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EVALUATION OF EPIDEMIOLOGY AND ANIMAL DATA FOR RISK ASSESSMENT: CHLORPYRIFOS DEVELOPMENTAL NEUROBEHAVIORAL OUTCOMES

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Developmental neurobehavioral outcomes attributed to exposure to chlorpyrifos (CPF) obtained from epidemiologic and animal studies published before June 2010 were reviewed for risk assessment purposes. For epidemiological studies, this review considered (1) overall strength of study design, (2) specificity of CPF exposure biomarkers, (3) potential for bias, and (4) Hill guidelines for causal inference. In the case of animal studies, this review focused on evaluating the consistency of outcomes for developmental neurobehavioral endpoints from in vivo mammalian studies that exposed dams and/or offspring to CPF prior to weaning. Developmental neuropharmacologic and neuropathologic outcomes were also evaluated. Experimental design and methods were examined as part of the weight of evidence. There was insufficient evidence that human developmental exposures to CPF produce adverse neurobehavioral effects in infants and children across different cohort studies that may be relevant to CPF exposure. In animals, few behavioral parameters were affected following gestational exposures to 1 mg/kg-d but were not consistently reported by different laboratories. For postnatal exposures, behavioral effects found in more than one study at 1 mg/kg-d were decreased errors on a radial arm maze in female rats and increased errors in males dosed subcutaneously from postnatal day (PND) 1 to 4. A similar finding was seen in rats exposed orally from PND 1 to 21 with incremental dose levels of 1, 2, and 4 mg/kg-d, but not in rats dosed with constant dose level of 1 mg/kg-d. Neurodevelopmental behavioral, pharmacological, and morphologic effects occurred at doses that produced significant brain or red blood cell acetylcholinesterase inhibition in dams or offspring.

Over the past 10 years, three cohort studies of pregnant women and their children were conducted investigating associations between levels of chlorpyrifos (CPF) or metabolites in maternal urine or umbilical cord blood and developmental neurobehavioral outcomes (Table 1). In addition, numerous animal studies on CPF-induced developmental neurotoxicity (DNT) effects were published in the peer-reviewed literature (see Tables 5–12). In view of these observations, the objective of this review was to evaluate the results of the CPF-associated developmental neurobehavioral studies from epidemiologic and in vivo animal data within a risk assessment framework relevant to current uses of CPF in the United States.

Approaches to evaluating epidemiologic studies have become increasingly important to regulatory agencies, as evidenced by the U.S. Environmental Protection Agency (U.S. EPA) draft proposed Framework for Incorporating Human Epidemiologic and Incidence Data in Health Risk Assessment (U.S. EPA 2010).

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TABLE 1. Summary of Study Characteristics

Study Characteristic	Columbia Center for Children's Environmental Health (CCCEH)	Center for Health Assessment of Mothers and Children of Salinas (CHAMACOS)		Mt. Sinai Children's Environmental Health Cohort Study
Author (citation)	Rauh et al., 2006	Eskenazi et al., 2007	Young et al., 2005	Engel et al., 2007
Location	New York City, NY, USA	Salinas Valley, CA, USA		New York City, NY, USA
Study design	Cohort	Cohort		Cohort
Enrollment years	1998–2002	1999–2000		1998–2001
Maternal eligibility	Pregnant nonsmokers, self-identified as black or Dominican, registered at the OB-GYN clinics at NY Presbyterian Medical Center and Harlem Hospital prior to 20 weeks gestation, 18–35 years old, free from diabetes and hypertension, no diagnosis of HIV, did not use drugs, and lived in the area for ≥1 year.	Pregnant Spanish or English speaking women, receiving prenatal care at one of six community clinics that served farm workers with plans to deliver at Natividad Medical Center, <20 weeks gestation at enrollment, ≥18 years old, eligible for Medi-Cal (California's Medicaid health care program).		Pregnant, primiparous, singleton pregnancies, receiving prenatal care from Mount Sinai Hospital, no underlying health conditions that might predispose to giving birth to high risk infants
Study size[a]	254	447	381	311
Outcome measurements (assessment tool)[b]	BSID:MDI; BSID:PDI; CBCL	BSID:MDI; BSID:PDI; CBCL	BNBAS	BNBAS
Outcome variable format	*BSID*: Continuous and categorical measurement of BSID:MDI and BSID:PDI (low: ≤85; high>85) *CBCL*: Categorical, using 98th percentile of the national normative sample in each domain as cutoff	*BSID*: Continuous measurement of BSID:MDI and BSID:PDI. *CBCL:* Categorical, using 93rd percentile of the national normative sample in each domain as the cutoff	*BNBAS*: Continuous measure of BNBAS domains. Categorical measure of abnormal reflexes (>3 vs. ≤3)	*BNBAS*: Continuous measure of BNBAS domains.
Timing of outcome assessment	*BSID:* Age 12, 24, 36 months *CBCL:* Age 36 months	*BSID:* Age 6, 12, 24 months *CBCL:* Age 24 months	*BNBAS*: Age ≤2 months	*BNBAS*: Within ≤5 days of delivery

Study Characteristic	Columbia Center for Children's Environmental Health (CCCEH)	Center for Health Assessment of Mothers and Children of Salinas (CHAMACOS)		Mt. Sinai Children's Environmental Health Cohort Study
Author (citation)	Rauh et al., 2006	Eskenazi et al., 2007	Young et al., 2005	Engel et al., 2007
Testing conditions	". . . each child was tested under controlled conditions in the study office by a trained bilingual research assistant, checked for reliability. Five different testers conducted a total of 1101 BSID-II assessments over the course of the 3-year study period. Every effort was made to maximize reliability in scoring by using standardized training procedures and regular quality control. Interrater reliability for the 24-month BSID-II assessments was $r = 0.92$, on the basis of double-scoring of a random 5% of the sample."	"Both [BSID] scales were administered in Spanish and/or English by psychometricians blind to exposure. Psychometricians were trained using standardized protocols and were supervised for quality assurance by a clinical neuropsychologist. Assessments were performed in a private room at the CHAMACOS research office or in a recreation vehicle (RV) modified to be a mobile testing facility."	"The BNBAS was administered once to each infant and in accordance with the BNBAS protocol, by four examiners trained to reliability by a BNBAS certified trainer. Examiners were blind to the infant's exposure status. Approximately 30% of the assessments included in the final sample were completed prior to discharge from the hospital; the remainder was performed at the CHAMACOS research office or in the participant's home. Assessments were performed away from the mother in a private room with low-level light and noise control."	"The BNBAS was administered before hospital discharge (n =311) by one of four examiners. Examiners were either trained and certified by the Brazelton Institute or trained by a certified examiner. Examinations took place in a quiet, semidarkened, warm room adjacent to the neonatal nursery, or in the mother's private room. The BNBAS was not administered if the infant was admitted to the Neonatal Intensive Care Unit (n = 21); if the infant was delivered and discharged over a weekend (n = 43); if the parent refused (n = 5); if the infant was not testable (n = 2); or if study personnel were unavailable (n = 22)."
Exposure measurement (Chemical/Metabolite[c] & biological specimen)	CPF in umbilical cord blood[d]	TCPy in maternal urine DEPs & DAPs in maternal and child urine	DEPs & DAPs in maternal urine	DEPs & DAPs in maternal urine
Timing of exposure assessment (approx.)	At delivery	During pregnancy (maternal prenatal): 14 and 26 weeks gestation; an average of these two measures was calculated Postnatal (child): age 6, 12, and 24 months	During pregnancy (maternal prenatal): 14 and 26 weeks gestation; an average of these two measures was calculated Post-delivery (maternal): 7 days postpartum	During pregnancy (maternal prenatal): 31 weeks gestation

(*Continued*)

TABLE 1. Continued

Study Characteristic	Columbia Center for Children's Environmental Health (CCCEH)	Center for Health Assessment of Mothers and Children of Salinas (CHAMACOS)		Mt. Sinai Children's Environmental Health Cohort Study
Author (citation)	Rauh et al., 2006	Eskenazi et al., 2007	Young et al., 2005	Engel et al., 2007
Exposure variable format for analysis	Categorical: >6.17 pg/g vs. ≤6.17 pg/g	Continuous: (nmol/L – $\log_{10}$) Categorical: (<LOD (ref), below median, above median detectable level)	Continuous: (nmol/L – $\log_{10}$)	Continuous: (nmol/L – $\log_{10}$)
Detection limit of chemical or metabolite	LOD = 0.5–1 pg/g; 80 samples below LOD Median CPF levels not provided.	TCPy detected in 91% of prenatal maternal samples (averaged). Prenatal (maternal): Median TCPy = 3.5 ug/L Percent with DEPs and DAPs detected not provided. Geometric means: Maternal (prenatal) (average) DEPs = 18.1 nmol/L, DAPs = 114.9nmol/L Geometric means: Child (postnatal) 6 mos. DEPs = 10.6 nmol/L, 12 mos. DEPs = 15.2 nmol/L, 24 mos. DEPs = 10.5 nmol/L, 6 mos. DAPs = 45.5 nmol/L, 12 mos. DAPs = 59.5 nmol/L, 24 mos. DAPs = 70.9 nmol/L,	DEPs and DAPs detected in 100% of prenatal samples (averaged) Prenatal (maternal): Median DEPs = 21 nmol/L Median DAPs = 132 nmol/L	DEPs detected in 89% of samples; DAPs detected 97% of samples Prenatal (maternal): Median DEPs = 24.7 nm/L Median DAPs = 82.0 nm/L

Study Characteristic	Columbia Center for Children's Environmental Health (CCCEH)	Center for Health Assessment of Mothers and Children of Salinas (CHAMACOS)		Mt. Sinai Children's Environmental Health Cohort Study
Author (citation)	Rauh et al., 2006	Eskenazi et al., 2007	Young et al., 2005	Engel et al., 2007
Information Obtained from Questionnaire / Interview	Demographic characteristics, home characteristics, lifetime residential history, history of active/passive smoking, occupational history, maternal education, income level, alcohol/drug use during pregnancy, history of residential pesticide use. Test of Nonverbal Intelligence, Home Observation for Measurement of the Environment (HOME)	Family demographics, work histories of household members, maternal behaviors (smoking, alcohol, drug use), maternal medical history (previous pregnancies) Peabody Picture Vocabulary Test (PPVT), Center for Epidemiologic Studies Depression Scale, Home Observation for Measurement of the Environment (HOME)	Family demographics, work histories of household members, maternal behaviors (smoking, alcohol, drug use), maternal medical history (previous pregnancies)	Environmental exposures, sociodemographic information, medical history, lifestyle factors.
Method of Assessing Confounding	"Covariates were included in models as possible confounders if they (1) had a significant association with level of pesticide exposure and any measure of developmental outcomes in this sample, (2) altered the estimate of chlorpyrifos effect by ≥10%, or (3) had been identified as confounders in comparable studies."	"Covariates were selected for these analyses if they were related to conditions of testing; related to neurodevelopment in the literature; and associated ($p < 0.10$) with most outcomes; or consistently related to neurodevelopment in the literature even if not in our data." "In addition to the variables we included, we examined the potential confounding effects of several other variables suggested by the literature but they did not markedly alter the observed associations." "For simplicity, the same set of covariates was used for CBCL models with three exceptions: maternal depression, found to be important ($p < 0.10$), was added, and psychometrician and assessment location were dropped, because scores were based on maternal report. "	"Covariates were initially selected based on their predictive value of BNBAS performance reported in the literature." "Covariates from this (initial) list were selected for final regression models if they were related to each of the seven BNBAS cluster scores, unadjusted for other variables ($p < 0.15$)."	"Backward elimination was used to arrive at the final adjusted models. Covariates were eliminated if their exclusion caused less than a 20 percent change in the beta coefficient of the full model."

(Continued)

TABLE 1. Continued

Study Characteristic	Columbia Center for Children's Environmental Health (CCCEH)	Center for Health Assessment of Mothers and Children of Salinas (CHAMACOS)		Mt. Sinai Children's Environmental Health Cohort Study	
Author (citation)	Rauh et al., 2006	Eskenazi et al., 2007	Young et al., 2005	Engel et al., 2007	
Maternal Demographic Characteristics					
Race/ethnicity White Black Latina Dominican Other	 ~42%[f] ~58%[f]	 ~100%	 ~100%	 20.3% 27.3% 51.1% – 1.3%	
Maternal age	mean years ± SD 24.6 ± 4.9[f]	Age group 18–24 25–29 30–34 ≥ 35	% 44.5% 31.1% 16.3% 8.1%	mean years ± SD 26 ± 5	Age group <20 20–24 25–29 30–34 ≥35	% 35.7 32.5 12.2 15.1 4.5
Did not complete high school	34.6%*	80.5%	81.4%	31.5%	
Married/living as married/living with infant's father	17%*	81.9%	81.1%	52.4%	

[a]Number of observations in analyses may be less, depending on the infant's attained age at the time of testing, availability of exposure data, testing data, and covariate data
[b]Outcome assessment tool: BNBAS: Brazelton Neonatal Behavioral Assessment Scale; BSI-MDI: Bayley Scales of Infant Development – Mental Development Index; BSI-PDI: Bayley Scales of Infant Development – Psychomotor Development Index; CBCL: Child Behavior Checklist
[c]Chemicals/Metabolites: DAPs = total dialkyl phosphates; DEPs = diethylphosphate; CPF: chlorpyrifos; TCPy = 3,5,6-trichloro-2-pyridionol
[d]Maternal blood was used when cord blood was unavailable.
[e]Maternal smoking during pregnancy or maternal residence with a smoker during pregnancy.
[f]Whyatt et al. 2004
*Calculated from values presented in Table 1 (Rauh_2006) for high and low exposure categories.
Abbreviations: LOD = limit of detection

It is also important that regulatory agencies consider published animal studies that evaluate neurodevelopmental endpoints, although investigations may not have followed U.S. EPA (1998a) DNT guideline requirements for sample size (10 males and 10 females from 20 litters; 1 male or female/litter), number of dose levels (>2), or relevant route of exposure (preferably oral). Furthermore, the animal studies can contribute to understanding the biological plausibility of outcomes that have been associated with human exposures in epidemiologic studies.

Previous reviews of CPF written within a risk assessment context include two expert panel reports by Clegg and van Gemert (1999a; 1999b), and a more recent expert panel review (Eaton et al. 2008). Other reviews also reported developmental outcomes associated with various classes of pesticides (Eskenazi et al. 1999; 2008; Weselak et al. 2007), but with limited discussion specific to potential CPF associations from epidemiologic studies. The Eaton et al. (2008) review summarized results of epidemiologic and toxicology studies, including in vivo and in vitro neurodevelopmental studies, critically evaluated human exposure studies, and estimated dermal, oral, and inhalation exposures to children and/or adults in the general population based on currently approved uses. A primary focus of the Eaton et al. (2008) review was to compare dose levels producing acetylcholinesterase activity (AChE) inhibition with concentrations at which in vitro or in vivo neurodevelopmental findings were reported. These levels were also compared with exposure concentration to which the general population may be exposed based upon currently approved uses for CPF. While comprehensive, the Eaton et al. (2008) review provided narrative summaries of findings from the epidemiologic cohort studies, rather than presenting the measures of association and corresponding confidence intervals, thus making it difficult to evaluate the precision of effect estimates and the consistency of the direction and magnitude of associations for similar measures at similar ages across studies.

The present review encompasses previous reviews and further contributes new analyses by providing (1) in-depth analyses of the analytical epidemiologic studies focused on neurobehavioral outcomes in infants and young children with comparisons of methodologies (including exposure measurement) and quantitative results, consideration of the potential role of systematic error (bias), and application of guidelines recommended by Hill (1965) and others for evaluating causality, (2) systematic critical analyses of the in vivo developmental neurobehavioral and neuropharmacology studies that includes assessment of methods and evaluation of pattern of negative and positive findings, and (3) integrative evaluation of the infancy/early childhood neurobehavioral findings with the animal data. The studies evaluated in this review included (1) epidemiologic studies of pregnant women that examined associations of CPF exposure with neurobehavioral measures in their infants and young children published before June 2010 (Table 1) and (2) in vivo DNT animal studies with emphasis on neurobehavioral, neuropharmacological and neuropathology endpoints at lower dose levels (<10 mg/kg-d) (see Tables 5–12).

APPROACH TO EVALUATION OF CPF STUDIES FOR HUMAN HEALTH RISK ASSESSMENT

The following steps were undertaken in our systematic review of published CPF animal and epidemiologic developmental neurobehavioral studies.

1. Identify uses and exposure patterns (including frequency, concentration, duration, and routes of exposure) in humans for CPF such that relevance of animal studies can be determined.
2. Assess evidence for possible modes of action (MOA). In the case of CPF, risk assessments are currently based upon an established MOA that is acetylcholinesterase (AChE) inhibition, and this needs to be compared to other reported endpoints.

3. Incorporate knowledge of the metabolism and pharmacokinetics of CPF in the evaluation of exposure measures in humans (e.g., studies evaluating outcomes associated with organophosphate [OP] metabolites that include metabolites unrelated to CPF need to be excluded or be given lower weight in the assessment).
4. Define approach and standards for review of human and animal studies.
   a. Human: Patterns of associations within and across different studies were evaluated with respect to the following viewpoints (Hill 1964): consistency, dose response, strength of association, and temporality (exposure precedes outcome). Frequency, concentration, timing (e.g., pregnancy, delivery, and early infancy), and duration of exposure also need to be described and compared across studies with respect to the results. It needs to be emphasized that lack of association must also be considered. For nonrandomized epidemiologic studies, consideration of sources of bias and their potential role in influencing study results is of critical importance.
   b. Animal: The following standards were used as guiding principles for evaluating the animal studies, and are consistent with current standards for neurodevelopmental toxicology (Adams 2010; Maurissen 2010). (1) Effects related to litter of origin need to be accounted for in design and statistical procedures, because maternal influences during gestation and lactation may exert significant impact on developmental effects (DeSesso et al. 2009; Holson and Pearce 1992; Holson et al. 2008). (2) A minimum of six animals per treatment condition is needed to provide minimal confidence in the results (Chapin et al. 2008, 235). Ten animals per gender are required for behavioral studies by U.S. EPA guidelines for DNT testing (U.S. EPA 1998a). (3) Dose-response evaluations need to include more than two doses. (4) The time of testing need to be balanced across dose groups and other factors if data are to be pooled in the statistical analyses (Maurissen 2010). (5) If similar tests were conducted at multiple ages, the statistical analyses should account for repeated measurement, in order not to inflate degrees of freedom (Holson et al. 2008; Chapin et al. 2008, 235). (6) Compare contemporary historical control data with CPF generated values to assess reliability of the methodology, variability in control behavior, and presence of adverse effect. This is particularly important when evaluating animal studies with limited dose response data.
5. Extract and summarize all analyses conducted (both significant and non-significant findings), key methodological features, conditions of exposure (e.g., duration, timing and concentration), and direction and magnitude of any associations in the human and animal studies so that conclusions about the weight of evidence will be specific.

USES AND HUMAN EXPOSURE

Chlorpyrifos (CPF) is a widely used OP insecticide, primarily approved for uses in agricultural pest management (crop protection) in the United States today. Most nonagricultural uses such as residential control of insect pests such as cockroaches and termites, and animal use including flea and tick control were phased out in the United States in 2001 (U.S. EPA 2002) and in the European Union in 2005 (Official Journal of the European Union Legislation 2007). Hence, exposures of pregnant women, infants, and children in the general population to CPF occur primarily through the diet and are estimated to be less than 1×10^{-2} μg/kg-d in children and $4–6 \times 10^{-3}$ μg/kg-d in adults (Eaton et al. 2008). Therefore, it is unlikely that inhalation, dermal, or secondary oral (nondietary) CPF exposures contribute significantly in the general population today (Eaton et al. 2008).

Exposures to agricultural applicators including women of childbearing age have been estimated by the U.S. Environmental Protection Agency (1999) to be 1.1–9.8 μg/kg-d based on biomonitoring data for mixers, loaders, or applicators using tractors or airplanes to apply liquid or granular formulations. Higher daily doses of 3–23 μg/kg-d were estimated by the U.S. EPA for manual solo applicators using backpack or hand wand sprayers.

Alexander et al.(2006) reported the 90th percentile for CPF exposure from a single application to be 7.4 μg/kg for applicators and 2.2–2.8 μg/kg for children living on the farm. Maximal levels of CPF exposure were estimated to be 16.3, 4.1, 6.3, and 2.5 μg/kg, respectively, for applicators, spouses, children older than 12 yr, and children younger than 12 yr. These estimates were based on levels of 3,5,6-trichloro-2-pyridinol (TCPy), a specific metabolite of CPF, in urine collected continuously over a 4-d period immediately after CPF was applied. Although this study estimated exposure after only one d of application, it provides an estimate of the order of magnitude of exposure to the farm family members who were not applicators.

Populations living in agriculturally intensive areas have potential exposures to outdoor air concentrations ranging from 15–100 ng/m^3 (Eaton et al. 2008), which results in approximate estimates of daily doses of 6×10^{-3} to 4×10^{-2} μg/kg-d assuming exposure of 4×10^{-4} and 3×10^{-4} μg/kg-d per 1 ng/m^3 for children and adults, respectively. Using conservative assumptions, the absorbed dermal exposures of 2.5 to 4×10^{-3} μg/kg-d were estimated based on measures in homes that recently used CPF for pest control, or in agricultural homes (Eaton et al. 2008). Nondietary oral intake rate for children are roughly estimated to be 1.4×10^{-3} μg/kg-d in homes of farm workers and applicators based on an assumption that a 20-kg child consumes 50 mg of dust/soil containing 700 ng CPF/g house dust (Eaton et al. 2008).

Although most residential uses are no longer permitted today, epidemiologic studies have been published involving previously allowed residential exposures. Lowe et al. (2009) estimated CPF residential exposures to pregnant women to be 0.15 μg/kg-d based on physiologically based pharmacokinetic (PBPK)/pharmacodynamic modeling and using umbilical cord blood levels reported by Whyatt et al. (2005) for mothers living in New York City during a period when residential exposures to CPF were allowed. These estimates are consistent with postapplication residential air monitoring for CPF that indicated levels of approximately 50–400 ng/m^3 (Eaton et al. 2008), which are approximated to result in daily doses of 0.02–0.16 μg/kg-d for children based on an assumption of 4×10^{-4} μg/kg-d exposure to CPF per 1 ng/m^3 CPF air concentration (Eaton et al. 2008). Exposure to adults would be lower based on the assumption that adults are exposed to 3×10^{-4} μg/kg-d per 1 ng/m^3 CPF (Eaton et al. 2008).

In summary, exposure to the general population including children and women of childbearing age today is primarily through the diet and in the 10^{-2} to 10^{-3} μg/kg-d dose range. Applicators and their families may be potentially exposed immediately after agricultural application in the range of 10^0 and 10^1 μg/kg. Other estimates for exposure to women and children living in agriculturally intensive areas are in the 10^{-2} μg/kg-d dose range. Although residential uses are no longer permitted, estimates for daily exposure directly to children and pregnant women when residential uses were allowed are in the 10^{-1} to 10^{-3} μg/kg-d dose range following dermal, inhalation, and/or nondietary oral exposures. Several of these estimates are based on a number of assumptions that may require further examination. However, for the purposes of this review, these estimates provide an initial basis for comparison of doses used in animal studies with possible human CPF exposure levels as recommended by Maurissen (2010).

MODE OF ACTION (MOA) AS CURRENT BASIS FOR RISK ASSESSMENT

The best characterized MOA for acute neurotoxic effects of CPF is inhibition of acetylcholinesterase (AChE) activity. In order to become an effective AChE inhibitor, CPF is

converted to chlorpyrifos oxon (CPO) through oxidative metabolism, which then binds to and inhibits AChE. This binding produces inhibition of AChE leading to increased acetylcholine (ACh) levels within synaptic clefts, hyperstimulation of the cholinergic system, and adaptive decreases in muscarinic acetylcholine receptor (mAChR) binding (Eaton et al. 2008).

In addition to AChE inhibition in the brain, spinal cord, and peripheral nervous system, CPO binds to and inhibits red blood cell (RBC) AChE, and plasma butyrylcholinesterase (BuChE). Although the physiological role of BuChE and RBC AChE is not clear, levels of inhibition of the activity of these enzymes are currently used in the U.S. as critical toxicity endpoints for human health risk assessment, with RBC AChE inhibition considered more relevant than BuChE inhibition.

Age-related differences between adults and offspring in magnitude, time of onset, and/or recovery of brain and RBC AChE inhibition were reported following acute dermal, oral, or subcutaneous (sc) administration (Abu-Quare et al. 2001; Moser et al. 1998; Moser and Padilla 1998; Pope and Chakraborti 1992). Although neonatal pups were more sensitive than adults to higher acute CPF doses (>10 mg/kg), there was less or absence of differential sensitivity to CPF induced inhibition of AChE at lower (<10 mg/kg-d) repeated exposures (Zheng et al. 2000; Liu et al. 1999). Following repeated gestational exposures, maternal brain and RBC AChE inhibition was greater in dams than fetus (Mattsson et al. 2000; Lassiter et al. 1998 1999). As discussed later in reviewing the animal literature, cholinergic and other noncholinergic MOA were proposed for adult and developmental neurotoxic effects (Aldridge et al. 2003; 2004; Betancourt et al. 2006; 2007; Betancourt and Carr 2004; Liu and Pope 1998; 1999; Pope 1999; Richardson and Chambers 2005; Slotkin et al. 2006; 2007a; Zamora et al. 2008).

Although it appears that animals exposed to CPF during gestation recover from brain AChE inhibition more rapidly than adults due to increased synthesis of AChE, there may be long-lasting effects on cholinergic and noncholinergic components (Eaton et al. 2008; Lassiter et al. 1998; 1999; Richardson and Chambers 2004; 2005; Slotkin et al. 2001; 2002; 2004; 2006). Therefore, this in-depth review of the developmental neurobehavioral animal studies compared dose levels that produced behavioral and neuropharmacological effects in offspring, with CPF concentrations inducing brain or AChE inhibition RBC or plasma BuChE inhibition in offspring or dams. In our view, from a risk assessment perspective, this dose comparison serves to determine whether regulations based on AChE or BuChE inhibition are protective of adverse effects that may be due to other undefined MOA.

CPF METABOLISM AND OP BIOMARKERS OF EXPOSURE

An understanding of the relative specificity of OP metabolites as potential biomarkers of CPF is critical in weighing the evidence associating CPF exposure with neurobehavioral outcomes in the human studies. The metabolism and pharmacokinetics of CPF were described in detail elsewhere (Eaton et al. 2008; Timchalk et al. 2002; 2006). Briefly, CPF is metabolized to a number of metabolites including chlorpyrifos-oxon (CPO), the primary toxic active metabolite, TCPy, and diethylphosphate (DEP) and diethylthiophosphate (DETP). These metabolites or their glucuronic or sulfate conjugates are excreted in the urine (Barr and Angerer 2006). Detoxification of the oxon is brought about by several enzymes including paraoxonases and carboxylesterases. Paraoxonase-1 (PON1) status based on PON1 levels or activity and polymorphism may modulate toxicity of CPF and CPO, but "at lower-level exposures to CPF [<0.5 mg/kg] other esterase detoxification pathways would be capable of compensating for the inter-individual differences in CPOase activity due to PON1Q192R polymorphism" (Cole et al. 2005).

The degree of specificity of the different biomarkers measured in human studies to CPF exposure is as follows (Barr and Angerer 2006; Bravo et al. 2004):

- CPF and CPO are measured in blood and are biomarkers of highest specificity for CPF.

- TCPy is the most common urinary biomarker of CPF exposure, with important limitations. For example, TCPy is also a metabolite of CPF-methyl and triclopyr (Barr and Angerer 2006; Whyatt et al. 2009). In addition, TCPy itself can be present in food, the environment, or homes, as a result of breakdown products from an application of CPF or chlorpyrifos-methyl (Barr and Angerer 2006; Eaton et al. 2008; Whyatt et al. 2009). Finally, significant intraindividual variability in repeat urine samples from the same individual was noted (Whyatt et al. 2009).
- DEPs represent a broad class of OP metabolites of CPF and other OP containing ethyl groups. DEPs include DEP, DETP, and diethyldithiophosphate (DEDTP). Only DEP and DETP are metabolites of CPF. As a class, DEPs are relatively nonspecific as exposure markers for CPF in urine because other OP can also be degraded into DEPs.
- DMPs are a broad class of urinary dimethylphosphate metabolites that are *not* metabolites of CPF. Although DMPs are useful as potential biomarkers for several methyl OPs including malathion and CPF-methyl (a pesticide distinct from CPF), these are not biomarkers for CPF. Dialkylphosphates (DAPs) are a broad class of OP urinary metabolites, which include both DEPs and DMPs. Therefore, DAPs are not specific biomarkers for CPF because they include metabolites that cannot be formed from CPF.

Biomarkers measured in blood provide the highest sensitivity for measuring exposure to the parent compound (Barr and Angerer 2006). Since this analytic method was only recently introduced, and obtaining urine samples is more feasible and affordable, measuring metabolite levels in urine is a common practice. Urinary metabolite levels are sensitive to the timing of collection, which may lead to misclassification of exposure. For example, Scher et al. (2007) found that results from the first morning void often overpredicted pesticide concentrations based on 24-h urine samples, which may be associated with the pharmacokinetics of the biomarker in the urine.

In summary, the use of urinary metabolites (TCPy, DEPs, DAPs) as indicators of CPF exposure needs to be considered with caution, because they may indicate exposure to metabolite residues and not necessarily exposure to the parent compound of concern (Barr and Angerer 2006; Eaton et al. 2008; Lu et al. 2005). Observed associations between levels of nonspecific metabolites and human health outcomes need to be interpreted cautiously with respect to the ability to make inferences regarding CPF specifically (Bravo et al. 2004).

ANALYSES OF NEUROBEHAVIORAL OUTCOMES DERIVED FROM EPIDEMIOLOGIC STUDIES

Scope of the Review and Literature Search

Our review includes peer-reviewed epidemiologic studies published in English through May 31, 2010, using the following search terms: "chlorpyrifos," "TCPy," "organophosphate," "organophosphorus," "neuro," "neurotoxicity syndromes," "neurotoxic," "neurotoxins," "neurotoxicity," "neurologic," "neurological," "nervous system," "neurobehavior," "neurobehavioral," "behavior," "motor skills," "psychomotor" "cognitive," "cognition," "cognitive development," or "impaired cognitive function," "motor development," "intelligence," or "autism," "parental," "parent," "neonate," "neonatal," "prenatal," "pregnancy," "pregnant," "fetus," "fetal," "maternal," "developmental," "child," "children," "teen," "adolescent," "utero." In addition, the reference lists were cross-checked in recent reviews on CPF to identify any relevant papers that might have been missed by our search terms.

Studies that were included in this review investigated potential associations between exposure to CPF during critical periods of brain development and neurodevelopmental outcomes in neonates, infants, or young children. Studies based upon self-reported exposure to CPF, measured exposure in the home environment (e.g., air, soil, dust), or biomarkers of exposure were eligible, provided that exposure

was evaluated in terms of its potential association with a neurobehavioral measure in infants or young children.

Population Parameters

Studies with populations potentially exposed to CPF during critical periods of brain development, such as in utero, infancy, and early childhood, were included. Only studies that were conducted on neonates, infants, toddlers, or young children were also included.

Exposure Measurements

The CPF biomarkers reported in this review were measured and quantified from biological specimens obtained from women during pregnancy or from their child after delivery. This included measuring CPF in umbilical cord blood, as well as measuring TCPy, DEPs, and DAPs in maternal or child urine (Table 1). In addition, two of the cohort studies also reported results for DMPs (Engel et al. 2007; Eskenazi et al. 2007; Young et al. 2005). It is important to note that DMPs are not biomarkers of CPF. Therefore, the results associated with DMPs are not systematically reported and tabulated, but are discussed when relevant to understanding results for DAPs. The use of DAPs, which include both DEPs and DMPs, may not be reflective of exposure to CPF.

The cohort studies utilized a questionnaire to obtain demographic personal and exposure information on study participants including personal habits (i.e., smoking, alcohol consumption, and drug use), maternal medical history, and environmental exposures (including pesticides) among the study participants (Table 1). The extent to which information ascertained from the questionnaires was analyzed or included (e.g., as covariates) in final multivariate models is summarized in Table 1.

Studies that inferred CPF exposure but did not directly quantify CPF levels were excluded. This included studies that evaluated CPF exposure based solely on the residential location of study participants or that estimated exposures from ambient air on a group or community level. Studies that reported CPF poisoning were excluded, as were any that reported toxic exposure levels above the approved levels of standard use. Studies that described CPF biomonitoring but did not report specific health outcomes were evaluated but not included in our final review.

Outcome Measurements

Studies reporting neurobehavioral outcomes including measures of psychomotor and mental development and assessment of potential behavioral problems were evaluated. The epidemiologic studies with respect to CPF that assessed the neurobehavioral outcomes used the following tools:

Bayley Scales of Infant Development II: Mental Development Index (BSID:MDI) The BSID:MDI assesses general cognitive development and higher order mental processing, with 178 individual items that measure memory, habituation, generalization, classification, vocalizations, visual preference, visual acuity skills, problem solving, early number concepts, language, and social skills and development (Black and Matula 2000; Sattler 2001; Strauss et al. 2006). Standardized index scores have a mean of 100 and a standard deviation of 15, and range from 50 to 150 (mean score $\pm$ $3\frac{1}{3}$ standard deviations). Infants with standardized scores between 70 and 84 on the BSID:MDI are classified as having "mildly delayed performance" (Black and Matula 2000). Infants with scores <70 are classified as having "significantly delayed performance." The Mental Scale has 22 item sets, designated by age, and each set has about 27 items (range: 20–36 items).

Bayley Scales of Infant Development II: Psychomotor Development Index (BSID:PDI) The BSID:PDI assesses overall motor development and contains 111 items that measure quality of movement, sensory integration, motor planning, fine and gross motor skills (e.g., rolling, crawling, creeping, sitting, standing, walking, running, jumping, prehension [act of holding, seizing, or grasping], use of writing implements, imitation of hand movements), and perceptual–motor integration

(Black and Matula 2000; Strauss et al. 2006). Standardized scores have a mean of 100 and a standard deviation of 15, and range from 50 to 150 (mean score $\pm$ $3\frac{1}{3}$ standard deviations). Classifications for "mildly delayed performance" and "significantly delayed performance" are similar to the MDI. The Psychomotor Scale has 22 item sets, designated by age, and each set has about 17 items (range: 14–21 items).

Brazelton Neonatal Behavioral Assessment Scale (BNBAS) The BNBAS groups the measurement of behavioral abilities and reflexes into the following seven domains: habituation, orientation, motor performance, range of state, regulation of state, autonomic stability, number of abnormal reflexes, and type of abnormal reflexes (Brazelton and Nugent 1995). It has been used extensively as a research instrument; however, it was originally designed to be a clinical instrument, and there are separate guidelines for research versus clinical uses of the BNBAS. The test is suitable for infants up to 2 mo of age and was initially developed to help parents and child care workers understand the language of newborn infants, including coping capacities and adaptive strategies. This test is also referred to as the Neonatal Behavioral Assessment Scale (NBAS).

Child Behavior Checklist (CBCL) The CBCL 1.5–5 is a paper-and-pencil instrument administered to caregivers, usually parents, of children ages 1.5 to 5 yr of age to measure emotional and behavioral functioning as indicated by the frequency of certain behaviors during the previous 2 mo (Achenbach and Rescorla 2000; Rescorla 2005). CBCL items are scored on a 3-point scale as follows: 0 = not true, 1 = somewhat or sometimes true, and 2 = very true or often true, within the past 2 mo (Rescorla 2005). The CBCL's seven empirically derived syndrome scales (emotionally reactive, anxious/depressed, somatic complaints, withdrawn, sleep problems, attention problems, aggressive behavior) were developed through factor analysis of data from the general pediatric population (Rescorla 2005). In addition, five DSM-oriented scales (affective problems, anxiety problems, pervasive developmental problems, attention deficit/hyperactivity problems, oppositional defiant problems) were developed to be relevant to the commonly used *Diagnostic and Statistical Manual of Mental Disorders,* 4th edition (DSM-IV) (American Psychiatric Association 2000) diagnostic categories, although they are not directly equivalent to a DSM diagnosis (Rescorla 2005). For each syndrome and DSM-oriented scale, a child's score is obtained by summing the ratings for the items that compose the syndrome and comparing to the normative sample as follows: <93rd percentile = normal range, 93rd to 97th percentile = borderline clinical range, >97th percentile = clinical range. The following three scales were evaluated by studies included in our review: Attention Problems syndrome scale (empirically derived), the DSM-oriented Attention-Deficit/Hyperactivity Disorder (ADHD) scale, and the DSM-oriented Pervasive Developmental Disorder (PDD) scale.

CHARACTERISTICS OF EPIDEMIOLOGIC STUDIES

Four publications from three cohort studies evaluated potential associations between CPF exposure and neurodevelopmental outcomes (Engel et al. 2007; Eskenazi et al. 2007; Rauh et al. 2006; Young et al. 2005; see Table 1).

Center for Health Assessment of Mothers and Children of Salinas

The research objective of this center (CHAMACOS; Eskenazi et al., 2007; Young et al. 2005) is to evaluate health effects (e.g., growth and development) of exposure to pesticides and other factors in the environment among pregnant women and their offspring who reside in Salinas Valley, California, an agricultural community. Study participants are predominantly low-income Latina women who enrolled in the study between October 1999 and October 2000 during their first trimester of pregnancy. Approximately 531 pregnant women were followed to birth;

however, fewer were available for analysis (i.e., <400), depending on the neurobehavioral outcome, age being evaluated, and the metabolite measurement. Information was not available in either paper in order to calculate overall participation rates. Urine samples were collected from mothers twice during pregnancy and thrice from children. Analyses relevant to our review from this center evaluated associations between urinary metabolites TCPy, DEPs, and DAPs, and the BSID:MDI, BSID:PDI, CBCL, and BNBAS. DEPs and DAPs were modeled as continuous variables. TCPy levels were categorized into three groups: less than limit of detection (<LOD), below the median detectable level, and above the median detectable level (Eskenazi et al. 2007).

Mount Sinai Center for Children's Environmental Health and Disease Prevention Research

The enrolled cohort (Engel et al. 2007) includes mothers recruited during pregnancy who are multi-ethnic (about 51% Latina, 27% black, 20% white), inner-city (New York) residents, and who gave birth between May 1998 and July 2001. Maternal blood and urine samples were collected once during the third trimester. Engel et al. (2007) reported that 33% of eligible women approached agreed to participate; 404 women had available birth data and 285 of these women had usable data on DAPs and DEPs. Engel et al. (2007) evaluated the association between DEPs and DAPs in maternal urine and the BNBAS for mother–infant pairs. The metabolites were modeled as continuous variables. Engel et al. (2007) also analyzed the potential interaction among PON1 activity, DEPs or DAPs, and abnormal reflexes (one of the seven BNBAS clusters of behaviors). One route of inactivation of CPO is through hydrolysis by PON1 to form TCPy.

Columbia Center for Children's Environmental Health Study participants (CCCEH; Rauh et al., 2006) are nonsmoking, inner-city (New York) mothers and their infants born between February 1998 and May 2002, who self-identified as black or Dominican. Rauh et al. (2006) evaluated associations between CPF in umbilical-cord blood and the BSID:MDI, BSID:PDI, and CBCL in up to 254 mother–infant pairs in which the child had reached age 36 mo (out of 536 "active participants"). Overall participation rates could not be calculated based on the information provided in the paper.

Rauh et al. (2006) reported in their methods section that there was "no indication of either a linear or nonlinear dose-response relationship between CPF and developmental outcomes" in preliminary analyses. The study implemented a cutoff point of CPF exposure at 6.17 pg/g, and CPF cord blood levels were dichotomized above or below that value. There were 204 participants in the <6.17 pg/g exposure group and 50 in the >6.17 pg/g exposure group (Table 1). The level of this threshold was obtained by first categorizing the CPF levels as undetectable or detectable. The CPF levels above detection were then categorized into tertiles, and the lower bound of the highest tertile was 6.17 pg/g. Rauh et al. (2006) justified the decision to implement this threshold in their analysis based on three factors. First, their previous results showed a statistically significant association between the highest tertile of CPF exposure and birth weight (Perera et al. 2003; Whyatt et al. 2004). Second, preliminary analyses showed that there were statistically significant differences between the PDI scores of children in the highest tertile of CPF exposure and scores for each of the other exposure groups. Finally, preliminary analysis also showed statistically significant differences between the MDI scores of children in the highest tertile of CPF exposure and scores of those in the lowest tertile, but not compared to the undetectable group.

Cognitive and Motor Endpoints (BSID II: MDI and PDI)

Table 2 summarizes the reported associations between CPF, TCPy, DEPs, and DAPs, and results of the BSID:MDI and BSID:PDI for the two studies that used this assessment tool (Eskenazi et al. 2007; Rauh et al. 2006).

TABLE 2. Summary of results from the Bayley Scales of Infant Development II (BSID II)

	Columbia Center for Children's Environmental Health (CCCEH) Rauh et al., 2006	Center for Health Assessment of Mothers and Children of Salinas (CHAMACOS) Eskenazi et al., 2007	
Exposure measurement	**CPF in umbilical cord blood[a]**	**TCPy in maternal[b] urine**	**DEPs and DAPs in maternal[b] and child urine**
Exposure variable format	Categorical: (>6.17 pg/g- vs. ≤6.17 pg/g)	Categorical: (<LOD (reference), below median detected level, above median detected level)	Continuous: (nmol/L – $\log_{10}$)
Sample size for analysis	Range: 225 – 229	Not reported. TCPy detected in 91% of 445 maternal (prenatal) urine samples	Not reported. Urine metabolite measures available for 445 women (prenatal) and 373 children at 24 mo
BSID:MDI **β (95% CI)**; Positive associations (β >0) indicate biomarkers associated with higher (better) MDI scores			
6 mo	Not measured	<Median: 0.24 (−2.12, 2.60) ≥Median: 0.08 (−2.29, 2.44)	Maternal DEPs: −0.16 (−1.96, 1.65) Child DEPs: 0.24 (−0.78, 1.25) Maternal DAPs: −1.15 (−2.89, 0.59) Child DAPs: −0.17 (−1.23, 0.90)
12 mo	−0.344 (SE = 1.66)	<Median: −0.45 (−3.67, 2.76) ≥Median: −0.65 (−3.88, 2.58)	Maternal DEPs: −1.14 (−3.51, 1.22) Child DEPs: **1.89 (0.21, 3.58)*** Maternal DAPs: −1.34 (−3.59, 0.92) Child DAPs: 1.36 (−0.05, 2.78)
24 mo	−1.480 (SE = 2.03)	<Median: −1.02 (−5.34, 3.31) ≥Median: −1.94 (−6.26, 2.37)	Maternal DEPs: −0.85(−3.98, 2.27) Child DEPs: 1.02 (−0.52, 2.57) Maternal DAPs: **−3.54 (−6.59, −0.49)*** Child DAPs: **2.37 (0.50, 4.24)***
36 mo	−3.327 (SE = 1.76)	Not measured	Not measured
Covariates included in the regression models	Race, gender, maternal education, maternal IQ, gestational age, prenatal ETS exposure	Psychometrician, location, exact age at assessment, gender, breast-feeding duration, HOME score, household income above poverty threshold, parity, maternal PPVT.	Psychometrician, location, exact age at assessment, gender, breast-feeding duration, HOME score, household income above poverty threshold, maternal PPVT.

(Continued)

TABLE 2. (Continued)

	Columbia Center for Children's Environmental Health (CCCEH) Rauh et al., 2006	Center for Health Assessment of Mothers and Children of Salinas (CHAMACOS) Eskenazi et al., 2007	
Exposure measurement	**CPF in umbilical cord blood**[a]	**TCPy in maternal**[b] **urine**	**DEPs and DAPs in maternal**[b] **and child urine**
"**Mental Delay**" (BSID:MDI ≤ 85)[c], **OR (95% CI)**; Positive associations (OR>1) indicates biomarkers associated with MDI scores ≤ 85 (worse scores)			
12 mo 24 mo 36 mo	1.22 (0.48–3.06) 1.75 (0.86–3.60) **2.37 (1.08–5.19)***	Not reported Not reported Not measured	Not reported Not reported Not measured
Covariates included in the regression models	Race, gender, maternal education, maternal IQ, gestational age, prenatal ETS exposure, home environment		
BSID: PDI **β (95% CI)**; Positive associations (β>0) indicates biomarkers associated with higher (better) PDI scores			
6 mo	Not measured	<Median: −0.56 (−4.03, 2.91) ≥Median: −0.21 (−3.69, 3.27)	Maternal DEPs: 0.02 (−2.63, 2.67) Child DEPs: 0.60(−0.89, 2.09) Maternal DAPs: −0.71 (−3.28, 1.86) Child DAPs: 0.39 (−1.18, 1.97)
12 mo	−3.30 (SE = 2.11)	<Median: −0.70 (−5.26, 3.86) ≥Median: −1.62 (−6.20, 2.96)	Maternal DEPs: 0.30 (−3.03, 3.63) Child DEPs: 1.91 (−0.46, 4.27) Maternal DAPs: −0.60 (−3.77, 2.57) Child DAPs: 1.22 (−0.78, 3.21)
24 mo	1.17 (SE = 1.98)	<Median: −2.65 (−6.50, 1.21) ≥Median: −2.72 (−6.57, 1.12)	Maternal DEPs: −0.86 (−3.64, 1.92) Child DEPs: 0.30 (−1.07, 1.67) Maternal DAPs: −1.28 (−4.01, 1.46) Child DAPs: 1.06 (−0.62, 2.74)
36 mo	**−6.46 (SE = 2.18)***	Not measured	Not measured
Covariates included in the regression models	Race, gender, maternal education, maternal IQ, gestational age, maternal ETS exposure	Psychometrician, location, exact age at assessment, gender, breast-feeding duration, HOME score, household income above poverty threshold, parity, maternal PPVT.	Psychometrician, location, exact age at assessment, gender, breast-feeding duration, HOME score, household income above poverty threshold, maternal PPVT.

	Columbia Center for Children's Environmental Health (CCCEH) Rauh et al., 2006	Center for Health Assessment of Mothers and Children of Salinas (CHAMACOS) Eskenazi et al., 2007	
Exposure measurement	**CPF in umbilical cord blood**[a]	**TCPy in maternal**[b] **urine**	**DEPs and DAPs in maternal**[b] **and child urine**
"**Psychomotor Delay**" (BSID:PDI ≤ 85)[c] **OR (95% CI)**; Positive associations (OR>1) indicates biomarkers associated with PDI scores ≤ 85 (worse scores)			
12 mo 24 mo 36 mo	1.88 (0.78,4.53) 1.01 (0.37,2.76) **4.52 (1.61,12.70)***	Not reported Not reported Not measured	Not reported Not reported Not measured
Covariates included in the regression models	Race, gender, maternal education, maternal IQ, gestational age, prenatal ETS exposure, home environment		

Abbreviations: DAPs = total dialkyl phosphates; DEPs = diethylphosphates; DMPs = dimethylphosphates; ETS: environmental tobacco smoke, HOME = Home Observation for Measurement of the Environment; PPVT = Peabody Picture Vocabulary Test; OR = odds ratio, CI = confidence interval.

a Maternal blood was used when cord blood was unavailable.

b Maternal samples were collected during pregnancy.

c Threshold for mental and psychomotor "delay" corresponds to 1 standard deviation and was defined by Rauh et al. (2006).

*p<.05, significance bolded for emphasis.

The CCCEH study, reported by Rauh et al. (2006), was the only study that evaluated the parent compound of CPF in umbilical-cord blood, and approximately 20% of the study population had a CPF level in the upper exposure category (>6.17 pg/g). Mean scores (± SD) on the BSID:MDI for the total study group were 94.0 (±9.8), 85.1 (±12.4), and 89.6 (±11.4) for ages 12, 24, and 36 mo, respectively. Corresponding mean scores (± SD) for the PDI by age group were 96.2 (±12.2), 97.4 (±11.5), and 100.5 (±13). There was no statistically significant association between CPF exposure (>6.17 pg/g compared to <6.17 pg/g) and MDI scores at 12, 24, or 36 mo or PDI scores at 12 or 24 mo of age (Table 2). A statistically significant inverse association was observed for the PDI at 36 mo, with children in the higher exposure category scoring lower than children in the lower exposure category ($\beta = -6.46$, SE = 2.18) (Rauh et al. 2006).

Rauh et al. (2006) also created a two-level categorical variable to characterize performance on the BSID. An assessment of mental and psychomotor delay, defined by the authors as BSID scores ≤85, concluded no marked association between CPF exposure and these specific delays at 12 or 24 mo. However, at 36 mo, the odds of "mental delay" and "psychomotor delay" were statistically significantly higher among the children in the higher exposure group ($n = 50$) compared to children in the lower exposure group ($n = 204$), which included the lower 2 tertiles and undetectable groups (odds ratio [OR] = 2.37, 95% confidence interval [CI]: 1.08–5.19 and OR = 4.52, 95% CI: 1.61–12.7, respectively) (Rauh et al. 2006).

Rauh et al. (2006) compared the distribution of CPF umbilical cord blood levels (at birth) and 36-mo BSID scores for children born during "preban" (before January 2000), "midban" (or "phase-out") (January 2000 to December 2000), and "postban" (January 2001 and later) periods. CPF levels as mean log-transformed CPF levels at delivery were 0.92 pg/g, 0.81 pg/g, and 0.9 pg/g for the preban, midban, and postban periods, respectively. The corresponding mean 36-mo BSID:MDI scores were 87.1, 91.7, and 89.5, respectively. The increase in mean MDI scores from preban to midban periods was statistically significant, whereas the subsequent decrease was not. Comparisons of PDI scores yielded a similar pattern (albeit statistically nonsignificant), with mean scores of 97.3, 101.8, and 99.4, for preban, midban, and postban periods, respectively. These observed decreases in cord blood and personal air sample CPF levels coupled with increases in 36-mo MDI and PDI scores when comparing preban with midban time periods may suggest a crude dose-response pattern. However, changes were smaller and were statistically nonsignificant when comparing preban to postban levels (Rauh et al. 2006). This analysis was based on different groups of participants that were tested at different time periods and did not control statistically for factors that may have changed over time and may be associated with these variables (including, but not limited to, maternal IQ and HOME score). Thus, correlations of CPF levels with changing BSID scores before and after the ban need to be interpreted with caution.

Eskenazi et al. (2007) also used the BSID:MDI and BSID:PDI assessment tools in the CHAMACOS study; however, potential exposure to CPF can only be estimated indirectly from metabolites TCPy, DEPs, and DAPs in maternal and child urine. In the CHAMACOS study, there were no statistically significant associations between TCPy and BSID:MDI or BSID:PDI at 6, 12, or 24 mo. The only statistically significant finding for DEPs indicated a positive association ("better" performance) between child DEPs and the MDI at age 12 mo ($\beta = 1.89$, 95% CI: 0.21–3.58). There was a statistically significant positive association between child DAPs and BSID:MDI at 24 mo, whereas a statistically significant inverse association was reported between maternal DAPs and BSID:MDI at 24 mo ($\beta = -3.54$, 95% CI: −6.59 to −0.49). In addition, maternal DMPs was associated inversely ($\beta = -3.64$, 95% CI: −6.36 −0.91) and child DMPs was associated positively ($\beta = 2.01$, 95% CI:

0.24 - 3.78) with MDI at 24 mo. Thus, the observed associations between DAPs and performance on the BSID:MDI are not likely to be a result primarily of exposure to CPF (Eskenazi et al. 2007).

Both the CCCEH (cord blood CPF; Rauh et al. 2006) and the CHAMACOS (maternal TCPy and DEPs; Eskenazi et al. 2007) studies administered the BSID II at ages 12 and 24 mo, and showed no statistically significant associations. The CCCEH study reported associations between CPF and indications of poorer performance on the MDI and PDI at age 36 mo. The inverse association between CPF and the BSID:MDI in the CCCEH study was statistically significant only in the analysis that modeled both the exposure and the outcome as dichotomous variables (Table 2). The CHAMACOS study did not evaluate children at 36 mo, preventing direct comparison of study findings. The decision to use a dichotomous exposure variable in the CCCEH study was based in part on results from previous analyses, including analyses of CPF and birth weight (Whyatt et al. 2004). Rauh et al. (2006) stated, "The most highly exposed group and the undetectable group had lower mean MDI and PDI scores than did the middle levels." Greenland (1998) offers the following advice regarding the categorization of exposure data: "Ideal categories would be such that any important differences in risk will exist between them but not within them." Thus, it may not have been appropriate to combine the "undetectable" group with the lower two tertiles in the analyses. Furthermore, one cannot assume a monotonic dose-response association based on statistical evaluation of a dichotomous variable. Therefore, the findings presented by the CCCEH study investigators do not necessarily support a dose-response association between increasing CPF and decreasing performance on the BSID MDI or PDI.

The CCCEH study (Rauh et al. 2006) was the only study that presented results for dichotomized BSID outcomes, in addition to using a continuous variable. The cutoff point of 85 was based on one standard deviation below the standardized mean score (standardized mean = 100; SD = 15) (Black and Matula 2000). In a normal distribution, one would expect approximately 16% of scores to be more than one standard deviation below the mean—that is, below 85. In the CCCEH study, 15.7, 49.3, and 32.9% had MDI scores below 85 ("mild or significant mental delay") at ages 12, 24, and 36 mo, respectively, and 14, 13.2, and 10.5% had PDI scores below 85 ("mild or significant psychomotor delay") at the same ages, respectively. Thus, the distribution of PDI and MDI scores appear to differ in this cohort. As described previously, mean MDI scores tended to be lower than mean PDI scores. Further evaluation of categorical associations of CPF with BSID scores distinguishing "mildly delayed" from "significantly delayed" performance on the MDI may be informative.

Behavioral Endpoints From the CBCL

Both the CHAMACOS and the CCCEH cohort studies evaluated potential associations between cord-blood CPF (Rauh et al. 2006) or maternal and child urinary metabolites (Eskenazi et al. 2007) with outcomes measured by the CBCL, albeit at different ages (Table 3). In the CCCEH cohort study, Rauh et al. (2006) reported a statistically significant association among children in the higher CPF exposure group ($n = 50$) compared to children in the lower exposure group ($n = 204$) and results from the CBCL assessed at age 36 mo. This includes increased OR for attention problems (OR = 11.26, 95% CI: 1.79–70.99), ADHD (OR = 6.5, 95% CI: 1.09–38.69), and PDD (OR = 5.39, 95% CI: 1.21–24.11) (Rauh et al. 2006). Although relatively high, the estimated OR for these outcomes are imprecise, as indicated by the broad 95% CI.

In the CHAMACOS study, neither maternal TCPy nor maternal DEPs was statistically significantly associated with any of the CBCL outcomes measured at 24 mo (Eskenazi et al. 2007). However, child metabolite levels of DEPs and DAPs were associated with a statistically significant, approximately 70%, increase

TABLE 3. Summary of results from the Child Behavior Checklist (CBCL)

	Columbia Center for Children's Environmental Health (CCCEH)	Center for Health Assessment of Mothers and Children of Salinas (CHAMACOS)	
	Rauh et al., 2006	Eskenazi et al., 2007	
Exposure measurement	**CPF in umbilical cord blood[a]**	**TCPy in maternal[b] urine**	**DEPs and DAPs in maternal[b] and child urine**
Exposure variable format	Categorical: (>6.17 pg/g vs. ≤6.17 pg/g)	Categorical: (<LOD (reference), below median detected level, above median detected level)	Continuous: (nmol/L – $\log_{10}$)
Sample size for analysis	228	Not reported. TCPy detected in 91% of 445 maternal urine samples	Not reported. Urine metabolite measures available for 445 women (prenatal) and 373–405 children depending on timing (age) of follow-up testing.
Age at assessment	36 mo	24 mo	
CBCL[c] **OR (95% CI):** Positive associations (OR >1) indicate biomarkers are associated with maternal reports indicating more CBCL problems.			
Attention problems	**11.26 (1.79–70.99)***	No statistically significant associations, data not reported	Maternal DEPs: 0.78 (0.26, 2.31) Child DEPs: 1.02 (0.61, 1.71) Maternal DAPs: 0.77 (0.27–2.24) Child DAPs: 1.41 (0.75, 2.64)
ADHD problems	**6.50 (1.09–38.69)***	No statistically significant associations, data not reported	Maternal DEPs: 0.59 (0.21, 1.68) Child DEPs: 1.18 (0.72, 1.94) Maternal DAPs: 1.34 (0.50, 3.59) Child DAPs: 1.11 (0.61, 2.03)
PDD problems	**5.39 (1.21–24.11)***	No statistically significant associations, data not reported	Maternal DEPs: 0.88 (0.37, 2.07) Child DEPs: **1.72 (1.12, 2.64)*** Maternal DAPs: 2.25 (0.99, 5.16) Child DAPs: **1.71 (1.02, 2.87)***
Covariates included in the regression models	Race, gender, maternal education, maternal IQ, gestational age, prenatal ETS exposure, home environment	Not reported	Gender, exact age at assessment, breast-feeding duration, HOME score, household income above poverty threshold, parity, maternal PPVT, maternal depression

Abbreviations: DAPs = total dialkyl phosphates; DEPs = diethylphosphates; DMPs = dimethylphosphates; ETS: environmental tobacco smoke, HOME = Home Observation for Measurement of the Environment; PPVT = Peabody Picture Vocabulary Test; OR = odds ratio, CI = confidence interval; ADHD = attention deficit hyperactivity disorder; PDD = pervasive developmental disorder; CPF = chlorpyrifos.

[a]Maternal blood was used when cord blood was unavailable.

[b]Maternal blood was collected during pregnancy.

[c]Measured at 36 mo in Rauh et al. (2006) and 24 mo in Eskenazi et al. (2007). Rauh et al. 2006 dichotomized the participants' CBCL scores at the 98th percentile and Eskenazi 2007 dichotomized the participants' CBCL scores at the 93rd percentile.

*$\mathbf{p < .05}$ significance bolded for emphasis.

in risk of maternal-reported PDD (OR = 1.72, 95% CI: 1.12–2.64 and OR = 1.71, 95% CI: 1.02–2.87, respectively) (Eskenazi et al. 2007). Eskenazi et al. (2007) reported positive significant ($p \leq .05$) association between maternal levels of DAPs and PDD (OR = 2.25, 95% CI: 0.99–5.16). However, because Eskenazi et al. (2007) noted statistically significant associations with maternal DAPs and DMPs (OR = 2.19; 95% CI:1.05–4.58), but not with DEPs or TCPy, evidence indicated that exposures to pesticides other than just CPF may be involved.

"Pervasive Developmental Disorder" (PDD) on the CBCL includes affirmative responses to items such as "avoids eye contact," "unresponsive to affection," and "rocks head, body" (Eskenazi et al. 2007). PDD is one of the "DSM-IV oriented scales" on the CBCL, and is consistent with though not equivalent to autism disorder and Asperger's disorder (Rescorla 2005; Eskenazi et al. 2007). In the CHAMACOS study, Eskenazi et al. (2007) combined children with scores in the clinical range (>97th percentile) with those in the borderline clinical range (>93rd percentile) for the CBCL outcomes. For PDD, 29.6% ($n = 105$) of the children were categorized as being within the clinical or borderline PDD range. Of these, 51 (14.4% overall) were in the clinical range for PDD.

In the CCCEH study, the classification of PDD used by Rauh et al. (2006) appears to be a 98th percentile cutoff point, thereby restricting their definition of PDD to "clinical" only. Rauh et al. (2006) reported that 4.7% of the 228 children included in the CBCL analyses were categorized as having "PDD problems." This corresponds to 11 children overall, with 4 children in the higher exposure group and 7 in the lower exposure group (numbers estimated based on reported percentages). Despite these relatively small numbers for analysis, the proportion of children with maternal-reported PDD is relatively high, particularly in the CHAMACOS study. The extent to which maternal-reported PDD in these studies may correspond to a clinical diagnosis of PDD, or to other related behavioral diagnoses, is unknown and is an important consideration as these cohorts continue to be followed. Preliminary data from the CHARGE case-control study of genetic and environmental risk factors for autism suggest that misclassification of PDD in epidemiologic cohort studies is likely (Hertz-Picciotto et al. 2006).

Interpretation of the CBCL data from the CCCEH and CHAMACOS study should bear in mind that assessment was conducted at different ages, analyses were based on different cutoff points for defining CBCL problems, and small numbers of outcomes produced imprecise effect estimates. Misclassification of the CBCL outcomes is also of concern.

Neonatal Behavioral Outcomes on BNBAS

Two studies—the CHAMACOS and Mt. Sinai study—reported associations between maternal urinary metabolites and the BNBAS (Table 4). The BNBAS was administered to newborns in the CHAMACOS study within 2 mo of delivery (Young et al. 2005). The median age at administration of the BNBAS was 3 d, with an interquartile range (IQR) between 1 and 26 d postdelivery. Young et al. (2005) provided results for the total sample and also stratified the infants based on the timing of their assessment (≥ or <3 d after delivery). In the Mount Sinai study, the BNBAS was administered to newborns within 5 d of delivery (Engel et al. 2007). Therefore, the data reported by Engel et al. (2007) may be most comparable with data <3 d after delivery reported in Young et al. (2005).

There was no meaningful or statistical association between maternal DEPs and DAPs and number of abnormal reflexes in analyses of newborns tested within 3 d of birth. However, maternal DEPs were associated inversely and significantly with autonomic stability among infants tested within 3 d of birth, including tremor, startling, and the color of the infant's skin (Young et al. 2005). DEPs and DAPs were associated positively and significantly with number of abnormal reflexes in analyses of

TABLE 4. Summary of results from the Brazelton Neonatal Behavioral Assessment Scale (BNBAS)

	Center for Health Assessment of Mothers and Children of Salinas (CHAMACOS)		Mt. Sinai Children's Environmental Health Cohort Study	
	Young et al., 2005		Engel et al., 2007	
Exposure measurement	**DEPs in maternal urine**	**DAPs in maternal urine**	**DEPs in maternal urine**	**DAPs in maternal urine**
Exposure variable format	Continuous: (nmol/L – $\log_{10}$)	Continuous: (nmol/L – $\log_{10}$)	Continuous: (nmol/L – $\log_{10}$)	Continuous: (nmol/L – $\log_{10}$)
Sample size for analysis	Range: 175–381 (full sample)		Range: 144–253	
BNBAS (Assessment < 3 days after birth) β (95% CI)			**BNBAS (within 5 days of delivery) β** (95% CI)	
Habituation	0.47 (–0.05, 0.99)	0.10 (–0.40, 0.60)	0.17 (–0.23, 0.57)	0.08 (–0.30, 0.46)
Orientation	−0.11 (–0.65, 0.43)	−0.02 (–0.53, 0.49)	−0.11 (–0.41, 0.20)	−0.03 (–0.34, 0.28)
Motor	0.08 (–0.17, 0.33)	0.04 (–0.20, 0.28)	0.05 (–0.08, 0.17)	0.05 (–0.08, 0.17)
Range of State	−0.21 (–0.54, 0.12)	0.11 (–0.21, 0.43)	0.04 (–0.12, 0.19)	0.02 (–0.14, 0.17)
Regulation of State	−0.08 (–0.52, 0.37)	−0.07 (–0.50, 0.36)	−0.05 (–0.30, 0.21)	−0.03 (–0.28, 0.23)
Autonomic Stability	0.31 (0.01, 0.61)*	−0.09 (–0.38, 0.20)	−0.15 (–0.38, 0.08)	−0.11 (–0.33, 0.12)
Number of Abnormal Reflexes	0.08 (–0.16, 0.32)	−0.01 (0.24, 0.22)	**RR[a]=1.49 (1.12, 1.98)***	RR[a]=1.32 (0.99, 1.77)
BNBAS (Assessment ≥ 3 days after birth[b]) β (95% CI)				
Habituation	0.20 (–0.43, 0.83)	0.06 (–0.54, 0.66)	NR	NR
Orientation	−0.33 (–0.73, 0.08)	−0.13 (–0.54, 0.27)	NR	NR
Motor	0.17 (–0.05, 0.38)	−0.07 (–0.28, 0.15)	NR	NR
Range of State	0.20 (–0.21, 0.62)	−0.02 (–0.44, 0.40)	NR	NR
Regulation of State	−0.24 (–0.72, 0.24)	−0.10 (–0.58, 0.37)	NR	NR
Autonomic Stability	−0.16 (–0.47, 0.14)	−0.19 (–0.49, 0.12)	NR	NR
Number of Abnormal Reflexes	**0.37 (0.09, 0.64)***	**0.53 (0.23, 0.82)***	NR	NR

BNBAS (full sample[b]) β (95% CI)				
Habituation	0.33 (–0.06, 0.72)	0.03 (–0.34, 0.40)	–	–
Orientation	−0.32 (–0.66, 0.03)	−0.17 (–0.50, 0.17)	–	–
Motor	0.10 (–0.06, 0.27)	−0.03 (–0.19, 0.14)	–	–
Range of State	−0.02 (–0.27, 0.24)	0.09 (–0.16, 0.34)	–	–
Regulation of State	−0.15 (–0.47, 0.17)	−0.07 (–0.39, 0.24)	–	–
Autonomic Stability	0.06 (–0.15, 0.27)	−0.16 (–0.36, 0.05)	–	–
Number of Abnormal Reflexes	**0.22 (0.04, 0.40)**∗	**0.23 (0.05, 0.41)**∗	–	–
Covariates∗∗				
Habituation	Age at BNBAS, smoking, alcohol, method of delivery, minutes fed since BNBAS, BNBAS interviewer.		Drug use during pregnancy, examiner, PON1 enzyme tertiles, urinary creatinine.	
Orientation	Age at BNBAS, BNBAS interviewer, number of prenatal care visits.		Pre-pregnancy BMI, examiner, neonatal jaundice, PON1 enzyme tertiles, urinary creatinine.	
Motor	Age at BNBAS, poverty level, gestational age at initiation of prenatal care, BNBAS interviewer.		Age at BNBAS, caffeine consumption during pregnancy, drug use during pregnancy, examiner, PON1 enzyme tertiles, urinary creatinine.	
Range of State	Age at BNBAS, number of prenatal care visits, gestational age at initiation of prenatal care, alcohol, BNBAS interviewer.		Age at BNBAS, examiner, PON1 enzyme tertiles, urinary creatinine.	
Regulation of State	Age at BNBAS, pre-pregnancy BMI, infant sex, parity, caffeine use, BNBAS interviewer.		Maternal education, examiner, PON1 enzyme tertiles, urinary creatinine.	
Autonomic Stability	Age at BNBAS, infant sex, parity, vitamin use, minutes since fed at BNBAS, BNBAS interviewer, illicit drug use during pregnancy.		Age at BNBAS, examiner, smoking during pregnancy, PON1 enzyme tertiles, urinary creatinine.	
Number of Abnormal Reflexes	Age at BNBAS, maternal age at delivery, smoking, vitamin use, BNBAS interviewer, mean diastolic and systolic blood pressure.		Examiner, anesthesia during delivery, PON1 enzyme tertiles, urinary creatinine.	

Abbreviations: DAPs=total dialkyl phosphates; DEPs=diethylphosphates; CI=confidence interval; RR=relative risk.

[a]"Poisson regression was used to analyze the relation between biomarker levels and the number of abnormal reflexes because of the count nature of the data."

[b]In the CHAMACOS study (Young et al. 2005), the median age at administration of the BNBAS was 3 days, with an interquartile range between 1 and 26 days post-delivery.

∗**p<.05,** significance bolded for emphasis.

∗∗The same covariates were included in the models for DEPs and DAPs within each paper.

the full CHAMACOS study sample, and analyses of infants evaluated 3 d or more after birth (Table 4). Results for the remaining BNBAS domains were variable and statistically nonsignificant, regardless of age at testing.

In the Mt. Sinai cohort study, Engel et al. (2007) reported a statistically significant positive association between maternal levels of DEPs, but not DAPs, and number of abnormal reflexes (Table 4). Results for the remaining domains were variable and statistically nonsignificant. Engel et al. (2007) reported that the OR for number of abnormal reflexes did not increase monotonically and were not statistically significant for the highest versus lowest category comparisons based on analyses of quartiles of DEPs and DAPs.

Engel et al. (2007) evaluated the interaction between PON1 activity and urinary metabolites on number of abnormal reflexes. The investigators considered lower tertiles of PON1 activity to be "slower OP metabolizers." Therefore, the implicit assumption is that slower OP metabolizers might have higher levels of CPO and other oxons. Engel et al. (2007) noted a statistically significant interaction between levels of DAPs and PON1 activity on the relative risk of abnormal reflexes. Engel et al. (2007) reported that for DAPs and DMPs, infants in the lowest tertile of PON1 activity displayed a significantly increased relative risk of abnormal reflexes (DAP RR = 2.38, 95% CI: 1.37–4.15; DMP RR = 1.96, 95% CI: 1.27–3.03). A statistically significant interaction was also observed between DMPs and the middle tertile of PON1 activity. In contrast, there was no statistically significant interaction between DEP metabolite level and PON1 activity. Therefore, the interaction between DAPs and PON1 activity is not likely to reflect consequences of CPF exposure.

The BNBAS findings that are more relevant to CPF are the results from the analyses of DEPs. Both studies with data reported one or more statistically significant associations between maternal DEPs and number of abnormal reflexes. However, the implication of these findings is not clear. Neither study reported statistically significant findings for any of the other six BNBAS measures, including motor performance. The association in the CHAMACOS study was approximately null and statistically nonsignificant among infants examined within 3 d of birth. Thus, results from the two studies are not consistent when infants of comparable ages of examination were analyzed. Testing conditions varied between the two studies (Table 1), particularly test location, with all infants in the Mt. Sinai study evaluated prior to discharge, compared with only 30% of infants in the CHAMACOS study. Finally, both studies evaluated DEPs and DAPs, which are not specific markers of CPF. Neither study analyzed associations between TCPy, a more specific urinary biomarker for CPF, and BNBAS outcomes. In a later publication from the CHAMACOS study, Eskenazi et al. (2007) reported relatively low correlations between DEPs and TCPy ($r = .1$ to $.2$).

Interpretation of Epidemiologic Studies

Review of the three epidemiologic cohort studies with data potentially relevant to CPF exposure and neurobehavioral outcomes indicated relatively few statistically significant findings in the direction of adverse effects, and no consistent patterns of adverse association across studies. One exception may be the positive association between DEPs and number of abnormal reflexes observed in both the Mt. Sinai study (Engel et al. 2007) and the CHAMACOS (full sample) study (Young et al. 2005). However, the lack of association between DEPs and number of abnormal reflexes among infants evaluated within 3 d of birth in the CHAMACOS study (beta = 0.08) is inconsistent with the statistically significant positive association reported by the Mt. Sinai study for infants evaluated within 5 d of delivery (RR = 1.49). Furthermore, DEPs reflect exposure to several pesticides besides CPF.

Interpretation of this group of epidemiologic studies needs to take into consideration study similarities and differences, and strengths and limitations. There

are insufficient data to address the specific research question regarding CPF and neurobehavioral outcomes in infants and young children. Isolating and quantifying the potential effect of CPF is challenging for several reasons. As shown in Tables 2–4, the results were from a number of regression models, which varied by factors including the biomarker/metabolite measured, the age of the infant/toddler, and the neurobehavioral test instrument. Thus, it is also important to consider the probability that some statistically significant findings may have occurred by chance. In addition, noncausal associations may also be produced as a result of nonrandom error (bias), including measurement error and confounding.

Measurement Error All studies used quantitative biomarkers of exposure, which is clearly desirable because they are more objective measures compared to questionnaires. Only one study, the CCCEH study, actually measured the parent compound in blood. This study was limited, however, by measuring CPF at one point in time (i.e., in umbilical cord blood at the time of delivery). The other two cohort studies used urinary metabolites of CPF that also reflect exposures to other OP. Eskenazi et al. (2007) examined TCPy in addition to DAPs and DEPs, but observed no statistically significant associations with this metabolite. Although TCPy is more specific than the DAPs, TCPy may reflect other exposures (e.g., chlorpyrifos-methyl, TCPy) and may not represent all routes of exposure equally well (Needham 2005).

All three cohort studies relied on one or two specimen collections and it cannot be assumed that these data will accurately represent exposure(s) during pregnancy. Eskenazi and colleagues (2004; 2007) reported high within-person variability for urinary metabolites measured at different times in both mothers and their children. In addition, Eskenazi et al. (2007) found that the proportion of CHAMACOS study participants with biomarker levels above the limit of detection was 71% at the baseline pregnancy measurement and 82% at the second measurement. The average of these two measures was reported as 91%, which ultimately becomes the reference group for future analysis in this publication. However, using an average limit of detection is likely to lead to misclassification of the exposure and also is likely to mask the exposure level during the different times of brain development throughout the gestational process.

Confounding Confounding might produce spurious findings or mask "real" effects. It is important to consider the extent to which any factors that predict performance on the BSID-II, or a mother's assessment of her child on the CBCL, or an infant's behavior during evaluation on the BNBAS, are related to factors associated with use of CPF or to factors that determine the degree of exposure to CPF. Such factors may include personal characteristics of the mother, including her age, education level, depressive symptoms, etc. If these factors are not measured and controlled in the analyses, the results may be confounded. In considering factors that are associated with levels of CPF in umbilical cord blood, it is important to evaluate the conditions that may be related to the reasons for being exposed to CPF or other OP (e.g., cockroach infestation associated with poor housing maintenance, hygiene, and care environment; extent of participation in agricultural work), as well as factors related to higher versus lower exposure (e.g., following instruction labels regarding proper use). These factors could also be related to the learning and developmental environment, which may affect the test scores in the child, or may influence the mother's interpretation or reporting of her child's behavior.

It is also important to consider the constellation of established predictors of performance on the BSID, scores on the CBCL, or scores on the BNBAS, and to evaluate these as potential confounders, particularly those that could conceivably be associated with CPF levels. For example, in their consideration of potential confounders, Eskenazi et al. (2007) stated that selection of covariates for the multivariate BSID-II models was based on whether the variables were

"related to conditions of testing, related to neurodevelopment in the literature and associated ($p < 0.1$) with most outcomes . . . or consistently related to neurodevelopment in the literature even if not in our data." In the CCCEH study, Rauh et al. (2006) stated that "Covariates were included in models as possible confounders if they (1) had a significant association with level of pesticide exposure and any measure of developmental outcomes in this sample, (2) altered the estimate of CPF effect by $\geq$ 10%, or (3) had been identified as confounders in comparable studies."

There were several differences in the factors that were adjusted for between Rauh et al. (2006) and Eskenazi et al. (2007). For example, Rauh et al. (2006) did not appear to measure or adjust for factors related to the BSID-II testing environment, such as the examiner or the exact age at examination. These factors are known to influence test scores (Black and Matula 2000) and were measured and adjusted for by Eskenazi et al. (2007). As another example, maternal depression, a significant factor in the Eskenazi et al. (2007) CBCL models, was not evaluated by Rauh et al. (2006). Thus, there are concerns about potential uncontrolled or residual confounding.

Causal Inference Distinguishing causal from noncausal associations in epidemiology is challenging because of the observational (i.e., nonrandomized) study designs and the inevitable role of bias. Hill proposed several viewpoints to consider when evaluating the evidence from a body of epidemiologic literature, including strength of the association, consistency, dose-response, and biological plausibility (Hill 1965). The associations (OR) between CPF and maternal-reported CBCL outcomes were relatively strong in the CCCEH study by Rauh et al. (2006), but the imprecision indicated by the accompanying CI should not be ignored. As pointed out by Poole (2001), inference and decision making should prefer estimates with narrow confidence intervals over small *p* values, "which are least vulnerable to the play of chance. These are the results for which, by virtue of intentional or accidental features of our research methods, our studies provide the most evidence." In contrast to the results from the CCCEH study, the association between maternal DEPs and PDD was inverse and statistically nonsignificant in the CHAMACOS study (Eskenazi et al. 2007).

For the most part, other observed associations were not particularly strong. There was no clear evidence for consistency, but given the limited number of studies, different types of exposure (home use versus agricultural), variability in exposure measure, timing of exposure measure, timing of outcome measure, and modeling of exposure and outcome for analysis, only a limited degree of consistency would be possible to establish. While there were some statistically significant results for analyses of continuous exposure and outcome variables, it was not always clearly indicated whether the presence of nonlinear associations was also explored. Rauh et al. (2006) and Engel et al. (2007) did describe data that indicated that some associations were not monotonic. Use of dichotomous exposure variables in the CCCEH study precluded the ability to assess exposure-response associations in that cohort (Rauh et al. 2006). Temporality and an argument for biological plausibility were supported in the three cohorts.

Summary

Therefore, data from these three cohorts did not support a causal association between CPF and adverse neurobehavioral outcomes in infants or young children. Additional research in this area is needed in order to assess causality with more certainty. Challenges to future research include limited exposure among most individuals, difficulties in estimating exposure during critical periods of development, and consistency of timing and outcomes measured across multiple studies to allow comparisons. Nevertheless, additional analyses from the existing cohorts to fully examine dose response, repeated measures of outcomes, assessment of test–retest reliability with outcome measures, correlations among all available exposure

measures (self-report, biomarker, air monitoring data), and additional follow-up time and testing of the children will provide helpful information.

ANALYSES OF DEVELOPMENTAL NEUROBEHAVIORAL ANIMAL STUDIES

Scope of the Review and Literature Search

PubMed was searched using the terms "chlorpyrifos" and ("offspring" or "neonatal" OR "maternal" OR "in utero" OR "development" OR "developmental" OR "pregnancy" OR "pregnant" OR "gestational" OR "newborn" OR "prenatal" OR "perinatal" OR "teratology" OR "fetus" OR "fetal" OR "maternal" OR "age-dependent" or "age dependent" or "age sensitivity") AND ("brain" OR "neuron" OR "nervous" OR "neurotoxic*" OR "neurolog*" OR "neurobehavior*" OR "neurodevelopment" OR "developmental neurotoxic*" OR "motor" OR "cognition" OR "cognitive" OR "behavior" OR "receptor" OR "neurotransmitter" OR "cholinesterase" OR "cerebellum" OR "hippocampus" OR "striatum" OR "cortex"). In addition, the reference list for Eaton et al. (2008) was cross-checked for additional papers meeting our inclusion criteria.

Our review focused on repeated-dose in vivo DNT animal studies in which CPF alone (i.e., not mixtures, not in sequence with other pesticides, and not as a formulation) was administered in a clearly defined vehicle to pregnant dams, lactating dams, and/or pups prior to weaning. Studies using oral, inhalation, dermal, and sc routes of exposure to CPF were included, but not those using intravenous or direct injections into the brain.

The primary purpose of this review is to provide an in-depth weight-of-evidence evaluation of the absence and presence of effects and direction of change reported for neurobehavioral endpoints. Neuropharmacologic and morphologic endpoints were evaluated for evidence of MOA directly linking effects of these endpoints with neurobehavioral outcomes, and relative sensitivity compared to brain or RBC AChE inhibition.

This review focused on evaluating effects at lower dose levels (i.e., less than 10 mg/kg). Studies conducted with doses near the maximum tolerated dose level, producing significant systemic toxicity or lethality, or designed to compare LD50s were not included. Standard developmental and reproduction studies were not included unless AChE activity inhibition or neurobehavioral, neuropharmacologic, and neuropathologic/morphologic endpoints were evaluated.

Sixty-one research papers measured effects of repeated exposures of CPF during development prior to weaning on behavioral, neuropharmacologic (AChE inhibition, neurotransmitter receptor binding, neurotransmitter levels), and/or brain pathology endpoints. Twenty-three papers included behavioral outcomes, 4 of which were excluded because the test material was a CPF formulation (Muto et al. 1992), test subjects were mutant mice (Laviola et al. 2006), or dose levels were greater than 10 mg/kg-d (Chanda and Pope 1996; Chakraborti et al. 1993). Of the remaining 19 behavioral studies, there were 7 oral studies (Braquenier et al. 2010; Carr et al. 2001; Johnson et al. 2009; Maurissen et al. 2000; Venerosi et al. 2006; 2009; 2010), 1 dermal study (Abou-Donia et al. 2006), 3 sc studies with peanut oil as vehicle (Jett et al. 2001; Ricceri et al. 2006 [oral for gestation, sc for postnatal]; Venerosi et al. 2008), and 8 sc studies with dimethyl sulfoxide (DMSO) as vehicle (Aldridge et al. 2005a; Dam et al. 2000; Billauer-Haimovitch et al. 2009; Haviland et al. 2010; Icenogle et al. 2004; Levin et al. 2001; 2002; Ricceri et al. 2003). Seven research papers included histopathology or morphometry evaluations of brain areas, one of which was excluded because CPF exposure was 40 mg/kg-d (Amira et al. 2005). Forty-four papers evaluated neuropharmacologic outcomes following developmental

exposures to repeated CPF doses less than 10 mg/kg-d.

Comparison of Experimental Design and Statistical Analyses

Using the standards and approaches described earlier in the section entitled "Approach to Evaluation of CPF Studies," the experimental design and statistical analyses were evaluated. Key studies representative of groups of investigators with similar experimental design and dosing regimens are discussed next for laboratories evaluating multiple behavioral outcomes.

The oral DNT rat study by Maurissen et al. (2000) was the most robust study in terms of number of dose levels tested (3 dose groups and control), number of pups (1 male or female/litter) and litters (20 litters/dose group) tested, and relevant route of exposure. In this study, dams were dosed from gestational day 6 to postnatal day 11 (GD 6–PND 11). Data for each behavioral endpoint from both sexes were pooled into a single analysis of variance (ANOVA). This study was designed so that the litter was the unit of analysis, the time of testing was balanced across sex and dose level, and experimental bias of subjective measures was controlled (e.g., blind observations for learning and memory test). A related study by Mattsson et al. (2000) measured AChE or BuChE inhibition in 10 litters/dose group (1 male or female/litter) from brain, RBC, or plasma samples collected 2–4 h after dosing.

Three oral neurobehavioral studies dosed mouse or rat pups directly with three CPF dose levels or vehicle (Braquenier et al. 2010; Johnson et al. 2009; Carr et al. 2001). Braquenier et al. (2010) tested 8–10 female mice/dose group (1 female/litter). Of the three behavioral tests scored by observers (locomotor activity, light/dark box test, elevated plus maze), only the elevated plus maze was scored blind to treatment level (Braquenier et al. 2010). For the two oral rat studies, Carr et al. (2001) and Johnson et al. (2009) dosed the low-dose group with a constant dose level, and exposed the mid- and high-dose groups to incremental dose regimens. Carr et al. (2001) dosed 2 pups/sex/litter from 5 litters/dose group every other day, and included litter as a factor in the statistical analysis. Observations of the open field activity were conducted blind to treatment level. Johnson et al. (2009) used a within litter design, in which all seven exposures (corn oil vehicle, three CPF doses, and three methylparathion doses) were represented within each litter, to the extent possible. The potential for cross-contamination was not addressed. The sample size for each dose group was 9–14 rats/sex/dose level. Statistical analysis included the litter as the experimental unit of analyses, and day or week of testing was included as a repeated-measures factor. There was no indication of whether observers for the radial arm maze were blind to treatment (Johnson et al. 2009).

Oral neuropharmacologic studies by Guo-Ross et al. (2007) and Richardson and Chambers (2003; 2004; 2005) included two or three dose levels other than the control, and litter was the individual unit of analysis. AChE inhibition was measured in brain samples collected 4–6 h after dosing (Guo-Ross et al. 2007; Richardson et al. 2005). The selection of pups from litters and the number of litters represented was not always clearly indicated (Guo-Ross et al. 2007; Richardson and Chambers 2003 2004; Tang et al. 1999). The sample size of three to four or four to six pups per dose group was small even for mechanistic biochemical endpoints. However, the use of multiple dose levels and/or times of sacrifice allowed data to be evaluated for consistency in dose-response and temporal patterns. Statistical analyses for these neuropharmacologic studies did not appear to take into consideration repeated measures, and a correction for multiple comparisons was not included in the analyses. An oral neuropharmacologic study by Eels and Brown (2009) evaluated dopamine neurochemistry in one oral CPF dose group receiving an incremental dosing regimen (1.5 to 6 mg/kg, PND 1–21). This study was considered inadequate because it did not report sample size for pups or litters.

There was one dermal neurobehavioral study that tested only one CPF exposure level. The litter was not the experimental unit (10 pups/sex from 5 litters; Abou-Donia et al. 2006). Observers were blind to treatment level.

Slotkin et al. (2001; 2002; 2004; 2006) and collaborators (Aldridge et al. 2003; 2004; 2005a; 2005b; 2005c; Icenogle et al. 2004; Levin et al. 2001; 2002; Qiao et al. 2002; 2003; 2004; Raines et al. 2001; Roy et al. 2004; 2005; Slotkin and Seidler 2005; 2007a; 2007b; Song et al. 1997) dosed rats sc with CPF in DMSO. For 4 of the 5 neurobehavioral studies, 1 offspring/sex/dose group from 8–10 litters was tested (Aldridge et al. 2005a; Icenogle et al. 2004; Levin et al. 2001; 2002). For one of the neurobehavioral studies, the pup was the individual unit of analyses with 5–7, 23–24, and 40–46 pups/sex/dose group (2 pups/sex/litter) for locomotor skills, reflex righting, and negative geotaxis, respectively (Dam et al. 2000). The litter of origin was confounded in studies that exposed dams during gestation because pups were randomized at birth to create newly constituted litters within a treatment level. In addition, male and female offspring from the same reconstituted litter (1 pup/sex/litter) were pooled together in a multivariate statistical analysis that did not include litter as a factor. Dams were repeatedly rotated every few days to control for differences in maternal care. Aldridge et al. (2005) and Dam et al. (2000) indicated that the observers for behavioral tests were blind to treatment level, but Levin et al. (2001; 2002) and Icenogle et al. (2004) did not.

The oral mouse study by Braquenier et al. (2010) only tested female offspring, and included 3 CPF-treated groups plus control and 8–10 pups tested per dose group. Behaviors on the elevated plus-maze, but not the light/dark box test, were recorded by observers blind to treatment.

Oral and sc mouse studies conducted by Ricceri et al. (2003; 2006) and Venerosi et al. (2006; 2008; 2009; 2010) dosed approximately 10 pups/dose level. With the exception of Ricceri et al. (2006), these studies indicated that observers were blind to treatment level for at least one of the behavioral measures. Mice were exposed to only one CPF dose level in 2 oral studies (6 mg/kg-d; Venerosi et al. 2009; 2010) and 1 sc study (3 mg/kg-d in peanut oil; Venerosi et al. 2008). Ricceri et al. (2003) dosed mice postnatally with two CPF dose levels sc in DMSO. For each oral group of dams dosed during pregnancy (vehicle, 3 mg/kg-d, or 6 mg/kg-d), Ricceri et al. (2006) and Venerosi et al. (2006) dosed the pups (vehicle, 1 mg/kg-d, or 3 mg/kg-d) sc and orally, respectively, using a within-litter design such that in total nine different treatment groups were established. The litter was the experimental unit of analyses for most studies, although not always for behavioral endpoints examined after weaning (Ricceri et al. 2003). The decision tree for the statistical analyses was unclear because results of post hoc tests were reported even though main effects were not significant (Ricceri et al. 2003; 2006; Venerosi et al. 2009). Frequency, duration, and latency for many behavioral parameters were analyzed. Corrections for the multiple comparisons were reported in two papers (Ricceri et al. 2003; 2006).

Relevance of Route, Vehicle, and Age of Exposure to Risk Assessment

The route and age of exposure, as well as the vehicle used, need to be taken into account in the interpretation and application of the results in animals for human health risk assessment. The relevance of different routes of exposure depends on the specific population of concern and uses of CPF. In the United States today, the primary route of exposure for the general population is dietary. The majority of the animal studies on CPF can be divided into the following periods of exposure: (1) gestational exposures to the dam, (2) gestational and postnatal exposure to the dam, so that pups are potentially exposed via lactation, (3) gestational exposures followed by direct exposures to the pups, or (4) postnatal exposures directly to the pup without any maternal exposures. Our review evaluates outcomes following gestational exposures separately from those

following direct exposure to pups for each outcome because the developmental period of exposure is an important consideration when evaluating the results.

Although different routes of exposure or vehicles may be used in mechanistic studies, the relevance of the methods must be evaluated when these data are considered for setting acceptable exposure levels for human risk assessment. Oral administration of test material is relevant to dietary exposures, which is the primary route of exposure for CPF to the general population. However, bolus gavage dose of CPF in corn oil may result in peak blood and brain levels that may not be similar to those measured following dietary exposures, the primary route of exposure to children in the United States where residential uses are not allowed. The sc route of exposure is not a human route of exposure. One rationale for using the sc route of exposure in animal studies is that it "avoids the potential confounds of differential rates of gastrointestinal absorption between compounds or ages and first-pass effects on bioavailability" (Slotkin et al. 2006). However, when evaluating data for purposes of human health risk assessment, first-pass effects on bioavailability are important considerations.

Carr and Nail (2008) studied the effects of route, vehicle, and volume of exposure on brain AChE inhibition 4 h after CPF oral and sc exposure to 5 mg/kg to pups from PND 10 to 16. Overall, sc DMSO injections of CPF increased regional brain AChE inhibition by 16–29% compared to levels in pups dosed orally. Carr and Nail (2008) reported statistically significant decreases in body weight after d 4 of sc injections but not after gavage dosing. Thus, sc DMSO injections of CPF increased toxicity compared to corn oil gavage doses as measured by AChE inhibition and body weight decreases. Carr et al. (2001) also pointed out that sc injection could mimic dermal exposure, but "it does not appropriately consider the disposition and metabolism . . . when penetration through the skin is a determining factor."

Marty et al. (2007) measured the blood PK for CPF and its major metabolite TCPy following gavage and sc dosing to pups on PND 5. Significant (56 ± 36%) radiolabeled CPF remained at the site of sc injection at the time to maximum concentration (Tmax = 2 h). Smith et al. (2009) compared CPF PK using oral and sc exposures and different vehicles in adult male rats. Orally administered CPF underwent much more extensive metabolism than CPF administered sc, consistent with hypothesis that oral CPF is rapidly absorbed and extensively metabolized in the gut and liver, resulting in greater first-pass metabolism. In addition, PBPK model simulation in adults show that sc administration in corn oil and DMSO is predicted to have "greatly prolonged inhibition of ChE activity in the brain relative to oral exposure," due to "slow release of CPF from the injection site depot and localized brain CPF metabolism to CPF-oxon" (Smith et al. 2009).

DMSO, by itself, perturbs behavior, neuropathology, or neuropharmacology at volumes administered as a vehicle. Specifically 20-μl injections of DMSO in PND 4 rats produced endocrine and neurochemical changes similar to or additive with chlordecone (Rosecrans et al. 1984; Uphouse et al. 1982). DMSO exacerbated the neuropathological effects of soman in adult rats at 1-ml/kg doses (Ballough et al. 2008), which is the equivalent volume used in CPF sc rat studies (Aldridge et al. 2003; Levin et al. 2002). These studies indicated that use of DMSO as a vehicle is a potential confounder for determination of cellular and pharmacologic effects produced by chemicals.

In summary, the relevance of sc injections of CPF in DMSO to dietary exposures for risk assessment is called into question due to (1) differences in PK resulting from a depot of test material at the site of the local sc injection including bypassing first pass metabolism, (2) the possible impact of DMSO itself on neurotoxicity, and (3) resultant effects on toxicology endpoints that may not be representative of human dietary exposures to CPF.

Neurobehavioral Domains

This section evaluates consistency of effects across different laboratories for similar behavioral domains. Tables 5–10 summarize

neurobehavioral effects of CPF exposures on selected behaviors for which there were data from different laboratories. An exception was made for spontaneous alternation, measured only by one laboratory (Table 8), because this behavior was considered to be related to other measures associated with learning and memory and/or activity in a novel environment. The major methodological issues of concern were discussed in the previous section and were taken into consideration in the overall assessment.

Developmental Landmarks, Neurodevelopmental Reflex, and Ultrasonic Vocalizations Prior to Weaning Overall, CPF exposures did not produce a consistent pattern of adverse effects on developmental landmarks, neurodevelopmental reflex and ultrasonic vocalization (USV) measured prior to weaning at doses below 5 mg/kg-d (Table 5).

Gestational exposures Maurissen et al. (2000) reported effects on developmental landmarks in rats only at the highest dose level of 5 mg/kg, which was maternally toxic and produced 25% pup mortality and decreased pup body weight before culling on PND 4. Billauer-Haimovitch et al. (2009) noted no marked effects in mice at doses from 1 to 10 mg/kg-d following gestational sc exposures. The sample size for the latter study was not reported and half the pups were cross-fostered.

In mice, 6-mg/kg CPF exposure from GD 14 to 17 exerted little impact on pup growth and sensorimotor development (Table 5B; Venerosi et al., 2009). There was a numerical trend toward delayed appearance of hindlimb grasping and decreased total daily qualitative score (comprising all seven reflex measures including hind limb grasping) (Venerosi et al. 2009). Venerosi et al. (2009) concluded that "a definitive conclusion about a retarding CPF effect cannot be drawn" based on this data. There were statistically significant decreases in pivoting frequency and duration and increases in duration of "immobility" at PND 12, but no significant effect on frequency or duration of crossing, head moving and wall climbing (Table 5B). The significance of the decreases in pivoting to overall motor abilities is uncertain given the low control values (approximately 3–4 frequency and 2–3 s). Thus far, the evidence for adverse effects on sensorimotor development is not compelling.

Exposures to 6 mg/kg from GD 14 to 17 decreased USV and higher peak frequency of calls at PND 10 but not PND 4 and 7 in male mouse pups isolated from the dam for 4 min (Table 5C; Venerosi et al., 2009). The CPF-treated mice had 80 and 15 USV calls/min on PND 7 and 10, respectively. The controls had 70 and 40 USV calls/min, respectively. It is premature to conclude that the fall in USV at 6 mg/kg-d (the only dose level tested) following gestational exposures is an adverse effect based on a study testing only one dose level. In addition, the overall pattern of rapid decrease in USV in mice at 6 mg/kg Venerosi et al. (2009) is consistent with the ontogenic profile for control mice in two other studies from this laboratory. Specifically, Ricceri et al. (2003) observed a rapid decline in USV calls in control pups (approximately 125, 80, and 20 USV calls/min on PND 5, 8, 11, respectively). Calamandrei et al. (1999) reported USV decreasing from approximately 70 to 20 USV/min on PND 7 and PND 11, respectively.

Postnatal exposures Johnson et al. (2009) noted no significant effects of oral low (1 mg/kg-d), mid (incremental 1, 2, and 4 mg/kg-d) and high (incremental 1.5, 3, and 6 mg/kg-d) doses of CPF from PND 1 to 21 on 5 physical development parameters (pinna detachment, downy fur development, hair growth, incisor eruption and eye opening) and 5 neurodevelopmental reflex parameters (surface righting, negative geotaxis, cliff avoidance, free fall righting, and acoustic startle) (Table 5A). This was a robust study measuring effects of direct dosing to pups on physical and reflex development at three dose levels that produced statistically significant hippocampal AChE inhibition (14–53%) on PND 20.

Ricceri et al. (2003) reported no significant effects of 1 and 3 mg/kg-d CPF (sc DMSO) from PND 1 to 4 on USV or homing behavior in mice (Table 5C). Dam et al. (2000) showed that 1 mg/kg-d CPF (sc DMSO) injected in rats from PND 1 to 4 increased surface righting

TABLE 5A. Developmental landmarks/neurodevelopmental reflex in the RAT (oral and sc)

Author	Test	Age Exposure	Age Testing	Sample Size Per Dose Level (Yes/No litter is unit)	Measure*	Magnitude of Dose Tested, mg/kg-day				
						0.3	1	1 to 4[a]	5.0	1.5 to 6[b]
Maurissen et al., 2000	Dev't landmarks	GD7-PND11	PND1	All 8 pups/ litter; 20 litters/dose	Pinna unfolding	0 [c]	0		(↑ day)[d] n.s. delay	
	Not blind	**ORAL corn oil**	PND 11 until criterion	1 M or F /litter; 20 litters	Eye opening	0	0		0	
			PND 27 until criterion	Yes, litter is unit	Vaginal opening	F0	F0		F↑ day delay	
			PND 38 until criterion		Preputial Separation	M0	M0		M(↑ day)[d] n.s. delay	
Johnson et al., 2009	Physical and Neurodev't reflex	PND1-21	Each day beginning on PND 1	12–14 pups/litter; 20 litters/dose	Pinna detachment		0	0		0
		ORAL corn oil		Within litter design	Downy fur development		0	0		0
	Not Blind	Incremental dose regimen for mid and high dose groups		Yes, litter is unit	Hair growth		0	0		0
					Incisor eruption		0	0		0
					Eye opening		0	0		0
					Surface righting		0	0		0
					Negative geotaxis		0	0		0
					Cliff avoidance		0	0		0
					Free fall righting		0	0		0
					Acoustic startle		0	0		0
Dam et al., 2000	Neurodev't reflex	PND1–4	PND3–4	23–24/group; 2 pups/sex/litter Litter is not unit	Surface righting reflex (time)		M0 F↑			
	Videotaped Blind	**SC DMSO**	PND 5–8	37–46/group;2 pups/sex/litter Litter is not unit	Negative geotaxis (# animals making 180° turn)		M0 F↓			

[a]Incremental dose level of 1.0 (PND1–5),2.0 (PND6–13) and 4.0 (PND14–20) mg/kg-day.
[b]Incremental dose level of 1.5 (PND1–5),3.0 (PND6–13) and 6.0 (PND14–20) mg/kg-day.
[c]all entries without "M" and/or "F" indicates analyses is based on males and females combined.
[d]( ) indicate effect in parenthesis is not statistically significant (n.s.); authors defined significance at p = 0.02 for dev't landmarks.
*If statistical analyses or measures were described in methods, then it was included and listed as "0" if not reported to be statistically significant.

TABLE 5B. Developmental landmarks/neurodevelopmental reflex in the MOUSE (oral and sc)

Author	Test	Age and Route of Exposure	Age Testing	Sample Size Per Dose Level (Yes/No litter is unit)	Measure*	Magnitude of Dose Tested, mg/kg-day				
						1	3	5	6	10
Billauer-Haimovitch et al., 2009	Developmental landmarks and neurodev't reflex Not blind	GD 9–18 **SC** **DMSO**	Not stated	1 pup/sex/litter; # litters and # pups not stated Litter is not unit. M & F from same litter pooled in analysis, and litter was not a factor	Surface righting response (day)	0[a]	0	0		0
					Startle response (day)	0	0	0		0
					Appearance of fur (day)	0	0	0		0
					Eye opening (day)	0	0	0		0
					Ear opening (day)	0	0	0		0
Venerosi et al., 2009	Neurodev't reflex Blind	GD 14–17 **ORAL** **peanut oil**	PND 3,6, 9, 12, 15	1 pup/sex/litter; 18 litters control and 16 litters treated Yes, litter is unit Litter was a factor in the statistical analysis	Surface righting reflex (time): shortest of 3 measures				0[a]	
					Surface righting reflex (time): longest of 3 measures				0	
					Grasping reflex (mean angle of falling)				0	
					Forelimb toothpick reflex				0	
					Cliff aversion				0	
					Hindlimb toothpick reflex				(↓)[b] $p = 0.06$	
					Forelimb pencil reflex				0	
					Total daily score (sum of all 7 reflex scores)				(↓) $p = 0.06$)	

[a]all entries without "M" and/or "F" indicates analyses is based on males and females combined.

[b]( ) indicate effect in parenthesis is not statistically significant, author defined significance to be $p<0.05$.

*If statistical analyses or measures were described in methods, then it was included and listed as "0" if not reported to be statistically significant.

TABLE 5C. Neonatal behaviors in the MOUSE (oral and sc)

Author	Test	Age and Route of Exposure	Age of Testing	Sample Size Per Dose Level (Yes/No litter is unit)	Measure	Magnitude of Dose Tested, mg/kg-day		
						1.0	3.0	6.0
Venerosi et al., 2009	Ultrasonic vocalization 4-min Automated confirmed by observational detection	GD15–18 **ORAL** **Peanut Oil**	PND 4, 7,10	1 male/litter; 15 litters/group Yes, litter is unit	# calls			M0 PND4&7 M↓ PND10
					Duration of calls			M0 PND4&7 M↓ PND10
					frequency			M0 PND4&7 M↑ PND10
Ricceri et al., 2003	Ultrasonic vocalization 3-min Not blind	PND1–4 **SC** **DMSO**	PND 5, 8, 11	10 males and 10 females from unstated # litters Yes, litter is unit Litter was a factor in the statistical analysis	# calls	0[a]	0	
					Interaction with age of testing	0	0	
Ricceri et al., 2003	Homing 3-min to reach goal (wood shavings from home cage) Not blind	PND 12 **SC** **DMSO**	PND 12	10 males and 10 females from unstated # litters Yes, litter is unit. Litter was a factor in the statistical analysis	Time to reach nest area	0[a].	(↓)[b]	
					Time spent in nest area	0	0	
					Locomotor activity during 5-min test	0	0	

[a]all entries without "M" and/or "F" indicates analyses is based on males and females combined
[b]authors state no effect, but noted that pups tended to have shorter latencies

reflex time and decreased the number of animals meeting the criterion for negative geotaxis (180° turn on an inclined surface) in female mice but not male mice (Table 5A). Brain AChE inhibition was 75% at 2 h after dosing on PND 1 (Dam et al. 2000). The study by Dam et al. (2000) was limited because only one CPF dose level in DMSO was tested, and the litter was not the experimental unit for data analysis.

Motor Activity There were mixed results (increases, decreases, and no effect) on motor activity (Table 6), as measured in the open field or automated devices. Some studies reported a tendency for increased activity as measured by increased total session activity, decreased intersession habituation, and/or reduced linear trend (slope) of the habituation curve. However, as discussed next, the weight of evidence does not support a consistent effect at lower exposure levels (i.e., <5 mg/kg) following gestational, lactational, or direct postnatal exposures to the pup.

Gestational exposures with or without postnatal exposures Maurissen et al. (2000) reported a decrease in automated motor activity at PND 13 and increases at PND 17, 21, and 60 in rats following gestational and lactational exposures to 5 mg/kg, but not at 1 or 0.3 mg/kg (Table 6A). None of these effects at 5 mg/kg were statistically significant. The lack of effect at lower doses is consistent with two oral mouse studies, in which no marked effects on observer-measured open-field activity (number of line crossing) were demonstrated following in utero exposure to 0.2–6 mg/kg (Braquenier et al. 2010; Venerosi et al. 2009) (Table 6C).

In contrast, Ricceri et al. (2006) found increases in frequency of crossing in an open field at PND 70 in offspring of dams dosed orally with CPF during gestation. In this study, mice were dosed with nine possible combinations of gestational gavage exposures to dams (0, 3, or 6 mg/kg-d) and postnatal sc (peanut oil) injection to pups (0, 1, or 3 mg/kg-d) were tested (Table 6C). Specifically, prenatal exposure to 3 mg/kg-d in combination with postnatal sc exposure to 3 mg/kg, but not 1 mg/kg or saline, produced an elevation in activity during the first 5 min of the 20-min session (Table 6C: 3/3 dose group). In addition, elevations in activity were observed in mice exposed in utero to 6 mg/kg-d and postnatally to vehicle or 1 mg/kg-d (Table 6C: 6/0 and 6/1 dose group), but not in mice exposed in utero to 6 mg/kg-d and postnatally to 3 mg/kg-d (Table 6C: 6/3 dose group). Ricceri et al. (2006) postulated that the higher postnatal dose of 6 mg/kg-d offset the effects of prenatal doses.

Icenogle et al. (2004) and Levin et al. (2002) dosed rat dams with 1 or 5 mg/kg CPF sc in DMSO during early (GD 9–12) or later (GD 17–20) gestation, and reported effects in opposite directions (Table 6B). Icenogle et al. (2004) noted greater habituation at 5 but not at 1 mg/kg-d, and significant decrease in activity in 2 of 12 time blocks toward the end of the session. This resulted in a higher linear trend (faster rate of decrease) in males and females at 5 mg/kg-d. In contrast, Levin et al. (2002) showed less habituation at 1 and 5 mg/kg, as measured by a lower linear trend (slower rate of decrease) in females only.

Caution is needed in evaluating data converted into linear trend values, which is defined in the figure legends as the "slope of decrease in activity over consecutive 5-min blocks" (Icenogle et al. 2004; Levin et al. 2002). The linear trend analysis is based on fitting linear functions to nonlinear habituation curves with insufficient information provided on goodness of fit to indicate how well they represent the actual data. Based on evaluation of the actual habituation data, there were no marked effects on habituation at 1 mg/kg-d following GD 9–12 exposures (Icenogle et al. 2004, Figure 2), nor at 1 or 5 mg/kg-d following GD 17–20 exposures (Levin et al. 2002, Figure 2). The linear trend values for the CPF groups were approximately 1.2 (GD 17–20, 1 and 5 mg/kg) and 1.6 (GD 9–12, 5 mg/kg). These values are within control linear trend values ranging from 1.2 to 1.6 for three studies published by the same laboratory (Levin et al. 2001 2002; Icenogle et al. 2004).

One dermal study conducted by the Abou-Donia et al. (2006) measured effects of CPF

TABLE 6A. Motor activity in the RAT (oral corn oil)

Author	Test	Age Exposure	Age Testing	Sample Size Per Dose Level (Yes/No litter is unit)	Measure*	Magnitude of Dose Tested, mg/kg-day					
						0.3	1	3	5	3, 6[a]	3,6,12[a]
Maurissen et al., 2000	Home Cage Activity (1 hour) Automated Time of testing balanced across dose level and sex	GD6-PND10 **Oral corn oil**	PND 13	N = 20 litters; 1 pup/sex/litter Yes, litter is unit, Litter was a factor in the statistical analysis	Total Activity	0[b]	0		(↓)[c]		
					Habituation	0	0		0		
			PND 17		Total Activity	0	0		(↑)		
					Habituation	0	0		0		
			PND 21		Total Activity	0	0		(↑)		
					Habituation	0	0		0		
			PND 60		Total Activity	0	0		(↑)		
					Habituation	0	0		0		
Carr et al., 2001	Open Field (3 min for PND 10,12; 6 min for PND 14–20) Video camera, measures from 2 technicians averaged for each rat; blind and semi-balanced	PND 1–21 (pups dosed every other day) **Oral corn oil**	PND 10, 12, 14, 16, 18, 20	2 males and 2 females per litter from 5 litters Yes, litter is unit, Litter was a factor in the statistical analysis	Line crosses (front 2 paws)			M0 F0 for each of 6 ages		M0 F0 for each of 6 ages	M0 F0 for each of 6 ages
			PND 25					M0 F0		M↓ F↓	M↓ F↓
			PND 30					M0 F0		M↓ F↓	M↓ F↓

[a]Carr 2001 dosed animals with incrementally higher doses as animals got older
[b]All entries without "M" and/or "F" indicates analyses is based on males and females combined.
[c]( ) indicates changes reported by the authors were not statistically significant
* If statistical analyses or measures were described in methods, then it was included and listed as "0" if not reported to be statistically significant

TABLE 6B. Motor activity in the RAT (sc DMSO)

Author	Test	Age and route of Exposure	Age Testing	Sample Size per Dose Level (Yes/No Litter is Unit)	Measure*	Magnitude of Dose Tested, mg/kg-day	
						1	5
Icenogle et al., 2004	Figure-8 maze (1 hour with 12 5-min blocks) Automated	GD 9–12 **sc DMSO**	Wk 4–8	10 M +10 F; 10 litters (1 rat /sex/ reconstituted litter) Litter of origin is not unit due to randomization of pups to dams at birth.	Habituation[a] (CPF x Block)	0	↑ (↓ activity time block 9, 11of 12)
					Linear trend of habituation[b],	0	↑ slope
					Quadratic trend of habituation*	0	0
Levin et al., 2002	Figure-8 maze (1 hour with 12 5-min blocks) Automated	GD 17–20 **sc DMSO**	Wk 4–6	10 M +10 F; 10 litters (1 rat /sex/ reconstituted litter) Litter of origin is not unit due to randomization of pups to dams at birth.	Mean activity	0	0
					Habituation (CPF x Block)	M0 F(△)[d]	M0 F(△)[d]
					Linear trend of habituation	M0 F↓ slope	M0 F↓ slope
					Quadratic trend of habituation*	0	0
Levin et al., 2001	Figure-8 maze (1 hour with 12 5-min blocks) Automated	PND 1–4 (initial pooled analysis with PND 11–14, see below) **sc DMSO**	Wk 4–6	10 M + 10 F; on average there was 1 rat/sex/litter Litter is not unit for initial pooled analysis combining M & F	Mean activity	0	
					Habituation (CPF x Block)	0	
Levin et al., 2001	Figure-8 maze (1 hour with 12 5-min blocks) Automated	PND 11–14 (initial pooled analysis with PND 1–4; see above) **sc DMSO**	Wk 4–6	10 M + 10 F; on average there was 1 rat/sex/litter Litter is not unit for initial pooled analysis combining M & F	Mean activity		0
					Habituation (CPF x Block)		Interaction not reported
					Linear trend of habituation		↓ slope

(*Continued*)

TABLE 6B. Continued

Author	Test	Age and route of Exposure	Age Testing	Sample Size per Dose Level (Yes/No Litter is Unit)	Measure*	Magnitude of Dose Tested, mg/kg-day	
						1	5
Dam et al., 2000	Open field (5-minute) Videotaped and blind to treatment	PND 1–4 **sc DMSO**	PND 21	5–7 pups/sex from unstated number of litters (Methods state 2 pups/sex/litter) Litter is not unit	Number of squares crossed	M↓F0	
					Rearing	M↓F0	
					Self-grooming	M0 F0	
			PND 30	Same as for PND 21	Number of squares crossed	M↓F0	
					Rearing	M↓F0	
					Self-grooming	M0 F0	
Dam et al., 2000	Open field (5-minute) Videotaped and Blind to treatment	PND 11–14 **sc DMSO**	PND 21	5–7 pups/sex from unstated number of litters (Methods state 2 pups/sex/litter) Litter is not unit	Number of squares crossed		M0 F0
					Rearing		M0 F0
					Self-grooming		M0 F0
			PND 30	Same as for PND 21	Number of squares crossed		M0 F0
					Rearing Self-grooming		M↑ F0 M0 F0

[a]Habituation results reported for specific dose level if authors indicated statistically significant post-hoc intersession differences following significant dose x time interaction for the habituation curve. Otherwise, see footnote "d"

[b]Linear trend of habituation was described by authors as "slope of decrease in activity over consecutive 5-minute block" so that ↑ slope could indicate less habituation or increased activity towards end of session.

[d]△ indicates a significant interaction between dose and 5-min intervals, but no post-hoc group differences reported.

*If statistical analyses or measures were described in methods, then it was included and listed as "0" if not reported to be statistically significant

TABLE 6C. Motor activity in the MOUSE (oral or sc)

Author	Test	Age and Route of Exposure	Age Testing	Sample Size per Dose Level (Yes/No Litter is Unit)	Measure*	Magnitude of Dose Tested, mg/kg-day					
Dose to dams						0.2	1		5		
Braquenier et al., 2009	Open field # line crossings (5-min daily testing for 8 days) Not blind	GD15-PND14 **ORAL Corn oil**	Not stated, after weaning Yes, litter is unit	9–10 F; 1 female pup per litter	Mean activity over 8 days	F0	F0		F0		
					Habituation over 8 days	F0	F0		F0		
Dose to dams									6		
Venerosi et al., 2009	Open field (5-minutes) duration(d) & frequency (f) analyzed Blind	GD 14–17 **ORAL Peanut oil**	PND 12	1 pup/sex/litter; 13 litters control and 14 litters treated Yes; litter was factor in analysis	Crossing				0		
					Immobility				↑d & f[a]		
					Head moving				0		
					Wall climbing				0		
					Pivoting				↓d & f		
					Self-grooming				0		
Dosed dams GD 15–18 (gavage) with peanut oil, 3 or 6 mg/kg and dosed pups PND 11–14 (sc) with vehicle, 1 or 3 mg/kg: First number indicates dose during gestation/second number indicates postnatal dose						3/0	3/1	3/3	6/0	6/1	6/3
Ricceri et al., 2006	Open field (20-minute divided in 4 5-min blocks) Video duration(d) & frequency (f) analyzed Not blind	GD15–18 **ORAL Peanut oil** & PND 11–14 SC Peanut oil	PND 70	10 males from unstated number of litters Yes; litter was factor in analysis	Crossing	M0	M0	M↑f first 5 min	M↑f	M0	M0
					Wall rearing	M0	M0	M0	M0	M0	M0
					Self-grooming	M0	M0	M0	M0	M0	M0
					Immobility	M0	M0	M0	M0	M0	M0

(*Continued*)

TABLE 6C. Continued

Author	Test	Age and Route of Exposure	Age Testing	Sample Size per Dose Level (Yes/No Litter is Unit)	Measure*	Magnitude of Dose Tested, mg/kg-day					
Dosed dams GD 15–18(oral) with peanut oil and dosed pups PND 11–14 (sc) with vehicle, 1 or 3 mg/kg: First number indicates dose during gestation/second number indicates postnatal dose							0/1	0/3			
Ricceri et al., 2006	Open field (20 minutes divided into 4 5-min blocks) Video, Not blind duration(d) & frequency (f) analyzed	PD 11–14 **SC peanut oil** (with oral peanut oil GD 15–18)	PD 70	10 males from unstated number of litters Yes; litter was factor in analysis	Crossing		M0	M↑f first 5 min			
					Wall rearing		M0	M0			
					Self-grooming		M0	M0			
					Immobility		M0	M0			
Dose to pups							1	3	6		
Ricceri et al., 2003	Automated square arena (20 minutes)	PND 1-4 **SC DMSO**	PND 25	Control 13–17 pups 1 mg/kg 8–14 pups 3 mg/kg 10–16 pups #litters/group not stated. Litter not unit of analysis	Locomotion (horizontal)		0	0			
					Rearing (vertical) Time in central area		0 0	0 0			
					Time in peripheral area		0	0			
Ricceri et al., 2003	Automated square arena (20 minutes)	PND 11–14 **SC DMSO**	PND 25	Control 12–18 pups 1 mg/kg 15–18 pups 3 mg/kg 12–14 pups #litters/group not stated. Litter not unit of analysis	locomotion (horizontal)		(↑)[b]	(↑)[b]			
					Rearing (vertical)		0	0			
					Time in central area		0	0			
					Time in peripheral area		0	0			

[a]d=duration; f=frequency
[b]Post-hoc tests were conducted and were statistically significant, although main effect was not significant at p=0.06
*If statistical analyses or measures were described in methods, then it was included and listed as "0" if not reported to be statistically significant

on subjective measures of motor strength and coordination that are related, but not directly comparable to, tests of motor activity. This study exposed dams (5/dose group) dermally to 1 dose level of 1 mg/kg-d CPF in 70% ethanol and measured effects in 1 offspring/sex/litter when they were adults (PND 90) on subjective motor function tests that were conducted blind to treatment level. There were no significant effects on beam-walk time or beam walking ability. However, there was a reduction in rat's fore-paw grip time (gripping of a 5-mm-diameter wood dowel held horizontally) in both males and females, and a decrease in females but not males in the angle at which the animal began to slip backward as the incline plane was raised from an initial horizontal position. Significant increases in female brain AChE were measured at PND 90. AChE was not measured at or soon after the time of exposure. This study is limited by testing only one dose level with a low number of animals. Therefore, these results need to be considered preliminary results from a pilot study that needs to be repeated using a larger sample size, more dose levels, and more objective measures of motor function and strength.

In summary, the overall weight of evidence suggests that there are no marked effects on motor activity following oral or sc gestational exposure to 1 mg/kg-d in rats or mice. Effects reported at 5 or 6 mg/kg include both increases and decreases in activity (Table 6). One dermal study that did not meet our standards for sample size (<6 litters) and number of dose levels (<2) reported effects on motor function at 1 mg/kg-d CPF. As described in detail earlier, these differences may be related to differences in species, route of exposure, vehicle, and duration and type of activity measures.

Postnatal exposures Carr et al. (2001) dosed pups every other day from PND 1 to 21 in a small-scale gavage study (10 males and 10 females from only 5 litters per dose level; Table 6A). Although the sample size of five litters did not meet our standards, this study included three dose levels and measured activity at several ages, allowing for evaluation of dose-response and temporal patterns. There was a consistent dose-related decrease in open-field activity in offspring on d 25 and 30, but not at earlier ages. There were no marked effects on motor activity at 3 mg/kg, but decreases in activity following staggered dose regimen (every other day administration) of 3 increasing to 6, and 3 to 6 to 12 mg/kg-d.

In contrast, with sc CPF doses in DMSO to pups, Levin et al. (2001) reported a statistically significant decrease in linear trend of the habituation data at 5 mg/kg (PND 11–14) but not at 1 mg/kg (PND 1–4), which was interpreted as a reduction in habituation consistent with an increase in activity (Table 6B). However, there was no statistically significant main effect of CPF, and the statistics for the CPF × Block interaction and post hoc tests for each time block were not reported. In comparing the CPF and control habituation curves, the means and standard errors overlapped for all but 2 (3rd and 12th) of twelve 5-min intersession time blocks. Thus, there was no marked effect of CPF on the pattern of habituation, and the statistically significant decrease in linear slope function used to represent the scalloped-shaped habituation curves is not considered an adverse effect.

Studies by Ricceri et al. (2003; 2006) suggest that CPF increases activity in mice following postnatal sc exposures (Table 6C). Ricceri et al. (2003) reported a rise in total distance traveled at PND 25 following sc injections of 1 and 3 mg/kg-d CPF in DMSO at PND 11–14 but not PND 1–4 exposures. The statistical significance was based on analyses that considered the individual pup and not the litter as the experimental unit, and was based on "post hoc" *t*-tests that were conducted despite lack of significant main effect on the ANOVA (Ricceri et al. 2003). In a subsequent study, Ricceri et al. (2006) injected mice from PND 11 to 14 with 1 and 3 mg/kg-d CPF in peanut oil. There was greater frequency of crossing on PND 70 following PND 11–14 exposure to 3 but not 1 mg/kg-d (litter was considered the experimental unit of analysis). Thus, the increase in activity following sc injections

of 1 mg/kg-d in DMSO was not replicated, although this may be due to differences in vehicle, age of testing, activity device, and statistical methods.

In summary, postnatal exposures to CPF dose levels above 3 mg/kg-d produced increases or decreases in activity depending on the species, method of measuring activity, and route of exposure. There were no consistent patterns of treatment-related effects on motor activity following postnatal doses below 3 mg/kg-d.

Novelty-Induced Activity, Plus Maze and Chocolate Milk Consumption Overall, there were no consistent patterns of effects on novelty-induced activity (Table 7) and behavior, or on activity in open arms and center area, which is considered by some investigators to be correlated with effects on "anxiety"-like or "depressive" behavior.

Gestational exposures with or without postnatal exposures Braquenier et al. (2010) reported an increase in **"**anxiety**"** in female mouse offspring (male mice were not tested) after oral exposures of dams during gestation and lactation to 1 mg/kg, but not to 0.2 or 5 mg/kg (Table 7A), indicating lack of dose response relationship. Specifically, oral doses of 1 mg/kg decreased the time spent in the open arm of the elevated plus maze, without effects on total number of arm entries (considered by authors to reflect locomotor activity). In addition, oral exposure of CPF decreased time in the center of the light side of the dark/light box at 1 but not 5 mg/kg-d (Braquenier et al. 2010).

In contrast, Ricceri et al. (2006) found no marked effects on time in open or closed arm in male and female mice dosed orally in utero to 3 or 6 mg/kg (Table 7C), and found a decrease in the number of head-dips in males only at 3 but not 6 mg/kg-d. Icenogle et al. (2004) also demonstrated no significant change in the time spent in the open arm versus the closed arm in rat offspring exposed in utero from GD 9 to 12 to sc injections of 1 or 5 mg/kg-d CPF in DMSO (Table 7B).

The conflicting results may be due to differences in duration of exposure and age of testing or species. Nevertheless, these studies do not support a consistent dose-related pattern of effect on activity level on plus maze or on behaviors potentially related to "anxiety."

Postnatal exposures Ricceri et al. (2006) reported a statistically significant increase in time spent in the open arms in female mice injected sc with 3, but not 1 mg/kg-d (Table 7C). There were no marked effects in male mice, and there were no effects on number of center crosses following sc injections of 1 or 3 mg/kg from PND 11 to 14 (Table 7C). Ricceri et al. (2006) considered these findings as a reduction in **"**anxiety responses**"** consistent with sex-selective effects reported in rats by Aldridge et al. (2005).

Aldridge et al. (2005a) reported that PND 1–4 sc exposure to CPF increased time in open arms and number of center crosses in male but not female rats at 1 mg/kg (Table 7B), the only dose level tested. Although "sex-selective," the effects were in the opposite sex from that reported by Ricceri et al. (2006). Furthermore, the males in the 1-mg/kg-d CPF treated group from the Aldridge et al. (2005a) study spent approximately 17% of time in open arms, which is comparable to control animals from 2 other studies reported by this laboratory using identical procedures and age of testing (PND 50–56). Specifically, Roegge et al. (2008) found that control males and females spent approximately 21 and 23% time in the open field, respectively. Timofeeva et al. (2008) observed approximately 16% of time in open arms for males and females combined. In contrast, Aldridge et al. (2005a) reported that the concurrent control males and females spent, respectively, 3 and 20% of time in open arms. The control data from all three studies indicate that there are no consistent sex-specific differences between male and female control rats, and a wide range of control male values.

In general, the statistically significant measures of automated novelty test resulted in no marked effects on novelty preference following sc injections of 3 mg/kg (1 mg/kg not tested) for PND 1–4 or PND 11–14 (Ricceri et al. 2003; Table 7C). The only effects were statistically significant increases in activity at

TABLE 7A. Novelty/plus maze in the MOUSE (oral corn oil)

Author	Test	Age and Route of Exposure	Age Testing	Sample Size Per Dose Level (Yes/No litter is unit)	Measure*	Magnitude of Dose Tested, mg/kg-day		
						0.2	**1**	**5**
Braquenier et al., 2009	Elevated plus maze (5 min) Blind review of video sequences	GD 15–PND 14 Oral Corn Oil	PND 80	9–10 F; 1 female pup per litter yes, litter is unit	Time spent in open versus closed arm	0	(↓)[a] ANOVA p=0.10; Dunnett p<0.02	0
					Number of entries in open arms	0	↓	0
	Dark/Light (5 min) Not blind		PND 72	9–10 F; 1 female pup per litter yes, litter is unit	% in Dark relative to total time	0	0	0
					% in Center of light relative to time in light	0	↓	0
					# crossings between compartments	0	(↓)[a] ANOVA p=0.08; Dunnett p<0.02	0

[a](↓) overall ANOVA not statistically significant, but post-hoc tests were statistically significant.
*If statistical analyses or measures were described in methods, it was included and listed as "0" if not reported to be statistically significant.

TABLE 7B. Novelty/plus maze in the RAT (sc DMSO)

Author	Test	Age and Route of Exposure	Age Testing	Sample Size Per Dose Level (Yes/No litter is unit)	Measure*	Magnitude of Dose Tested, mg/kg-day	
						1	**5**
Icenogle et al., 2004	Elevated plus maze Not blind	GD 9–12 sc DMSO	Not stated	10 M +10 F; 10 litters (1 rat /sex/ reconstituted litter)	Time spent in open versus closed arm	0	0
				Litter of origin is not unit due to randomization of pups to dams at birth.	Center crossing	0	↑
Aldridge et al., 2005a	Elevated plus maze Not blind	PND 1–4 sc DMSO	PND 52–53 (Wk 7)	1 pup/sex/ litter; 9 litters	Open arm time	M↑ F0 (reduced "anxiety")	
				Litter is not unit for initial pooled analysis combining M & F	Center crossing	M↑ F0	

*If statistical analyses or measures were described in methods, it was included and listed as "0" if not reported to be statistically significant.

TABLE 7C. Novelty and plus maze in the MOUSE

Author	Test	Age and Route of Exposure	Age Testing	Sample Size Per Dose Level (Yes/No litter is unit)	Measure*	Magnitude of Dose Tested, mg/kg-day		
Dosed GD15–18 with 3 or 6 mg/kg and Dosed PND 11–14 with vehicle: First number indicates dose during gestation to dams/second number indicates postnatal dose to pups							3/0	6/0
Ricceri et al., 2006	Elevated Plus Maze Not blind	**GD 15–18 gavage CPF in peanut oil to dams** PND 11–14 sc peanut oil (no CPF)	4 months	Not clear, assume 10 M and 10 F; yes, litter was factor in analyses	Total		0	0
					Center crossing		0	0
					Open arm		0	0
					Closed arm		0	0
					Head dipping		M↓ F0	M0 F0
					Stretch-attend		0	0
Dosed GD 15–18 with vehicle and Dosed PND 11–14 with vehicle, 1 or 3 mg/kg: First number indicates dose during gestation to dams/second number indicates postnatal dose to pups						0/1	0/3	
Ricceri et al., 2006	Elevated Plus Maze Not blind	GD 15–18 gavage peanut oil (no CPF) **PND 11–14 sc CPF in peanut oil**	PD 60	10 males from unstated number of litters yes, litter was factor in analyses	Total	0	0	
					Center crossing	0	0	
					Open arm	M0 (F↑) (reduced "anxiety")	M0 F↑ (reduced "anxiety")	
					Closed arm	0	0	
					Head dipping	0	0	
					Stretch-attend	0	0	

(Continued)

TABLE 7C. Continued

Author	Test	Age and Route of Exposure	Age Testing	Sample Size Per Dose Level (Yes/No litter is unit)	Measure*	Magnitude of Dose Tested, mg/kg-day		
Dose to pups						1	3	6
Ricceri et al., 2003	Automated novelty test with black and white chambers (25 minute test: one 5-min block familiar; four 5-min blocks partition open)	PND 1–4 sc DMSO	PND 35	Control 18 pups 1 mg/kg not tested 3 mg/kg 16 pups # litters/ dose group not stated litter not unit	Day 1 activity rate (25 min)		0	
					Day 4 activity familiar side		0	
					Day 4 activity partition open		↑(2nd 5-min block)	
					Time in white		0	
					Time in black		0	
					Activity rate white		0	
					Activity rate black		0	
					#passages/ minute		0	
					Latency to enter novel		0	
Ricceri et al., 2003	Automated novelty test with black and white chambers (25 minute test: one 5-min block familiar; four 5-min blocks partition open)	PND 11–14 sc DMSO	PND 35	Control 17 pups 1 mg/kg not tested 3 mg/kg 17 pups #litters/dose group not stated litter not unit	8 measures as described above for PND 1–4 in Ricceri 2003		0 for all 8 measures described above for PND 1–4	
					Day 4 activity partition open		↑ (4th 5-min block)	

*If statistical analyses or measures were described in methods, it was included and listed as "0" if not reported to be statistically significant

3 mg/kg-d (the only dose level tested) during one of the 5-min time bins after the mice were exposed to the novel environment. However, similar changes in motor activity were not seen, especially during the beginning of the sessions, when the activity device is a novel environment to the animals (Table 6).

Aldridge et al. (2005a) reported effects of PND 1–4 sc injection of 1 mg/kg-d CPF on the consumption of chocolate milk compared to water in both male and female rats tested at PND 54. The control male and female rats in this study clearly demonstrated a preference for chocolate milk compared to water with chocolate milk to water consumption ratios of approximately 4.5 and 5.3, respectively. The CPF-treated male and female rats had chocolate milk–water preference ratios of approximately 3.5, which was lower than for the concurrent control but higher than for controls from other studies by this laboratory (all non-CPF studies) reporting milk–water preference ratios of approximately 1.2–1.75 (Roegge et al. 2008; Timofeeva et al. 2008). The lack of dose-response data and wide range of control values indicate that this finding is not appropriate for risk assessment purposes.

The apparent CPF effect on chocolate milk consumption and increased time spent in the open arm was described as "changes in 5HT-related behaviors that resemble animal models of depression," citing papers using an olfactory bulbectomized (OB) animal model of depression (Aldridge et al. 2005a). However, Kelly et al. (1997) and Mar et al. (2000) caution that the data generated using the OB model cannot be attributed to any particular neurotransmitter system. In addition, Slotkin et al. (1999) previously found no effects on chocolate milk consumption in young adult OB rats (13 wk old), an age commonly used for this model of depression (Kelly et al. 1997).

In summary, there are no consistent patterns of adverse effect on novelty-induced activity, plus maze (Table 7), and chocolate milk consumption at any dose level, especially at 1 mg/kg-d.

Spontaneous Alternation Spontaneous alternation (Table 8) was measured by Levin et al. (2001; 2002) and Icenogle et al. (2004) using a T-maze without any positive or negative reinforcement. There was a consistent lack of effect on spontaneous alternation, the primary endpoint for this test, following sc (DMSO) gestational exposures to the dam or postnatal exposures to the pups. Levin et al. (2002) found a 3- to 5-s decrease in latency in the first of 5 trials following gestational exposures to 1 and 5 mg/kg-d CPF. This effect was considered evidence of hyperactivity. However, there was no evidence of increased activity in the Figure-8 maze motor activity tests, as discussed previously. The biological significance of this decrease in 3- to 5-s latency is uncertain, and there was no consequence to the animals for the time it took to perform the test.

Learning and Memory

Gestation exposures with or without postnatal exposure Maurissen et al. (2000), Icenogle et al. (2004), and Levin et al. (2002) evaluated the effects of CPF gestational exposures on learning and memory of offspring (Table 9). Maurissen et al. (2000) dosed rat dams by gavage with corn oil throughout gestation and early lactation, and tested the same 1 pup/litter from 18 litters/dose group on a T-maze delayed spatial alternation test at weaning and as adults (Table 9A). There were no significant effects on learning or retention at dose levels of 0.3, 1, or 5 mg/kg-d at PND 22–24 or on long-term memory and retention at PND 61–90. Icenogle et al. (2004) and Levin et al. (2002) injected dams with 1 and 5 mg CPF/kg-d sc in DMSO and tested the offspring using a radial arm maze (RAM), with 12 of 16 arms baited to assess working memory and 4 arms unbaited to test reference memory (Table 9B).

Icenogle et al. (2004) reported increased errors at 5 but not 1 mg/kg-d following exposures to dams from GD 9 to 12 (Table 9B). The increased number of errors at 5 mg/kg-d occurred in the first and third block of trials out of 6 for working memory, and only in the first block for reference memory. In contrast, Levin et al. (2002) found that GD 17–20 exposures to dams resulted in increased working and reference memory errors in female offspring at 1 but

TABLE 8. Spontaneous alternation in the RAT (s.c. DMSO)

Author	Test	Age and Route of Exposure	Age Testing	Sample Size Per Dose Level (Yes/No litter is unit)	Measure	Magnitude of Dose Tested, mg/kg-day	
						1	5
Icenogle et al., 2004	Spontaneous Alternation T-maze Not blind	GD 9–12 **s.c. DMSO**	Wk 4–8	10 M +10 F; 10 litters	Spontaneous alternation	0	0
				Litter is not unit Litter of origin is not known due to randomization of pups to dams at birth	Latency	↓ Trial 1 of 5 (3 sec)	↓ Trial 1 of 5 (5 sec)
Levin et al., 2002	Spontaneous Alternation T-maze Not blind	GD 17–20 **s.c. DMSO**	Wk 4–6	10 M +10 F; 10 litters	Spontaneous alternation	0	0
				Litter is not unit Litter of origin is not known due to randomization of pups to dams at birth	Latency	↓ Trial 1 of 5	↓ Trial 1 and 2 of 5
Levin et al., 2001	Spontaneous Alternation T-maze Not blind	PND 1–4 and PND 11–14 **s.c. DMSO**	Wk 8–13	10 M + 10 F; "on average there was 1 rat/sex/litter"	Spontaneous alternation	0 (PND 1–4)	0 (PND 11–14)
		analysis for both periods pooled together because no dose × period interaction		Litter is not unit for initial pooled analysis combining M & F	Latency Data pooled together in different ways	1 and 5 mg/kg-day ↑ 3 sec / trial (average) both sexes ↑ latency during 3rd session in males (1 mg/kg-day PND 1–4 data pooled together with 5 mg/kg-day PND 11–14, males and females pooled together and also analyzed for each sex separately	

TABLE 9A. Learning and memory (rat oral corn oil)

Author	Test	Age and Route of Exposure	Age Testing	Sample Size per Dose Level (Yes/No litter is unit)	Measure*	Magnitude of Dose Tested mg/kg-day			
						0.3	1	3	5
Maurissen et al., 2000	Spatial delayed alternation	GD 6–PND10	PND 22–24	N=16 litters; 1 male OR 1 female pup per litter from 16 litters	% correct	0 [b]	0		0
			[a]85% free-feeding weight		acquisition	0	0		0
					retention	0	0		0
	Blind	**Oral, corn oil**	PND 61–90	Yes, litter is unit	% correct	0	0		0
	No feed restrction		[a] 85% free-feeding bodyweight		acquisition	0	0		0
					retention	0	0		0
Incremental dose regimen							1	1, 2 & 4[c]	1.5, 3 & 6[d]
Johnson et al., 2009	Radial arm maze	PND1–21	PND36–60	Within-litter design for methyl-parathion and chlorpyrifos doses	Working errors analyzed by week		M↑[e] F0	M↑[e] F0	M↑ F0
					Working error overall		M0F0	M0F0	M↑ F0
	Not Blind	**Oral, corn oil**	[a]gradual 95–90—80% of feed	Yes, litter is unit, N=10–14 rats/sex/dose group	Reference errors analyzed by week		M0 F0	M0 F↓	M↑ F↓
	Gradual feed reduction (95%-90% to 80% over 4 weeks				Reference errors cumulative		M0 F0	M0 F↓	M↑ F↓
					Response time (seconds per entry)		0	0	0

[a]Animals were feed restricted based on free-feeding weight or amount of feed
[b]Entries without "M" and/or "F" indicate analyses was based on pooling data for males and females
[c]Incremental dosing regimen from 1.0 (PND1–5) to 2.0 (PND6–13) to 4.0 (PND14–20) mg/kg-day
[d]Incremental dosing regimen from 1.5 (PND1–5) to 3.0 (PND6–13) to 6.0 (PND14–20) mg/kg-day
[e]Increase errors (3.2 vs. 2.7 in controls approximated from graph) during 4th week but not first 3 weeks. No overall effect on total working errors at low and mid dose
*If statistical analyses or measures were described in methods, it was included and listed as "0" if not reported to be statistically significant

TABLE 9B. Learning and memory (rat sc DMSO or peanut oil)

Author	Test	Age and Route of Exposure	Age Testing	Sample Size Per Dose Level (Yes/No litter is unit)	Measure*	Magnitude of Dose Tested, mg/kg-day			
						0.3	1	5	7
Icenogle et al., 2004	Radial Arm Maze	GD 9–12	Wk 8–13	10 M +10 F; 10 litters	Working error		0[b]	↑[b] (trial block1 & 3 of 6)	
	Not blind	**sc DMSO**	85% feed[a]	Litter of origin is not unit due to randomization of pups to dams at birth	Reference error		0	↑ (trial block 1 of 6)	
Levin et al., 2002	Radial Arm Maze	GD 17–20	Wk 8–13	10 M +10 F; 10 litters	Working error		M0 F↑	M0 F0	
	Not blind	**sc DMSO**	85% feed[a]	Litter of origin is not unit due to randomization of pups to dams at birth	Reference error		M0 F↑	M0 F0	
Aldridge et al., 2005a	Radial Arm Maze	PND1–4	Wk 9–14	1 pup/sex/ litter; 9 litters	Working error		M0 F↓		
	Blind	**sc DMSO**	15 g/day[a]	Litter is not unit for initial pooled analysis combining M & F	Reference error		M↑ F↓		
Levin et al., 2001	Radial Arm Maze	PND 1–4	Wk 8–13	10 M + 10 F; "on average there was 1 rat/sex/litter"	Working error		M↑(block1 of 6) F↓		
	Not blind	**sc DMSO** (pooled analysis with PND 11–14)	85% feed[a]	Litter is not unit for initial pooled analysis combining M & F[c]	Reference error		M↑(block1 of 6) F↓		

(*Continued*)

TABLE 9B. (Continued)

Author	Test	Age and Route of Exposure	Age Testing	Sample Size Per Dose Level (Yes/No litter is unit)	Measure*	Magnitude of Dose Tested, mg/kg-day			
						0.3	1	5	7
Levin et al., 2001	Radial Arm Maze	PND 11–14	Wk 8-13	10 M + 10 F; "on average there was 1 rat/sex/litter"	Working error			0	
	Not blind	**sc DMSO** (pooled analysis with PND 1–4)	85% feed[a]	Litter is not unit for initial pooled analysis combining M & F[c]	Reference error			0	
Jett et al., 2001	Morris Water Maze	PN 7,11,15	PND 24–28	10M and 9–10F from 2–4 litters	Escape latency	↑[b] day1 only			↑
	Not blind	**sc peanut oil every 4 days**		litter is not unit	Probe time	0			↓ (less time in correct quad)
Jett et al., 2001	Morris Water Maze latency	PND 22,26	PND 24–28	4M + 3-4F from 2–4 litters	Escape latency	↑			↑ magnitude of change same as low dose
	Not blind	**sc peanut oil**		offspring dosed after weaning	Probe time	↓			↑ magnitude of change same as low dose

[a]Animals were feed restricted

[b]Entries without "M" and/or "F" indicate analyses was based on males and females pooled together in a single analyses because there was no significant interaction between dose and sex.

[c]Pups dosed PND 1–4 1 mg/kg-day were combined with PND 11–14 5 mg/kg-day and males combined with females in the initial pooled analysis.

*If statistical analyses or measures were described in methods, it was included and listed as "0" if not reported to be statistically significant

TABLE 9C. Learning and memory (mouse sc DMSO)

Author	Test	Age Exposure	Age Testing	Sample Size Per Dose Level Yes/No litter is unit)	Measure*	1	3	5	10
Billauer-Haimovitch et al., 2009	Morris Maze Video monitored, not blind	GD9-18 **sc DMSO**	PD 75	1 pup/sex/litter; 7–17 litters/group Litter is not unit. Half pups cross-fostered at birth; males and females from same litter pooled together; litter was not a factor in the analysis	Latency	↑[a,b] (deficit)	↑[a] (deficit)	0[a]	0[a]
Haviland et al., 2010	Radial Arm Maze Not blind	GD17-20 **sc DMSO**	PD 60 Mice were fasted	8 litters/dose group Yes, litter is unit # pups / litter was not reported	Working error	0		0	
					Reference error	0		0	
	Foraging Maze Not blind	GD17-20 **sc DMSO**	PD 60	8 litters/dose group Yes, litter is unit # pups / litter was not reported	Food recognition: Food pieces found per reward baited arm	M↑[c] F↓ (male improve, female deficit)		M0[c] F↓ (female deficit)	
					Food Position : # correct looks left where bait is per total looks in baited arm	M0[c] F↓ (female deficit)		M0[c] F↓ (female deficit)	
					Foraging activity	M0 F0		M0 F0	
Ricceri et al., 2003	Passive Avoidance Not blind	PD 1–4 **sc DMSO**	PD 60	8 or 9 males with at least 1 male/litter; total 36 litters for 4 groups Pup and not litter was the unit	Trials to criterion	M0	M0		
					Latency to step through	M0	M0		
					24 hr. retention	M0	M0		
Ricceri et al., 2003	Passive Avoidance No blind	PD 11–14 **sc DMSO**	PD 60	7–10 males with at least 1 male/litter; total 36 litters for 4 groups Pup and not litter was the unit	Trials to criterion	M0	M0		
					Latency to step through	M0	M0		
					24 hr. retention	M0	M0		

[a]Entries without "M" and/or "F" indicate analyses was based on males and females pooled together in a single analyses because there was no significant interaction between dose and sex.
[b]Inspection of data indicates significant overlap of learning curve with controls
[c]Increase (↑) means improvement in learning compared to controls. Decrease (↓) means deficit or delay in learning compared to controls
*If statistical analyses or measures were described in methods,it was included and listed as "0" if not reported to be statistically significant

not 5 mg/kg-d, and considered these findings to be "sex-selective effects on behavioral development." Levin et al. (2002) speculated that, at a higher dose level of 5 mg/kg, "cholinergic actions may serve to offset adverse, non-cholinergic effects on brain development."

Another possibility not discussed by Levin et al. (2002) is that the concurrent control females could have an unusually low number of errors based on comparison with two other studies from the same laboratory (Aldridge et al. 2005a; Levin et al. 2001). In these two other studies, Levin et al. (2001) and Aldridge et al. (2005a) reported that control females normally have higher errors than males, and that in these studies CPF disrupted the normal sex differences in activity so that males and females had the same activity level. In fact, the higher number of female errors compared to male errors at the 1 mg/kg-d dose level in Levin et al. (2002) is consistent with the sex differences in control animals observed in these two other studies from the same laboratory (Levin et al. 2001; Aldridge et al. 2005a). Thus, the non-monotonic rise in error in the 1-mg/kg-d GD 17–20 females in Levin et al. (2002) may be more a reflection of variability in control female behavior than an effect of CPF.

Billauer-Haimovitch et al. (2009) used a Morris Water Maze to study effects of sc injection of CPF to mice during GD 9–18 (Table 9C). Statistical significant increases in latency were measured at 1 and 3 mg/kg-d but not at 5 or 10 mg/kg-d. Billiauer-Haimovitch et al. (2009) indicated that there was an overall significant CPF effect, based on ANOVA, which was "individually significant at 1 and 3 mg/kg-d." However, the results of the Tukey tests for post hoc analysis mentioned in the methods section were not reported. Based on inspection of the figure, there was overlap of the learning curves for the controls, 1, 5, and 10 mg/kg-d for the first 3 d, with the largest difference between the controls and the 1-mg/kg-d group on the fourth and last day estimated to be 3–4 s. Thus, this study does not provide evidence of a treatment-related effect of CPF.

Haviland et al. (2010) tested mice on an 8-arm (6 baited, 2 unbaited) RAM and a "novel" foraging food maze test (modification of RAM) following sc injection of CPF at 1 or 5 mg/kg-d in DMSO from GD 17 to 20 (Table 9C). CPF did not affect learning on the traditional RAM, but these data may be unreliable because the controls did not learn on this maze. Based on the results of the "novel" maze test, CPF diminished foraging food recognition learning in females (dose-dependent effects at 1 and 5 mg/kg/d) and enhanced foraging learning in males (1 but not 5 mg/kg-d) (Table 9C). Haviland et al. (2010) provided inadequate information on methods (Table 9c). The statistical analysis of the behavior was described as a "mixed procedure accounting for autocorrelation of repeated measures." If this refers to the repeated-measures ANOVA as recommended by Holson et al. (2008), the statistical significances for the main effect and interaction were not provided. Given the limitations of this study and the novelty of the test procedure, this study provides preliminary evidence of effect of in utero exposure on learning in female but not male offspring.

In summary, there are mixed results on learning and memory tests following CPF exposures during gestation. The differences could be due to differences in species, route of exposure, vehicle, age of exposure, time of testing, and the behavioral test. However, based on detailed evaluation of the methods and results, examination of dose-response relationships, and consideration of historical control data, the overall weight of evidence does not support a consistent pattern of effects on learning and memory across studies at 1 mg/kg-d.

Postnatal exposures Aldridge et al. (2005a) and Levin et al. (2001) reported that performance on the RAM in females improved, and males exerted either no effect or a deficit in performance following sc injection of 1 mg/kg CPF in DMSO to pups from PND 1–4 (Table 9B). Thus, a decrease in female error rate (improved performance) was replicated in two separate studies by the same laboratory and was considered to be evidence that CPF disrupts sexually dimorphic behaviors by improving female performance to be comparable to that of the males (Aldridge et al. 2005a; Levin et al. 2001). No marked

effects were reported following sc injection of 5 mg/kg CPF in DMSO from PND 11 to 14 (Levin et al. 2001).

Johnson et al. (2009) dosed CPF daily with incrementally increasing oral doses from PND1 to 21 (Table 9A). The lowest dose level was 1 mg/kg from PND1 to 21, the mid-dose level incrementally elevated doses from 1 to 2 to 4 mg/kg, and the highest dose level incrementally rising doses from 1.5 to 3 to 6 mg/kg. Beginning on PND 36, working and reference memory were tested using a radial maze. In females, decreased errors (improved performance) were observed in reference memory at the mid- and high-dose levels, but there were no marked effects on working memory. In males, there were increased overall errors in working and reference memory at the highest dose. Johnson et al. (2009) reported statistically significant increases in working memory errors during the fourth week but not in the first 3 wk of testing at the low and mid doses. These effects do not appear to be adverse effects because of the small differences in number of errors (3.2 [low] to 3.3 [mid] errors vs. 2.7 [control] errors), and the lack of a consistent dose-response pattern over the 4-wk period. Statistically significant hippocampal AChE inhibition was measured in all treatment groups at PND 20. AChE inhibition persisted in the mid- and high-dose groups for up to 19 d following exposure.

An increase in escape latency (deficit in performance) was measured in rats (males and females combined) tested from PND 24 to 28 by Jett et al. (2001) on the Morris Water Maze test following sc doses of 0.3 (d 1 only) and 7 mg/kg in peanut oil on PND 7, 11, and 15 (Table 9B). Although not meeting our inclusion criteria for studies because animals were dosed after weaning, a significant increase in latency was also measured from PND 24 to 28 following sc doses of 0.3 and 7 mg/kg on PND 22 and 26. This study was limited because pups from only two to four reconstituted litters/dose level were used, with all pups from the same litter dosed with the same dose level. Finally, only 60% of control animals were able to meet the criterion on this test. It is notable that AChE levels were measured 3 h after injections, but no brain AChE inhibition was measured at 7 mg/kg, a dose level that should have resulted in significant AChE inhibition. Taking all these factors into consideration, this study is not sufficiently reliable for risk assessment purposes.

Ricceri et al. (2003) tested effects of postnatal sc injections of CPF in DMSO to mice during PND 1–4 or PND 11–14 (Table 9C), and found no significant effects on passive avoidance at 1 or 3 mg/kg-d (data were not shown).

In summary, a decrease in errors in females (improvement in learning and memory) and a tendency toward increased errors in males were noted in two postnatal exposure studies following sc injection of 1 mg/kg-d CPF (only dose tested) (Aldridge et al. 2005a; Levin et al. 2001). Johnson et al. (2009) measured similar effects at higher oral dose levels (incremental oral doses spanning from 1 to 4 or 1 to 6 mg/kg-d) inducing 50% AChE inhibition in the hippocampus, but did not observe effects at 1 mg/kg.

Pharmacologic Challenge Pharmacologic challenge studies (Table 10) were conducted to determine whether CPF changes the involvement of underlying neurotransmitter systems that support different behaviors. Many of the studies were conducted in rats injected sc with CPF (Table 10B). CPF attenuated the effects of scopolamine (muscarinic antagonist) to elevate the number of errors on the RAM following gestational (Icenogle et al. 2004; Levin et al. 2002) or PND 11–14 exposures (Levin et al. 2001), but not PND 1–4 exposures (Table 10B; Levin et al. 2001). In comparing the effects of scopolamine across the different studies, it appears that the variability in the data is high, and scopolamine did not consistently produce dose-response increase in number of errors in controls (Levin et al. 2001, Fig 4; 2002, Figures 5, 6, and 7). Ketanserin elevated the number of errors on the RAM with increasing doses of ketanserin in animals treated with 1 mg/kg-d of CPF from PND 1–4 (Aldridge et al. 2005a). These same doses of ketanserin exerted no marked effect on saline-treated

TABLE 10A. Pharmacologic challenge in the MOUSE (oral peanut oil)

Author	Test	Age and Route of Exposure	Age Testing	Sample Size Per Dose Level of Challenge Drug (Yes/No litter is unit)	Challenge Drug	Measure*	Effect of Challenge Drug on Controls	Effect of CPF on the Effect of Challenge Drug Magnitude of CPF Dose 6 mg/kg-day
Venerosi et al., 2010	Forced Swimming Test Videotaped and blind Not balanced	GD 15–18 **Oral Peanut Oil**	adulthood	12M+6F from unstated number of litters Litter included as factor in analysis	Fluvoxamine (5HT uptake inhibitor) 30 mg/kg i.p.	Swimming frequency	M↑ F0	M↓ (Attenuate)
						Swimming duration	M↑ F↑	M↓ F↓ (Attenuate)
						Struggling frequency	M↑ F0	M↓ (Attenuate)
						Struggling duration	M↑ F0	M↓ (Attenuate)
						Floating duration	M↓ F(↓) p=0.07	M and F Attenuate
Venerosi et al., 2010	Maternal Aggression Male mouse placed in home cage with the dam and her empty nest Videotaped and blind frequency(f), duration (d), and latency (l) measured for each parameter	GD 15–18 **Oral Peanut Oil**	Females only, 8 days after delivery of pups	5–7 F from unstated number of litters Litter included as factor in analysis	Fluvoxamine (5HT uptake inhibitor) 30 mg/kg i.p.	Fighting behavior – attack (3 different cage areas) f,d,l	F↓ f and d for 2–3 cage areas	F attenuate duration of in-nest attack Note: CPF also ↓ baseline levels
						Social behavior – social sniffing (3 subtypes) f,d,l	F↑ duration inactivity in-nest	F attenuate duration inactivity in-nest
						Non-social behavior (3 subtypes) f,d,l	F0	No effect of fluvoxamine, but CPF increased basal levels in 1 of 3 subtypes

TABLE 10B. Pharmacologic challenge in the RAT (sc DMSO)

Author	Test	Age and Route of Exposure	Age Testing	Sample Size Per Dose Level (Yes/No litter is unit)	Challenge Drug	Measure[a]	Effect of Challenge Drug on Controls	Effect of CPF on the Effect of Challenge Drug Magnitude of CPF dose, mg/kg-day	
								1	5
Icenogle et al., 2004	Radial Arm Maze Not blind doses for each challenge drug given in counterbalanced order	GD 9–12 **sc DMSO**	Wk 8–13	10 M +10 F; 10 litters (1 rat /sex/ reconstituted litter) Litter of origin is not unit due to randomization of pups to dams at birth.	Scopolamine (0, 0.04, 0.08, 0.16 mg/kg sc)	Working error	↑error	0[b]	↓ (attenuate errors induced by scopolamine)
						Reference error	↑error	Not reported	Not reported
					Mecamylamine (0, 1.25, 2.5, 5 mg/kg sc)	Working error	0	0	0
						Reference error	0	0	0
Levin et al., 2002	Radial Arm Maze Not blind doses for each challenge drug given in counterbalanced order	GD 17–2 **sc DMSO**	Wk 4–6	10 M +10 F; 10 litters (1 rat /sex/ reconstituted litter) Litter of origin is not unit due to randomization of pups to dams at birth.	Scopolamine (0, 0.04, 0.08, 0.16 mg/kg sc)	Working error	↑error	M0 F↓ (attenuate errors induced by scopolamine based on linear trend analysis)	M0 F0
						Reference error	0	M0 F0	M0 F0
						Latency	0	↑ (enhance effect of scopolamine to increase latency – more evident in females)	0
					Mecamylamine (0, 1.25, 2.5, 5 mg/kg sc)	Working error		0	0
						Reference error		0	0
						Latency		0	0

(Continued)

TABLE 10B. Continued

Author	Test	Age and Route of Exposure	Age Testing	Sample Size Per Dose Level (Yes/No litter is unit)	Challenge Drug	Measure[a]	Effect of Challenge Drug on Controls	Effect of CPF on the Effect of Challenge Drug Magnitude of CPF dose, mg/kg-day	
								1	5
Aldridge et al., 2005a	Radial Arm Maze Not blind doses for challenge drug given in counterbalanced order	PND1–4 **sc DMSO**	Wk 16–17	1 pup/sex/ litter; 9 litters Litter is not unit for initial pooled analysis combining M & F	Ketanserin (0, 0.5, 1, 2 mg/kg sc)	Working error	0	↑ errors	
						Reference error	0	↑errors	
Levin et al., 2001	Radial Arm Maze Not blind doses for each challenge drug given in counterbalanced order	PND 1–4 (analysis pooled with PND 11–14) **sc DMSO**	Wk 14–17	10 M + 10 F; "on average there was 1 rat/sex/litter" Litter is not unit for initial pooled analysis combining M & F	Scopolamine (0, 0.04, 0.08, 0.16 mg/kg sc)	Working error	↑error	0	
						Reference error	↑error (graph shows M0 F↑)	0	
					Mecamylamine (0, 1.25, 2.5, 5 mg/kg sc)	Working error	0	0	
						Reference error	0	0	
Levin et al., 2001	Radial Arm Maze Not blind doses for each challenge drug given in counterbalanced order	PND 11–14 (analysis pooled with PND 1–4) **sc DMSO**	Wk 14–17	10 M + 10 F; "on average there was 1 rat/sex/litter" Litter is not unit for initial pooled analysis combining M & F	Scopolamine (0, 0.04, 0.08, 0.16 mg/kg sc)	Working error	↑error		0
						Reference error	↑error		M0 F↓ (F: attenuate errors at 0.16 mg/kg scopolamine)
					Mecamylamine (0, 1.25, 2.5, 5 mg/kg sc)	Working error	0		0
						Reference error	0		0

[a]Radial arm maze measures include working memory errors (entry into a formerly baited arm), reference memory errors (entry into unbaited arm), latency (total session time in seconds divided by total number of arm entries in units of seconds per entry)

[b]"0" means that developmental exposure to CPF did not change the effect of the challenge drug.

*If statistical analyses or measures were described in methods, then it was included and listed as "0" if not reported to be statistically significant

animals, similar to that observed by Levin et al. (2005). Ketanserin is a potent 5HT2 receptor antagonist, but also has effects on other 5HT, α-adrenergic, and histamine receptors (Levin et al. 2005). These data support hypotheses requiring further testing that CPF produces alterations in cholinergic, serotonergic, and/or other neuropharmacologic systems that are unmasked by pharmacologic challenge.

One oral study was conducted by Venerosi et al. (2010) in mice gavaged with a single dose level (6 mg/kg-d) of CPF during gestation (Table 10A). Attenuation of the effect of a single 40-mg/kg ip dose of fluvoxamine (5HT uptake inhibitor) to increase swimming duration or frequency (i.e., number of swimming intervals) on a forced swim test was measured following 6-mg/kg CPF exposure during gestation. Enhanced swimming duration or frequency in controls was interpreted as less "depressive-like" behavior in controls. Fluvoxamine also reduced duration of maternal aggression while in the nest. The baseline behavior (i.e., response following acute vehicle for fluvoxamine) of CPF and control animals was different for several forced swimming and maternal aggression parameters, making it more challenging to interpret the fluvoxamine effects. Venerosi et al. (2010) speculated that the acute effects of fluvoxamine are due to either "changes in constitutive 5HT levels or overactivation/ hypoactivation of specific 5-HT receptor families," yet no neuropharmacologic evaluations were conducted.

In summary, these data form the basis for developing hypotheses for further testing. Further testing needs to include concurrent measurements of neuropharmacologic endpoints based on clearly defined hypothesis. The pharmacologic challenge data need to be replicated using multiple doses of CPF, using an oral route of exposure, and avoiding DMSO as the vehicle.

Social and Agonistic Behaviors

Four studies measured multiple parameters for many social agonistic or maternal behaviors in adolescent or adult mice exposed during gestation and/or prior to weaning (Ricceri et al. 2003,2006; Venerosi et al. 2006, 2008).

Venerosi et al. (2006) measured USV and frequency and duration of different social recognition behaviors in female offspring. This study exposed pups prenatally (GD 15–18; 0, 3, or 6 mg/kg) and postnatally (PND 11–14, 0, 1, or 3 mg/kg), resulting in nine different groups, each dosed with one combination of gestational and postnatal oral doses. Of 9 possible dose combinations, the only statistically significant effect was (1) an increase in USV by female adult mouse during social interaction with another female mouse and (2) a rise in the frequency of social investigation in mice exposed gestationally to 6 mg/kg and postnatally to vehicle. The lack of effects in mice exposed gestationally to 6 mg/kg and postnatally to 1 or 3 mg/kg indicates a lack of dose-response relationship. The biological function of the USV emission by the female mouse during social interaction with another female is "poorly understood" (Venerosi et al. 2006). Thus, further research and replication of findings are needed before these data may be considered relevant for human health risk assessment.

A similar study by Ricceri et al. (2006) also involved nine combinations of the same doses, except that postnatal sc (not oral) injections were used. Ricceri et al. (2006) found that 1 and 3 mg/kg (PND 11–14) CPF enhanced maternal-like behaviors in "virgin females" exposed to pups from different mothers. This is an unreliable effect, given the artificial conditions of the test and the fact that there were no effects on latency, frequency, and duration of nest building and number of pups retrieved to the nest. The relevance of these data to human health risk assessment is questionable.

Venerosi et al. (2008) exposed mice from PND 11 to 14 to a single sc dose of 3 mg/kg-d in peanut oil. Measures of sociability, nest-building activity, and maternal behavior were analyzed in dams. The main finding was that females showed alterations in different aspects of the maternal behavior repertoire following artificial conditions of repeated daily

removal of nest and pups. These were alterations on individual parameters, and a large number of key maternal behaviors were unaffected (Venerosi et al. 2008). For example, latency to first licking episode on postpartum day 1 and latency to start nest building was lower at 3 mg/kg. However, there was no significant effect on quantitative or qualitative nest features, or on licking durations and frequencies. Decreased combined time crouching and nursing (an apparent a posteriori analysis) was measured, but no marked differences were found in the frequencies, latencies, or durations of the other pup-directed behaviors such as retrieving, sniffing, and nest building. There were also no significant effects on social behavior and social preferences at adolescence. Taken together, there appears to be a low level of concern for the patterns of alterations measured in mice dosed postnatally with 3 mg/kg-d.

Ricceri et al. (2003) studied the effects of postnatal injections of 1 and 3 mg/kg-d CPF sc in DMSO. Both doses were used for PND 1–4 and PND 11–14 exposure periods. For PND 1–4 exposures, frequency of aggressive grooming increased at 3 but not 1 mg/kg-day, although the main effect of treatment was not statistically significant. For PND 11–14, male agonistic responses appeared significantly enhanced throughout the test period after both doses of CPF. The agonistic responses included aggressive, defensive, and submissive behaviors. Therefore, conclusions regarding aggressive behaviors specifically require further analyses of data that were not presented. However, these results are consistent with enhanced agonistic behaviors reported by Ricceri et al. (2006) at 3 mg/kg-d CPF sc in peanut oil, as described next.

In the Ricceri et al. (2006) study, the frequency and duration of six male agonistic behavior parameters were analyzed following GD 15–18 gavage and/or PND 11–14 sc injections of CPF in peanut oil. Following prenatal injections, a significant increase in offensive posture frequency at 6 but not 3 mg/kg/d was found. For postnatal injections, a significant rise in frequency and duration of attack duration at 3 but not 1 mg/kg-d. However, this significant elevation in frequency was based on post-hoc comparisons conducted in the absence of significant main effect on the ANOVA. The duration and incidence of these findings at 3 mg/kg-d was, respectively, 6 versus 2.5 (treated vs. control) s of attack response per 5 min and 3.5 versus 2 attacks per 5 min. It is noteworthy that no effects on aggressive or agonistic behaviors were reported at 1 mg/kg-d when peanut oil was used instead of DMSO as the vehicle (Ricceri et al. 2006 compare with Ricceri et al. 2003).

In summary, multiple social behavior parameters were measured, and the frequency and duration for each of these parameters were statistically analyzed. The guidance from the U.S. EPA Neurotoxicity Risk Assessment Guidelines to risk assessors on similar type of observational data involving a large number of endpoints (i.e., functional observational battery) applies when considering these data for risk assessment purposes (U.S. EPA 1998b). The relevance of statistically significant test results needs to be evaluated according to "the number of signs affected, the dose(s) at which effects are observed, and the nature, severity and persistence of the effects and their incidence in relation to control animals" (U.S. EPA 1998b).

Taking into account the overall patterns of perturbations of behavioral parameters related to social behaviors, the magnitude of change or low incidence of the behaviors, and the number of multiple comparisons statistically analyzed, the biological significance of these alterations in social behaviors to humans is uncertain. Based on the data thus far, 1 mg/kg-day appears to be a no-observed-adverse-effect level (NOAEL) for perturbations in mouse social, agonistic and maternal behaviors.

Ricceri et al. (2006) measured brain and serum cholinesterase (ChE) activity in offspring at PND 15 after oral exposure from GD 15–18 and/or sc injections from PND 11–14. The mice were sacrificed 24 hr after postnatal exposures, and more than 15 d after last CPF injection for mice receiving only prenatal CPF exposures. There was a 20–50% decrease in

serum BuChE activity in all groups dosed postnatally with either 1 or 3 mg/kg-d. As expected, there was no brain or serum ChE inhibition in PND 15 pups dosed prenatally with vehicle, 3 or 6 mg/kg-d, and postnatally with vehicle. Ricceri et al. (2003) reported significant pup brain AChE inhibition 1 hr following PND 1–4 (but not PND 11–14) sc doses of 1 and 3 mg/kg-d. In contrast to the large number of rat studies conducted by multiple laboratories at more optimal sacrifice times (2–6 hr) after exposure, RBC and brain AChE inhibition has not been adequately characterized in mice following CPF exposures. From a risk assessment standpoint, 1 mg/kg-d is an effect level for RBC or brain AChE inhibition or plasma BuChE following postnatal exposures to mice or rats. Thus, behavioral perturbations reported in mice exposed to 1–6 mg/kg-d occurred at dose levels considered to be effect levels for CPF based on serum, RBC or brain ChE inhibition.

Neuropharmacologic Endpoints

Many studies evaluated the effects of gestational and/or postnatal exposures of CPF on the cholinergic and other neurotransmitter systems. Although the emphasis of this review is on neurobehavioral effects, understanding potential MOA is important in determining whether the most sensitive endpoints are being used for risk assessment and in understanding the weight of evidence relevant to neurobehavioral outcomes.

Cholinergic Effects The cholinergic system plays an important role in brain development beginning during the embryonic period and continuing into the postnatal period (Brimijoin and Koenigsberger 1999; Lauder and Schambra 1999). Therefore, inhibition of AChE during development by CPF exposure may have the potential to produce alterations in neuronal development. Alterations in cholinergic endpoints such as choline acetyl transferase (ChAT), mAChR, high-affinity presynaptic choline transporter (HAChT), vesicular Ach transporter (VAChT), and choline transporters were found (Chakraborti et al. 1993; Chambers 2003; 2004; 2005; Qiao et al. 2002; 2003; 2004; Richardson and Slotkin et al. 2001; Tang et al. 1999; Zhang et al. 2002). Although alterations in some of these cholinergic parameters were more persistent than AChE inhibition, the significance of these neuropharmacologic alterations (some less than 20% at lower doses) in terms of causal relationship with functional effects has not been established (Carr et al. 2001; Guo-Ross et al. 2007; Richardson and Chambers 2005). Nevertheless, decreases in mAChR binding (as measured by maximal binding sites B_{max}) and HAChT (as measured by ^{3}H-hemicholonium-3 binding) were considered to be among the more sensitive mechanistic endpoints that may potentially result in alterations in neurologic functions.

Richardson and Chambers (2003; 2004; 2005) measured effects of CPF on cholinergic neurochemistry, including brain AChE inhibition, mAChR, HAChT, VAChT, and ChAT. These studies indicated that gestational exposure to 7 but not 3 or 1 mg/kg-d CPF results in long-term (e.g., 1 mo) alterations of presynaptic cholinergic endpoints at a time when brain AChE inhibition had returned to control levels. Richardson and Chambers (2005) dosed pups from PND 1 to 21 by gavage (corn oil) with a low dose, 1.5 mg/kg-d, and high dose, 1.5 mg/kg, increasing to 3 and then 6 mg/kg. Both dosage levels resulted in persistent inhibition of AChE and alterations in mAChR density and reductions in presynaptic cholinergic endpoints.

One dermal study by Abdel-Rahman et al., (2004) found no marked effects on ligand-binding densities of nicotinic and mAChRs in cortex at PND 60 in offspring of dams exposed dermally to 0.1 mg/kg-d CPF in ethanol. Increases in AChE activity (not inhibition) were found in midbrain regions in males and brainstem regions in females, while other brain regions in both sexes showed no significant effect. AChE activity was not measured during or soon after the period of exposure.

Slotkin et al. (2001) and Qiao et al. (2002; 2003; 2004) measured ability of CPF to alter cholinergic endpoints such as ChAT, HAChT

and mAChR following exposures to dams during GD 9–12 or GD 17–20, or directly to pups on PND 1–4 or PND 11–14. A large number of measures were analyzed in males and females at different ages and multiple brain areas. There were some alterations (mostly decreases) in HAChT binding, ChAT activity, and mAChR binding at doses of 1 or 5 mg/kg-d following gestational and/or postnatal exposures. Song et al. (1997) and Qiao et al. (2002) measured rat pup brain AChE inhibition 24 h after the last sc injections of CPF in DMSO. Brain AChE inhibition was measured in pups from dams exposed for GD 17–20 to 5 mg/kg-d (>40%), 2 mg/kg-d (around 20%), but not 1 mg/kg-d (Qiao et al. 2002). Brain AChE inhibition (25%) was measured in pups directly injected PND 1–4 with 1 mg/kg-d CPF (Song et al. 1997). Even greater AChE inhibition (75%) was found in pups sacrificed 2 h after the first sc injection of 1 mg/kg CPF on PND 1 (Dam et al. 2000).

In general, brain AChE inhibition from oral or sc in dams during gestation or pups dosed postnatally is an equally or more sensitive endpoint compared to other cholinergic alterations measured in rat and mouse pups. At present, the relationship between CPF effects on cholinergic measures and behavioral effects is not clearly understood.

Noncholinergic Neuropharmacologic Effects Several studies reported that CPF produced long-lasting alterations of serotonergic, dopaminergic, and possibly noradrenergic systems after gestational or postnatal sc injections of 1 or 5 mg/kg-d (Aldridge et al. 2003; 2004; 2005b; 2005c; Dam et al. 1999; Raines et al. 2001; Slotkin et al. 2002; Slotkin and Seidler 2005; 2007a; 2007b [referred to as Slotkin et al. lab]). These studies measured a number of serotonergic endpoints, including 5HT1a and 5HT2 receptors, as well as 5HT transporter (5HTT) binding in multiple brain regions at different ages. Noradrenergic and dopaminergic endpoints were also measured, but in fewer studies. Alterations occurred in different directions and magnitudes and include increasing, decreasing or flat dose-response relationships depending on the specific parameter, age of exposure, age of sacrifice, dose, and brain regions (Slotkin et al. lab).

In general, these studies used 1 pup/sex/litter from 6 litters, 2 dose levels (1 and 5 mg/kg in DMSO, sc) for the gestational exposures (Aldridge et al. 2004; 2003; Slotkin and Seidler 2007b 2005b), and only 1 dose level postnatally (1 mg/kg for PND 1–4, PND 2–5, and 5 mg/kg for PND 11–14; Dam et al. 1999; Raines et al. 2001; Seidler 2005; 2007a; Slotkin and Aldridge et al. 2005b; 2005c; Slotkin et al. 2002). Limitations regarding the sc route of exposure, DMSO as the vehicle, and lack of dose-response data need to be considered in evaluation of results for risk assessment purposes. In addition, caution is needed in interpreting the biological significance of these noncholinergic data due to methodological issues described next.

Biological significance of statistical significant pooled data require further evaluation of specific measures A major challenge in evaluating consistency of results within and across studies is that the statistical analyses were based on data pooled together across multiple brain regions, exposure durations, ages, and neuropharmacologic endpoints (Slotkin et al. lab). Lower order analyses (e.g., group comparisons) were conducted depending on which interactions were statistically significant. For example, Aldridge et al. (2004) concluded that exposure to a low dose of CPF (GD 9–12) "elicited a significant overall elevation of 5HT1a, 5HT2 and 5HTT ligand binding without statistical distinction by region, measure or sex." In other words the statistical significance was based on statistical analysis that pooled together data for multiple 5HT receptor ligands and brain regions and both sexes. However, inspection of the graphed data indicates that many of the changes in individual measures in this and other studies (Slotkin et al. lab) for each sex, period of exposure, neuropharmacologic measure, and/or brain region may be of questionable biological significance. Some of these individual effects that were statistically significant in the pooled analyses are <10–20% alterations from control and/or have large standard errors within 10%

of the *x* axis representing 0 % change from control.

Biological significance of percent changes require evaluation of control values Most of the neuropharmacologic effects are reported as percent changes. The statistical analysis was based appropriately on the actual data, not on percent differences from control (Slotkin et al. lab). Control data were reported in some but not all papers, or in nonstandard units of measure (sometimes not corrected for protein level or wet weight of the brain region), making it more difficult to assess the reliability of measures.

If control levels are too small to measure reliably, this may result in apparent large percent differences from control. For example, Aldridge et al., (2005b) reported a statistically significant 40–50% decrease in dopamine levels in the hippocampus at PND 60 at 1 and 5 mg/kg-d, with no changes in the striatum, cortex, midbrain, or brainstem following gestational exposure to CPF. However, the small dopamine (DA) values for hippocampus in controls raise doubts about the biological significance of the 40–50% changes from control. Indeed, in later publications, Slotkin et al. (2002) state that DA levels were not measured in the hippocampus, because they were considered too low to be reliable. Similarly, in control animals, Dam et al. (1999) and Aldridge et al. (2003) demonstrated that levels of several cholinergic, noradrenergic, and DA endpoints were low from GD 17 to PND 5 and then rapidly rose (depending on brain area) from PND 5 to PND 20. Thus, evaluation of biological significance of percent changes from control measured during these early periods should take into consideration low control values and normal control variation (e.g., historical control values) during this period of rapid change.

Receptor binding assays use subsaturating ligand concentrations The serotonergic binding assays and the majority of those for cholinergic binding for CPF were made with single ligand concentrations that are at or slightly above the K_d value and below full saturation (Aldridge et al. 2005c). This is in contrast to approaches used by Richardson and Chambers (2004) for cholinergic endpoints, in which a single saturating concentration was used based on preliminary kinetic studies conducted in the laboratory under identical experimental conditions, or by Tang et al. (2003), in which different concentrations of ligands were used to calculate K_d and B_{max}.

Although this method of using subsaturating concentrations might be appropriate as a screen in preliminary-hypothesis-generating experiments, it is important to rigorously confirm that these alterations are biologically meaningful by conducting a full kinetic examination or employing a sufficient single concentration (i.e., $10 \times K_d$) that will allow for an accurate estimation of B_{max}. Thus, further confirmation is needed to ensure that reported differences from control (<10–25%) are not artifacts of the screening methods used.

Data are inadequate to establish MOA, and risk assessments based on RBC AChE inhibition are protective of non-AChE inhibition effects At present, data support hypotheses requiring further testing that there may be alternative MOA for developmental neurotoxic effects other than brain AChE inhibition. For the reasons outlined earlier, data are insufficient to consider serotonergic or other neuropharmacologic as a MOA for behavioral or other developmental outcomes. In addition, many of the neuropharmacologic changes studies did not measure AChE inhibition concurrently in the experiments. However, Song et al. (1997) and Qiao et al. (2002) reported significant offspring brain AChE inhibition following postnatal exposures to 1 mg/kg CPF (Table 11). Maurissen et al. (2000) and Mattsson et al. (2000) also found significant inhibition of brain and RBC AChE inhibition and of plasma BuChE in dams following oral exposure to 0.3 or 1 mg/kg CPF from GD 6 to 20. Therefore, all effects reported at 1 mg/kg following gestational or postnatal sc exposures occur at dose levels that would be considered in risk assessments to produce brain or RBC AChE or plasma BuChE inhibition in pregnant dams or offspring. Thus, it cannot be ruled out that these "non-AChE inhibition" effects were a result of AChE

TABLE 11. CPF effects on brain or RBC AChE inhibition in rats

	Selected Rat Brain or RBC AChE inhibition[a] for developmental period of exposure			
	GD 9–12 (dams exposed)	GD 17–20 (dams exposed)	PND 1–4 (pups exposed)	PND 10–16 or 11–14 (pups exposed)
CPF s.c. exposures (Brain AChE inhibition in rat offspring)	**1 and 5 mg/kg:** Not measured	**1 mg/kg:** 0 (24 hr) **2 mg/kg:** 20% (24 hr) (Qiao et al., 2002) **5 mg/kg:** 50% (24 hr) (Qiao et al., 2002)	**1 mg/kg:** 25% (24 hr) Song et al., 1997 **1 mg/kg:** 75% (2 hr after PND1 dose) Dam et al., 2000	PND 10–16 **5 mg/kg** 73% (4 hr) Carr and Nail, 2008 PND 11–14 **5 mg/kg** >65% (24 hr) Song et al., 1997
CPF oral exposures (Brain AChE inhibition in rat offspring)	GD 6–20 **1 mg/kg:** 0 (4 hr) **5 mg/kg:** 60% (4 hr) (Mattsson et al., 2000)			PND 10–16 **5 mg/kg** >58% (4 hr) Carr and Nail, 2008
CPF oral exposures (RBC AChE inhibition in dams)	GD 6–20 **0.3 mg/kg:** >25% dam RBC AChE Inhibition (4 hr) **1 mg/kg:** >80% dam RBC AChE inhibition (4 hr) (Mattsson et al., 2000)			

[a]Greatest effect from selected publication was reported, with 0 indicating no effect

inhibition that occurred daily during the period of dosing.

This review focuses on neuropharmacologic (e.g., serotonergic, noradrenergic, dopaminergic) findings. Other mechanistic in vitro and in vivo studies were comprehensively reviewed by Eaton et al. (2008). Eaton et al. (2008) conducted an expert panel review of the in vitro and in vivo mechanistic studies and concluded that "the weight of evidence from animal studies and in vitro mechanistic studies suggests that many of the neurodevelopmental effects of CPF are secondary to inhibition of AChE in target tissues such as the developing brain, although plausible alternative mechanisms have been proposed, based on in vitro studies" (102). The U.S. EPA (2011) recent preliminary human health risk assessment of CPF stated that "although multiple mechanisms have been proposed, a coherent mode of action with supportable key events, particularly with regard to dose-response and temporal concordance, has not yet been elucidated."

From a risk assessment perspective, data thus far indicate that these "non-AChE inhibition" effects occur at doses that produced RBC or brain AChE inhibition and/or plasma BuChE inhibition in adults, dams, or offspring. Although there is insufficient evidence to determine whether neuropharmacologic effects represent different MOA, risk assessments based on AChE inhibition are likely to be protective of these potentially different cholinergic or noncholinergic MOA.

Neuropathologic and Morphometric Measurements

Although neuropathologic and morphometric measurements are not a focus of this review, the main findings are briefly discussed because they may provide additional weight of evidence. There were one oral gestation/lactation study (Maurissen et al., 2000), three dermal gestational studies (Abdel-Rahman et al., 2003; 2004; Abou-Donia et al. 2006); and two sc (DMSO) postnatal studies (Roy et al., 2004; 2005) evaluating effects of CPF on histopathology or morphometric measures in the brain. With the exception of the oral study by Maurissen et al. (2000), all other studies tested only one dose level.

Briefly, alterations in morphometric measurements and other quantitative measures (e.g., length, thickness, neuron and glial cell counts) were found at 5 mg/kg-d following either oral GD 6–PND 10 (Maurissen et al. 2000) or sc PND 11–14 exposures (Roy et al. 2004 2005). These effects occurred at doses that are known to produce significant brain or RBC AChE and plasma BuChE inhibition in the dams during gestation and >65% brain AChE inhibition in pups following direct dosing to pups (Table 11). In addition, Maurissen et al. (2000) reported that 5 mg/kg-d produced maternal toxicity such as decreased bodyweight gain and clinical signs of AChE inhibition such as tremors. In the pups, this was accompanied by an increase in mortality between PND 1 and 4 (1% in controls vs. 25% at 5 mg/kg-d), and decreases in body and brain weight.

Since this article is focused on neurobehavioral endpoints, it is of interest that Roy et al. (2005) reported a number of "subtle morphologic changes in the hippocampus" including number of neurons and glia, and neuronal cell diameter. The hippocampus is related to learning and memory function. These morphologic changes included decreased layer thickness and neuron and glial cell count in the hippocampus following PND 11–14 sc injections of 5 mg/kg-d CPF. Levin et al. (2001) also dosed rats from PND 11–14 with sc injections of 5 mg/kg-d CPF, and found no effect on RAM working or reference memory, but did not report any histopathology examination of the hippocampus. Similarly, Maurissen et al. (2000) observed no effects on a delayed spatial alternation test of learning and memory following GD 6–PND 10 maternal doses of 0.3 to 5 mg/kg-d CPF, and no morphometric effects on the hippocampus. The differences in morphometric measurements may be related to differences in the types of brain measurements made and the route and period of exposures. At present, neuropathology data cannot be directly linked to any behavioral effects.

Decreases (approximately 12%) in number of surviving Purkinje cells and increases (approximately 8%) in glial fibrillary acidic protein (GFAP) immunostaining in cerebellum were also noted in offspring at 1 mg/kg-d (Abou-Donia et al. 2006) but not 0.1 mg/kg-d dermal exposures during gestation by the same laboratory (Abdel-Rahman et al. 2003; 2004). The GFAP measures were based on methods of approximating areas with targeted levels of pixels from digitized images of GFAP immunostained sections. In contrast, Garcia et al. (2002) found no effects on GFAP levels measured from brain homogenates following doses of 1–10 mg/kg from GD 17 to 20. These conflicting results may also be due to differences in duration and route of exposure, age of sacrifice, and method of measuring GFAP levels. The functional relevance of the findings from Abdel-Rahman et al. (2003; 2004) remains unknown.

EVALUATION OF EPIDEMIOLOGY AND ANIMAL NEUROBEHAVIORAL DATA FOR RISK ASSESSMENT

The purpose of this review was to provide in-depth analyses of CPF exposure and developmental neurobehavioral outcomes from the published human and animal in vivo peer-reviewed papers within the context of human health risk assessment. Previous approaches provided a summary of significant findings appropriate for comparisons of dose levels at which potential adverse effects were reported. In contrast, this review concentrated on providing a critical analysis of the neurobehavioral studies for risk assessment purposes. An important aspect of the approach used in reviewing the data is that methods and results (absence and presence of findings) were systematically tabulated by dose level and period of exposure. In addition, historical control data was considered as an additional perspective.

In the animal studies, the lowest dose level at which alterations in developmental neurobehavioral, neuropharmacologic, and neuropathologic/morphometric endpoints have been reported more frequently is 1 mg/kg-d (1000 μg/kg-d). The weight of evidence for neurobehavioral effects at 1 mg/kg-d is not compelling when taking into consideration dose response, study methodology, pattern of effects, and consideration of both the absence and presence of findings from different studies. There were a greater number of findings reported following exposure to 3–6 mg/kg-d. Significant RBC or brain AChE inhibition either in dams or in pups was produced at and below these dose levels. Therefore, risk assessments based on AChE inhibition are likely to be protective of these behavioral effects. As discussed in the introduction of this review, exposures to the general population today are primarily dietary in the 10^{-2} to 10^{-3} μg/kg-d dose level, which is more than 5 orders of magnitude below the animal dose levels. Estimates for daily exposure to pregnant women and children following previously allowed residential uses are in the 10^{-1} to $10-^{3}$ μg/kg-d dose range (Lowe et al. 2009; Eaton et al. 2008). Estimates for agricultural worker exposures are in the 10^{1} μg/kg-d dose range.

Epidemiology studies are also considered for use in risk assessment. However, the dose-response data for CPF are not reliable for human health risk assessment, largely because of the paucity of exposure data available from each study. Further, in the studies with multiple sources of data (e.g., biomarker data and air monitoring samples), correlations among these measures were weak ($r \sim .2$) (Whyatt et al. 2005). Furthermore, while data from the biomarkers of CPF at a single time point provide some indication of short-term exposure, understanding long-term patterns of use could add valuable information regarding exposures within and across critical stages of development in utero and after birth. In addition, although measures of nonspecific metabolites may be more objective quantitative measures of exposure to OP in general as compared to self-reported exposure information, such measures cannot form the basis for causal conclusions about CPF or other specific chemicals. At present, the epidemiology studies are useful to consider for hazard characterization.

Based on the epidemiologic studies reviewed thus far, the evidence for causality is weak. As new studies are published, there are four important principles in evaluating the CPF literature that are important to consider: (1) relative strengths and limitations of the studies, particularly in their ability to provide informative data about associations between CPF exposure and neurobehavioral and other relevant outcomes, taking into account potential bias and with consideration of the evidence for and against causality; (2) differences in exposure settings across the studies (Needham 2005); (3) measurement and characterization of exposure, keeping in mind that different biomarkers have different degrees of specificity with respect to CPF, and thus, a statistically significant result for DAPs and DMPs in the absence of a similar finding for DEPs is not likely to reflect exposure to CPF; and (4) weight of evidence for biological plausibility needing to be considered, rather than selecting individual findings from human studies and trying to find a "match" with animal data.

The conclusion of this review is that there is insufficient evidence that human neurodevelopmental exposures to CPF result in adverse neurobehavioral effects in infants and children based on studies that estimated CPF exposure using measures derived from maternal or child urine, umbilical cord blood, or personal air monitoring samples. In looking for consistent patterns across studies, and certainly across species, one needs to guard against simply accumulating a list of positive, statistically significant, yet potentially random findings. With this caveat in mind, two examples are discussed.

An increasing number of abnormal reflex in newborn infants was the only one of seven BNBAS domains that was found to be statistically significant in two epidemiologic studies evaluating DEPs and DAPs in maternal urine (Engel et al. 2007; Young et al. 2005). Young et al. (2005) and Engel et al. (2007) relate these outcomes to animal results such as significant alterations in cliff avoidance and righting reflex at postnatal days 1 and 3 following sc gestational exposures to 25 mg/kg-d reported by Chanda and Pope (1996). The route of exposure and dose level are not relevant to humans. Dose levels of 5 and 25 mg/kg-d produced greater than 60% brain AChE inhibition (Chanda and Pope 1996; Mattsson et al. 2000; Maurissen et al. 2000) and maternal toxicity (Maurissen et al. 2000, Qiao et al. 2002). The overall weight of evidence indicates that CPF does not exert effects on neurodevelopmental landmarks and reflexes in animals tested prior to weaning at doses below 5 mg/kg-d (Table 5). Therefore, the conclusion that these studies provide biological plausibility for abnormal reflexes in newborn humans is not appropriate.

As a second example, one of the epidemiology studies reported a statistically significant association (with wide 95% confidence interval) between cord blood CPF and maternal-reported PDD problems at age 36 mo (Rauh et al. 2006). Animal models can evaluate only certain aspects of heterogeneous complex neurologic disorders. At present, there are no definitive animal behavioral models for PDD or autism, and it is not appropriate to make direct comparisons between specific behavioral effects of CPF in animals with autism. Furthermore, none of the epidemiologic studies in this review were able to evaluate outcome misclassification by obtaining clinical confirmation information regarding PDD, ADHD, or other behavioral problems or disorders measured by maternal reports on the CBCL.

CONCLUSIONS

This review evaluated the published human epidemiology and animal literature that described potential associations between CPF exposure and developmental neurobehavioral outcomes, emphasizing their role in informing risk assessment. The epidemiologic studies do not support a causal association between CPF exposure to mothers and adverse neurobehavioral outcomes in infants or young children. Only one study evaluated associations between potential child (postnatal) CPF based on urine metabolites

and neurobehavioral outcomes and similarly did not provide strong evidence in support of causality. These cohorts are being followed into early and middle childhood, permitting further evaluation.

Taking into consideration both oral and sc animal behavioral studies, data indicate that most of the alterations of neurobehavioral, neuropharmacologic, or morphologic parameters occur at exposure levels that also produce brain or RBC AChE or plasma BuChE inhibition in adults or pups. Based on animal studies reviewed in this article, the no-observed-effect level (NOEL) for RBC AChE inhibition is <0.3 mg/kg-d following gestational exposures to pregnant rats. The U.S. EPA estimated a BMDL10 of 0.03 mg/kg-d for RBC AChE inhibition (U.S. EPA 2011). Therefore, the most sensitive endpoint for CPF is RBC AChE inhibition. Taking into consideration consistency of outcomes across studies, and strength of experimental design and methods for risk assessment purposes, the NOAEL for behavioral effects is 1 mg/kg-d. There is strong evidence from the animal literature that AChE inhibition (RBC or brain from adult or offspring) is a sensitive endpoint that is protective of neurobehavioral, neuropharmacologic, and morphologic alterations that were measured following gestational, lactational, and/or early postnatal exposures to 1 to 6 mg/kg-d.

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ACRONYMS

5HT	serotonin
AChE	acetylcholinesterase
ADHD	attention deficit hyperactivity disorder
AUC	area under the curve
BMDL10	benchmark dose lower confidence limit for 10% effect
BNBAS	Brazelton Neonatal Behavioral Assessment Scale
BSID	Bayley Scales of Infant Development II
BSID:MDI	Bayley Scales of Infant Development II – Mental Development Index (sometimes abbreviated to just MDI)
BSID:PDI	Bayley Scales of Infant Development II – Psychomotor Development Index (sometimes abbreviated to just PDI)
BuChE	butyrylcholinesterase
CBCL	child behavior checklist
CCCEH	Columbia Center for Children's Environmental Health
CHAMACOS	Center for Health Assessment of Mothers and Children of Salina
ChAT	choline acetyl transferase
ChE	cholinesterase (used when referring to both AChE and BuChE)
CI	confidence interval
CPF	chlorpyrifos
CPO	chlorpyrifos-oxon
d	day or days
DA	Dopamine
DAPs	total group of dialkylphosphates (includes DEPs and DMPs)
DEP	diethylphosphate
DEPs	total group of diethylphosphates (DEP, DETP, DEDTP)
DETP	diethylthiophosphate
DEDTP	diethyldithiophosphate
DMPs	total group of dimethylphosphates
DMS-IV	*Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition*
DMSO	dimethyl sulfoxide
EPA	U.S. Environmental Protection Agency
GD	gestational day
GFAP	glial fibrillary acidic protein
HAChT	high affinity presynaptic choline transporter
min	minute or minutes
mAChR	muscarinic cholinergic receptor
MDI	mental development index (same as BSID:MDI)
mg	milligram
NBAS	Neonatal Behavioral Assessment Scale
ng	nanogram
NHANES	National Health and Nutrition Examination Survey
NIEHS	National Institute of Environmental Health Sciences
NOEL	no-observed-effect-level
NOAEL	no-observed-adverse-effect level
OP	OP insecticides
OR	odds ratio
PBPK	physiologically based pharmacokinetic (model)
PDD	pervasive development disorders
PDI	psychomotor development index (same as BSID:PDI)
PND	postnatal day
PON1	paraoxonase
RBC	red blood cell
RR	risk ratio
sc	subcutaneous(ly)
SEM	standard error of the mean
TCPy	3,5,6-trichloro-2-pyridionol
USV	ultrasonic vocalizations
VAChT	vesicular Ach transporter
μg	microgram