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Developmental neurotoxicity of the organophosphorus insecticide chlorpyrifos: from clinical findings to preclinical models and potential mechanisms

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Abstract

Organophosphorus (OP) insecticides are pest-control agents heavily used worldwide. Unfortunately, they are also well known for the toxic effects that they can trigger in humans. Clinical manifestations of an acute exposure of humans to OP insecticides include a well-defined cholinergic crisis that develops as a result of the irreversible inhibition of acetylcholinesterase (AChE), the enzyme that hydrolyzes the neurotransmitter acetylcholine (ACh). Prolonged exposures to levels of OP insecticides that are insufficient to trigger signs of acute intoxication, which are hereafter referred to as subacute exposures, have also been associated with neurological deficits. In particular, epidemiological studies have reported statistically significant correlations between prenatal subacute exposures to OP insecticides, including chlorpyrifos, and neurological deficits that range from cognitive impairments to tremors in childhood. The primary objectives of this article are: (i) to address the short- and long-term neurological issues that have been associated with acute and subacute exposures of humans to OP insecticides, especially early in life (ii) to discuss the translational relevance of animal models of developmental exposure to OP insecticides, and (iii) to review mechanisms that are likely to contribute to the developmental neurotoxicity of OP insecticides. Most of the discussion will be focused on chlorpyrifos, the top-selling OP insecticide in the United States and throughout the world. These points are critical for the identification and development of safe and effective interventions to counter and/or prevent the neurotoxic effects of these chemicals in the developing brain.

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For over half a century, organophosphorus (OP) insecticides have been among the most heavily and ubiquitously used insecticides throughout the world, with chlorpyrifos (CPF) leading the market for many years. Unfortunately, similar to other xenobiotics, these insecticides are toxic to humans, and their widespread usage has become a major global public health concern. Yet, given their effectiveness against insects, ease of application, and low cost, their use is predicted to grow worldwide through 2022 (Grand View Research, 2014).

The fatalities and poor health outcomes resulting from exposures of humans of all ages to high doses of OP insecticides are well documented and result primarily from the common action of these chemicals and/or their metabolites as irreversible inhibitors of acetylcholinesterase (AChE), the enzyme that hydrolyzes the neurotransmitter acetylcholine (Dharmani and Jaga 2005). Cases of accidental and intentional acute OP poisoning occur throughout the world, including the United States (US) (Jaga and Dharmani 2003). However, they are particularly insidious in developing countries, where OP insecticides are readily available, poorly regulated, and account for hundreds of thousands of deaths every year (Buckley *et al.* 2004; Gunnell *et al.* 2007).

There are also concerns regarding the health effects of long-term exposures of humans throughout the world to levels of OP insecticides that are insufficient to trigger overt signs of acute intoxication (Jaga and Dharmani 2003; Bouvier *et al.* 2005). Different research groups have reported that these subacute OP exposures are associated with neurological deficits in adults (reviewed in Jamal *et al.* 2002; Levin and Rodnitzky 1976; Ross *et al.* 2013) and, as it will be further discussed in the next section, in children (reviewed in Eaton *et al.* 2008; Engel *et al.* 2011; Reiss *et al.* 2015; Rosas and Eskenazi 2008). In fact, out of concern of the potential dangers posed by subacute exposures of developing children to CPF, in 2000 the Environmental Protection Agency (EPA) restricted the household use of this OP insecticide in the US (Lemus and Abdelghani 2000). As of 2006, however, CPF could still be detected in 78% of homes surveyed in the US (Stout *et al.* 2009). In addition, agricultural exposures in the United States were not addressed by the EPA restriction, and biological markers of CPF exposure, including the CPF metabolite 3,5,6-trichloro-2-pyridinol (TCPY) in urine and the parent compound (CPF) in blood serum, have been detected in samples from American agricultural workers and their families (Fenske *et al.* 2002; Eskenazi *et al.* 2004, 2007; Huen *et al.* 2012). Thus, chronic subacute exposures to CPF remain a serious public health concern, particularly for children, in the United States and throughout the world.

Epidemiological assessment of the developmental neurotoxicity of CPF

Exposure to CPF is a major issue during pregnancy because, similar to other hydrophobic compounds, this insecticide readily crosses the placenta (Abdel-Rahman *et al.* 2002) and, as such, has the potential to induce untoward effects in the developing organism.

Longitudinal epidemiological studies carried out in a multiethnic inner-city population of children traced statistically significant correlations between prenatal subacute exposures that resulted in CPF concentrations > 6.17 pg/g in cord blood collected at birth and: (i) reduced weight and length at birth (Perera *et al.* 2003), (ii) impaired cognition and motor function, attention deficit hyperactive disorder, and developmental problems at the age of 3 (Rauh *et al.* 2006), (iii) deficits in working memory and reduced full-scale intelligence quotient at the age of 7 (Rauh *et al.* 2011), and (iv) childhood tremors at the age of 11 (Rauh *et al.* 2015). Some authors have argued that levels of CPF in umbilical cord blood are too low to cause biologically meaningful AChE inhibition (critical reviews of the subject can be found in Eaton *et al.* 2008; Reiss *et al.* 2015). While this is true, mechanistic studies that will be discussed later in this review have provided evidence that CPF can interact with and change the activity of non-AChE targets. Therefore, the neurodevelopmental toxicity of CPF may develop in the absence of significant AChE inhibition. In addition, one cannot rule out the possibility that *in utero* levels of CPF are substantially higher than those measured in umbilical cord because the half-life of CPF has been estimated to be approximately 27 h (Timchalk *et al.* 2002). Umbilical cord levels of CPF provide a snapshot of the degree of exposure rather than an accurate assessment of the total prenatal OP burden experienced by the developing organism *in utero*.

In studies that used urine or blood level of TCPY as a biomarker of CPF exposure, either no associations or only weak associations were traced between prenatal CPF exposure and fetal growth indices (Eskenazi *et al.* 2004; Whyatt *et al.* 2004) or cognitive deficits in children (Fortenberry *et al.* 2014). Although the presence of TCPY in blood and/or urine reflects exposure to CPF, levels of the metabolite do not necessarily correlate with the internal CPF dose, that is, the amount of CPF absorbed by an organism. Specifically, CPF can be hydrolyzed to TCPY in the environment (Morgan *et al.* 2005; Lu *et al.* 2006). As such, measured levels of TCPY in body fluids or tissue represent not only the amount of TCPY generated by the metabolism of CPF *in vivo* but also the amount of TCPY absorbed together with CPF. In cases in which the amount of TCPY absorbed from external sources overwhelms the amount of TCPY produced by the *in vivo* breakdown of CPF, blood or urine levels of TCPY do not accurately correlate with levels of CPF absorbed and, consequently, do not correlate with the magnitude of the biological effects of CPF. This emphasizes the limitations of some biomarkers of exposure in health risk assessments (Ryan *et al.* 2007).

Prenatal exposures that resulted in CPF concentrations ≥ 4.39 pg/g in cord blood collected at birth have also been associated with significant structural abnormalities in the brain of 7–9-year-old children when compared with age-matched children who were either not exposed to CPF during pregnancy or experienced prenatal exposures that resulted in cord blood CPF levels < 4.39 pg/g. These abnormalities included a significant enlargement of the mesial surface of the superior frontal gyrus bilaterally in addition to frontal and parietal cortical thinning (Rauh *et al.* 2012). In addition, the statistically significant positive correlations normally traced between the full-scale intelligence quotient (FSIQ) and the surface area of the superior temporal, inferior frontal, inferior precentral, and inferior postcentral gyri bilaterally, and the precuneus of the left hemisphere were either absent or reversed among children, particularly boys, who experienced prenatal exposures producing cord levels of CPF ≥ 4.39 ng/g (Rauh *et al.* 2012). This finding lent support to the hypothesis that

disruption of the structural integrity of the brain can be an important determinant of the cognitive deficits associated with prenatal exposure to CPF.

The limitations of the epidemiological studies reviewed above, including assessment of biomarkers of exposure only at birth rather than throughout pregnancy, lack of an ideal biomarker of exposure or effect, and the potential influence of other risk factors on the measured neurological outcomes, have been extensively discussed in critical reviews published earlier (Eaton *et al.* 2008; Reiss *et al.* 2015). However, some findings strongly indicate that the association between cord blood levels of CPF and neurodevelopmental outcomes cannot be ignored. First, following the government-mandated ban of residential CPF use in 2001, decreased use of CPF was accompanied by significant decreases in CPF levels in both breathing air samples and cord blood. Specifically, maternal personal air samples collected over 48 h during the third trimester of pregnancies in 1999 and 2002 dropped from 17.2 to 4.8 ng/m^3 (Whyatt *et al.* 2005). Likewise, CPF levels in umbilical cord blood sampled from deliveries in 1999 and 2002 dropped from 6.9 to 1.3 pg/g (Whyatt *et al.* 2005). In parallel, the significant inverse correlations between CPF levels in umbilical cord plasma and birth weight and length observed in a cohort of children born before the mandated ban were not detected in a cohort of children born after the ban (Whyatt *et al.* 2005). The monotonic relationships traced between cord blood levels of CPF and neurological or morphological outcomes in these and other studies support the notion that a biological gradient exists between the level of CPF exposure and the biological response (Whyatt *et al.* 2005; Rauh *et al.* 2006, 2012). Second, infants born after the ban had significantly better Mental Development Index and Psychomotor Development Index scores than those born before the ban (Rauh *et al.* 2006).

Developmental toxicity also has been observed in children born to mothers who are exposed during gestation to levels of OP insecticides that cause marked inhibition of serum cholinesterase. Specifically, Farahat *et al.* (2016) compared the birth outcomes of pregnant women who lived in close proximity to or worked in agricultural fields in the Menoufia governorate in Egypt to those of pregnant women living in the governorate capital. The activity of serum cholinesterase measured in blood collected at 20–22 weeks of pregnancy from women in the rural cohort was approximately 73% lower than that measured in blood collected from women in the urban cohort. Gestational age at delivery was shorter in the rural cohort compared to the urban cohort. In addition, birth weight was lower and head circumference was smaller in the rural than in the urban cohort. In the rural cohort, there was a statistically significant correlation between serum cholinesterase activity and reduced gestational age at delivery, low birth weight, and small head circumference. Additional studies with larger samples, analyses of both serum cholinesterase and red blood cell (RBC) AChE activities at multiple time points during pregnancy, and clinical follow up of children's neurodevelopment will help to establish the health risks for pregnant women at risk of exposure to cholinesterase-inhibiting levels of OP insecticides. This is especially critical for agricultural communities in the developing world, where use of these insecticides is not well regulated.

Given the inherent limitations of epidemiological studies, controlled preclinical studies using translationally relevant animal models can be extremely useful to trace cause-

consequence relationships between exposures to specific toxicants and health outcomes. They can also play a critical role in the identification of mechanisms that contribute to the pathological conditions triggered by a given toxicant, and lay the groundwork for the discovery of potential interventions to mitigate the health issues resulting from an exposure to that toxicant.

Preclinical assessment of the developmental neurotoxicity of CPF in rats and mice

Studies of different rodent models have provided evidence supporting the notion that subacute exposures of the developing mammalian brain to CPF result in significant neurobehavioral alterations, including cognitive deficits and locomotor impairments.

Developmental subacute exposure of mice and rats to CPF results in an increase, a decrease, or no change in locomotor activity. The outcome is dependent upon the CPF dose, the age of the animals when they are tested, the time of exposure, and the animal species, among other factors (see Table 1). For instance, Dam *et al.* (2000) reported that male rats exposed on postnatal day (PND) 1–4 to CPF [1 mg/kg/day, subcutaneous (s.c.)] and tested in open fields on PND21 and PND30 presented lower locomotor activity than sex- and age-matched rats exposed to vehicle. Rats injected with CPF (1 mg/kg/day, s.c.) on PND1–4 presented no change in locomotor activity when tested in the figure 8 apparatus on PND35 (Levin *et al.* 2001). Finally, an increase in locomotor activity was observed when rats that had been neonatally exposed to the same CPF dose regimen were tested in the elevated plus maze on PND52–53 (Aldridge *et al.* 2005). These results suggest that developmental subacute exposure to CPF triggers an age-dependent alteration in the locomotor activity of rats, with hypolocomotion in pre-adolescent ages giving way to hyperlocomotion in young adulthood. In fact, there is evidence in the literature that behavioral deficits induced by another OP insecticide – parathion – wax and wane with the age of rats (Levin *et al.* 2010). However, one cannot rule out that the different types of test apparatuses also influenced the outcomes of the studies.

In 2014, Levin and collaborators reported that 35-day-old male rats that were exposed to CPF (1 mg/kg/day, s.c.) on PND1–4 exhibited higher locomotor activity than their control counterparts when tested in the figure 8 apparatus. The CPF dose regimen, the rat strain, the age at which the animals were tested, and the testing apparatus in this study were the same as those in the 2001 study, in which the authors reported that the locomotor activity of 35-day-old rats had not been impacted by the neonatal CPF exposure. The apparent discrepancy could be reconciled by the fact that offspring in the 2014 study were born to dams that had been injected with saline once a day between gestation days (GD) 17 and 19. Results obtained from CPF-exposed offspring of rats injected with saline during pregnancy were compared to those obtained from offspring of dams that had also been injected with saline. Therefore, potential effects of the prenatal injections were controlled for. However, potential interactions between the neonatal injections of CPF and the stress imparted by the prenatal injections of saline were not. Aldridge *et al.* (2005) reported that neonatal rats exposed to CPF present hyperlocomotion in young adulthood. Thus, it is possible that the stress induced

by the prenatal saline injections could have precipitated the early development of hyperlocomotion in the CPF-exposed rats.

The alterations observed in the locomotor activity of mice and rats subjected to different developmental CPF exposures do not correlate well with the degree of brain AChE inhibition measured at 24 h after the last dose (see Table 1). Because AChE activity in the developing brain can quickly recover between repeated exposures to CPF, in part because of the rapid turnover of the enzyme, measurement of enzyme inhibition 24 h after the last dose underestimates the actual degree of inhibition induced by CPF (Lassiter *et al.* 1998). Thus, in their study, Dam *et al.* (2000) measured AChE activity in different brain regions 2 and 4 h after rats were exposed to CPF (1 mg/kg/day, s.c.) on PND1. Brain AChE was inhibited by approximately 60% among males and 20% among females. This higher degree of brain AChE inhibition 2–4 h after the administration of the first dose of CPF in males than females could have affected the brain development of males more pronouncedly such that only males presented reduced locomotor activity when tested at 21 or 30 days of age in open fields (Dam *et al.* 2000). However, it remains unclear whether AChE inhibition is in fact the main driving mechanism of the developmental neurotoxicity of CPF, because female but not male rats presented righting reflex deficits and impaired negative geotaxis when tested at PND5–8 (Dam *et al.* 2000).

Preclinical studies have also reported that developmental subacute exposure of rats and mice to CPF, in different vehicles and through different routes of administration, results in spatial learning and memory deficits that are sexually dimorphic (Table 2). Whether the impairments are more pronounced in males or females depends on the time at which the animals are exposed to CPF. In general, subacute exposure of rats and mice to CPF exclusively during the prenatal period results in cognitive deficits that are more pronounced among females than males (Levin *et al.* 2002; Haviland *et al.* 2010). In contrast, cognitive deficits resulting from neonatal (with or without prenatal) subacute exposure of rats to CPF are more pronounced among males than females (Levin *et al.* 2001; Aldridge *et al.* 2005; Johnson *et al.* 2009; Gómez-Giménez *et al.* 2017).

In one study, rat pups born from dams continuously exposed to CPF (0.3–5 mg/kg/day, p.o. gavage) during gestation and lactation presented no learning or memory deficits (Maurissen *et al.* 2000). One cannot rule out that those results could have been confounded by the rapid and robust effects that gavage has on stress-related responses (Balcombe *et al.* 2004) and the long-lasting effects of maternal stress on cognitive functions of offspring (Richetto and Riva 2014). However, even more importantly, the battery of behavioral tests used by Maurissen *et al.* may not have been sufficiently sensitive to detect the cognitive domain(s) affected by CPF. For instance, the authors concluded that gestational followed by lactational exposure of rats to CPF had no effect on spatial memory, because it did not reduce the percentage of correct choices pre-adolescent and adult rats made in a spatial delayed alternation task in a T-maze. This is a classic spatial working memory task in which rats are required to remember the spatial location of a reward, in this study a food pellet, within a short delay (Tsutsui *et al.* 2016). Using the same task, Chen *et al.* (2012) also reported that the percentage of correct choices made by adult mice prenatally exposed to CPF (1 or 5 mg/kg/day, s.c.; GD13–17) was not significantly different from that made by control mice.

However, Chen *et al.* introduced a correction procedure in to the task. If the animals made an error choice, they were given a chance to shift their selection in consecutive trials during which the same arm was kept baited until the animals made a correct choice. A win-shift error was counted every time the animals did not shift their choice after they had selected a correct arm in the previous trial. On the other hand, a lose-shift error was counted if the animals repeated an incorrect choice made in the previous trial. While the inability to use win-shift failures is associated with working memory deficits, the inability to use lose-shift strategies is suggestive of perseverative behavior or executive function deficits (Zhang *et al.* 2013). The prenatal CPF exposure had no effect on the percentage of win-shift errors. However, it did increase the percentage of lose-shift errors made by the mice in the task (Chen *et al.* 2012). Since these results suggest that cognitive deficits induced by prenatal exposure are function-specific, the negative results reported by Maurissen *et al.* have to be interpreted with a great deal of caution.

Translational relevance of preclinical models of the developmental neurotoxicity of OP insecticides

The advancement of research in the field of developmental OP neurotoxicity rests on the use of models carefully selected for: (i) elucidation of endpoints of toxicity and their dose–response relationships, (ii) identification of the toxicodynamics of the different OP insecticides, and (iii) characterization of mechanisms of action underlying the developmental neurotoxicity of these insecticides. Since all models are approximations, selecting an appropriate model for research is indeed a challenging task and must be driven by the test hypothesis and scientific rationale, while considering humane and ethical endpoints.

Undoubtedly, research conducted in rats and mice in the past decades has critically increased our understanding of the sensitivity of the developing mammalian brain to the neurotoxic effects of CPF and other OP insecticides. These animal models present a number of advantages to toxicological research, including: (i) wide availability, (ii) manageable handling, maintenance, and breeding, (iii) large litter sizes, (iv) validated neurobehavior assays, and (v) well-established genomic sequences that facilitate genetic manipulations. However, as discussed in the following paragraphs, there are limitations associated with these models that can be overcome by the use of additional models.

Rats and mice are notoriously unsuitable to high-throughput large-scale screening of large numbers of toxicants. Animal models such as the zebrafish are generally used for this purpose. In fact, methods are being developed and validated for the use of zebrafish in the characterization of the persisting neurobehavioral impairments caused by developmental exposure to CPF and other OP insecticides (Eddins *et al.* 2010; Richendrfer and Creton 2015).

There is also a shift in the temporal brain development of rats and mice compared to humans (Dobbing and Sands 1970, 1973). While at birth humans display advanced neural and perceptual development, rats and mice are comparatively immature (Dobbing and Sands 1979). Based on brain growth spurt and development of the GABAergic system in the brain,

rat postnatal days (PNDs) 2–7 equate to the human fetal age during the third trimester of pregnancy (Clancy *et al.* 2007 and refs. therein). Consequently, *in utero* exposure of rats and mice to toxicants does not target the same developmental stages of the brain as those targeted by *in utero* exposure of humans. This has been carefully taken into account in the assessment of the developmental neurotoxicity of CPF and other toxicants in mice and rats. To target the period of brain development that corresponds to the last trimester of human pregnancy, researchers expose rats during the first postnatal week to the toxicants of interest. As such, potential effects that interactions between the toxicants and the placenta would have on the developing fetus in the last trimester of human pregnancy are missed.

The structure of the human placenta is also quite distinct from that of the rat and mouse placenta. While in humans the placenta is hemomonochorial, that is, it has a single layer of trophoblasts, in rats and mice the placenta is hemotrichorial, that is, it has a triple trophoblastic layer, making it difficult to compare placental transfer of chemicals in humans to that in rats and mice (Carter 2007).

Finally, while rats and mice have high levels of circulating carboxylesterases, which metabolically inactivate OP compounds, humans have low levels of these enzymes (de Jong *et al.* 1993). Since age-related differences in sensitivity of rats to CPF have been correlated with age-dependent expression of these enzymes (Benke and Murphy 1975; Moser *et al.* 1998), the age- and species-dependent expression of carboxylesterases need to be taken into account in physiologically based pharmacokinetic and pharmacodynamics models developed to convert biologically active doses of CPF from rats and mice of various ages to humans.

It is in this context that the guinea pig emerges as a potentially useful animal model to address specific questions related to the developmental neurotoxicity of OP insecticides. First, brain growth spurt in humans and guinea pigs is predominantly a prenatal event (Dobbing and Sands 1970, 1973). Second, once developed, the overall brain structure of guinea pigs is remarkably similar to that of humans, particularly in limbic regions that are known to play a critical role in cognitive processing, including the hippocampus, and in the Circle of Willis, an arterial polygon that sits at the base of the brain and supplies blood to the brain and surrounding structures (Librizzi *et al.* 1999). Third, the sensitivity of guinea pigs to OP compounds is also more similar to that of non-human primates and humans than to that of rats and mice (see Pereira *et al.* 2014 and references therein), in part because levels of circulating carboxylesterases are markedly lower in guinea pigs than in rats or mice. Finally, the guinea pig has a hemomonochorial placenta with a fetal/maternal transport barrier very similar to that of the human placenta (Mess 2007). Therefore, the guinea pig can be a valuable model for elucidating the involvement of the placenta in regulating the neurotoxicity of OP insecticides in the developing organism. They can also be suitable to aid in the translation of biologically active doses of CPF to humans and in the identification of medical interventions that used during pregnancy can effectively prevent the neurotoxic effects of the insecticide during the prenatal brain growth spurt.

Developmental neurotoxicity of CPF in guinea pigs

In recent years, our laboratory has assessed the developmental toxicity of CPF in guinea pigs (Mullins *et al.* 2015; Mamczarz *et al.* 2016). In those studies, guinea pigs were exposed *in utero* to CPF (25 mg/kg/day formulated in peanut oil, 10 days, starting on approximate GD53–55) during the period of brain growth spurt and rapid brain myelination (Dobbing and Sands 1970).

The CPF dose regimen used in the guinea pig study captured important features associated with occupational OP exposures of humans. First, the daily s.c. injections of the guinea pigs were intended to recapitulate the repetitive nature of the human occupational exposures (Farahat *et al.* 2011). Second, the s.c. route was used to approximate the slow sustained release of CPF from the dermal route, a prevalent route of occupational human exposures (Cattani *et al.* 2001; Fenske *et al.* 2012). Third, the dose of CPF injected in the pregnant guinea pigs was selected to model a scenario in which occupational human exposure to levels that produce no overt signs of acute toxicity may be presumed safe.

Although CPF-exposed guinea pig dams presented no clinical signs of acute OP intoxication, they had approximately 75% lower AChE activity in RBC than control animals at the time of delivery. As mentioned earlier, there are several reports that RBC AChE activity can be reduced by as much as 40–80% from baseline in workers who otherwise present no overt sign of OP intoxication (Ames *et al.* 1989; Lakew and Mekonnen 1998; Ohayo-Mitoko *et al.* 1999; Farahat *et al.* 2011; Singleton *et al.* 2015). Thus, the CPF dose regimen administered to the pregnant guinea pigs translates into levels that can be experienced by workers who handle OP insecticides throughout the world.

As reported in Mamczarz *et al.* (2016), on the day they were born, guinea pigs prenatally exposed to CPF (25 mg/kg/day, s.c., 10 days starting on ~ GD52) had significantly lower RBC AChE and plasma butyrylcholinesterase activities than their control counterparts. This finding demonstrated that CPF crossed the placenta and reached the fetuses. Brain butyrylcholinesterase was also markedly inhibited in different brain regions of CPF-exposed pups, indicating that CPF crossed the fetal blood–brain barrier as well. However, AChE activities in different brain regions of CPF-exposed pups were not significantly different from those measured in control offspring (Mamczarz *et al.* 2016).

The degree of RBC AChE inhibition following continued exposure to OP compounds is a result of cumulative inhibition of the enzyme in the RBCs. As such, it does not necessarily reflect the degree of AChE inhibition in tissues, where recovery of AChE activity between exposures is a result of both reactivation of the inhibited enzyme and synthesis of new enzyme (Mason 2000). Since RBCs do not have a nucleus and, therefore, do not synthesize proteins, RBC AChE activity is only recovered when new RBCs come into circulation, and the half-life of RBCs is approximately 120 days (D'Alessandro *et al.* 2010). In the brain, on the other hand, the half-life of AChE is approximately 2–3 days (Wenthold *et al.* 1974). However, the possibility cannot be ruled out that AChE is not significantly inhibited in the brain of the CPF-exposed offspring, because CPF and/or CPF-oxon can interact with high affinity with molecular targets other than AChE in the brain (Terry 2012).

Cholinergic activity in the hippocampus controls locomotor activity and habituation (Izquierdo *et al.* 1992; Leussis and Bolivar 2006). Thus, the finding that neither locomotor activity nor locomotor habituation was affected by the prenatal CPF exposure could be explained, at least in part, by the finding that AChE was not significantly inhibited in the brain of the guinea pigs (Mamczarz *et al.* 2016). Sprague–Dawley rats tested at different ages after being exposed to CPF (5 mg/kg/day, s.c.) between PND 11 and 14 or (3 mg/kg/48 h, p.o.) between PND1 and 21 also presented no changes in locomotor activity (Dam *et al.* 2000; Carr *et al.* 2001; Levin *et al.* 2001). It is important to note that the ages at which the rat pups were exposed to CPF in these studies covered a period of brain growth spurt comparable to that experienced by fetal guinea pigs during the last third portion of gestation (Dobbing and Sands 1970). In addition, the CPF dose regimens administered to the rats in those studies resulted in variable degrees of total cholinesterase inhibition in the brain that did not exceed 35% 1 day before and 20% 1 day after the last CPF dose (Dam *et al.* 2000; Carr *et al.* 2001).

To assess the impact of prenatal exposure to CPF on cognitive functions, the guinea pigs were subjected to the classical non-cued version of the Morris water maze. Starting on approximate PND38, guinea pigs were trained to escape onto a hidden platform during five consecutive trial days. Among control animals, time to escape onto the hidden platform (hereafter referred to as escape latency) and distance to reach the platform significantly decreased with increased number of training days, with control male outperforming control female guinea pigs (Mamczarz *et al.* 2016). Both male and female guinea pigs prenatally exposed to CPF presented learning deficits in this task; they swam longer distances and times to escape onto the hidden platform (Fig. 1). However, CPF-exposed male guinea pigs were more severely affected than their female counterpart, such that the task was no longer sexually dimorphic among guinea pigs prenatally exposed to the insecticide (Mamczarz *et al.* 2016).

After completion of the behavioral tests, the structural integrity of the brain of female offspring that had been prenatally exposed to CPF or peanut oil was analyzed by means of *in vivo* magnetic resonance imaging methods that included T2-weighted images and diffusion kurtosis imaging, as described in Mullins *et al.* (2015). That study revealed that prenatal exposure of female guinea pigs to CPF resulted in a significant reduction of brain volume, specifically in the frontal brain regions that included the striatum. It also provided evidence that, compared to control age- and sex-matched offspring, female offspring prenatally exposed to CPF presented in the striatum, amygdala, and corpus callosum: (i) decreased fractional anisotropy, and (ii) increased mean and radial diffusivity (Fig. 2). These results led to the hypothesis that prenatal exposure of female guinea pigs to CPF disrupts the axonal integrity and/or results in demyelination within the striatum, amygdala, and corpus callosum. In support of this hypothesis was the finding that the intensity of Luxol Fast Blue staining, which has been used to clarify the role of myelination in various disease states (Deshmukh *et al.* 2013), was significantly reduced in the lateral amygdala following the prenatal CPF exposure (Mullins *et al.* 2015).

Diffusion kurtosis imaging measures obtained from the striatum, amygdala, and corpus callosum were significantly correlated with the performance of the guinea pigs in the Morris

water maze (Mullins *et al.* 2015; see also Fig. 2). These correlations do not establish a cause-consequence relationship between the structural brain damage and the behavioral impairment presented by the guinea pigs exposed prenatally to CPF. However, they align well with reports that lesions in the striatum, amygdala, and corpus callosum result in cognitive deficits in laboratory animals and humans (Block *et al.* 1993; Sauerwein and Lassonde 1994; Galliot *et al.* 2010; Chida *et al.* 2011).

The findings that guinea pigs subjected to prenatal subacute exposure to CPF presented spatial learning deficits that were sexually dimorphic and correlated with disruption of the structural integrity of different brain regions are in line with reports that: (i) correlations between prenatal CPF exposure and cognitive deficits in children are generally stronger among boys than girls (Marks *et al.* 2010; Horton *et al.* 2012; Rauh *et al.* 2012), and (ii) normal correlations between FSIQ and the surface area of different brain regions were either absent or reversed among children, particularly boys, who experienced prenatal exposures producing cord blood levels of CPF ≥ 4.39 ng/g (Rauh *et al.* 2012). However, it is important to note that the CPF dose regimen administered to the pregnant guinea pigs caused marked inhibition of RBC AChE, whereas RBC AChE is not markedly inhibited following environmental exposures of humans to CPF. A dose–response relationship analysis is necessary to determine the dose dependence of the developmental neurotoxicity of CPF in the guinea pig model and establish the relevance of the model for human environmental exposures.

Perspectives on potential mechanisms underlying the developmental neurotoxicity of OP insecticides: emphasis on CPF

As mentioned earlier, irreversible inhibition of AChE in the peripheral and central nervous systems contributes to the cholinergic syndrome induced by an acute exposure to OP insecticides (reviewed in Pereira *et al.* 2014). However, as discussed here, several lines of evidence suggest that additional compound-specific mechanisms of action contribute not only to the acute toxicity of high doses of these insecticides but also to the neurotoxic effects that develop following continued low-level exposures, particularly in the developing brain.

Based on the notion that AChE is the primary molecular target accounting for the acute toxicity of OP insecticides, the LD50 of these insecticides should directly correlate with their IC50 to inhibit the enzyme and/or with the rate of reactivation of the inhibited enzyme. However, that is not always the case (e.g., Santhoshkumar *et al.* 1996). In addition, if AChE inhibition were the sole mechanism underlying the acute toxicity of OP compounds, mice with a null mutation in the gene that encodes AChE would be resistant to the toxicity of these chemicals. Instead, $AChE^{-/-}$ mice are more sensitive than wild-type mice to the acute toxicity of OP compounds, including CPF-oxon (Lockridge *et al.* 2005). In addition, while treatment of wild-type mice with the muscarinic antagonist atropine counters the acute toxicity of OP compounds, treatment of $AChE^{-/-}$ mice does not (Duysen *et al.* 2001).

Based on the assumption that AChE inhibition is a common mechanism underlying the developmental neurotoxicity of OP insecticides, one could also predict that exposure of a developing organism to any OP insecticide would trigger exactly the same effect with a

biological gradient proportional to the degree of the inhibition of the catalytic enzyme activity. This is, however, not the case. Exposure of developing organisms to different OP insecticides triggers different effects. For instance, a statistically significant up-regulation of the serotonin (5HT) receptor subtypes 5HT1 and/or 5HT2 is observed in different brain regions of adult rats exposed neonatally to CPF (1 mg/kg/day, s.c.; PND1–4), with the effects being more pronounced among males than females (Aldridge *et al.* 2004). In contrast, a statistically significant down-regulation of 5HT1 receptors is noted in the brain of male (but not female) adult rats exposed neonatally to doses of the OP insecticide diazinon (0.5 mg/kg/day, s.c.; PND1–4) (Slotkin *et al.* 2008) that cause similar degree of AChE inhibition as that induced by CPF.

Numerous studies have demonstrated that CPF can directly interact with and change the activity of serine hydrolases, including carboxylesterases, muscarinic receptors, cannabinoid receptors, and such structural proteins as tubulin (reviewed in Jett and Lein 2006; Terry 2012). The paragraphs that follow discuss how some of these molecular interactions and hitherto unexplored mechanisms may contribute to the developmental neurotoxicity of CPF.

Some studies have proposed that disruption of the structural and functional integrity of the brain following exposure to low levels of CPF may be a result of disruption of axonal transport and outgrowth mediated by tubulin and related structural proteins (Howard *et al.* 2005; Prendergast *et al.* 2007; Grigoryan *et al.* 2008; Yang *et al.* 2008; Middlemore-Risher *et al.* 2011). This proposal was built upon the initial *in vitro* demonstration that CPF oxon, among other OP compounds, binds covalently to tubulin and disrupts tubulin polymerization, with 5–10 μM CPF-oxon decreasing and 25 μM CPF-oxon increasing microtubule length and density (Prendergast *et al.* 2007; Grigoryan *et al.* 2008, 2009). Subsequent studies demonstrated that organophosphorylated tubulin and disrupted microtubule structures could be detected in the brain of adult female mice treated with doses of CPF that did not cause significant AChE inhibition (3 mg/kg/day, 14 days, s.c.) (Jiang *et al.* 2010). Direct covalent binding of CPF to kinesin has also been proposed to explain the concentration-dependent inhibition by CPF (IC50 ≈ 9 μM) and CPF-oxon (IC50 ≈ 2 μM) of kinesin-dependent microtubule motility observed *in vitro* (Gearhart *et al.* 2007). One can hypothesize that disruption of axonal transport and outgrowth resulting from direct interactions of CPF and/or CPF-oxon with structural proteins in the developing brain can generate abnormal patterns of neuronal connectivity and, thereby, contribute to the neurobehavioral alterations reported to be associated with developmental exposure to CPF.

The work of Yang *et al.* (2008) demonstrated that, at concentrations that did not inhibit the catalytic activity of AChE, CPF (1 nM) and CPF oxon (1 pM) inhibited axonal outgrowth in primary cultures of rat and mice DRG. The inhibitory effect of CPF or CPF oxon on axonal outgrowth was: (i) smaller in magnitude in primary DRG cultures from $AChE^{+/-}$ mice than in primary DRG from wild-type mice, (ii) absent in primary cultures of dorsal root ganglia of $AChE^{-/-}$ mice, and (iii) restored when primary DRG cultures from $AChE^{-/-}$ mice were transfected with the full-length wild-type AChE. Taken together these findings reveal that the ability of CPF and CPF oxon to inhibit axonal outgrowth: (i) is independent of their ability to block the catalytic activity of AChE, and (ii) cannot be solely explained by the direct interactions of CPF and/or CPF-oxon with structural proteins. They also suggest that

CPF/CPF-oxon-induced inhibition of the morphogenic activity of AChE may contribute to the neurotoxicity of concentrations of the insecticide that are insufficient to inhibit the catalytic activity of the enzyme.

Previous studies demonstrated that, with IC50s of approximately 22 nM and 14 nM, CPF-oxon displaces binding of the m2 muscarinic receptor ligand [3H]-cismethyldioxolane from rat striatal membranes and of the cannabinoid receptor CB1 ligand [3H]CP559940 from mouse brain membranes, respectively (Huff *et al.* 1994; Quistad *et al.* 2002). While these binding studies suggested that CPF-oxon can directly interact with m2 and CB1 receptors, they did not elucidate whether the interaction leads to receptor activation or inhibition. In 2001, however, Olivier *et al.* (2001) reported that CPF-oxon blocked forskolin-induced cAMP production in cerebral cortical slices, and it did so more potently in slices from 7-day-old rats than from adult rats. Since the effect was only partially blocked by the non-selective muscarinic agonist atropine, CPF-oxon-induced suppression of cAMP signaling in the cerebral cortex may be mediated, at least in part, by the ability of CPF-oxon to interact with and activate m2 muscarinic receptors and CB1 receptors. During fetal life, CB1 receptor signaling regulates neural progenitor cell differentiation and guides axonal migration and synaptogenesis (reviewed in Fride *et al.* 2009). Thus, it remains to be determined whether CB1 is a molecular target that contributes to the developmental neurotoxicity of CPF.

In vivo studies have reported that changes in downstream signaling involving neurotrophins could contribute to the developmental neurotoxicity of CPF. A significant reduction in the expression of neurotrophins in the superfamily of fibroblast growth factor has been observed in the brainstem and forebrain of 5-day-old rats exposed to CPF (1 mg/kg/day, s.c.) between PND1 and PND4 (Slotkin *et al.* 2007). A transient reduction in the levels of nerve growth factor has also been noted in the forebrain of rat pups gavaged with CPF (1.5–3 mg/kg/day) between PND1 and PND6 (Betancourt and Carr 2004). Although the molecular mechanisms underlying these effects remain to be elucidated, CPF-induced down-regulation of fibroblast growth factor and nerve growth factor expression in the developing brain can certainly contribute to suppression of neurite outgrowth, cell differentiation, and neuronal repair, all of which are largely regulated by these neurotrophins (Rydel and Greene 1987; Limke *et al.* 2003; Bernd 2008).

Additional mechanisms that have been proposed to contribute to the developmental neurotoxicity of CPF and have been discussed in the literature include: (i) exacerbated oxidative stress (Crumpton *et al.* 2000; Jett and Navoa 2000; Slotkin and Seidler 2010), (ii) imbalanced intracellular Ca^{2+} homeostasis (Giordano *et al.* 2007), (iii) increased signaling mediated by inflammatory mediators, such as interleukins and cytokines (Tian *et al.* 2015), and (iv) increased activity/expression of protein kinases, including protein kinase C and mitogen-activated kinases (Slotkin and Seidler 2009; Zhang *et al.* 2015).

In more recent years, attention has been directed to epigenetic mechanisms, which play critical roles in the development of the nervous systems, as potential determinants of the etiology of neurological disorders resulting from exposure of the developing brain to toxicants such as heavy metals and OP insecticides (Senut *et al.* 2012; Kim *et al.* 2016).

Among the epigenetic modifications most studied to date are DNA methylation, histone modifications, and non-coding RNAs (reviewed in Bannister and Kouzarides 2011; Esteller 2011; Roidl and Hacker 2014).

Exposure of pregnant mice to CPF-methyl (4, 20, 100 mg/kg/day, p.o.; GD7–12), an OP insecticide chemically related to but less acutely toxic than CPF, has been shown to cause a dose-dependent hypomethylation of the *H19* gene in different organs of the fetuses on GD13 (Shin *et al.* 2015). Demethylation of the imprinting control region of this gene has been associated with intrauterine and postnatal growth retardation (Murphy *et al.* 2012). If exposure of developing fetuses to toxicologically relevant doses of CPF also leads to hypomethylation of the *H19* gene, this mechanism could prove to be an important determinant of the developmental toxicity of this insecticide.

A recent study also demonstrated that *in vitro* exposure of proliferating and differentiating human neuronal progenitor cells to CPF induced a concentration-dependent hypermethylation of histone H3 on the lysine (K) 4 residue (H3K4) that became statistically significant at 57 μM (Kim *et al.* 2016). H3K4 methylation controls the expression of a number of pluripotency-associated genes during the differentiation of embryonic stem cells into neural stem cells and neurons (Roidl and Hacker 2014). Thus, it remains to be determined whether CPF-induced increased methylation of H3K4 is also observed following exposure of developing fetuses to CPF and, if so, whether it contributes to the developmental neurotoxicity of the insecticide.

No study has assessed whether the expression of non-coding RNAs is affected by exposure of the developing brain to CPF. However, an increased expression of the non-coding micro RNAs miRNA-132 and miRNA-212 has recently been observed in the hippocampi of young adult rats exposed for 21 days to doses of CPF (10 mg/kg/day, s.c.) that caused significant inhibition of brain AChE activity but were not sufficient to trigger overt signs of acute toxicity (Lee *et al.* 2016). These miRNAs play an important role in synaptogenesis, as indicated by the findings that: (i) miRNA-132 over-expression in forebrain neurons increases synaptic density and (ii) loss of miRNA-132/212 suppresses spine formation and reduces dendritic length and branching in newborn hippocampal neurons (Hansen *et al.* 2010; Magill *et al.* 2010). Therefore, if CPF were to induce up-regulation of these miRNAs in the developing brain as well, this mechanism could contribute to improper neuronal wiring that would culminate in neurological deficits later in life.

Another question that remains unexplored relates to whether potential effects of CPF in the placenta create an environment that is detrimental to the healthy development of the fetuses. In the placenta, a proper balance between a pro- and an anti-inflammatory environment, which is maintained in part by placental muscarinic and nicotinic receptors (Satyanarayana 1986; Paparini *et al.* 2015), is essential to nurture fetal growth. There is evidence that at high micromolar concentrations CPF and CPF-oxon disturb the redox balance in and induce apoptosis of human placental JEG-3 cells (Saulsbury *et al.* 2008; Chiapella *et al.* 2013). However, no study has assessed whether toxicologically relevant concentrations/doses of CPF impair the ability of the placenta to support normal fetal development and, if so, by what mechanism.

Conclusions

The epidemiological studies reviewed herein have reported statistically significant correlations between prenatal exposures to CPF and postnatal neurological complications, particularly cognitive deficits that are also associated with disruption of the structural integrity of the brain. Based on scientific evidence provided by these and other studies, the US EPA has given serious consideration to a potential ban of all uses of CPF in the United States. A major limitation of epidemiological studies, however, lies on the fact that they are generally not suitable to establish cause-consequence relationships between exposures and health outcomes. It is in this context that preclinical studies become extremely relevant. Various preclinical research groups throughout the world have consistently demonstrated that CPF is a developmental neurotoxicant. The developmental CPF neurotoxicity, which is well supported by studies using different animal models, routes of exposure, vehicles, and testing methods, is generally characterized by cognitive deficits and disruption of the structural integrity of the brain. Nevertheless, there is still controversy as to whether the effects observed in animal models can be extrapolated to humans exposed to low levels of CPF.

Researchers have argued that the doses of CPF reported to induce developmental neurotoxicity in animals are orders of magnitude greater than incidental environmental exposures in humans (Juberg 2012; Reiss *et al.* 2015). However, based on a fundamental principle of pharmacology and toxicology, the concentration of a xenobiotic at its site of action and the affinity of the xenobiotic for the molecular target(s) that mediate its effects are the primary drivers of the biological effect of that chemical. Although the concentration of a xenobiotic at its site of action correlates with the dose, this relationship is dependent upon the xenobiotics, pharmacokinetics, which is species specific. Many studies that attempted to translate rodent to human levels of CPF exposure did so on the basis of the pharmacokinetics of CPF in these species (reviewed in Eaton *et al.* 2008). Others have developed physiologically based pharmacokinetic and pharmacodynamic models that take into account the pharmacokinetics of CPF and use the ability of CPF to block AChE in the different species as a measure of the pharmacodynamics of CPF (e.g., Poet *et al.* 2017). Unfortunately, as discussed in the previous section, it is unclear to what extent, if any, AChE inhibition contributes to the developmental neurotoxicity of CPF. In addition, as discussed in the previous section, there is evidence that CPF interacts directly with and changes the activity of non-AChE targets. The challenge is to determine the contribution of these targets to the developmental neurotoxicity of CPF. Therefore, the use of AChE inhibition as the pharmacodynamic parameter in physiologically based pharmacokinetic and pharmacodynamic models is likely to result in inaccurate translation of animal to human doses of CPF (or vice-versa). Until the mechanism(s) underlying the developmental neurotoxicity of CPF is(are) identified, a comparison of the CPF effects (rather than doses) across different species seems to be more appropriate. Such comparison may lead to the identification of a biomarker of effect that, together with a biomarker of exposure, will more accurately guide the human health risk assessment for CPF.

Undoubtedly, addressing the questions that remain unanswered regarding the developmental neurotoxicity of CPF is critically needed to provide the basis for the creation and

enforcement of programs to better monitor and control the agricultural, industrial, and domestic use and handling of CPF throughout the world.

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Abbreviations

ACh	acetylcholine
AChE	acetylcholinesterase
BChE	butyrylcholinesterase
CPF	chlorpyrifos
DKI	diffusion kurtosis imaging
DMSO	dimethyl sulfoxide
EPA	Environmental Protection Agency
FA	fractional anisotropy
FGF	fibroblast growth factor
GD	gestation day
H3K4	lysine 4 of histone 3
MD	mean diffusivity
miRNA	micro RNA
MRI	magnetic resonance imaging
NGF	nerve growth factor
OP	organophosphorus
p.o	*per os*
PND	postnatal day
RBC	red blood cell
RD	radial diffusivity
s.c	subcutaneous
TCPY	3,5,6-trichloro-2-pyridinol

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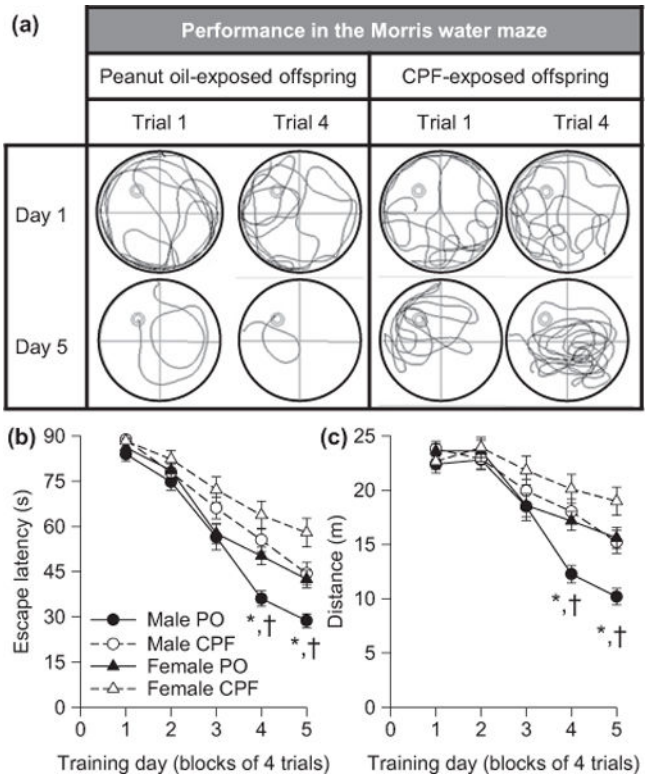

Fig. 1.

Learning deficits presented by guinea pigs prenatally exposed to chlorpyrifos (CPF). Learning performance of offspring born to pregnant guinea pigs that had been injected with peanut oil (PO) or with CPF (25 mg/kg/day for 10 days starting on approximate GD53–55) was examined in the Morris water maze, as described in Mamczarz *et al.* (2016). (a) Swim paths of a vehicle (peanut oil)-exposed and a CPF-exposed male offspring on the 1st and 4th trials of the first and last days of training to find the hidden platform in the Morris water maze. The swim path of the male guinea pig prenatally exposed to peanut oil became shorter with training as the animal learned to use the contextual cues to find the platform. In contrast, the swim path of the male prenatally exposed to CPF did not improve substantially with training. (b and c) Graphs show mean escape latency (b) and distance (c) traveled by CPF- and peanut oil (PO)-exposed offspring per training day. Results are presented as mean ± standard error of the mean. A random effect ANOVA model revealed that: (i) among control animals, learning performance was sex dimorphic, with performance being better among control males than control females, and (ii) prenatal exposure to CPF impaired learning of male and female offspring, with the effect being more pronounced among males. According to Tukey– Kramer post hoc test for pairwise comparisons: $*p < 0.05$ PO males versus CPF males; $\dagger p < 0.05$ PO males versus PO females. Details of the analysis are provided in the article by Mamczarz *et al.* (2016), from which this figure was adapted.

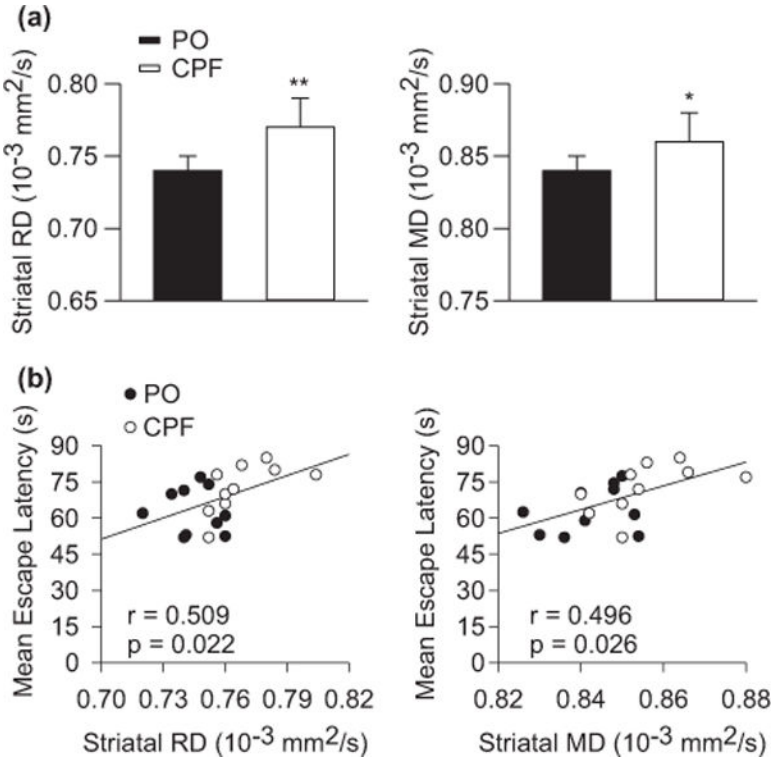

Fig. 2.

Diffusion kurtosis imaging (DKI) parameters (mean diffusivity, MD and radial diffusivity, RD) obtained from the striatum of female guinea pigs prenatally exposed to chlorpyrifos (CPF) or peanut oil and correlation of these parameters with the learning performance of the animals. (a) Mean striatal RD and MD measured from female guinea pigs born to dams exposed to peanut oil or CPF (25 mg/kg/day for 10 days starting on approximate gestation day 53–55). Results are presented as mean and standard error of the mean. *$p < 0.05$; **$p < 0.01$. (b) Scatterplots of the correlation between striatal DKI measures and mean escape latency measures of CPF- and peanut oil-exposed offspring. Filled circles are data from CPF-exposed offspring, whereas open circles are data from peanut-oil exposed offspring. Details on the statistical analysis are provided in the article by Mullins *et al.* (2015), from which this figure was adapted.

Table 1

Locomotor phenotypes observed following developmental exposure of mice and rats to CPF

CPF dose regimen	Vehicle	Species/strain	Time of exposure	Testing age	Type of test	Sex tested	Locomotor activity[a]	Rate of habituation	Sex affected	Brain AChE inhibition 24 h after last dose (%)[b]	Ref
1 or 5 mg/kg/day; s.c.	DMSO	SD rats	GD17–20	PND35	Figure-8	M/F	↔	↑	NA	1 mg/kg: NS 5 mg/kg: 45%[c]	*1*
3 or 6 mg/kg/day; p.o.	Peanut Oil	CD-1 mice	GD15–18	PND120	Social recognition	F	↔	NR	NA	3 mg/kg: NS 6 mg/kg: 40%	*2*
3 or 6 mg/kg/day; p.o.	Peanut Oil	CD-1 mice	GD15–18	PND60	Open field	M	↑ (6 mg/kg)	NR	M	3 mg/kg: NS 6 mg/kg: 40%	*3*
1 or 3 mg/kg/day; s.c.	Peanut Oil	CD-1 mice	PND1–4	PND25	Open field	M/F	↔	NR	NA	NS	*4*
1 mg/kg/day; s.c.	DMSO	SD rats	PND1–4	PND35	Figure-8	M/F	↔	NR	NA	NR	*5*
1 mg/kg/day; s.c.	DMSO	SD rats	PND1–4	PND21 & 30	Open field	M/F	↓	NR	M	20–25%[d]	*6*
1 mg/kg/day; s.c.	DMSO	SD rats	PND1–4	PND35	Figure-8	M/F	↑	↔	M	20–25%[d]	*7*[e]
1 mg/kg/day; s.c.	DMSO	SD rats	PND1–4	PND52–53	Plus Maze	M/F	↑	NR	M	20–25%[d]	*8*
5 mg/kg/day; s.c.	DMSO	SD rats	PND11–14	PND21 & 30	Open field	M/F	↔	NR	NA	65%[d]	*6*
5 mg/kg/day; s.c.	DMSO	SD rats	PND11–14	PND35	Figure-8	M/F	↔	↓	M/F	NR	*5*
1 or 3 mg/kg/day; s.c.	Peanut Oil	CD-1 mice	PND11–14	PND25	Open field	M/F	↑	NR	M/F	NS	*4*
1 or 3 mg/kg/day; s.c.	Peanut Oil	CD-1 mice	PND11–14	PND60	Open field	M	↑ (3 mg/kg)	NR	M	NS	*3*
1 or 3 mg/kg/day; s.c.	Peanut Oil	CD-1 mice	PND11–14	PND120	Social recognition	F	↔	NR	NA	NS	*2*
3 mg/kg/48 h; p.o.	Corn oil	SD rats	PND1–21	PND10–30	Open field	M/F	↔	NR	NA	NR[f]	*9*[g]

AChE, acetylcholinesterase; CPF, chlorpyrifos; GD, gestation day; NA, not applicable; NR, not reported; NS, not significant; SD, Sprague–Dawley; s.c., subcutaneous; p.o., *per os* (oral gavage); PND, postnatal day; M, male; F, female.

References:

[1] Levin *et al.* 2002

[2] Venerosi *et al.* 2006

[3] Ricceri *et al.* 2006

[4] Ricceri *et al.* 2003

[5] Levin *et al.* 2001

[6] Dam *et al.* 2000

[7] Levin *et al.* 2014

[8] Aldridge *et al.* 2005

[9] Carr *et al.* 2001.

[a] Locomotor activity refers to the total distance traveled or the number of photobeam breaks in specific apparatuses.

[b] Data available for earlier time points are discussed in the paper.

[c,d] Degree of brain AChE inhibition is reported in Qiao *et al.*, 2002[c] and Song *et al.*, 1997[d].

[e] In this study, dams received one s.c. injection of vehicle per day between GD17 and 19.

[f] AChE activity in different brain regions was inhibited by approximately 30% on PND20 and 25% on PND25.

[g] In this study, male and female rats born from dams exposed to higher doses of CPF (escalating from 3 mg/kg on PND1 to either 6 mg/kg or 12 mg/kg on PND21) presented reduced locomotor activity on PND 25 and PND30.

Table 2

Cognitive phenotypes observed following developmental exposure of mice and rats to CPF

CPF dose regimen	Vehicle	Species/strain	Time of exposure	Testing age	Type of test	Sex tested	Spatial learning	Memory	Sex affected	Brain AChE inhibition 24 h after last dose (%)	Ref
1 or 5 mg/kg/day; s.c.	DMSO	ND4 mice	GD17–20	PND60	Foraging maze	M/F	↓	↓	F	NR	1
1 or 5 mg/kg/day; s.c.	DMSO	SD rat	GD17–20	Young adulthood	16-arm radial maze	M/F	↓	↓	F	NR	2
1 mg/kg/day; s.c.	DMSO	SD rats	PND1–4	PND64	16-arm radial maze	M/F	↓	↓	M[a]	20–25%[b]	3
0.1, 0.3, or 1 mg/kg/day; oral diet	Corn Oil and sweet jelly	Wistar rats	GD7 through lactation (PND21)	PND60–90	MWM 8-arm radial maze	M/F	↓	↓	M[a]	NR	4
1, 4, or 6 mg/kg/day[c]; p.o.	Corn Oil	SD rats	PND1–21	PND36–60	12-arm radial maze	M/F	↓	↓	M	NR[d]	5
1 mg/kg/day; s.c.	DMSO	SD rats	PND1–4	Young adulthood	16-arm radial maze	M/F	↓	↓	M	20–25%[a]	6
0.3, 1, or 5 mg/kg/day; p.o.	Corn Oil	SD rats	GD6 through lactation (PND10)	PND22–24 PND 61–90	T-maze delayed spatial alternation	M/F	NA	↔[e]	NA	NR[f]	7
1 or 5 mg/kg/day, s.c.	DMSO	ICR mice	GD13–17	PND45–60	T-maze delayed spatial alternation	M/F	NA	↓[g]	M	NR	8

AChE, acetylcholinesterase; CPF, chlorpyrifos; GD, gestation day; NA, not applicable; NR, not reported; NS, not significant; SD, Sprague–Dawley; s.c., subcutaneous; p.o., *per os* (oral gavage); PND, postnatal day; MWM, Morris water maze; M, male; F, female.

References: Haviland *et al.* (2010); Levin *et al.* (2002); Aldridge *et al.* (2005); Gómez-Giménez *et al.* (2017); Johnson *et al.* (2009); Levin *et al.* (2001); Maurissen *et al.* (2000); Chen *et al.* (2012).

[a] Performance of female rats exposed to the highest CPF dose was significantly better than that of control females in the radial mazes.

[b] Degree of brain AChE inhibition is reported in Qiao *et al.*, 2002.

[c] The medium and high doses were escalated to starting from the lower dose of 1 or 1.5 mg/kg, respectively, and doubling it on PND6 and again on PND14.

[d] On PND20, hippocampal AChE was inhibited by 14%, 50%, and 53% among rats that had been exposed to the low, medium, and high CPF dose, respectively.

[e] Outcome measured: % correct first choice.

[f] On GD20, maternal brain AChE activity was inhibited by approximately 18% and 90% in the groups that had been exposed to the medium and the high CPF dose, respectively.

[g] Outcomes measured: % correct first choices; mean number of win-shift errors; mean number of lose-shift errors. Outcome affected: mean number of lose-shift errors.