Australian Government

Australian Pesticides and
Veterinary Medicines Authority

Reconsideration of chlorpyrifos: supplementary toxicology assessment report

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EXECUTIVE SUMMARY

Chlorpyrifos is a broad spectrum, non-systemic organophosphorous insecticide with contact, stomach and respiratory action. It acts by inhibiting the enzyme, acetyl cholinesterase (AChE), which is important for the transmission of nerve signals. Chlorpyrifos was first introduced for use in Australia in the mid-1960s.

The Australian Pesticides and Veterinary Medicines Authority (APVMA) commenced a reconsideration of chlorpyrifos due to concerns about toxicology, occupational health and safety (OHS), residues, trade, environment, efficacy and crop safety. These issues were assessed and an interim review report released in 2000, along with the implementation of various regulatory measures.

The scope of this supplementary toxicological assessment included a consideration of new studies published between 2000 and 2016, with a focus on the potential for chlorpyrifos to cause developmental or behavioural neurotoxicity. Following a comprehensive assessment of these new studies, it was concluded that there is no evidence to indicate potential neurodevelopment effects reported in some studies to occur at or below doses that inhibit AChE activity. Further, animal studies consistently indicated that there is no age-related differential sensitivity to chlorpyrifos. On this basis, cholinesterase inhibition remains the most sensitive and relevant adverse effect caused by chlorpyrifos and is therefore the most appropriate endpoint for the establishment of health based guidance values used to protect the entire population including pregnant women, infants and children.

The current acceptable daily intake (ADI) for chlorpyrifos is 0.003 mg/kg bw/d, based on the no observed adverse effect level (NOAEL) of 0.03 mg/kg bw/d for the inhibition of plasma cholinesterase activity in a 28 day study in humans, and applying a 10-fold safety factor to account for potential variability in sensitivity. The current acute reference dose (ARfD) for chlorpyrifos is 0.1 mg/kg bw, based on the NOAEL of 1 mg/kg bw for the inhibiton of erythrocyte AChE activity in a single dose human study, and applying a 10-fold safety factor to account for potential variability in sensitivity. Based on an assessment of the new published studies, the current ADI and ARfD for chlorpyrifos remain appropriate.

Chlorpyrifos is currently in Schedule 6 of the Standard for the Uniform Scheduling of Medicines and Poisons (SUSMP), except when included in Schedule 5 or in prepared potting or soil mixtures containing 100 g or less of chlorpyrifos per cubic metre. The Schedule 5 entry includes chlorpyrifos in aqueous preparations containing 20% or less of microencapsulated chlorpyrifos, when in controlled release granular preparations containing 10% or less of chlorpyrifos and when in other preparations containing 5% or less of chlorpyrifos except in prepared potting or soil mixtures containing 100 g or less of chlorpyrifos per cubic metre. Based on an assessment of the new published studies, the poison schedule remains appropriate.

1 INTRODUCTION

1.1   Previous toxicological assessment of chlorpyrifos

The toxicology of chlorpyrifos has been extensively reviewed by international agencies and the Australian Department of Health. This supplementary report focuses on new neurotoxicity studies, including epidemiological studies, published since the APVMA's 2000 toxicological evaluation of chlorpyrifos. Further information regarding the toxicology of chlorpyrifos can be found at www.apvma.gov.au/sites/default/files/publication/14746-chlorpyrifos-irr-toxicology.pdf.

1.2   Mechanism of mammalian toxicity

Chlorpyrifos is an organophosphorus insecticide or 'OP', and like all chemicals belonging to this group, it kills insects by interfering with the nervous system. It also has the potential to kill humans (and other mammals) by the same mechanism of interference with the brain, spinal cord and peripheral nerves. Following absorption, chlorpyrifos is bioactivated by the microsomal cytochrome P450 system(s) within the bodies of vertebrates and insects to the active oxon (phosphate ester) metabolite, which is approximately three orders of magnitude more potent than parent chlorpyrifos. The majority of bioactivation takes place in the liver.

The types of adverse effects that can occur in humans depend entirely on the level of exposure, with a spectrum of increasingly more severe effects occurring as the level of exposure increases. This spectrum ranges from effects on biochemical parameters in the blood and brain, clinical signs (nausea, vomiting, diarrhoea, dizziness, confusion, salivation, muscle twitching, laboured breathing, lethargy and coma) to death. These same adverse effects also occur in laboratory animal species exposed to organophosphates (e.g. fenthion), and on this basis, studies conducted using laboratory animals provide information relevant to effects on humans.

The inhibition of an enzyme critical for transmitting nerve signals is accepted by toxicologists, chemical regulators and the World Health Organisation (WHO) as the most sensitive adverse effect resulting from exposure to OPs, including chlorpyrifos. This enzyme, called acetylcholinesterase (AChE), is found in both the brain and blood and is specifically involved in maintaining normal nerve function. The statistically significant inhibition of this enzyme by greater than 20% below baseline is considered adverse and forms the basis of the health standards set for most OPs around the world. If the level of inhibition of AChE gets too high, people will begin displaying overt signs of poisoning.

For Australian workers using OPs, Safe Work Australia and WorkSafe WA recommend that health monitoring be undertaken before starting, during and after working with OPs. This analysis includes the measurement of AChE in blood in addition to urine for the presence of metabolites (breakdown products). If the level of AChE activity drops too low then workers may no longer be able to continue using these types of pesticides until their blood AChE level has normalised.

For the general public that may be exposed to chlorpyrifos residues in food, dietary health standards are set based on the same adverse effect on AChE in blood used to protect workers. Two health standards can be set: a) the dose that is safe to consume in a single meal (Acute Reference Dose—ARfD) and b) the dose that is safe to consume on a daily basis over a lifetime (Acceptable Daily Intake—ADI). In Australia, a different form of the cholinesterase enzyme, plasma non-specific butyryl cholinesterase (BChe), has historically been used as a more conservative endpoint on which to base the ADI.

1.3   Current health based guidance values

ADI

The current Australian ADI for chlorpyrifos is 0.003 mg/kg/d, based on the no observed adverse effect level (NOAEL) of 0.03 mg/kg/d for the inhibition of plasma cholinesterase activity in a human volunteer study (Coulston et al, 1972) and using a 10–fold safety factor.

ARfD

The current Australian ARfD for chlorpyrifos is 0.1 mg/kg bw/d, based on the NOAEL of 1 mg/kg/d for the inhibition of erythrocyte AChE activity in a human volunteer study (Coulston et al, 1972) and using a 10–fold safety factor.

Poison schedule

Chlorpyrifos is in Schedule 6 of the Standard for the Uniform Scheduling of Medicines and Poisons (SUSMP), except a) when included in Schedule 5; or b) in prepared potting or soil mixtures containing 100 g or less of chlorpyrifos per cubic metre.

Chlorpyrifos is in Schedule 5 when in a) aqueous preparations containing 20% or less of microencapsulated chlorpyrifos; b) controlled release granular preparations containing 10% or less of chlorpyrifos; or c) other preparations containing 5% or less of chlorpyrifos, except in prepared potting or soil mixes containing 100 g or less of chlorpyrifos per cubic metre.

2 SUPPLEMENTARY TOXICOLOGY REPORT

2.1 Scope

Since the publication of the APVMA's 2000 toxicological review of chlorpyrifos, new studies were published in the scientific literature suggesting that chlorpyrifos is associated with developmental neurotoxicity. These include studies conducted in laboratory animals and epidemiological studies, which have examined statistical associations between the use of chlorpyrifos and adverse outcomes at the population level. Developmental neurotoxicity is the adverse effect on the structure or function of the nervous system during development *in utero* or in early life, which persists throughout life—this may manifest as changes in biochemical parameters, behaviour or structures in the brain or nervous tissue.

Chlorpyrifos, like all OPs, is neurotoxic, and therefore it is possible that direct exposure of the fetus at a sufficiently high enough level could have adverse effects on the nervous system. From a human risk assessment perspective, the critical consideration is that if chlorpyrifos does cause developmental neurotoxicity, whether it occurs at doses lower than that inhibiting cholinesterase activity, which is known to be the most sensitive adverse effect (see below).

New published scientific literature relevant to the scope of the toxicological review of chlorpyrifos was identified using the search strategy described in Table 1.

Table 1:   Summary of online literature search strategy

Tool	Search characteristics		
	Combined search criteria (search field)	Date	Result
PubMed *	'Chlorpyrifos' (all) AND '2012 to present' (date of addition in PubMed) AND 'neuro*' (all)	3 November 2015	35 references
	'TCP' (all)	20 November 2015	71 references
Google Scholar #	'Chlorpyrifos and developmental neurotoxicity' AND '2013 to 2015' (custom date range)	3 November 2015	N/A

* PubMed; http://www.ncbi.nlm.nih.gov/pubmed; # Google Scholar: https://scholar.google.com.au/, TCP = 3,5,6-trichloro-2-pyridinol, N/A: Not applicable

2.2   Neurotoxicity studies in laboratory animals

Mice

Study 1: Venerosi et al, 2009

Reference**:** Venerosi A, Ricceri L, Scattoni ML, Calamandrei G (2009). Prenatal chlorpyrifos exposure alters motor behaviour and ultrasonic vocalisation in CD-1 mouse pups. Environ Health 8:12

Forty pregnant CD-1 female mice were administered a gavage dose of chlorpyrifos at 6 mg/kg bw/d from GD14–17. The use of a single dose prevents the establishment of a dose-response relationship and therefore limits the power of this study to detect biologically meaningful effects. This dose when previously tested in the same laboratory induced 75% serum and 60% brain AChE inhibition in pregnant females and 20% inhibition in serum AChE activity in offspring at birth. Assessment of early development was completed using a reflex-battery on postnatal (PN) days (PND), *viz.* PND3, 6, 9, 12 and 15 for assessment of somatic sensorimotor maturation. Ultrasonic emissions (USV) were tested on PND4, 7 and 10, and pups motor skills were assessed in a spontaneous activity test on PND12.

No signs of systemic toxicity, weight loss or altered litter parameters (sex ratio, live births, litter weight) occurred. Maternal behaviour was assessed during and after separation of a male pup from each litter on PND4. Chlorpyrifos-treated dams exhibited statistically significant increases in duration of licking response and wall-rearing, and decreased digging behaviours upon return of the pup. No other pup or non-pup oriented behaviours were reported to have been altered.

One pup/sex/litter was assessed for somatic development on PND3, 6, 9, 12 and 15. Treated male pups exhibited statistically significant reductions in body length on PND12 and 15, but there were no decreases in bodyweight gain on the same days. Neurobehavioural development was assessed in the same group by modified Fox battery and no treatment-related effects were evident.

USV was assessed to monitor neurobehavioural development, emotional/affective states, and serotonergic and oxytocinergic neurotransmission. USV measurements on PNDs 4, 7 and 10 showed significant decrease in both the number and duration of calls of exposed male pups on PND10. On PND10, chlorpyrifos exposure also delayed the onset of the first call and peak frequency (kHz) of calls increased ($p < 0.05$).

On PND12, spontaneous behaviour was used to evaluate age-specific mobility. Statistically significant decreases in frequency and duration of pivoting as well as increases in frequency and duration of immobility were observed in chlorpyrifos exposed pups. Other behaviours (grooming, head moving, and wall climbing and crossing) were not affected.

Study 2: Braquenier et al, 2010

Reference: Braquenier JB, Quertemont E, Tirelli E, Plumier JC (2010). Anxiety in adult female mice following perinatal exposure to chlorpyrifos. Neurotoxicol Teratol. 32(2): 234–239.

Pregnant mice were administered gavage doses of chlorpyrifos at 0 (vehicle only), 0.2, 1 or 5 mg/kg bw/d from gestation day (GD) 15 to PND 14. Following a recovery period of several weeks (PND14–72), the anxiety of adult female pups was evaluated using neurobehavioural tests (elevated plus-maze and light/dark box tests). Preliminary results indicated alteration of anxiety levels during developmental exposure only in female pups—therefore only females (one per litter) were assigned to behavioural tests in follow-up experiments. Anxiety was assessed by spontaneous locomotion, light/dark box test, and the elevated plus-maze test.

There was no treatment-related effect on bodyweight gain in pregnant females or in offspring at PND70, or on spontaneous locomotor activity (8–10/group) were observed at any dose. A light/dark box test on PND72 was used to assess anxiety in offspring. Time in the centre of the light section was taken to indicate low anxiety, while time in the dark section and switches between the light and dark sections was taken to indicate higher anxiety. There were no significant effects observed at any doses.

The proportion of entries into the open arms of the plus-maze was counted and time spent on the open arms was measured. Decreases in these metrics compared to the control were taken to indicate anxiety. A significant decrease in time spent on the open arms was observed in 1 mg/kg group, but not for other doses. Similarly, for the mean number of entries into the maze measurement, only the 1 mg/kg group was significantly lower than the control. In the absence of an effect at the highest dose, these changes were not considered to be treatment-related. Total number of open and closed arm entries, taken as a measure of overall locomotor activity, did not differ between groups. Brain AChE activity was only measured on PND1. In the high dose group (5 mg/kg bw/d), a 14% reduction in comparison to vehicle controls was reported.

Overall this study indicated no effect of chlorpyrifos doses on neurobehavioural parameters in mice exposed *in utero*.

Study 3: Cole et al, 2012

Reference: Cole TB, Fisher JC, Burbacher TM, Costa LG, Furlong CE (2012). Neurobehavioural assessment of mice following repeated postnatal exposure to chlorpyrifos-oxon. Neurotoxicol Teratol. 34(3): 311–322

The study involved the use of Paraoxonase1 (PON1) knockout (PON1$^{-/-}$) mice and mice expressing either the human PON1R192 or PON1Q192 transgene in place of endogenous mouse PON1. Six male mice from each genotype were administered 0 (vehicle control—dimethyl sulfoxide, DMSO), 0.35 or 0.50 mg/kg bw/d subcutaneous injection (s.c.) of chlorpyrifos-oxon from PND4–21. Other mice were selected from cohorts of male and female mice from each of the four genotypes (ie a total of 8 cohorts). These animals were injected subcutaneously with 0, 0.15, 0.18, 0.25, 0.35 or 0.50 mg/kg bw/d chlorpyrifos between GD4–22. Preliminary testing identified that ≥ 0.625 mg/kg bw/d chlorpyrifos-oxon was lethal to PON1$^{-/-}$ tgHuPON1Q192 mice after 3–5 days of dosing. One male and one female from each litter (and from each genotype) were analysed for brain AChE activity. Bodyweights of all mice were measured daily prior to dosing and dates of appearance of developmental landmarks (eye opening, pinna detachment, hair growth) were noted. Cerebral examination was conducted using

ribonucleic acid (RNA) extraction, labelling and microarray hybridization followed by processing using microarray to measure gene expression (Affymetrix GeneChip).

Significant male bodyweight reductions occurred in mice exposed to 0.50 mg/kg bw/d chlorpyrifos on PND21 (8.13 ± 0.33 g vs. 9.78 ± 0.50 g; p = 0.027). The authors reported no overt toxic effects and no delay in developmental landmarks (eye opening, pinna detachment, hair growth) followed dosing. Changes in gene expression of unclear biological relevance included the following:

- PON1 was expressed in the cerebellum of PON1$^{+/+}$, tgHuPON1Q192, and tgHuPON1R192 mice, but not PON1$^{-/-}$ mice.

- PON2 expression was also reduced in the PON1$^{-/-}$ cerebellum, regardless of treatment (an unexpected result).

- PON2 mRNA levels were 17–33% lower in the PON1$^{-/-}$, tgHuPON1Q192, and tgHuPON1R192 mice, compared to the PON1$^{+/+}$ mice.

Study 4: Shalaby et al, 2013

Reference: Shalaby MA, Abo-El-Sooud K, Hamoda AA (2013). Assessment of Toxicity of Chlorpyrifos Insecticide on Fetuses and Suckling Pups of Rats. Insight Ecology 2(1): 1–7

The study aimed to investigate the adverse effect of chlorpyrifos on rat fetal development and suckling pups till weaning age. The authors specified that the chlorpyrifos used in the study was in the form of 50% EC (purchased from KANZA group). However, it is unclear from the details in the study whether chlorpyrifos has been administered as mg/kg bw/d ai or product. Further, no information was provided on the excipients or vehicle used in the study. Though the authors aimed to investigate effects in pups of dams administered with chlorpyrifos from GD15 to PND21, deaths of all pups occurred before PND21 on PND15 and PND17. Gross examination of these pups was not conducted after death and consequently, the cause of the deaths was not determined. The authors reported morphological abnormalities in fetuses from dams administered chlorpyrifos from GD6–15 but due to the lack of reporting detail and other study limitations, these findings cannot be clearly attributed to treatment.

Study 5: Wang et al, 2013

Reference: Wang L, Ohishi T, Akane H, Shiraki A, Itahashi M, Mitsumori K, Shibutani M (2013). Reversible effect of developmental exposure to chlorpyrifos on late-stage neurogenesis in the hippocampal dentate gyrus in mouse offspring. Reprod Toxicol. 38: 25–36

Pregnant Crl:CD Sprague Dawley (SD) rats were exposed to chlorpyrifos in the diet from GD10 to PND21 at 0, 4, 20 or 100 ppm (15/dose). Mean daily intakes of chlorpyrifos during gestation were 0.6 ± 0.1, 3.2 ± 1.1 or 16.5 ± 7.0 mg/kg bw/d at 4, 20 or 100 ppm, respectively. During lactation, intakes were 1.7 ± 0.4, 8.3 ± 2.0 or 38.6 ± 8.0 mg/kg bw/d. Pups were observed up to PND77. Dams were examined daily for clinical signs. Bodyweight and food consumption were recorded throughout the exposure period. Following scheduled termination, organ weights were recorded and cholinesterase analysed. In male offspring only, the following was studied: serum thyroid-related hormone concentration; cell apoptosis and proliferation in the sub granular zone (SGZ) of the hippocampal dentate gyrus; neuronal progenitors and CHRM1 distribution in the SGZ; distribution of neurons and astrocytes in the dentate hilus; gene expression of markers in the dentate gyrus.

Dams exhibited no significant treatment-related effect in response to chlorpyrifos exposure. Food consumption was unaffected during gestation. During lactation, there were significant decreases ($p < 0.05$) in food consumption for all doses on PND19, but it was not considered an adverse effect in isolation. Chlorpyrifos did not affect any litter parameters or maternal bodyweight at necropsy on PND21. Relative liver weight at 4 ppm ($p < 0.05$) and kidney weight at 100 ppm ($p < 0.01$) were significantly elevated but in the absence of a dose-response relationship were not considered treatment-related.

Bodyweights of male pups were significantly lower ($p < 0.05$) at all doses at the following ages: 4 ppm: between PND49–70; 20 ppm: between PND21–70; and 100 ppm: between PND28–70. The study authors reported that these changes were not present at PND77, however it is noted that the control group bodyweight was lower on PND77 compared to PND70. Bodyweights of female pups were unaffected by exposure. Organs were weighed at PND21 and 77. Significant ($p < 0.01$) increases in relative brain, liver and kidney weights were observed in male pups from the 20 ppm group but in the absence of an effect at the highest dose were not considered treatment-related. Chlorpyrifos exposure did not affect body or organ weights in female pups. There was no treatment-related effect on thyroid hormones in male pups.

Cholinesterase activity was decreased in dams and male offspring on PND21: in red blood cells (RBC) and plasma at ≥ 4 ppm and in brain at 100 ppm. This effect was sustained until PND77 in male pups. No female pups were tested.

Proliferating and apoptotic cell indices were assessed in the SGZ of male offspring. There were no significant differences in the number of proliferating cell nuclear antigen (PCNA)-positive cells in the SGZ for any dose groups on either PND21 or PND77, or in the number of Terminal dUTP nick end labeling (TUNEL)-positive cells.

Neuronal progenitors and Chrm1 distribution in the SGZ were also assessed. On PND21, chlorpyrifos exposure did not affect the number of PAX6+ or Tbr2+ cells (type 2 progenitor cells), but the number of DCX+ cells (representing Types 2b/3 progenitors and post-mitotic immature granule cell populations in the SGZ) decreased significantly ($p < 0.05$) at doses ≥ 20 ppm. There was no significant difference in the number of CHRM1+ cells. On PND77, there were no significant changes observed.

Testing of the distribution of neurons and astrocytes in the dentate hilus of male offspring indicated that on PND21, only the 100 ppm dose was affected—there was a significant ($p < 0.05$) decrease in the number of NeuN+ cells observed in the hilus. There were no treatment-related changes in the distributions of reelin+ or NeuN+ cells or GFAP+ astrocytes after chlorpyrifos exposure at any other dose.

None of the genes (Pcna, CASP3, Bax, Bcl2, PAX6, Tbr2, CHRM1) analysed had altered expression on PND21, except for DCX, which decreased at chlorpyrifos exposure greater than 20 ppm.

Study 6: Lee et al, 2015

Reference: Lee I, Eriksson P, Fredriksson A, Buratovic S, Viberg H (2015). Developmental neurotoxic effects of two pesticides: Behavior and biomolecular studies on chlorpyrifos and carbaryl. Toxicol Appl Pharmacol 288(3): 429–438

To emulate the fat content of mouse milk (~14%) for physiologically appropriate absorption and hence distribution, egg lecithin and peanut oil mixture (1:10) was sonicated with water to yield a 20% (w/w) fat emulsion vehicle (Keller and Yeary, 1980; Palin et al, 1982). Chlorpyrifos was dissolved in this vehicle to achieve desired doses of

the pesticide. Three sets of mice were used for 1) protein analysis, 2) behavioural analysis and 3) AChE inhibition assay analysis.

The mice used for protein analysis were administered a single gavage dose of chlorpyrifos (5 mg/kg bw) on PND10 or vehicle alone. Mice were killed at 24 h (neonatal age) or 4 months (adult age) post-exposure. The hippocampus and cerebral cortex brain regions were collected and snap frozen until used for analysis of proteins (CaMKII, GAP–43, GluR1, PSD–95, synaptophysin and tau). Five to eight animals for each treatment group and timepoint were used.

The mice used for the behavioural analysis were administered a single gavage dose of chlorpyrifos: 0.1, 1.0 or 5.0 mg/kg bw (treatment group) or vehicle alone (control group) at PND10. At 2 and 4 months of age, mice were subjected to three behavioural tests (locomotion, rearing and total activity) and subsequently after 4 months of age, animals were killed for brain tissue sampling (12 pups/timepoint). The animals were also monitored for any signs of toxicity during the experimental period.

The mice used for AChE-inhibition assay were administered a single oral dose of chlorpyrifos (5 mg/kg bw) (treatment group) or vehicle alone (10 mL/kg vehicle) (control group) on PND10. Mice were euthanised 1, 3, 6, 12, 24 or 36 h after exposure and the whole brain (without cerebellum) was dissected out, snap frozen in liquid nitrogen and stored in 80°C until analysis (4 pups/group/timepoint).

There were no clinical signs of toxicity and the bodyweight gains were not affected in any of the treatment groups, although no data for toxicity or bodyweight was reported by the authors.

The authors reported significant decrease (42% in comparison to control group, $p ≤ 0.01$) in the CaMKII level in the hippocampus and also a significant decrease (50% in comparison to control group, $p ≤ 0.05$) in the level of synaptophysin in the cerebral cortex. Other proteins (GAP–43, GluR1, PSD-95 and tau) were not significantly effected in the hippocampus or cerebral cortex of the neonates exposed to chlorpyrifos.

There were no treatment-related effects on any of the protein levels, in either hippocampus or cerebral cortex of adult mice.

The authors reported that the treatment group exposed to the high (5 mg/kg bw) dose of chlorpyrifos exhibited a significantly ($p ≤ 0.01$) decreased activity for all three behavioural measures (locomotion, rearing and total activity) during the first 20 min period (0–20 min) in 2 month old mice, compared to the control, low and middle chlorpyrifos dose groups. These activities returned to normal during the later timepoints (20–40 and 40–60 min). Similar results were reported for spontaneous behaviours of 4 month old mice. Despite these behavioural changes, there were no reported changes in brain AChE activity between chlorpyrifos treatment group (5 mg/kg bw/d) and their time-matched controls at 1, 3, 6, 12, 24 or 36 h in the AChE inhibition assay. The study authors proposed that the results support the hypothesis that behavioural and cognitive effects may be introduced by an alternative cholinergic mechanism.

Rats

Study 1: Levin et al, 2001

Reference: Levin ED, Addy N, Nakajima A, Christopher NC, Seidler FJ, Slotkin TA (2001) Persistent behavioural consequences of neonatal chlorpyrifos exposure in rats. Dev Brain Res. 130(1): 83–89

Each group contained 10 rats/sex/treatment group; chlorpyrifos was injected subcutaneously at 1 mg/kg bw/d on PND1–4 (early exposure) and 5 mg/kg bw/d on PND11–14 (late exposure). The behavioural test was conducted during adolescence and adulthood after postnatal treatment with chlorpyrofos with the T-maze spontaneous alternation test (weeks 4–6), figure 8 locomotor activity test (weeks 4–6), radial-arm maze test (weeks 8–13), and the radial-arm maze test with rats challenged by scopolamine and mecamylamine (weeks 14–17).

For the T-maze test, no treatment-related effects were noted regarding the measure of percent alternation. On PND1–4, the chlorpyrifos treated groups had 84 ± 6% alternation compared to 86 ± 4% alternation for control group. On PND11–14, the percent alternation values were 83 ± 6% (treatment) and 82 ± 5% (control), respectively. Chlorpyrifos-treated males but not females exhibited significantly slower response latency in the T-maze.

There were no significant treatment-related effects in locomotor activity (Figure-8 apparatus) averaged over the 1 h test period. No effects on habituation in animals treated with chlorpyrifos during the early exposure period (PND1–4), but chlorpyrifos slowed the rate of habituation over the course of the first test session (weeks 4–6) in both sexes.

For the learning test assessed in the radial-arm maze, chlorpyrifos-treated rats exhibited impaired cognitive performance (including both working memory errors and reference memory errors) in male rats in the early exposure period of PND1–4, but not in late exposure period of PND11–14. However, chlorpyrifos treated female rats exhibited improved working and reference memory in the early exposure period of PND1–4 (but not in late exposure period of PND11–14). For working memory, chlorpyrifos did not alter the effects of scopolamine in either sex after both the early and late exposure periods. However, female rats pre-exposed to chlorpyrifos in late exposure period of PND11–14 showed decreased reference memory errors when challenged with scopolamine at PND14–17. Rats pre-exposed to chlorpyrifos had no effects when challenged by mecamylamine. The opposite results observed in males and females suggests, without a plausible biological explanation that they are unlikely to be treatment-related. The authors concluded that the cognitive effects observed were likely to be cholinergic synaptic function related, although AChE activity was not measured in the study.

Study 2: Aldridge et al, 2003

Reference: Aldridge JE, Seidler FJ, Meyer A, Thillail I, Slotkin TA (2003). Serotonergic systems targeted by developmental exposure to chlorpyrifos: Effects during different critical periods. Environ Health Perspect. 111(14): 1736–1743

Four treatment windows were investigated, *viz.* neural tube stage (GD9–12), late gestational period (GD17–20), postnatal neuronal differentiation and synaptogenesis (PND1–4, PND11–14). The doses were selected to avoid impairment of maternal and fetal growth as previous studies (by other authors) showed significant cholinesterase inhibition in the fetal brain at doses of 5 mg/kg bw chlorpyrifos and above.

Control rats showed an increase in 5HT1A receptors in the brainstem and forebrain from GD17–PND20, rising in parallel (similar level of effect in both areas of the brain). An increase in 5HT1B receptors was also seen in the control group, with the increase observed in the forebrain 3-fold greater than the brainstem by PND20. Serotonin-transporter (5HTT) binding showed regional specificity during development; at GD17 the whole brain value was 147 fmol/mg protein which rose to 240 in the brainstem but remained at a similar level of 118 in the forebrain on GD21.

Early gestational treatment: Dams (5–7 in total) were injected subcutaneously with either 0 (DMSO vehicle), 1 or 5 mg/kg bw/d chlorpyrifos on GD9–12. Fetal tissues were obtained on GD17 (whole brain analysis) and GD21 (forebrain separated and remainder termed brainstem). Only one fetus was analysed per dam. No signs of systemic toxicity were observed. There were no treatment-related effects on brain or bodyweight. Dose-dependent reductions in 5HT1A and 5HT2 receptor binding at GD17 ($p < 0.006$) were observed across all regions of the brain. At GD21 rebound elevations were noted in all three parameters but the observations were only statistically significant in the brainstem. There were no treatment-related effects on basal or forskolin stimulated AC (adenylyl cyclase) activity, although chlorpyrifos treatment enhanced the inhibitory effect of 5HT in this treatment group.

Late gestational treatment: Dams were injected subcutaneously with either 0 (vehicle control), 1, 2, 5, 10, 20 or 40 mg/kg bw/d of chlorpyrifos (DMSO vehicle) on GD17–20. Fetal tissues were sampled on GD21. Only one fetus was analysed per dam. Elevations in 5HT1A, 5HT2, 5HTT receptor binding were observed at all doses tested. In the brainstem, chlorpyrifos elicited significant elevations in 5HT1A and 5HT2 receptor binding, as well as in [3H] paroxetine binding to the 5HTT site. In each case significant effects were obtained only with doses ≥ 10 mg/kg bw/d, a dose which exceeded the threshold for systemic toxicity (a point of difference as compared to the doses administered earlier in the gestational period). A statistically significant ($p < 0.0001$) dose dependent inhibition of AC activity in response to 5HT was noted in both brainstem and forebrain at all doses tested.

Postnatal treatments were made to pups born on GD22. Pups were randomised on the day after birth and redistributed to dams so that litter size was 10 pups. Randomisation was repeated after several days to randomly distribute effects of maternal care. Pups were given 1 mg/kg bw/d of chlorpyrifos on PND1–4 or 5 mg/kg bw/d chlorpyrifos on PND11–14. Animals were selected either 24 hours or 6 days after treatment finished for tissue sampling (tissue from PND5 and 10 was used for early postnatal treatment, and PND15 and 20 for late postnatal treatment). For all postnatal samples the forebrain and brain stem separation was made as per the description for GD21. Only 1/sex/litter was used for each determination (5–7 for each treatment group at each age for each sex).

Early postnatal period treatment group: Chlorpyrifos treatment to newborn rats on PN1–4 did not result in statistically significant increases in 5HT1A and 5HT2 receptor binding when evaluated 24 h after the last dose, and were smaller than those observed after gestational exposure.

Late postnatal period treatment group: Similar effects to those observed in early postnatal rats were noted for the older postnatal female rats (PND11–14). On PND20 receptor binding was subnormal across brain regions and subtypes.

The authors concluded that the results indicated that chlorpyrifos affects the development of signalling proteins, and their ability to elicit cellular response for one of the major neurotrophic monoamines, 5HT during a discrete critical period of development. The observed effects in animals treated during the gestational period were more pronounced than effects seen in animals treated during the postnatal period. While the study provides information that may be relevant to the future derivation of a mode of action for the developmental effects of chlorpyrifos, it does not provide a suitable basis for the establishment of endpoints for use in regulatory risk assessment as the route of exposure (s.c) is not relevant to humans

Study 3: Meyer et al, 2004

Reference: Meyer A, Seidler FJ, Aldridge JE, Tate CA, Cousins MM, Slotkin TA (2004). Critical periods for chlorpyrifos-induced developmental neurotoxicity: alterations in adenylyl cyclase signaling in adult rat brain regions after gestational or neonatal exposure. Environ Health Perspect. 112(3): 295–301

Four developmental stages were examined, as follows:

1. pregnant dams were injected with 0 (vehicle only control), 1 or 5 mg/kg bw/d over GD9–12 (during fetal neural tube development)

1. pregnant dams were injected with 1 mg/kg bw/d or 5 mg/kg bw/d GD17–20

2. pups were injected with 0 or 1 mg/kg bw/d on PND1–4

3. pups were injected with 0 or 5 mg/kg bw/d on PND11–14.

On the day of birth, all pups were randomised within their respective treatment groups to maintain a litter size of 10. Dams were rotated among litters to distribute any differences in caretaking randomly across litters. No changes in suckling or maternal caretaking were observed. Brains were dissected on PND60 into cerebral cortex, hippocampus, striatum, midbrain, brainstem and cerebellum. Hippocampus and striatum were not examined in pups prenatally exposed. AC effects were evaluated by a) βAR (beta-adrenergic receptors) binding, and b) measurement of AC basal enzymatic signalling, in response to direct stimulation by the agonists forskolin and Mn^{2+}, and response to indirect stimulation by the βAR agonist, isoproterenol.

Between GD9–12, no exposure related effects on βAR binding in any brain region were reported for either dose. At 5 mg/kg bw/d, there was an increase in AC activity in response to forskolin ($p < 0.0006$), Mn^{2+} ($p < 0.003$), and isoproterenol ($p < 0.04$).

From GD17–20, study authors reported a sex-dependent effect on βAR binding in adulthood. Significant sex-dependent treatment-related effects were noted in different brain regions at both 1 and 5 mg/kg bw/d. Due to the reported sex differences, data was subdivided by study authors. In males at 5 mg/kg bw/d, several treatment related effects were noted, including small elevations of binding in the striatum and midbrain and depression in the cerebellum. Females showed significant overall reductions in binding across all brain regions at both doses.

On GD17–20, chlorpyrifos exposure resulted in an increase of AC in the hippocampus. In the striatum, males had increased AC effects, while females had reductions in AC measured by response to AC stimulants (forskolin and Mn^{2+}). In the midbrain no significant effects were reported for either sex, and in the cerebellum, females (but not males) had significant elevations at both doses across all stimulants.

Chlorpyrifos exposure on PND1–4 did not affect βAR binding at PND60. However, there was a reported significant elevation of AC that was dependant on sex, brain region and AC measure. Lower-order analysis of brain regions indicated that the cerebral cortex had elevated response to forskolin and Mn^{2+}, and the brainstem showed elevated response to isoproterenol ($p < 0.05$) in the absence of sex differences. The cerebellum showed a sex-specific effect: in males, decreases in basal activity and response to isoproterenol, and main treatment effect (βAR binding) in females.

On PND11–14, chlorpyrifos treated rats, predominantly females, showed significant decreases in βAR binding. Measured AC activities were consistent across brain regions, but showed sex differences. In males, a significant decrease in basal and isoproterenol response was reported. In females, the effect on βAR binding was significant, with decreases in basal and forskolin response. A significant overall tissue-specific signalling decrease was observed in the cerebral cortex of females. Sex-specific alterations in AC activity ratios were also detected: in males, isoproterenol was suppressed relative to forskolin, and this was significant in the striatum and cerebellum. In females, isoproterenol was suppressed relative to forskolin and forskolin relative to Mn^{2+}.

It should be noted however, that the doses that induced these effects were higher than those that caused inhibition of AChE activity, thus the relevance of these findings from a regulatory perspective is limited.

Study 4: Roy et al, 2004

Reference: Roy TS, Seidler FJ, Slotkin TA (2004). Morphologic effects of subtoxic neonatal chlorpyrifos exposure in developing rat brain: regionally selective alterations in neurons and glia. Dev Brain Res. 148(2): 197–206

Rats were administered either chlorpyrifos in DMSO (s.c.) at 5 mg/kg bw/d or vehicle on PND11 through to 14 (12 litters/group), and on PND15 (6 control pups and 4 treated pups, 1/litter) and PND20 (6 pups/group, 1/litter). Morphometric measurements were carried out using standard equipment and criteria for assessing brain tissue slides. Septal nucleus and striatum slides were selected at the level of caudal limit of the anterior commissure. Somatosensory cortex slides were selected from the level of the anterior commissure. Glial cells were assessed including microglia, astrocytes and oligodendrocytes.

Chlorpyrifos treated rats did not display gross or obvious morphological differences, however quantitative morphologic alterations in the three brain regions were reported. In the septal nucleus, chlorpyrifos increased the number of neurons on PN20. In contrast, the striatum showed a significant overall reduction in neuron numbers, whereas the somatosensory cortex was relatively unaffected. In the somatosensory cortex the relative distribution of large pyramidal neurons and smaller, non-pyramidal neurons was significantly different to controls at PN20 ($p < 0.001$). In the absence of a functional effect on the nervous system, the biological relevance of these changes is unclear.

Study 5: Aldridge et al, 2005

Reference: Aldridge JE, Levin ED, Seidler FJ, Slotkin TA (2005). Developmental exposure of rats to chlorpyrifos leads to behavioural alterations in adulthood, involving serotonergic mechanisms and resembling animal models of depression. Environ Health Perspect. 133(5): 527–531

Nine rats/sex/treatment group were used; with a maximum 1/sex/litter (36 pups total). Pups were randomly reassigned to dams and dams were rotated among litters. Litter sizes were culled to 10/litter, and weaned on PND21. On PND1–4, pups were exposed to subcutaneous injection of either 1 mg/kg bw/d chlorpyrifos dissolved in DMSO or DMSO only (control group). The timing of the dosing was selected to coincide with a heightened sensitivity to long-term effects on biomarkers of 5HT synaptic function.

All behavioural tests were performed during the normal dark phase (the most active period) although areas were lit so that rats could assess visual cues. Elevated plus maze test was performed at PND52–53. The proportion of time spent on the open arm was taken to indicate lower anxiety. The number of passages through the centre of the maze was counted as a measure of locomotor activity. Anhedonia was tested on PND54; rats could choose between bottles of water or chocolate milk, and the ratio of chocolate milk: water consumption was measured. Rats were trained on the 16–radial-arm maze for 5 weeks beginning on PND64 (week 9). During training, a food reward was always placed on the same 12 arms. Repeated entries into an arm from which the rat had consumed all the food was counted as an error of working memory, and entry into an arm that had never been baited was counted as a reference memory error. Response latency after release into the maze was also measured. During weeks 16–17, chlorpyrifos-treated and control rats were also administered with ketanserin 20 minutes before maze trials, at subcutaneous doses of 0, 0.5, 1 or 2 mg/kg bw/d in 1 mL/kg saline vehicle.

Chlorpyrifos-treated rats exhibited sex-related differences in the control group; males spent a shorter period of time in the open arms than females and control males were less active than control females (as assessed by the centre crossing). There was no significant difference in the time spent in the open arms or in the number of centre crossings of the elevated plus maze between control and treated females. Treated males spent more time in the open arms, and made a higher number of centre crossings in the elevated plus maze compared to control values. These changes brought the total measures for treated males to a similar number to those observed in treated females.

The control animals showed a preference for chocolate milk over water (both males and females). Chlorpyrifos treated animals showed a decreased preference for chocolate milk as compared to the control group, but there were no significant differences in total fluid consumption.

During training, control males showed fewer working and reference memory errors than control females ($p < 0.008$). Chlorpyrifos treated males showed an increase in working memory errors as compared to controls whilst chlorpyrifos treated females showed a decrease in working memory errors compared to controls. There was no significant difference in working memory errors between treated sexes; chlorpyrifos treatment removed the sex related differences in maze performance. The same effects were seen for reference memory errors; a sex related difference was observed in the control group with males showing fewer errors than females. There was an increased number of errors in the treated males and a decreased number of errors in females, and no significant difference between treated males and females. There was no treatment-related effect on latency (time/arm entry).

Ketanserin exposure was not associated with any changes to working memory error rates or reference memory error rates in the control group. Ketanserin exposure was reported to be associated with increased working memory and reference memory error rates in the chlorpyrifos treatment group. For reference memory errors there was a statistically significant increase as compared to controls at the 0.5 and 2 mg/kg bw doses of ketanserin. For working memory errors, a significant increase was observed at all three doses with clear dose-response relationship.

The study authors postulated that the results indicate that rats exposed to chlorpyrifos learn the radial arm maze via alternate non-cholinergic mechanisms and that 5HT may be involved in these adaptations. It was commented that the effects of the 5HT antagonist (ketanserin) on the chlorpyrifos treated rats demonstrate the reliance of the chlorpyrifos-treated rats on this alternative mechanism. In the absence of measurement of AChE activity, the biological relevance of these observations is unclear.

Study 6: Roy et al, 2005

Reference: Roy TS, Sharma V, Seidler FJ, Slotkin TA (2005). Quantitative morphological assessment reveals neuronal and glial deficits in hippocampus after a brief subtoxic exposure to chlorpyrifos in neonatal rats. Dev Brain Res. 155(1): 71–80

Rats were administered either chlorpyrifos in DMSO s.c at 5 mg/kg bw/d or vehicle on PND11 through to 14 (12 litters/group), and on PND15 (6 control pups and 4 treated pups, 1/litter) and 20 (6 pups/group, 1/litter). Morphometric measurements were carried out using standard equipment and criteria for assessing brain tissue slides. Morphometric measurements were carried out by a modification of the fractionator method. The first section was selected at random from all of the sequential sections cut within each designated region (5 μm thick). From the first section, every fifth section was evaluated (5 sections/animal). For each section, all the neuronal and glial profiles were counted. At least 100 cell profiles were evaluated for each region at a given age for every animal in both treatment groups. To standardize measurements, only neurons exhibiting a well-stained nucleus with a clear nucleolus were counted. The values obtained in a given animal were averaged (geometric mean) to produce a single number, so that the '*n*' in each case represents the number of animals, not the number of cells or sections.

Chlorpyrifos treated rats did not display gross or obvious morphological differences in corresponding hippocampal regions. Quantitative morphometry did identify significant differences from the control group. A significant overall reduction in the total number of neurons and glia was reported ($p < 0.05$) as well as a reduction in the layer thickness in all layers assessed ($p < 0.0003$). In addition, on PND20, an increase to the neuron/glia ratio ($p < 0.5$) indicative of selective gliotoxicity was evident. This was accompanied by perikaryal swelling and enhanced development of astrocytic processes.

It is noted that more detailed quantitative analysis of observed morphological changes would be required to fully evaluate potential treatment related effects. As a result, this study is considered to be of limited regulatory value.

Study 7: Guo-Ross et al, 2007

Reference: Guo-Ross SX, Chambers, JE, Meek EC, Carr RL (2007). Altered muscarinic acetylcholine receptor subtype binding in neonatal rat brain following exposure to chlorpyrifos or methyl parathion. Toxicol Sci. 100(1): 118–127

The study used analytical grade chlorpyrifos (> 98% purity) in corn oil administered by oral gavage. Each treatment group contained between 8–10 pups with an even distribution of sexes/group. The day of birth was designated PND0. The pups received (0.5 mL/kg bw) by oral gavage of:

1. control group—corn oil

2. chlorpyrifos low dose group—1.0 mg/kg bw/d from PND1–8

3. chlorpyrifos medium dose group—1.0 mg/kg bw/d from PND1–5 and 2.0 mg/kg bw/d from PND6–8

4. chlorpyrifos high dose group—1.5 mg/kg bw/d from PND1–5 and 3.0 mg/kg bw/d from PND6–8.

Pups were sacrificed at 4 hours post-treatment at PND4 or PND8.

During the study, the AChE activity and mAChR levels on PND4/PND8 were analysed, and mAChR levels were measured in the anterior forebrain (containing the cerebral cortex and corpus striatum), the posterior forebrain (containing the hippocampus), and the medulla/pons to determine the most vulnerable region of the brain.

Four radioligands including 3H-Pirenzepine, 3H-AF-DX 384, 3H-4-DAMP and 3H-QNB were used to measure the different receptor subtype densities to determine which were the most susceptible.

The study authors reported the following results:

- No clinical signs of toxicity were observed.

- There was a significant reduction in bodyweight gain (between 17–22% when compared to the control) within the high dose group on both PND4 and PND8.

- Chlorpyrifos significantly inhibited AChE activity on PND4 (between 17–45% within all dose groups; $\alpha = 0.05$). However, on PND8, a statistically significant inhibition of ChE activity was only seen in the medium and high dose groups (-47% and -65%, respectively)

- A significant decrease ($\alpha = 0.05$) in total mAChR binding was observed on PND4 in the high dose group (-14%) compared to control values

- In the anterior forebrain:

  - on PND4, there were no significant differences in the M1, M2/M4 or M3–subtype levels between control and chlorpyrifos treated rats; but there was a significant decrease in the total ligand binding (-14%)

  - on PND8, a significant decrease in all subtype levels was observed in the high dose group (between 12 to 14% compared to control)

  - on PND8, there was a significant decrease in total ligand binding in the medium dose group (-7%) and high dose group (-12%).In the posterior forebrain:

  - on PND4, there were no significant differences in the M1, M2/M4 or M3–subtype levels between control and chlorpyrifos treated rats; but there was a significant decrease in the total ligand binding (-17%)

  - on PND8 there were no significant differences in the M1, M2/M4–subtype levels between control and chlorpyrifos treated rats, but there was a decrease ($\alpha = 0.05$) in M3–subtype levels (-16%) and total ligand binding (-9%) in the high dose group

- In the medulla/pons:

  - on PND4, no significant effects were seen in the M1, M2/M4–subtype levels or total ligand binding, but a significant decrease in M3–subtype levels (-16%) was observed in the high dose group

  - on PND8, significant decreases were observed for the M1–subtype in the high dose group (-28%), the M2/M4–subtype for the low, medium and high dose groups (-9%, -12% and -17%, respectively), the M3–subtype in the low, medium and high dose groups (-15%, -16% and -17%, respectively), and in total ligand binding for the medium and high dose groups (-9% and -13%, respectively)

Study 8: Johnson et al, 2009

Reference: Johnson FO, Chambers JE, Nail CA, Givaruangsawat S, Carr RL (2009). Developmental Chlorpyrifos and Methyl Parathion Exposure Alters Radial-Arm Maze Performance in Juvenile and Adult Rats. Toxicol Sci. 109(1):132–42

Groups of SD rats were administered incremental doses of chlorpyrifos (< 99% purity) (or control) by oral gavage between PND1–20 (Table 2). Dosing was designed to avoid mortality and overt signs of toxicity but to maintain moderate levels of whole brain AChE inhibition throughout the exposure period.

Table 2:   Incremental Dose (oral gavage) regimen by treatment group

Treatment (mg/kg bw/d)	PND1–5	PND6–13	PND14–20
Control (*n* = 13F, 14M)	Corn oil	Corn oil	Corn oil
Chlorpyrifos low dose (*n* = 10F, 12M)	1.0	1.0	1.0
Chlorpyrifos medium dose (*n* = 10F, 12M)	1.0	2.0	4.0
Chlorpyrifos high dose (*n* = 11F, 12M)	1.5	3.0	6.0

PND: postnatal day, n: number; F = female, M = male

Hippocampal AChE activity was measured in male and female rats sacrificed on PND2, 30, 40, 50 and 60. Physical and reflex development, bodyweight pinna detachment, downy fur development, hair growth, incisor eruption and eye opening were measured from PND1 onwards. Reflex tests included surface righting, negative geotaxis, cliff avoidance, free fall righting, and acoustic startle. A twelve arm circular (radial) platform with 12 arms extending radially from the centre was used to test working and reference memory.

The behavioural experiments were conducted between PND29 and 60 to cover the juvenile and adolescent phases of development. Habituation occurred for 4 days from PND29. Training was conducted for 4 days per week for a total of 4 weeks (16 days total). Entry into an unbaited arm was scored as a reference memory error, entry into a baited arm was scored as correct and re-entry into a baited arm (after removal of the bait) was scored as a working memory error.

The authors reported the following results:

- No clinical signs of toxicity were observed in treated rats. There was a statistically significant decrease in bodyweight observed in the medium dose group on PND46 but in the absence of a dose-response relationship was not considered treatment-related.

- No significant effects were observed on developmental parameters and reflex development measures.

- Following exposure, hippocampal AChE activity was significantly inhibited in the low, medium and high dose groups (14%, 50% and 53%, respectively) on PND20. Decreased hippocampal AChE activity was measured at PND30 (17% and 23% for medium and high dose groups respectively) and PND40 (17% and 20% for medium and high dose groups respectively). Hippocampal AChE activities in chlorpyrifos treated rats were not significantly different from controls at PND50, 28 days post-dosing.

- Males in the control group appeared to learn the maze faster than females; a significant decrease in working memory errors was seen between the first and second week in males but improvement was seen in females between the second and third weeks. Similar results for reference memory were also noted in these groups; a significant decrease in reference memory errors was seen between the first and second week in males but improvement was seen in females between the second and third weeks.

- There was no effect on the working memory of females treated with chlorpyrifos.

- There was a treatment-related effect on the working memory of males; there was a greater number of working memory errors in high dose males during all four weeks of testing, and in low and medium dose males during the fourth (final) week of testing.

- There were changes to reference memory observed in females; significantly fewer errors were made in medium and high dose females during week two and in medium dose females during week 4. The medium and high dose females had a significant reduction in total reference memory errors.

- There were changes to reference memory observed in males; significantly more reference errors were made during the second week in medium and high dose males. The high dose males had a significant increase in total reference memory errors.

- Chlorpyrifos appeared to alter both short and long-term memory, a process that is known to require synthesis of new proteins and strengthening of neuronal connections.

The observation of deficits in learning and memory at the lowest dose tested in males led the authors to conclude that the mechanisms by which developmental OP exposure induces aberrant behaviour may include dysfunction of both cholinergic and non-cholinergic systems, and it is possible that these dysfunctions do not occur simultaneously. The authors do not explore alternative mode of actions, although it should be noted that learning and memory deficits were observed at doses which also resulted in reductions in hippocampal ChE activity.

Study 9: Lopez-Crespo et al, 2009

Reference: Lopez-Crespo GA, Flores P, Sanchez-Santed F, Sanchez-Amate MC (2009). Acute high dose of chlorpyrifos alters performance of rats in the elevated plus-maze and the elevated T-maze. Neurotoxicology 30(6): 1025–1029

Seventy-two male Wistar albino rats were exposed to 250 mg/kg bw chlorpyrifos or vehicle-only control in a single s.c. dose. For the plus-maze test, numbers of open and closed arm entries were counted. For the T-maze, two factors were measured: from a start in the closed arm, the time to leave was measured (avoidance latency; two trials named avoidance 1 or 2), and from the start in an open arm, the time spent to leave was measured (escape latency; one trial). Time spent on open arms was used as a proxy for lower anxiety, and time on closed arms for higher anxiety.

No information was provided regarding any clinical signs of toxicity and no AChE activity was recorded.

When rats were tested by elevated plus-maze 5 d after treatment, chlorpyrifos-exposed rats made significantly more entries into, and spent more time in, the open arms. Total locomotor activity was not affected. Increased open arm entries were taken to indicate lower anxiety.

When tested by elevated T-maze two days after treatment, avoidance latencies (time taken for the rat to leave a closed arm) were investigated. In the second avoidance latency test, treated rats exhibited higher latencies than the base-line trial ($p < 0.05$). Escape latencies in chlorpyrifos-treated rats were significantly higher ($p < 0.05$) indicating lower anxiety.

When the latency avoidance experiments were conducted on a second cohort 5 d post-treatment, the latencies of rats were lower for the second test of avoidance ($p < 0.05$) when compared to the first experiment. Control rat latencies increased with trials (avoidance 1 vs. baseline, $p < 0.05$; avoidance 2 vs. baseline, $p < 0.001$). No significant effects of trial or treatment were detected for escape trials.

Study 10: Middlemore-Risher et al, 2010

Reference: Middlemore-Risher ML, Buccafusco JJ, Terry AV (2010). Repeated exposures to low-level chlorpyrifos results in impairments in sustained attention and increased impulsivity in rats. Neurotoxicol Teratol. 32(4): 415–24

Rats (2 month old) were trained to perform the 5C-SRTT then treated subcutaneously with chlorpyrifos at a dose of 18 mg/kg bw/d for 14 days, or every second day for 30 days. Cohorts were either used in daily behavioural tests, which were conducted throughout the dosing period and 30 day washout period to assess recovery. Another cohort was euthanized 24 h after the last dose and subject to AChE assays. Rats were competent in the 5–SRTT prior to onset of the dosing regimen, as assessed by correct/incorrect responses, omitting responses and latency to collect rewards. Magazine latency and number of trials completed were also assessed, with no significant results reported. For AChE assays, blood serum was collected and brains were dissected into pre-frontal cortex, cortex, hippocampus, striatum and basal forebrain.

After 14 days, chlorpyrifos-treated rats were significantly less accurate than controls ($n = 8$–10). The effect was detected by post-hoc analysis for each day ($p < 0.05$) and persisted 19 days into the washout period. Chlorpyrifos treatment was associated with significant higher premature responses (impulsivity; % of responses made after trial begun but before light stimulus) during sessions 6, 18, 19, 20, and 21. Chlorpyrifos treatment also significantly decreased the percentage of perseverative responses (Compulsivity; % nose pokes made after correct response but before collecting reward) on sessions 6, 8, 9, 10, 11 and 12 ($p < 0.05$). Significant chlorpyrifos–related increases in the latency to correct (throughout treatment and washout period) and incorrect responses (through treatment period only) were also observed.

After 30 days of exposure every second day to 18 mg/kg ($n = 6$–8/session), a significant decrease in accuracy was observed in chlorpyrifos-treated rats. Post-hoc analysis indicated that chlorpyrifos-treated rats were significantly ($p < 0.05$) impaired (increases in omissions) relative to control rats during sessions 7, 11–42, 44, 47 and 57. For premature responses, an increased number of responses from session 21 to the end of the study were reported. For perseverative responses, significantly lower response rates were observed in treated rats during sessions 6, 10, 11 and 12, but there was an increase in the response rate during the washout period when compared to controls. The mean latencies to correct and incorrect responses increased during chlorpyrifos exposure which returned to pre-study levels during the washout period. Regarding stimulus durations, rats exposed to chlorpyrifos were significantly less accurate at 0.5 s, and a decrease in latency to collect reward was reported at 0.25 s; no other significant differences were noted in the latencies, omissions or measures in impulsive or compulsive behaviours.

Cholinesterase activity in plasma was decreased compared to control by 21% for daily chlorpyrifos treated rats and by 31% for second-daily chlorpyrifos treated rats. After 30 days of washout, levels recovered to 80–89% of controls. AChE activity in brain homogenates was significantly decreased in all brain regions for both treatment regimes. For daily treatment: pre-frontal cortex, 59.4% of control; cortex, 46.6%; hippocampus, 63.3%; striatum, 55.9%; and basal forebrain 23.5%. For second-daily treatment: pre-frontal cortex, 44.6% of control; cortex, 54.5%; hippocampus, 50.9%; striatum, 48.7%; and basal forebrain 25.9%. After washout period, only the striatum and basal hindbrain had a significant reduction in ChE activity when compared to control animals.

Study 11: Marty et al, 2012

Reference: Marty, MS, Andrus, AK, Bell, MP, Passage, JK, Perala, AW, Brzak, KA, Bartels, MJ, Beck, MJ, Juberg DR (2012). Cholinesterase inhibition and toxicokinetics in immature and adult rats after acute or repeated exposures to chlorpyrifos or chlorpyrifos-oxon. Regul Toxicol Pharmacol. 63(2): 209–224

Crl:CD(SD) rats were used: female-only adults (63 days old at start of study), as well as male and female pups (4 or 5 days old at start of study). Rats were either exposed to a single acute dose, followed by clinical observations for behavioural neurotoxicity, or repeated doses at the same level for 11 days, with evaluations of functional observational battery (FOB) and motor activity after the 10th dose. Further details of the dosing regimes are provided below. No treatment-related effects on neurobehavioural endpoints or bodyweight were detected.

Preliminary range-finding and time-of-peak cholinesterase inhibition studies were used to determine appropriate doses of chlorpyrifos and chlorpyrifos-oxon and post-exposure times to use in later experiments, based on RBC and brain ChE inhibition. Time-of-peak brain AChE inhibition in PND11 pups was 6 h after exposure to chlorpyrifos in corn oil, 8 h for chlorpyrifos in milk, and 4 h for chlorpyrifos-oxon in corn-oil. In adults, peaks were 8 h for chlorpyrifos in corn oil or diet and 4 h for chlorpyrifos-oxon in corn oil. These timepoints were used for all later cholinesterase activity measurements.

In the acute dose-response studies, 7–8 animals/dose were used for each study. Adult females were administered either chlorpyrifos (diet or by gavage in corn oil) at doses of 0, 0.05, 0.1, 0.5, 2.0 or 10 mg/kg, or chlorpyrifos-oxon in corn oil at doses of 0, 0.01, 0.05, 0.1, 0.5 or 1.0 mg/kg. Male and female PND11 pups were given chlorpyrifos by gavage in corn oil or milk at 0, 0.05, 0.1, 0.5, 2.0 or 5 mg/kg, or chlorpyrifos-oxon by gavage in corn oil at 0, 0.005, 0.01, 0.05, 0.1, or 0.5 mg/kg. RBC, brain and plasma cholinesterase activity were then measured at the times-of-peak inhibition points established earlier, in absolute terms (corn oil only) and relative to mean blood concentrations of chlorpyrifos, chlorpyrifos-oxon and 3,5,6-trichloro-2-pyridinol (TCP) (milk/diet and corn oil).

In adults, the highest dose of 10 mg/kg chlorpyrifos in corn oil (gavage) gave significant ChE inhibition in RBC (15.7% of control), brain (42.5%) and plasma (13.2%). At 2 mg/kg in corn oil, cholinesterase was significantly inhibited in RBC (80.6% of control) and plasma (45.8%). At 10 mg/kg in diet, rats exhibited significant inhibition of AChE in RBC (3.1% of control), brain (76.3%) and plasma (13.5%). At 2 mg/kg in diet, significant inhibition was seen in RBC only (47.7% of control), which was a greater effect than seen with the gavage dose. No significant effects were seen at lower doses by either intake route. Chlorpyrifos-oxon in corn oil did not inhibit brain AChE at any dose. At the highest dose of 1 mg/kg, significant inhibition of cholinesterase was seen in RBC and plasma (both 23.8% of control). At the second highest dose of 0.5 mg/kg, inhibition was 63.4% and 44.2% of control, respectively. No significant effects were seen at lower doses.

In pups, significant inhibition of cholinesterase was observed at 5 mg/kg in corn oil (RBC, 11.7–13.6% of control; brain, 44.5–49%; plasma, 13.2–22%) and at 2 mg/kg (RBC, 64.3–69% of control; plasma, 49–53%). For chlorpyrifos administered in milk, 5 mg/kg chlorpyrifos significantly inhibited cholinesterase in RBC (21.7–28.6% of control), brain (43.6–58.2%) and plasma (21.5–29.2%). In milk, 2 mg/kg chlorpyrifos significantly inhibited cholinesterase in RBC (females only: 72.7% of control) and plasma (56.5–61.1%). No significant effects were seen at lower doses. As with adults, chlorpyrifos-oxon in corn oil did not inhibit brain AChE at any dose. Cholinesterase activity was significantly inhibited in RBC at the highest dose of 0.5 mg/kg (52.9–53.8% of control) and in plasma (48.6–51.1%) and in plasma only at the second highest dose (0.1 mg/kg: 79.3–81.8% of control).

For chlorpyrifos, the NOAEL was 0.5 mg/kg bw for single acute chlorpyrifos exposure in adults and pups. For chlorpyrifos-oxon, the NOAEL was 0.1 mg/kg for RBC AChE inhibition in PND11 pups, and for RBC and plasma cholinesterase in adults (brain inhibition was not observed at any dose).

Blood analyses of adult rats (4/dose) indicated an approximate dose-proportional relationship for chlorpyrifos/chlorpyrifos-oxon and TCP (in corn oil or diet). Blood concentration of chlorpyrifos was detectible at doses greater than 0.5 mg/kg chlorpyrifos (in corn oil) or 2 mg/kg (in diet), while blood chlorpyrifos-oxon levels were at or below the level of quantitation for all chlorpyrifos and chlorpyrifos-oxon doses. Enzyme activities decreased non-linearly with dose as described above, with no significant effect of vehicle.

Blood analyses of pooled male and female PND11 pups (4/dose) indicated an approximate proportional relationship between chlorpyrifos dose and TCP, when exposure was by corn oil or milk. Blood chlorpyrifos levels was approximately dose-proportional at doses ≤ 2 mg/kg bw. Blood chlorpyrifos levels increased by 466–fold (corn oil) and 165–fold (milk) for a 100-fold increase in dose (0.5 to 5 mg/kg bw). Blood chlorpyrifos-oxon levels were not detectable at any oral dose of chlorpyrifos-oxon, and detectable only at doses of chlorpyrifos greater than 0.5 mg/kg (in corn oil) or 2 mg/kg (in milk). Enzyme activities decreased non-linearly with dose as described above, with no significant effect of vehicle.

Pups had higher blood concentrations of chlorpyrifos than adults at similar high doses (e.g. at 5 mg/kg bw, pups had 3–fold higher blood chlorpyrifos levels than adults administered 10 mg/kg bw). Pups had detectable levels of chlorpyrifos-oxon at these dose levels (5 vs. 10 mg/kg bw in adults) and lower levels of TCP (67% of adult levels at these doses). At a dose of 2 mg/kg bw, PND11 pups had 10–fold (chlorpyrifos) and 2-fold (TCP) higher blood levels than adults, indicating that pups were metabolizing proportionately more chlorpyrifos at this lower dose. TCP concentrations were higher in pups than adults for matched chlorpyrifos-oxon dose (significance not tested), although TCP increased at a lower rate than dosage increases in pups. When blood was sampled at the same time for adults and pups (4 h after 0.5 mg/kg bw dose), pups had 1.6–fold higher blood TCP levels than adults but similar levels of inhibition of RBC and plasma cholinesterase activities. At the lower dose of 0.1 mg/kg, pups had 1.7–fold higher TCP than adults, and significant inhibition of plasma ChE, while adults did not exhibit plasma ChE inhibition.

In the repeat-dose study, the same experiments were undertaken and the same parameters measured, after exposing rats to 11 repeated doses of chlorpyrifos or chlorpyrifos-oxon. For all rats, the dosing regimen was 0, 0.05, 0.1, 0.5, 1.0 or 3.5 mg/kg bw/d chlorpyrifos in corn oil, or 0, 0.01, or 0.5 mg/kg bw/d chlorpyrifos-oxon in by gavage in corn oil. Pups (8/sex/dose) were dosed from PND11–21 and adults (8/dose) from PND70–80 days old.

In adults, the highest dose of 3.5 mg/kg chlorpyrifos significantly inhibited cholinesterase E in RBC (activity 2.7% of control), brain (31%) and plasma (11.6%). Similar significant decreases were observed at 1 mg/kg (RBC, 27.2% of control; brain, 91.1%; plasma, 30.7%), with no brain AChE inhibition at 0.5 mg/kg bw/d (RBC, 80.5% of control; plasma 54.1%). Only the highest dose of chlorpyrifos-oxon significantly decreased cholinesterase activity in adults (RBC, 12.6% of control; plasma 24%).

In pups, there were no gender-specific effects reported. The highest dose of 3.5 mg/kg bw chlorpyrifos significantly inhibited cholinesterase in RBC (8.4–12.1% of control values), brain (32.1–41%) and plasma (20.8–28.7%). In adults, similar significant decreases were seen at 1 mg/kg bw (RBC, 38.7–56% of control; brain, 71.7–81%; plasma, 55.9–56.5%). No effect on brain AChE was observed at 0.5 mg/kg, while RBC cholinesterase activity was 62.2–81.8% of control; and plasma 71.3–76.8%). Again, as with adults, only the highest dose of chlorpyrifos-oxon significantly decreased cholinesterase activity in pups (RBC, 13.7–15.8% of control; plasma 38.3–39%). chlorpyrifos-oxon did not affect brain AChE activity at any dose tested.

After 11 repeated exposures, the NOAEL was 0.1 mg/kg bw/d chlorpyrifos in adults and pups based on the level of inhibition of RBC and plasma cholinesterase at the next highest dose. Inhibition of brain AChE activity was reported at 1 mg/kg bw/d in adults and pups. For chlorpyrifos-oxon, 0.01 mg/kg bw/d was the NOAEL in pups and adults based on significant inhibition of RBC and plasma cholinesterase E activity at 0.5 mg/kg bw/d (brain inhibition not observed at any dose).

Blood analyses of adult rats (4/dose) indicated an approximately proportional relationship between chlorpyrifos dose and blood TCP. Blood chlorpyrifos was detectable only at chlorpyrifos doses above 0.5 mg/kg, and blood chlorpyrifos-oxon was not detected. Only two doses of chlorpyrifos-oxon were used, and blood chlorpyrifos-oxon was not detected at either dose. TCP was approximately dose proportional (ie 62x TCP concentration increase for 50x increase in chlorpyrifos-oxon dose).

Blood analyses of pups were again pooled (4/sex/dose) and indicated an approximately proportional relationship of chlorpyrifos dose with blood TCP. Again, only two doses of chlorpyrifos-oxon were used, and blood chlorpyrifos-oxon was not detected at either dose. The concentration of TCP was less than dose proportional (ie 20x TCP concentration increase for 50x increase in chlorpyrifos-oxon dose). Blood TCP was therefore relatively higher (compared to chlorpyrifos-oxon) for adults at the dose of 0.5 mg/kg, and lower for pups at 0.01 mg/kg.

The NOAEL for chlorpyrifos was 0.5 mg/kg bw for acute doses, and 0.1 mg/kg bw/d for repeated doses, in both adults and pups. The NOAEL for chlorpyrifos-oxon was 0.1 mg/kg bw/d for adults and 0.05 mg/kg bw/d for pups. Chlorpyrifos-oxon did not inhibit brain AChE activity at any dose tested. The vehicle substance did exert an effect on the levels of chlorpyrifos and chlorpyrifos metabolites in blood. After acute exposure, pups had higher blood concentrations of chlorpyrifos and metabolites than adults at proportionately lower doses.

Study 12: Reiss et al, 2012

Reference: Reiss R, Neal B, Lamb JC, Juberg DR (2012). Acetylcholinesterase inhibition dose-response modeling for chlorpyrifos and chlorpyrifos-oxon. Regul Toxicol Pharmacol. 63(1): 124–131

The authors conducted their analysis in four steps; collation of dose-response data to be used for benchmark dosing (BMD), benchmark dosing to assess model fits, benchmark dosing of a number of different dose-response sets from different studies and selection of a point of departure for both acute and chronic assessment.

The BMD is defined as the dose that corresponds to a specific change in an adverse response compared to the response in unexposed subjects, and the lower 95% confidence limit is termed the benchmark dose level (BMDL).

The authors also considered the studies included in the United States Environmental Protection Agency (US EPA) Science Advisory Panel analysis of chlorpyrifos; however, they note that they did not have access to the primary data. BMDs were estimated using US EPA methodology for organophosphates as outlined in the OP cumulative risk assessment report (US EPA, 2006).

In pups: For acute BMD modelling, the available data sets included Marty et al (2012), Betancourt and Carr (2004), Zheng et al (2000), Moser et al (2006), and Timchalk et al (2006). These studies measured AChE at different PNDs (including PND1, 5, 11 and 17). The authors reported that the age of the pups is important relative to AChE activity. Measurements at PND1 were considered equivalent to human *in utero* exposure and measurement at PND17 was considered too far from PND11 to combine datasets, noting that the rodent CNS predominantly matures in the postnatal period and permeability of the BBB is known to decrease significantly around this period. On this basis only primary data from Marty et al (2012) were included in the BMD analysis for acute dosing to pups.

In adults: For BMD analysis of acute dose to adults, data were combined from Marty et al (2012) and Zheng et al (2000). The authors noted that the BMD for gavage administration of chlorpyrifos in corn oil were lower for brain AChE inhibition and higher for RBC AChE inhibition compared to diet. Brain AChE was regarded as the most toxicologically relevant endpoint and a measure of actual toxicity rather than a surrogate (such as RBC AChE). Due to the variability of RBC AChE, a 20% cut off for RBC is applied, while the cut-off for brain is 10% AChE for the purposes of assessing toxicological significance. These values are considered to be appropriate by the APVMA.

For pups exposed to repeated doses, the authors used only the BMD estimates from Marty et al (2012). The authors report a BMD_{20} (dose that cause 20% inhibition) for RBC AChE inhibition of 0.30 mg/kg bw/d and a $BMDL_{20}$ of 0.26 mg/kg bw/d. For brain AChE inhibition, BMD_{10} estimates were 0.58 mg/kg bw/d for males and 0.65 mg/kg bw/d for females. When data sets were combined for both sexes for brain AChE inhibition, the reported BMD_{10} was 0.62 mg/kg bw/d and the $BMDL_{10}$ was 0.46 mg/kg bw/d.

For adults exposed to repeated doses, the authors combined data sets from several studies (Marty et al, 2012; Zheng et al, 2000; Hoberman, 1998; and Mattsson et al, 1998; 2000).

The authors compared BMD and BMDL (at 20% inhibition) across these studies and then combined the datasets excluding data from Zheng et al (reason not provided) to estimate a BMD_{20} of 0.16 mg/kg bw/d ($BMDL_{20}$ 0.13 mg/kg bw/d). The lowest individual BMD_{20} of 0.12 mg/kg bw/d was obtained from Hoberman (1998).

Finally, the authors conducted a meta-analysis using the Marty et al (2012) data to determine BMDs for regulatory purposes. There was no statistical difference between pups and adults, so the meta-analysis was performed with the combined datasets. Table 3 summarises the results of the meta-analysis. BMD and BMDLs are as recommended by the authors for regulatory purposes.

Table 3: Study authors recommended Benchmark Doses of chlorpyrifos for regulatory purposes

Duration	Benchmark dose			
	RBC AChE inhibition		Brain ChE inhibition	
	BMD_{20}	BMDL	BMD_{10}	BMDL
Acute dose (mg/kg bw)	1.0	0.90	1.7	1.3
Repeat dose (mg/kg bw/d)	0.21	0.19	0.67	0.53

Study 13: Terry et al, 2012

Reference: Terry AV, Beck WD, Waner S, Vandenhuerk L, Callahan PM (2012). Chronic impairments in spatial learning and memory in rats previously exposed to chlorpyrifos or diisopropylfluorophosphate. Neurotoxicol Teratol. 34(1): 1–8

Male Wistar albino rats were exposed (s.c.) to a dose of chlorpyrifos at 10 or 18 mg/kg bw or vehicle (8–10 rats/dose). Doses were selected as sub-threshold levels for behavioural effects based on previous work in the same laboratory. Exposure took place every second day for 30 days, and was followed by a 50 day OP-free washout before spatial learning tests. Tests were: a) radial arm maze (RAM), comprising a habituation phase, acquisition/win-shift training (food reward shifts position), and delayed non-match to position (DNMTP) testing (food reward placed in unvisited arms of the maze after a lockout delay of varying time); b) Water maze repeated acquisition, comprising a hidden platform task and a visible platform task.

Rats were exposed to 18 mg/kg bw chlorpyrifos and tested for plasma and brain cholinesterase activities during the washout period, on days 0, 14, 30 and 45 after the last dose. On the final day of dosing (washout day 0), brain AChE was inhibited in chlorpyrifos treated rats to 50% of control, and plasma ChE to 20%. At day 14, brain AChE decreased to ~40% of control. By day 45, AChE had recovered to control levels in both brain locations.

RAM testing took place over 10 days, starting on day 50 of washout. For win-shift testing, there was a significant main effect of chlorpyrifos dose ($p < 0.03$) and treatment day ($p < 0.001$; ie learning did occur over the 10 days), with no day x dose interaction. Post-hoc analysis indicated that both doses of chlorpyrifos were associated with statistically significant impairment of performance on one or more days of testing (day 3 for 18 mg/kg group; days 10 and 11 for 10 mg/kg bw group). For the DNMTP testing, there was a significant main effect related to dose ($p < 0.04$) and delay in performing tasks ($p < 0.01$; ie longer delays decreased performance), without a dose x day effect. Post-hoc analysis indicated that both doses of chlorpyrifos were associated with significant impairment of performance following delays (15 min, 3 h and 6 h delays) for the 10 mg/kg bw dose group ($p < 0.05$), and after a 6 h delay for the 18 mg/kg bw dose group ($p < 0.05$).

The water maze test began on day 140 of washout and comprised two phases of 5 d sessions of the hidden platform task and two phases of 5 d sessions of the visible platform task. For the hidden platform task, all rats progressively learned to locate the hidden platform. In chlorpyrifos treated rats, there was a modest dose-related effect during phase one. On day one of phase one, latency to locate the platform was significantly higher for rats exposed to 10 mg/kg chlorpyrifos vs. controls ($p < 0.05$). On day five of phase one, latency was significantly higher for rats exposed to 18 mg/kg chlorpyrifos vs. controls ($p < 0.05$). No effects were seen in phase two. No effects were observed in rats performing the visible platform task, and swim speeds were not impaired by chlorpyrifos exposure.

Study 14: Ohishi et al, 2013

Reference: Ohishi T, Wang L, Akane H, Itahashi M, Nakamura D, Yafune A, Mitsumori K, Shitbutani M (2013). Reversible effect of maternal exposure to chlorpyrifos on the intermediate granule cell progenitors in the hippocampal dentate gyrus of rat offspring. Reprod Toxicol. 35: 125–136

Pregnant Crl:CD (SD) rats were exposed to chlorpyrifos in the diet at 0, 70 ppm (9.18 mg/kg bw/d), 14 ppm (1.86 mg/kg bw/d) and 2.8 ppm (0.36 mg/kg bw/d) (n = 8/dose). The lowest dose was estimated to be 500–fold higher than estimated consumption of chlorpyrifos by the general population in Japan. Exposure took place over GD10 to PND21. Pups were studied up to PND77. Effects of chlorpyrifos were determined by: physical parameters (weight, food consumption; external differentiation and organ weights of offspring); ChE activity; plasma concentration of thyroid-related hormones; behavioural parameters; and cell apoptosis, proliferation and morphometric analysis in the dentate gyrus.

Dams were weighed daily during gestation and every 3–4 days during lactation. Dams exhibited no significant effect of chlorpyrifos exposure during gestation, but during lactation, exhibited significant ($p < 0.05$) transient weight increases for the 14 ppm and 70 ppm chlorpyrifos exposed groups on PND11. Food consumption by dams increased significantly ($p < 0.05$) on GD14 and GD17 in the 14 ppm group and on GD17 in the 70 ppm group. Food consumption did not change during lactation. These marginal increases in bodyweight and feed consumption are not considered adverse.

Pups were weighed every 3–4 days from birth throughout the experimental period. Offspring from the 14 ppm treatment group exhibited significant ($p < 0.05$ to $p < 0.01$) increases in bodyweight compared to control, with timing of weight gain varying with sex. No significant increases occurred in the 2.8 or 70 ppm groups. No changes in food consumption were reported. Eruption of incisors was earlier in the 14 ppm group, and eyelid opening was earlier in the 14 ppm and 2.8 ppm groups. No other significant differences in external differentiation (pinna detachment, vaginal opening, balanopreputial gland cleavage) or organ weight was seen up to PND77. In the absence of a dose-response relationship, these findings were not considered treatment-related.

In dams, cholinesterase activity was examined on PND21, ie the last day of exposure (n = 8/dose). Significant inhibition was seen in the brain (~80%), RBC (~75%) and plasma (~75%) for the 70 ppm dose ($p < 0.01$), in RBC (~65%) and plasma (~60%) for the 14 ppm dose ($p < 0.01$), and the RBC (~15%) for the 2.8 ppm dose ($p < 0.05$). In male pups at PND21 (n=6/dose), significant inhibition ($p < 0.01$ or 0.05) of ChE was observed in the brain (~50%), RBC (~65%) and plasma (~65%) for the 70 ppm dose, and in plasma (~25%) only at the 14 ppm dose. No inhibition was induced by 2.8 ppm in male pups, or at any dose at PND77. Female offspring were not analysed.

At PND21, plasma concentration of thyroid related hormones T3, T4 and TSH was assayed in dams (n = 8), with only T3 having a significantly ($p < 0.05$) increased level in the 14 ppm group. Male offspring showed no significant changes. Female offspring were not analysed.

Both male and female offspring were subjected to behavioural tests. Observations of motor activity (8/sex/dose) over an hour showed a significant increase at 0–10 min ($p < 0.05$) and decrease at 40–50 min ($p < 0.01$) in males from the 14 ppm group. There were no statistically significant changes in the other dosage groups, or in females. In water-filled multiple T-maze tests, males (8/dose) from the 14 and 70 ppm dose groups exhibited a longer elapsed time in the straight course. There was also a statistically significant decrease in errors at the second trial on the second day in males of the 2.8 ppm group, but not in higher doses or in females. There were no treatment-related differences for any groups in sensory and reflex functional examinations, grip strength, detailed clinical signs or manipulative tests.

Neurogenesis was assessed by the distribution, proliferation and apoptosis of granule cell lineages in the sub granular zone (SGZ) of the hippocampal dentate gyrus and the distribution of reelin-producing interneurons in the hilus of the hippocampal dentate gyrus.

Apoptotic and proliferating cell indices in the dentate SGZ were assessed by immunohistochemistry for proliferating cell nuclear antigen (PCNA) and TUNEL-positive cells (8 males/dose/timepoint). On PND21, a statistically significant decrease was observed in the number of PCNA-positive cells in the 70 ppm exposure group. There were no statistically significant differences in PCNA- or TUNEL-positivity for the other dosage groups, or for any dosage at PND77.

Morphometric analysis of cells in the SGZ, dentate hilus and nicotinic acetylcholine receptor alpha 7 (NAChRα7)-positive and NeuN-negative cells in the SGZ were performed by immunohistochemistry on PND21 and PND77 ($n = 8$ males/dose/timepoint).

For cells in the SGZ: on PND21, in the 70 ppm exposure group a significant decrease ($p < 0.05$) was observed in the number of Tbr2–positive cells (Tbr2 is a marker for type-2 progenitor cells). There were no significant differences in the numbers of DCX- or GFAP-positive cells in any of the treatment groups at PND21, and no changes in Tbr2–, DCX- or GFAP-positive cells at PND77 (DCX is a marker for type-2b and type-3 progenitor cells and immature granule cells and GFAP is a marker for astrocytes and type-1 progenitor cells).

For cells in the dentate hilus, neither the number of cells expressing reelin (a glycoprotein critical for neuronal migration and positioning) nor NeuN (a marker for post-mitotic neurons) was significantly altered at any dose on PND21 or 77.

NAChRα7–positive/NeuN-negative cells were assayed in PND21 pups to examine the effects of cholinergic stimulation on neurogenesis in the SGZ, because NAChRα7 is a reported target of chlorpyrifos. NAChRα7–positive/NeuN-negative cells were sparsely distributed in the SGZ, and there were no treatment-related significant differences in their numbers.

Study 15: Vatanparast et al, 2013

Reference: Vatanparast J, Naseh M, Baniasadi M & Haghdoost-Yazdi H (2013). Developmental exposure to chlorpyrifos and diazinon differentially affect passive avoidance performance and nitric oxide synthase-containing neurons in the basolateral complex of the amygdala. Brain Res. 1494: 17–27

Pregnant female Wistar rats were treated with chlorpyrifos (s.c.) at 1 mg/kg bw/d on GD15–18. Control animals received equivalent injections of vehicle control (DMSO 1 mL/kg). Pups received 1 mg/kg bw/d s.c. on PND1–4. Pups were weaned at PND23 and behavioural evaluations conducted on at least 12 pups/sex.

The following behavioural tests were conducted; locomotor activity study (open field experiment on PND60), passive avoidance (PA) test and retention test (PND60–63 travel through dark to light compartment). Passive avoidance is an indicator of fear memory in rats. The animals learn to avoid a specific place, in this case, a dark area with aversion stimulus (electrical pulse to foot). The basolateral complex (BLC) of the amygdala, is thought to be central to learning fear-motivated tasks, such as passive avoidance (PA). The BLC is also thought to be indirectly involved with memory consolidation, an important aspect of PA. Nitric oxide (NO) has a well-known role at the cellular level for memory consolidation. NO is synthesised *in vivo* by nitric oxide synthase, an enzyme that has three isoforms. The inhibition of neuronal NO synthase (nNOS), one isoform, is thought to inhibit learning and induce memory impairment. The study authors evaluated the effects of exposure on PA and nNOS expressing neurons in the BLC of amygdala (via a NADPH-d positive/nNOS-immunoreactive staining assay) in 4 rats/sex on PND60. ChE inhibition was not measured.

The authors reported the following findings:

- No treatment-related effects on locomotor activity occurred.

- Prenatal and postnatal exposure to chlorpyrifos had no effect on the acquisition of PA. Prenatal exposure to chlorpyrifos did show a reduction in PA retention with increases in time spent in the dark, but not for postnatally exposed rats. The finding that postnatal chlorpyrifos exposure did not affect PA retention was in contrast to effects reported in other studies that report effects on spatial learning and memory (Levin et al, 2001).

- Prenatal exposure to chlorpyrifos did not affect cell numbers in the lateral (LM) or basomedial (BM) amygdala, but the basolateral (BL) amygdala neural count was significantly higher than the controls. Conversely, postnatal exposed rats had a significant loss of neurons in the BL amygdala, but there were no effects on LM, BM or PA performance.

Study 16: Chen et al, 2014

Reference: Chen WQ, Zhang YZ, Yuan L, Li YF, Li J (2014). Neurobehavioral evaluation of adolescent male rats following repeated exposure to chlorpyrifos. Neurosci Lett. 570: 76–80

Male SD rats (12/dose) were treated with chlorpyrifos (purity not stated) in olive oil subcutaneously at 0 (olive oil only), 2.5, 5, 10 or 20 mg/kg bw/d for 10 consecutive days from PND27–36. The authors state that this is the 'accepted adolescent period in rats'.

Behavioural assessments included:

1. Forced swimming test (FST) (PND37–38): Rats were placed in a cylinder of warm water and their immobility time was measured.

2. Open field test (PND43): This measured locomotor activity as the number of crossings and rearings in a standard arena over 5 min.

3. Novelty-suppressed feeding test (NSFT) (PND44–46): No food was provided to rats for 48 h, then a food pellet was placed in a novel (specifically designed) plastic box 76 cm square, and the time taken for the rats to begin to eat was measured (feeding latency).

4. Learned helplessness (LH) test (PND48–52): This involved applying computerised electric shocks on the grid floor of a modular shuttle box. Helplessness induction on day 1 of this test delivered an inescapable shock and this was followed over 4 consecutive days by conditioned avoidance trials. The number of avoidance and escape responses was recorded automatically, and the number of escape failures was stated by the authors to be a measure of helplessness behaviour.

There was no positive control in this study, and it is not clear whether the methods had been validated previously by the study authors.

There were no cholinergic signs observed during the study. In the FST, there was a significant increase in immobility time at 10 mg/kg bw/d only, and no effect was evident at 20 mg/kg bw/d. In the absence of a dose-response relationship, this finding was not considered treatment-related. No differences in locomotor activity between treated and control groups were reported by the study authors in the open field test. The NSFT test results were inconsistent, with the lowest dose showing increased feeding latency time, while the latency at the 20 mg/kg dose was lower than the control. In the LH test, chlorpyrifos induced a significantly increased number of escape failures, which appeared to be dose related, although control rats had higher numbers of escape failures on day 5 compared to those from the lowest dose group (5 mg/kg bw/d). On day 5 of the test, the number of escape failures was 6.9, 3.8, 13.2, 15.6 and 19.3 at 0, 5, 10 and 20 mg/kg bw/d, respectively. A similar trend was also noted on earlier days (3 and 4).

The study authors concluded that the results support 'the epidemiological findings that pesticide-exposed populations are susceptible to depression'. However, the mixed results among the tests are not conclusive, hindered by the lack of a positive control and insufficient evidence of the use of validated methods. As such, this study is considered to be of limited regulatory value.

Study 17: Levin et al, 2014

Reference: Levin ED, Cauley M, Johnson JE, Cooper EM, Stapleton HM, Ferguson PL, Seidler FJ, Slotkin TA (2014). Prenatal dexamethasone augments the neurobehavioral teratology of chlorpyrifos: significance for maternal stress and preterm labor. Neurotoxicol Teratol. 41: 35–42

On GD17, the animals were randomly assigned to four different treatment groups comprising 12–14 dams involving the following dosing regimes:

1. controls (prenatal saline + postnatal DMSO)

2. dexamethasone (prenatal dexamethasone + postnatal DMSO)

3. chlorpyrifos (prenatal saline + postnatal chlorpyrifos)

4. combination (prenatal dexamethasone+ postnatal chlorpyrifos).

On GD17, 18, and 19 the groups received s.c of saline vehicle or dexamethasone. Parturition occurred on GD22 which was taken as PND0. The litter sizes were culled to 10 (5/sex) and pups were randomised within the treatment groups to dams with the same treatment as the birth dam to ensure standard nutrition.

Chlorpyrifos (> 98% pure, in DMSO) or vehicle control (DMSO) was administered subcutaneously to pups at 1 mg/kg bw/d from PND1–4.

Rats were subject to a number of behavioural tests covering a wide range of behavioural functions including cognition (novel object recognition test), emotional response (novel environment feeding test) and spontaneous behaviour (Figure-8 apparatus locomotor and T-maze exploration tests). All rats were tested in the same sequence of tests and at least a week interval was allowed between tests to minimise carryover behavioural effects.

The tests were carried out in the following order:

1. T-maze exploration (PN week 4)

2. figure-8 apparatus locomotor test and its habituation (PN week 5)

3. novel environment feeding test (PN week 7)

4. bovel object recognition test (PN week 9).

The study authors indicated that due to the reported effect of chlorpyrifos on thyroid structure and circulating T4 levels (De Angelis et al, 2009), chlorpyrifos concentrations in the brain of rats at PND4 and PND150 were analysed by selecting rats on those days (8–12 rats/group). For PND150, only the cerebellum was sampled, while the whole brain was analysed on PND4.

Dexamethasone pre-treatment caused a significant reduction in weight gain in the pregnant dams (10%) compared to control animals, as well as up to a 15% reduction in offspring weight during the postnatal period.

No significant effect of chlorpyrifos was found in the T-maze exploration test between the chlorpyrifos only treatment group (27.3 ± 0.9 s/trial) or chlorpyrifos/dexamethasone (24.8 ± 0.9 s/trial) and control group (27.1 ± 0.7 s/trial).

The mean activity level over the Figure-8 Maze test showed a significant main effect of treatment × sex interaction ($p < 0.01$), thus the study authors considered males and females separately. In males, chlorpyrifos treatment alone was found to evoke significant locomotor hyperactivity ($p < 0.05$) in the Figure-8 maze test compared to controls. In females, locomotor activity was not significantly affected by chlorpyrifos only treatment. However, females were found to be significantly more active than males ($p < 0.0005$) in the control group. Treatment of chlorpyrifos alone was reported to reduce this sex difference in locomotor activity, however the significance of this observation was not reported.

Four measures were taken for the Novelty-Suppressed Feeding test—latency to begin feeding, amount of food eaten, number of feeding bouts and time spent feeding. No significant effects on the measures were reported for the chlorpyrifos only treatment group. However combined treatments resulted in significant effects on the feeding test results.

All rats were reported to spend a greater amount of time investigating the novel vs. familiar object ($p < 0.0005$), validating the Novel object recognition test. The authors also found a significant interaction of treatment and sex ($p < 0.05$) and therefore carried out separate analysis for males and females. Males in the chlorpyrifos only treatment group exhibited a significant increase in time spent exploring the objects ($p < 0.05$), while females were reported not to exhibit any significant effects for all treatments.

No significant differences were reported for brain T4 levels between the chlorpyrifos treatment group and the control group on PND4 or PND150.

Given the subcutaneous exposure route and the pre-existing reported sexual differences in activity of the control group, this study is considered to provide only limited evidence to contribute to the overall weight of evidence.

Study 18: Grabovska and Salyha, 2015

Reference: Grabovska S, Salyha Y (2015). ADHD-like behaviour in the offspring of female rats exposed to low chlorpyrifos doses before pregnancy. Arh Hig Rada Toksikol. 66(2): 121–7

Three groups of adult female Wistar rats ($n = 3$) received doses of chlorpyrifos (99.9% pure in refined sunflower oil) using an oral probe at 5 (test group 1), 10 (test group 2) or 15 mg/kg bw/d (test group 3) for 30 consecutive days. All rats were weighed, numbered and kept under standard conditions for four months before pregnancy. A fourth group ($n = 3$) was administered a single dose of 30 mg/kg bw chlorpyrifos on GD6 which is noted to have been used as a subtoxic dose that does not cause immediate symptoms but can lead to adverse effects in a number of studies. A fifth control group of females received pure sunflower oil. No information has been provided on feed consumption or on mating procedures.

Groups 1, 2, 3 and control had one pregnancy in each group which resulted in 14, 12, 10 and 13 pups respectively. Group 4 had two pregnancies resulting in 21 (12 + 9) pups.

On PND21, pups were divided into experimental groups and housed in separate cages and numbered. At two months of age, the offspring were subjected to the following behavioural tests:

- Open field test—used to assess emotionality, exploratory activity and anxiety rate. One rat was placed in the test area at a time for 3 minutes with behaviour recorded. The testing area was cleaned after each test to avoid influence of other animals. These tests were repeated 10 days after the first testing.

- Dark/light box test—behavioural test to assess emotionality, exploratory activity and anxiety rate. The authors used the apparatus as per Bourin and Hascoe (2003). Each rat was put into the light chamber for 3 minutes and its behaviour observed.

- Extrapolation escape test—used to assess cognitive function. One rat was placed in the test area at a time and subsequently placed in the test area again 30 minutes after the first test. These tests were repeated 10 days after the first testing.

Signs of maternal or pup toxicity were not reported in the study.

The authors reported significantly higher outer horizontal (49.00 ± 4.69 s) and vertical (14.75 ± 0.85 s) activity and hole sniffing for the 15 mg/kg bw/d chlorpyrifos group in the open field test compared to control (16.13 ± 3.27 s, 10.00 ± 2.21 s and 0.25 ± 0.16 s respectively). In the repeated open field test, the 15 mg/kg bw/d chlorpyrifos group was also reported to show significantly lower anxiety rate (long and short grooming, defecation and freezing). Significantly higher outer horizontal activity (42.50 ± 6.50 s), vertical activity (12.75 ± 2.06 s), long grooming (0.25 ± 0.25 s) and hole sniffing (2.50 ± 1.04 s) were reported for the 15 mg/kg bw/d chlorpyrifos group in the repeat test. No freezing, defecation or short grooming was observed for the 15 mg/kg bw/d chlorpyrifos group in the repeat test which was significantly different to the control values (0.63 ± 0.26 s, 1.88 ± 0.77 s and 66.13 ± 19.45 s respectively). Considering the absence of freezing, defecation and short grooming, the author's state that the results suggest hyperactivity disorder and that OP pesticide exposure of the dam may lead to ADHD in children. No significant results for other groups were reported for the open field test.

The 15 mg/kg bw/d chlorpyrifos group were also reported to exhibit significant difference in the number of peeks out of the hole (6.25 ± 0.63 s), compared to control (1.75 ± 0.67 s) for the dark/light box test. The authors state that other differences between the treatment groups were not significant.

For the extrapolation escape test, the 10 mg/kg bw/d chlorpyrifos (successful attempts ratio: 0.43 ± 0.20) and the 30 mg/kg groups (successful attempts ratio: 0.41 ± 0.15) were reported to show significantly fewer successful attempts than controls (successful attempts ratio: 0.75 ± 0.16) in the repeat tests. In the first test, the 5 mg/kg bw/d group was reported to display motor hyperactivity.

It is noted that the number of offspring that underwent each test was not reported. While clinical signs of toxicity were not reported, the authors note a high offspring mortality, with only four offspring in the 15 mg/kg bw/d chlorpyrifos group reaching maturity and being tested. Further, the authors' state that data within groups were variable, leading to high statistical error and non-significant results. Based on the inconsistent reporting of study parameters, and lack of consistency of results, this study is considered to be of limited regulatory value.

Study 19: Lopez-Granero et al, 2013

Reference: Lopez-Granero C, Canadas F, Cardona D, Yu Y, Gimenez E, Lozano R, Avila DS, Aschner M, Sanchez-Santed F (2013). Chlorpyrifos-, Diisopropylphosphoro fluoridate-, and Parathion-Induced Behavioural and Oxidative Stress Effects: Are They Mediated by Analogous Mechanisms of Action? Toxicol Sci. 131(1): 206–216

Rats were administered a single subcutaneous dose of 250 mg/kg bw chlorpyrifos, or corn oil vehicle control. Water maze (tests spatial training and reference memory for spatial cues) and locomotor testing (vertical/horizontal movements) were performed.

Cholinergic signs were not observed for any OP. Chlorpyrifos treatment resulted in weight loss at 48 h, and decreased vertical movements and distance travelled at 24 h. These decreases were significant ($p < 0.05$).

A water maze was used to evaluate spatial learning. Rats ($n = 79$) were treated with chlorpyrifos (17–21/group) and subject to spatial training and testing after 72 h, comprising a spatial task, a probe test, reinstatement task, reversal task, and a visual task, in the same order. Tasks were undertaken over a period of eight days.

No treatment-related effects related to chlorpyrifos exposure were noted for most of the behavioural tasks, with the exception of the reversal task, for which a significant group effect for chlorpyrifos treated rats was observed ($p < 0.05$): these rats displayed higher escape latencies than controls. This was reported for the other OPs. For the visual task, no significant differences were observed.

Induction of oxidative stress in the hippocampus was evaluated by measurement of lipid peroxidation products, F2–IsoPs, at 1, 3, 24, 48 h or 11 d after exposure to the four treatments ($n < 10$ for all timepoints). The post-11 day animals were sourced from the maze trials. For chlorpyrifos treated rats, levels of F2–IsoPs were elevated significantly overall ($p < 0.05$), with increases for all timepoints ($p < 0.05$, except for 11 d, $p < 0.01$). To evaluate pro-inflammatory response, PGE2 was evaluated in the samples. All OP treatments resulted in significantly elevated PGE2 levels at 11 d compared to controls ($p < 0.05$). Data for other timepoints were not reported.

There were correlations between escape latencies for spatial training and F2–IsoPs, but not PGE2, for the second ($r = 0.605$, $p < 0.01$), third ($r = 0.370$, $p < 0.05$) and fourth sessions ($r = 0.348$, $p < 0.05$).

Soluble- and particulate-specific AChE activity and Acylpeptide hydrolase (APH) activity was also measured 3 h, 24 h and 48 h after OP exposure. Chlorpyrifos ($p < 0.05$) exposure induced significant inhibition of soluble and particulate AChE activity at all of the timepoints ($p < 0.05$). APH activity was not affected. No significant differences in relative abundance of forms of AChE were observed for any treatment or timepoint.

The effect of OP treatments on alternative splicing of AChE mRNA was also investigated. Levels of AChE-R 'read-though' soluble variant and AChE-S 'synaptic', membrane-bound or soluble variant mRNAs were measured. No changes to mRNA expression were observed for chlorpyrifos treated rats.

Guinea pigs

Study 1: Mullins et al, 2015

Reference: Mullins RJ, Xu S, Pereira E, Pescrille JD, Todd SW, Mamczarz J, Albuquerque EX, Gullapalli RP (2015). Prenatal exposure of guinea pigs to the organophosphorus pesticide chlorpyrifos disrupts the structural and functional integrity of the brain. Neurotoxicology 48: 9–20

The study involved administration of a daily s.c. dose of 25 mg/kg bw chlorpyrifos dissolved in peanut oil, for 10 consecutive days to pregnant dams during GD50–60 ($n = 4$). The dose used in this study was approximately 20 times below the oral LD50 of chlorpyrifos (504 mg/kg) for guinea pigs. Based on the findings from a previous study by Shih and McDonough (1997), the cumulative dose was stated to be below the threshold for reliable OP-induced seizure in rats. The control group received a single daily dose of 0.5 mL/kg peanut oil ($n = 5$) for 10 days. No positive control group was included in the study. After chlorpyrifos treatment, potential signs of toxicity were monitored every 15 minutes for two hours and then every hour for the next eight hours.

The guinea pigs were born on GD67–72 and weaned at PND15–20. As noted by the authors, previous investigations have shown that chlorpyrifos-induced cognitive deficits were more prominent in females than in male offspring (Levin et al, 2002; Haviland et al, 2010). For this reason, only female young adult guinea pigs underwent behavioural testing and magnetic resonance imaging scans (MRI). It is noted that the current study was not double-blinded.

The investigators employed the widely used Morris Water Maze test to examine the cognitive behaviour of the guinea pigs at PND40–45 (de Groot et al, 2001; Dringenberg et al, 2001; Filliat et al, 2002; Byrnes et al, 2004; Lewejohann et al, 2010). In this test, both control and exposed groups were trained to find the platform in four 90 second trials with 15 second inter-trial intervals for five days. *In vivo* MRI was used to assess the structural integrity of the brain performed in anaesthesised animals at PND65–76. The investigators used a small animal MRI which allows for higher magnetic field strength and higher spatial resolution (Pirko et al, 2005). Analyses were conducted using three acceptable MRI parameters: conventional T2–weighted images (volumetric measurement of the forebrain); diffusional kurtosis imaging[1] (fractional anisotropy[2] and diffusivity—white matter integrity); and kurtosis metrics[3] (cellular microstructure integrity).

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[1] Kurtosis imaging—An advanced neuroimaging technique that estimates the kurtosis of the water diffusion probability function. [2] Fractional anisotropy—A measure of white matter integrity. [3] Kurtosis metrics—A statistical measure of whether the data are peaked or flat relative to a normal distribution. If kurtosis = 0, means uniform distribution of water diffusion. If kurtosis > 0, means non-uniform distribution of water diffusion.

To confirm the MRI findings, the investigators performed Luxol Fast Blue staining of brain cryosections following the completion of the behavioural testing and imaging. This histological technique is commonly used to visualise myelination.The authors reported that the learning index (mean escape latency of the five days training) of the young adult guinea pigs prenatally-exposed to chlorpyrifos ($n = 10$) was significantly longer compared with controls. The learning ability of these animals to escape onto the hidden platform was significantly impaired. Chlorpyrifos treatment caused a significant reduction in the forebrain volume, including parenchymal and internal cerebrospinal fluid (4.9%; $p = 0.004$) and striatal volume (8.3%; $p = 0.005$) compared to control animals, however, the biological relevance of this finding is unclear.

In animals prenatally exposed to chlorpyrifos ($n = 10$), the corpus callosum showed significant reduction in fractional anisotropy ($p = 0.014$) and in the following kurtosis measures: mean kurtosis ($p = 0.009$); axial kurtosis (parallel to direction of axon) ($p = 0.008$); and radial kurtosis (perpendicular to the direction of the axons) ($p = 0.023$). Reductions in these parameters are generally associated with disrupted white matter integrity. The study authors report that the axonal integrity or myelination of the striatum and amygdalae of the chlorpyrifos-exposed guinea pigs appeared to be compromised. In the striatum, fractional anisotropy was reduced ($p = 0.006$), while other diffusivity measures such as mean diffusivity, radial diffusivity (measure of myelin integrity), and radial kurtosis were reported to increase ($p = 0.012$, $p = 0.002$, $p = 0.048$). In the amygdalae, fractional anisotropy was also reduced ($p = 0.002$), and the mean and radial diffusivity measures were increased ($p = 0.024$, $p = 0.002$).

Compared with controls ($n = 6$), the intensity of the Luxol Fast Blue staining was significantly lower ($p < 0.05$) in the lateral nucleus of the amygdalae of the guinea pigs prenatally-exposed to chlorpyrifos ($n = 7$), suggesting reduced myelination.

Consistent with these findings, the investigators reported a positive significant correlation between the forebrain volume and fractional anisotropy measures ($r = 0.462$; $p = 0.041$), mean kurtosis ($r = 0.041$; $p = 0.046$), and axial kurtosis ($r = 0.475$; $p = 0.034$). Additionally, the mean escape latency and the striatal mean and radial diffusivity were positively correlated ($r = 0.535$, $p = 0.015$; and $r = 0.497$, $p = 0.026$, respectively). In the amygdalae, the escape latency was positively correlated with the mean diffusivity ($r = 0.494$; $p = 0.027$) and radial diffusivity ($r = 0.560$; $p = 0.010$), and negatively correlated with fractional anisotropy ($r = -0.520$; $p = 0.012$), mean kurtosis ($r = -0.473$, $p = 0.035$) and axial kurtosis ($r = -0.552$, $p = 0.012$). Negative correlations were reported between the mean escape latency and the diffusional kurtosis imaging analysis of the corpus callosum.

2.3   Epidemiological studies

This review considers epidemiological research from three prospective birth cohort studies, as well as results from two smaller cohorts. These cohorts include:

- the Mothers and Newborn Study of North Manhattan and South Bronx performed by the Columbia Children's Centre for Environmental Health at Columbia University, known as the CCCEH Cohort

- the Mount Sinai Inner-City Toxicants, Child Growth and Development Study, known as the Mount Sinai Cohort

- the Center for Health Assessment of Mothers and Children of Salinas Valley conducted by researchers at University of California Berkeley, known as CHAMACOS Cohort.

The cohort studies examined pregnant women for possible statistical associations between *in utero* exposure to chlorpyrifos and adverse neurodevelopmental outcomes of their children.

CHAMACOS Cohort

Study 1: Eskenazi et al, 2004

Reference: Eskenazi B, Harley K, Bradman A, Weltzien E, Jewell NP, Barr DB, Furlong CE, Holland NT (2004). Association of *in utero* organophosphate pesticide exposure and fetal growth and length of gestation in an agricultural population. Environ Health Perspect. 112–10: 1116–1124

Pregnant women constituting the CHAMACOS cohort were recruited within an agricultural community in the Salinas Valley of California. Eligible women were over 18 years old, less than 20 weeks pregnant at enrolment, English or Spanish speaking, Medi-Cal eligible; and planning to deliver at the Natividad Medical Center, a county hospital located in the town of Salinas. Of 1,130 eligible women, 601 (53.2%) agreed to participate in the study. After losses due to miscarriage, moving, or dropping from the study before delivery, birth weight information was available for 538 women. Among these women, several were excluded from the study for various reasons including: presence of diabetes (26), hypertension (15), twin birth (5) or stillbirth (3). Another woman was excluded for out of range birth weight (less than 500 g). The final study sample included 488 women.

The study authors reported statistics on socio demographic and medical characteristics of the study sample. These data were sourced through three interviews (two during pregnancy and one shortly after delivery) and from medical records. The reported data included the women's age (average of 25 years old), parity (67% of the sample were multiparous), marital status (80% married or living as married), education (79% had not graduated from high school), preferred language (88% preferred Spanish), country of birth (84% born in Mexico), family income (compared to US federal poverty thresholds, > 60% of the women were below the poverty threshold and > 96% were below twice the threshold), drug use during pregnancy (6% smoked, 2% used unspecified drugs, 1% drunk alcohol), body mass index related statistics were tabulated (21% overweight or obese; 38% underweight, 41% normal weight), history of previous pregnancies; and working status during pregnancy (28% worked in the fields and another 14% had other jobs in agriculture, 85% had agricultural workers living at home during their pregnancy).

Exposure to OP pesticides was assessed by measuring biological parameters: (i) organophosphate dialkyl phosphate metabolites in maternal urine during pregnancy, (ii) seven different pesticide-specific metabolites in maternal urine during pregnancy, including TCP, which is specific for chlorpyrifos and chlorpyrifos methyl; and (iii) cholinesterase (ChE) in whole blood and butyrylcholinesterase (BChE) in plasma. All analyses were conducted according to published protocols, or similar procedures. Samples for ChE and BChE measurement were collected from mothers (at the time of two interviews during pregnancy, and at delivery); and from the umbilical cord collected at delivery. The first and second interview and blood sampling occurred between gestation week 4 to 29 (mean of 13 weeks) and between gestation week 18 to 39 (mean of 26 weeks), respectively. The rationale to analyse ChE and BChE was to provide an indirect estimation of organophosphate exposure, as high doses of organophosphate pesticides (and/or of carbamates) are known to depress these enzymatic activities. It is noted that there are several limitations with the data reported, which add uncertainty in considering potential neurodevelopmental effects of chlorpyrifos exposure. The authors did not discuss the passage of OP across the placental barrier, or how measurement of OP metabolites in the mother's urine relate to *in utero* exposure. The only data which appear directly relevant to *in utero* conditions are the measurement of ChE and BChE in the umbilical cord. These measures may not be reflective of OP exposure, as it is noted that 'n-Methyl carbamate use

in Monterey County in the year this study was conducted exceeded 100,000 lb (California EPA, 2002) and may be a major contributor to ChE levels in this population'.

The total concentration of dialkyl phosphate metabolites in maternal urine was used to estimate OP exposure. For data analyses, the two measures of each urinary metabolite were averaged for each woman. It is noted that there was large within person variability reported by the authors, to the extent that the two separate measurements were not correlated. The use of means is this instance may introduce bias and the subsequent statistical analysis conducted by the authors is considered to be questionable.

Only values relevant to chlorpyrifos (ie TCP values) are reported in this evaluation. TCP was measured in the urine of 482 women, its concentration was found above the limit of detection (0.26 µg/L) in the urine of 76.3% of these women. Measures performed on urine samples collected at pregnancy interviews were used to calculate average TCP concentrations for each woman. The median TCP value was 3.3 µg/L and the range was 0.2 to 56.1 µg/L. Based on their average value, women were assigned to one of three groups: no detectable levels (control group, 41 women), detectable levels below the median (220 women), and detectable levels above the median (221 women).

Offspring related parameters measured included infant birth weight, crown-heel length, head circumference, ponderal index (ratio of birth weight to birth length), and length of gestation.

Linear regression models were used to test for associations between exposure measurements and length of gestation, birth weight, body length, head circumference, and ponderal index. The study authors also investigated possible correlations between exposure measurements and low birth weight (< 2500 g), preterm delivery (<37 weeks gestation), and births considered to be small for gestational age (SGA).

No adverse associations were found between TCP and parameters of fetal growth or length of gestation.

A small positive correlation was found between dialkyl phosphate metabolite levels during pregnancy and ChE activity in maternal blood just before delivery and in the umbilical cord. The authors commented that this finding was unexpected, and that 'the absence of a negative correlation between dialkyl phosphate metabolites and ChE is perhaps partially caused by substantial measurement error in both measures'. It is noted that correlations between OP measures and *in utero* were tested with statistical analyses, but further details are scarce. As such, there is a great deal of uncertainty associated with the conclusions. This is largely due to questionable treatment of raw data and preparation for analyses, and the lack of detail provided regarding the techniques used for statistical analyses.

The authors found that decreases in gestational duration were associated with OP pesticide exposure (ie dimethyl phosphates, but not diethyl phosphates) and whole blood ChE activity. The clinical significance of the finding is not clear, considering the rate of preterm delivery in the study cohort (6.4%) is lower than that reported for Mexican-born women in the United States (10%). The authors concluded that the results of this study failed to demonstrate an adverse relationship between fetal growth and *in utero* OP pesticide exposure. In fact, increases in body length and head circumference were reported for some of the pesticides.

Study 2: Eskenazi et al, 2007

Reference: Eskenazi B, Marks AR, Bradman A, Harley K, Barr DB, Johnson C, Morga N, Jewell NP (2007). Organophosphate Pesticide Exposure and Neurodevelopment in Young Mexican-American Children. Environ Health Perspect. 115(5): 792–798

Participants were from the CHAMACOS cohort, for which mother's recruitment and selection up to delivery has been described elsewhere (see Study 1: Eskenazi et al, 2004). In the Eskenazi et al (2007) study, children and their mothers were tested for an additional 24 months post-delivery. The authors indicated the number of children were further excluded from the study based on various criteria, such as the lack of a neurodevelopmental assessment (71), lack of dialkyl phosphate (DAP) metabolites measurement (3), not being singleton (8), or due to a medical condition (3; Down syndrome, deafness, hydrocephalus). It is noted that there are some inconsistencies with the number of mothers reported in the Eskenazi (2004) vs. (2007) reports.

Most maternal characteristics of the cohort have been described previously (Eskenazi et al, 2004). Additional cohort characteristics in this study were determined during three assessments conducted 6, 12 or 24 months post-delivery. The new cohort characteristics included the children's sex (226 F and 221 M) and the number of breast feeding mothers (208, 124 and 30 at 6, 12 and 24 months, respectively; 96.6% children were initially breast-fed, about 50% and 29% were still breast-fed at 6 and 12 months, respectively).

Exposure to OP pesticides was assessed by measuring metabolites in maternal and child urine: three dimethyl (DM) phosphate metabolites (dimethylphosphate, dimethylthiophosphate, dimethyldithiophosphate); and three diethyl (DE) phosphate metabolites (diethylphosphate, diethylthiophosphate, and diethyldithiophosphate). Maternal urine generally collected at the time of two pregnancy interviews (mean of 14.0 and 26.6 weeks gestation) was also analysed for metabolites specific to malathion [malathion dicarboxylic acid (MDA)], and chlorpyrifos (TCP). Measures were conducted according to previously validated methods. Only values relevant to chlorpyrifos (ie TCP values) are reported in this evaluation. Measurements below limit of detection (LOD) of 0.26 µg/L were inputted a value of $LOD/\sqrt{2}$. As noted in the previous report of Eskenazi et al (2004), there are significant uncertainties associated with using the concentration of TCP in maternal urine during pregnancy to estimate developmental exposure to chlorpyrifos. Further, the authors comment that 'any measurement of OPs in urine or blood reflect exposure during the brief (usually <48 hr) antecedent period and therefore may not accurately reflect exposure throughout the entire critical period of neurodevelopment'.

Urine from a total of 445 women was assessed for TCP (442 samples collected at the first pregnancy interview and 419 samples collected at the second pregnancy interview). TCP was detected above the LOD in 71% and 82% of the first and second interview samples, respectively. By combining measures on urine samples collected at both pregnancy interviews, TCP was detected at least once in the urine of 91% of the women. Median values were determined for women with at least one TCP measurement above the LOD. Median TCP values for samples collected at the first and second interviews; and for the average values were 3.76, 4.60 and 3.54 µg/L, respectively.

The levels of contaminants other than OP metabolites were also measured including dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyldichloroethylene (DDE), β hexachlorocyclohexane, hexachlorobenzene, and polychlorinated biphenyls (PCBs) in maternal blood samples taken before delivery. Lead was measured in umbilical cord blood collected at delivery.

Various children's neurodevelopmental and behavioural parameters were assessed according to published methodologies. Children performance on the Bayley Scales of Infant Development (BSID) was determined at 6, 12 and 24 months (396, 395, and 372 infants, respectively). The BSID comprises both the Mental Development Index (MDI) which characterises a variety of cognitive abilities and the Psychomotor Development Index (PDI) that characterises large muscle and fine motor coordination. For Bayley scales, 'problem' (potentially indicative of clinically significant outcome) is defined as scoring more than one standard deviation below the mean, ie < 85. Bayley PDI scores at 6, 12 and 24-months respectively (mean ± SD) were 96.4 ± 10.6, 106.0 ± 12.6, and 97.5 ± 10.6, and MDI scores were 95.7 ± 7.0, 100.6 ± 8.9, and 85.9 ± 11.8. The proportion of MDI scores < 85 increased at 24 months with 50% < 85 relative to 3–4% on earlier assessments. The significance of that finding is unclear as the study authors commented that the Bayley Scales 'may not be a clinically valid tool in Spanish or in Latino immigrant communities'; and that 'similar findings of drops in MDI scores at 24 months of age have been observed in a cross-sectional study of children in semiurban Mexico and in African-American and Dominican children in New York City'.

Children's emotional/behavioural problems and competencies were assessed through a questionnaire, the Child Behaviour Checklist (CBCL) administered to 336 mothers at 24 months. Three scales were examined, *viz.*, the Attention Problems syndrome scale, which includes such items as 'can't concentrate' and 'can't sit still'; the DSM-oriented Attention-Deficit/Hyperactivity Disorder (ADHD) scale, which additionally includes such items as 'gets into everything'; and the DSM-oriented Pervasive Developmental Disorder (PDD) scale, which includes such items as 'avoids eye contact,' 'rocks head, body,' and 'unresponsive to affection'. The study authors indicated that a score considered to be of 'clinical' significance is > 98th percentile of the national normative sample, and of 'borderline' clinical significance is > 93rd percentile. On the CBCL, more children scored in the clinical range on the DSM-oriented pervasive developmental disorder scale (14.4%) than the national reference sample (≤ 3%) (binomial test *p* < 0.0001), although similar numbers to the expected proportion of children scored in the clinical range on the attention problems syndrome (2.0%) and ADHD (3.3%) scales. The study authors noted that 'because the CHAMACOS population consisted mostly of children from low-income families, they were already at risk for poorer neurodevelopment.'

The children's environment was also assessed according to published methodologies including: the Infant-Toddler Home Observation for Measurement of the Environment (HOME) instrument which was completed at 6 and 12 months of age, and 32 of 45 items were completed at 24 months. Few children lived in home environments considered to be of low quality in terms of stimulation and interaction (HOME scores < 26) at 6 (8%) and 12 (< 1%) months of age. Mother's scholastic abilities were assessed at six months using the Peabody Picture Vocabulary Test (PPVT). The average maternal PPVT score was in the low normal range, averaging 86 ± 21. Maternal depression was assessed at 12 months using the Center for Epidemiologic Studies Depression Scale (CES-D). Half of the mothers had symptoms of depression one year postpartum and mothers who reported depressive symptomatology had nearly 3–fold odds for reporting that their 2–year-old had attention problems and ADHD.

The study results were analysed to investigate possible correlations between parameters of pesticides/contaminants exposure and children's neurodevelopmental and behavioural parameters. Because a large proportion of women had non-detectable levels of TCP, the study authors categorised levels into three groups: < LOD for both pregnancy measurements, and for those with at least one detectable level, below and above the median of the average pregnancy level. No significant associations were observed between TCP (surrogate marker for chloropyrifos exposure) and any Bayley or CBCL outcomes.

Mount Sinai Cohort

Study 1: Berkowitz et al, 2004

Reference: Berkowitz S G, Wetmur J G, Birman-Deych E, Obel J, Lapinski R H (2004). *In utero* pesticide exposure, maternal paraoxonase activity, and head circumference. Environ Health Perspect. 112(3): 388–391

Mothers were recruited during early pregnancy from the Prenatal Clinic and two private practices at Mount Sinai Hospital during March 1998–March 2002 (ie during four years). Only primiparas with singleton births were included in the study. In addition, the mothers subject to the following circumstances were excluded from the study; first prenatal visit after 26 weeks of gestation; serious chronic diseases such as diabetes, hypertension, or thyroid disease or those who developed a serious pregnancy complication that could affect fetal growth and development; and consumed more than two alcoholic beverages per day or who used illegal drugs. Mothers and infants were also excluded if the child was born with a congenital malformation or severe prematurity (< 1,500 g or < 32 weeks of gestation). A total of 479 prenatal patients were recruited, however 75 of these were excluded from the study because of medical complications, very premature births, delivery of an infant with birth defects, inability to collect biologic specimens before birth, change of hospital or residence outside New York, or refusal to continue to participate. The final sample size for this analysis consisted of 404 births.

Participants' data were linked to a computerized perinatal database at Mount Sinai Hospital and information on delivery characteristics and birth outcome, including birth weight, length, head circumference, gestational age, and infant sex were obtained from this database. Standardized clinical techniques were used to measure birth weight, length, and head circumference.

Maternal blood samples were obtained during the third trimester at the time of routine venipuncture and maternal urine samples were also obtained at the same time. Cord blood samples were obtained at birth. To adjust for intrapersonal variability in urine dilution, analysed concentrations were normalized to creatinine, which is considered to be a common and appropriate method. Paraoxonase 1 (PON1) is an enzyme that acts as a phase-II detoxifying system and is important in the metabolism of OP pesticide. Specifically, PON1 can detoxify the chlorpyrifos-oxon before it can inhibit acetylcholinesterase in the peripheral and central nervous systems. Therefore, samples were used to determine maternal and infant PON1 enzyme activity and PON1 polymorphisms. Three phenolic metabolites of pesticides were determined in urine: 1) TCP, a chlorpyrifos metabolite (one of the most commonly detected pesticide metabolites), 2) phenoxybenzoic acid (PBA), a possible metabolite of several pyrethroid insecticides, including sumithrin, permethrin, and cypermethrin and 3) pentachlorophenol (PCP).

The authors reported that the patients were drawn predominantly from East Harlem, but also from other parts of New York City. It was reported that 35.4% of the women were under the age 20. The largest racial/ethnic group was Hispanics (49.8%), followed by African-Americans (27.7%) and whites (21.0%). The medians (and interquartile ranges) for the creatinine-corrected metabolites were 11.5 (1.8–35.4) µg/g creatinine for TCP, 19.8 (4.8–62.9) µg/g creatinine for PBA, and 8.0 (2.6–32.3) µg/g creatinine for PCP. The authors reported no statistically significant correlation between maternal PON1 activity and head circumference of children whose mothers had TCP levels either below or above the LOD. Controlling for birth weight or birth length did not alter these results. Similar trends across all three racial/ethnic groups were observed upon stratification by race/ethnicity; however, the association was higher for African-Americans. Excluding preterm births did not affect the mean head circumferences for those with TCP levels either below or above the LOD. A significant positive trend ($p = 0.004$) was observed with head circumference when maternal PON1 activity was considered alone (ie without level of TCP); the adjusted means were 33.5 cm for the low PON1, 33.9 cm for medium PON1, and 34.1 cm for high PON1 activity.

Table 4: Key data relevant to the study ie adjusted mean ± SD of fetal growth indices, maternal PON activity and TCP level (reproduction of Table 4 from Berkowitz et al, 2004)

		Birth weight[a] (g)		Birth length[a] (cm)		Head circumference[a] (cm)	
		Mean ± SD	No.	Mean ± SD	No.	Mean ± SD	No.
TCP < LOD	Low PON	3237 ± 456	76	50.3 ± 2.3	75	33.6 ± 1.8	76
	Medium PON	3255 ± 436	62	50.1 ± 2.2	62	33.7 ± 1.7	62
	High PON	3337 ± 444	71	50.3 ± 2.3	71	34.1 ± 1.7	70
TCP > LOD	Low PON	3278 ± 395	47	50.9 ± 2.3	46	33.3 ± 1.5*	47
	Medium PON	3327 ± 406	57	51.0 ± 2.3	57	34.0 ± 1.5	57
	High PON	3270 ± 409	55	50.8 ± 2.4	55	34.1 ± 1.6	55

[a]Adjusted for race/ethnicity, infant sex, and gestational age. *p = 0.014, SD = Standard deviation, PON = Paraoxonase enzyme, TCP = 3,5,6-trichloro-2–pyridinol

No trends were observed for birth weight or birth length with respect to TCP (Table 4) or the other metabolite levels when maternal PON1 levels were taken into account. Infant PON1 activity did not show association with any of the fetal growth measures. The study reported that no association was seen between maternal and infant genotype and PON1 activity. Results also suggest that neither maternal nor infant genotypes were associated with head circumference or length at birth.

As head size (brain weight and head circumferences) has been found to be predictive of subsequent IQ and cognitive ability, the authors suggest that the observed reductions in head circumference indicate that chlorpyrifos may have a detrimental effect on fetal neurodevelopment in mothers who exhibit low PON1 activity. It should be noted however, that it is unclear what effects, if any, the observed deviations in head circumference in this study may have on IQ or behaviour. Additionally, only the relationship between TCP and head circumference was explored in the study, although three pesticides and their metabolites (TCP, PBA and PCP) were present in the maternal urine samples. The potential influence of other pesticides and effects on head circumference were not examined or reported.

In summary, maternal levels of the chlorpyrifos metabolite TCP above the LOD, combined with low maternal PON1 activity were associated with a small reduction in head circumference. Maternal PON1 activity, but not PON1 genetic polymorphisms, was associated with reduced head circumference. The observed reductions in outcome parameters appear to be of little clinical significance.

Study 2: Engel et al, 2007

Reference: Engel SM, Berkowitz GS, Barr DB, Teitelbaum SL, Siskind J, Meisel SJ, Wetmur JG, Wolff MS (2007). Prenatal Organophosphate Metabolite and Organochlorine Levels and Performance on the Brazelton Neonatal Behavioural Assessment Scale in a Multiethnic Pregnancy Cohort. Am J Epidemiol. 165: 1397–1404

Delivery characteristics and birth outcomes were obtained from a perinatal database from the Mount Sinai Department of Obstetrics, Gynecology and Reproductive Science. The Brazelton Neonatal Behavioural Assessment Scale (BNBAS) was administered before hospital discharge ($n = 311$), but not all subjects were analysed. 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 ($n = 22$). It is noted that this may have introduced bias and limited the validity of the associations and conclusions.

Maternal urine samples were analysed for six dialkylphosphate metabolites (a prenatal biomarker for organophosphate metabolites) and MDA. Metabolite values within a group (diethyl or dimethyl) were correlated, with the study authors reporting that six regression models were developed for subjects with a complete set of metabolites. The model was used to estimate missing metabolite values from other measured metabolites. It is noted that the imputation of values rather than normalising for the samples may have resulted in the skewing of results and incorrect assumptions. Diethyl- and dimethylphosphate metabolites were then summed on a molar basis (as nm/L) to obtain total diethylphosphates (DEP) and total dimethylphosphates (DMP), and combined to calculate total dialkylphosphates (DAP) levels. Urine samples that contained less than 20 mg/dL of creatinine ($n = 26$) were excluded from organophosphate metabolite analyses. A random subset ($n = 194$) (distributed equally by maternal race/ethnicity) of maternal peripheral blood samples from the entire cohort was analysed for PCBs and DDE. Total lipids (g/L) were calculated by using cholesterol and triglycerides determined on 174 plasma samples with sufficient volume. Of the infants who were administered the BNBAS, 151 had PCB and DDE levels measured prenatally.

The BNBAS included 28 behavioural and 18 primitive reflex items. The seven-cluster scoring method was used to simplify interpretation of the data by dividing infant behaviour into seven domains: 1) habituation ($n = 183$), ability to respond to and inhibit discrete stimuli while asleep; 2) orientation ($n = 282$), attention to visual and auditory stimuli and quality of overall alertness; 3) motor ($n = 311$), motor performance and quality of movement and tone; 4) range of state ($n = 310$), a measure of infant arousal and state lability; 5) regulation of state ($n = 309$), ability to regulate state in the face of increasing levels of stimulation; 6) autonomic stability ($n = 310$), signs of stress related to homeostatic adjustments of the central nervous system; and 7) number and type of abnormal primitive reflexes ($n = 311$).

PON1 activity was measured, and dialkylphosphate models were adjusted for PON1 enzyme level tertiles and run both with and without adjustment for urine creatinine. Possible interaction among PON1, ΣDAP level, and abnormal reflexes were also analysed. Exploratory analyses of the association between multiple abnormal reflexes and prenatal pesticide levels, for those exhibiting two or more abnormal reflexes was also carried out using multivariable logistic regression models. Authors indicated that when necessary, over-dispersion in the data was corrected by introducing a scale parameter estimated by deviance divided by degrees of freedom. It is noted that while correction of overdispersion is standard practice, it may cause underestimation of the standard errors, which was not addressed in the paper.

The authors indicated that there were strong and consistent associations between organophosphate exposure (as measured by prenatal urinary organophosphate metabolites) and abnormal primitive reflexes. The study authors indicated that MDA levels above the limit of detection were associated with a 2.24–fold increase in the number of abnormal reflexes (95% confidence interval: 1.55, 3.24) in a Poisson regression model adjusted for examiner, anesthesia, PON1 enzyme level, and creatinine. The authors report that similarly, relative to the first quartile, quartiles 2–4 of total DAP levels were also associated with an increased proportion of abnormal reflexes, although the associations were variable.

The authors reported that there was a strong interaction between PON1 expression levels and ΣDMP (but not ΣDEP) on risk of abnormal reflexes. Infants born to women in the first (interaction $p = 0.002$) and second (interaction $p = 0.01$) tertiles (slower metabolizers) had a greater risk of abnormal reflexes than infants of those in the highest tertile (fast metabolizers). For women in the highest tertile of PON1 expression, increased exposure did not result in increased risk of abnormal reflexes. The authors also mentioned that the results provided additional evidence that prenatal levels of OP pesticide metabolites are associated with abnormalities in primitive reflexes, which are a critical marker of neurological integrity.

Some of the acknowledged limitations of the study include the possibility that chlorpyrifos alone may not have driven the effects observed in the study because the class of DMP includes metabolites from several different organophosphate pesticides. The study authors mentioned that the subjects in this cohort were enrolled between 1998 and 2002, which overlaps with the period when the residential use of chlorpyrifos and diazinon was being phased out. However, in 1997, chlorpyrifos was the most heavily used insecticide by pest control operators according to the New York City Housing Authority. The study also discussed that the results reflect only pesticide metabolites and it is yet to be confirmed how these levels relate to the parent organophosphate. One major limitation discussed by the authors, was that the analysed metabolites have short half-lives, and the study involved only a single urine sample taken during the third trimester. It was noted that this effect may be small, if the sources and patterns of exposure (e.g. residential pesticide use or exposure from a food source) are unchanged throughout the pregnancy. However, it remains unclear what the effects could be if chlorpyrifos exposure occurs for a longer time period or a specific time period during pregnancy. Because the study identified the presence of various pesticides including organophosphate and organochlorine metabolites in urine samples at the same time, it is also unclear if co-existence of other pesticides might have modified the abnormal reflexes reported in the study. Therefore, it is unclear whether there was a direct relationship between prenatal chlorpyrifos exposure and neonatal behaviour.

Study 3: Engel et al, 2011

Reference: Engel SM, Wetmur J, Chen J, Zhu C, Barr DB, Canfield RL, Wolff MS (2011). Prenatal Exposure to Organophosphates, Paraoxonase 1, and Cognitive Development in Childhood. Environ Health Perspect 119: 1182–1188

The authors applied the Bayley Scales of Infant Development, 2nd edition (BSID-II), on the Mount Sinai cohort at approximately 12 (n = 200) and 24 months (n = 276). The BSID-II scale uses age-standardized norms of mental and psychomotor development indices, MDI and PDI respectively. The MDI rates the child's cognitive ability in a number of areas, including memory, habituation, problem solving, early number concepts, generalization, classification, vocalizations, language, and social skills. The PDI rates the child's fine and gross motor coordination. Children were invited to undertake Wechsler psychometric intelligence (WPPSI) tests for IQ according to their ages.

Maternal urine samples were collected and analysed for six dialkylphosphate (DAP) metabolites and missing metabolite levels were imputed in the same manner as described above in Engel 2007. PON activity was also measured and PON1 polymorphism were measured using maternal and child DNA. The study examined (1) interactions between prenatal organophosphate exposure and tertiles of PON1 activity in maternal prenatal peripheral blood and child cord blood, (2) interactions between prenatal organophosphate exposure and the maternal or child PON1 polymorphism, (3) analysis that combined the Full Scale Intelligence Quotient (FSIQ), Perceptual Reasoning, Verbal Comprehension, and Processing Speed composite scores from children who attended at least one of the Wechsler psychometric intelligence exams.

The study authors indicated that at the 12 month BSID-II exam, the estimated effect of organophosphate metabolites on the MDI was strongly heterogeneous by race/ethnicity for the ΣDAP and ΣDMP metabolites. Increasing ΣDAP and ΣDMP tertiles of exposure were associated with a decrease in the MDI in non-whites (blacks and Hispanics). Conversely, a reverse pattern was observed for whites, where higher exposure was associated with better MDI. According to the authors, there was no relationship between organophosphate metabolites and the PDI at 12 months overall, and no interaction with race/ethnicity for any of the metabolite groups. The study reported that at the 24 month BSID-II exam, effect estimates were not heterogeneous by race/ethnicity, and metabolite levels were not associated with changes in PDI outcomes.

Study authors examined the interaction between PON1 polymorphism and ΣDAP/ΣDMP. They identified that among blacks and Hispanics, the effects of ΣDAP, ΣDEP, and ΣDMP varied considerably according to PON1 genotype at 12 months, but not 24 months. At 12 months only, children of mothers with the PON1 QR/RR genotype experienced decline of the MDI with increase in ΣDAP or ΣDMP or ΣDEP biomarker level (approximately 5–point decline with each log10 unit increase of ΣDAP or ΣDMP and a 2–point decline in MDI for each log10 unit increase in ΣDEP) which was monotonic across tertiles. There were either no effects among children of mothers with QQ genotype (slow-catalytic-activity genotype) or estimations did not follow a monotonic pattern.

It was reported that among the children of mothers with the PON1 QQ genotype (slow-catalytic-activity genotype), increasing tertiles of ΣDAP, ΣDEP, and ΣDMP exposure was generally associated with a monotonic decline in the combined WISC-IV/WPPSI-III FSIQ and Perceptual Reasoning domains, after adjusting for sex, race/ethnicity, maternal education, language in the home, alcohol use in pregnancy, batch and season of urine collection and urinary creatinine. Authors indicated that no consistent patterns were observed in the QR/RR genotype group. However, it was noted that there was considerable imprecision in all estimates and that the first-versus third-tertile contrasts for Perceptual Reasoning were significantly different (at $p < 0.05$) for ΣDAP and ΣDMP.

Some of the acknowledged limitations of the study included inconsistency with reported effects on developmental parameters between different genotypes. The authors' proposed explanation for this issue was that the PON1 gene is involved in multiple physiological processes, including organophosphate metabolism but also in lipid peroxidation and oxidative stress, which may impact neurodevelopment independently and/or jointly with organophosphate exposure. It is also discussed in the study that heterogeneity according to maternal race/ethnicity may indicate differences in exposure sources (potentially influenced by social situations such as housing conditions and fresh fruit/vegetable consumption) rather than any underlying susceptibility. Concerns were also raised regarding the use of urinary metabolites for the estimation of organophosphorous pesticide exposure. The authors indicated that in recent studies it was found that more than half of the samples tested contained more preformed ΣDAP residues than parent organophosphate pesticides and that that ΣDAP residues were produced by abiotic hydrolysis, photolysis, or plant metabolism. Direct intake of the metabolite without the active oxon, rather than the parent pesticide, does not inhibit cholinesterase activity. Thus, for subjects for whom the primary source of pesticide exposure is fresh fruit and vegetable consumption, use of urinary metabolite concentrations as an indication of parent compound exposure may result in significant misclassification of exposure. Additionally the authors recognized that organophosphate biomarkers were measured at one time during pregnancy but other time-related variability may result in additional misclassification of exposure. The study showed stronger QR gene associations for ΣDMP, not ΣDEP (into which chlorpyrifos and diazinon both metabolize) with FSIQ, Perceptual Reasoning, and Working Memory. A possible justification for this provided by the authors is that PON1 status may indirectly influence methyl organophosphate metabolism when multiple organophosphate exposures are involved.

In summary, the study authors reported that prenatal ΣDAP levels were linked with a decrease in mental development at 12 months among African-Americans and Hispanics. These associations were stronger in the children whose mothers carried the PON1 QR genotype. In later childhood, increasing prenatal ΣDAP and ΣDMP biomarkers were associated with decrease in perceptual reasoning in the maternal PON1 QQ genotype (genotype leading to slow catalytic activity for chlorpyrifos-oxon), with a monotonic trend consistent with greater decrements upon increased prenatal exposure.

The study identified the co-exposure of the organophosphorous and organochlorine metabolites throughout the duration of the study, and it is unclear whether chlorpyrifos alone had a role to play in modifying the FSIQ and Perceptual Reasoning of the children because the metabolites analysed are the derivatives of multiple parent compounds. This study is therefore considered to be of limited regulatory value.

Columbia (CCCEH) Cohort

Study 1: Whyatt et al, 2002

Reference: Whyatt RM, Garfinkel, R, Hoepner LA, Holmes D, Borjas M, Williams MK, Reyes A, Rauh V, Perera FP and Camann DE (2002). Residential Pesticide Use during pregnancy among a cohort of Urban Minority Women. Environ Health Perspect. 110(5): 507–389

This study involved analysis of questionnaire data on pesticide use in the home during the pregnancy of 316 African-American and Dominican women residing in northern Manhattan and the South Bronx. Additionally, 72 women underwent personal air monitoring for 48 h during their third trimester of pregnancy to determine exposure levels to 21 pesticides and/or degradants. The women included in this study were part of ongoing prospective cohort study of African-American and Dominican women conducted by the Columbia Centre of Children's Environmental Health (Columbia Cohort, CCCEH).

The study mostly included pregnant African-American and Dominican (≤ 20th week) women aged 18–35 years, and registered at New York Presbyterian Medical Centre and Harlem Hospital, in New York City. Women who smoked, had a history of drug abuse, diabetes, hypertension, or HIV infection were excluded from participation in the study, as were women who resided in New York City for less than 1 year or were employed outside of their residential address during enrolment between February 2001 and May 2004. Of the women who were screened, 870 of 1706 (47%) met the eligibility criteria. Of these, 70% agreed to participate. Full enrolment was complete once prenatal monitoring and questionnaires had been completed, as well as blood samples had been collected.

Questionnaires were administered by a trained research worker during the third trimester, and included information on demographics, home characteristics, residential history, smoking, occupation, alcohol and drug use and the history of residential pesticide use (see table below, reproduced from study).

Table 5:   Demographic characteristics of the study (reproduced from Whyatt et al, 2002)

Characteristics	Total cohort (n = 316)	Sample[a] (n = 266)		Statistic[c]	Personal ambient air monitoring, 48 h (n = 72)	Statistic[d]
		Complete data[b] (n = 231)	Incomplete data[b] (n = 35)			
Age	24.5 ± 5.2	24.9 ± 4.9	25.1 ± 7.3	$p = 0.9$, t-test	24.2 ± 5.2	$p = 0.3$, t-test
Ethnicity						
African-American	136/316 (43%)	96/231 (42%)	15/35 (43%)	$\chi^2 = 0.02$, $p = 0.9$	38/72 (53%)	$\chi^2 = 2.5$, $p = 0.11$
Dominican	180/316 (57%)	135/231 (58%)	20/35 (57%)		34/72 (47%)	
Community						
Harlem	138/316 (44%)	99/231 (43%)	15/35 (43%)	$\chi^2 = 1.2$, $p = 0.5$	32/72 (44%)	$\chi^2 = 2.4$, $p = 0.3$

Characteristics	Total cohort (n = 316)	Sample[a] (n = 266)		Statistic[c]	Personal ambient air monitoring, 48 h (n = 72)	Statistic[d]
		Complete data[b] (n = 231)	Incomplete data[b] (n = 35)			
Washington Heights	110/316 (35%)	82/231 (35%)	15/35 (43%)		20/72 (28%)	
South Bronx	68/316 (22%)	50/231 (22%)	5/35 (14%)		20/72 (28%)	
Marital status						
Never married	220/315 (70%)	158/231 (68%)	24/34 (71%)	$\chi^2 = 0.07$, $p = 0.8$	53/71 (75%)	$\chi^2 = 0.9$, $p = 0.3$
Education						
< High school	103/315 (33%)	68/230 (30%)	13/35 (37%)	$\chi^2 = 0.8$, $p = 0.4$	24/72 (33%)	$\chi^2 = 0.01$, $p = 0.9$
Annual household income						
< $10,000	135/303 (45%)	95/220 (43%)	16/34 (47%)	$\chi^2 = 0.2$, $p = 0.7$	31/72 (44%)	$\chi^2 = 0.06$, $p = 0.8$
Medical recipient	279/316 (88%)	204/231 (88%)	33/35 (94%)	$\chi^2 = 1.1$, $p = 0.3$	63/72 (88%)	$\chi^2 = 0.0$, $p = 1.0$

Age is reported as mean ± SD; other data are number of subjects (%) in each category. [a]Women reporting that pest control measures were used during pregnancy .[b]Data on eight specific measures. [c]Comparing women with complete versus incomplete data on the eight specific pest control methods. [d]Compared with the women in the total cohort without prenatal monitoring for pesticide levels; analyses included the 68 women with air monitoring results ffrom whom we also collected questionnaire data on use of pest control methods in the home.

Personal ambient air monitoring was conducted using a backpack containing a personal monitor sampling 4 litres/minute of air. On average 11.5 m^3 of air was drawn through the sampler. Air samples were analysed for polycyclic aromatic hydrocarbons (PAHs) and 21 pesticides. Additionally, study authors included air sample results from a previous substudy monitored between September 1998 and August 1999.

Of the 72 personal air samples collected, 4/21 pesticides were detected in 100% of the air samples, chlorpyrifos (range 0.7–193 ng/m^3, median of 9.9), diazinon (2.0–6010 ng/m^3, median 24.5), propoxur (3.9–1380 ng/m^3, median 33.1) and the fungicide, o-phenylphenol 5.7–743 ng/m^3, median 23.7). Further, four pesticides were detected in more than 1/3 of the samples, but at lower concentrations: piperonyl butoxide (an indicator for exposure to pyrethrins); the synthetic pyrethroid permethrin, and the organochlorines (4,4'–DDT) and chlordane. The study authors reported that the other pesticides were either not detected (malathion, aldrin, dicofol, dieldrin, endosulfan, and endrin) or detected in ≤ 10% of the samples (methyl parathion, dichlorvos, carbaryl, cyfluthrin, lindane, methoxychlor, and folpet).

Study 2: Perera et al, 2003

Reference: Perera FP, Rauh V, Tsai, WY, Kinney P, Camann D, Barr D, Bernert T, Garfinkel R, Tu YH, Diaz D, Dietrich J, Whyatt RM (2003). Effects transplacental exposure to environmental pollutants on birth outcomes in a multiethnic population. Environ Health Perspect 111(2): 201–205

This study aimed to evaluate the effects of prenatal exposure to common urban pollutants: environmental tobacco smoke (ETS), airborne PAH and pesticides on birth weight, length and head circumference after controlling for the effects of known physical, biological, and toxic determinants of fetal growth.

A sample of 263 non-smoking African-American and Dominican women were evaluated for effects on birth outcomes when exposed to PAHs, ETS and chlorpyrifos.

Participants were from the CCCEH cohort, with exclusion criteria as specified in the assessment of Whyatt et al (2002) above. Participants were only included in the current analysis if they have valid prenatal personal monitoring data on PAHs, cord or maternal blood samples, complete questionnaire data and birth outcome data. Additionally, seven participants that exhibited plasma cotinine concentrations > 25 ng/mL were excluded to rule out active smoking.

Prenatal personal PAHs assessment was conducted over two consecutive days using a personal backpack monitor. Maternal blood was collected within one day postpartum, and umbilical cord blood was collected at delivery. Measurement of fetal growth was collected from medical records.

All study participants had detectable levels of one or more PAHs, ranging from 0.36–36.47 ng/m^3. Between 42 and 45% of mothers and newborns had cotinine levels between 0.05 and 25 ng/mL, indicative of ETS exposure. Chlorpyrifos was detected in 98% of maternal blood, and 94% of cord samples with means of 7.6 pg/g in cord blood and 1 pg/g in maternal blood. Among African-Americans, prenatal exposure to PAHs was associated with lower birth weight and smaller head circumference, however the variability in these parameters were greater in the African-American group compared to Dominican group. Chlorpyrifos was associated with significantly decreased birth weight among the African-American (3299 ± 548.7 g) but not the Dominican group (3348.5 ± 449.4 g) (see Table below, reproduced without modification from Perera et al, 2003), despite the mean chlorpyrifos exposure values being similar. The study authors reported that chlorpyrifos was associated with reduced overall birth length, but not with head circumference. There appears to be much uncertainty associated with this finding due to the small sample size, lack of significance by t-test for the individual data and absence of reporting of individual data. Interestingly, it was also reported that African-Americans gave birth at a lower gestational age (39 weeks) as compared to the whole or Dominican group (39.3 or 39.6 weeks, respectively). Lower gestational age is associated with the reduction in birth weight, birth length and head circumference. But, in the absence of individual data, it is difficult to confirm the effect of gestational age on these other parameters. It is also important to note that chlorpyrifos was the only pesticide measured in the current cohort, and therefore potential effects of other chemicals were not considered.

Table 6:   Demographic and exposure characteristics of the population[a] **(reproduced from Perera et al, 2003)**

	All (n = 263)	African-American (n = 116)	Dominican (n = 146)
Maternal age (year)[b]	24.6 (5.2) (n = 262)	24.1 (5) (n = 115)	25 (5.3) (n = 146)
Maternal education (%)			
< High school	32.8	31	34.3
High school	45.3	46.6	44.3
> High school	21.9	22.4	24.4
Maternal environmental tobacco smoke (ETS)			
(% reporting smoker in the home)	42.8	51.8*	35.9
Maternal alcohol consumption			
(% drank alcohol during pregnancy)	24	12.4*	33.3
Maternal height (cm)[b]	162.6 (8.3) (n = 263)	164.5 (8.4) (n = 116)*	161 (8) (n = 146)
Maternal prepregnancy weight (kg)[b]	67 (16.5) (n = 263)	71.6 (19.7) (n = 116) *	63.3 (12.4) (n = 146)
Gestational age (weeks)[b]	39.3 (1.5) (n = 263)	39 (1.7) (n = 116)*	39.6 (1.2) (n = 129)
Newborn birth weight (g)	3382.6 (499.2) (n = 262)	3299 (548.7) (n = 115)[c]	3348.5 (449.4) (n = 146)
Newborn birth length (cm)[b]	50.9 (2.7) (n = 259)	50.8 (3.4) (n = 115)[c]	51.1 (2.2) (n = 143)
Newborn head circumference (cm)[b]	34.1 (1.6) (n = 248)	33.8 (1.9) (n = 111)[c]	34.3 (1.2) (n = 136)
Sex of newborn (% females)	51.5	48.3	54.1
Plasma cotinine (ng/mL)[b,d]	0.5 (2.4) (n = 254)	0.6 (2) (n = 110)*	0.5 (2.7) (n = 143)
Inhalation PAH (ng/m^3)[b]	3.7 (3.6) (n = 263)	3.5 (2.8) (n = 116)	3.9 (4.1) (n = 146)
Plasma chlorpyrifos (CPF) (pg/g)[b]	7.5 (7.5) (n = 113)	8 (6.3) (n = 57)	7.1 (8.5) (n = 56)

[a]*Subjects with prenatal monitoring data on PAH, either cord or maternal blood sample, complete questionnaire data, and birth outcome data. There were no significant differences between the overall parent population and the present subset in terms of demographic, questionnaire-derived, and birth outcome variables shown in the table.* [b]*Mean (SD). Arithmetic means are presented for ease of comparison with other studies; however, the reported analyses are based on log-transformed data.* [c]*By Multivariate Hotelling's t-test, at least one of these outcomes (weight, length, head circumference) was significantly lower in African-Americans ($p < 0.001$).* [d]*Subjects with cotinine >25 ng/mL were excluded from analysis. Cotinine represents the level in cord blood or, if unavailable, the level in maternal blood, using the formula provided in the text. *$p \leq 0.01$ for African-American vs. Dominican (Student's t-test for maternal height, prepregnancy weight, and gesta-tional age; χ^2 for ETS exposure and alcohol; Mann-Whitney for cotinine).*

The study authors concluded that exposure to environmental pollutants at levels encountered in New York City between the years 1998 and 2004 adversely affected fetal development. The study involved analysis of plasma cotinine (product of nicotine detoxification) and chlorpyrifos and inhalation PAH. No other pesticides were measured for the purposes of this analysis. The findings are considered inconclusive due to the small sample size, lack of statistical significance and failure to consider the potential impacts of other pesticides on the observed effects on fetal development.

Study 3: Rauh et al, 2006

Reference: Rauh VA, Garfinkel R, Perera, FP, Andrews HF, Hoepner L, Barr DB, Whitehead R, Tang D, Whyatt, W (2006). Impact of prenatal chlorpyrifos exposure on neurodevelopment in the first 3 years of life among inner-city children. Pediatrics 118(6): e1845–1859

The purpose of this study was to continue the investigation of previously reported (Perera et al, 2003; Whyatt et al, 2004) inverse associations between umbilical cord chlorpyrifos levels and birth weight and length of infants. The present study investigated longer term associations between prenatal chlorpyrifos exposure and developmental endpoints at 3 years of age. The investigation was part of an ongoing prospective cohort of inner city (New York) African-American and Dominican women and their children by the CCCEH.

The authors introduced a working hypothesis for the link between chlorpyrifos exposure and neurodevelopmental toxicity. Included in the proposed modes of action include:

- inhibition of AChE to down regulate muscarinic receptors and/or act as a neurotropic factor during brain development

- inhibition of adenylate cyclase signalling cascade to decrease brain DNA and RNA synthesis and suppress neurite outgrowth

- action through noncholinergic mechanisms at doses that cause only minimal AChE inhibition.

The cohort originally consisted of non-smoking women (classified by self-report and validated with blood cotinine levels less than 15 ng/mL), 18 to 35 years of age, who self-identified as African-American or Dominican and who registered at the obstetrics/gynaecology clinics at New York Presbyterian Medical Centre and Harlem Hospital by the 20th week of pregnancy. Eligible women were free of diabetes mellitus, hypertension, known HIV infection and documented or reported drug abuse and had resided in the area for at least one year.

The cohort was reported to include a total of 536 active participants including 254 of the children which had reached the age of 3 years. The following data was collected from the cohort; prenatal maternal interview data, biomarkers of chlorpyrifos exposure levels from maternal and/or cord blood samples obtained at delivery, postnatal observational data on the quality of the home caretaking environment and a neurobehavioural outcome evaluation at 12, 24 and 36 months.

The subjects were grouped into two groups (high and low exposure) based on a preliminary analysis of chlorpyrifos levels in cord blood. A dichotomized exposure variable was used, classifying participants into high exposure (> 6.17 pg/g) or lower exposure (≤ 6.17 pg/g). The preliminary analysis categorised chlorpyrifos levels into three groups: non-detects, low and high exposure groups. The non-detects and highest group had lower MDI and PDI scores than the middle exposure group.

This study employed the methods of Whyatt et al (2005), who reported that the maternal and umbilical cord blood levels of chlorpyrifos were strongly correlated ($r = 0.76$; $p < 0.001$). In the instances where umbilical cord blood was not collected (12% of participants), the mother's values were used, based on the formula derived from the regression analyses described by Whyatt et al (2005). All regression models included inputs for prenatal exposure, gender, ethnicity, gestational age at birth, quality of the home caretaking environment, maternal educational level (high school degree vs. no high school degree), and maternal IQ.

The Bayley Scales of Infant Development II (BSID-II) were used to assess cognitive and psychomotor development at 12, 24 and 36 months of age. The BSID-II is a widely used, normative value-referenced, developmental test for young children that is used frequently to diagnose developmental delay and is known to be sensitive to the effects of toxic exposures such as low-level, intrauterine, lead exposure. Each scale provides a developmental quotient (raw score/chronological age), which generates a continuous MDI and a corresponding PDI.

The 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. The PDI assesses overall motor development and contains 111 items that measure quality of movement, sensory integration, motor planning, fine and gross motor skills, and perceptual motor integration (Black and Matula, 2000; Strauss et al, 2006). Standardized scores for the MDI and PDI have a mean of 100 and a standard deviation of 15, and range from 50 to 150. Delayed development is scored as less than or equal to 85 with a standardised cut-off point of 1 standard deviation.

The characteristics of the study population is shown below (Table 7), reproduced from Rauh et al (2006).

Table 7:   Demographics and key data relevant to the study, reproduced from Rauh et al (2006)

Characteristics	High Exposure (n = 50)		Lower Exposure (n = 204)	
	Proportion %	Mean ± SD	Proportion %	Mean ± SD
Maternal characteristics				
Ethnicity—Black	24.2		75.8	
Ethnicity—Dominican	14.9		85.1	
Age, y		24.6 ± 5.3		25.1 ± 5.1
Married	12.2		18.2	
No high school degree	40.0		33.3	
Maternal IQ[a]		84.3 ± 12.4		87.3 ± 13.7
HOME score		38.5 ± 6.0		39.78 ± 5.7
Infant characteristics				
Birth weight, g		3239.6 ± 558.1		
Birth length, cm		50.0 ± 2.4		51.1 ± 3.6

Characteristics	High Exposure (*n* = 50)		Lower Exposure (*n* = 204)	
	Proportion %	Mean ± SD	Proportion %	Mean ± SD
Birth head circumference, cm		34.0 ± 1.7		34.4 ± 1.8
Gestational age, wk		39.2 ± 1.4		39.3 ± 1.5
Male	48.0		46.1	
Prenatal exposure to residential ETS	56.0		31.9	

ETS = environmental tobacco smoke

The raw scores for developmental delay as reproduced from the original study is provided in Table 8, and a summary of the results reported by the authors is shown in Table 9. The main reported finding was that children of the high exposure group (chlorpyrifos levels of 6.17 pg/g plasma and above) scored on average, 6.5 points lower on the PDI and 3.3 points lower on the MDI at 3 years of age.

Table 8:   BSID-II Means and Proportion delayed at 12, 24 and 36 months according to chlorpyrifos exposure level (*n* = 254), reproduction of Table 2 from Rauh et al (2006)

Domain	Total	High Exposure Group	Low Exposure Group	*p*
MDI score; mean ± SD				
12 month	94.0 ± 9.8	93.7 ± 9.6	94.1 ± 9.8	0.810
24 month	85.1 ± 12.4	83.7 ± 12.2	85.5 ± 12.5	0.396
36 month	89.6 ± 11.4	87.4 ± 10.1	90.1 ± 11.7	0.155
PDI score, mean				
12 month	96.2	93.4	97.0	0.074
24 month	97.0	98.7	96.6	0.274
36 month	100.5	95.7	101.6	0.006
Mild/significant mental delay %[a]				
12 month	15.7	17.0	15.4	0.468
24 month	49.3	60.0	46.7	0.076
36 month	32.9	45.5	29.9	0.048
Mild/significant psychomotor delay %[a]				
12 month	14.0	21.7	12.1	0.078
24 month	13.2	13.0	13.3	0.595
36 month	10.5	24.4	7.1	0.002

[a]*MDI or PDI score of ≤ 85 are counted as developmental delay.*

Table 9:   Summary of results from Rauh et al (2006)

	12 months (*n* = 228-9)	24 months (*n* = 225-7)	36 months (*n* = 228)	Comment
Model 1 chlorpyrifos exposure > 6.17 pg/g vs. ≤ 6.17 pg/g. Race, gender, maternal education, maternal IQ, gestational age and prenatal environmental tobacco smoke exposure (primary data log transformed)				
MDI	B = -0.34 (SE = 1.66; *p* = 0.836)	B = -1.48 (SE = 2.03; *p* = 0.466)	B = -3.33 (SE = 1.76; *p* = 0.60)	Not significant
PDI	B = -3.30 (SE = 2.11; *p* = 0.12)	B = -1.17 (SE = 1.98; *p* = 0.56)	B = -6.46 (SE = 2.18; *p* = 0.003)	Significant at 36 months
Model 2				
Model 1 together with HOME environment evaluation (primary data log transformed)				
MDI	OR = 1.22 (95% CI: 0.48, 3.06)	OR = 1.75 (95% CI: 0.86, 3.60)	OR = 2.37 (95% CI: 1.08, 5.19)	Significant at 36 months
PDI	OR = 1.88 (95% CI: 0.78, 4.53)	OR = 1.01 (95% CI: 0.37, 2.76)	OR = 4.52 (95% CI: 1.61, 12.70)	Significant at 36 months

B = Unstandardised regression coefficient; MDI = Mental Development Index; PDI = Psychomotor Development Index; IQ = Intelligent quotient; OR = odds ratio; SE = Standard error

A significant association was also reported by the authors for the covariates race/ethnicity and gestational age. The proportion of black to Dominican mothers varied considerably in this study. Of the high exposure group, 24% (12 of 50) were black while 75.8% (159 of 204) of the low exposure group were black. In addition, 56% of the African-American high exposure group (28 of 50) had prenatal exposure to residential ETS versus 31.9% in the low exposure group (65 of 204).

In summary, at 36 months, mean PDI and MDI scores of the high and low exposure groups differed by 7.1 and 3.0 points respectively, and the proportion of delayed development in high exposure children was 5 times greater for PDI and 2.4 times greater for MDI, increasing the likelihood of children requiring early intervention services. This study suggests that exposure to chlorpyrifos during pregnancy is associated with delayed mental development and psychomotor development in infants.

Study 4: Whyatt et al, 2007

Reference: Whyatt RM, Garfinkel, R, Hoepner LA, Holmes D, Borjas M, Williams MK, Reyes A, Rauh V, Perera FP, Camann DE (2007). Within- and Between-Home Variability in Indoor-Air Insecticide Levels during Pregnancy among an Inner-City Cohort from New York City. Environ Health Perspect. 115: 383–389

This study assessed within- and between-home variability in indoor-air levels of various insecticides measured over the final two months of pregnancy of a subset of participants in the Columbia Mother's and Newborn Study, a cohort of women from New York City (Columbia Cohort, CCCEH).

Enrolment criteria are reported in previous studies (Perera et al, 2003; Whyatt et al, 2002; 2003). Nine insecticides (the OPs chlorpyrifos, diazinon, malathion and methyl parathion; the carbamates propoxur, bendiocarb and carbofuran; and the pyrethorids cis and transpermethrin) and piperonyl butoxide (an adjuvant associated with pyrethroid formulations) were measured in 48–h personal air samples and 2 wk integrated indoor air samples collected sequentially for 7.0 ± 2.3 wks (337 air samples).

Values below the LOD were assigned a value of half of the LOD. Greater than 45% samples had pesticide levels > LOD, for which means SDs were calculated. Before statistical analyses, pesticide values were log-transformed to normalise their distributions.

Sixty women (61%) reported using pest control during the 7–week air sampling period. Of the air samples collected from 102 homes (from 32nd week of pregnancy), chlorpyrifos (range 0.4–171 ng/m^3), diazinon (0.4–641 ng/m^3), and propoxur (0.4–317 ng/m^3) were detected in 99–100% of samples. Similarly, in personal air samples, chlorpyrifos (0.4–83.4 ng/m3), diazinon (0.4–427.5 ng/m^3), and propoxur (0.4–342.9 ng/m^3) were detected in 99–100% of samples. The values within homes remained relatively stable over time, however between-home variability was high and accounted for 88% of the variance in the indoor air levels of propoxur, 92% in chlorpyrifos and 94% in diazinon. Between 2001 and 2004, chlorpyrifos levels declined 5-fold after implementation of a voluntary cancellation by registrants of indoor residential chlorpyrifos use. Interestingly, diazinon levels also declined during this period.

An observed association between average indoor air levels of diazinon and piperonyl butoxide and women's self-report of pesticide usage over the seven weeks of sequential indoor air sampling was reported. Interestingly, no similar finding was reported for chlorpyrifos, despite chlorpyrifos being detected in indoor air samples from all homes assessed including those measured 2.5 years after the implementation of the voluntary cancellation of indoor chlorpyrifos use.

Study 5: Whyatt et al, 2009

Reference: Whyatt RM, Garfinkel, R, Hoepner LA, Andrews H, Holmes D, Williams MK, Reyes A, Diaz, D, Perera FP, Camann DE, Barr DB (2009). A Biomarker Validation Study of Prenatal Chlorpyrifos Exposure within an Inner-City Cohort during Pregnancy. Environ Health Perspect. 117(4): 559–567

This study used a subset of pregnant mothers and newborns from a pre-existing CCCEH cohort to evaluate trends over time in multiple biomarkers of prenatal chlorpyrifos exposure. Retail sales of chlorpyrifos for indoor use were not permitted in the US from December 2001 onwards. Previous analysis of chlorpyrifos levels in personal and indoor air samples showed a significant decrease in chlorpyrifos levels, as expected, from 2001–2004. The current study was designed to validate a variety of biomarkers for prenatal chlorpyrifos exposure during this time when exposure levels were decreasing after removal from the home indoor market. In this study, chlorpyrifos was measured in prenatal maternal and umbilical cord blood, and TCP, a known chlorpyrifos biomarker, was measured in prenatal maternal and newborn urine and meconium samples.

The current cohort included 102 African-American and Dominican pregnant non-smoking women aged 18-35 years residing in inner-city New York. All women were unemployed to eliminate confounding factors related to potential pesticide exposure in the workplace. Samples were collected between 2001 and 2004. Women who smoked, had a history of drug abuse, diabetes, hypertension, or HIV infection were excluded from participation in the study, as were women who resided in New York City for less than one year or who were employed outside the home at the time of enrolment (for the purposes of this validation study).

Air: Integrated 2–week indoor air samples were collected every two weeks, beginning at the 34th week of pregnancy and continuing until delivery. Personal air samples were collected over 48 h during the 32nd week of pregnancy. Air samples were analysed by gas chromatography mass spectrometry (GC/MS) for chlorpyrifos, and results were as previously reported by Whyatt et al, 2007.

Urine: Repeat prenatal maternal spot urine samples were collected at the end of each 2–week period of indoor air sampling, beginning at the 34th week of pregnancy and continuing until delivery. A total of 253 repeat samples were collected from 97 of 102 women. Urine was also collected the day after delivery from 73 women and 59 newborns. An aliquot was separated for creatinine measurement. TCP was released by enzyme hydrolysis and quantified by high performance liquid chromatography (HPLC)/tandem MS. The LOD for TCP in urine was 0.26 ng/mL.

Blood: Umbilical cord blood was collected from 65 newborns, and maternal blood was collected from 92 women within 2 days post-partum. Paired samples were available for 64 of the mothers and newborns. Chlorpyrifos was quantitated by GC/high-resolution MS. The LOD for chlorpyrifos in blood was 0.5–1 pg/g plasma.

Meconium: Meconium (first intestinal discharges of the newborn infant typically consisting of epithelial cells, mucus and bile) was collected from 83 newborns, and TCP was quantified by HPLC/tandem MS. The LOD of TCP in meconium was 0.2 ng per 0.5 mg sample. The relative standard distribution of the meconium controls was 16%, which is considered to be acceptable.

Analyses of chlorpyrifos and TCP were done at the Centers for Disease Control and Prevention (CDC), according to published quality control protocols. Positive and negative controls were applied for approximately one in every 10 samples. Biological samples were shipped twice a year on dry ice, but the stability of the analytes over the duration of the 4–year study was not stated. The statistical analyses were comprehensive and appear to be appropriate.

A large proportion of samples were below the LOD and therefore, in addition to quantitative measurements, the data were ranked and assessed non-parametrically.

For the TCP urine analyses in prenatal maternal samples, the numbers of subjects by year from 2001–2004 were 32, 25, 26 and 14 (total = 97), respectively. Mean TCP concentration in prenatal urine were 1.4 ± 1.8 µg/g creatinine. The proportion of subjects with TCP concentrations > LOD in one or more of repeat samples decreased significantly over time and was 0.91, 0.84, 0.31 and 0.29, in each year respectively ($p < 0.001$ for years 2001–2002 vs. 2003–2004). The data were ranked (for the purpose assigning percentiles) after adjusting for creatinine, and the mean ranks also decreased significantly from 65.2 in 2001 to 32.9 in 2004 for ($p < 0.001$).

By comparison, measured chlorpyrifos in integrated indoor air samples also decreased from 10.0 to 3.2 ng/m^3 among subjects enrolled in 2001–02 compared with 2003–4 ($p = 0.002$). In maternal personal air samples for the same years, chlorpyrifos decreased from 8.1 to 3.7 ng/m^3 ($p = 0.04$). Spearman's rank correlations were positive and often statistically significant between chlorpyrifos in repeat 2–week integrated indoor air samples and TCP levels in repeat maternal spot urine samples collected during 2001–2002. There were insufficient samples > LOD in 2003–2004 for correlation analyses.

TCP urine concentrations showed high intra-individual variability among the five prenatal and postnatal samples. For example, at the 75th percentile, TCP urine concentrations were 2.0, 1.7, 2.1, 4.3 and 1.5 µg/g creatinine while at the 95th percentile the values were 6.2, 4.2, 6.4, 6.8 and 4.5 µg/g creatinine. This intra-individual variability limits the usefulness of this parameter as an individual biomarker of chlorpyrifos exposure. The authors reported that 57% of the total variability can be explained by within-subject variability.

TCP was detected in 42% of the postnatal maternal urine samples, but no TCP was detected in newborn urine. Therefore it was concluded that there was no contamination of meconium samples by TCP in the urine.

For chlorpyrifos analyses in maternal blood samples, the numbers of subjects by year from 2001–4 were 29, 22, 26 and 15 (total = 92), respectively. The number of participants for which cord blood samples were taken was 21, 14, 20 and 10 (total = 65), respectively. Chlorpyrifos was detected in 19–29% of maternal and cord blood collected in 2001–2002. By contrast, both maternal and umbilical cord blood chlorpyrifos levels were below the LOD in all samples collected after 2002. Not unexpectedly, between 2001–2002 and 2003–2004 there was a significant decrease in detections of chlorpyrifos in maternal blood ($p < 0.001$) and umbilical blood ($p = 0.005$). Maternal and umbilical cord blood levels were highly correlated.

For TCP analyses in meconium, the numbers of subjects by year were 28, 21, 21 and 13 (total = 83). The proportion of samples with TCP > LOD was 0.64 in 2001 and 0.24 in 2002. None was detected in samples from 2003 or 2004. Between 2001–2002 and 2003–2004 there was a significant decrease in detection frequencies for TCP in meconium ($p < 0.001$). TCP levels in meconium were weakly correlated with chlorpyrifos in maternal and umbilical cord blood and with TCP levels in maternal urine samples. The authors suggested that TCP in meconium may provide a valid biomarker of prenatal exposure.

Study 6: Rauh et al, 2011

Reference: Rauh V, Arunajadai S, Horton M, Perera F, Hoepner L, Barr DB, Whyatt R (2011). Seven-year neurodevelopmental scores and prenatal exposure to chlorpyrifos, a common agricultural pesticide. Environ Health Perspect 119(8): 1196–1201

The study consisted of 265 New York City children, who were part of an ongoing prospective cohort study, undertaken by CCCEH including inner-city mothers and their newborn infants. The women were non-smokers, aged 18–35, had an African-American or Dominican background and were free of diabetes, hypertension and known HIV, documented drug abuse, and had resided in the area for at least one year. From the 725 consenting women, 535 were active participants in the ongoing cohort study at the time of this report, and 265 of their children had reached the age of seven years with complete data on the following: 1) prenatal maternal interview data; 2) biomarkers of prenatal chlorpyrifos exposure level from maternal and/or cord blood samples at delivery; 3) postnatal covariates; and 4) neurodevelopmental outcomes.

Prenatal chlorpyrifos exposure was measured by collecting umbilical cord blood plasma within two days post-delivery. Analysis included chlorpyrifos in plasma, as well as lead and cotinine to examine environmental tobacco smoke exposure. PAH exposure was measured by personal air monitoring during the 3rd trimester. The 7-year neurodevelopment assessment was done using the Wechsler Intelligence Scales for Children (WISC-IV) as described by Wechsler (2003). The instrument measured four areas of mental functioning:

1. Verbal Comprehension Index (measure of verbal concept formation, a good predictor of school readiness)

2. Perceptual Reasoning Index (measures non-verbal and fluid reasoning)

3. Working Memory Index (assesses children's ability to memorize new information, hold it in short-term memory, concentrate, and manipulate information) and

4. Processing Speed Index (assesses ability to focus attention and quickly scan, discriminate, and sequentially order visual information).

A FSIQ score was obtained by combining these four indices. Data was analysed using linear regression models to estimate associations. Effect estimates, 95% confidence intervals, and *p*-values were calculated for all analytic procedures with results considered significant at $p < 0.05$. No means or standard deviations for overall IQ or for any other of the subsets were provided.

As reported by the study authors, chlorpyrifos exposure levels ranged from non-detectable (43%, *n* = 115) to 63 pg/g as measured in the umbilical cord blood. The retention rate for the full cohort was 82% at the 7–year follow-up, with no socio-demographic differences between participants reported. Measurements in the umbilical cord blood plasma showed that higher prenatal chlorpyrifos exposure was associated with decreased cognitive functioning for only two indices; FSIQ and Working Memory. On average, for each standard deviation increase in exposure (4.61 pg/g), FSIQ declined by 1.4%, and Working Memory declined by 2.8%. The dose-effect relationships between chlorpyrifos exposure and log-transformed Working Memory Index and FSIQ scores were linear across the range of exposures in the study population, with no evidence of a threshold. While considered statistically significant, the toxicological (biological) significance of correlations between FSIQ, working memory and exposure was considered negligible. Further, in the US EPA critique of this study (US EPA, 2014b), it was suggested that the regression analysis was highly dependent on the outliers (7/102 children with exposures $\geq$ 15 pg/g, and 2/102 with exposures $\geq$ 20 pg/g).

No significant correlation was noted between umbilical cord lead and chlorpyrifos level or WISC-IV scores among the 89 children with lead data available. There was no significant association between birth weight and any of the WISC-IV Indexes and it was not included in the final models. There were no significant association between chlorpyrifos and any of the potential or final covariates, including maternal educational level, maternal IQ, quality of the home environment, or other chemical exposures measured during the prenatal period (environmental tobacco smoke and PAHs).

The Working Memory Index was the measure that was most strongly associated with chlorpyrifos exposure. As Working Memory is less likely to be affected by socioeconomic or cultural conditions than full-scale IQ (Wechsler, 2003, as cited in Rauh et al, 2011), it offers a useful, more targeted measure of possible neurotoxic effects on brain function. Sensitivity analysis was undertaken to determine if the observed chlorpyrifos effect on the Working Memory Index was partially explained by its effect on general intelligence. This was done by adding the

log-transformed General Ability Index, a general intelligence scale that does not include the Working Memory Index or Processing Speed Index, to the linear regression model. The study authors reported that as there was no evidence of interaction between chlorpyrifos and General Ability Index, the results suggest that Working Memory effect is targeted and does not depend upon level of general intelligence. Acknowledging that child performance on the Working Memory Index can be influenced by child behaviour problems (Wechsler 2003, as cited in Rauh et al, 2011), the study undertook a supplementary analysis to exclude any effect that behaviour problems may have on the Working Memory Index using a Child Behaviour Checklist developed by Sobel (1982). The study authors reported that there was no association between child behaviour problems and the Working Memory Index.

Some of the acknowledged limitations of the study include the sample size consisting of low-income, urban, minority children who may experience other unmeasured exposures or underlying health problems which could potentially confound or modify associations with pesticide exposure. It was also noted that due to the absence of mechanistic evidence linking brain anomalies to more refined neuropsychological testing, caution needs to be applied when interpreting the observed functional deficits. The authors were not able to directly compare the findings of this study with the results from the other epidemiologic studies that have relied on urinary OP concentrations as the biomarker of exposure.

Study 7: Helps et al, 2012

Reference: Helps S, (2012). Brain anomalies in children exposed prenatally to a common OP pesticide. Expert Commentary of Rauh et al, 2012 (Unpublished).

This expert commentary reviews the underlying methodological aspects of the Rauh et al (2012) studies. Helps (2012) states that the high-chlorpyrifos exposure group identified enlargement of a number of discrete brain regions including:

- bilateral superior temporal (social cognition)

- posterior middle temporal (attention and language comprehension)

- bilateral inferior postcentral gyri (sensory and motor processing)

- supramarginal gyrus and inferior parietal lobule (language)

- mesial wall of the right hemisphere, the superior frontal gyrus, gyrus rectus, cuneus, and precuneus (social cognition)

- enlargement of underlying white matter, with inward deformations in the dorsal and mesial surfaces of the left superior frontal gyrus (executive functioning).

Helps (2012) notes that cortical thickness was lower in the high-exposure group which may represent glial scarring and that enlargement of these brain structures is usually associated with epilepsy, memory deficits and dementia. The expert commentary did not report any deficiencies in the functional magnetic resonance imaging (fMRI) method employed for the Rauh et al (2012) study, and was in support of the hypothesis that low to moderate levels of exposure to chlorpyrifos during pregnancy may lead to long-term potentially irreversible changes in the brain structure of the child.

Study 8: Horton et al, 2012

Reference: Horton MK, Kahn LG, Perera F, Barr DB, Rauh V (2012). Does the home environment and the sex of the child modify the adverse effects of prenatal exposure to chlorpyrifos on child working memory? Neurotoxicol Teratol. 34(5): 534–541

This paper further examined the epidemiological investigation of the CCCEH by Rauh et al (2012). It primarily involved investigation of whether the quality of the home environment (ie parental nurturance and environmental stimulation) influenced the relationships previously reported between chlorpyrifos and IQ deficits (ie adverse effects of prenatal chlorpyrifos exposure on working memory at child age seven years). The authors state that it is 'the first investigation into factors that may inform an intervention strategy to reduce or reverse the cognitive deficits resulting from prenatal chlorpyrifos exposure'. The study used new social science research techniques to investigate how biological and social factors may exacerbate the negative effects of chemical exposures.

Participants were selected from among the cohort, where children had reached seven years of age at the beginning of the study and had a complete data set. Maternal interviews in the 3rd trimester of pregnancy (and follow-up within a year) included questions about demographics, residential history, living conditions, maternal education, maternal income and employment, illness, alcohol and drug use during pregnancy and chemical exposure (PAH, pesticides, lead, environmental tobacco smoke). In addition at three years of age home environments were evaluated using the 'HOME' inventory (a standardised 1 h observational interview undertaken by a trained researcher using a validated 'HOME' questionnaire). The HOME inventory was analysed (scored) to derive two variables; environmental stimulation and parental nurturance. The total score and the scores for individual variables were derived from a 55 item checklist with these items subcategorised by eight subscales.

The data analysis involved the investigation of potential associations between prenatal chlorpyrifos exposure, the quality of the home environment (Total HOME, as well as Parental Nurturance and Environmental Stimulation subscales), childhood IQ (WISC-IV) and demographic characteristics.

- The median prenatal chlorpyrifos level from analysis of umbilical cord blood is reported as 0.36 ng/g (range 0.25–32.1 ng/g). Forty percent of results were below the LOD—0.5 to 1.5 pg/g plasma (Whyatt 2003).

Multivariate linear regression models were used to examine associations between 'predictor variables' and working memory scores at seven years of age.

–   Predictor variables included:

–   prenatal chlorpyrifos × Total HOME score

–   prenatal chlorpyrifos × child sex and

–   Parental Nurturance × child sex.

The authors reported the following findings:

- There was no observed remediating effect of a high quality home environment (either parental nurturance or environmental stimulation) on the adverse effects of prenatal chlorpyrifos exposure on working memory.

- A borderline significant interaction between prenatal exposure to chlorpyrifos and child sex, with males experiencing a greater decrement in working memory than females following prenatal chlorpyrifos exposure.

- A borderline interaction between parental nurturance and child sex suggesting that, in terms of working memory, males benefit more from a nurturing environment than females.

- The results did not support the hypothesis that a high-quality home environment modifies the adverse effect of prenatal exposure to chlorpyrifos on working memory.

The authors reported the following limitations:

- The cohort comprised exclusively low-income, urban, Dominican and African-American children. The majority of households are headed by single mothers who, because of their role as sole caregivers, may nurture their children differently than partnered women.

- Certain covariates such as prenatal stress, a possible contributor to working memory in children (Entringer et al, 2010), were not included in the analysis.

- Chlorpyrifos exposure data were only collected at the time of delivery, it is impossible to draw any conclusions about the potentially differential effects of exposure at various stages of development.

- There was a high frequency of subjects with levels of chlorpyrifos below the LOD (40% of total).

Study 7: Rauh et al, 2012

Reference: Rauh VA, Perera FP, Horton MK, Whyatt RM, Bansal R, Hao X, Liu J, Barr DB, Slotkin TA, Peterson BS (2012). Brain anomalies in children exposed prenatally to a common organophosphate pesticide. Proc Natl Acad Sci USA 109(20): 7871–7876. With Supporting Information 10.173/pnas.1203396109

The CCCEH was monitoring cognitive health of the children of these women with relation to urban environmental toxicants. Previous studies from the CCCEH had found links between chlorpyrifos exposure and cognitive deficits in children at seven years old. In this publication, authors hypothesised that chlorpyrifos exposure related to cognitive deficits in children could be a result of altered morphological characteristics in brain regions (frontal, parietal, and lateral temporal) that subserve higher-cognitive functions. They set out to analyse this by comparing low and high chlorpyrifos exposure groups and the morphological measures of the cerebral surfaces and cortical thickness, through MRI, and associated these to adverse full-scale IQ (FSIQ) scores and disruptions in normal sex differences in brain morphology.

METHODOLOGY AND CONFOUNDERS

The mothers of the participants were from a larger prospective cohort study (the CCCEH). Women were of African-American or Dominican background from low-income urban areas of New York City. The CCCEH study recruited women who were deemed 'low-risk' (free of diabetes, HIV, hypertension and no documented drug abuse). At birth, umbilical cord blood was collected and analysed for chlorpyrifos and other organophosphates, cotinine (a tobacco smoke biomarker) and metals (including lead); PAH were also measured from third-trimester personal-air monitoring (Whyatt et al, 2003 shows full list of substances tested). Despite lead levels being ~50% higher in the high chlorpyrifos exposure group (1.4 μg/dL to 0.8 μg/dL), the levels recorded are lower than the level of concern. The authors then grouped subjects into two chlorpyrifos exposure groups, low (< 4.39 pg/g) and high exposure (≥ 4.39 pg/g) (US EPA, 2014a). The validity of measuring chlorpyrifos exposure at a single timepoint test rather than longitudinal testing, and thus any potential differences in effects from chlorpyrifos acute and chronic exposure were not evaluated.

Cognitive assessment was performed at 7 yr ± 1 m using the Wechsler Scales of Intelligence for Children (WISC-IV), a common method of testing cognition in children between 6 and 16 years and has also been widely used in lead neurotoxicity studies (refer to previous study, Rauh et al (2011) for further details). It is noted that the qualifications of the researchers administering the WISC-IV questionairres were not specified.

The selection of study participants from the CCCEH cohort of 369 children was contingent on strict parameters and was considered appropriate. For subjects to be included for the full battery of measures, chlorpyrifos, PAH, ETS and WISC-IV testing were required to be completed (this was not the case for previous studies using the same cohort). From the 369 participants, 70 had high chlorpyrifos levels (≥ 4.39 pg/g), only 28 of which had low or no measurable PAH and ETS levels. On the other hand, 99 subjects had low chlorpyrifos levels (< 4.39 pg/g) and low or no measurable levels of PAH and ETS, 38 of which were randomly selected. Of this subset, only 20 participants from each the high and low chlorpyrifos exposure groups (and no measurable levels of PAH and ETS) had MRI data which was used for further analysis. No significant differences in common socio-demographic characteristics were reported between groups and each group was fairly representative of the larger CCCEH cohort. Furthermore, no significant difference in blood lead values was reported between each exposure group (only 13 and 15 subjects had detections of lead for the high and low chlorpyrifos groups respectively).

To study changes in cortical brain morphology the authors employed high-resolution MRI technology. This allowed measurements of overall brain size, distances between points on cerebral surfaces, cortical thickness and differences in underlying white matter. Processing of images was performed by utilising ANALYZE 8.0 Biomedical Imaging Resource and an 'in-house software'. Expert reviewers in the US EPA assessment stated that ANALYZE 8.0 software is in common use in biomedical imaging research and the method of assessment did not deviate from common practices (US EPA, 2014a). The images were processed to assess; cortical grey matter segmentation, morphological maps of the cerebral surface, and cortical thickness. The study focussed on forebrain morphology given the role of the forebrain in cognition. The MRI image readers were blinded to chlorpyrifos exposure and hemisphere orientation providing objective assessments. However, the use of other common MRI analytical software/protocols to cross-reference the data may have helped to further validate the findings of this study. It is also important to note that MRI imaging (5.9–11.2 yr) may have been performed at a different time to the FSIQ testing (7 yr). This limitation impacts the direct comparison between FSIQ and brain morphology as it is important that these two tests were completed within the same time period.

To compare brain images between subjects, the authors employed a two-step process of selecting a *template brain*. First, a brain that was the closest representation of the average subject of the cohort of forty was chosen, ie child age, weight and height. Subsequently, the remaining 39 subjects' brains were then compared to this initial template (in accordance to those set out by the Consortium on Brain Imaging) and distances between points on the cerebral surface were measured. The brain that most closely reflected the average of cerebral surface measurements was chosen as the *template brain*. The purpose behind choosing a *template brain* rather than a 'synthetic, average brain' was increase image clarity. A template brain chosen from the cohort was deemed superior by study authors to a synthetic average as it has greater sharpness of brain anatomy, thus making clearer measurements for comparison. The authors acknowledge that selection of a *template brain* can skew (either positively or negatively) the interpretation of any differences in measurements between two groups depending on the whether the *template brain* exhibits distances that are higher or lower than an average brain. The study authors did not indicate which exposure group the template brain was derived from.

The rationale for having two dose groups [low (< 4.39 pg/g) and high (≥ 4.39 pg/g)] was to maintain a level of statistical power within the small cohort and given the extensive inclusion/exclusion criteria. No dose-response relationship could be determined between the two groups, as no negative control group (chlorpyrifos exposure < 1 pg/g) was included in the study protocol. This is a significant limitation, noting that the study authors reported that low exposure (< 4.39 pg/g) had an effect on brain morphology.

The study authors reported that children in the high chlorpyrifos exposure group exhibited general enlargements of certain cortical regions, enlargements of white matter and cortical thinning compared to the low chlorpyrifos group. These cortical regions have been reported to be important for social cognition, attention and language comprehension, sensory and motor processing, as well as executive functioning (Helps, 2012).

Of interest is that there was no significance increase in the overall brain size (either unadjusted or adjusted for age, sex and height) between high and low-chlorpyrifos exposure groups (as is seen in animal studies). Juberg (2012) in correspondence to Rauh et al (2012) suggested that the animal studies involved doses of 5 mg/kg bw/d while the estimated dose of chlorpyrifos in the Columbia cohort was 0.027 µg/kg/d and general population exposure is estimated to be 0.01 µg/kg/d.

The study authors reported that the brains of the high-chlorpyrifos exposure group were significantly enlarged bilaterally in the superior temporal, posterior middle temporal, and inferior postcentral gyri, and superior frontal gyrus, gyrus rectus, cuneus, and precuneus in the mesial views of the right hemisphere. Further, it is reported that the enlargement is largely attributable to the white matter as presented in supplementary information. The studies by *Roy et al (2004; 2005) were cited as evidence from experimental animal studies of morphological changes (subtle changes in cortical thickness and neuronal and glial cell proportions in the septal nucleus, striatum, somatosensory cortex, and hippocampus).

For the low-chlorpyrifos exposure group, the study authors report a positive relationship between increased distance measures on the cerebral surface and increased FSIQ, but these observations are inversely related among the high chlorpyrifos exposure group with greater regional brain size associated with lower FSIQ scores. The authors do not comment on the significance of this finding, but generally discuss a link between these areas of the brain and cognition.

The study authors reported that the preliminary analyses indicates that the high-chlorpyrifos exposure group did not show expected sex differences in the right inferior parietal lobule and superior marginal gyrus, and there was a reversal of expected sex differences in the right mesial superior frontal gyrus. The authors state that this is consistent with exposure effects on disruption of normal behavioural sexual dimorphisms, as is reported in animal models. Also reported was frontal and parietal cortical thinning in the high exposure group compared to the low dose group.

CONCLUSION

The overall conclusion of the study is that there are significant associations of prenatal exposure to chlorpyrifos at levels observed with routine (non-occupational) use and below the threshold for any signs of acute exposure, with structural changes in the developing human brain observed.

There were methodological issues with the MRI data analysis, such as the lack of validation of the computation method used and further information regarding the source of the template brain. The authors also acknowledge other limitations of the study, one of which is the modest sample size including only 40 children.

The sample size makes it difficult to detect within-group correlations and to test multiple interactions of exposure with other variables. The reason for this sample size was the study requirement that participants have minimal or no prenatal exposure to ETS and PAH, as these are known neurotoxicants and therefore are potential confounders in the assessment of the neurodevelopmental effects of chlorpyrifos.

Another limitation of the study is that the cognitive assessment was limited to a standard, broadband performance measure where more sensitive and functionally specific measures of cognitive and behavioural functioning may yield more anatomically relevant correlations of those measures with regional effects of chlorpyrifos on brain structure.

The supporting information document provides a technical overview of the methodology used in the above Rauh et al (2012) study including the MRI Acquisition, Image Segmentation and overview of the Analysis of Surface Morphologies. Details are also provided for the steps used in the selection of the template brain and the statistical modelling procedures used in the population of participants (Rauh et al, 2011).

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*Study evaluated in the Supplementary report on developmental neurotoxicity and behavioural toxicity. Implications for current Australian Public Health Standards (Department of health, 2016).

US EPA REVIEW OF CCCEH COHORT STUDIES

In 2014, the US EPA published a revised human health risk assessment for registration review for chlorpyrifos, which updated the previous US EPA review undertaken in 2011 (US EPA, 2011). This revised assessment includes new evidence from experimental toxicology and epidemiology with respect to ChE inhibition and neurodevelopmental outcomes. Key issues that the US EPA were aiming to address through this review included investigation of whether chlorpyrifos causes long-term effects from prenatal and/or early life-stages exposure and the doses at which any adverse effects occurred relative to those associated with RBC AChE inhibition. As part of this process, the US EPA met with the CCCEH researchers to gather information to address limitations identified in published reports (Rauh et al (2011); (2006); Whyatt et al (2004)). The US EPA specifically requested additional information to ascertain if AChE inhibition would have occurred with the reported adverse outcomes and the role of other environmental chemicals (lead, PAHs etc.) in the reported adverse effects. Information provided by the researchers indicated that the CCCEH study design did not incorporate pre- or post-pesticide use/exposure measurement in the study protocol.

Further, the quality of the written questionnaires was considered to be low, hence it was difficult to ascertain the pattern and frequency of OP pesticide use among the cohort participants. However this information was later sourced directly from the New York City Department of Health, to enable the US EPA to undertake appropriate analysis (US EPA, 2014a).

In regards to the confounding issue of lead exposure, Rauh et al (2006) demonstrated that blood lead levels and cord blood chlorpyrifos levels are very weakly correlated; hence the US EPA concluded that adverse effects are unlikely to be related to lead exposure. This conclusion was later supported by information from the US State Health Department that indicated blood lead levels decreased by 92% between 1995 and 2008. Considering that it is known that lead exposure results in different changes in MRI images (affecting grey matter) as compared to those exposed to chlorpyrifos (affecting white matter), the confounding issue relating to lead was dismissed. The effect of postnatal exposure to ubiquitous PAH via air pollutants was determined to be beyond the scope of the CCCEH studies, and thus remain unknown. Similarly, the effects relating to exposure to different OPs could not be ascertained.

EXPERT REVIEW OF RAUH ET AL (2012). (US EPA, 2014B)

A separate review of Rauh et al (2012) paper was undertaken by US EPA (2014b). Some of these comments have been included in the OCS critical appraisal of the Rauh et al (2012) study above. Additional comments by the US experts have been included here for completeness:

1. Reanalysis of the MRI data using different analytical programs that use different methods and assumptions, and an average brain approach would allow one to establish consistency in the qualitative findings.

2. One expert commented on the high exclusion rate due to poor quality of MRIs scans in the high (20/28) and low dose (20/38) groups, that this was understandable considering that the patients were young children, and calling for greater details regarding the methods employed to reduce motion during MRI scan.

3. Another expert indicated that the scales used in the Rauh et al, studies were appropriate, but advised caution, noting that the scales were standardised using data collected from the generalised population not from a subset of the population (Dominican, African-American, predominantly residing in the northeast and having a relatively low income).

4. It was noted by another expert that the distributions of the BSID-II scores for both PDI and MDI were reported in the Rauh et al (2006) paper for both the low and high exposure groups. The PDI scores appear to be relatively similar to normal population for this instrument, while the MDI scores for the lower exposure group range from 6–15 points less than PDI scores at age 12–24 months of age. Therefore, the sample of children was already below the average score for MDI when the MDI tool was standardized. This expert further indicated that it was impossible to compare the WISC-IV scores to the population norms because no information was provided regarding the distribution of WISC-IV scores (ie mean and standard deviation) in the Rauh et al (2011) paper. Further the expert opined that means and standard deviations for these scores should be presented for either a non-exposed or a non-exposed combined with low exposed group and these should be compared to a moderate or high exposed group similar to BSID-II in the Rauh et al (2006) study.

Overall, the fMRI and statistical methods employed by Rauh et al (2012) were also positively reviewed by US EPA (2014b) as reported above.

2.4   Appraisal of literature reviews (non-meta analytical)

Study 1: Zhao et al, 2005

Reference: Zhao Q, Gadagbui B, Dourson M (2005). Lower birth weight as a critical effect of chlorpyrifos: a comparison of human and animal data. Regul Toxicol Pharmacol. 41(1): 55–63

In this review article authors investigated in the light of epidemiological study findings of impaired fetal development whether ChE inhibition was still the most sensitive indicator of adverse effects and thus the basis for the point of departure for human health risk assessment.

In order to investigate the central question, the review authors conducted an evaluation of three epidemiology studies in a 'side-by-side comparison'. The three studies reviewed included Whyatt et al (2004), Eskenazi et al (2004) and Berkowitz et al (2004). The study by Whyatt et al was one of the earlier reports from the prospective cohort of inner city (New York) African-American and Dominican women and their children (more recently reported by Rauh et al, 2006; 2011 and 2012).

Table 10: Summary of basic characteristics of the three epidemiology studies reviewed by Zhao et al, 2005

	Whyatt et al, 2004	Eskenazi et al, 2004	Berkowitz et al, 2004
Institution/Affiliation	Columbia Centre for Children's Environmental Health	University of California—Berkley, Salinas Valley California	Mt Sinai Hospital (east Harlem) New York City
Population	African (58%) and Dominican (42%)	Majority Hispanic	Hispanics (predominantly Puerto Rican), African, whites
Chlorpyrifos levels	Maternal blood, cord blood	Maternal blood, cord blood and urine (TCP) measured PON1 activity	Maternal blood, cord blood and urine (TCP) measured PON1 activity
Zhao comments on associations reports	Association between cord blood chlorpyrifos and fetal birth weight but not head circumference. However there is no association between chlorpyrifos in maternal personal air samples and fetal growth indices.	Diethyl phosphates and TCP were not associated with fetal bodyweight changes; there was no association between maternal or fetal blood or plasma ChE inhibition and fetal birth weight, length and head circumference.	There is no association between TCP and fetal growth indices; Maternal PON1 activity is associated with head circumference when urine TCP > LOD

ChE = Cholinesterase, LOD = Limit of detection, PON1 = Paraoxonase 1, TCP = 3,5,6-trichloro-2-pyridinol

The Zhao et al, 2005 review provide a summary of seven animal developmental/reproductive studies: four studies in rats (1 Fischer 344, 3 SD) and 3 two-generation reproductive toxicity studies. The review authors note the decreased fetal birth weight is seen at ≥ 5.0 mg/kg bw/d. Doses of ≥ 5 mg/kg/d cause maternal toxicity such as decreased bodyweight, lactation and brain AChE inhibition. The authors discuss findings by James et al (1988) of a decrease in fetal weight at ≥ 0.5 mg/kg bw/d, but noted that JECFA have concluded that this finding is more likely to be due to an increased litter size rather than chemical toxicity. The authors conclude that given the significant maternal toxicity observed at the same treatment dose as the decreased in fetal birth weight, this is likely to be secondary to maternal toxicity caused by chlorpyrifos exposure.

The authors discuss available *in vivo* studies (Garcia et al, 2002 and Qiao et al, 2002) conducted via the parental administration-route during early and late gestation in support of the above conclusion, where bodyweight changes after s.c. treatment on GD17–20 occurred at higher doses than those found to be associated with maternal toxicity.

Several points on a comparison of chlorpyrifos internal dose between human and animal studies is provided below:

- The authors analysed the results of Mattsson et al (2000) that reported blood chlorpyrifos concentrations of g0, 3 and 109 ng/g on GD20 in dams receiving daily chlorpyrifos treatment of 0, 0.3, 1.0 or 5 mg/kg bw/d. Blood chlorpyrifos concentrations and ChE levels in plasma and RBCs up to PD22 are also reported. The three main points of the analysis reported by the authors include:

  - A clear dose-response relationship exists between inhibition of ChE activities and both plasma and RBC chlorpyrifos concentrations.

  - The NOAEL for the fetus and pups for both plasma and RBC ChE inhibition are 1 ng chlorpyrifos/g blood.

- The authors of the Whyatt et al (2004) study reported that the umbilical cord blood concentration was 2.5 pg/g which is 400 times lower than the NOAEL (ChE inhibition) in rat fetuses.

The authors also note that there is a comparable sensitivity between animals and humans to chlorpyrifos ChE inhibition, concluding that 'exposure to chlorpyrifos encountered by the human population living in New York City would cause decreased fetal birth weight or body length. But direct comparison of experimental animal neonatal information indicates that ChE inhibition is a more sensitive indicator of effect than reduced bodyweight and that neonates are equally or perhaps less sensitive to ChE inhibition than their maternal parent'.

Study 2: Prueitt et al, 2011

Reference: Prueitt RL, Goodman JE, Bailey LA, Rhomberg LR (2011). Hypothesis-based weight-of-evidence evaluation of the neurodevelopmental effects of chlorpyrifos. Crit Rev Toxicol. 41(10): 822–903

Prueitt et al (2011) published a detailed review paper presenting a hypothesis-based weight of evidence approach of the epidemiology and animal toxicity data for chlorpyrifos and any causal relationship between chlorpyrifos and adverse birth outcomes. Further, consideration was given to the appropriate critical endpoint to support a regulatory assessment of chlorpyrifos.

The evaluation was conducted as a submission to US EPA made by Gradient; an environmental and risk sciences consulting firm and funded by Dow Chemical (aspects of the evaluation are also summarised in Goodman et al (2012)), which is also evaluated separately.

In addition to a review of studies and causal relationship assessment the paper discusses a hypothesis-based weight of evidence framework to assess and analyse evidence.

The review can be summarised within the following descriptive categories:

- the methodology proposed and described as hypothesis-based weight of evidence

- review of epidemiology studies by endpoint

- review of toxicology studies by endpoint

- review of mechanistic studies

- weight of evidence conclusions on the potential neurodevelopmental toxicity of chlorpyrifos.

METHODOLOGY—HYPOTHESIS-BASED WEIGHT OF EVIDENCE

The method while represented by the review authors as a new approach, is complementary to a conventional weight of evidence appraisal in toxicology. This approach has been incorporated within the World Health Organisation/International Programme on Chemical Safety mode of action/human relevance framework, as a structured approach to assess hypothesised modes of action using human and animals studies (Meek et al, 2013).

Prueitt et al (2011) examined whether the available evidence supports a causal association between low chlorpyrifos exposures (below the acetylcholinesterase (AChE) activity inhibition threshold) and neurodevelopmental effects.

Review of Epidemiology Studies: The authors provided an overview of the reviewed papers in a summary table which provide the statistical summaries of each relationship examined.

The evaluated neurodevelopmental endpoints included newborn head circumference, cognitive and behavioural outcomes using Bayley Scale of Infant Intelligence (12, 24, 36 months old), infant neurodevelopment using Brazelton Neonatal Behavioural Assessment Scale (BNBAS) (≤ 2 months of age), Weschler Scale of Intelligence (7 yo) and child behavioural outcomes. These endpoints were considered with a range of chlorpyrifos exposure measurements and estimates including maternal prenatal and postnatal blood sampling, newborn cord blood sampling, ambient air sampling, maternal urinary concentration of TCP, maternal and child urinary concentration of DEP and DEPT.

An overview of the cohort study findings by endpoint evaluated, children's age, the summary of results and the review author's appraisal is outlined below (Table 11).

Table 11: Summary of results, postnatal endpoints and age of assessment

Endpoint	Age evaluated	Summary of results	Analysis
Newborn head circumference	0–6 months	Negative (all 4 cohorts; 6 studies)	Studies of associations between chlorpyrifos exposure and newborn head circumference have reported consistently null results.
Infant neurodevelopment (BSBAS)	0–6 months	Negative (abnormal abdominal reflexes in 2/2 cohorts1)	Studies examining infant neurobehaviour reported no clinically relevant association between increasing maternal urinary levels of DEPs and BNBAS scores.
Cognitive and motor development			
Bayley scale of infant intelligence	12 months	Negative (3/3 cohorts)	Studies examining chlorpyrifos exposure and cognitive and motor development are not consistent with respect to the exposure metric and timing of outcome assessment.
	2 years	Negative (3/3 cohorts)	
	3 years	Positive (1/1 cohorts)	
Weschler scale of intelligence2	7 years	Positive in one cohort working memory but not verbal comprehension, perceptual reasoning or processing speed	

Endpoint	Age evaluated	Summary of results	Analysis
	7–9 years	Negative	
Child behavioural outcomes3	2 years	Positive (1/1)	Studies examining behavioural outcomes in children with chlorpyrifos exposure reported few results in the clinical range.
	3 years	Positive 1/1, attention problems	

[1] BNBAS is designed to only potentially identify gross neurologic abnormalities; more than three abnormal results in reflexes is considered clinically relevant. This study only examined newborns once (potential misclassification bias) and did not include births that occurred on weekends (potential selection bias).

[2] Unclear whether results adjusted for socioeconomic status or child behavioural problems

[3] Results based on maternal reports (potential reporting bias and misclassification bias); the test has low sensitivity for inattention for children of the age group examined.

Based on the analysis of the results of the cohort studies, Prueitt et al (2011) concluded the evidence was not sufficiently robust to support a causal relationship between chlorpyrifos and neurodevelopmental effects. The conclusions were drawn based largely on the factors summarised in Table 12.

Table 12: Summary of causal assessment of epidemiological evidence by Prueitt et al (2011)

Cohort	Evaluation criteria	Comments
General Conclusions	Consistency/Clinical Significance	The clinical significance of reported associations for cognitive and motor development is questionable as most of the results fell within normal ranges.
		The significance of child development scores from maternal reports (ie to measure attention problems, ADHD, PDD) are difficult to compare to the standardised DSMO –IV oriented scale and are therefore not considered equivalent to a diagnosis for these conditions.
	Dose-response/Exposure issues	Inadequate information on exposure and dose-response to conclude causal association.
		Many studies used an indirect measure of exposure or were based on potential multiple chemical exposures.
	Confounding and bias	Many of the studies were unable to rule out alternative explanations for the associations—many additional neurodevelopmental causes or relationships were not considered or adjusted for in analysis.
		Bias was introduced in the methodology and it is unclear as to whether this was accounted for in the final results.
	Power	Most studies have a low chance of observing an association based on the small sample sizes used.

Review of Animal Studies: The review authors considered 25 papers investigating potential association between chlorpyrifos and neurodevelopmental responses in experimental animals (rodents, predominantly SD rats and CD-1 mouse).

Prueitt et al (2011) reviewed studies where the exposure periods generally ranged from GD1 to the post-weanling period, up to PND25 over a range of pre- and postnatal timepoints. The doses ranged from 0.03 to 40 mg/kg bw chlorpyrifos and were administered via subcutaneous or intraperitoneal injection, oral gavage or dermally. There were no inhalational studies evaluated.

The studies examined endpoints including social behaviour (including maternal behaviour), emotion and anxiety, motor function (including locomotor activity, neuromuscular and neuromotor function, and sensorimotor reflexes), and cognitive function (ie learning and memory). In addition, many of the studies reviewed included brain (and other) AChE activity.

The findings by endpoint are summarised in Table 13.

Table 13: Summary by neurodevelopmental endpoint of key Prueitt et al (2011) findings from review of animal studies

Endpoint	Doses tested (mg/kg bw)	Key findings
Social and maternal behaviour—three studies by the same research group; exposure via oral gavage or subcutaneous injection		
Socio-agonistic behaviour	CD-1 mice (*n* = 10/dose) Dams—0, 3, 6 on GD15–18 via oral gavage Pups—0,1,3 PND11–14 via subcutaneous injection	Increased offensive upright posture (M pups) during PND75–80 at prenatal exposure of 6 mg/kg bw/d. Increased frequency and duration of attacks (M pups) at postnatal exposure of 3 mg/kg bw/d. No effect on brain AChE activity during pre or postnatal dosing, or on GD19 and PND15. Maternal behaviour: Increased frequency and duration of crouch response, decreased frequency but increased duration of licking and decreased sniffing at ≥ 1 mg/kg bw/d PND11–14.
	CD-1 mice (*n* = 7–10/dose) Dams—0, 3, 6 on GD15–18 via oral gavage Pups—0, 1, 3 on PND11–14 via subcutaneous injection	Increased aggressive grooming (M) at 1 mg/kg bw/d PND1–4 Increased aggressive response frequency at ≥ 1 mg/kg bw/d PND1–4 Increased aggressive response frequency at ≥ 1 mg/kg bw/d PND11–14, but higher at 1 mg/kg bw/d than 3 mg/kg bw/d Transient effects on AChE inhibition at ≥ 1 mg/kg bw/d PND1–4
	CD-1 mice (*n* = 12–15/dose) Pups (F)—0, 3 on PND11–14 via subcutaneous injection mated on PND60	Increased latency to licking pups, decreased defensive posture increased digging duration, increased investigative behaviour, increased latency for nesting.
Emotion and Anxiety—nine studies consisting of exposure via oral gavage or subcutaneous injection		
Anxiety and distress calling in pups		Increased anxiety only observed in studies with oral c exposures of 6 mg/kg bw/d on GD15–18 or at 1 mg/kg bw/dy on GD15−PND14. Effects at 1 mg/kg bw/d GD15−PND14 were not dose dependent, as no effects were observed at 5 mg/kg bw/d (which inhibited AChE activity in the brain).
Anxiety (elevated plus maze test)		
Anxiety (light/dark box test)		
Mood (forced swim test)		
Motor Function—seventeen studies consisting of exposure via oral gavage, subcutaneous injection, nursing, intraperitoneal injection or dermally.		
Locomotor activity		Mostly negative results across neurodevelopmental tests. Positive results were noted at doses that inhibited AChE activity

Endpoint	Doses tested (mg/kg bw)	Key findings
Neuro-muscular and neuromotor function		Most subcutaneous exposures report negative effects.
		One study reported deficits in neuromotor function in the rotorod test with i.p. exposure to a chlorpyrifos formulation (Dursban) at 0.03 mg/kg bw/d prenatally and 0.1 mg/kg bw/d postnatally, however this may have been due to the presence of xylene in the formulation.
		Positive results were noted at doses that inhibited AChE activity.
Cognitive function—ten studies evaluating one or more tests assessing exploratory behaviour, learning or memory; exposure via oral gavage, via nursing or subcutaneous injection.		
Learning and memory (T-maze test)		No effects up to 5 mg/kg bw/d administered either pre or postnatally.
Memory (radial arm maze)		Generally no effects at doses up to 6 mg/kg bw/d administered either pre or postnatally. One study reported AChE activity inhibition at exposures ≥ 1 mg/kg bw/d. One study reported an increase on working and reference errors in females only at 1 mg/kg bw/d PND1–4.
		One study reported an increase in reference errors in males only at 1 mg/kg bw/d PND1–4.
Habituation (Figure-8 apparatus)		In one study involving administration at 5 mg/kg bw/d (GD9–12), increased habituation was observed. In one study at ≥ 1 mg/kg bw/d GD17–20 females had slower habituation than controls.
		In one study slower habituation was noted at 5 mg/kg bw/d PND11–14 which was not seen at 1 mg/kg bw/d PND1–4.
Other cognitive function tests		Transient effects were seen in both sexes at 1 mg/kg bw/d GD17–20 in the foraging maze test. No effects were seen at 3 mg/kg bw/d PND1–4 or PND11–14 in passive avoidance learning or novelty-seeking behaviour, AChE inhibition was noted from ≥ 1 mg/kg bw/d. Transient effects in new cage exploration were noted at 3 mg/kg bw/d PND11–14.
		Transient effects were seen at 7 mg/kg bw/d PND7, 11, 15 or PND22, 26 in the Morris swim test. An effect in the probe version of the swim test showed learning deficiencies at 7 mg/kg bw/d PND22, 26 that did not increase with dose. AChE activity was measured on PND28 and showed no inhibition of activity.

AChE = Acetylcholinesterase;GD = Gestational Day; M = male; PND = Postnatal day

Observations of adverse neurodevelopmental effects in animals were inconsistent and transient across different studies and did not demonstrate consistent dose-response relationships. The majority of studies reporting effects involved administration by s.c. injection, which is not a dose-route relevant to human exposure. Importantly, studies involving oral administration reported minimal neurodevelopmental effects, and these occurred at doses that have also been associated with AChE inhibition. It was concluded that the animal data does not support adverse neurodevelopmental effects of chlorpyrifos at doses below those associated with systemic toxicity or AChE inhibition.

EVALUATION OF MECHANISTIC DATA

Because AChE inhibition is not observed in humans at low exposures of chlorpyrifos, alternative modes of action have been proposed that act below the AChE inhibition threshold and are dependent on parent compound effects rather than the mode of action regulated by chlorpyrifos-oxon.

A summary of an appraisal undertaken by Prueitt et al (2011) is outlined below.

Table 14: Summary of mechanistic data evaluated by Prueitt et al (2011)

Mode of action and summary	Summary of data
Neuronal differentiation *In vitro* doses of ≥ 1μM and *in vivo* doses of 1 or 5 mg/kg bw/d (prenatal and postnatal) have effects on neuronal differentiation and function. These effects are seen at doses with and without inhibition of AChE activity.	*In vitro*: • At 1 μg/mL—inhibition of nerve growth factor-induced neurite outgrowth in rat PC12 cells. • At ≥ 0.001 μM (in the presence of CYP450 inhibitor)—inhibition of axon outgrowth in embryonic rat sympathetic neurons; suggesting effects due to parent compound • At 1–10 μM—enhanced BMP-induced dendritic growth (at levels of AChE inhibition, however TCP also causes these effects and does not inhibit AChE) • At 0.001–10 μM—decreased axonal length in primary sensory neurons from embryonic rat dorsal root ganglia (AChE inhibition occurred at ≥ 0.1 μM; effects on axon length required presence of AChE) • At 5 μM—reduced choline acetyltransferase (ChAT) in PC12 cells only occurred in cells at the start of the differentiation phase *In vivo:* • At 1 mg/kg bw/d PND1–4—decreased synaptic proliferation in cerebellum (rat) • At 1 mg/kg bw/d PND1–4 and 5 mg/kg bw/d PND11–14—increased synaptic activity of norepinephrine and dopamine in cerebellum>forebrain and brainstem (rat) • At 1 mg/kg bw/d GD17–20—slight change in synaptic proliferation; suppression of forebrain synaptic activity in offspring with recovery by weaning; deficits were noted in learning and memory at adolescence/adulthood (rat) • At 1 mg/kg bw/d GD9–12—increased synaptic proliferation; decreased hippocampic and striatum activity in adolescence/adulthood (rat)

Mode of action and summary	Summary of data
Oxidative stress *In vitro* doses of ≥ 1 µM and *in vivo* doses shown to inhibit AChE activity chlorpyrifos exposure induces oxidative stress in neuronal cells, especially in cells undergoing differentiation	*In vitro*: • At 0.5–50 µg/mL (1.4–142 µM) for 10 minutes—dose dependent increase in ROS in PC12 cells; not seen in cells treated with chlorpyrifos-oxon; effects were transient for parent compound at 24–72 hours exposure • At ≥ 1 µM—increased lipid peroxidation in undifferentiated and NGF-differentiated PC12 cells; lack of enhancement seen in differentiated cells indicates a non-cholinergic response • At ≥ 30 µM—dose dependent increase in CG-4 (oligodendrocyte progenitor) cell death; transient increase in ROS; protection of cells seen with addition of Vit E. • At 30 µM—larger and more widespread transcriptional change in mRNA levels of genes in differentiating PC12 cells; increased effects on expression of genes for excitotoxic cell death in undifferentiated PC12 cells *In vivo*: • At doses above the AChE activity threshold (not specified) GD17–20 or PND1–4—no increase in lipid peroxidation (rat) • At 5 mg/kg bw/d PND11–14—increased lipid peroxidation in forebrain and cerebellum in males only (rat)
cAMP-related cell signalling *In vitro* exposure disrupts and reprogrammes AC signal transduction pathway which *in vivo* is only seen at levels that inhibit AChE activity	*In vitro:* • At 60 pM—increased pCREB levels in rat cortical neurons; inhibition of AChE noted at ≥ 1 µM • At 1–10 nm—increased pCREB levels in rat hippocampal neurons; not seen in astrocytes at up to 10 µM; effects not changed with addition of CYP450 inhibitor • At 30 µM—decreased basal, fluoride-stimulated and forskolin-stimulated AC activity in differentiating PC-12 cells; not protected by AChE receptor inhibitor or Vit E; addition of theophylline restored AC activity • At 50 µM—decreased AC signalling in differentiating PC12 cells; effects reversed within 4 days *In vivo:* • At 1 mg/kg bw/d PND1–4—decreased AC activity at PND10 in brainstem, cerebellum and heart but not PND5; not as severe when treated at 5 mg/kg bw/d PND11–14; AChE inhibition noted at ≥ 1 mg/kg bw/d PND1–4 which resolved within 5 days (rat) • At 5 mg/kg bw/d GD9–12—effects (not specified) on AC signalling pathway; transient effects at GD17–20 between sex, exposure time and brain region

Mode of action and summary	Summary of data
Serotonergic dysfunction	*In vivo:*
Effects only seen at levels that inhibit AChE activity	• At ≥ 1 mg/kg bw/d GD9–12—increased serotonin and dopamine in cerebral cortex at PND30; increased serotonin transporter (5HTT), serotonin (5–hydroxytryptamine, 5HT) receptors, 5HT1A and 5HT2 in cerebral cortex, midbrain and brainstem in adulthood (rat)
	• At ≥ 1 mg/kg bw/d GD17–20—increased serotonin activity in brain regions with 5HT projections or cell bodies in adolescent males; seen in females at 5 mg/kg bw/d GD17–20 (above threshold for AChE inhibition); similar effects for dopamine activity at a lower magnitude with no sex difference (rat)
	• At ≥ 1 mg/kg bw/d GD9–12—increased 5HT1A, 5HT2, serotonin in males in brain regions with 5HT projections in adulthood
	• At 5 mg/kg bw/d—decreased serotonin levels; increased serotonin activity; increased dopamine turnover in cerebral cortex, striatum and midbrain (rat)
	• At ≥ 1 mg/kg bw/d—decreased dopamine in hippocampus (rat)
	• At ≥ 1 mg/kg bw/d PND1–4—increased serotonin receptors in adulthood with enhanced effects seen in males and brain regions with 5HT cell bodies; increased 5HTT in brainstem, decreased in all other brain areas examined; increased 5HT activity; decreased dopamine level and activity in cerebrocortical area, increased in the striatum; increase dopamine activity in the midbrain; behavioural tests showed males with feminized test scores
	• At 5 mg/kg bw/d PND11–4—smaller increases in %HT1A and 5HT2 in adulthood; same decreases in 5HTT levels; no effects seen on 5HT or dopamine

5HT = Serotonin (5–hydroxytryptamine); 5HTT = Serotonin transporter (5–hydroxytryptamine transporter);

AChE = Acetylcholinesterase; BMP = Bone morphogenetic proteins; CYP450 = Cytochromes P450; GD = Gestation day;

PND = Postnatal day; ROS = reactive oxygen species

Prueitt et al (2011) concluded that there is little evidence to support any of the alternative modes of action for neurodevelopmental outcomes as the effects seen are at levels where inhibition of AChE activity is expected or *in vitro* exposure levels correspond to approximately 1000-fold higher than those *in vivo*. It is also noted that the weight of evidence suggests that there is no indication of causation in epidemiology studies linking chlorpyrifos to adverse neurodevelopmental effects. Further, it is also suggested that the effects seen at doses below the threshold of AChE inhibition are not relevant for humans as *in vitro* effects are not replicated *in vivo* without inhibition of AChE.

Study 3: Goodman et al, 2012

Reference: Goodman JE, Prueitt RL, Rhomberg LR (2012). Incorporating low-dose epidemiology data in a chlorpyrifos risk assessment. Dose Response 11(2): 207–219

Goodman et al (2012) published a review paper evaluating whether AChE inhibition is a critical endpoint for the regulatory assessment of low level exposures to chlorpyrifos that may result in neurodevelopmental effects. The paper discusses the use of epidemiology data in considering the selection of the point of departure for chlorpyrifos relying on the information reported in the previous review by Prueitt et al (2011) (which is evaluated for the purposes of this report below).

In particular, it was investigated whether there is potentially a non-cholinergic mechanism of toxicity evident at exposure levels below AChE inhibition, and if so, what entity is driving the toxicity, the parent compound chlorpyrifos or the metabolite chlorpyrifos-oxon.

The review authors state that much of the data pertaining to a non-cholinergic mechanism are from *in vitro* studies with little *in vivo* data to support effects at low exposures to chlorpyrifos. Additionally, it is noted that many *in vitro* studies had inherent confounding experimental factors that may be contributing to observed biological effects (e.g. using DMSO vehicle and poor assay specificity).

It was reported that the single *in vivo* human study which reported adverse biological effects (at 1 mg/kg bw/d on GD17–20) did not provide evidence of the absence of AChE inhibition. Further, animal studies that investigated the neurodevelopmental effects were of limited regulatory value due to the number of doses administered and the subcutaneous route of exposure employed that has no relevance to human exposure (subcutaneous injection of chlorpyrifos that circumvents first-pass liver metabolism in humans). Studies with a combination of oral and subcutaneous exposure showed no adverse effects in neurodevelopmental tests but a dose-response was associated with AChE inhibition.

In cohort studies in children the exposure estimates are at least 1000-fold lower than those used in the experimental animal studies or in *in vitro* mechanistic studies where AChE inhibitions were reported. In the cohort studies that reported adverse neurodevelopmental effects, significant methodological deficiencies were noted, such as bias, confounding, exposure misclassification, statistical artefact or lack of dose-response, resulting in a lack of robust evidence to support a causal relationship with chlorpyrifos exposure.

Overall, there is little data and inconsistent effects seen in *in vitro*, *in vivo* and epidemiology studies to support a non-cholinergic mode of action.

Based on the data (and its limitations) the review authors concluded that the current approach of protecting against AChE inhibition is applicable to risk assessment for the general population including sensitive life stages (ie developing nervous system). The authors further note that no additional uncertainty factors should be applied to protect against non-cholinergic mechanisms of action on the developing nervous system as the data show no robust evidence to support a non-cholinergic mechanism acting at levels below AChE inhibition.

Study 4: Li et al, 2012

Reference: Li AA, Lowe KA, McIntosh LJ, Mink PJ (2012). Evaluation of epidemiology and animal data for risk assessment: chlorpyrifos developmental neurobehavioural outcomes. J Toxicol Environ Health B Crit Rev. 15(2): 109–184

This review aimed to conduct a review of epidemiological and animal studies of chlorpyrifos neurodevelopmental toxicity. The authors approach to the review was to provide:

- In-depth analyses of the analytical epidemiologic studies focused on neurobehavioural outcomes in infants and young children with comparisons of methodologies (including exposure measurement) and quantitative results. Tables comparing the three main cohorts (Columbia Centre for Children's Environmental Health, Centre for Health Assessment of Mothers and Children of Salinas and the Mt Sinai Children's Environmental Health Cohort Study) are provided. These cohorts have been subject to epidemiological studies of pregnant women that examined associations of chlorpyrifos exposure with neurobehavioural measures in their infants and young children published before June 2010.

- Review of *in vivo* developmental neurobehavioural and neuropharmacology studies that includes assessment of methods and evaluation of patterns of negative and positive findings and focussing on neurobehavioural, neuropharmacological and neuropathology endpoints at lower dose levels (<10 mg/kg/d).

- Integrative evaluation of the infancy/early childhood neurobehavioural findings with the animal data.

The review assessed; overall strength of study design, specificity of chlorpyrifos exposure biomarkers, potential for bias, Hill guidelines for causal inference including a focus on concordance with animal study findings and biological plausibility.

The major findings reported in this review include:

- Estimates within cohort studies reporting 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 non-dietary oral exposures. These estimates are reported in years before chlorpyrifos was banned in the US for domestic use. These estimates are in part based on the use of urinary metabolites that may indicate exposure to metabolite residues and not exposure to the parent compound.

- The authors concluded that data from these three cohorts did not support a causal association between chlorpyrifos and adverse neurobehavioural outcomes in infants or young children. The reasons include; weak associations with measured endpoints, lack of consistency, limited number and size of studies, issues in exposure estimations and dose-response grouping. The authors note that temporality and biological plausibility are supported in the three cohorts.

- The authors concluded that the s.c. route of exposure is of limited value for risk assessment purposes. The reasons provided include; differences in pharmacokinetics resulting from a depot of test material at the site of the local s.c. injection including bypassing first pass metabolism, the possible impact of DMSO itself on neurotoxicity, and resultant effects on toxicology endpoints that may not be representative of human exposures to chlorpyrifos.

- The authors review toxicity studies by endpoint and conclude that chlorpyrifos is not associated with motor skill neurotoxicity. The review of memory and learning studies concludes that there are 'mixed results' on learning and memory tests. The authors conclude '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'. Of significant note is the author's commentary of Johnson et al (2009) as this is an oral study reporting memory and learning deficiencies. The authors did not consider the effects observed to be adverse given that there is a small difference in the number of errors (although Johnson reported a significant error rate in male pups) and lack of consistent dose-response. It was also noted that the effects were observed at doses which also caused significant hippocampal AChE inhibition.

- The authors reviewed behavioural toxicity studies and concluded that 'overall patterns of perturbations of behavioural parameters related to social behaviours, the magnitude of change or low incidence of the behaviours, and the number of multiple comparisons statistically analysed, the biological significance of these alterations in social behaviours to humans is uncertain. Based on the data thus far, 1 mg/kg bw/d appears to be a no-observed-adverse effect level (NOAEL) for perturbations in mouse social, agonistic and maternal behaviours'.

- The authors reviewed studies investigating potential modes of action. These were discussed as either cholinergic or non-cholinergic. The authors concluded that there was insufficient evidence to support any alternative mode of action other than RBC, brain AChE inhibition and/or plasma BChE inhibition in adults, dams, or offspring. The authors support the use of AChE inhibition in regulatory risk assessment as these are 'likely to be protective of these potentially different cholinergic or noncholinergic MOA'.

Study 5: Mink et al, 2012

Reference: Mink PJ, Kimmel CA, Li AA (2012). Potential effects of chlorpyrifos on fetal growth outcomes: implications for risk assessment. J Toxicol Environ Health B Crit Rev. 15(4): 281–316

This literature review (including published articles up to May 31, 2011) describes the consideration of epidemiology and animals studies for investigating a potential link between chlorpyrifos exposure and fetal growth indices. Eight published studies were included that reported on four cohort studies.

The four cohort studies are: 1) Columbia Centre for Childrens Environmental Health (CCCEH) (Perera et al, 2003; Rauh et al, 2006; Whyatt et al, 2004; 2005), the Mount Sinai Centre for Childrens Environmental Health and Disease Prevention Research (Berkowwitz et al, 2004; Wolff et al, 2007), the Centre for Health Assessment of Mothers and Children of Salinas (CHAMACOS) (Esenazi et al, 2004), and the New Jersey Cohort of Pregnant Women and their children (Barr et al, 2010).

The scope of the literature search included epidemiology studies which investigated associations between *in utero* exposure to chlorpyrifos and several growth endpoints (such as head circumference, birth weight and length, longitudinal growth parameters, abdominal circumference, and weight index). Studies that inferred chlorpyrifos exposure (not measured and quantified) were excluded. Animal studies were included when the route of exposure was relevant to human exposures and the minimum regulatory requirements (three dose levels and 20 litters/dose) were met to ensure confidence in the data.

The review authors indicated that the four epidemiological cohort studies do not indicate a consistent strong association between biomarkers of chlorpyrifos exposure during pregnancy and measures of fetal growth. The review authors noted that the effects on development seen in animal studies occurred in the presence of maternal toxicity, as evidenced by clinical symptoms and/or AChE inhibition.

In conclusion, the review authors did not identify any causal association between chlorpyrifos exposures during pregnancy and fetal growth measures based on a consideration of the data from all four cohort studies, and that this data was not sufficient for use as a point of departure for risk assessment. It was noted that there was strong evidence from the animal studies that effects on fetal and birth weight occur at doses several orders of magnitude higher than those estimated in the human epidemiology studies and only where maternal toxicity is present. Based on a consideration of both the epidemiology and animal data they concluded that the most sensitive endpoint for risk assessment is RBC AChE inhibition.

The assumption that RBC AChE inhibition remains the most appropriate risk assessment endpoint is based on a level of 10% inhibition. It should be noted that the Department of Health considers RBC AChE inhibition of 20% or greater to be biologically significant.

Study 6: Juberg et al, 2013

Reference: Juberg DR, Gehen SC, Coady KK, LeBaron MJ, Kramer VJ, Lu H, Marty MS (2013). Chlorpyrifos: Weight of evidence evaluation of potential interaction with the estrogen, androgen or thyroid pathways. Regul Toxicol Pharmacol. 66(3): 249–63

The US EPA commenced an Endocrine Disruptor Screening Program (EDSP) in the late nineties to evaluate 73 priority chemicals for potential interactions with the estrogen, androgen and thyroid hormone pathways. Chlorpyrifos was one the initial priority compounds selected for screening. Its selection was not based on effects criteria but for its exposure potential.

The EDSP program consists of a two-tiered analytical testing and screening approach. Tier 1 screening phase includes 11 assays designed to identify substances with potential to interact with hormone systems. Tier 2 consists of a series of tests designed to determine causal-, effects- and dose-response relationships.

This paper summarises relevant chlorpyrifos Tier 1 testing results and provide a weight of evidence based conclusion on the potential of chlorpyrifos as an endocrine disruptor.

In order to produce a scientifically valid and transparent weight of evidence evaluation of the potential for chlorpyrifos to disrupt hormone systems, the following approach was used:

1. literature search and data gathering

2. reliability evaluation using Toxicological Data Reliability Assessment Tool (ToxRTool; US EPA 2011)

3. data organisation using OECD (2010) 5–tiered conceptual framework for the Testing and Assessment of Endocrine Disrupting Chemicals

4. organisation of data into endocrine specific pathways.

Results of the 11 EDSP Tier 1 screening assays are summarized below; adapted from Juberg et al (2013). The screening results indicate that chlorpyrifos:

In vitro assays

- negative for estrogen binding (at concentrations up to 10^{-3} M).

- induced a weak increase in estrogen receptor mediated transactivation (at *in vitro* concentrations which are significantly higher than *in vivo* blood concentrations that inhibit RBC and brain ChE activities in adult female rats).

- androgen receptor binding assay results were ambiguous at 10^{-3} M concentration (higher than *in vivo* blood levels that inhibit RBC and brain ChE activities in adult female rats).

- altered steroidogenesis at *in vitro* concentrations (higher than *in vivo* blood levels that inhibit RBC and brain ChE activities in adult female rats).

- classifiable as non-inhibitor of aromatase activity.

In vivo assays

- Overall uterotrophic assays showed no indication of estrogenicity at doses ≤ 4 mg/kg bw/d.

- Deemed negative for both androgenic and anti-androgenic activity in Hershberger assay.

- No evidence of endocrine activity in the female pubertal assays at concentrations up to 2.0 mg/kg bw/d.

- No evidence of endocrine activity in the male pubertal assays at concentrations up to 2.0 mg/kg bw/d.

- Showed no signs of advanced or asynchronous development in exposed tadpoles and was determined 'likely thyroid inactive'.

- Short term fish reproduction assays found a statistically significant decrease in fecundity at study termination in all treatment groups (with chlorpyrifos concentrations as low as 0.251 µg/L). Reduced brain ChE activity was observed in males (mid and high dose) and females (all treated groups).

Table 15: Summary of chlorpyrifos EDSP Tier 1 assay results (Juberg et al, 2013)

No.	Tier 1 assay	Potential endocrine MoA detected	Concentration or dose levels	Assay results	Assay LOAEL	Comments
In vitro						
1	ER Binding	E (Anti E)	10^{-10}–10^{-3} M	Negative	NA	Not interactive at ER
2	ER transactivation assay	E	10^{-10}–10^{-4}M	Weak positive	10^{-5} and 10^{-4} M	10–25% induction (relative to 1 nm E2)
3	AR binding	A (Anti A)	10^{-10}–10^{-3}M	Equivocal	10^{-4} and 10^{-3} M	Lower *in vivo* blood concentration causes marked brain and RBC ChE inhibition
4	Steroidogenesis	T & E steroidogenesis	10^{-10}–10^{-4}M	Positive	10^{-5} and 10^{-4} M	T increased and E2 decreased; lower *in vivo* blood concentration causes marked brain and RBC ChE inhibition
5	Aromatase	E steroidogenesis	10^{-10}–10^{-3}M	Negative	NA	Non-inhibiting
In vivo						
6	Uterotrophic	E	0, 0.5, 1.5 or 4 mg/kg bw/d	Negative	NA	23% decrease in bodyweight gains, 4 mg/kg bw/d causes RBC ChE inhibition
7	Hershberger	A (Anti A); DHT steroidogenesis	0, 1, 6 or 12 mg/kg bw/d	Negative	NA	RBC ChE inhibition at all dose levels, brain ChE inhibition at 6 and 12 mg/kg bw/d
8	Female pubertal	E (Anti E); E steroidogenesis; HPG axis; HPT axis	0, 0.5, 1 or 2 mg/kg bw/d	Negative	NA	RBC ChE inhibition at all dose levels, brain ChE at 2 mg/kg bw/d
9	Male pubertal	A (Anti A); T steroidogenesis; HPG axis, HPT axis	0, 0.5, 1 or 2 mg/kg bw/d	Negative	NA	RBC ChE inhibition at all dose levels, brain ChE at 2 mg/kg bw/d
10	Amphibian metamorphosis	HPT axis	0, 0.215, 0.881, 3.68 or 13.6 µg/L	Negative	NA	Limb and tail ChE inhibition at 3.68 and above; no thyroid histopathology at any dose level
11	Fish short term reproduction	E(Anti E); A (Anti A); T & E steroidogenesis; HPC axis	0, 0.251, 0.812 or 3.02 µg/L	Positive (with ChE inhibition)	NA	Brain ChE inhibition at all doses in females and 0.812 and above in males.

E: estrogenicity/estradiol; ER = Estrogen receptor; A: Androgenicity; AR = Androgen receptor; T: testosterone; DHT: dihydrotestosterone; HPG: hypothalamic pituitary gonadal; HPT: hypothalamic pituitary thyroid; ChE: cholinesterase

ESTROGENICITY AND THYROID PATHWAYS

The study authors concluded that there was no indication (from a diverse set of biological markers) that chlorpyrifos perturbs estrogenic endpoints or pathways in mammalian species (four different *in vitro*, and three different *in vivo*, pathways were investigated). Similar results were noted for the thyroid pathways using three different *in vivo* assays.

Based on the considered material, whilst there are some equivocal and weakly positive *in vitro* data, the *in vivo* data suggested that there is no overall endocrine disrupting effect of chlorpyrifos via the estrogenic, androgenic or thyroid pathways. This article provides supporting information that ChE inhibition is the more sensitive endpoint for chlorpyrifos toxicity as compared to endocrine disruption.

2.5   Other epidemiological studies

Study 1: Barr et al, 2010

Reference: Barr DB, Ananth CV, Yan X, Lashley S, Smulian JC, Ledoux TA, Hore P, Robson MG (2010). Pesticide concentrations in maternal and umbilical cord sera and their relation to birth outcomes in a population of pregnant women and newborns in New Jersey. Sci Total Environ. 408 (2010) 790–795

This study involved a prospective cohort analysis of pesticide exposures in maternal and fetal compartments. The study recruited 150 mothers and their newborns at Saint Peter's University Hospital in New Brunswick. Subjects were recruited from July 2003 to May 2004 and it represented a convenience sample of non-consecutive cases based on availability of research personnel for recruitment. Eligible subjects for recruitment included 1) women with singleton pregnancies and non-anomalous fetus scheduled for an elective caesarean birth at term (≥ 37 weeks) and 2) if the haemoglobin level was ≥ 8 mg/dL. Subjects were excluded from the study if there was evidence for labour or rupture of membranes at the time of operative delivery and if they were taking medications that could potentially interfere with metabolism of environmental chemicals.

Maternal serum and umbilical cord sera were collected. The following pesticide compounds were analysed in both maternal and umbilical cord sera: chlorpyrifos, diazinon, carbofuran, chlorothalonil, dacthal, metolachlor, trifluralin and diethyl-m-toluamide (DEET). A questionnaire for pest control use, frequency of use in the pre-operative, holding area, maternal age, gravidity, race, maternal pre-pregnancy BMI, infant sex and gestational age was collected.

Study authors reported that the vast majority of the population were non-smoking (96%) and over half of the participants reported using some type of pesticide during pregnancy. When data was controlled for confounding variables, there was no association between birth weight/length and chlorpyrifos. The study did not examine PON1 activity, and consequently, the results could not be compared with the other existing cohorts for correlation of exposure and head circumference. Some of the other acknowledged limitations of the study were that 1) the samples were collected at birth, thus the exposures measured do not necessary precede the outcome measured in this cohort, and (2) many of the concentrations were near the LOD.

This study found no correlation between chlorpyrifos exposure and head circumference, birth weight or abdominal circumference, which is inconsistent with some of the previous cohort study findings. However, it should be noted that the study has only considered exposure relative to one timepoint, ie at the time of birth and also there is no discussion on potential effects of co-exposure to chlorpyrifos and other pesticides. This study is considered to be of limited regulatory value, for several reasons as outlined above including lack of PON1 activity measurement, the fact that sampling was limited to a single point in time (at birth), the high amount of results at or below the LOD and failure to examine potential impacts of co-exposure to other pesticides.

Study 2: Shelton et al, 2014

Reference: Shelton J F, Geraghty E M, Tancredi D J, Delwiche L D, Schmidt R J, Ritz B, Hansen R L, Hertz-Picciotto I (2014). Neurodevelopmental disorders and prenatal residential proximity to agricultural pesticides: the CHARGE study. Environ Health Perspect. 122: 1103–1109.

This study evaluated the possible statistical association between prenatal residential proximity to pesticides (including chlorpyrifos) and autism spectrum disorders (ASD) or developmental delay (DD) (Childhood Autism Risks from Genetics and Environment (CHARGE) study).

The CHARGE study is a population-based study of ASD, DD and typical development (TD). There were 970 participants, for which commercial pesticide application data from the California Pesticide Use Report (1997–2008) were linked to the addresses during pregnancy (pre-conception and pregnancy periods, beginning three months before conception and ending with delivery). This study is unique as the authors evaluated links between mental disorders in children whose mothers were exposed to chlorpyrifos during five distinct timepoints during pregnancy, *viz.*, 1) pregnancy as whole, 2) pre-conception, 3) 1st trimester, 4) 2nd trimester and 5) 3rd trimester. Amount of active ingredient applied for various other pesticides and chlorpyrifos were aggregated within 1.25 km, 1.5 km, and 1.75 km buffer distances from the home. Multinomial logistic regression was used to estimate the odds ratio (OR) of exposure comparing confirmed cases of ASD ($n = 486$) or DD ($n = 168$) with TD ($n = 316$). The CHARGE survey weights were designed to correct for the non-socio-demographically representative participation, ie the differences in participants vs. non-participants with regard to key socio-demographic factors such as maternal education, insurance payment type, birth regional center, birth place of mother and child race. Survey weights were based on the probability of participation in the study.

The authors reported that in the unweighted study populations, there were no significant differences in proportion exposed, but once the survey weights were applied, both AD and DD case populations showed higher proportions of exposure (as described below) than TD controls. According to the authors, exposure to chlorpyrifos applications (any vs. none) within 1.5 km of the home during the three months before conception resulted in 14.4% and 18.4% exposure proportion for ASD and DD, respectively compared to 12.4% exposure proportions for TD once the CHARGE survey weights were applied.

The study authors reported that for models evaluating the exposure to chlorpyrifos as a continuous variable, each 100 lb (45.4 kg) increase in the amount applied over the course of pregnancy (within 1.5 km of the home) was associated with higher prevalence of ASD, but no association was detected with DD.

The authors suggested that these findings supported the results of two previous studies linking ASD to gestational exposure of agricultural pesticides (Roberts et al, 2007, study evaluated in the Supplementary report on developmental neurotoxicity and behavioural toxicity. Implications for current Australian Public Health Standards (Department of health, 2016) and Eskenazi et al, 2007).

Some of the acknowledged limitations of the study were:

1. the approach used to estimate exposure in the study did not include all potential sources of exposure to the pesticides such as non-agricultural sources

2. potential errors such as reporting to the database, the assumption of homogeneity of exposure and potential geocoding errors.

Although the study reported a link between chlorpyrifos exposure to mothers and neurodevelopmental disorders in their children, the study did not examine if the co-exposure to various other pesticides at the same time, could also play a role in the development of these disorders. The extent of exposure, including systemic exposure is also unclear as the study did not observe any metabolites of specific pesticides in the mothers or the children (e.g. cord blood).

In summary, the authors suggested that living near agricultural areas or being exposed to chlorpyrifos and other pesticides during gestation increases the risk of developing neurodevelopmental disorders such as ASD and DD. However, given the significant limitations in the study design reported above, this findings are considered inconclusive.

2.6   Occupational exposure studies

Study 1: Kim et al, 2013

Reference: Kim H, Lim Y, Yang J, Shin D, Ham H, Choi B and Lee J (2013). Health risk assessment of exposure to chlorpyrifos and dichlorvos in children at childcare facilities. Sci Total Environ. 444: 441–450

The authors measured chlorpyrifos in indoor air, indoor dust, surface wipe of indoor objects, and hand wash water of children at childcare facilities in various districts in South Korea to determine accurate indoor conditions of the facilities. The childcare facilities consisted of 40 home day-care centres, 42 day-care centres, 44 kindergarten classrooms, and 42 indoor playgrounds in 6 cities including; Seoul and Busan, Daejeon, Suwon, Yeosu (industrial), Asan (provision farming city). The measurements occurred over two seasons July 2007 to September 2007 (summer) and January 2008 to February 2008 (winter).

Chlorpyrifos residues were measured in indoor air using a low volume vacuum pressure pump (GVAC, Gast, USA) to collect air at a height of 1.5 m above the floor level at a flow rate of 16 L/min. A 125 mm filter paper was used to collect samples of indoor dust. The official US EPA A3051 analysis method was used to process the indoor dust samples. Chlorpyrifos surface residue levels were measured using polyester wipes which were rubbed twice back and forth over a 20 cm long area to collect indoor dust samples on the surface of indoor objects, including floor mats, desks, chairs, and toys. Hand wash residues of chlorpyrifos were also collected. To collect hand washing residue, all of the participating children first washed their hands with distilled water prior to spending more than 5 h in the childcare facility. At 5 h hands were washed and residues collected.

Samples were analysed by gas chromatography with a mean extraction efficiency values of 102.9 ± 6.4% for chlorpyrifos. The limit of detection was 0.001 µg/m^3 for indoor air, 0.001 ng/g for indoor dust, 0.001 µg/m^2 for surface wipes and 0.001 µg/hand for hand wash water.

Chlorpyrifos was not detected in indoor dust or wash water in any of the childcares investigated. Surface wipe samples only detected chlorpyrifos in 14.3% of home day-care (mean 0.003 µg/m^2 maximum 0.026 µg/m^2) and did not detect chlorpyrifos in day-care centres, kindergarten classrooms or indoor playgrounds. Chlorpyrifos was measured in low frequencies (1–2.5%) in child care facilities. The highest detection was in a kindergarten classroom at 0.058 µg/m^3 (the mean was 0.029 µg/m^3). The overall mean and maximum result for all facilities were 0.027 and 0.058 µg/m^3.

A Lifetime Average Daily Exposure (LADE) was calculated using exposure pathways and pesticide exposure scenarios in young children. The exposure equations and input parameters are transparently described in the paper. Exposure times of five and 10 h/d were used. Exposure estimates were calculated using probabilistic (Monte Carlo) distribution functions. The lifetime average daily doses were not presented in the paper.

Study 2: Berent et al, 2014

Reference: Berent S, Giordani B, Albers J W, Garabrant DH, Cohen S S, Garrison RP, Richardson RJ (2014). Effects of occupational exposure to chlorpyrifos on neuropsychological function: A prospective longitudinal study. Neurotoxicology 41: 44–53.

A prospective longitudinal study design was used to compare neurobehavioural function over one year period among 53 chlorpyrifos workers and 60 control group workers. The study evaluated two groups (chlorpyrifos workers and a control group) of workers at the Dow Chemical Company in Midland, Michigan, on two occasions, i) baseline and ii) one year later. The study design has been described previously by Albers et al (2004). The chlorpyrifos workers had almost a decade of exposure to chlorpyrifos, and there was no evident movement of workers from chlorpyrifos-related jobs. The control group included workers involved in the manufacturing of Saran (a clear plastic film wrapping material) who had no current or recent occupational exposure to chlorpyrifos and no exposure between the baseline and one year evaluations. Chlorpyrifos exposure was assessed by two methods; by review of industrial hygiene records and by biological assessment during the year between the baseline and second examinations by urinary excretion of TCP. RBC AChE activity was also measured at baseline and at one year. The urine TCP level was reported as a weighted average of four overnight collections of TCP corrected for creatinine. Quantitative and qualitative measures were used, and potential confounders were identified and tested for possible inclusion in the statistical models. Neurobehavioural function was assessed by neuropsychological tests covering various behavioural domains that may be adversely affected by exposure to chlorpyrifos. Neuropsychological tests included tests for the following domains: 1) general ability, 2) attention/information processing, 3) memory-visual, 4) memory-verbal, 5) problem solving, 6) psychomotor and 7) personality/mood.

The study authors indicated that the chlorpyrifos group were comparable to the control group at study baseline in terms of age, sex, body mass index (BMI) and anxiety level. The authors suggested that chlorpyrifos workers had significantly ($p < 0.0001$) higher cumulative chlorpyrifos exposure (64.16 mg/m^3 days) compared to the control group (0.69 mg/m^3 days); and interim chlorpyrifos exposure were also significantly ($p < 0.0001$) higher in the chlorpyrifos workers (6.13 mg/m^3 days) than the control group (no chlorpyrifos detected), meaning none of the control group subjects had any identifiable exposure to chlorpyrifos in their jobs during the study period.

According to the authors, urine TCP was significantly ($p < 0.0001$) higher in the chlorpyrifos group (192.13 mg/g; 32-fold higher than control) compared to the control group (6.19 mg/g), which was consistent with elevated chlorpyrifos exposure among the chlorpyrifos workers. Interesting to note that the level of chlorpyrifos exposure was 32-fold higher in exposed group when compared to controls (TCP levels), but the cumulative exposure was between 6 and 10-fold higher than controls. RBC AChE levels were similar for both groups at the baseline examination (6923.19 vs. 6966.77 m/mL; $p = 0.77$) and the second examination (7148.55 vs. 7252.74; $p = 0.48$), indicating that no inhibition of AChE activity was associated with work in the chlorpyrifos exposed areas in comparison to the control group. It is not clear when the RBC AChE activity levels were measured, or whether the AChE monitoring was matched with TCP monitoring in terms of time and subject. Low chlorpyrifos exposure levels could have resulted in transient inhibition of AChE activity which had returned to baseline before measurements were determined. In other studies in humans, chlorpyrifos exposures of 7 mg/d have led to decreases in AChE activities

The study authors mentioned that neurophysiological outcomes showed significant ($p = 0.03$) group effects for the Memory-Verbal domain, with the chlorpyrifos group performing better than the referent group on this domain. The chlorpyrifos exposed group performed significantly better in 2000 than in 1999 (time effect) on four domains which included Attention/Information Processing, Problem Solving, Psychomotor, and Personality/Mood ($p < 0.01$ for all four domains). However, group-by-time interactions for any of the domains were not different.

Authors reported a significant ($p = 0.03$) difference between groups, with better estimates for one of the components of the Memory-Verbal domain (ie story immediate recall) by the chlorpyrifos group than the control group. The results also showed a significant ($p = 0.04$) group-by-time interaction for one of the component of attention/information processing (ie movement time) domain in the chlorpyrifos group. It is noted that no information was provided on the level of blinding of researchers undertaking the neurophysiological testing.

The authors noted that the subjects were well educated and worked in professional environments with high standards of personal protective equipment (PPE). However, the results may not be reflective of different work environments involving different personnel, equipment, and safety regulations. The results may also not be relevant to the expected exposure which may occur in occupations involving application of chlorpyrifos pesticides. The authors also acknowledge that the neurobehavioural tests used in the study might be limited in terms of functional areas observed and sensitivity in identifying potential impairments. It was also recognized that subjects were not observed over a sufficient period of time to identify and monitor a more comprehensive range of neurobehavioural measures. No information was provided regarding the potential cofounders of this study, such as alcohol consumption, smoking habits, social status of the male subjects etc. The monitor of neurophysiological behaviours is limited and appears unvalidated. The experience of the researchers undertaking these assessments was not reported.

This study did not look at the effects of exposure of chlorpyrifos in workers such as pesticide applicators. All the subjects included in this study were adult male; therefore, it is unclear if the chlorpyrifos related neurophysiological observations might have a gender preference or its effect on the neurobehaviour if the exposure occurred during the fast phase of nervous system development, ie fetal or juvenile phase. Sample size in this study was also small (less than 100), so the statistical power of this study was limited.

In summary, the study authors suggested that chlorpyrifos exposure during the manufacturing process does not affect neurophysiological parameters in workers. However, the regulatory value of the study is limited due to various limitations as reported above.

Study 3: Rohlman et al, 2014

Reference: Rohlman DS, Ismail AA, Abdel-Rasoul G, Lasarev M, Hendy O, Olson JR (2014). Characterizing exposures and neurobehavioral performance in Egyptian adolescent pesticide applicators. Metab Brain Dis. 29(3): 845–855

Twenty one adolescent employees of the Egyptian Ministry of Agriculture involved in spraying cotton crops with pesticides were included in this study. Twenty other adolescents from the same villages were recruited as controls. Age and educational year reached were similar in both applicators and non-applicators groups, as summarised in the table below. The gender of the participants was not provided.

Table 16: Age and educational year for applicators and non-applicators

Participant's characteristic		Range	Mean (SD)	Median
Age (year)	Non-applicator	12–18	15.5 (1.5)	16
	Applicator	12–18	15.5 (2.1	15
Education (year)	Non-applicator	6–12	9.5 (1.5)	10
	Applicator	6–11	8.9 (1.6)	9

The authors indicated that 'pesticides, equipment and calibration procedures were standardised under the control of the Egyptian Ministry of Agriculture'. Applicators also performed mixing/loading; they typically sprayed chlorpyrifos based insecticides five hours a day and 4–5 days per week during two fortnights between June and August. Spraying was performed with backpack sprayers, 90% applicators reported wearing long pants and long sleeves during application, 48% reported spraying barefooted. No applicator reported wearing PPE such as gloves, goggles or respirator.

Chlorpyrifos exposure was assessed through analysis of urine and blood samples. Spot urine samples were collected at the beginning of the second chlorpyrifos spraying fortnight. Samples were then analysed for TCP, a specific metabolite of chlorpyrifos. Creatinine was also analysed and served as an internal standard. Blood samples (time of collection was unspecified) were analysed for AChE and BChE activity. All analyses were performed according to previously published methods. The median TCP value was 3.6 times higher in applicators' urine than in the urine of controls. Applicators had lower AChE and BChE than non-applicators, but the differences were not found to be significant. Urine and blood analyses are summarised in Table 17 below.

Table 17: Urine and blood analyses of applicators and non-applicators

Analysis		Range	Mean (SD)	Median
Urine TCP (µg/g creatinine)	Non-applicator	2.4–64.9	9.4 (14.3)	4.8
	Applicator	4.9–125	33.6 (36.6)	17.5
BChE (U/mL)	Non-applicator	1.1–3.0	1.7 (0.4)	1.6
	Applicator	0.1–2.7	1.5 (0.8)	1.7
AChE (U/g Hgb)	Non-applicator	23.5–30.8	26.7 (1.9)	26.6
	Applicator	17.7–29.8	25.4 (3.6)	26.3

TCP = 3,5,6-trichloro-2-pyridinol; BChE = Butyrylcholinesterase; AChE = Acetylcholinesterase

All study participants completed a battery of neurobehavioural tests described in various publications. The functions tested included memory, attention/short-term memory, sustained attention, motor speed/coordination, information processing speed, complex visual-motor/executive function, verbal abstraction and perception. After adjusting for age and education, applicators performed worse than non-applicators on 12 of the 19 tests examined. After correction for multiple comparisons, the difference between applicators and controls was significant for two tests: match-to-sample, which is a memory test ($p = 0.087$) and similarities, which is a verbal abstraction test ($p < 0.001$).

The study authors investigated possible correlations between the markers of chlorpyrifos exposure and performance at the neurobehavioural tests. They found no significant correlation. Similarly, they found no significant correlation between the urine (TCP) and blood (AChE and BChE) markers of chlorpyrifos exposure.

The study authors commented on two aspects of the study that may impact on the significance of the results. Firstly, the small size of the population samples used limits the study's statistical power. Secondly, the authors noted that the level of TCP in the non-applicators group was five times greater than the levels observed in US teenagers, indicating that their control group (who lived near treated fields) may have been exposed to significant environmental exposure. These aspects of the study limit the study's regulatory value.

Study 4: Andersen et al, 2015

Reference: Andersen HR, Debes F, Wohlfahrt-Veje C, Murata K, Grandjean P (2015). Occupational pesticide exposure in early pregnancy associated with sex-specific neurobehavioural deficits in the children at school age. Neurotoxicol Teratol. 47: 1–9

The study was designed to investigate the potential health effects of prenatal pesticide exposure in cohorts of children whose mothers were occupationally exposed to mixtures of modern pesticides in greenhouses during the first trimester of pregnancy. The authors stated that the study was 'part of an ongoing prospective study on the effects of pesticide exposure in early pregnancy on the growth and development of children'. The authors hypothesised that impaired neurodevelopment in school age children is correlated with occupational exposure to pesticides in early pregnancy. The authors compared neurodevelopment between children born from mothers exposed to pesticides during early pregnancy and unexposed mothers.

This study has limited regulatory value due to the nature of the pesticide exposure. Indeed, greenhouses used around 200 pesticide formulations comprising of 124 active pesticide constituents. Greenhouses commonly used pesticides including OPs (e.g. dichlorvos, dimethoate, chlorpyrifos), pyrethroids (deltamethrin, fenpropathrin) and carbamates (methiocarb, methomyl). Therefore, it is not possible to determine whether statistical associations with adverse health outcomes can be attributed specifically to chlorpyrifos

3 DISCUSSION

The purpose of this assessment was to evaluate new laboratory animal and human epidemiological studies to determine whether exposure to chlorpyrifos could lead to neurotoxicity, including adverse neurobehavioural outcomes, at doses lower than those which inhibit AChE activity. Whether chlorpyrifos causes neurotoxicity via mechanisms other than AChE inhibition is not in itself relevant unless it occurs at lower levels of human exposure and would therefore not be covered by current Australian health-based guidance values.

3.1   Evaluation methodology

Consistent with the scientific method, the APVMA has used a weight-of-evidence approach to evaluate the new studies. To conduct an initial quality assessment of each individual study, the study design was assessed, taking into account OECD (Organisation for Economic Co-operation and Development) or national test guidelines where appropriate. In a weight-of-evidence assessment, any observation should be reproducible: the strength of any finding will be increased if it can be replicated under the same conditions in more than one laboratory. Plausible patterns in the hierarchy of the results will also strengthen the finding—ie where a finding *in vitro* is reproduced *in vivo*.

In toxicological science, there are a number of criteria that are used to determine whether effects, such as those on the nervous system, are treatment-related and adverse:

- *Dose-response relationship*—the number of animals or subjects showing the effect and/or the severity of the effect should increase with dose. There should be a progression to a more severe state of toxicity as the dose and duration of dosing increases.

- *Consistency of the effect*—the effect should be observed consistently across studies of similar exposure duration and sexes (in unusual cases an effect may be sex-specific). Additionally, an effect should be corroborated by related toxicological endpoints—for example, increases in malignant neoplasms should be preceded by cellular changes that should be observed at lower doses or following shorter exposure durations.

- *Statistical significance*—differences between treated groups and the concurrent control group should be statistically significant. However, statistical significance on its own does not imply biological significance and the absence of statistical significance also does not necessarily mean the absence of an effect (for example a rare type of tumour may be highly biologically relevant).

- *Biological plausibility*—an observed effect needs to be mechanistically plausible based on the characteristics of the chemical and principles of biology/physiology.

- *Natural variation and incidental findings*—the normal range of natural variation of a parameter in the test species needs to be understood through the use of age- and sex-matched historical control data. It is critical that this normal range of biological variation is documented and understood.

The APVMA considered aspects of study design and reporting that may either increase or decrease confidence in the data. The presence of a dose-response relationship, consistency and reproducibility were considered to increase confidence in the data, while any unexplained inconsistencies and significant deviations from international test guidelines were considered to reduce confidence in the data. Thus, those studies that demonstrated a dose-response relationship, adhered to international test guidelines (where appropriate) and were consistent and reproducible within and/or between laboratories were given more weight in the assessment.

3.2   Laboratory animal studies

A range of new neurotoxicity studies conducted in laboratory animal models and *in vitro* test systems were evaluated. These studies supplement those already evaluated as part of the 2000 toxicological assessment of chlorpyrifos. While the new studies provide additional useful information, in general they had a number of features which limit their use for human risk assessment purposes. This includes the use of dose routes not relevant to human exposure scenarios (such as administration by the subcutaneous route), the testing of single doses (which precludes the establishment of a dose-response relationship important for determining treatment-related effects), the lack of a positive control to validate the assay, the mechanistic nature of the experiments (without any concurrent investigation of AChE inhibition), and the lack of specificity of the behavioural parameters analysed. While a number of alternative (non-cholinergic) modes of action have been proposed by some study authors, there are currently no reliable animal models that demonstrate that these mechanisms are relevant to the observed neurodevelopmental effects observed in some rodent studies.

Mice

In mice, inconsistent neurobehavioural changes were reported by Venerosi et al (2009) but due to the use of a single-dose (which prevents the establishment of a dose-response relationship) and the absence of the analysis of cholinesterase activity, the results are difficult to interpret. Braquenier et al (2010) found no effect of chlorpyrifos on anxiety in adult female mice when exposed to chlorpyrifos during gestation. Changes in expression in the brain of mice of human PON1 genes following repeated subcutaneous administration of chlorpyrifos were of unclear biological relevance. The study of Shalaby et al (2013), which investigated the effect of chlorpyrifos on fetal development, was of limited value due to the lack of reporting detail. In mice exposed to chlorpyrifos in the diet during gestation and lactation, inhibition of cholinesterase activity was accompanied with some changes in the distribution of neurons and astrocytes in the brain (Wang et al, 2013). In mice exposed to chlorpyrifos on postnatal day 10, neurobehavioural changes were not associated with decreased AChE activity or alterations in cerebral proteins—this led the authors to propose that the results were due to a non-cholinergic mechanism (Lee et al, 2015).

Rats

In a mechanistic study in rats, subcutaneous administration of chlorpyrifos during different periods of development resulted in the disruption of signalling proteins in the brain (Aldridge et al, 2003). In a follow-up study by Aldridge (2005), changes in behaviour occurred inconsistently, with sometimes opposing effect observed in males and females. However, in both studies the absence of the analysis of cholinesterase activity limited the biological relevance of the observations. Oral exposure of rats to chlorpyrifos during the first week after birth resulted in the inhibition of AChE activity in various regions of the brain accompanied with changes in mRNA levels (Guo-Ross, 2007). Deficits in learning and memory observed in male rats exposed orally to chlorpyrifos during the first three weeks after birth co-occurred with inhibition of AChE activity (Johnson, 2009).

Levin et al (2001) found opposing effects on cognition in male and female rats injected subcutaneously with chlorpyrifos during the first few weeks after birth—while the authors suggested that these effects were cholinergic synaptic-function related, AChE activity was not measured in the study. A subsequent study by the same group (Levin et al, 2014), also using subcutaneous exposure, reported some changes in behaviour in males but not females, but again no analysis of AChE was undertaken. Meyer et al (2004) reported alterations in adenylyl cyclase signalling in adult rat brain following gestational or neonatal injection of chlorpyrifos at doses higher than those causing AChE inhibition. Repeated subcutaneous exposure of young rats resulted in impairments in sustained attention and increased impulsivity concomitant with AChE inhibition (Middlemore-Risher et al (2010). Dietary exposure of maternal rats to chlorpyrifos caused reversible changes in intermediate granule cell progenitors in the brains of offspring in addition to AChE inhibition (Ohishi et al, 2013)

Studies conducted by Roy et al (2004 and 2005) reporting cellular changes in the brain of rat pups following subcutaneous injection with chlorpyrifos were not accompanied by any functional impairment of the nervous system. Gestational exposure of female rats via the subcutaneous route had no effect on locomotor or behavioural tests in pups, and caused inconsistent changes in cell numbers in the brain (Vataparast et al, 2013). A neurobehavioural evaluation of adolescent male rat pups injected subcutaneously with chlorpyrifos reported inconsistent effects on behaviour that were inconclusive (Chen et al, 2014). In a non-validated model, ADHD-like behaviour was reported in the offspring of pregnant female rats that were administered chlorpyrifos orally for 30 days (Grabovska and Salyha 2015). However, due to the lack of reporting detail, inconsistent results and high mortality, the study was considered to be of limited regulatory value. Lipid peroxidation in association with AChE inhibition was increased in the brain following a single, high subcutaneous dose of chlorpyrifos (Lopez-Granero et al, 2013).

In a study designed to examine age-related differences in AChE inhibition, the NOAEL following an acute dose was 0.5 mg/kg bw in both adults and pups, while the NOAEL for chlorpyrifos-oxon was 0.1 mg/kg bw in adults and 0.05 mg/kg bw in pups (Marty et al, 2012). Benchmark-dose modelling of erythrocyte and brain AChE data was undertaken by Reiss et al (2012), who determined that there was no difference between rat pups and adults. A study conducted in male rats exposed subcutaneously to chlorpyrifos reported effects on special learning and memory that persisted after AChE had returned to baseline levels (Terry et al, 2012).

Guinea pigs

A single study conducted in guinea pigs exposed subcutaneously to chlorpyrifos found changes in learning in conjunction with statistically-significant changes in myelination in the brain (Mullins et al, 2015). However, the biological relevance of these findings is difficult to interpret as the analytical method (MRI) used to analyse cellular integrity is not typically applied to neurotoxicity studies conducted in laboratory animals. Therefore concerns like validation of the method (including the use of positive controls), historical control data (to determine the normal range of biological) and inadequate group size ($n = 4$) are significant issues in the study.

Conclusion

Numerous potential molecular targets have been proposed through *in vitro* studies evaluating cytotoxic, other enzymatic or macromolecule synthesis effects, signal transduction pathways or oxidative stress mechanisms. However, the doses required to cause significant inhibition of AChE are well below those required to cause adverse effects in these *in vitro* studies. Additionally, the biological and ultimately toxicological significance of these endpoints remains unclear. More recently, additional *in vitro* mechanistic studies have been reported to show adverse effects at very low concentrations (in nanomoles), below the concentration that would inhibit AChE, such as, inhibition of neuronal growth or decreasing axonal length (considered in the review by Pruiett et al, 2011). However, whether such effects would occur *in vivo* with low dose exposure has yet to be determined. The Goodman (2012) review concludes that there is little evidence that the proposed non-cholinergic mechanisms occur at doses lower than those that cause inhibition of AChE activity. Until animal models demonstrate the sensitivity of these measures *in vivo*, the weight of evidence currently supports the continued use of AChE as a point of departure for establishing health-based guidance values.

3.3   Epidemiological studies

The APVMA has critically appraised the cohort studies and concludes that in general the majority were well designed and the analysis supports the calculated statistical associations between *in utero* chlorpyrifos exposure and adverse neurodevelopmental effects observed at birth and through childhood (age 7 years). However, it is difficult to conclude that chlorpyrifos is the only contributor to these outcomes as some of the studies actually confirmed co-exposure to other OPs.

The epidemiology studies critically appraised during this review vary widely regarding the methodology, the subpopulations and the types of cognitive functions evaluated. Some results are affected by sources of bias and/or other confounding factors that make it difficult to establish a definitive causal link between exposure and effect in isolation. However, for the three main prospective birth cohorts, robust study designs that included assessing chlorpyrifos exposure via various methods (questionnaires, air monitoring) as well as biomarkers have added to the weight of evidence assessment of the potential for neurodevelopmental and/or behavioural effects following exposure to chlorpyrifos. Further, many of the developmental outcomes were assessed using previously validated clinical tools. With the exception of one-time measurement of prenatal exposure and the effect of exposures to mixtures, the studies addressed the common confounders and forms of bias providing support to a weight of evidence assessment for chlorpyrifos.

One of the major uncertainties for these epidemiological studies is the reliability of the measurement of exposure. Across the three cohorts, TCP, parent chlorpyrifos and DEP were used to measure the level of chlorpyrifos exposure. The following sections provide study summaries to address some of the identified cofounders in the epidemiological studies.

3.4   Summary of main epidemiology study findings

Several epidemiology studies on three large cohorts have been considered as part of the chlorpyrifos toxicology assessment. Studies of the CHAMACOS cohort by Eskenazi et al (2004 and 2007) found no association between *in utero* exposure to organophosphorus pesticides and fetal growth or infant development as measured with

Bayley Scales of infant development (including MDI and PDI indices or child behaviour checklist outcomes. Studies of the Mount Sinai cohort by Berkowitz et al (2004) and Engel et al (2007 and 2011) reported some links between high maternal levels of various biomarkers of pesticide exposure (including the chlorpyrifos metabolite TCP and other OP pesticide metabolites DAP and DMP) and slight reductions in newborn head circumference, an increase in abnormal reflexes and some general decreases in mental development. The clinical significance of reported alterations in newborn head circumference were not considered to be clinically significant, and there is also significant uncertainty around the potential confounding effects of other organophosphorus pesticides or other environmental contaminants/sources of these metabolites. The use of urinary TCP as a direct biomarker of chlorpyrifos exposure is also questionable given that there are alternative potential sources other than chlorpyrifos.

Studies of the CCCEH cohort have reported several findings around potential neurodevelopmental effects of exposure to environmental contaminants and pesticides amongst children residing in the New York area. These include estimated exposure to chlorpyrifos and analysis of neurodevelopmental outcomes for mothers and children born immediately before and after the time that retail sales of chlorpyrifos for indoor use were voluntarily cancelled in December 2001. A study by Whyatt et al (2002) found that chlorpyrifos was detected (along with three other pesticides) in all personal air samples taken from 72 women from the larger sample of 231. These results were largely confirmed by Whyatt et al (2007) who reported that almost all taken during the final two-months of pregnancy included detectable levels of chlorpyrifos. Sixty-percent of these women had reported using pesticides. Whyatt et al (2009) conducted further analyses and found that certain biomarkers including chlorpyrifos in postnatal maternal and umbilical cord blood, TCP in postpartum meconium, and both pre and postnatal maternal urine were reliable dosimeters to differentiate between groups with prenatal chlorpyrifos exposure differing by a factor of two or more. No association was found between expected chlorpyrifos exposure and newborn urine samples. Perera et al (2003) published a study on the CCCEH cohort reporting that exposure to environmental contaminants in New York City from 1998 to 2004 was associated with reduced birthweight amongst African-Americans, but not Dominicans despite estimated exposure being comparable between groups. The study involved analysis of plasma cotinine and chlorpyrifos, and inhalational PAH but no other pesticides were measured. The findings with respect to chlorpyrifos were considered inconclusive due to the inconsistency between ethnic groups, lack of statistical significance, and failure to consider the potential confounding effects of other pesticides (known to be in use at the time) on the observed effects on fetal development. Further analysis regarding estimated prenatal exposure to chlorpyifos and neurodevelopment and behaviour was undertaken at various stages of infant development by Rauh et al (2006, 2011 and 2012). Rauh et al (2006) reported that by three years of age, highly exposed children (determined by measurement of chlorpyrifos in umbilical cord blood) exhibited mental and motor delays that increased in effect over time. At three years of age, children were also reported to be more likely to display attention problems such as ADHD and PDD. In a follow up analysis of the cohort, Rauh et al (2011) examined the relationship between prenatal chlorpyrifos exposure and neurodevelopment among a cohort of children at 7 years of age. The study reported a reduction in working memory (2.8%) and FSIQ (1.4%), associated with increases in cord levels equivalent to one standard deviation (4.61 pg/g). While the study methodology was generally considered to be robust, the clinical relevance of the findings was unclear as the relationship between the known toxicological properties of chlorpyrifos, brain anomalies and associated neuropsychological testing measures are not well understood. To address these uncertainties, Rauh et al (2012) published a study examining the effects of chlorpyrifos on morphological characteristics of the developing human brain using MRI techniques. The authors hypothesised that previously reported cognitive deficits associated with prenatal chlorpyrifos exposure in children could be a result of altered morphological characteristics in brain regions (frontal, parietal, and lateral temporal) that subserve higher-cognitive functions.

Overall, the analyses of the CCCEH cohorts reported some associations between the levels of chlorpyrifos in the umbilical cord blood and altered birth outcomes (dimensions and weight etc.), various mental development and psychomotor measures and structural changes in the developing brain. It should be noted that domestic indoor uses of chlorpyrifos (including many products available to householders) used in the New York area at the time are not available in Australia, where such uses are highly restricted to professional commercial operations involving limited application to cracks and crevices for control of common indoor pests such as cockroaches, silverfish and spiders. However, it is important to investigate the findings further, as estimated exposure in the CCCEH cohort (0.027 µg/kg bw/d) is well below the most sensitive effect observed in relevant clinical toxicology studies (0.03 mg/kg bw/d reported by Coulston et al, 1972).

The analyses of developmental chlorpyrifos exposure by Rauh (2006) involved the categorisation of high (> 6.17 pg/g) and low (≤ 6.17 pg/g) exposure groups based on a single analysis of chlorpyrifos in umbilical cord blood. It is not clear whether a single measure of umbilical cord blood at birth would accurately reflect critical exposures throughout pregnancy and critical developmental periods, especially considering that an orally administered dose of chlorpyrifos is typically eliminated in the urine within four days (Rauh et al, 2006). Based on this, it could be considered that cord blood levels of chlorpyrifos only represented a short period of exposure prior to birth.

Interestingly, Whyatt et al (2009) found no detectable levels of TCP in newborn infant urine, adding some uncertainty around fetal exposure to chlorpyrifos. Additionally, there was no information on postnatal chlorpyrifos exposure after birth, so the noted effects on neurodevelopment were solely based on an estimation of prenatal exposure with significant limitations around longer term representation. It should be noted that statistical analyses were conducted by Whyatt et al (2009) comparing intra- and inter-individual variability in indoor and maternal air sample measurements and correlating various biomarkers of chlorpyrifos exposure over time such as meconium TCP, and maternal and umbilical cord blood. This additional information provides some support that umbilical cord blood taken at birth may represent longer term exposure during critical stages of fetal development, although the lack of information on postnatal exposure to chlorpyrifos, lead, or any other environmental or pesticide chemicals remains a significant limitation. The categorisation (dichotomization) of chlorpyrifos umbilical cord blood levels into high and low exposure groups for the purposes of the analyses were successful in revealing some significant differences between the two exposure groups, but did not provide enough information to allow a full exploration of a dose-response relationship. It is noted that the level of blood chlorpyrifos measured in the CCCEH cohort was not particularly high compared to other US populations, with some including those reported by the Cincinnati Red Cross, actually reporting significantly higher mean concentrations as background levels. These findings are not unexpected, considering that it has been estimated that approximately two-third of chlorpyrifos exposure for mothers of the CCCEH cohort may have come from the diet (Eaton et al, 2008). Further, the concentrations and estimated exposures of mothers in the cohort are considered to be at least an order of magnitude lower than those associated with the most sensitive clinically significant effects on ChE inhibition, including those observed by Coulston et al (1972), which is considered to be the most sensitive *in vivo* biological effect observed for chlorpyrifos in humans.

Observed effects on mental and physical performance as measured by the BSID-II results were inconsistent over the duration of the phase out and eventual cancellation of residential chlorpyrifos use, casting doubt over whether chlorpyrifos alone, or potentially another pesticide, environmental contaminant or lifestyle factor also played a role in the observed developmental effects. It is also noted that there are known limitations in the validity of standardised developmental testing of infants in the first three years. Whyatt et al (2002 and 2007) found that chlorpyrifos was detected in almost all indoor and personal air samples taken during the later stages of pregnancy. These chlorpyrifos levels were reported to decline significantly, as expected, after the voluntary cancellation of indoor residential chlorpyrifos use in December 2001. It is noted that three other pesticides including diazinon (another organophosphorous insecticide), propoxur (carbamate) and o-phenylphenol (organic fungicide) were also found in 99–100% of air samples, and interestingly maternal air samples of both diazinon and propoxur also reduced significantly during the (seemingly unrelated) chlorpyrifos phase out period.

Although the researchers accounted for many covariates in their analyses of the CCCEH cohort, it cannot be ruled out that some potential confounding factors may have contributed to the observed effects. Covariates factored into the analyses included ethnicity, gender, gestational age, maternal education, HOME score and ETS exposure. No other pesticides or environmental contaminants were considered. It is noted that alcohol intake was considered to be relatively high in the CCCEH cohort but was not rigorously examined. Alcohol is known to be an independent factor in many (if not all) of the adverse developmental outcomes reported (Eaton et al, 2008). Several other pesticides which were commonly used in the New York City area and were detected in air samples at comparable frequency and levels to chlorpyrifos were also not factored into many of the analyses of the CCCEH cohort.

Results for exposure and neurodevelopmental outcomes were categorised over time to add weight to the observations by reflecting the potential effects of the residential phase out and voluntary cancellation that was in progress around the time. These time periods included births during the preban period (before January 2000), the midban or phase out period (January 2000 to December 2000) and the postban period (January 2001 onwards). The authors reported that mean chlorpyrifos cord blood levels at delivery significantly decreased over time, although the APVMA notes that the mean concentration during the postban period was in fact higher (0.90 pg/g), than at the midban period (0.81 pg/g). Similarly, significant increases in 36 month BMID-II (MDI and PDI) scores were reported from the preban to midban period, but this pattern did not continue into the postban period. The APVMA notes that the time periods used to categorise the results with relation to the residential phase out and cancellation may not have accurately reflected the availability and potential exposure to chlorpyrifos at the time. The US EPA (2001) specified that the technical registrants had agreed to cancel most residential uses in June 2000, before the cancellation came into effect on December 31 2001, after which, distribution and sale of these products was prohibited. This means that residential chlorpyrifos products (including those available to householders) may have been available after the time period used by Rauh et al (2006) to assess 'postban' chlorpyrifos cord concentrations for births after January 2001.

Rauh et al (2011) reported reductions in IQ associated with increasing levels of chlorpyrifos in cord blood at birth. The APVMA note that while the study methodology is robust, the clinical relevance of the findings was unclear as the relationship between the known toxicological properties of chlorpyrifos, brain anomalies and neuropsychological testing measures are not well understood. It is also noted that the sampled demographic represents a high risk group, and other factors may have contributed to the observed learning difficulties. A follow-up study by Rauh et al (2012) hypothesised that the cognitive deficits in children could have resulted from altered morphological characteristics in brain regions (frontal, parietal, and lateral temporal) that subserve higher-cognitive functions. The authors reported that there were significant associations between prenatal exposure to chlorpyrifos at levels observed with routine (non-occupational) use which are below the threshold for any signs of acute exposure, and structural changes in the developing human brain. The APVMA considers that there were methodological issues with the MRI data analysis, such as the lack of validation of the computation method used and further information regarding the source of the template brain. The authors also acknowledged other limitations of the study, one of which is the modest sample size including only 40 children. The sample size makes it difficult to detect within-group correlations and to test multiple interactions of exposure with other variables. The reason for the small sample size was the study requirement that participants have minimal or no prenatal exposure to ETS and PAH, as these are known neurotoxicants and therefore are potential confounders in the assessment of the neurodevelopmental effects of chlorpyrifos. Another limitation of the Rauh (2011 and 2012) studies is that the cognitive assessment was limited to a standard, broadband performance measure where more sensitive and functionally specific measures of cognitive and behavioural functioning may have yielded more anatomically relevant correlations of those measures with regional effects of chlorpyrifos on brain structure observed in MRI scans.

US EPA (2014b) conducted a detailed appraisal of the Rauh et al (2012) study and generally concurred with the authors' conclusions that there were associations with chlorpyrifos concentrations in umbilical cord blood at birth and structural changes in the developing human brain. However, it was noted that increased research into the area is required with more sophisticated MRI methods to enable morphological observations to be linked with specific functional outcomes as observed in the CCCEH cohort. They concluded that 'while the strengths and limitations of the studies would be more likely to lead to an under-estimation of the true effect, the possibility of false positive associations cannot be entirely ruled out' and further state 'the presence of factors which may have over-estimated effects, the lack of consistency in many domains (e.g. fetal growth), and the lack of a clear mechanism of action may argue against a true association'. Overall, the current database supports the conclusion from the 2008 Panel that 'chlorpyrifos likely played a role in the birth and developmental outcomes noted in the three cohort studies. It cannot be stated with certainty, however, that chlorpyrifos is the sole contributor to these effects' (US EPA, 2014a).

Several literature reviews including those by Goodman et al (2012), Prueitt et al (2011) and Li et al (2012) dismissed the adverse findings of the CCCEH cohort studies due to what were described as significant methodological limitations. It should be noted however, that the literature reviews did not consider more recent cohort studies, such as Rauh et al (2012) which sought to support the reported links between routine chlorpyrifos exposure and observed psychological and behavioural outcomes, by examining physical brain alterations through the use of sophisticated MRI techniques.

The APVMA considers that studies of the CCCEH cohort had several strengths and some limitations to consider in interpreting and incorporating the study results into a contemporary assessment of chlorpyrifos toxicology. Statistical analyses of several measures of chlorpyrifos exposure over time added some confidence that the single measurement of cord chlorpyrifos levels may have reflected longer term exposure over key stages of fetal development, although these are considered tenuous, and do not include any attempt to estimate exposure to chlorpyrifos (or any other known neurotoxic substance) after birth. Detailed analyses of the potential confounding effects of lifestyle factors such as socio-economic status and exposure to other environmental contaminants (such as lead, tobacco smoke) strengthened the observed results, however, there is significant uncertainty over whether other factors (including other pesticides, environmental contaminants or lifestyle factors) may have contributed. Monitoring and categorisation of chlorpyrifos exposure and neurodevelopmental outcomes over time provided some supporting evidence that there was a correlation between the residential chlorpyrifos phase out/cancellation and improved exposure and neurodevelopmental outcomes, although the APVMA notes that these were inconsistent. The small sample sizes may have also led to underpowered statistical assessments, and the ability of the study population to be extrapolated to the general population is unknown. These limitations hindered the establishment of a definitive dose-response relationship for adverse neurodevelopmental and/or perturbations for behavioural parameters. Several reported effects observed in the CCCEH cohort were also inconsistent with, or contradicted by findings from other epidemiology studies of 'at risk' populations including those conducted on the CHAMACOS and Mount Sinai cohorts.

However, due to justified concerns regarding the reported outcomes for infants of the CCCEH cohort, it was considered prudent to ensure that the chlorpyrifos toxicology assessment considered all available literature on the subject, including a detailed appraisal of the most contemporary clinical neurodevelopmental toxicity studies of animals. For this reason, the APVMA has undertaken a comprehensive assessment of additional neurodevelopmental studies of animals as part of this supplementary report to investigate reported sensitivity of women of child bearing age and infants, and examine the potential for effects to be occurring at doses below those which have long been associated with the most sensitive effects on cholinesterase inhibition in humans. After reviewing the most contemporary literature on the neurodevelopmental toxicity of chlorpyrifos in animals, including studies investigating potential modes of action through cholinergic or non-cholinergic pathways, there appears to be insufficient evidence to support a definitive mode of action other than AChE inhibition in adults, dams, or offspring exposed to chlorpyrifos. On this basis, the APVMA considers that the use of AChE inhibition as the key endpoint for the chlorpyrifos regulatory risk assessment remains appropriate. For this reason, the current health based guidance values (ADI and ARfD) for chlorpyrifos remain appropriate and continue to be protective of all population subgroups, including women of child bearing age and infants.

3.5   Conclusion

On the basis of the new information reviewed during this assessment, AChE inhibition remains the most sensitive toxicological endpoint on which to establish health-based guidance values for chlorpyrifos. Thus the current ADI and ARfD for chlorpyrifos remain appropriate.

4 SUPPLEMENTARY DOCUMENTS

4.1   Studies scrutinised but not evaluated

1. Bouchard MF, Chevrier J, Harley KG, Kogut K, Michelle V, Calderon N, Trujillo C, Bradman A, Barr DB, Eskenazi B (2011). Prenatal Exposure to Organophosphate Pesticides and IQ in 7–Year-Old Children. Environ Health Perspect. 119(8): 1189–1195

The study was designed to determine the association between prenatal and postnatal exposure to OP pesticides and cognitive abilities in school-age children from the CHAMACOS cohort. The study measured DAP metabolites in urine collected during pregnancy and at several postnatal timepoints. The cognitive ability of seven years old children was assessed by administering the Wechsler Intelligence Scale for Children (4th edition).

The study measured DAP which is a metabolite of OP pesticides. Data specific to chlorpyrifos were not provided in this study. The study was therefore considered but not evaluated as it does not hold any regulatory value.

2. Bradman A, Barr DB, Clause Henn BG, Drumheller T, Curry C, Eskenazi B (2003). Measurement of pesticides and other toxicants in amniotic fluid as a potential biomarker of prenatal exposure: a validation study. Environ Health Perspect. 111(14): 1779–1782

This study was conducted to validate the application in human amniotic fluid of analytical methods previously developed in urine to measure various pesticides metabolites, including OP metabolites. The study was not evaluated as it is purely methodological and presents no results directly relevant for the present review.

3. Carr RL, Adams AL, Kepler DR, Ward AB, Ross MK (2013). Induction of endocannabinoid levels in juvenile rat brain following developmental chlorpyrifos exposure. Toxicol Sci. 135(1): 193–201

It is known that developmental exposure to chlorpyrifos inhibits the enzymes monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH) in the brain. These enzymes mediate degradation of the endogenous cannabinoids 2-arachidonoylglycerol (2–AG) and anandamide (AEA) in the brain, and this study investigated whether this enzyme inhibition is persistent or whether the endocannabinoids accumulate in the brain.

Chlorpyrifos (99% pure) in corn oil or at 1, 2.5 or 5 mg/kg bw/d was given by oral gavage for 7–10 day old rats (n=6–8 per group), which, according to the authors, corresponds to a time of significant brain maturation. The doses were selected based on an oral repeated dose NOAEL of 0.75 mg/kg bw/d for inhibition of brain ChE activiy and the oral repeated dose NOAEL of 4.5 mg/kg bw/d for toxicity to postnatal rats. Forebrains were collected at 4, 12, 24 and 48 h after the last exposure, and assayed for ChE activity, the endocannabinoids AEA and 2–AG, and the enzyme activity of MAGL and FAAH. All dosages inhibited ChE activity and enzyme levels. All doses resulted in elevated endocannabinoid levels.

This study was of no regulatory value because there were no data for plasma or RBC ChE levels and thus no correlation between brain and RBC ChE could be determined.

4. Chen XP, Chen WF, Wang DW (2014). Prenatal organophosphates exposure alternates the cleavage plane orientation of apical neural progenitor in developing neocortex. PLoS 9(4): e95343

This study was designed to elucidate the direct effects of three compounds, including chlorpyrifos, on the cleavage plane orientation of apical neural progenitors, ie on the morphology of developing embryo brains. Pregnant mice were daily injected with chlorpyrifos s.c. at 2 mg/kg bw, from embryo day E7.5 to E11.5. Cleavage plane orientation was determined at E16. The study authors found the embryos brain cleavage plane orientation to be significantly altered by the dams' chlorpyrifos pre-treatment. The proportion of vertical, oblique and horizontal orientation was $79.4 \pm 1.06\%$, $14.7 \pm 0.73\%$ and $5.4 \pm 0.53\%$ in controls; and $72.7 \pm 0.76\%$, $21.4 \pm 1.12\%$ and $5.6 \pm 0.59\%$ in the chlorpyrifos group, respectively. The differences in vertical and oblique orientation were found to be significant ($p < 0.01$).

This study was considered but not evaluated as it holds no regulatory value due to both the route of exposure and the dose used. Subcutaneous injection is not a relevant route of exposure to assess chlorpyrifos risk on human health; such exposure does not reflect the high level of metabolic transformation undergone by chlorpyrifos after oral or dermal absorption. The daily dose used in this study (2 mg/kg bw) is several orders of magnitude higher than the ADI for chlorpyrifos (0.003 mg/kg bw).

5. Eskenazi B, Huen K, Marks A, Harley KG, Bradman A, Barr DB, Holland N (2010). PON1 and Neurodevelopment in Children from the CHAMACOS Study Exposed to Organophosphate Pesticides *in utero*. Environ Health Perspect. 118(12): 1189–1195

The study was designed to determine 1) the relationship between PON1 (a key enzyme involved in the metabolism and detoxification of OP pesticides) genotypes and enzyme measurements and child neurobehavioural development, 2) if PON1 modified the association of *in utero* exposure to OP and neurobehaviour.

This study was of no regulatory value due to the nature of the DAP metabolites measured. The study measured six DAP metabolites (three dimethyl phosphate metabolites derived from pesticides such as malathion, oxydemeton-methyl, and dimethoate, and three diethyl phosphate metabolites derived from pesticides such as diazinon, chlorpyrifos, and disulfoton). The nature of the metabolites studied does not allow for direct assessment of the effect of chlorpyrifos on human health.

6. Harley KG, Huen K, Aguilar Schall R, Holland NT, Bradman A, Barr DB, Eskenazi B (2011) Association of Organophosphate Pesticide Exposure and Paraoxonase with Birth Outcome in Mexican-American Women. PLoS One 6(8): e23923

The study was designed to assess the effect of PON1, a key enzyme involved in the metabolism and detoxification of OP pesticides on fetal growth and length of gestation in the CHAMACOS cohort. The study also investigated the interaction of maternal DAP metabolites of OP pesticides levels and PON1.

This study was of no regulatory value due to the nature of the DAP metabolites measured. The study measured six DAP metabolites (three dimethyl phosphate metabolites derived from pesticides such as malathion, oxydemeton-methyl, and dimethoate, and three diethyl phosphate metabolites derived from pesticides such as diazinon, chlorpyrifos, and disulfoton). The nature of the metabolites studied does not allow for direct assessment of the effect of chlorpyrifos on human health.

7. Hernandez CM, Beck WD, Naughton SX, Poddar I, Adam BL, Yanasak N, Middleton C, Terry AV Jr (2015). Repeated exposure to chlorpyrifos leads to prolonged impairments of axonal transport in the living rodent brain. Neurotoxicology. 47:17–26

This study evaluated the effects of chlorpyrifos on axonal transport in the brain of living rats using manganese enhanced magnetic resonance imaging (MEMRI). The study was found to have no regulatory value for the following reasons:

•   Chlorpyrifos was administered s.c., which is not a relevant route of exposure for evaluation of the human health risk subsequent to chlorpyrifos exposure, as this does not reflect the high level of metabolic transformation undergone by chlorpyrifos after oral or dermal absorption.

•   The daily chlorpyrifos dose used in this study (3 or 18 mg/kg bw) was several orders of magnitude higher than the ADI for chlorpyrifos (0.003 mg/kg bw), therefore the doses used are unlikely to be comparable to human exposure.

•   The study authors have not addressed potential confounding factors, therefore the study would likely be found inconclusive if fully assessed. In particular, all MEMRI experiments in this study were conducted under isoflurane anaesthesia; the authors did not discuss how the well-established disruption of axonal transport by isoflurane would affect their results.

8. Marks AR, Harley K, Bradman A, Kogut K, Barr DB, Johnson C, Calderon N, Eskenazi B (2010). Organophosphate pesticide exposure and attention in young Mexican-American children: the CHAMACOS study. Environ Health Perspect. 118(12): 1768–1774

The study was designed to assess the relationship between exposure to OP pesticides (measured by urinary DAP metabolites in pregnant women and their children) and attention-related outcomes among the CHAMACOS cohort.

This study was considered to have no regulatory value due to the nature of the DAP metabolites measured. The study measured six DAP metabolites (three dimethyl phosphate metabolites derived from pesticides such as malathion, oxydemeton-methyl, and dimethoate, and three diethyl phosphate metabolites derived from pesticides such as diazinon, chlorpyrifos, and disulfoton). The nature of the metabolites studied does not allow to directly assessing the effect of chlorpyrifos on human health. Therefore, the study was evaluated but does not hold any regulatory value for the purpose of this review.

The US EPA, 2014 report concludes that there are suggestive detrimental association between prenatal OP exposure (as measured by maternal urinary metabolite levels) and attentional difficulties at age five years using three different measures of this neurodevelopmental outcome. However the OCS concludes that this change cannot be directly related to chlorpyrifos exposure.

9. Mullen BR, Khialeeva E, Hoffman DB, Ghiani CA, Carpenter EM (2012) Decreased reelin expression and organophosphate pesticide exposure alters mouse behaviour and brain morphology. ASN Neuro. 18;5(1): e00106

This study investigated genetic and environmental factors believed to be involved in autism. The authors examined the interaction between the reduction in the expression of the extracellular matrix protein reelin and prenatal exposure to chlorpyrifos-oxon (CPO) in mice with reduced reelin expression.

The study was of no regulatory value for the following reasons.

- The study was conducted on homozygous and heterozygous reeler mice (reeler mice have reduced reelin expression). It is not clear how results obtained on these specific mice may be extrapolated to the Australian population.

- The authors administered chlorpyrifos-oxon (but not chlorpyrifos) to the pregnant mice and studied effects on the progeniture.

- The dose used and the route of administration are not described in detail, thus direct extrapolation for human health risk assessment is not possible.

- Chlorpyrifos-oxon was administered during three days to the pregnant mice with a pump in which the reservoir contained ca 0.6 mg chlorpyrifos-oxon (OCS note: considering a mice weight of 20 g, the maximal dose delivered over three days may be approximately 30 mg/kg bw, but the authors did not specify the exact dose, nor the mice weight).

- The amount of chlorpyrifos-oxon actually delivered and the location of pump delivering chlorpyrifos-oxon are not specified. Therefore, the relevance of administrating CPO to assess chlorpyrifos' effects is not clear (OCS note: the CPO dose administered to the pregnant mice reduced AChE activity in the fetuses' brain).

10. Muller M, Hess L, Tardivo A, Lajmanovich R, Attademo A, Poletta G, Simoniello MF, Yodice A, Lavarello S, Chialvo D, Scremin O (2014). Neurologic dysfunction and genotoxicity induced by low levels of chlorpyrifos. Neurotoxicology. 45: 22–30

This study examined the effects of s.c. administration of chlorpyrifos in sunflower oil at 0 (vehicle control), 0.1, 1 or 10 mg/kg bw/d daily for seven days in rats (n = 4-10/group). Measurements included the plasma activity of several B-esterases, electroencephalograms, auditory startle responses and comet assays.

This study had no regulatory value due to the repeated low-dose subcutaneous administration that cannot be extrapolated to human exposure levels. In addition, there were significant variations in the pattern of biomarkers, and the relevance to human exposure cannot be determined.

11. Peris-Sampedro F, Salazar JG, Cabré M, Reverte I, Domingo JL, Sánchez-Santed F, Colomina MT (2014). Impaired retention in AβPP Swedish mice six months after oral exposure to chlorpyrifos. Food Chem Toxicol. 72: 289–94

This study was conducted on a mouse model of Alzheimer's disease (Tg2576). The authors investigated the long-term effect of repeated chlorpyrifos doses on these mice' spatial learning and memory; and on the level of amyloid beta protein in these mice frontal cortex and hippocampus.

The study was found to have no regulatory value due to the difficulty in extrapolation from an animal model for Alzheimer's disease to the general population.

12. Ray A, Liu J, Ayoubi P, Pope C (2010). Dose-related gene expression changes in forebrain following acute, low-level chlorpyrifos exposure in neonatal rats. Toxicol Appl Pharmacol. 248(2): 144–55

This study aimed to examine potential pathways leading to developmental neurotoxicity by studying gene expression profiles in the forebrains of neonatal rats at 24 h following single dose oral gavage of chlorpyrifos in peanut oil at 0 (vehicle control) or 0.1, 0.5, 1 or 2 mg/kg bw to 7 day-old rats.

This study was considered to hold no regulatory value because there were no data relating to inhibition of plasma or RBC ChE activity, hence no correlation between variations in gene expression and the surrogate human biomarkers could be determined.

13. Slotkin TA, Seider FJ (2012). Developmental neurotoxicity of organophosphates targets cell cycle and apoptosis, revealed by transcriptional profiles *in vivo* and *in vitro*. Neurotoxicol Teratol. 34(2): 232–41

The study examined the effect of chlorpyrifos on the transcription of genes reputed to be involved in the control of cell cycle and apoptosis. Chlorpyrifos (1 mg/kg bw/d in DMSO) was injected s.c. to neonatal rats on PND1–4. The brainstem and forebrain of rats were harvested on PND5 and genes expression was assessed using microarrays. The effect of chlorpyrifos (30 µM in DMSO) on the transcription of the same genes was also examined *in vitro* using either differentiated or undifferentiated PC12 cells. The authors concluded that chlorpyrifos altered the expression of genes involved in cell cycle and apoptosis, particularly around the time of neurodifferentiation initiation. Importantly, according to the authors, these findings occurred at chlorpyrifos doses that elicit less than 20% ChE inhibition, hence independently of cholinergic hyperstimulation.

This study was considered of low regulatory value on the following grounds,

- The difficulty in extrapolating s.c. administration of 1 mg/kg bw chlorpyrifos in rats to human exposure levels through routes of exposure relevant to human health.

- Although the altered gene expression determined in the study may be compatible with a mechanism for altered neurobiological development, the clinical relevance of this effect is unclear.

- Significant RBC AChE inhibition has been reported in various dietary animal studies containing chlorpyrifos at doses significantly lower than 1 mg/kg bw, which weakens the authors' interpretation that their observations are independent of cholinergic mechanism of action.

14. Slotkin TA, Skavicus S, Seidler FJ (2015). Prenatal drug exposures sensitize noradrenergic circuits to subsequent disruption by chlorpyrifos. Toxicology. 338: 8–16

This study aimed at exploring whether prenatal exposure to nicotine or dexamethasone (commonly used in the management of preterm labor) modifies the effects of postnatal chlorpyrifos exposure to noradrenergic systems in the cerebellum. A group of pregnant rats was administered dexamethasone at 0.2 mg/kg bw/d during GD17–19; another group of pregnant rats was administered nicotine bitartrate at 3 mg/kg bw/d during GD4–21. Offsprings of these groups and of control groups were administered chlorpyrifos s.c. at 1 mg/kg bw/d during PND1–4. The authors then measured the concentration of β-adrenergic receptors (βARs) in different parts of the rats' brain at PND30, 60, 100 and 150. βARs concentration was determined by [125I]-iodopindolol binding to the tissues' membrane fraction.

This study was considered to have no regulatory value on the following grounds,

- The s.c. administration of chlorpyrifos is not relevant for human health assessment, as it does not reflect the high level of metabolic transformation undergone by chlorpyrifos after oral or dermal absorption.

- The only parameter assessed in the study was the concentration of βARs in specific parts of rats' brain; by itself, those endpoints do not provide direct evidence of adverse health effect.

15. Young JG, Eskenazi B, Gladstone EA, Bradman A, Pedersen L, Johnson C, Barr DB, Furlong CE, and Holland NT (2005). Association Between *In Utero* Organophosphate Pesticide Exposure and Abnormal Reflexes in Neonates. NeuroToxicology 26(2): 199–209

This study aimed to assess the relationship between *in utero* and early postnatal OP exposure and neonatal neurobehaviour in humans, as measured by seven clusters (habituation, orientation, motor performance, range of state, regulation of state, autonomic stability, and reflex) on the BNBAS. Exposure was assessed by measurement of OP DAP metabolites in urine. Six OP DAP metabolites were measured in maternal urine: three dimethylphosphate metabolites (dimethylphosphate, DMP; dimethyldithiophosphate, DMDTP; dimethylthiophosphate, DMTP); and three diethylphosphate metabolites (diethylphosphate, DEP; diethyldithiophosphate, DEDTP; diethylthiophosphate, DETP). Chlorpyrifos was only one of several parent OP pesticides identified for the measured diethylphosphate metabolites. The study was not evaluated as it provided no data specific to chlorpyrifos.

APPENDIXES

ABBREVIATIONS

Time	
d	Day
h	Hour
min	Minute
wk	Week
yr	Year
yo	Year old
Weight	
bw	Bodyweight
g	Gram
kg	Kilogram
µg	Microgram
mg	Milligram
ng	Nanogram
pg	Picogram
Length	
cm	Centimetre
m	Metre
µm	Micrometre
mm	Millimetre
nm	Nanometre
Dosing	
i.p.	Intraperitoneal
mg/kg bw/d	Milligram/kilogram bodyweight/day
s.c.	Subcutaneous

Frequency	
kHz	Kilohertz
Volume	
L	Litre
mL	Millilitre
µL	Microlitre
Concentration	
M	Molar
fmol	Femtomole, 10^{-15} of a mole
ppm	Parts per million
Clinical chemistry, haematology	
RBC	Red blood cell
T3	Liothyronine
T4	Thyroxine
Terminology	
2-AG	2-arachidonoylglycerol
5HT	Serotonin (5–hydroxytryptamine)
5HTT	Serotonin transporter (5–hydroxytryptamine transporter)
5–SRTT	5–Serial Reaction Time Task
βAR	Beta-adrenergic receptors
AC	Adenylyl cyclase
ACh	Acetylcholine
AChE	Acetylcholinesterase
ADHD	Attention deficit hyperactivity disorder
ADI	Acceptable Daily Intake
AEA	Anandamide
APH	Acylpeptide hydrolase

AR	Androgen receptor
ARfD	Acute Reference Dose
ASD	Autism spectrum disorder
BChE	Butyrylcholinesterase
BLC	Basolateral complex
BMD	Benchmark dose
BMDL	Benchmark dose level
BMI	Body mass index
BMP	Bone morphogenetic proteins
BNBAS	Brazelton Neonatal Behavioural Assessment Scale
BSID	Bayley Scales of Infant Development
CaMKII	Calcium calmodulin-dependent protein kinase II
CBCL	Child Behaviour Checklist
CCCEH	Columbia Centre of Children's Environmental Health
CDC	Centers for Disease Control and Prevention
CES-D	Center for Epidemiologic Studies Depression Scale
CHAMACOS	Centre for Health Assessment of Mothers and Children of Salinas
CHARGE	Childhood Autism Risks from Genetics and Environment
ChAT	Choline acetyltransferase
ChE	Cholinesterase
CNS	Central nervous system
CPO	Chlorpyrifos-oxon
CYP	Cytochromes P450
DAP	Dialkyl phosphate
DD	Developmental delay
DDE	Dichlorodiphenyldichloroethylene
DE	Diethyl

DEDTP	Diethyldithiophosphate
DEET	Diethyl-m-toluamide
DEP	Diethylphosphate
DETP	Diethylthiophosphate
DMP	Dimethylphosphate
DMSO	Dimethyl sulfoxide
DMTP	Dimethylthiophosphate
DNA	Deoxyribonucleic Acid
DNMTP	Delayed non-match to position
E	Embryo day
ETS	Environmental tobacco smoke
ER	Estrogen receptor
F2–IsoP	F_2–isoprostanes
F	Female(s)
FAAH	fatty acid amide hydrolase
fMRI	Functional Magnetic Resonance Imaging
FOB	Functional Observational Battery
FSIQ	Full-scale IQ score
FST	Forced swimming test
GC/MS	Gas Chromatography Mass Spectrometry
GD	Gestational Day
GFAP	Glial fibrillary acidic protein
HIV	Human immunodeficiency virus
HOME	Home Observation for Measurement of the Environment
HPLC	High Performance Liquid Chromatography
IQ	Intelligence Quotient
LADE	Lifetime Average Daily Exposure

LD	Lactation day
LD_{50}	Lethal Dose 50
LH	Learned helplessness
LOAEL	Lowest Observed Adverse Effect Level
LOD	Limit of detection
M	Male(s)
mAChR	Muscarinic acetylcholine receptor
MAGL	Monoacylglycerol lipase
MDI	Mental Development Index
Mean ± SD	Mean ± standard deviation
MEMRI	Manganese enhanced magnetic resonance imaging
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
NADPH-d	Dihydronicotinamide adenine dinucleotide phosphate diaphorase
NeuN	Neuronal Nuclei
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOAEL	No Observed Adverse Effect Level
NSFT	Novelty-suppressed feeding test
OP	Organophosphorus pesticide
PA	Passive avoidance
PAH(s)	Polycyclic aromatic hydrocarbon(s)
PBA	Phenoxybenzoic acid
PCBs	Polychlorinated biphenyls
PCNA	Proliferating cell nuclear antigen
PCP	Pentachlorophenol

pCREB	Phosphorylated cAMP response element-binding protein
PDD	Pervasive Developmental Disorder
PDI	Psychomotor Development Index
PGE2	Prostaglandin E2
PN	Postnatal
PND	Postnatal Day
PON/PON1	Paraoxonase/Paraoxonase 1
PPE	Personal protective equipment
PPVT	Peabody Picture Vocabulary Test
PSD-95	Postsynaptic Density Protein 95
RNA	Ribonucleic acid
SD	Sprague Dawley (rats)
SGZ	Sub granular zone
TCP	3,5,6-trichloro-2-pyridinol
TD	Typical development
TSH	Thyroid-stimulating hormone
USV	Ultrasound vocalisations
Vit	Vitamin
WISC-IV	Wechsler Intelligence Scales for Children
WPPSI	Wechsler psychometric intelligence
Organisations & publications	
APVMA	Australian Pesticides and Veterinary Medicines Authority
IPCS	International Programme on Chemical Safety
OCS	Office of Chemical Safety
OECD	Organisation for Economic Cooperation and Development
SUSMP	Standard for the Uniform Scheduling of Medicines and Poisons
US EPA	United States Environmental Protection Agency

| WHO | World Health Organisation |

REFERENCES

Aldridge JE, Seidler FJ, Meyer A, Thillail I, Slotkin TA (2003). Serotonergic systems targeted by developmental exposure to chlorpyrifos: Effects during different critical periods. Environ Health Perspect. 111(14): 1736–1743

Aldridge JE, Levin ED, Seidler FJ, Slotkin TA (2005). Developmental exposure of rats to chlorpyrifos leads to behavioural alterations in adulthood, involving serotonergic mechanisms and resembling animal models of depression. Environ Health Perspect. 133(5): 527–531

Andersen HR, Debes F, Wohlfahrt-Veje C, Murata K, Grandjean P (2015). Occupational pesticide exposure in early pregnancy associated with sex-specific neurobehavioral deficits in the children at school age. Neurotoxicol Teratol. 47: 1–9

Barr DB, Ananth CV, Yan X, Lashley S, Smulian JC, Ledoux TA, Hore P, Robson MG (2010). Pesticide concentrations in maternal and umbilical cord sera and their relation to birth outcomes in a population of pregnant women and newborns in New Jersey. Science of the Total Environment 408: 790–795

Berent S, Giordani B, Albers JW, Garabrant DH, Cohen SS, Garrison RP, Richardson RJ (2014). Effects of occupational exposure to chlorpyrifos on neuropsychological function: a prospective longitudinal study. Neurotoxicology. 41: 44–53

Berkowitz GS, Wetmur JG, Birman-Deych E, Obel J, Lapinski RH, Godbold JH, Holzman IR, Wolff MS (2004). *In utero* pesticide exposure, maternal paraoxonase activity, and head circumference. Environ Health Perspect. 112(3): 388–391

Braquenier JB, Quertemont E, Tirelli E, Plumier JC (2010). Anxiety in adult female mice following perinatal exposure to chlorpyrifos. Neurotoxicol Teratol. 32(2): 234–239

Chen WQ, Zhang YZ, Yuan L, Li YF, Li J (2014). Neurobehavioral evaluation of adolescent male rats following repeated exposure to chlorpyrifos. Neurosci Lett. 570: 76–80

Cole TB, Fisher JC, Burbacher TM, Costa LG, Furlong CE (2012). Neurobehavioural assessment of mice following repeated postnatal exposure to chlorpyrifos-oxon. Neurotoxicol Teratol. 34(3): 311–322

Engel SM, Berkowitz G S, Barr DB, Teitelbaum S L, Siskind J, Meisel SJ, Wetmur JG, Wolff M S (2007). Prenatal Organophosphate Metabolite and Organochlorine Levels and Performance on the Brazelton Neonatal Behavioural Assessment Scale in a Multiethnic Pregnancy Cohort. Am J Epidemiol (2007)165: 1397–1404

Engel SM, Wetmur J, Chen J, Zhu C, Barr DB, Canfield RL, Wolff MS (2011). Prenatal Exposure to Organophosphates, Paraoxonase 1, and Cognitive Development in Childhood. Environ Health Perspect 119: 1182–1188

Eskenazi B, Harley K, Bradman A, Weltzien E, Jewell NP, Barr DB, Furlong CE, Holland NT (2004). Association of *in utero* organophosphate pesticide exposure and fetal growth and length of gestation in an agricultural population. Environ Health Perspect. 112–10: 1116–1124

Eskenazi B, Marks AR, Bradman A, Harley K, Barr DB, Johnson C, Morga N, Jewell NP (2007). Organophosphate Pesticide Exposure and Neurodevelopment in Young Mexican-American Children. Environ Health Perspect. 115(5): 792–798

Garcia SJ, Seidler FJ, Qiao D, Slotkin TA (2002). Chlorpyrifos targets developing glia: effects on glial fibrillary acidic protein. Dev Brain Res 133: 151–161

Grabovska S, Salyha Y (2015). ADHD-like behaviour in the offspring of female rats exposed to low chlorpyrifos doses before pregnancy. Arh Hig Rada Toksikol. 66(2): 121–7

Goodman JE, Prueitt RL, Rhomberg LR (2012). Incorporating low-dose epidemiology data in a chlorpyrifos risk assessment. Dose Response 11(2): 207–219

Guo-Ross SX, Chambers, JE, Meek EC, Carr RL (2007). Altered muscarinic acetylcholine receptor subtype binding in neonatal rat brain following exposure to chlorpyrifos or methyl parathion. Toxicol Sci. 100(1): 118–127

Helps S (2012). Brain anomalies in children exposed prenatally to a common OP pesticide. Expert Commentary of the paper by Rauh et al, 2012

Horton MK, Kahn LG, Perera F, Barr DB, Rauh V (2012). Does the home environment and the sex of the child modify the adverse effects of prenatal exposure to chlorpyrifos on child working memory? Neurotoxicol Teratol. 34(5): 534–541

Johnson FO, Chambers JE, Nail CA, Givaruangsawat S, Carr RL (2009). Developmental Chlorpyrifos and Methyl Parathion Exposure Alters Radial-Arm Maze Performance in Juvenile and Adult Rats. Toxicol Sci. 109(1): 132–42

Juberg DR, Gehen SC, Coady KK, LeBaron MJ, Kramer VJ, Lu H, Marty MS (2013). Chlorpyrifos: Weight of evidence evaluation of potential interaction with the estrogen, androgen or thyroid pathways. Regul Toxicol Pharmacol. 66(3): 249 263

Kim HH, Lim YW, Yang JY, Shin DC, Ham HS, Choi BS, Lee JY (2013). Health risk assessment of exposure to chlorpyrifos and dichlorvos in children at childcare facilities. Sci Total Environ. 444: 441–450

Lee I, Eriksson P, Fredriksson A, Buratovic S, Viberg H (2015). Developmental neurotoxic effects of two pesticides: Behavior and biomolecular studies on chlorpyrifos and carbaryl. Toxicol Appl Pharmacol 288(3): 429–438

Levin ED, Addy N, Nakajima A, Christopher NC, Seidler FJ, Slotkin TA (2001). Persistent behavioural consequences of neonatal chlorpyrifos exposure in rats. Dev Brain Res. 130(1): 83–89

Levin ED, Cauley M, Johnson JE, Cooper EM, Stapleton HM, Ferguson PL, Seidler FJ, Slotkin TA (2014). Prenatal dexamethasone augments the neurobehavioral teratology of chlorpyrifos: significance for maternal stress and preterm labor. Neurotoxicol Teratol. 41: 35–42

Li AA, Lowe KA, McIntosh LJ, Mink PJ (2012). Evaluation of epidemiology and animal data for risk assessment: chlorpyrifos developmental neurobehavioural outcomes. J Toxicol Environ Health B Crit Rev. 15(2): 109–184

Lopez-Crespo GA, Flores P, Sanchez-Santed F, Sanchez-Amate MC (2009). Acute high dose of chlorpyrifos alters performance of rats in the elevated plus-maze and the elevated T-maze. Neurotoxicology 30(6): 1025–1029

Lopez-Granero C, Canadas, F, Cardona D, Yu Y, Gimenez E, Lozano R, Avila DS, Aschner, M, Sanchez-Santed F (2013). Chlorpyrifos-, Diisopropylphosphorofluoridate-, and Parathion-Induced Behavioural and Oxidative Stress Effects: Are They Mediated by Analogous Mechanisms of Action? Toxicol Sci. 131(1): 206–216

Marty MS, Andrus AK, Bell MP, Passage JK, Perala AW, Brzak KA, Bartels MJ, Beck MJ, Juberg DR (2012) Cholinesterase inhibition and toxicokinetics in immature and adult rats after acute or repeated exposures to chlorpyrifos or chlorpyrifos-oxon. Regul Toxicol Pharmacol. 63(2): 209–224

Meyer A, Seidler FJ, Aldridge JE, Tate CA, Cousins MM, Slotkin TA (2004). Critical periods for chlorpyrifos-induced developmental neurotoxicity: alterations in adenylyl cyclase signaling in adult rat brain regions after gestational or neonatal exposure. Environ Health Perspect. 112(3): 295–301

Middlemore-Risher ML, Buccafusco JJ, Terry AV (2010). Repeated exposures to low-level chlorpyrifos results in impairments in sustained attention and increased impulsivity in rats. Neurotoxicol Teratol. 32(4): 415–24

Mink PJ, Kimmel CA, Li AA (2012). Potential effects of chlorpyrifos on fetal growth outcomes: implications for risk assessment. J Toxicol Environ Health B Crit Rev. 15(4): 281–316

Mullins RJ, Xu S, Pereira EF, Pescrille JD, Todd SW, Mamczarz J, Alburquerque EX, Gullapalli RP (2015). Prenatal exposure of guinea pigs to the organophosphorus pesticide chlorpyrifos disrupts the structural and functional integrity of the brain. Neurotoxicology 48: 9–20

Ohishi T, Wang L, Akane H, Itahashi M, Nakamura D, Yafune A, Mitsumori K, Shitbutani M (2013). Reversible effect of maternal exposure to chlorpyrifos on the intermediate granule cell progenitors in the hippocampal dentate gyrus of rat offspring. Reprod Toxicol. 35: 125–136

Perera FP, Rauh VA, Tsai W, Kinney P, Camann D, Barr D, Bernert T, Garfinkel R, Tu Y, Diaz D, Dietrich J, Whyatt RM (2003). Effects of transplacental exposure to environmental pollutants on birth outcomes in a multiethnic population. Environ Health Perspect 111(2): 201–205

Prueitt RL, Goodman JE, Bailey LA, Rhomberg LR (2011). Hypothesis-based weight-of-evidence evaluation of the neurodevelopmental effects of chlorpyrifos. Crit Rev Toxicol. 41(10): 822–903

Rauh VA, Garfinkel R, Perera FP, Andrews HF, Hoepner L, Barr DB, Whitehead R, Tang D, Whyatt RW (2006). Impact of prenatal chlorpyrifos exposure on neurodevelopment in the first 3 years of life among inner-city children. Pediatrics 118(6): 1845–1859

Rauh V, Arunajadai S, Horton M, Perera F, Hoepner L, Barr DB, Whyatt R (2011). Seven-year neurodevelopmental scores and prenatal exposure to chlorpyrifos, a common agricultural pesticide. Environ Health Perspect 119(8): 1196–1201

Rauh VA, Perera FP, Horton MK, Whyatt RM, Bansal R, Hao X, Liu J, Barr DB, Slotkin TA, Peterson BS (2012). Brain anomalies in children exposed prenatally to a common organophosphate pesticide. Proc Natl Acad Sci USA 109(20): 7871–7876. With Supporting Information 10.173/pnas.1203396109

Reiss R, Neal B, Lamb JC, Juberg DR (2012). Acetylcholinesterase inhibition dose-response modeling for chlorpyrifos and chlorpyrifos-oxon. Regul Toxicol Pharmacol. 63(1): 124–131

Rohlman DS, Ismail AA, Abdel-Rasoul G, Lasarev M, Hendy O, Olson JR (2014). Characterizing exposures and neurobehavioral performance in Egyptian adolescent pesticide applicators. Metab Brain Dis 29(3): 845–855

Roy TS, Seidler FJ, Slotkin TA (2004). Morphologic effects of subtoxic neonatal chlorpyrifos exposure in developing rat brain: regionally selective alterations in neurons and glia. Dev Brain Res. 148(2): 197–206

Roy TS, Sharma V, Seidler FJ, Slotkin TA (2005). Quantitative morphological assessment reveals neuronal and glial deficits in hippocampus after a brief subtoxic exposure to chlorpyrifos in neonatal rats. Dev Brain Res. 155(1): 71–80

Shalaby MA, Abo-El-Sooud K, Hamoda AA (2013). Assessment of Toxicity of Chlorpyrifos Insecticide on Fetuses and Suckling Pups of Rats. Insight Ecology 2(1): 1–7

Shelton JF, Geraghty EM, Tancredi DJ, Delwiche LD, Schmidt RJ, Ritz B, Hansen RL, Hertz-Picciotto I (2014). Neurodevelopmental disorders and prenatal residential proximity to agricultural pesticides: the CHARGE study. Environ Health Perspect. 122(10): 1103–1109

Terry AV, Beck WD, Waner S, Vandenhuerk L, Callahan PM (2012). Chronic impairments in spatial learning and memory in rats previously exposed to chlorpyrifos or diisopropylfluorophosphate. Neurotoxicol Teratol. 34(1): 1–8

Vatanparast J, Naseh M, Baniasadi M & Haghdoost-Yazdi H (2013). Developmental exposure to chlorpyrifos and diazinon differentially affect passive avoidance performance and nitric oxide synthase-containing neurons in the basolateral complex of the amygdala. Brain Res. 1494: 17–27

Venerosi A, Ricceri L, Scattoni ML, Calamandrei G (2009). Prenatal chlorpyrifos exposure alters motor behaviour and ultrasonic vocalisation in CD-1 mouse pups. Environ Health 8:12

Wang L, Ohishi T, Akane H, Shiraki A, Itahashi M, Mitsumori K, Shibutani M (2013). Reversible effect of developmental exposure to chlorpyrifos on late-stage neurogenesis in the hippocampal dentate gyrus in mouse offspring. Reprod Toxicol. 38: 25–36

Whyatt RM, Garfinkel, R, Hoepner LA, Holmes D, Borjas M, Williams MK, Reyes A, Rauh V, Perera FP, Camann DE (2002). Residential Pesticide Use during pregnancy among a cohort of Urban Minority Women. Environ Health Perspect. 110(5): 507–514

Whyatt RM, Garfinkel, R, Hoepner LA, Holmes D, Borjas M, Williams MK, Reyes A, Rauh V, Perera FP, Camann DE (2007). Within- and Between-Home Variability in Indoor-Air Insecticide Levels during Pregnancy among an Inner-City Cohort from New York City. Environ Health Perspect. 115: 383–389

Whyatt RM, Garfinkel R, Hoepner LA, Andrews H, Holmes D, Williams MK, Reyes A, Diaz D, Perera FP, Camann DE, Barr DB (2009). A biomarker validation study of prenatal chlorpyrifos exposure within an inner-city cohort during pregnancy. Environ Health Perspect. 117(4): 559–567

Zhao Q, Gadagbui B, Dourson M (2005). Lower birth weight as a critical effect of chlorpyrifos: a comparison of human and animal data. Regul Toxicol Pharmacol. 41(1): 55–63

Studies cited but not evaluated in this assessment

Albers JW, Berent S, Garabrant DH, Giordani B, Schweitzer SJ, Garrison RP, Richardson RJ (2004). The effects of occupational exposure to chlorpyrifos on the neurologic examination of central nervous system function: a prospective cohort study. J Occup Environ Med. 46: 367–378.

Bradman A, Barr DB, Clause Henn BG, Drumheller T, Curry C, Eskenazi B (2003). Measurement of pesticides and other toxicants in amniotic fluid as a potential biomarker of prenatal exposure: a validation study. Environ Health Perspect. 111(14): 1779–1782

Betancourt AM, Carr RL (2004). The effect of chlorpyrifos and chlorpyrifos-oxon on brain cholinesterase, muscarinic receptor binding, and neurotrophin levels in rats following early postnatal exposure. Toxicol Sci. 77(1): 63–71

Black MM, Matula K (2000). Essentials of Bayley Scales of Infant Development—II Assessment. John Wiley and Sons, New York, USA

Bouchard MF, Chevrier J, Harley KG, Kogut K, Michelle V, Calderon N, Trujillo C, Bradman A, Barr DB, Eskenazi B (2011). Prenatal Exposure to Organophosphate Pesticides and IQ in 7–Year-Old Children. Environ Health Perspect. 119(8): 1189–1195

Bourin M, Hascoët M (2003). The mouse light/dark box test. Eur J Pharmacol 463(1): 55–65

Byrnes ML, Richardson DP, Brien JF, Reynolds JN, Dringenberg HC (2004). Spatial acquisition in the Morris water maze and hippocampal long-term potentiation in the adult guinea pig following brain growth spurt--prenatal ethanol exposure. Neurotoxicol Teratol 26(4): 543–51

California EPA (2002). Pesticide Use Reporting 2001 Summary Data. Sacramento, CA. Department of Pesticide Regulation, California Environmental Protection Agency. Available: http://www.cdpr.ca.gov/docs/pur/pur01rep/01_pur.htm (accessed 28/03/2017)

Carr RL, Adams AL, Kepler DR, Ward AB, Ross MK (2013). Induction of endocannabinoid levels in juvenile rat brain following developmental chlorpyrifos exposure. Toxicol Sci. 135(1): 193–201

Chen XP, Chen WF, Wang DW (2014). Prenatal organophosphates exposure alternates the cleavage plane orientation of apical neural progenitor in developing neocortex. PLoS 9(4): e95343

Coulston F, Golberg L & Griffin T (1972). Safety evaluation of Dowco 179 in human volunteers. Institute of Experimental Pathology and Toxicology, Albany Medical College, Albany, New York. USA. Dated March 1972. Sponsor: Dow Agrosciences; Submission 238; A3162/5, Box 43

De Angelis S, Tassinari R, Maranghi F, Eusepi A, Di Virgilio A, Chiarotti F, Ricceri L, Venerosi Pesciolini A, Gilardi E, Moracci G, Calamandrei G, Olivieri A, Mantovani A (2009). Developmental exposure to chlorpyrifos induces alterations in thyroid and thyroid hormone levels without other toxicity signs in CD-1 mice. Toxicol Sci. 108(2): 311–19

de Groot DM, Bierman EP, Bruijnzeel PL, Carpentier P, Kulig BM, Lallement G, Melchers BP, Philippens IH, van Huygevoort AH (2001). Beneficial effects of TCP on soman intoxication in guinea pigs: seizures, brain damage and learning behaviour. J Appl Toxicol. 21 Suppl 1: S57–65

Department of Health (2016). Acceptable Daily Intakes for Agricultural and Veterinary Chemicals: Current as of 31 March 2016. Available: http://www.health.gov.au/internet/main/publishing.nsf/content/ocs-adi-list.htm (Accessed 28/03/2017).

Dringenberg HC, Richardson DP, Brien JF, Reynolds JN (2001). Spatial learning in the guinea pig: cued versus non-cued learning, sex differences, and comparison with rats. Behav Brain Res. 124(1): 97–101

Eaton DL, Daroff RB, Autrup H, Bridges J, Buffler P, Costa LG, Coyle J, McKhann G, Mobley WC, Nadel L, Neubert D, Schulte-Hermann R, Spencer PS (2008). Review of the toxicology of chlorpyrifos with an emphasis on human exposure and neurodevelopment. Crit Rev Toxicol. Suppl 2: 1–125

Entringer S, Buss C, Wadhwa PD (2010). Prenatal stress and developmental programming of human health and disease risk: concepts and integration of empirical findings. Curr Opin Endocrinol Diabetes Obes. 17(6): 507–516.

Eskenazi B, Huen K, Marks A, Harley KG, Bradman A, Barr DB, Holland N (2010). PON1 and Neurodevelopment in Children from the CHAMACOS Study Exposed to Organophosphate Pesticides *in utero*. Environ Health Perspect. 118(12): 1189–1195

Filliat P, Foquin A, Lallement G (2002). Effects of chronic administration of huzerpine A on memory in guinea pigs. Drug Chem Toxicol. 25(1): 9–24

Harley KG, Huen K, Aguilar Schall R, Holland NT, Bradman A, Barr DB, Eskenazi B (2011) Association of Organophosphate Pesticide Exposure and Paraoxonase with Birth Outcome in Mexican-American Women. PLoS One 6(8): e23923

Haviland JA, Butz DE, Porter WP (2010). Long-term sex selective hormonal and behavioural alterations in mice exposed to low doses of chrlopyrifos *in utero*. Reprod Toxicol. 29(1): 74–9

Hernandez CM, Beck WD, Naughton SX, Poddar I, Adam BL, Yanasak N, Middleton C, Terry AV Jr (2015). Repeated exposure to chlorpyrifos leads to prolonged impairments of axonal transport in the living rodent brain. Neurotoxicology. 47: 17–26

Hoberman AM (1998). Developmental neurotoxicity study of chlorpyrifos administered orally via gavage to Crl:CD.BR VAF/Plus presumed pregnant rats. Sponsor: Dow Agrosciences, protocol No.: K-044793109. Performing laboratory: Argus Research Laboratories, Pennsylvania. Study ID: 304001, Dated May 1, 1998. [Dow submission No.: 11709] GLP

James MO, Yan Z, Cornett R, Jayanti VM, Henderson GN, Davydova N, Katovich MJ, Pollock B, Stacpoole PW (1998). Pharmacokinetics and metabolism of [^{14}C]dichloroacetate in male Sprague-Dawley rats. Identification of glycine conjugates, including hippurate, as urinary metabolites of dichloroacetate. Drug Metab Dispos. 26(11): 1134–43

Juberg DR (2012). Differentiating experimental animal doses from human exposures to chlorpyrifos. Proc Natl Acad Sci USA. 109(33): E2195

Keller WC, Yeary RA (1980). A comparison of the effects of mineral oil, vegetable oil, and sodium sulfate on the intestinal absorption of DDT in rodents. Clin. Toxicol. 16: 223–231

Levin ED, Addy N, Baruah A, Elias A, Christopher NC, Seidler FJ, Slotkin TA (2002). Prenatal chlorpyrifos exposure in rats causes persistent behavioral alterations. Neurotoxicol Teratol. 6: 733–41

Lewejohann L, Pickel T, Sachser N, Kaiser S (2010). Wild genius—domestic fool? Spatial learning abilities of wild and domestic guinea pigs. Front Zool. 7: 9

Marks AR, Harley K, Bradman A, Kogut K, Barr DB, Johnson C, Calderon N, Eskenazi B (2010). Organophosphate pesticide exposure and attention in young Mexican-American children: the CHAMACOS study. Environ Health Perspect. 118(12): 1768–1774

Mattsson JL, Maurissen JP, Spencer PJ, Brzak BS & Zablotny CL (1998). Effects of chlorpyrifos administered via gavage to CD rats during gestation and lactation on plasma, erythrocyte, heart and brain cholinesterase, and analytical determination of chlorpyrifos and metabolites. Sponsor: Dow Agrosciences. Performing laboratory: Dow—Health & Environmental Research Laboratories, Michigan.

Mattsson JL, Maurissen JP, Nolan RJ, Brzak KA (2000). Lack of differential sensitivity to cholinesterase inhibition in fetuses and neonates compared to dams treated perinatally with chlorpyrifos. Toxicol Sci. 53(2): 438–446

Meek ME, Boobis A, Cote I, Dellarco V, Fotakis G, Munn S, Seed J, Vickers C (2013). New developments in the evolution and application of the WHO/IPCS framework on mode of action/species concordance analysis. J Appl Toxicol. 34(1): 1–18

Moser VC, Simmons JE, Gennings C (2006). Neurotoxicological interactions of a five-pesticide mixture in preweanling rats. Toxicol Sci. 92(1): 235–245

Mullen BR, Khialeeva E, Hoffman DB, Ghiani CA, Carpenter EM (2012) Decreased reelin expression and organophosphate pesticide exposure alters mouse behaviour and brain morphology. ASN Neuro.18; 5(1): e00106

Muller M, Hess L, Tardivo A, Lajmanovich R, Attademo A, Poletta G, Simoniello MF, Yodice A, Lavarello S, Chialvo D, Scremin O (2014). Neurologic dysfunction and genotoxicity induced by low levels of chlorpyrifos. Neurotoxicology. 45: 22–30

Palin KJ, Wilson CG, Davis SS, Phillips AJ (1982). The effects of oil on the lymphatic absorption of DDT. J. Pharm. Pharmacol., 34: 707–710

Peris-Sampedro F, Salazar JG, Cabré M, Reverte I, Domingo JL, Sánchez-Santed F, Colomina MT (2014). Impaired retention in AβPP Swedish mice six months after oral exposure to chlorpyrifos. Food Chem Toxicol. 72: 289–94

Pirko I, Fricke S, Johnson A, Rodriguez M, Macura S (2005). Magnetic resonance imaging, microscopy, and spectroscopy of the central nervous system in experimental animals. NeuroRx. 2(2): 250–264

Prueitt RL, Goodman JE, Bailey LA, Rhomberg LR (2011). Hypothesis-based weight-of-evidence evaluation of the neurodevelopmental effects of chlorpyrifos. Crit Rev Toxicol. 41(10): 822–903

Qiao D, Seidler FJ, Padilla S, Slotkin TA (2002). Developmental neurotoxicity of chlorpyrifos: what is the vulnerable period? Environ Health Perspect 110: 1097–1103

Ray A, Liu J, Ayoubi P, Pope C (2010). Dose-related gene expression changes in forebrain following acute, low-level chlorpyrifos exposure in neonatal rats. Toxicol Appl Pharmacol. 248(2): 144–55

Roberts EM, English PB, Grether JK, Windham GC, Somberg L, Wolff C (2007). Maternal residence near agricultural pesticide applications and autism spectrum disorders among children in the California Central Valley. Environ Health Perspect 115: 1482–1489

Shih TM, McDonough JH Jr (1997). Neurochemical mechanisms in soman-induced seizures. J Appl Toxicol. 17(14): 255–264

Slotkin TA, Seider FJ (2012). Developmental neurotoxicity of organophosphates targets cell cycle and apoptosis, revealed by transcriptional profiles *in vivo* and *in vitro*. Neurotoxicol Teratol. 34(2): 232–41

Sobel ME (1982). Asymptotic confidence intervals for indirect effects in structural equation models. In: S Leinhardt (Ed.) Sociological Methodology: American Sociological Association, Washington, D.C., USA. pp 290–312

Strauss E, Sherman EMS, Spreen OA (2006). A compendium of neuropsychological tests: Administration, norms and commentary, 3rd Ed. Oxford University Press, New York, USA

Timchalk C, Poet TS, Kousba AA (2006). Age-dependent pharmacokinetic and pharmacodynamic response in preweanling rats following oral exposure to the organophosphorus insecticide chlorpyrifos. Toxicology 220(1): 13–25

Wechsler D (2003). Wechsler Intelligence Scale for Children, 4th Edition. Psychological Corporation, San Antonio, Texas, USA

Whyatt RM, Barr DB, Camann DE, Kinney PL, Barr JR, Andrews HF, Hoepner LA, Garfinkel R, Hazi Y, Reyes A, Ramirez J, Cosme Y, Perera FP (2003). Contemporary-use pesticides in personal air samples during pregnancy and blood samples at delivery among urban minority mothers and newborns. Environmental Health Perspectives 111(5): 749–756

Whyatt RM, Rauh V, Barr DB, Camann DE, Andrews HF, Garfinkel R, Hoepner LA, Diaz D, Dietrich J, Reyes A, Tang D, Kinney PL, Perera FP (2004). Prenatal insecticide exposures and birth weight and length among an urban minority cohort. Environ Health Perspect. 112(10): 1125–1132

Whyatt RM, Camann D, Perera FP, Rauh VA, Tang D, Kinney PL, Garfinkel R, Andrews H, Hoepner L, Barr DB (2005). Biomarkers in assessing residential insecticide exposures during pregnancy and effects on fetal growth. Toxicology and Applied Pharmacology 206(2): 246–254

Wolff MS, Engel S, Berkowitz G, Teitelbaum S, Siskind J, Barr DB, Wetmur J (2007). Prenatal pesticide and PCB exposures and birth outcomes. Pediatr Res. 61(2): 243–50

Zheng Q, Olivier K, Won YK, Pope CN (2000). Comparative cholinergic neurotoxicity of oral chlorpyrifos exposures in preweanling and adult rats. Toxicol Sci. 55(1): 124–32

US EPA (2001). Interim Reregistration Eligibility Decision for Chlorpyrifos. United States Environmental Protection Agency, Washington, D.C., USA. Available: https://nepis.epa.gov/Exe/ZyPDF.cgi/200008BM.PDF?Dockey=200008BM.PDF (accessed 28/03/2017)

US EPA (2006). Revised Organophosphorous Pesticide Cumulative Risk Assessment. United States Environmental Protection Agency, Washington, D.C., USA.

US EPA (2011). Chlorpyrifos Preliminary Human Health Assessment for Registration Review. United States Environmental Protection Agency, Washington, D.C., USA. Available: https://archive.epa.gov/oppsrrd1/registration_review/web/html/index-299.html (accessed 28/03/2017)

US EPA (2014a). Chlorpyrifos: Revised Human Health Risk Assessment for Registration Review. United States Environmental Protection Agency, Washington, D.C., USA. Available: https://www.regulations.gov/document?D=EPA-HQ-OPP-2008-0850-0195 (accessed 28/03/2017)

US EPA (2014b). Federal Letter-Review of Chlorpyrifos Epidemiology Studies. United States Environmental Protection Agency, Washington, D.C., USA. Available: https://www.regulations.gov/document?D=EPA-HQ-OPP-2008-0850-0170 (accessed 28/03/2017)

Young JG, Eskenazi B, Gladstone EA, Bradman A, Pedersen L, Johnson C, Barr DB, Furlong CE, and Holland NT (2005). Association Between *In Utero* Organophosphate Pesticide Exposure and Abnormal Reflexes in Neonates. NeuroToxicology 26(2): 199–209