consistently increased choice for the small-immediate reinforcer (Cardinal et al. 2000; Floresco et al. 2008). In addition, a study in which dopaminergic input to the OFC was selectively lesioned resulted in increased choice of smaller-more immediate rewards (Kheramin et al. 2004). However, lesioning the dopaminergic input to NAC did not alter DD choice (Winstanley et al. 2005), suggesting that dopaminergic innervation has a differential effect in different regions important for DD performance.

At the same time, other neurotransmitter systems, particularly the serotonergic system, have roles in DD performance. Administration of either the 5-HT\textsubscript{1A/1B} agonist eltoprazine (van den Bergh 2006) or the 5-HT\textsubscript{1A} agonist 8-OH-DPAT (Winstanley et al. 2005) increased choice of the larger-delayed reward. Other studies have focused on the relationship between the dopaminergic and serotonergic systems in DD performance. Winstanley et al. (2003) injected a serotonergic neurotoxin intracerebroventricularly to deplete forebrain serotonin levels by over 85%. While this manipulation did not directly affect DD choice, it attenuated the ability of AMPH to increase choice. In the study by Winstanley et al. (2005) in which dopaminergic input to the NAC was lesioned, 8-OH-DPAT was unable to increase choice for the larger-delayed reward post-lesioning. This suggests that an interaction between the serotonergic and dopaminergic systems occurs in the NAC, and that the NAC is unable to function normally in DD task.
performance if serotonergic input increases concurrently with impaired dopaminergic functioning. Winstanley et al. (2006b) also used microdialysis techniques to quantitate dopamine and serotonin turnover in the mPFC and OFC during DD performance. It was found that dopamine turnover increased in both regions while serotonin efflux increased only in the mPFC. Thus performance on DD is not only influenced by dopaminergic signaling, but also by other neurotransmitter systems.

Despite the large body of knowledge that exists about the DD task, it has received little use as a means to explore the effects of toxicant exposure on response inhibition. A literature search did not locate any studies involving PCB or PBDE exposure and DD. Because it is known that PCBs affect response inhibition, as discussed in Chapter 1, the current experiment used the DD task to assess whether PCBs and PBDEs affect an important aspect of response inhibition: impulsive choice. It was hypothesized that developmental PCB or PBDE exposure would result in more impulsive DD performance as compared to controls. This would manifest as a shift of the discounting curve towards greater choice of smaller-immediate rewards as compared to controls as the delays to the larger reward increased.

Because DD is sensitive to dopaminergic manipulation and because PCBs and PBDEs perturb dopaminergic signaling, as discussed before, it was hypothesized that developmental PCB or PBDE exposure would result in a shift in the dose response curve to dopaminergic agents. More specifically, based on in vivo evidence that PCBs result in lower synaptic concentrations of dopamine (Seegal et al. 2002), AMPH administration to PCB- or PBDE-exposed rats was hypothesized to improve DD performance at lower AMPH doses and impair performance to a lesser extent at higher
AMPH doses than in controls. Also, FLU was predicted to impair DD performance to a greater extent in contaminant-exposed subjects than in controls.

2. Methods

2.1. Animals and Exposure

Details of the perinatal exposure paradigm of experimental subjects to PCBs and PBDEs are discussed in Chapter 3. In brief, Long Evans rat dams were allocated between 5 treatment groups: control, 3 mg/kg PCBs, 6 mg/kg PCBs, 11.4 mg/kg PBDEs, and 22.8 mg/kg PBDEs. Dams were exposed daily beginning 4 weeks prior to breeding and continuing through post-natal day 20 (PND 20). Pups were weaned from the dams on PND 21 and male-female pairs of pups from each litter were allocated to begin behavioral testing on PND 90. Two litters were not assigned to the DD study: 1 litter had all female pups and 1 litter had only 1 male-female pair that was assigned to the DRL study (Chapter 3). Starting at PND 70, food restriction commenced so that food pellets could be used as reinforcement for the behavioral task, as detailed in Chapter 3. Sixty-four male-female pairs completed behavioral testing. Their allocation to treatment groups was as follows: 12 pairs of control, 14 pairs of 3 mg/kg PCB, 13 pairs of 6 mg/kg PCB, 11 pairs of 11.4 mg/kg PBDE, and 14 pairs of 22.8 mg/kg PBDE.

2.2. Behavioral Testing Procedures

Apparatus

Behavioral testing was conducted in 8 automated operant chambers (Med Associates; St. Albans, VT) housed in sound attenuated cubicles, each ventilated by a
fan. All operant chambers contained 3 stimulus cue lights, positioned above the 2 retractable response levers and the centrally located pellet trough. The levers, which were 6 cm above the floor, were located symmetrically on both sides of the pellet trough. An external pellet dispenser delivered 45 mg AIN-76A purified rodent food pellets (TestDiet; Richmond, IN). An 80 decibel white-noise generator masked extraneous sounds. A house light positioned at the rear of the chamber provided general illumination. The experimental contingencies were programmed using Medstate Notation behavioral programming language (Med Associates; St. Albans, VT).

**Autoshaping (Training Phase 1)**

Daily sessions of operant testing were conducted 6 days per week, excluding Sundays. Beginning at PND 90, an autoshaping program (training phase 1) was used to train the subjects to press the response levers. The contingencies of the program and the criterion for advancement to the next phase were the same as in DRL training phase 1 (see Chapter 3 for details). Autoshaping was completed in 3.1 sessions on average (range 2 to 8).

**Fixed Ratio Training (Training Phase 2)**

The next training phase elicited lever pressing in response to cue light illumination. The contingencies of the program and the criterion for advancement to the next phase were the same as in DRL training phase 2 (see Chapter 3 for details). This phase was completed in 3 sessions on average (all subjects completed in 3 days, except for 2 that were mistakenly tested for 2 days and 2 that took 4 and 5 days to meet criterion).
**DD Training (Training Phases 3-5)**

Training on the DD task took place over 3 more phases. The purpose of the third phase was to introduce alternating levers and time-controlled trials. This phase consisted of 60 trials/session with each trial lasting 40 seconds. Animals were required to initiate each trial with a nosepoke into the food trough, when the center cue light was illuminated. Also, a 10-second limited hold on lever presses was introduced, wherein a subject had 10 seconds to nosepoke or the trial was counted as an omission. If the subject initiated a trial with a nosepoke, the center cue light was extinguished and a single response lever was extended at random. If the subject pressed the lever, a food pellet was immediately delivered, and the lever retracted. After the next nose poke, the opposite lever was extended. An extended lever was always accompanied by an illuminated cue light. For this and all subsequent phases of DD training and testing, the house light was on during the times the subject could nosepoke or lever press, but off for the remainder of the time. Progression to the next phase of testing required $\geq 40$ successful responses, with at least 20 responses on the left lever and 20 responses on the right lever. This phase was completed in 2.1 sessions on average (range 1 to 6).

The fourth training phase consisted of two sessions where rats were introduced to large and small reinforcers. Half of the subjects were assigned to the left lever as the large reinforcer lever, and the other half to the right lever. This phase also consisted of 60 trials/session (5 blocks of 12 trials), but the length of each trial was increased to 80 seconds. The first 2 trials of each block were *forced choice trials* in which the large reinforcer lever (resulting in 4 pellets if pressed) and the small reinforcer lever (resulting
in 1 pellet if pressed) were alternately introduced. Both levers were presented in the remaining 10 trials of each block, termed *choice trials*. There was no delay in delivery of either the small or large reinforcers in this phase. All subjects were tested for 2 sessions on this phase.

Delays to the large reinforcer were introduced in the fifth testing phase. Parameters of this phase remained the same as in the fourth phase, but delays to the larger reinforcer increased across the 5 trial blocks, progressing from 0 seconds in the first block of 12 trials to 5, 10, 20, and then 40 seconds over the course of the 5 blocks of trials in each session (see Figure 4.1). Importantly, if the subject chose the delayed reinforcer, the cue light above that lever remained illuminated until the delay was complete and delivery of the food pellets began. Performance was evaluated for the development of a sensitivity to delay, according to the criteria discussed below. All subjects were tested for a minimum of 30 sessions on this phase (average 33.5, range 26 to 35) except for 2 who were tested for 26 and 29 sessions.

**Drug Challenges (Phase 6)**

The experimental parameters for the drug trials were identical to those in phase 5. Drug doses were selected based on their previous use in similar behavioral experiments (Cardinal et al. 2000;Floresco et al. 2008; Sable et al. 2009). Drugs and dosages were the same as those used in the DRL drug trials (see Chapter 3). In short, FLU dosages were 0, 0.05, 0.125, and 0.25 mg/kg. AMPH dosages were 0, 0.25, 0.5, and 1.0 mg/kg. Intraperitoneal injections of FLU and AMPH were administered 30 and 10 minutes,
respectively, before testing, based on their pharmacokinetic properties (Jorgensen et al. 1969; Kunh and Schanberg 1978).

Injections were administered in 3 successive blocks: FLU, then AMPH, then FLU/AMPH combined (COMBO). Each dose within a block was administered once to each subject, with the dosing order individually randomized using a balanced Latin Square design. Drugs were administered on Tuesdays and Fridays, with 7 calendar days between each block of injections. Additional saline injections were given for the two days before the FLU block, one day before the AMPH block, one day before the COMBO block, and one day after the COMBO block. The first additional saline injection was performed to acclimate the subjects to the injection procedure. Results from the subsequent additional saline days were examined to assess whether baseline performance remained stable across the drug trials.

2.3. Data Analysis

All statistical analyses were conducted using SPSS for Windows (version 20.0, SPSS Inc.; Chicago, IL) with statistical significance set at $p<0.05$. For some repeated-measures factors, a sphericity violation was noted. In these instances a Greenhouse-Geisser correction was used to reduce the risk of a Type I error if $\epsilon$ was $<0.75$ and a Huynh-Feldt correction was used if $\epsilon$ was $\geq0.75$ (Rogan et al. 1979). Analyses requiring these corrections are reported using the adjusted degrees of freedom rounded to the nearest integer. When significant main effects or interactions were obtained, post hoc one-way ANOVA followed by Tukey’s least significant difference (LSD) analyses were performed. Data are reported as mean ± SEM.
Four dependent measures were evaluated. *Choice ratio* was defined as the number of lever presses for the larger, delayed reinforcer divided by the total number of lever presses for both delayed and immediate reinforcers (i.e., the percent choice of the delayed reinforcer). Omissions occurred when a subject either did not make a nose poke to initiate a trial or did not make a choice when the levers were extended. *Total omissions* included both omission types across all trials (choice and forced choice). *Trial initiation latency* was defined as the time at the beginning of a trial from when the cue light above the pellet trough was illuminated to when the subject poked its nose into the food trough to initiate the trial. *Choice latency* was defined as the time from when the levers extend to when the subject pressed one of the levers.

**Analyses of Training**

Choice ratios for each delay during the Training Phase 5 were averaged across 6 consecutive daily sessions for each subject to form five 6-session-blocks of training (30 days). Choice ratios were then analyzed via a 5 (block) x 5 (delay) x 2 (sex) x 5 (treatment group) repeated-measures ANOVA, with block, delay, and sex (nested within litter; Hughes 1979) as within-subjects factors. Criteria that a sensitivity to delay had developed were met when there was either a significant main effect of delay or a significant block x delay interaction, and when *post-hoc* analysis confirmed significantly smaller choice ratios for one or more of the longer delays.

Examination of the final session-block of training (Block 5) was undertaken to document baseline performance just prior to commencing the drug challenges. Choice ratios were analyzed with a 5 (delay) x 2 (sex) x 5 (treatment group) repeated-measures
ANOVA, with delay and sex (nested within litter) as within-subjects factors. Total omissions, trial initiation latency, and choice latency were each analyzed with a 2 (sex) x 5 (treatment group) mixed ANOVA, with sex (nested within litter) as a within-subjects factor.

**Analyses of Drug Trials**

Choice ratios from drug challenges were analyzed with 4 (drug dose) x 5 (delay) x 2 (sex) x 5 (treatment group) repeated-measures ANOVAs, with treatment group as a between-subjects factor. Total omissions, trial initiation latency, and choice latency were each analyzed with 4 (drug dose) x 2 (sex) x 5 (treatment group) repeated-measures ANOVAs, with treatment group as a between-subjects factor.

It was noted that especially 0.125 and 0.25 mg/kg FLU caused more omissions in some subjects than 0 or 0.05 mg/kg FLU, with choice for an entire block of trials for a delay sometimes being omitted. When a subject omits an entire block of trials, the choice ratio is reported by the software as 0%, which incorrectly implies that the subject chose only the immediate reinforcer for that delay, when in fact no choices were made. The percent of individual blocks in which all 10 trials were omitted was examined for each drug dose and delay. Doses and delays in which all trials were omitted by > 10% of subjects were as follows: 0.25 mg/kg FLU at 40 sec (42% of subjects omitted all trials), at 20 sec (28%), at 10 sec (22%); 0.125 mg/kg FLU at 40 sec (13%); and 0.25/1.0 mg/kg FLU/AMPH (13%). To address this concern, the 40 sec delay for FLU was removed from the statistical analysis of choice latency due to excessive omissions. Also, whenever a subject omitted all 10 trials at a given delay and drug dose, mean
replacement was performed for the subject’s choice ratio by averaging choice ratio for all other subjects of the same sex at the same dose and delay.

Choice ratios from the saline administration days one day before the FLU block, one day before the AMPH block, one day before the COMBO block, and one day after the COMBO block were analyzed via a 4 (saline day) x 5 (delay) x 2 (sex) x 5 (treatment group) repeated-measures ANOVA, with treatment group as a between-subjects factor.

3. Results

3.1. DD Training

Analysis of choice ratio during the 5 6-session-blocks of DD training did not show a significant treatment effect but did reveal significant main effects of block [F(2,143)=12.1, p<0.001] and delay [F(2,89)=695.7, p<0.001] and a significant block x delay interaction [F(4,265)=51.9, p<0.001]. Neither the main effect of sex nor other interactions were significant. Post-hoc analysis confirmed that during the first session-block (Block 1, testing days 1-6) a sensitivity to delay developed as evidenced by a decrease in choice ratio as delay to reinforcement increased (see Figure 4.2, Block 1). Once the sensitivity to delay developed, it was maintained throughout the experiment, as all subsequent repeated-measures ANOVAs on choice ratio were significant for the main effect of delay [all F≥374.5, all p<0.001].

Analysis of choice ratio during Block 5 did not show a significant treatment effect but did reveal a main effect of delay [F(2,100)=433.3, p<0.001] (see Figure 4.2, Block 5). Neither sex (see Figure 4.3) nor any of the interactions were significant. Choice ratio decreased from 88.2 ± 1.4% at 0 sec delay to 18.1 ± 2.4% at 40 sec delay. Total
omissions were minimal during Block 5 (< 1 omission per session). Neither treatment group nor sex had significant effects on omissions. Trial initiation latency was not significantly affected by treatment group or sex (see Figure 4.4). Average initiation latency was 1.15 ± 0.03 seconds. Treatment group did not significantly affect choice latency, but sex did have a significant effect [F(1,59)=4.5, \( p=0.039 \)], with females choosing faster than males (0.97 ± 0.03 sec versus 1.07 ± 0.04 sec; see Figure 4.4). Because sex differences in choice ratios and trial initiation latencies were seen during the drug trials, performance for both sexes on these measures are shown to allow for comparisons between pre-drug and drug performance.

### 3.2. Drug Trials

#### Choice Ratio

With FLU administration, the main effect of PCB/PBDE treatment group and interactions with treatment were not significant, but significant effects of drug dose [F(3,167)=24.1, \( p<0.001 \)] and delay [F(2,111)=444.8, \( p<0.001 \)] on choice ratio were seen, as well as a significant drug dose x delay interaction [F(6,353)=3.0, \( p=0.008 \)]. Sex did not significantly affect choice ratio, nor were other interactions with sex significant. Post-hoc analysis comparing choice ratio at the different drug doses within each delay revealed significant differences at 5 and 10 sec delays (see Figure 4.5). Choice ratio at the highest FLU dose (0.25 mg/kg) was smaller than at the control dose at 5 and 10 sec delays (\( p=0.001 \) at 5 sec, \( p=0.024 \) at 10 sec) and also smaller than the 0.05 mg/kg dose at the 5 sec delay (\( p=0.007 \)).
With AMPH administration, treatment group effects and interactions with treatment group were not significant, but significant effects of drug dose \( F(2,131)=4.7, p=0.009 \) and delay \( F(2,138)=419.5, p<0.001 \) on choice ratio were seen, as well as a significant drug dose x delay interaction \( F(7,433)=8.3, p<0.001 \). There was a significant effect of sex \( F(1,59)=4.4, p=0.039 \), but sex did not significantly interact with other factors. *Post-hoc* analysis comparing choice ratio at the different drug doses within each delay revealed significant differences at 0, 10, and 20 sec delays (see Figure 4.6). At the 0 sec delay, 1 mg/kg AMPH resulted in a smaller choice ratio than the control \( p=0.003 \) and 0.05 mg/kg \( p=0.026 \) doses, whereas at the longer delays 1 mg/kg AMPH increased choice ratio above that of control \( p=0.016 \) at 10 sec, \( p=0.001 \) at 20 sec).

Because there were not significant interactions of sex with other factors, further *post-hoc* analysis was not performed. Inspection of the graphs (see Figure 4.7) reveals that although AMPH increased choice ratio in both sexes as the delays lengthened, choice ratio was always smaller for females than males at each AMPH dose.

The statistical findings from the COMBO trials were similar to those from AMPH trials. While the main effect of treatment group and the interactions involving treatment group were not significant, drug dose \( F(3,161)=3.9, p=0.013 \) and delay \( F(2,139)=460.4, p<0.001 \) significantly affected choice ratio, and the interaction for dose x delay was significant \( F(8,449)=2.1, p=0.042 \). Sex \( F(1,59)=7.1, p=0.01 \) and an interaction between dose x sex \( F(3,152)=2.8, p=0.048 \) were also significant, but no other significant interactions were observed. *Post-hoc* analysis of choice ratio of the different drug doses within each delay did not find any significant differences between COMBO doses (all \( p>0.17 \); see Figure 4.8). Inspection of the graphs (see Figure 4.9)
reveals that overall choice ratio was always smaller for females than males across COMBO doses.

**Total Omissions**

For FLU, PCB/PBDE treatment group did not have a significant effect on omissions ($p=0.105$), but a significant effect of drug dose [$F(1,86)=103.6, p<0.001$] was seen along with a significant drug dose x treatment group interaction [$F(6,86)=2.3, p=0.047$]. Sex did not have a significant effect, nor were other interactions of treatment or sex with other factors significant. Post-hoc analyses of differences in omissions between treatment groups within each FLU dose did not find any significant differences (all $p\geq0.065$). When differences in omissions between drug doses alone were analyzed, it was determined that increasing the FLU dose caused increases in omissions (see Figure 4.10). There were significantly more omissions with 0.125 and 0.25 mg/kg FLU than with control or 0.05 mg/kg FLU (all $p<0.01$), and 0.25 mg/kg FLU also caused more omissions than 0.125 mg/kg.

For AMPH, treatment group was not significant, but significant effects of drug dose [$F(2,103)=19.7, p<0.001$] and sex [$F(1,59)=6.5, p=0.014$] on omissions were seen, as well as a significant drug dose x sex interaction [$F(2,119)=9.0, p<0.001$]. Post-hoc analysis determined that in females only 1.0 mg/kg AMPH caused a significant increase in omissions over the smaller doses (all $p<0.001$; see Figure 4.11). Because AMPH did not have this effect in males, the number of omissions seen at 1.0 mg/kg in females was also significantly greater than at the same dosage in males ($p<0.001$).
With COMBO injections, although treatment group was not significant, a significant effect of drug dose \([F(2,122)=26.0, \ p<0.001]\) on omissions was seen. Sex did not have a significant effect, nor were any interactions of treatment or sex with other factors significant. COMBO 0.25/1.0 mg/kg FLU/AMPH was found on post-hoc analysis to result in significantly more omissions than with other dosages, while 0.125/1.0 mg/kg also resulted in more omissions than control (all \(p\leq0.001\); see Figure 4.12).

**Trial Initiation and Choice Latencies**

With FLU, PCB/PBDE treatment group was not significant, but drug dose significantly affected trial initiation latency \([F(3,148)=88.6, \ p<0.001]\). Sex did not affect initiation latency, nor were any interactions of treatment group or sex with other factors significant. Post-hoc analysis revealed that both 0.125 and 0.25 mg/kg significantly increased initiation latency, with latency at each of those doses differing from latencies at all other doses (all \(p<0.001\); see Figure 4.13). While treatment group was not significant for choice latency, FLU dose significantly affected choice latency \([F(2,119)=43.2, \ p<0.001]\). Sex did not have a significant effect, nor were any interactions of treatment group or sex with other factors significant for choice latency. Post-hoc analysis revealed that both 0.125 and 0.25 mg/kg FLU significantly increased choice latency, with latency at each of those drug doses differing from latencies at all other doses (all \(p<0.01\); see Figure 4.14).

Although treatment group was not significant for AMPH trials, the dose of AMPH significantly affected trial initiation latency \([F(2,110)=21.3, \ p<0.001]\). Also, sex \([F(1,59)=12.5, \ p<0.001]\) and the drug dose x sex interaction \([F(2,126)=11.9, \ p<0.001]\)
were significant. No other interactions of treatment or sex with other factors were significant. Post-hoc analysis revealed that initiation latency after 1.0 mg/kg AMPH was significantly longer than with all smaller doses in females as well as longer than the latency seen with the same dose in males (all $p \leq 0.001$; see Figure 4.15). AMPH 0.5 mg/kg in females also resulted in a longer initiation latency than control and the same dose in males (both $p < 0.05$). While treatment group was not significant, AMPH dose also significantly affected choice latency [$F(2,125)=32.5$, $p < 0.001$]. Sex did not affect choice latency ($p = 0.073$), nor were any interactions of treatment group or sex with other factors significant for choice latency. Post-hoc analysis determined that only 1.0 mg/kg resulted in significantly longer choice latency than all other AMPH doses (all $p < 0.001$; see Figure 4.16).

With COMBO administration, the main effects of treatment group [$F(4,59)=2.9$, $p = 0.029$], drug dose [$F(3,167)=42.7$, $p < 0.001$], and sex [$F(1,59)=4.7$, $p = 0.035$] on trial initiation latency were all significant. Post-hoc analysis of differences due to treatment group determined that the initiation latency in the 22.8 mg/kg PBDE group was significantly shorter than that of controls ($p = 0.034$), while the 6 mg/kg PCB group had a longer latency than that of both PBDE groups (both $p < 0.05$) but did not differ from that of controls (see Figure 4.17). The drug dose x sex interaction approached significance [$F(3,156)=2.7$, $p = 0.055$], and thus was examined post-hoc to better understand the effects of drug dose and sex on trial initiation latency. It was determined that in females 0.125/1.0 and 0.25/1.0 mg/kg significantly increased initiation latency compared to the lower COMBO doses, while this effect was only seen with 0.25/1.0 mg/kg in males (all $p < 0.05$; see Figure 4.18). The latencies of females did not differ from males when
compared at the same dosages. Although treatment group did not affect choice latency, COMBO drug dose significantly affected choice latency [F(3,165)=42.7, \( p<0.001 \)], and an interaction between drug dose x treatment group was also significant [F(11,165)=1.9, \( p=0.04 \)]. Sex did not have a significant effect, nor were other interactions significant. Post-hoc analyses of choice latencies between treatment groups at each COMBO dose were not significant. Post-hoc analysis of drug doses alone revealed that choice latencies at 0.125/1.0 and 0.25/1.0 mg/kg were significantly longer than at smaller dosages, while the 2 higher COMBO dosages also differed from each other (all \( p<0.05 \); see Figure 4.19).

Stability of Performance During Drug Trials

Treatment group was not significant for choice ratio across the 4 saline administration days, but saline day [F(3,177)=4.8, \( p=0.003 \)] and delay [F(2,129)=374.5, \( p<0.001 \)] were significant, as was a day x delay interaction [F(8,485)=3.4, \( p=0.001 \)]. Sex was not significant, nor were other interactions. To examine the day x delay interaction, choice ratios for each of the 4 days were compared at each delay. None of the post-hoc analyses were significant. Visual inspection of the data supports these findings.

4. Discussion

This is the first study to examine the effects of PCBs and PBDEs on impulsive choice. It was found that developmental exposure to PCBs or PBDEs did not result in impulsive choice on the DD task. Nor did contaminant-exposed subjects differ in their response to the dopaminergic drugs AMPH or FLU, with the exception that subjects in
the 22.8 mg/kg PBDE group were significantly faster to initiate trials than controls and subjects in the 6 mg/kg PCB group after combined exposure to FLU and AMPH. These findings are contrary to the experimental hypotheses.

**Lack of PCB/PBDE Treatment Effect on DD**

While it is possible that PCBs and PBDEs do not affect impulsive choice, it is also possible that the experimental methodology used to test impulsive choice in this study impaired the ability for differences to be detected between treated and control subjects. Whether or not the delay to the larger reward is cued has been shown to impact the experimental findings on DD tasks. In the current experiment the delay was cued. That is, if the subject chose the larger-delayed reward lever then the cue light above the lever remained on until the reward was delivered. Cueing the delay has been suggested to serve as a conditioned reinforcer that bridges the interval between choosing the delayed reward lever and the delivery of the reward (Cardinal et al. 2000).

A study of ADHD children performing DD illustrates how cueing the delay can greatly influence the experimental findings. Antrop et al. (2006) tested ADHD and non-ADHD children on both cued and uncued conditions of a DD task. It was found that under the uncued condition ADHD children chose the smaller-immediate reward much more often than non-ADHD children. However, under the cued condition, the choice for the larger-delayed reward increased in both groups so that the performance of the two groups was indistinguishable. Thus, a concern with the current study is that the cued DD task used in the study may actually have served to mask differences in performance between the treatment groups and the control group.
Unfortunately, the above explanation may not always hold true. In a study by Zeeb et al. (2010), male Long Evans rats were trained with either cued rewards or uncued rewards, and then subjects in each group were either classified as low impulsive (LI) or high impulsive (HI). As compared to the uncued condition, the cued condition resulted in a substantial increase in choice for the larger-delayed reward, but only in the LI subjects. Cueing the delay did not significantly improve the performance of the HI subjects. So it is possible that cueing the delay in rodents may improve choice ratio in only a subset of subjects. When subjects in the Zeeb et al. study were collapsed together to compare cued versus uncued conditions, there was a small increase in preference for the larger-delayed reward in the cued group as compared to the uncued group (Stan Floresco, personal communication, May 2013). Other studies in “normal” unexposed rats have found a minimal effect of cueing the delay on choice ratio when subjects were trained on either cued or uncued delays and the subjects were not subdivided by impulsivity (Cardinal et al. 2000; Slezak & Anderson 2009), and it was found that choice ratio did not differ between the cued and uncued groups. Thus, the effect of cueing the delay may vary based on the characteristics of the populations being examined in each study.

Another complicating factor, as suggested by the Zeeb et al. experiment, is the large degree of variation in performance between “normal” unexposed subjects on DD. Galtress et al. (2012) found that 55% of the variance in DD performance was due to individual differences, which was a greater effect than the delay to reward. In addition, differences in DD performance can also vary by strain of rat (Huskinson et al. 2012; Garcia & Kirkpatrick 2013), although individual differences also accounted for a greater
degree of variation than strain (Garcia & Kirkpatrick 2013). A large degree of variance within groups makes it more difficult to statistically detect differences between groups. So although the goal of this study was to have approximately 12 male-female pairs per treatment group, it is possible that this sample size was insufficient to detect effects caused by PCBs or PBDEs because of the large individual variability in DD performance. Because it is possible that factors such as cueing the delay, individual variation in performance, and strain of rat can either act alone or together to influence DD performance and the ability to detect differences between groups, it would be ideal if the study were repeated with a larger sample size using an uncued DD task.

**Drug Effects on DD**

Although contaminant exposure did not have an effect on choice ratio during the drug trials, the influence of sex and the effects of the drug doses on choice ratio were interesting. The effect of sex on choice ratio following AMPH treatment was unexpected, especially in that sex did not interact with other factors; rather, females chose the smaller-immediate reward more frequently across AMPH doses and delays, whereas there was no difference in choice ratio between sexes prior to the drug challenges. This suggests that females were more sensitive than males to the effects of AMPH on choice between immediate and delayed rewards. While other DD studies have not compared the effects of AMPH on performance between sexes, greater behavioral response to AMPH in female rodents has been demonstrated using locomotor activity assessment (Milesi-Halle et al. 2007; Simpson et al. 2012). In addition, estrogen, but not testosterone, increases dopamine release in the striatum and nucleus accumbens and
alters performance on dopamine-mediated tasks in female rats relative to males (Becker 1999). AMPH also differentially affected females in some of the other measures. AMPH increased total omissions at the highest dose and increased trial initiation latency at the 2 highest doses only in females. Interestingly, the sex difference in choice latency seen during Block 5, with females being significantly faster to choose a lever, was not seen during any of the drug trials. This suggests that both drugs obscured the baseline tendency of females to choose faster. Overall, these findings suggest that while AMPH may have affected impulsive choice, other factors such as diminished motivation to perform may have also come into play with AMPH, resulting in increased choice for the smaller-immediate reinforcers in females as compared to males.

Regarding the significant effect of AMPH dose on choice ratio, interactions with delay were only seen at the highest dose (1.0 mg/kg): AMPH decreased choice ratio at 0 sec delay but increased choice ratio at 10 and 20 sec delays. The decrease at 0 sec is concerning because a shift towards the smaller reward when there is no delay for the larger reward suggests that the drug may have affected the ability to distinguish between the experimental contingencies (Koffarnus et al. 2011). This makes interpretation of the findings difficult. However, such a scenario usually involves a shift of the entire discounting curve or a flattening of the curve at shorter delays (Madden & Johnson 2010; e.g., AMPH and apomorphine curves in Koffarnus et al. 2011). In the current study, there was a modest but significant decrement from 91% choice of the larger reward in the control group to 81% choice in the 1.0 mg/kg AMPH group. While this suggests that other factors, such as diminished motivation to perform or difficulty in
distinguishing experimental contingencies, may have come into play, a decrease in choice for the larger reward should have carried over to longer delays if these factors were the only explanation.

The increases in choice ratio at 10 and 20 sec delays with 1.0 mg/kg AMPH more likely represent a decrease in impulsive choice for the reasons discussed above. An increase in choice ratio at this dosage corresponds with findings in at least one other DD study (Huskinson et al. 2012), but is contrary to the findings of studies which found a decrease in choice ratio at 1.0 mg/kg (Evenden & Ryan 1996; Cardinal et al. 2000; van Gaalen et al. 2006; Slezak & Anderson 2009; Koffarnus et al. 2011). However, Cardinal et al. (2000) qualified the effects of 1.0 mg/kg AMPH in decreasing choice ratio as only being present when the delay was uncued. There was no effect of AMPH when the delay was cued. Also, although van Gaalen et al. (2006) reported that 1.0 mg/kg AMPH decreased choice ratio when the delay was cued, that dose actually did not increase choice ratio any more than 0.5 mg/kg AMPH. So 1.0 mg/kg may be a threshold dose at which the effect of AMPH on choice ratio may vary with experimental methodology or due to other factors, such as the rat strain. Huskinson et al. (2012) compared the baseline DD performance and response to AMPH in Lewis and Fischer 344 rats. It was concluded that the Lewis rats were more impulsive at baseline and, subsequently, demonstrated an increase in choice ratio at 1.0 mg/kg, as compared to the less impulsive Fischer 344 rats in which the same dosage decreased choice ratio. It is possible that Long Evans rats are more impulsive on the DD task than strains in the studies in which 1.0 mg/kg AMPH reduced choice ratio [Sprague Dawley (Evenden & Ryan 1996; Slezak & Anderson 2009; Koffarnus et al. 2011), Lister Hooded (Cardinal et
al. 2000), and Wistar (van Gaalen et al. 2006)], which could account for an increase in choice ratio at longer delays. However, no studies have compared the performance of Long Evans rats on DD with that of other strains. Nor have the effects of 1.0 mg/kg AMPH on the DD performance of Long Evans rats been reported before. Floresco et al. (2008) used Long Evans rats in their study, but the highest AMPH dose in that study was 0.5 mg/kg, which did not alter choice ratio.

An effect of sex on choice ratio was not seen with FLU, but an effect of dose was seen, with 0.25 mg/kg decreasing choice ratio in both sexes. The findings for FLU are similar to those seen in other DD studies (Cardinal et al. 2000; Wade et al. 2000; Floresco et al. 2008), but these other studies only tested male subjects. In the current study, dopaminergic blockade appeared to affect choice in a similar manner in both sexes. However, it is important to ask whether FLU actually increased impulsive choice at that dose or affected other components of task performance such as locomotion (Pitts & Horvitz 2000). FLU 0.25 mg/kg significantly increased trial omissions, to the extent that the data from the 40 sec delay was removed from the statistical analysis, and significantly increased latency to initiate trials by 0.7 sec and choice latency by 0.4 sec in both sexes. So it is likely that, to some extent, the changes seen with FLU involved alterations in locomotor activity or motivation. In support of this, it has been suggested that D1/D2 receptor antagonism produced by FLU diminishes the value of conditioned food reinforcers (Beninger & Ranaldi 1993; Wade et al. 2000). At the same time, 0.25 mg/kg FLU did not significantly decrease choice ratio at 0 sec delay, which suggests that the subjects still were willing to follow the experimental contingencies to maximize reward at the 0 sec delay.
In the COMBO drug trials, the effect of contaminant treatment on trial initiation latency was unanticipated since it was not seen prior to drug challenges or with AMPH or FLU alone. It is possible that both PBDE treatment groups were faster to initiate trials than the 6 mg/kg PCB group due to enhancement either of the ability to detect a signal (i.e., the light above the food tray illuminating) or of some other component of the response assessed by trial initiation latency. Whether PBDEs affect attentional processes in rodents has not been examined, while one study of developmental exposure to the commercial PCB mixture, Aroclor 1254 found no effect on signal detection (Bushnell et al. 2002). Because this treatment effect was only seen during the COMBO trials, it is not clear to what extent D1 and D2 receptors were stimulated by increased synaptic dopamine due to AMPH versus antagonized by FLU binding in order to produce the changes seen.

The effect of sex on choice ratio during COMBO trials mirrors that seen during the AMPH trials. Yet the effect also appeared to be tempered by FLU. Although drug dose of FLU/AMPH was not significant, visual inspection of the data showed a tendency for choice ratios at delays above 0 sec to increase as AMPH dose increased from 0.5 to 1.0 mg/kg, but then choice ratios decreased back to the levels seen with control injections as FLU dose increased from 0.125 to 0.25. The sex effect on trial initiation latency also mirrored that seen with AMPH, although it is not surprising that trial initiation latency and choice latency both increased as the doses of AMPH and then FLU increased, since both drugs independently increased latencies. There was not a sex effect on trial omissions, unlike the findings seen with AMPH alone. It is clear from the trials of FLU and AMPH alone that FLU is more potent than AMPH in causing
omissions. So in the COMBO trials the omissions were more likely due to the effect of FLU, which did not cause a sex difference in omissions, being tempered by the effect of AMPH.

5. Conclusions

Other than a finding of treatment group affecting trial initiation latency during trials in which AMPH and FLU were administered together, PCB or PBDE exposure did not affect performance on the DD task. The original hypothesis was that developmental exposures to PCBs or PBDEs would affect impulsive choice by decreasing choice ratio. There is concern that the version of the DD task used in this study, in which delays to delivery of the large rewards were cued, may have masked changes in choice ratio. A version of the task in which delays to reinforcement are not cued may be more sensitive to detecting changes in choice ratio when the effects of developmental exposure to contaminants are assessed.

Similarly, if the cued version of the task was inadequate to detect changes in choice ratio, then any differences between contaminant-exposed subjects and controls during the drug trials may have also been masked. Despite a lack of contaminant effects on choice ratio, interesting sex effects of AMPH on choice ratio were found which have not been previously reported. If preclinical studies are to be successfully extrapolated to human populations, then investigating sex differences in the DD paradigm is important.
6. Figures

Figure 4.1. Delay discounting task. Each daily session had 5 blocks of trials, with the delay to the larger reinforcer increasing between blocks. The rat chose between two levers, one associated with a smaller, immediate reward and the other associated with a larger, delayed reward. Delays were signaled by an illuminated cue light above the lever. Each trial lasted 80 seconds, regardless of choice. Modified from Winstanley et al. (2004).
Figure 4.2. Choice ratios from Blocks 1 and 5 during the fifth training phase. In both Block 1 and Block 5, choice ratios at 5, 10, 20, and 40 sec delays were significantly different than the choice ratio at 0 sec delay (all $p \leq 0.001$).

Figure 4.3. Choice ratios of female and male subjects from Block 5. There are no significant differences between the sexes. This figure is provided to allow comparison to differences in choice ratios between sexes that occurred during drug trials.
Figure 4.4. Trial initiation and choice latencies for both sexes during Block 5. Females chose which lever to press significantly faster than males. *$p \leq 0.05$. 
Figure 4.5. Choice ratio following administration of 0, 0.05, 0.125, and 0.25 mg/kg FLU. Choice ratio was significantly smaller than control with 0.25 mg/kg FLU at 5 and 10 sec delays. Also 0.25 mg/kg resulted in a smaller choice ratio than 0.05 mg/kg at 5 sec delay. Sex differences were not seen during the FLU trials. Data from the 40 sec delay was removed from the analysis due to excessive trial omissions caused by 0.25 mg/kg FLU. *p<0.05, ***p<0.001 as compared to control.

Figure 4.6. Choice ratio following administration of 0, 0.25, 0.5, and 1.0 mg/kg AMPH. At 0 sec delay, choice ratio was significantly smaller with 1.0 mg/kg AMPH than control or 0.05 mg/kg FLU. However, as the delay to reinforcement lengthened, choice ratio became significantly greater with 1.0 mg/kg AMPH than control. *p<0.05, **p<0.01, ***p≤0.001 as compared to control.
Figure 4.7. Sex had a significant effect on choice ratio during AMPH trials. Although increasing AMPH dose resulted in higher choice ratios as the delay to reinforcement lengthened in both sexes, choice ratios at each dose were smaller in females than males.
Figure 4.8. Choice ratio following administration of 0/0, 0.125/0.5, 0.125/1.0, and 0.25/1.0 mg/kg FLU/AMPH. There were no significant differences between doses at each delay.
Figure 4.9. Sex had a significant effect on choice ratio during drug COMBO trials. While the FLU/AMPH doses did not produce statistically different choice ratios at each delay, choice ratios at each dose were smaller in females than males.
Figure 4.10. Increasing FLU dosages increased omissions. Both 0.125 and 0.25 mg/kg caused significantly more omissions than 0 and 0.05 mg/kg. More omissions were also seen at 0.25 mg/kg than at 0.125 mg/kg. ** $p<0.01$, *** $p<0.001$ as compared to control.
Figure 4.11. AMPH increased the number of omissions in females only. 1.0 mg/kg AMPH resulted in significantly more omissions than with smaller dosages in females and when compared to the same dosage in males. ***$p<0.001$ as compared to control. +++$p<0.001$ as compared to same dosage in males.

Figure 4.12. FLU/AMPH together increased the number of omissions. Significantly more omissions occurred in combinations involving 1.0 mg/kg AMPH. ***$p<0.001$ as compared to control.
Figure 4.13. FLU increased trial initiation latency. Both 0.125 and 0.25 mg/kg increased initiation latency as compared to 0 and 0.05 mg/kg. A higher latency was also seen at 0.25 mg/kg as compared to 0.125 mg/kg.*** p<0.001 as compared to control.

Figure 4.14. FLU increased choice latency. Both 0.125 and 0.25 mg/kg increased choice latency as compared to 0 and 0.05 mg/kg. A higher latency was also seen at 0.25 mg/kg as compared to 0.125 mg/kg.** p<0.01, *** p<0.001 as compared to control.
Figure 4.15. AMPH increased trial initiation latency in females but not males. Latency was longer at 1.0 mg/kg than at all other dosages in females, and also at 0.5 mg/kg as compared to control in females. Initiation latency was also longer at the 2 higher dosages in females as compared to the respective dosages in males. *p<0.05, ***p<0.001 as compared to control. +p<0.05, +++p<0.001 as compared to same dosage in males.

Figure 4.16. AMPH increased choice latency at the highest dose. Latency at 1.0 mg/kg was longer than at the other doses. ***p<0.001 as compared to control.
Figure 4.17. Trial initiation latencies varied with treatment group during COMBO trials. Initiation latency in the 22.8 mg/kg PBDE group was significantly shorter than that of controls, while the 6 mg/kg PCB group had a longer initiation latency than that of both PBDE groups. Low PCB=3 mg/kg; High PCB=6 mg/kg; Low PBDE=11.4 mg/kg; High PBDE=22.8 mg/kg. *p<0.05.

Figure 4.18. Trial initiation latency increased with COMBO dosages. In females 0.125/1.0 and 0.25/1.0 mg/kg FLU/AMPH significantly increased initiation latency compared to smaller dosages, while in males only 0.25/1.0 mg/kg significantly increased initiation latency. Latencies of females did not differ from that of males when compared at the same dosages. **p<0.01, ***p<0.001 as compared to control.
Figure 4.19. Higher FLU/AMPH COMBO dosages lengthened choice latency. The 2 higher combination dosages significantly differed from smaller dosages and from each other. ***$p<0.001$ as compared to control.
7. References


Chapter 5. Effects of PCBs and PBDEs on Dopamine Transporter Expression in the Brain

1. Introduction

One mechanism by which polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) have been proposed to exert their effects on neurodevelopment is by influencing the levels of synaptic dopamine. The role of dopamine in response inhibition, and the general effects of PCBs and PBDEs were discussed in Chapter 1. This chapter will more closely focus on the effect of these contaminants on dopamine transporter (DAT), a neuroprotein that regulates synaptic dopamine.

DAT is a 12-transmembrane domain transporter protein that imports synaptic dopamine along its concentration gradient into the cytosol of the presynaptic terminal (Torres et al. 2003). DAT levels are much higher in the striatum as compared to other brain regions (Moll et al. 2000) due to the greater density of dopaminergic neurons in the striatum. Functionally, striatal DAT recycles synaptic dopamine, whereas in the prefrontal cortex norepinephrine transporter has a greater role than DAT in clearing synaptic dopamine (Yamamoto & Novotney 1998; Devoto et al. 2004). Vesicular monoamine transporter 2 (VMAT2), in turn, is a 12-transmembrane domain protein that imports cytosolic dopamine into the vesicles against its concentration gradient as part of the repackaging process (Wimalasena 2011). It is vital that both transporters function in concert to terminate synaptic signaling and to minimize cytosolic dopamine concentrations. Because dopamine serves as a neuromodulator, DAT dysfunction can
result in either excess or reduced levels of dopamine binding to D1 receptor, which has detrimental effects on many different cognitive functions (Seamans & Yang 2004). VMAT2 dysfunction can also alter the amount of dopamine released into the synapse, but it can additionally result in oxidative damage to dopaminergic neurons if excess dopamine, which is readily oxidized, accumulates in the cytosol (Guillot & Miller 2009).

There is evidence that PCBs reduce expression as well as impair the function of DAT and VMAT2. Two commercial PCB mixtures, Aroclors 1016 and 1260 decreased striatal DAT expression in adult male mice, but only Aroclor 1260 decreased VMAT2 expression in the same subjects (Richardson & Miller 2004). In a follow-up study, it was shown that exposure to a 1:1 mixture of Aroclor 1254 and 1260 decreased DAT and VMAT2 expression in the striatum. However the change in DAT expression was not due to altered DAT mRNA levels in the dopaminergic cell bodies in the ventral mesencephalon (Caudle et al. 2006). In the same study, DAT expression was unaltered in the PFC of mice. Recently, it was shown that DE-71 also decreased DAT and VMAT2 expression in the striatum of adult mice (Bradner et al. 2013). Of interest, these rodent study findings parallel those in a study of adult humans who were occupationally exposed to PCBs. In that study, higher serum PCB levels were associated with lower striatal DAT densities in women, but not men, as assessed by an advanced imaging technique (Seegal et al. 2010).

Complementing the studies of transporter expression, studies utilizing in vitro preparations of synaptosomes, to assess DAT function, and neurotransmitter vesicles, to assess VMAT2 function, have led to a better understanding of the effects of PCBs and PBDEs on functioning of these transporters. Aroclors 1242 and Aroclor 1254 were
shown to inhibit dopamine uptake into synaptosomes from rat brains (Mariussen & Fonnum 2001). Similar findings were seen in a study using synaptosomes of mice postnatally exposed to Aroclor 1254 (Tian et al. 2011). Furthermore, individual ortho-chlorinated (non-coplanar) PCB congeners, but not coplanar congeners, were shown to inhibit dopamine uptake into rat brain synaptosomes (Mariussen & Fonnum 2001) and vesicles (Mariussen et al. 2001). The same researchers then demonstrated that DE-71, but not more highly brominated PBDE mixtures, also inhibited synaptosomal and vesicular dopamine uptake (Mariussen & Fonnum 2003). Relevant to the current study, Fox River PCB Mix was shown to be 2-3 times more potent than DE-71 at inhibiting synaptosomal dopamine uptake in immature rats (Dreiem et al. 2010).

Building upon the current body of evidence, this study sought to determine whether the Fox River Mix and DE-71 alter DAT expression in 4 brain regions that are important for the performance of 2 behavioral tasks which are mediated through dopaminergic signaling. It was hypothesized that developmental PCB or PBDE exposure would decrease DAT expression at postnatal day (PND) 90 in 4 regions of the brain. Specific regions postulated to be affected were medial prefrontal cortex (mPFC), orbitofrontal cortex (OFC), nucleus accumbens (NAC), and dorsal striatum (dSTR). These regions play important roles in the differential reinforcement of low rates of responding (DRL) and delay discounting (DD) behavioral tasks used in this research (see Chapters 3 and 4).

2. Methods and Materials

2.1. Animals and Exposure
Details of the perinatal exposure of experimental subjects to PCBs and PBDEs were discussed in Chapter 3. In brief, Long Evans rat dams were allocated between 5 treatment groups: control, 3 mg/kg PCBs, 6 mg/kg PCBs, 11.4 mg/kg PBDEs, and 22.8 mg/kg PBDEs. Dams were exposed daily beginning 4 weeks prior to breeding through postnatal day (PND 20). Pups were weaned from the dams on PND 21 and male-female pairs of pups from each litter were allocated to behavioral testing or to western blot analysis of brains on PND 90. Pups allocated for western blot analysis were double- or triple-housed together with others of the same sex and same contaminant exposure group until PND 90. On PND 90, these subjects were euthanized by overexposure to CO₂ followed by decapitation. The brains were quickly removed, weighed, and then snap frozen in liquid nitrogen. The brains were stored at -80°C until western blot analysis was performed.

2.2. Brain Dissection

Each PND 90 brain was positioned on the frozen stage (-20°C) of a microtome so that successive coronal slices could be taken in an anterior to posterior direction. Landmarks from the Paxinos and Watson (1998) brain atlas were used to guide cutting, but it should be noted that the anterior-posterior distances noted in the atlas did not perfectly correspond with the distances in the specimens. Thus when distances from Bregma are noted, they are only intended to allow orientation to the appropriate diagrams in the brain atlas that show relevant landmarks.

Slices were removed until Bregma 5.20 mm was reached, at which point the sizes of the frontal cortices and the olfactory bulbs were approximately equal. Then a 1.75 mm
(females) or 1.85 mm (males) thick coronal slice was cut. The slice was placed on a glass slide with the anterior side facing down. Using a 2.0 mm circular tissue punch (Harris Uni-Core; Ted Pella, Inc.) a single punch of the mPFC was taken (see Figure 5.1) with the punch directed perpendicular to the plane of the slide. The punch was primarily centered on the prelimbic portion of the mPFC. From this same slice, two 2.0 mm circular punches of the OFC were taken (see Figure 5.1). The punches were centered over the ventral orbital portion of the OFC and directed in a ventro-medial and anterior direction to follow the contour of the ventral orbital OFC. All tissue punch specimens were immediately placed in closed vials and snap frozen with subsequent storage at -80° C until the time of western blot analysis.

Next, thin slices were taken and discarded until Bregma 1.70 mm was reached, at which point the two sides of the corpus callosum were < 1 mm apart. From this point, a 1.75 mm (females) or 2.0 mm slice (males) thick coronal slice was cut and placed on a glass slide with the posterior side facing down. Two 2.0 mm circular punches were taken with the punch directed perpendicular to the plane of the slide. The punch was centered over the NAC core, but also included NAC shell and some of the ventro-medial striatum (see Figure 5.2). From this same slice, two 2.0 mm punches of the dSTR were also taken (see Figure 5.2). Each punch was centered just ventral to the corpus callosum and then directed in a dorso-posterior direction so that the punch remained centered under the corpus callosum throughout.

2.3. Chemicals
Complete Mini Protease Inhibitor (PI) Cocktail Tablets were obtained from Roche. Pierce T-PER Tissue Protein Extraction Reagent, Pierce BCA Protein Assay Kit, Restore Plus Western Blot Stripping Buffer, and CL-XPosure X-ray film were obtained from Thermo Scientific. Corning Costar 96-Well Cell Culture Plates, Amersham Full-Range Rainbow Molecular Weight Markers, and Tris Buffered Saline 10x were obtained from Fisher Scientific. NuPAGE Novex 4-12% Bis-Tris Gels, NuPAGE LDS Sample Buffer 4x, NuPAGE Sample Reducing Agent 10x, NuPAGE MES SDS Running Buffer 20x, NuPAGE Antioxidant, NuPAGE Transfer Buffer 20x, and Invitrolon PVDF/Filter Paper Sandwiches (0.45 μM pore size) were obtained from Life Technologies. TWEEN 20 was obtained from Sigma-Aldrich. Blotting Grade Blocker nonfat dry milk was obtained from Bio-Rad Laboratories. Anti-Dopamine Transporter rabbit polyclonal antibody (#AB2231) and Goat Anti-Rabbit IgG, HRP-conjugate, polyclonal antibody (#12-348) were obtained from Millipore. These were used as the primary and secondary antibodies for dopamine transporter. Anti-Alpha Tubulin rabbit polyclonal antibody (#ab4074) and Goat Secondary Antibody to Rabbit IgG polyclonal antibody (#ab6721) were obtained from Abcam. There were used as the primary and secondary antibodies for alpha tubulin. 20x LumiGLO Reagent and 20x Peroxide were obtained from Cell Signaling Technology.

2.4. Western Blot Analysis

Only PND 90 brains were analyzed for this project. There were 5 male-female pairs of brains analyzed from each treatment group, except for the 3 mg/kg PCB group which had 6 pairs analyzed.
Protein Extraction and Quantitation

One PI tablet was dissolved in 10 mL T-PER. The samples from each brain region were suspended and homogenized in the following amounts of T-PER/PI: 190 μL for each mPFC sample; 375 μL for each pooled pair of OFC, NAC, and dSTR samples; 500 μL for each liver sample (see methods for negative control for more details on liver samples). The homogenized sample was then centrifuged at 10,000 x g for 5 minutes, at 4° C. The supernatant was used in the BCA protein assay. Also at this time, an aliquot of the supernatant from each sample was diluted 1:20 in T-PER/PI. The 1:20 dilution of protein sample would later be loaded into each lane of the gels.

The protein assay was performed using the directions for the microplate procedure provided in the instructions of the Pierce BCA Protein Assay Kit. In brief, for each sample, 10 μL of protein supernatant was diluted in 15 μL of TPER-PI so that the measured protein amounts were within the range of the standard curve. The samples and standards were added to wells of a microplate and incubated at 37° C for 30 minutes. The absorbance of the samples at 562 nm was then measured using a Multiskan Ascent microplate reader (Type 354; Thermo Scientific). Protein concentrations were calculated using Ascent Software (v. 2.6, Revision 3.1, Dec. 2003; Thermo Scientific).

In order to be able to compare protein expression between different gels, a protein standard that was put in one lane of every gel was created. This was done by preparing mPFC, OFC, and NAC samples from one subject using the methods described above. Then the supernatants from the 3 samples were combined and the concentration in the
resulting solution was analyzed. As with the other samples, a 1:20 dilution of the protein standard was used during the western blot procedure.

**Electrophoresis and Membrane Transfer**

A stepwise description of the methods used for western blotting is in the Appendix. In brief, for each sample, a volume equal to a target amount of 0.25 μg protein was added to reducing agent, sample buffer, and a sufficient quantity of T-PER/PI to attain 15 μL sample volume. For the molecular weight ladder, 5 μL of marker was used in place of 0.25 μg protein. Samples were denatured while heating at 70˚ C for 10 minutes. Then the protein ladder, protein standard, and protein samples were loaded into the lanes of the gel. Each gel contained samples from one brain region and at least one male-female pair of samples from each treatment group. The pairs of samples were randomized across gels. Once running buffer was added, gels were electrophoresed at 150 volts for 1 hour.

In preparation for membrane transfer, the PVDF membrane was soaked in methanol, water, and then transfer buffer. Filter paper, fiber pads, and each gel were soaked in transfer buffer. A “sandwich” was made with the gel and membrane apposed in the middle surrounded by filter paper and then fiber pads on the outside. Transfer buffer was added and the transfer module was electrophoresed in a cold room at 30 volts for 1.5 hours.

**Blocking and Probing**
The membrane was rinsed in Tris Buffered Saline and TWEEN 20 (T-TBS). T-TBS was used for all subsequent rinses. The membrane was blocked in 5% milk solution for 1 hour and then incubated with anti-DAT primary antibody at 1:2000 w/v in 5% milk overnight.

The next day an incubation of goat anti-rabbit secondary antibody (Millipore) at 1:2000 w/v in 1% milk was performed for 1 hour. The membrane was then treated with LumiGLO and chemiluminescence was captured on X-ray film. The membrane was subsequently stripped and then blocked again in 5% milk solution for 1 hour. It was then incubated with anti-alpha tubulin primary antibody at 1:10,000 w/v in 5% milk overnight.

On the third day the membrane was incubated with goat anti-rabbit secondary antibody (Abcam) at 1:2500 w/v in 2% milk for 1 hour. Then the membrane was treated with LumiGLO and chemiluminescence was captured on X-ray film.

2.5. Negative Experimental Control

The aim of the negative control was to demonstrate that the bands suggested to be DAT protein were not present in tissue that does not have a significant amount of DAT. Liver is poorly innervated by dopaminergic neurons (Eldrup et al. 1989). Because liver does not have appreciable levels of DAT protein, it has been used as a negative control for DAT by others (Maggos et al. 1997). The hypothesis for the negative control was that there would not be a band present in liver tissue at the molecular weight of DAT protein.

Liver from PND 21 had been stored at -80° C from when it was collected. The liver was allowed to thaw enough to allow 2.0 mm tissue punches to be taken from the
parenchyma. Western blotting was performed. It was determined that undiluted supernatant from protein extraction had to be used to detect alpha tubulin in liver samples.

2.6. Data Analysis

Following probing for DAT, bands were detected between the 76K and 102K molecular weight markers, which was the anticipated location for DAT (see Figure 5.3 for example). Following probing for alpha tubulin, bands were detected above the 52K molecular weight marker, which was the anticipated location for alpha tubulin (see Figure 5.4 for example). Densitometry of the bands was performed using ImageJ (version 1.46r, http://imagej.nih.gov/ij). Briefly, developed X-ray films were scanned (HP Scanjet 4850, Hewlett-Packard) and saved as 600 ppi in TIFF format. The densities of samples for DAT and alpha tubulin were determined and then standardized to the protein standard densities on each image to obtain relative densities for DAT and alpha tubulin. Then adjusted densities for the samples were determined by dividing the relative density of each sample by the relative density of the standard. Adjusted densities were used in the statistical analyses, which were conducted using SPSS for Windows (version 20.0, SPSS Inc.). Statistical significance was set at $p<0.05$. Adjusted densities for treatment groups were compared for each of the 4 brain regions using mixed ANOVAs with sex (nested within litter) as the within-subjects factor and treatment group as the between-subjects factor. Negative control data were not subjected to statistical analysis. Data are reported as mean ± SEM.
3. Results

DAT protein expression in mPFC, OFC, NAC, or dSTR was not significantly altered by PCB or PBDE exposure (see Figure 5.5). However, upon visual inspection, it appears that there was a tendency for DAT density to be increased for the 6 mg/kg PCB group in the striatum. Sex had a significant effect on DAT expression in the NAC, but not in the other 3 regions [F(1,21)=4.564, p=0.045] (see Figure 5.6). The adjusted density for DAT expression in NAC was decreased for females (1.250±0.108) versus males (0.986±0.096).

For the negative control experiment, there was not a detectable band in the location where a DAT band was expected for the liver sample (see Figure 5.7). However, there was an alpha tubulin band detected for the liver sample.

4. Discussion

Perinatal contaminant exposure did not alter DAT expression at PND 90 in the 4 brain regions examined: mPFC, OFC, NAC, dSTR. This is in contrast to the findings of earlier studies. Visual inspection of the results suggests a trend for DAT expression in dSTR to be greater in the 6 mg/kg PCB group. While the direction of the difference is in the opposite direction than was hypothesized, the striatum is the region where changes in DAT expression in PCB and PBDE exposure have consistently been reported (Richardson & Miller 2004; Caudle et al. 2006; Bradner et al. 2013). It should be noted that these findings are based upon 5-6 subjects from each sex per treatment group. Brains from additional PND 90 subjects have been archived, so analyzing additional samples is planned. If a significant increase in DAT expression in the striatum is
detected after increasing the sample size, it may suggest that there are differences in response of the dopaminergic system following perinatal PCB exposure, given that all of the studies cited above involved contaminant exposure in subjects that were young adults or older.

When considering why there was a lack of findings in the current study versus previous studies, closer examination of the methodologies of the prior studies reveals differences that may account for the negative findings in the current study. Richardson and Miller (2004) administered a single, very high dosage (500 mg/kg) of Aroclor 1016 or Aroclor 1260 to 8-10 month-old male mice and examined striatal DAT expression 1, 7, and 14 days later. Decreases in DAT expression were seen at all time points. Caudle et al. (2006) administered 7.5 or 15 mg/kg of Aroclor 1254:Aroclor 1260 to 8 week-old male mice for 3, 7, 14, or 30 days and then examined DAT expression in the striatum and prefrontal cortex the day after dosing was finished. No changes were seen after 3 or 7 days of exposure, and there were no changes in prefrontal DAT expression at either dose for up to 30 days of exposure. However, decreased striatal DAT expression was seen after 14 and 30 days of exposure at either dose. In the DE-71 study, 4 month-old mice administered 30 mg/kg for 30 days were shown to have decreased striatal DAT expression 1 day post-dosing (Bradner et al. 2013).

As already mentioned, one of the main ways the prior studies differ from the current one is that exposure occurred perinatally in the current study, while older subjects were exposed in the previous studies. In addition, the interval between when dosing was completed and when DAT expression was evaluated was from 1 day up to 2 weeks. In the current study, the last day of exposure was on PND 20 while the brains were
collected on PND 90. It is possible that there were changes in DAT expression present earlier that were resolved by the time of assessment. Another way that prior studies differ is that they were performed in mice while the current study utilized rats. Although similar findings were seen in rats and mice in studies of the effects of PCBs on DAT functioning (Mariussen & Fonnum 2001; Tian et al. 2011), species-differences in the effect of PCBs on DAT expression can not be completely discounted.

One more consideration raised by the prior studies is whether the doses of PCBs and PBDEs were sufficient to induce changes in DAT expression. It is clear that higher doses of PCBs cause changes more quickly: 500 mg/kg PCBs produced a decrease in DAT expression within 24 hours (Richardson & Miller 2004), while it took more than 7 days for 7.5-15 mg/kg PCBs administered daily for decreased DAT expression to develop (Caudle et al. 2006). It could be possible, given that it was the dams that were dosed with 3 or 6 mg/kg PCBs or 11.4 or 22.8 mg/kg PBDEs that the amount of either contaminant to cross the placenta to the pups was insufficient to cause changes in DAT expression.

The best way to try to answer questions about whether the exposure period, the interval after dosing, and dose itself contributed to the negative findings is to examine brains from PCB- and PBDE-exposed subjects earlier in development. This is possible because PND 21 brains from pups from all treatment groups were collected and archived. The next step should be to examine DAT expression on the PND 21 brains. The findings may address the concerns raised by examining PND 90 brains.

The finding of females having greater DAT density than males in the NAC, but not in the other 3 regions, is interesting. While there is sufficient evidence available that there
are sex differences in dopaminergic and, specifically, DAT functioning (Becker 1999; Dluzen & McDermott 2008), surprisingly limited information is available on sex differences in DAT expression in laboratory species. Significantly higher levels of DAT binding in the whole striatum and higher levels of DAT mRNA in the substantia nigra have been reported in females versus males (Morissette & Di Paolo 1993; Rivest et al. 1995). The evidence is even stronger for higher striatal DAT density in human females, as discussed by Wong et al. (2012). It should be acknowledged that the difference in DAT expression in the NAC in the current study was subtle. However, given that it was based on findings from 26 subjects of each sex it is unlikely to be a spurious finding. It is also not clear why a similar sex difference was not found in the dSTR, which is very richly innervated with dopaminergic neurons. It will be of interest to determine if there are any sex differences in the brains of PND 21 subjects.

5. Conclusions

While the other studies have reported decreased DAT density in rodent brains following PCB exposure, the current study did not find such differences subsequent to developmental exposure to PCBs or PBDEs. The brain regions in which DAT expression was examined are important for performance of the DRL and delay discounting tasks, which are modulated by dopaminergic signaling (see Chapters 3 and 4). Thus, the lack of PCB/PBDE-related findings in the western blot study is consistent with the negative results of the behavioral studies. Sex differences in DAT expression in NAC or prefrontal regions have not been examined before, so the finding of increased
DAT density in the striatum of females is new. However a sex difference in dSTR was not found, which differs from findings in the limited number of prior studies.

Several reasons exist for why DAT density may not have been affected in the current study, all of which reflect differences in experimental design from prior studies. At this point, an important next step will be to examine PND 21 brains for treatment- and sex-related differences in DAT expression. The brains from that time point will more closely match the time frame after the exposure period when DAT expression was examined in prior studies.
6. Figures

Figure 5.1. Locations of tissue punches of frontal cortices. One 2.0 mm punch was centered over the prelimbic (PrL) portion of the medial prefrontal cortex. Two 2.0 mm punches were centered over the ventral orbital (VO) portion of the orbitofrontal cortices. Figures modified from Paxinos and Watson (1998).
Figure 5.2. Locations of tissue punches of striatal regions. Two 2.0 mm punches were bilaterally centered over the nucleus accumbens core (AcbC). Two separate 2.0 mm punches were bilaterally centered over the dorsal striatum (CPu). Figures modified from Paxinos and Watson (1998).
Figure 5.3. Example of DAT bands in all 4 brain regions. These bands were consistently visible between molecular weights of 76K and 102K. The double bands were common in NAC and dSTR samples. The bands are those of the same female (F) subject on the left and the same male (M) subject on the right for each of the 4 brain regions. dSTR = dorsal striatum; mPFC = medial prefrontal cortex; NAC = nucleus accumbens; OFC = orbitofrontal cortex; std = protein standard.

Figure 5.4. Example of alpha tubulin bands in all 4 brain regions. These bands were consistently visible above the 52K marker. The bands are from the same subjects and the abbreviations are the same as in Figure 5.3.
Figure 5.5. Examining for differences in DAT expression in 4 brain regions. Neither PCB nor PBDE exposure significantly affected DAT density. Abbreviations are the same as in Figure 5.3. Low PCB = 3 mg/kg exposure group; High PCB = 6 mg/kg; Low PBDE = 11.4 mg/kg; High PBDE = 22.8 mg/kg.
Figure 5.6. Examining for differences in DAT expression in 4 brain regions. Sex had a significant effect on DAT expression in NAC only. Abbreviations are the same as in Figure 5.3. *$p<0.05$. 
Figure 5.7. In the negative control experiment, DAT was not detected in liver samples but alpha tubulin was. Both proteins were detected in brain samples. Lane 1: undiluted supernatant from liver extraction. Lane 2: supernatant from liver extraction diluted 1:10. Lanes 3-5: pooled protein standards from brain; lane 4 is the protein standard used throughout the experiment. A. There is no detectable DAT band at the appropriate position in lane 1 or lane 2 (liver). There is binding of DAT antibody to protein of a molecular weight much less than that of DAT in lane 1. The protein standards (brain) all have a DAT band in the appropriate position. B. Alpha tubulin was detected in the liver sample in lane 1 and the brain samples in lanes 3-5. The molecular weight marker is not included in B. for reasons similar to that stated in Figure 5.5. The asterisk (*) indicates the position of the alpha tubulin bands. The left-pointing arrowhead (⟨) points to the residual DAT bands after membrane stripping; the right-pointing arrowhead (⟩) points to the bound protein in lane 1 in A. Both arrowheads permit visualization of the position of the alpha tubulin bands relative to the DAT bands. Abbreviations are the same as in Figure 5.3.
7. References


Chapter 6: Conclusions

1. Discussion

The first specific aim of this research was to use a rodent model to assess the effects of developmental exposure to polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) on two distinct aspects of response inhibition: impulsive action and impulsive choice. The differential reinforcement of low rates of responding (DRL) task was used to assess impulsive action while the delay discounting (DD) task was used to assess impulsive choice. It was hypothesized that contaminant-exposed subjects would exhibit deficits on DRL and DD tasks as compared to controls.

It was found that PCB or PBDE exposure did not affect DRL performance. This is in contrast to a study by Sable et al. (2009) which found that developmental PCB exposure decreased the ratio of reinforced:non-reinforced lever presses. It is possible that differences in diet, contaminant dose, or prior experience account for the absence of significant findings in the current study. PCBs and PBDEs also did not affect choice ratio on the DD task. However, an important experimental manipulation, cueing the delay to delivery of the large reinforcer, may have affected subjects’ choice to the extent that the task was unable to elicit differences in performance. Providing a visual cue while the subject is waiting for the larger-delayed reward can make it easier for both high and low impulsive subjects to wait. The result is that choice ratio may increase in cued versus uncued tasks, especially with impulsive subjects, at longer delays. Another important factor which may have confounded the ability to detect treatment differences in either task is inter-individual variation in impulsivity. Much variation has been shown
to exist between subjects in performance of tasks that assess impulsive action (Dellu-Hagedorn et al. 2004) and impulsive choice (Galtress et al. 2012). As a result, the variance due to inter-individual differences in impulsivity reduces the ability of statistical analyses to detect a difference. Although efforts were made to have 12 pairs of male-female subjects per treatment group in this study, it is possible that a larger sample size may be needed to enable the detection of treatment-related effects when employing behavioral tasks in which there is sizeable inter-individual variation in impulsive performance.

The second specific aim was to use drug challenges to assess the role of perturbations in dopaminergic neurotransmission in mediating the effects of developmental exposure to PCBs and PBDEs on response inhibition. Amphetamine (AMPH) and the D1/D2 receptor antagonist flupenthixol (FLU) were parenterally administered to test the hypothesis that there would be shifts in the dose-response curves such that performance of contaminant-exposed subjects on DRL and DD tasks would be more improved by drugs that enhance dopaminergic neurotransmission (AMPH) and more disrupted by drugs that reduce dopaminergic neurotransmission (FLU).

Neither drug produced treatment-related differences on the primary measures used to assess impulsive performance on either DRL or DD task. It is possible that if the current experimental methods were not sensitive enough to detect a difference between treated subjects and controls, then differences in dopaminergic signaling between groups were not great enough to result in a greater or lesser degree of perturbation between treated subjects and controls. However, due to the number of trial omissions
produced by the highest FLU and AMPH dosages, further increases in dosage of either
drug would not be beneficial in trying to elicit differences between groups. A treatment
effect on trial initiation latency was detected in the FLU and AMPH combination drug
trials: the 22.8 mg/kg PBDE group was significantly faster than controls to initiate trials,
while the 6 mg/kg PCB group was significantly slower than both PBDE groups, but not
controls. This effect was not seen in testing that occurred prior to the drug challenges or
with FLU or AMPH alone, so it is not clear to what extent binding of dopamine to D1 and
D2 receptors under the combination drug condition differed from dopamine binding
during the single drug trials to produce the changes seen.

The third specific aim was to use western blot analysis to quantify expression of the
dopamine transporter (DAT) in specific regions of the prefrontal cortex and striatum
following developmental exposure to PCBs and PBDEs. Tissue punches of medial
prefrontal cortex, orbitofrontal cortex, nucleus accumbens, and dorsal striatum were
taken on postnatal day (PND) 90 from subjects perinatally exposed to PCBs or PBDEs
and controls. These regions were chosen because they are important for performance
of either DRL or DD tasks. It was hypothesized that DAT expression would be reduced
in the prefrontal cortex and striatum of subjects developmentally exposed to PCBs and
PBDEs.

Contaminant exposure did not alter DAT expression in any of the brain regions
examined. It is possible that the doses of PCBs and PBDEs used in the current study
were not sufficient to induce changes in DAT expression. It is also possible that
changes in DAT expression occurred, but that differences then normalized during the
interval between when contaminant exposure ceased (PND 20) and when the brains were evaluated (PND 90).

In conclusion, developmental PCB and PBDE exposure did not result in any significant changes in impulsive action or impulsive choice as assessed by the DRL and DD tasks; nor did FLU or AMPH differentially alter performance of PCB- or PBDE-treated groups on either task. Additionally, DAT expression did not differ between contaminant-exposed groups and controls in any of 4 different brain regions when assessed at PND 90. While it is tempting to conclude that the influence of PCBs and PBDEs on impulsive action and choice and DAT expression is minimal, a large body of experimental and epidemiologic evidence argues otherwise, as discussed in Chapter 1. It is more accurate to state that PCB and PBDE exposure did not result in impulsive behavior or altered DAT expression under the current experimental methodologies. Reasons were outlined above as to why certain experimental factors and the experimental methods used in these studies may have prevented differences from being discerned between treatment groups. Thus, it is difficult to draw conclusions about the effects of the PCB and PBDE mixtures used in this study on impulsive behavior and DAT expression. Because of these concerns, it is also difficult to extrapolate the current findings to whether PCBs and PBDEs influence the clinical manifestation of attention deficit hyperactivity disorder (ADHD).

2. Future Studies

It has been shown that cueing the delay during the DD task may enable more impulsive subjects to better tolerate delays to the larger reinforcer, resulting in greater
choice ratios at longer delays than if delays are uncued (Antrop et al. 2006). Because the current study used cued delays, an important consideration would be to assess whether differences become evident between contaminant-exposed groups and controls if a version of the DD task that does not cue delays is used. Another improvement on the current methodology would be to investigate whether differences in DAT expression between PCB- and PBDE-exposed groups and controls are evident in PND 21 brains. If differences were found, it would raise questions about whether changes in dopaminergic signaling are present earlier in the developmental period and then resolve as treated subjects mature. Regardless of differences in DAT expression, it would also be interesting to investigate whether PCB and PBDE exposure results in impulsive behavior at a younger age than that evaluated in the current studies. If there were positive findings, it would have interesting implications for the role of developmental contaminant exposure in ADHD, for which the prevalence decreases as subjects age and enter adulthood (Faraone et al. 2006).

Another interesting route of investigation would be to explore the potential for brominated flame retardants other than PBDEs to affect response inhibition and dopaminergic signaling. Hexabromocyclododecane (HBCD) and tetrabromobisphenol-A (TBBPA) are 2 high production level brominated flame retardants that are among those that have replaced PBDEs in industrial products (Kemmlein et al. 2009). Both HBCD and TBBPA have been shown to be more potent than PBDEs in impairing DAT and vesicular monoamine transporter 2 reuptake (Mariussen & Fonnum 2003). Both contaminants have also been shown to increase intracellular concentrations of free calcium, similar to PCBs and PBDEs (Dingemans et al. 2009; Hendriks et al. 2012).
HBCD has also been shown to alter latency to movement time in a haloperidol catalepsy test in rats, which is an experimental measure that is strongly influenced by dopaminergic signaling (Lilienthal et al. 2009). Using some of the methods developed in the current study to further assess the effects of HBCD and TBBPA on different aspects of dopaminergic neurotransmission will potentially further what is known about these two newer contaminants which are widely used but relatively unstudied.
3. References


Appendix: Western Blot Procedure

Running the Gel
For 15 well gels with 15 μL sample volume per well.
1) Prepare 1.5 μL reducing agent and 3.75 μL sample buffer per well so have total of 27 μL reducing agent + 67.5 μL sample buffer.
2) In 1.5 mL tubes, add T-PER/PI, then calculated amount of protein, then 5.25 μL of sample buffer + reducing agent combination. For ladder, add 5 μL of ladder instead of protein.
3) Mix and centrifuge briefly.
4) Heat at 70° C for 10 min. Mix and centrifuge briefly.
5) Make Running Buffer in 1000 mL container:
   a. 1 gel: 825 mL nanopure water + 43.4 mL NuPAGE MES SDS Running Buffer.
   b. 2 gels: 875 mL nanopure water + 46.1 mL NuPAGE MES SDS Running Buffer.
6) Make buffer for inside of gel compartment:
   a. 1 gel: pour 250 mL in conical container and add 625 μL antioxidant.
   b. 2 gels: pour 300 mL in conical container and add 750 μL antioxidant.
7) Remove gel from packaging and rinse with regular water.
8) Remove comb and white strip.
9) Use solution from step 6 and transfer pipette to flush wells in gel 3x.
10) Put gels in tank and seal. Make sure the open side of the gels face the inner compartment. Then add running buffer from step 6 to fill inner compartment.
11) Load lanes using long 200 μL pipette tip and pipetter set to 16.5 μL. Avoid bubbles. Suggested order for lanes is ladder, blank, universal standard, then 10-12 samples.
12) Pour rest of running buffer from step 5 into outer compartment to bring it to a level even with buffer in inner compartment.
13) Put lid on and run at 150 V for 1 hour.

Transfer
1) When gel is halfway through, prepare transfer buffer and other things needed for transfer.
2) Prepare transfer buffer in 500-1000 mL container
   a. 1 gel: 255 mL nanopure water, 15 mL NuPAGE Transfer Buffer (20X), 30 mL methanol, 300 μL antioxidant.
   b. 2 gels: 510 mL nanopure water, 30 mL NuPAGE Transfer Buffer (20X), 60 mL methanol, 600 μL antioxidant.
3) Fill a tray with methanol, one with water, and one with transfer buffer – just enough to soak membranes. Fill 2 more trays with buffer.
4) Remove PVDF membrane from between filter papers and write date and other info on it in pencil.
5) Soak membrane in methanol for 30 sec, then briefly agitate in the water, then soak in transfer buffer. Filter papers also soak in transfer buffer.
6) Soak sponges in transfer buffer.
7) Use gel knife to carefully crack open gel cassette. Pull the cassette off to expose the gel. Rinse gel with transfer buffer using a transfer pipette. Cut off the wells and the bottom of the gel below the blue streak.
8) Soak gel in transfer buffer for a few minutes.
9) Put gel on one filter paper and then make a sandwich in the transfer module: 3 sponges – filter paper – gel – PVDF membrane – filter paper – 3 sponges. Roll over topmost filter paper with pipette to remove air bubbles before putting the last 3 sponges in place.
10) Lock transfer module into gel tank and fill inner compartment with transfer buffer.
11) Fill outer chamber with ice cold water.
12) Put lid on and transfer in cold room at 30 V for 1.5 hours.

Blocking and Incubation for DAT then Developing
1) Prepare T-TBS (TBS with Tween 20) as needed: 1 L of 1X TBS and 1 mL of Tween 20. Stir for 1 hour.
2) Prepare 5% blocking milk: 2.5 g nonfat powdered milk per 50 mL T-TBS
3) Remove membrane from transfer module. Briefly rinse in T-TBS.
4) Block in 15 mL of 5% milk for 1 hour on orbital shaker.
5) Primary antibody: Add 7.5 μL of rabbit anti-DAT (Millipore) to 15 mL of 5% milk. Incubate overnight on orbital shaker in cold room. Wrap tray in saran wrap.
6) Rinse 3X in T-TBS for 10 min each time on orbital shaker.
7) Secondary antibody: Make 1% milk (3 mL of 5% milk + 12 mL T-TBS) and add 7.5 μL of goat anti-rabbit (Millipore). Incubate 1 hr on orbital shaker.
8) Rinse 3X in T-TBS for 10 min each time on orbital shaker. Turn on film developer 30 min ahead of time.
9) Supplies for dark room:
   a. 13.5 mL nanopure water in 15 mL tube
   b. Membrane in T-TBS
   c. Spare tray for treating membrane
   d. LumiGLO Reagents A and B
   e. 1000 mL capacity pipette and tips
   f. Tweezers
   g. Film and cassette
   h. Kim wipes
   i. Timer
   j. Keys
   k. Sharpie
10) LumiGLO is light sensitive so only do this under red light. Add 0.75 mL from clear bottle (Reagent B) then 0.75 mL from dark bottle (Reagent A).
11) Remove membrane from T-TBS and put in spare tray. Pour LumiGLO over membrane and rotate tray for 1 min.
12) Drain excess LumiGLO from membrane and put membrane between protector sheets in cassette. Wipe with Kim wipe to remove any air bubbles.
13) 30 sec exposure of film.
14) Crimp corner of film that is in corner of cassette so can go back and mark the ladder on the film after developing.
15) Run film through developer.
16) Decrease or increase exposure time of subsequent films as needed to attain bands that are visible but not too dark.

**Blocking and Incubation for Alpha Tubulin then Developing**

1) Strip antibodies from membrane by rinsing briefly in T-TBS then put membrane in 15 mL of Restore Plus Western Stripping Buffer for 15 min on orbital shaker.
2) Rinse 3X in T-TBS for 5 min each time on orbital shaker.
3) Block in 15 mL of 5% milk for 1 hour on orbital shaker.
4) Primary antibody: Add 10 μL of rabbit anti-alpha tubulin (Abcam ab4074) to 15 mL of 5% milk. Incubate overnight on orbital shaker in cold room. Wrap tray in saran wrap.
5) Rinse 3X in T-TBS for 10 min each time on orbital shaker.
6) Secondary antibody: Make 2% milk (6 mL of 5% milk + 9 mL T-TBS) and add 6 μL of goat anti-rabbit (Ab6721). Incubate 1 hr on orbital shaker.
7) Rinse 3X in T-TBS for 10 min each time on orbital shaker. Turn on film developer 30 min ahead of time.
8) Supplies for dark room:
   a. 13.5 mL nanopure water in 15 mL tube
   b. Membrane in T-TBS
   c. Spare tray for treating membrane
   d. LumiGLO Reagents A and B
   e. 1000 mL capacity pipette and tips
   f. Tweezers
   g. Film and cassette
   h. Kim wipes
   i. Timer
   j. Keys
   k. Sharpie
9) LumiGLO is light sensitive so only do this under red light. Add 0.75 mL from clear bottle (Reagent B) then 0.75 mL from dark bottle (Reagent A).
10) Remove membrane from T-TBS and put in spare tray. Pour LumiGLO over membrane and rotate tray for 1 min.
11) Drain excess LumiGLO from membrane and put membrane between protector sheets in cassette. Wipe with Kim wipe to remove any air bubbles.
12) 30 sec exposure of film.
13) Crimp corner of film that is in corner of cassette so can go back and mark the ladder on the film after developing.
14) Run film through developer.
15) Decrease or increase exposure time of subsequent films as needed to attain bands that are visible but not too dark.

**Preparing membrane for storage**

1) Assuming the bands for DAT and alpha tubulin turned out well, strip antibodies from membrane by rinsing briefly in T-TBS then put membrane in 15 mL of Restore Plus Western Stripping Buffer for 15 min on orbital shaker.
2) Rinse 3X in T-TBS for 5 min each time on orbital shaker.
3) Store in 4°C walk-in freezer in generous level of T-TBS with container sealed with saran wrap.