Published in final edited form as:

Neurotoxicology. 2014 July ; 43: 82–89. doi:10.1016/j.neuro.2013.12.009.

Low Level Chlorpyrifos Exposure Increases Anandamide Accumulation in Juvenile Rat Brain in the Absence of Brain Cholinesterase Inhibition

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Abstract

The prevailing dogma is that chlorpyrifos (CPF) mediates its toxicity through inhibition of cholinesterase (ChE). However, in recent years, the toxicological effects of developmental CPF exposure have been attributed to an unknown non-cholinergic mechanism of action. We hypothesize that the endocannabinoid system may be an important target because of its vital role in nervous system development. We have previously reported that repeated exposure to CPF results in greater inhibition of fatty acid amide hydrolase (FAAH), the enzyme that metabolizes the endocannabinoid anandamide (AEA), than inhibition of either forebrain ChE or monoacylglycerol lipase (MAGL), the enzyme that metabolizes the endocannabinoid 2-arachidonylglycerol (2-AG). This exposure resulted in the accumulation of 2-AG and AEA in the forebrain of juvenile rats; however, even at the lowest dosage level used (1.0 mg/kg), forebrain ChE inhibition was still present. Thus, it is not clear if FAAH activity would be inhibited at dosage levels that do not inhibit ChE. To determine this, 10 day old rat pups were exposed daily for 7 days to either corn oil or 0.5 mg/kg CPF by oral gavage. At 4 and 12 h post-exposure on the last day of administration, the activities of serum ChE and carboxylesterase (CES) and forebrain ChE, MAGL, and FAAH were determined as well as the forebrain AEA and 2-AG levels. Significant inhibition of serum ChE and CES was present at both 4 and 12 h. There was no significant inhibition of the activities of forebrain ChE or MAGL and no significant change in the amount of 2-AG at either time point. On the other hand, while no statistically significant effects were observed at 4 h, FAAH activity was significantly inhibited at 12 h resulting in a significant accumulation of AEA. Although it is not clear if this level of accumulation impacts brain maturation, this study demonstrates that developmental CPF exposure at a level that does not inhibit brain ChE can alter components of endocannabinoid signaling.

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Keywords

chlorpyrifos; developmental neurotoxicity; organophosphorus; endocannabinoid

1. Introduction

The organophosphorus (OP) insecticides are the most frequently used class of insecticides in the U.S. accounting for over ~35% of total insecticides used (Grube et al., 2011). The most heavily used OP insecticide is chlorpyrifos (CPF) in spite of the fact that CPF is restricted to agriculture use. Its household use was eliminated in 2000 due to concerns that exposure to OP insecticides exert greater neurotoxic effects in children as compared to adults (U.S. EPA, 2000). However, in agricultural communities, the potential for childhood exposure to CPF, as well as other OP insecticides, still exists (Koch et al., 2002; Arcury et al., 2007). Developmental exposure to OP insecticides has been suggested to have lasting negative impacts including decreased cognitive abilities and motor skills (Ruckart et al., 2004; Marks et al., 2010; Engel et al., 2011; Bouchard et al., 2011). Specifically, children exposed to CPF exhibit increased manifestation of attention deficit disorder and attention-deficit/hyperactivity disorder (ADHD) (Rauh et al., 2006), decreased working memory and IQ (Rauh et al., 2011), and altered brain morphology (Rauh et al., 2012).

The inhibition of brain cholinesterase (ChE), a serine esterase that degrades the widely distributed neurotransmitter acetylcholine (ACh), is considered the canonical target for OP insecticides. At higher levels of OP exposure, significant inhibition of brain ChE activity leads to accumulation of ACh and hyperactivity of the cholinergic system resulting in the disruption of normal physiological functioning. Therefore, it is logical to assume that if such an exposure occurred during critical periods of nervous system development, the resulting cholinergic hyperactivity could alter the process of maturation and establishment of the normal pathways and synapses that occurs during this period. However, the environmental levels of CPF to which children would typically be exposed would be unlikely to induce significant inhibition of ChE in the brain, and thus would not induce hyperactivity of the cholinergic system. Nonetheless, this does not mean that low level exposure to OPs such as CPF is safe. In fact, laboratory studies have reported adverse neurochemical and behavioral effects following exposure to CPF and other OP insecticides at levels that induce only minimal amounts of brain ChE inhibition and little hyperactivity in the cholinergic system (Levin et al., 2002; Slotkin et al., 2006; 2007; Timofeeva et al., 2008a; 2008b). This has led to the hypothesis that the developmental toxicological effects of OP insecticides involves a currently unknown “non-cholinergic mechanism of action”.

We have recently suggested that the endocannabinoid system could possibly be a non-cholinergic target that contributes to the developmental toxicity of OP insecticides. The endocannabinoid system consists of a group of neuromodulatory lipids that are involved in a variety of physiological processes including appetite, motor learning, synaptic plasticity, and pain sensation. They also play a pivotal role in normal brain development (for reviews see Harkany et al., 2008; Anavi-Goffer and Mulder, 2009). The two main endocannabinoids, arachidonylethanolamide (AEA or anandamide) and 2-arachidonoylglycerol (2-AG) (Figure 1), are not stored in vesicles due to their lipophilic nature but are synthesized and

released on demand in response to elevations in intracellular calcium (Devane et al., 1992; Di Marzo et al., 1994; Mechoulam et al., 1995; Sugiura et al., 1995; Stella et al., 1997). Endocannabinoid production can also be upregulated by group I metabotropic glutamate, nicotinic, and dopamine D2 receptor activation (Giuffrida et al., 1999; Stella and Piomelli, 2001; Ohno-Shosaku et al., 2002; Kim et al., 2002). In the brain, the endocannabinoids bind primarily to cannabinoid receptor 1 (CB1) resulting in their physiological actions, which can differ based on the brain region, second messenger machinery utilized, and type of synapse involved (for review see Kano et al., 2009). After activating the CB1 receptor, 2-AG and AEA are degraded primarily by the action of monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH), respectively.

It was demonstrated that acute exposure of adult mice to high dosages of OP compounds resulted in the inhibition of 2-AG and AEA hydrolysis in the brain (Quistad et al., 2001; 2002; 2006). Additional studies demonstrated that acute in vivo exposure of adult mice to CPF resulted in increased levels of 2-AG and AEA in the brain (Nomura et al., 2008; Nomura and Casida, 2011). Given the importance of the endocannabinoids in brain development, it is possible that developmental OP exposure could alter the normal endocannabinoid levels and result in deleterious effects on brain maturation. Therefore, we previously initiated investigations on the effects of repeated exposure to CPF in the brain of developing rats (Carr et al., 2011; 2013). Following 7 days of daily CPF exposure, 2-AG and AEA hydrolysis activities in the brain were inhibited in a dose-related manner at 4 h post-exposure, and the extent of inhibition from highest to lowest level was AEA hydrolysis > acetylcholine hydrolysis > 2-AG hydrolysis (Carr et al., 2011). Using the same exposure paradigm, we determined the peak time of inhibition for MAGL (4 h post-exposure) and brain ChE and FAAH (12 h post-exposure) and that levels of 2-AG and AEA were significantly elevated (Carr et al., 2013). However, significant brain ChE inhibition was still observed at all dosages administered and it was not clear whether CPF exposure can induce significant effects on endocannabinoid metabolism in the absence of brain ChE inhibition. Therefore, the goal of the current study was to continue this line of investigation by determining the extent of inhibition of endocannabinoid hydrolysis and accumulation of the endocannabinoids in brains of developing rats following repeated exposure to a dosage of CPF that does not inhibit brain ChE.

2. Materials and methods

2.1 Chemicals

Chlorpyrifos (> 99% purity) was a generous gift from DowElanco Chemical Company (Indianapolis, IN). DowElanco did not contribute to or have any control over the data presented in this publication. All other chemicals were purchased from Cayman Chemicals (Ann Arbor, MI) or Sigma Chemical Co. (St. Louis, MO).

2.2. Animal Treatment

Adult male and female Sprague Dawley rats [SD] were obtained from Harlan Laboratories (Prattville, AL) and used for breeding. All animals were housed in a temperature controlled environment ($22 \pm 2^\circ\text{C}$) with a 12-h dark-light cycle with lights on between 0700 and 1900

in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility. LabDiet rodent chow and tap water were freely available during the experimentation. All procedures were approved by the Mississippi State University Institutional Animal Care and Use Committee. Following parturition, male and female rat pups within the same litter were assigned to different treatment groups. There were always representative control animals of the same sex present in each litter to match the CPF treated animals. For this project, rats from 6 litters were used. The day of birth was considered as postnatal day 0 (PND 0).

Chlorpyrifos was dissolved in corn oil and administered at a volume of 1 ml/kg body weight by oral gavage (*per os*) every day from PND10 through PND16. This period corresponds to the period following birth in humans. This age range is beyond the period of the growth spurt (PND7 and below) but encompasses a time of significant brain maturation (Andersen, 2003; Counotte et al., 2011; Tau and Peterson, 2010). The dosage selected was 0.5 mg/kg. Oral gavage was performed using a 50- μ l tuberculin syringe equipped with a 1-inch 24-gauge straight intubation needle (Popper and Sons, Inc., New Hyde Park, NY) to deliver the solution to the back of the throat.

The dosage used in this study was designed to be below the level required to induce inhibition of brain ChE activity. It is below the oral repeated No Observed Effect Level (NOEL) for inhibition of brain ChE activity (0.75 mg/kg) and below the oral repeated NOEL for signs of toxicity (4.5 mg/kg) for postnatal rats as reported by Zheng et al. (2000). However, it is unclear if this dosage recapitulates the exposure levels in children.

Rat pups were sacrificed at 4 and 12 h after the last exposure to CPF. Brains were rapidly removed and dissected on ice to obtain the forebrain (excluding the medulla and cerebellum). Blood was collected by trunk bleeding to obtain serum. The forebrain was split into left and right hemispheres, which were frozen on a stainless steel plate on top of dry ice and maintained at -80°C until assay.

2.3. Enzyme Assays

One forebrain hemisphere was homogenized at 200 mg tissue weight per ml of cold 0.05 M Tris-HCl buffer containing 0.2 mM EDTA (pH 7.4 at 37°C) in a glass mortar using a Wheaton motorized tissue grinder and a Teflon pestle. For determination of ChE activity, an aliquot was diluted in cold 0.05 M Tris-HCl buffer (pH 7.4 at 37°C) to 40 mg tissue/ml. Forebrain (1 mg tissue/ml final concentration) and serum (12.5 μ l serum/ml final concentration) were diluted in assay buffer. The activity of ChE was measured spectrophotometrically using a modification (Chambers et al., 1988) of Ellman et al. (1961) with acetylthiocholine as the substrate (1 mM final concentration) and 5,5'-dithiobis(nitrobenzoic acid) as the chromogen. Serum carboxylesterase (CES) activity (0.625 μ l serum/ml final concentration) was measured spectrophotometrically using 4-nitrophenyl valerate as the substrate (0.5mM final concentration) and monitoring 4-nitrophenol, one of the hydrolysis products, as previously described (Carr and Chambers, 1991). ChE activity is defined as a mixture of acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8) activities since specific inhibitors or specific substrates were not utilized in the assay.

For the forebrain samples, an additional 1.0 ml aliquot of the 200 mg/ml homogenate was centrifuged at $1500 \times g$ for 10 min to obtain supernatant, which was diluted 3-fold for determination of AEA and 2-AG hydrolysis at 37°C. Following a 10 min preincubation period, AEA and 2-AG hydrolysis in forebrain (2.667 mg tissue/ml final concentration) were determined by incubation with substrate (either 50 μ M anandamide or 2-AG, respectively) for 10 min. The reaction was terminated by the addition of 4-volumes of cold acetonitrile containing 2.5 μ M of internal standard (arachidonic acid-d₈). Samples were placed in ice for 10 min and centrifuged at $21,000 \times g$ for 10 min to obtain supernatant. The amount of arachidonic acid formed was determined by LC-MS as previously described (Xie et al., 2010). For determination of MAGL and FAAH activity, corresponding tubes containing either 10 μ M of the specific MAGL inhibitor 4- nitrophenyl- 4- (dibenzo[d][1, 3]dioxol- 5- yl(hydroxy)methyl)piperidine- 1- carboxylate (JZL184) or of the specific FAAH inhibitor (3'- (aminocarbonyl)[1, 1'- biphenyl]- 3- yl)- cyclohexylcarbamate (URB597), respectively, were included during the preincubation period prior to adding substrate.

Protein concentrations of tissue extracts were quantified with the Folin phenol reagent using bovine serum albumin as a standard (Lowry et. al., 1951). Specific activities were calculated as nmole (ChE and CES) or pmole (FAAH or MAGL) product produced $\text{min}^{-1} \text{mg protein}^{-1}$.

2.4. Endocannabinoid Analysis

The remaining forebrain hemisphere was dounce homogenized in 2:1 v/v ethyl acetate:0.1 M potassium phosphate (pH 7.0) (total volume 6 ml) containing known amounts of deuterated internal standards (2-AGd₈ and AEA-d₈). The resulting emulsion was vortexed (1 min), centrifuged ($10,000 \times g$, 20 min), and the top organic layer was removed and dried under a stream of N₂. The residue was resolubilized in 1:1 v/v water:methanol (200 μ l). After filtration through centrifugal filters (0.1 μ m), a 10 μ l aliquot of the resolubilized lipid was injected onto an Acquity UPLC BEH C18 column (2.1 x 100 mm, 1.7 μ m), equipped with pre-column (2.1x 5 mm, 1.7 μ m), interfaced with a Thermo Quantum Access triple-quadrupole mass spectrometer. The mobile phase was a blend of solvent A (2 mM ammonium acetate/0.1% acetic acid in water) and solvent B (2 mM ammonium acetate/0.1% acetic acid in methanol). Analytes were eluted with the following gradient program: 0 min (95% A, 5% B), 0.5 min (95% A, 5% B), 5 min (5% A, 95% B), 6 min (5% A, 95% B), 7 min (95% A, 5% B), 8 min (95% A, 5% B). The flow rate was 0.4 mL/min and the entire column eluate was directed into the mass spectrometer via heated electrospray ionization in positive ion mode. Single reaction monitoring (SRM) of each analyte were as follows: 2AG, [M+NH₄]⁺ m/z 396.3>287.3; 2AG-d₈, [M+NH₄]⁺ m/z 404.3>295.3; AEA, [M+H]⁺ m/z 348>62; AEA-d₄, [M+H]⁺ m/z 352>66. Scan times were 0.2 s per SRM and scan width was 0.01 m/z. Collision energies and tube lens voltage were optimized using autotune software for each analyte by post-column infusion of individual compounds into 50% A/50% B mobile phase, pumping at a flow rate 0.4 ml/min. The source settings were as follows: spray voltage, +4000V; vaporizer temp, 350°C; capillary temp, 285°C; sheath gas pressure, 50 psi; aux gas pressure, 5 psi. Endocannabinoids are quantified by measuring the area under each chromatographic peak and comparing it to the area under the chromatographic peak for the

appropriate deuterated internal standard, followed by correction for the ionization efficiency of 2AG and AEA relative to their deuterated standards (empirically determined). The endocannabinoid amounts are normalized on the brain wet weight and expressed as pmol/g brain (AEA) or nmol/g brain (2-AG).

2.5. Statistical Analysis

All statistical analyses were performed using the SAS statistical package (SAS-Institute, 2009). For weight gain, the sphericity of the data was initially tested by analysis of variance (ANOVA) using the general linear model with a repeated measures paradigm and was found to violate the assumption of sphericity. Therefore, subsequent analysis by ANOVA using the mixed model (Littell et al., 1996) was conducted with a repeated measures paradigm with a Huynh-Feldt covariance structure (Huynh and Feldt, 1970), followed by separation of means using least significant difference. The analysis was designed to identify differences in the fixed effects (sex and treatment) and all possible interactions. Enzyme activity data and endocannabinoid data were tested using Shapiro-Wilk's test to check the normality of the residuals and homoscedasticity of data. Data were log-transformed where appropriate. Data were also tested for outliers using the modified Z-score (Iglewicz and Hoagli, 1993), PROC BOXPLOT, and four PROC ROBUSTREG tests using each of the four estimations available (M, LTS, S, MM). Data points identified as outliers in all six tests were removed from the dataset. Data were analyzed using the PROC MIXED procedure (Littell et al., 1996). For this model, treatment, time, and sex were considered as fixed effects and litter was treated as random effect. The model was designed to identify significant differences between sexes, times, and treatments and all possible interactions. In the initial overall analysis, there were no significant sex differences or significant sex $\times$ treatment or sex $\times$ treatment $\times$ time interactions for either the enzyme activities or endocannabinoid levels. Thus, males and females were pooled for mean separation, which was performed by Least Significant Difference (LSD). The criterion for significance was set at $p = 0.05$.

3. Results

No signs of overt toxicity or cholinergic hyperstimulation, such as lacrimation, diarrhea or tremors, were observed following CPF exposure. We have previously demonstrated that comparing differences in weight gain between treatment groups rather than body weights more effectively reveals the effect of treatment (Carr et al., 2011). At the dosage studied here, there was no significant difference in weight gain between the control group and the treatment group on any day (Figure 2).

When statistical analysis indicated that there was a significant overall effect of treatment on a particular biochemical parameter, there was no significant treatment $\times$ time interaction. This lack of a significant treatment $\times$ time interaction suggests that each parameter measured did not change significantly between 4 h and 12 h. Although the overall analysis indicated that treatment was significantly different from control, these results did not allow us to determine if a treatment group was significantly different from its corresponding control group at a specific time. Therefore, to better demonstrate the impact of treatment at each time, a lower level analysis for each parameter at each sampling time was performed

followed by means separation to identify differences between each treatment group and its corresponding control at each time.

With respect to serum CES activity levels (Figure 3A), there was a significant overall effect of treatment only ($p < 0.0001$). Lower level analysis indicated that serum CES activity was significantly inhibited at both 4 h ($p < 0.004$) and 12 h ($p < 0.0001$). CES activity was almost completely inhibited (94.2%) at 4 h, with a small non-significant amount of recovery occurring by 12 h.

With respect to serum ChE activity levels (Figure 3B), there was a significant overall effect of treatment ($p < 0.0001$) and time ($p < 0.0114$) but no significant interactions. Lower level analysis indicated that serum ChE activity was significantly inhibited at both 4 h ($p < 0.0001$) and 12 h ($p < 0.0001$). The level of inhibition of serum ChE activity was very similar at 4 h (32.6%) and 12 h (25.7%).

With respect to brain enzymes, there were no significant differences in forebrain ChE activity (Figure 4A) or forebrain MAGL activity (Figure 4B) between treated and control. However, there was a significant overall effect of treatment ($p < 0.0003$) on forebrain FAAH activity (Figure 4C). Lower level analysis of FAAH activity indicated that activity was significantly inhibited (25%) at 12 h ($p < 0.0001$), but not at 4 h even though there was observed inhibition of activity (13.5%).

With respect to endocannabinoid levels, there was no significant difference in forebrain 2-AG levels (Figure 5A) between treated and control. However, there was a significant overall effect of treatment ($p < 0.0125$) on forebrain AEA levels (Figure 5B). Lower level analysis of AEA amounts indicated that the level of AEA was significantly elevated (37%) at 12 h ($p < 0.0041$) but not at 4 h even though there was an increased amount above control levels (17.9%).

4. Discussion

With regards to the hypothesis that the adverse developmental effects of OP insecticides involves a currently unknown “non-cholinergic mechanism of action”, we report here that repeated exposures of juvenile rats to a dosage of CPF that did not inhibit forebrain ChE activity caused a significant inhibition of FAAH and accumulation of AEA in the forebrain. The time that these significant changes were detected is in agreement with our previous report that 12 h following administration of higher dosages of CPF is the time to peak effects on FAAH activity and AEA accumulation (Carr et al., 2013).

In laboratory rodents, it is believed that carboxylesterases play a protective role against OP poisoning by providing an alternative binding site for OP compounds, and that the lower level of CES activities in juveniles contribute to their increased sensitivity to OPs (Morgan et al., 1994; Moser et al., 1998; Atterberry et al., 1997; Karanth and Pope, 2000). Our data indicate that exposure to a low dosage of CPF can effectively saturate the plasma CES binding sites. This suggests that the inhibition of CES by the active metabolite of CPF, chlorpyrifos-oxon, would effectively remove those molecules from circulation thus preventing them from reaching the brain. However, a recent report that wild-type mice are

more susceptible to the toxic effects of moderate dosages of CPF than plasma CES-knockout mice does question the long thought protective role for CES in OP toxicity (Duysen et al., 2012). Regardless, although CES is an excellent marker of exposure in laboratory rodent models, humans do not have carboxylesterases in their plasma (Li et al., 2005) making its inhibition non-relevant in direct relation to human toxicity.

Blood cholinesterases are also sensitive markers for OP exposure. As evidenced here, serum ChE was inhibited following repeated exposure to CPF at dosages that did not inhibit brain ChE. Zheng et al. (2000) reported that exposure to CPF for 14 days beginning on PND7 produced a NOEL of 0.75 mg/kg for brain, red blood cell, and plasma ChE. While our brain ChE data are in agreement, our serum ChE data differ in that we observed significant inhibition with 0.5 mg/kg. The two studies differed with respect to blood collection methods (serum versus plasma) and methods to determine ChE activity (spectrophotometric versus radiometric). These could have possibly contributed to the discrepancy between the two studies but the basis for the difference is not clear.

Developmental exposure to exogenous cannabinoids can alter the normal development of multiple neurotransmitter systems, including catecholaminergic, serotonergic, GABAergic, glutamatergic, and opioid systems (Kumar et al., 1990; Molina-Holgado et al., 1996; 1997; Garcia-Gil et al., 1997; 1999; Vela et al., 1998; Fernandez-Ruiz et al., 2000; 2004; Hernandez et al., 2000; Suarez et al., 2004; Wang et al., 2006). Likewise, developmental exposure to OP insecticides alter the development of multiple neurotransmitter systems such as cholinergic, catecholaminergic, and serotonergic neurons (Dam et al., 1999; Raines et al., 2001; Slotkin et al., 2001; 2002; Meyer et al., 2004; 2005; Richardson and Chambers, 2005; Aldridge et al., 2003; 2004; 2005). While these studies collectively suggest that developmental exposure to either exogenous cannabinoids or OP insecticides can impact multiple neurotransmitter systems, there is a significant difference. Exogenous cannabinoid exposure would exert widespread cannabinoid-related effects in the brain. Exposure to OP insecticides at the levels utilized in the studies listed above would not only exert widespread cannabinoid-related effects in the brain with levels of both AEA and 2-AG being elevated (Carr et al., 2013), but activation of the cholinergic system would also be occurring. However, at very low level exposure to OP insecticides, such as the dosage used in the current study, the exposure would only impact AEA hydrolysis and exert actions only in specific brain regions where AEA hydrolysis plays an important role in endocannabinoid metabolism (Fegley et al., 2005; Bortolato et al., 2007). However, we could find no literature that described the developmental effects on neurotransmitter systems following low levels of FAAH inhibition.

Multiple potential biological targets have been proposed to play a role in the long term effects caused by exposure to OP compounds (Casida and Quistad, 2004). As first reported by Richards et al. (2000), acylpeptide hydrolase (APH) is more sensitive to inhibition by certain OP compounds than is ChE (Pancetti et al., 2007). APH has been suggested to play a role in cognitive function (Richards et al., 2000). It is located in the synaptic region (Sandoval et al., 2012) and its inhibition by dichlorvos has been demonstrated to enhance long term potentiation in hippocampal slices (Olmos et al., 2009). While exposure to CPF results in higher levels of ChE inhibition than APH inhibition in the early periods following

exposure, the inhibition of APH is much more persistent than is that of AChE (Quistad et al., 2005; Cardona et al., 2013; Lopez-Granero et al., 2013a). However, subsequent long term behavioral studies involving either a high dose acute exposure or a chronic feeding exposure to CPF did not find any association between APH activity and cognitive deficits observed (Lopez-Granero et al., 2013b; 2014). While APH has greater sensitivity to inhibition by certain OP compounds, its role in the long term effects of OP exposure is not clear. Similarly, no literature could be found regarding the ontogeny of APH activity in the brain.

With respect to endocannabinoid-mediated behavioral changes, developmental exposure to exogenous cannabinoids results in activation of the endocannabinoid system and leads to long term effects on anxiety-related behaviors in adults (Jiang et al., 2005; O'Shea et al., 2006). It is well known that the endocannabinoid system modulates emotion and anxiety (Moreira and Lutz, 2008; Taber and Hurley, 2009). With this in mind, previous studies on developmental CPF exposure have also reported altered anxiety-like behavior and emotionality in adult animals (Aldridge et al., 2005; Ricceri et al., 2006; Roegge et al., 2008; Timofeeva et al., 2008b; Venerosi et al., 2008; 2010). It is also known that specific inhibition of FAAH by the carbamate inhibitor URB597 during adolescence alters the levels of the CB1 receptor in adult rat brain (Marco et al., 2009) indicating that long term adverse effects may result as a consequence of developmental inhibition of FAAH. In a more recent study from the same laboratory, exposure of adolescent mice to URB597 increased anhedonia in adults (Macri et al., 2012) which is a behavior observed in adult rats exposed postnatally to several OP insecticides (Aldridge et al., 2005; Roegge et al., 2008). Therefore, although not directly related, some similarities between developmental disruption of endocannabinoid signaling and developmental OP insecticide exposure do appear to exist.

In conclusion, we have reported that repeated developmental exposure to CPF inhibits the degradation of endocannabinoids (Carr et al., 2011) and this inhibition results in the accumulation of those endocannabinoids in the brain (Carr et al., 2013). Here, we report that exposure to CPF, at a dosage that does not inhibit brain ChE activity, can selectively inhibit FAAH activity and cause AEA accumulation. Despite this provocative finding, it is unclear if inhibition of FAAH activity may be a potential mechanism of toxic action following very low level OP exposure. Consequently, it will be necessary to determine if the elevation of AEA levels, at the amounts reported in this study, alter the normal function of the brain in the OP exposed animals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Research was supported by the Mississippi Agricultural and Forestry Experiment Station (MAFES), the College of Veterinary Medicine, Mississippi State University, and NIH 5T35OD010432-12. This paper is MAFES publication #J-12463 and Center for Environmental Health Sciences publication #128. LC-MS/MS analyses was supported by R15ES015348-02.

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Highlights

- Repeated developmental chlorpyrifos exposure alters brain endocannabinoid signaling.
- Low levels of chlorpyrifos did not alter monoacylglycerol lipase activity or 2-AG levels.
- Low levels of chlorpyrifos inhibits fatty acid amide hydrolase and increases AEA levels.
- These effects occur in the absence of brain ChE inhibition.

ANADAMIDE
(N-arachidonylethanolamine)

2-AG
(2-Arachidonoylglycerol)

Figure 1.
Chemical structures of the two major endocannabinoids anandamide and 2-arachidonoylglycerol.

Figure 2. Rates of weight gain of rat pups exposed daily from postnatal day 10 through 16 to either corn oil (control) or 0.5 mg/kg chlorpyrifos (CPS). Values are expressed as weight $\pm$ SE ($n = 27-29$).

Figure 3.

Specific activity of (A) carboxylesterase (CES) and (B) cholinesterase (ChE) in the serum of rat pups exposed daily from postnatal day 10 through 16 to either corn oil (control) or 0.5 mg/kg chlorpyrifos (CPS). Values are expressed as mean $\pm$ SE ($n = 12-17$). Percent inhibition for each treatment group as compared to its respective control is presented in the oval overlaying the corresponding bar. Bars indicated with an asterisk (*) are statistically significant ($p < 0.05$) from control.

Figure 4. Specific activity of (A) cholinesterase (ChE), (B) monoacylglycerol lipase (MAGL), and (C) fatty acid amide hydrolase (FAAH) in the forebrain of rat pups exposed daily from postnatal day 10 through 16 to either corn oil (control) or 0.5 mg/kg chlorpyrifos (CPF). Values are expressed as mean $\pm$ SE ($n = 12-17$). Percent inhibition for each treatment group as compared to its respective control is presented in the oval overlaying the corresponding bar. Bars indicated with an asterisk (*) are statistically significant ($p < 0.05$) from control.

Figure 5.

Levels of (A) 2-arachidonylglycerol (2-AG) and (B) arachidonylethanolamide (AEA) in the forebrain of rat pups exposed daily from postnatal day 10 through 16 to either corn oil (control) or 0.5 mg/kg chlorpyrifos (CPF). Values are expressed as nmoles/g tissue $\pm$ SE for 2-AG and pmoles/g tissue $\pm$ SE for AEA ($n = 12-17$). Percent increase for each treatment group as compared to its respective control is presented in the oval overlaying the corresponding bar. Bars indicated with an asterisk (*) are statistically significant ($p < 0.05$) from control.