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COMPARATIVE EFFECTS OF PARATHION AND CHLORPYRIFOS ON EXTRACELLULAR ENDOCANNABINOID LEVELS IN RAT HIPPOCAMPUS: INFLUENCE ON CHOLINERGIC TOXICITY

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Abstract

Parathion (PS) and chlorpyrifos (CPF) are organophosphorus insecticides (OPs) that elicit acute toxicity by inhibiting acetylcholinesterase (AChE). Endocannabinoids (eCBs, N-arachidonylethanolamine, AEA; 2-arachidonoylglycerol, 2AG) can modulate neurotransmission by inhibiting neurotransmitter release. We proposed that differential inhibition of eCB-degrading enzymes (fatty acid amide hydrolase, FAAH, and monoacylglycerol lipase, MAGL) by PS and CPF lead to differences in extracellular eCB levels and toxicity. Microdialysis cannulae were implanted into hippocampus of adult male rats followed by treatment with vehicle (peanut oil, 2 ml/kg, sc), PS (27 mg/kg) or CPF (280 mg/kg) 6–7 days later. Signs of toxicity, AChE, FAAH and MAGL inhibition, and extracellular levels of AEA and 2AG were measured 2 and 4 days later. Signs were noted in PS-treated rats but not in controls or CPF-treated rats. Cholinesterase inhibition was extensive in hippocampus with PS (89–90%) and CPF (78–83%) exposure. FAAH activity was also markedly reduced (88–91%) by both OPs at both time-points. MAGL was inhibited by both OPs but to a lesser degree (35–50%). Increases in extracellular AEA levels were noted after either PS (about 2-fold) or CPF (about 3-fold) while lesser treatment-related 2-AG changes were noted. The cannabinoid CB1 receptor antagonist/inverse agonist AM251 (3 mg/kg, ip) had no influence on functional signs after CPF but markedly decreased toxicity in PS-treated rats. The results suggest that extracellular eCBs levels can be markedly elevated by both PS and CPF. CB1-mediated signaling appears to play a role in the acute toxicity of PS but the role of eCBs in CPF toxicity remains unclear.

Keywords

acetylcholinesterase; organophosphorus; pesticides; cholinergic; cannabinoid; neuromodulation

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Conflict of Interest

The authors declare there are no conflicts of interest.

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Introduction

Parathion (PS) and chlorpyrifos (CPF) are both organophosphorus insecticides (OPs, Mileson *et al.* 1998). While PS use has been banned in numerous countries including the United States, CPF remains one of the most commonly used insecticides throughout the world (Grube *et al.*, 2011). PS and CPF are protoxicants, bioactivated by cytochrome P450 isoforms to the oxygen analogs, paraoxon (PO) and chlorpyrifos oxon (CPO), both potent inhibitors of acetylcholinesterase (AChE, Sultatos *et al.*, 1983).

Inhibition of AChE leads to elevated levels of the neurotransmitter acetylcholine in synapses of the central and peripheral nervous systems and resulting signs of “cholinergic” toxicity (Pope *et al.*, 2005). Following acute subcutaneous exposure to PS or CPF in rats at dosages sufficient to elicit similar degrees of extensive AChE inhibition, marked differences in overt signs of toxicity are noted, i.e., parathion-treated rats exhibit characteristic signs including involuntary movements/tremors and autonomic signs (e.g., excessive salivation) while rats treated with CPF show no or minimal signs (Pope *et al.*, 1992; Liu and Pope, 1996; Karanth *et al.*, 2006). As relatively similar degrees of AChE inhibition are elicited by both OPs, we hypothesized that differential modulation of a “downstream” neurochemical process mediates the differences in toxic expression.

Endocannabinoids (eCBs, e.g., arachidonylethanolamine [AEA] and 2-arachidonoylglycerol [2AG]) are global neuromodulators produced from membrane lipids following neuron activation (Castillo *et al.*, 2012). The eCBs are released by postsynaptic neurons after which they diffuse across the synapse to activate cannabinoid CB1 receptors on the presynaptic terminal. CB1 activation leads to the inhibition of neurotransmitter release in a wide variety of neuron types and pathways.

Elevation of synaptic acetylcholine levels is pivotal in the development of cholinergic toxicity following AChE inhibition. The release of acetylcholine is inhibited by eCBs in a variety of brain regions (Gifford and Ashby, 1996; Gifford *et al.*, 2000; Tzavara *et al.*, 2003; De Groot *et al.*, 2006). Conversely, blocking CB1 receptors with the antagonist/inverse agonist SR 141716A increased hippocampal acetylcholine release (Gifford and Ashby, 1996; Kathmann *et al.*, 2001). Thus eCB signaling may modify cholinergic toxicity following exposure to an anticholinesterase by inhibiting acetylcholine release and decreasing neurotransmitter accumulation. We previously reported that the acute toxicity of paraoxon and another anticholinesterase, diisopropylfluorophosphate, is reduced by CB1 receptor agonists (Nallapaneni *et al.*, 2006, 2008; Wright *et al.*, 2010). Moreover, extensive AChE inhibition leads to recruitment of non-cholinergic signaling by other neurotransmitters (Shih *et al.*, 1991; Lallement *et al.*, 1991, 1992; Cassel and Fosbraey, 1996; Jacobsson *et al.* 1997), and such non-cholinergic signaling alterations may play a role in the ultimate expression of toxicity following anticholinesterase intoxication. Differential changes in eCB signaling could therefore contribute to selective neurochemical and neurological outcomes following exposures to anticholinesterases eliciting similar degrees of AChE inhibition.

The enzymatic degradation of AEA is mediated by the enzyme fatty acid amide hydrolase (FAAH, Cravatt *et al.* 1996, 2001; Egertova *et al.*, 2003). Monacylglycerol lipase (MAGL) is the primary enzyme involved in 2AG hydrolysis (Blankman *et al.*, 2007; Hashimoto-dani *et al.*, 2007; Savinainen *et al.*, 2012). Both of these enzymes are sensitive to inhibition by a number of organophosphorus anticholinesterases (Quistad *et al.*, 2001, 2006; Nallapaneni *et al.*, 2006, 2008; Casida *et al.*, 2008; Nomura and Casida, 2011). Of particular importance to our studies, CPO is more potent than PO at inhibiting both FAAH and MAGL (Quistad *et al.*, 2006; Crow *et al.*, 2012). We therefore hypothesized that greater inhibition of eCB-

degrading enzymes by CPO could lead to prolonged elevation of eCBs, more effective reduction of acetylcholine release, and thus less extensive signs of cholinergic toxicity in rats treated with CPF compared to PS.

In this study, adult male rats were treated with vehicle or dosages of PS and CPF that elicit similar degrees of extensive brain AChE inhibition. Cholinergic signs were compared among the treatment groups and AChE, FAAH and MAGL inhibition was measured at 2 and 4 days after dosing. Microdialysis in the hippocampus was used to measure the effects of OP exposure on extracellular levels of AEA and 2AG. In additional rats, the CB1 receptor antagonist/inverse agonist AM251 was given after OP exposure to evaluate the influence of CB1-mediated eCB signaling on the expression of toxicity. The results suggest that both PS and CPF can lead to substantial increases in extracellular eCBs, and that these neurochemical changes may contribute to the differential outcome following exposure to these two OPs.

Methods

Chemicals and Reagents

Parathion (O,O -diethyl-*p*-nitrophenyl-phosphorothioate) and chlorpyrifos (O,O -diethyl-3,5,6-trichloro-2-pyridinyl-phosphorothioate), >99% pure; GC/MS analysis, were purchased from ChemService (West Chester, PA). Acetylcholine iodide (acetyl-³H, specific activity 76.0 Ci/mmol) was purchased from Perkin Elmer (Wellesley, MA). [³H]Anandamide (ethanolamine 1-³H) specific activity 60 Ci/mmol), was purchased from American Radiochemical Company (St. Louis, MO). Arachidonoyl-1-thio-glycerol, anandamide and AM251 (*N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide) were purchased from Cayman Chemical (Ann Arbor, MI). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Guide cannulae (MD 2250) and microdialysis probes (MD 2204, 2 mm membrane) were purchased from Bioanalytical Systems Inc. (BAS, West Lafayette, IN).

Animals and Treatments

Male, Sprague-Dawley rats (3 months of age) were purchased from Harlan (Indianapolis, IN) and maintained in the AAALAC-accredited Animal Resources facility at Oklahoma State University. Animals were housed in polycarbonate cages with a 12-h:12-h light:dark cycle, allowed free access to food (PMI® Laboratory Rodent Diet 5001, PMI Feeds, Richmond, IN) and water throughout, and acclimated to the facility for 5–7 days prior to beginning the study. Surgical procedures and routine animal care were in accordance with protocols established in the NIH/NRC *Guide for the Care and Use of Laboratory Animals* and approved by the local Institutional Animal Care and Use Committee.

OP compounds were dissolved in peanut oil (100% pure; Lou-Ana brand, Ventura Foods, Opelousas, LA) and injected subcutaneously (sc) at a volume of 2 ml/kg. Rats were treated with vehicle, PS (27 mg/kg) or CPF (280 mg/kg). Functional signs of toxicity were recorded essentially as described by Moser *et al.* (1988) by a trained observer “blinded” to the treatment groups. SLUD signs (an acronym for salivation, lacrimation, urination and defecation) were graded as: 1 = normal (no secretions); 2 = mild one or multiple secretions; 3 = moderate multiple secretions; 4 = severe multiple secretions. Involuntary movements were scored as: 2 = normal quivering of vibrissae and head; 3 = fine head and neck tremors; 3.25 = more consistent tremors in head, neck and forelimbs; 3.5 = consistent tremors extending caudally from head to the midbody; 3.75 = tremors extending caudally to the hindlimbs; 4 = whole body tremors; 5 = myoclonic jerks.

Stereotaxic Surgery and Microdialysis

A guide cannula was surgically implanted into the left hippocampus. Animals were first anesthetized with a ketamine/xylazine (9:1) mixture (0.6 ml/kg, ip). The scalp was shaved and head positioned into a stereotaxic apparatus (Stoelting Co., Wood Dale, IL). The cannula was positioned using the coordinates: anterior-posterior, -3.8 mm, medial-lateral, +2.4 mm; dorsal-ventral, -2.0 mm from surface. Two screws were placed on each side of the cannula and dental cement was used to secure the cannula. Animals were allowed to recover for 6–7 days prior to OP treatment. On the day of microdialysis, rats were lightly anesthetized with isoflurane and the probe was rapidly inserted into the guide cannula. Rats were then transferred into a Rturn® animal chamber (BAS, West Lafayette, IN) and dialysis tubing was stabilized with a plastic collar. The dialysis probe was equilibrated for five hours by perfusion with artificial cerebrospinal fluid (aCSF: NaCl, 149 mM; KCl, 2.8 mM; CaCl₂, 1.2 mM; MgCl₂, 1.2 mM; ascorbic acid, 0.25 mM; D-glucose, 5.4 mM; hydroxypropyl- β -cyclodextrin, 30% (to increase eCB capture, Caille *et al.*, 2007)) at flow rate of 0.8 μ l/min using a syringe pump (MD 1101, BAS). Following equilibration, twelve fractions (15 min each) were collected into a refrigerated fraction collector. At the beginning of fraction 5, the perfusion buffer was switched to depolarizing conditions (NaCl, 43 mM; KCl, 100 mM; CaCl₂, 10 mM; MgCl₂, 1.2 mM; ascorbic acid, 0.25 mM; D-glucose, 5.4 mM; hydroxypropyl- β -cyclodextrin, 30%), and then back to non-depolarizing conditions at the beginning of fraction 9. All fractions were stored at -80°C until analysis. Cannula/probe placement was verified in all tissues by H&E staining.

Endocannabinoid Analysis

AEA and 2AG in microdialysates were analyzed by the method of Caille and coworkers (2007). Each dialysate was spiked with methanandamide prior to loading onto a pre-column (0.5 $\times$ 2.5 mm, Haisil HL C18 column, 5 μ m; Higgins Analytical, Mountain View, CA). After washout, flow through the pre-column was reversed and directed onto a 0.3 $\times$ 50 mm microbore analytical column (Haisil HL C18, 3 μ m) using 70% methanol mobile phase. Eluent was delivered into a mass spectrometer (1100MSD, Agilent Technologies, Santa Clara, CA) by a nanoelectrospray interface, run in positive selected ion monitoring mode.

Cholinesterase, Fatty Acid Amide Hydrolase and Monoacylglycerol Lipase Assays

Total cholinesterase activity in homogenates of right hippocampus (1:60 in phosphate buffered saline, PBS) was measured by the radiometric method of Johnson and Russell (1975) as reported previously (Pope *et al.*, 1991) using [³H]acetylcholine iodide as the substrate (1 mM final concentration). FAAH was measured by a modification of the method of Long and coworkers (2009). Homogenates in PBS (1:120, approximately 30 μ g protein) were incubated at 37 °C for 20 min with [³H]anandamide (10 μ M final concentration) in a final reaction volume of 0.4 ml. The reaction was stopped by adding 0.4 ml chloroform:methanol (1:1) and vortexing. Tubes were then centrifuged in a microcentrifuge (3,300 rpm for 10 min, Marathon 13K/M, Fisher, Chicago, IL). An aliquot of the upper aqueous phase (200 μ l) was removed, added to 4 ml Scintisafe® scintillation fluid, and counted.

MAGL was measured by a modification of the photometric method of Ulloa and Deutsch (2010), adapted for a plate reader. Homogenate in PBS (1:60, approximately 20 μ g protein) was added to each well of a 96-well plastic plate on ice. A solution (175 μ l) containing arachidonoyl-1-thio glycerol and the color reagent 5,5'-dithiobis-(2-nitrobenzoic acid) (both 100 μ M final concentration) was then added and the plate was immediately placed into the reader (SPECTRAmax 340PC, Molecular Devices, Sunnyvale, CA). The reaction was conducted in kinetic mode at 412 nm, 30°C for 5 min, with an initial lag time of 60 sec for equilibration. Data were collected every 20 sec and average reaction rates determined. Rate

of hydrolysis was calculated based on the molar extinction coefficient of the thiolate product ($14,150 \text{ M}^{-1} \text{ cm}^{-1}$, Ulloa and Deutsch, 2010), which is produced in direct proportion to substrate hydrolysis.

Total protein was estimated using the Bio-Rad (Bradford) protein assay with bovine serum albumin as the standard. Enzyme activities were expressed as nmole substrate hydrolyzed $\text{min}^{-1} \text{ mg}^{-1}$ protein and plotted as percent of control values.

Statistical Analysis

All statistical analyses were conducted using the Prism statistical package (version 5.0, GraphPad Software, La Jolla, CA). Functional signs data were evaluated at 2 and 4 days after dosing using the Kruskal-Wallis test, followed by Dunn's multiple comparisons. Enzyme and eCB data were analyzed at both 2 and 4 days after dosing using one-way ANOVA and Bonferroni Multiple Comparisons *post-hoc* tests. Depolarization-induced eCB release was estimated using the XY analysis, area under curve program.

Results

Figure 1 shows functional signs of toxicity (involuntary movements) at 2 and 4 days following treatment (vehicle, PS (27 mg/kg) or CPF (280 mg/kg)). There was a significant main effect of treatment on involuntary movements at both 2 (Kruskal-Wallis statistic = 26.51, $p < 0.0001$) and 4 (Kruskal-Wallis statistic = 50.81, $p < 0.0001$) days. Rats treated with PS exhibited significantly greater signs of toxicity at both time-points (Dunn's multiple comparisons, $p < 0.05$). All (38/38) parathion-treated rats showed involuntary movements, while no (0/27 and 0/39) control or CPF-treated rats showed signs. The relative lack of acute signs of toxicity in rats following high subcutaneous dosages of CPF has been noted previously (Pope *et al.*, 1992; Liu and Pope, 1996; Karanth *et al.*, 2006; Cardona *et al.*, 2011). We also recorded excessive secretions (SLUD signs) in these same animals. While median scores were not different among the treatment groups (i.e., all groups had median scores of 1), there was a main effect of treatment at both 2 (Kruskal-Wallis statistic = 10.57, $p = 0.0051$) and 4 (Kruskal-Wallis statistic = 8.387, $p = 0.0151$) days after dosing, with only PS-treated rats (6/23) showing signs.

Figure 2 shows hippocampal ChE, FAAH and MAGL activity at 2 and 4 days after dosing. While all three activities were significantly reduced by PS and CPF, there were no significant OP-selective effects. Relatively similar degrees of extensive brain regional ChE inhibition (78–90%) were noted between PS and CPF treatment groups. FAAH, the enzyme primarily responsible for metabolic degradation of AEA, was also extensively inhibited by PS and CPF (88–91%). MAGL, the principal enzyme in the hydrolysis of 2AG, was lesser but significantly inhibited (35–50%) by both PS and CPF.

The effects of PS and CPF on hippocampal extracellular AEA levels are shown in Figure 3. There was a main effect of treatment on AEA levels at 2 days after dosing (Figure 3A, $F=115$, $p < 0.0001$, 2 df). AEA levels were significantly elevated above controls by both PS ($t=7.188$) and CPF ($t=15.16$), and AEA levels were higher following CPF than PS exposure ($t=7.969$). There was also a main effect of treatment on AEA levels at 4 days after dosing (Figure 3B, $F=215.9$, $p < 0.0001$, 2 df). Again, AEA levels were significantly higher than controls in rats treated with either PS ($t= 9.925$) or CPF ($t= 20.77$), and there was a difference in AEA levels between PS- and CPF-treated rats ($t=10.85$).

Figure 4 shows the effects of PS and CPF on 2-AG levels in these same dialysates. At 2 days after dosing, there was a main effect of treatment on 2AG levels (Figure 4A, $F=14.04$, $p < 0.0001$, 2 df). In CPF-treated rats, 2AG levels were significantly higher than controls

($t=5.172$). While there was no significant effect of PS ($t=1.586$), 2AG levels at 2 days after dosing were different between PS and CPF-treated animals ($t=3.586$). At 4 days after exposure, there was no main effect of treatment on hippocampal 2AG levels (Figure 4B, $F=1.736$).

The above analysis of the effects of OPs on extracellular eCB levels considered all fractions (1–12) collected during the dialysis procedure. We also attempted to study depolarization-induced release of eCBs using an ionic pulse of potassium (100 mM) and calcium (10 mM) *via* the dialysis probe (Wiskerke *et al.*, 2012). Thus, we switched to depolarizing conditions at the start of fraction 5 to determine if eCB levels would be differentially elevated due to either FAAH or MAGL inhibition by the OPs. A delayed peak in eCBs was noted beginning at fraction 7. We therefore estimated depolarization-induced AEA and 2AG release by determining the area under the curve for fractions 7–11 for each animal, using each respective fraction 7 as the baseline (100%). Figure 5 shows depolarization-induced release of AEA at 2 (Figure 5A) and 4 (Figure 5B) days after dosing, while Figure 5C and 5D show depolarization-induced 2AG release at 2 and 4 days, respectively. There was a main effect of treatment on AEA release 2 days after dosing ($F=3.4$, $p<0.05$, 2 df), but no significant group-wise comparisons. No significant treatment effects were noted with AEA at 4 days or with 2AG release at either 2 or 4 days.

In additional rats, we evaluated the functional effects of the CB1 receptor antagonist/inverse agonist AM251 (3 mg/kg, ip, either at 24 hours after dosing or at 24, 48 and 72 hours after dosing) on the expression of toxicity following either PS or CPF. AM251 had no effect on functional signs (involuntary movements) following either vehicle or CPF (data not shown). Figure 6 shows the effects of AM251 on involuntary movements following PS exposure. Surprisingly, functional signs of PS toxicity were markedly decreased by AM251 given 24 hours after OP, and 3 daily doses had a greater effect than a single dose.

Discussion

The mechanism of acute toxicity for parathion (PS), chlorpyrifos (CPF) and other OPs is initiated by covalent phosphorylation of the active site of the enzyme acetylcholinesterase (AChE). AChE terminates cholinergic signaling by the rapid and efficient hydrolysis of the neurotransmitter acetylcholine within cholinergic synapses. Thus, inhibition of AChE leads to reduced catalysis and therefore elevated synaptic levels of acetylcholine. This in turn elicits excessive/prolonged stimulation of cholinergic receptors throughout the body, leading to cholinergic signs of toxicity including involuntary movements (e.g., tremors), excessive secretions (e.g., salivation), respiratory dysfunction and others.

Rats treated with PS showed marked involuntary movements, while exposure to chlorpyrifos was associated with essentially no overt signs of cholinergic toxicity (Figure 1). Similar findings have been reported previously (Pope *et al.*, 1992; Liu and Pope, 1996; Karanth *et al.*, 2006). As noted before, relatively similar degrees of AChE inhibition were noted at 2 and 4 days in these same animals (Figure 2A). Thus the differential toxicological response does not appear due to differences in target enzyme (AChE) inhibition.

Recently, enzymes which degrade endocannabinoids (i.e., fatty acid amide hydrolase, FAAH, and monoacylglycerol lipase, MAGL) have been shown to be inhibited by organophosphorus toxicants including CPO and PO. Of particular interest, CPO has been shown to be a more potent *in vitro* inhibitor of both FAAH and MAGL in mouse brain than PO (Quistad *et al.*, 2001, 2006). As the acute toxicity of PS and CPF is generally considered to be mediated by their active metabolites (PO and CPO), we hypothesized that acute exposure to either PS or CPF could lead to increased levels of eCBs, but with higher or more

prolonged levels following CPF exposure because of the greater inhibitory potency of its oxon (CPO) against these enzymes. Greater increases in brain eCBs could more effectively inhibit acetylcholine release and thereby block the expression of toxicity following CPF exposure.

Hippocampal FAAH activity was extensively inhibited *in vivo* by both PS and CPF (Figure 2B). FAAH inhibition was associated with significant increases in extracellular levels of AEA at both 2 (Figure 3A) and 4 (Figure 3B) days after dosing with either PS or CPF. Moreover, AEA levels were significantly higher in CPF- compared to PS-treated rats at both time-points.

MAGL was also inhibited by both PS and CPF (35–50%) but to a much lesser degree than FAAH (88–91% inhibition, Figures 2B, 2C). In a postnatal rat model, Carr and coworkers (2011) reported that repeated doses of CPF (5 mg/kg/day) from postnatal day 10–16 led to greater than 95% inhibition of forebrain FAAH activity but only 37% inhibition of MAGL. In mice, Quistad and coworkers (2006) reported that the highest dosage of CPF tested (30 mg/kg, ip) led to about 40% inhibition of brain MAGL activity. Using an activity-based gel assay to measure sensitivity of several enzymes to OP inhibitors, Nomura and coworkers (2008) reported almost complete inhibition of mouse brain MAGL activity following *in vivo* CPO exposure (4 mg/kg, ip). In *in vitro* studies, Quistad et al., (2001) previously reported an IC₅₀ for CPO of 34 nM for mouse brain MAGL inhibition. Crow and colleagues (2012) reported that human recombinant MAGL was very sensitive *in vitro* to CPO (IC₅₀ = 5 nM, 15 min at 37°C). In our hands, the IC₅₀ (20 min at 37°C) for CPO against rat brain MAGL is around 100 nM (Pope et al., 2010; unpublished results). Thus, while there are substantial differences in assay methods/conditions used among these various *in vitro* and *in vivo* studies, there is some suggestion that mouse and human MAGL may be more sensitive to inhibition by CPO than the rat enzyme.

In the present study, hippocampal 2AG levels were significantly increased only at 2 days after dosing with CPF (Figure 4A) while no significant changes in 2AG levels were noted with PS at 2 or 4 days, or with CPF at 4 days. AEA levels were more substantially affected by both CPF and PS at both 2 and 4 days after dosing (Figure 3), with higher levels in CPF-treated rats compared to rats given PS. Thus, these results suggest that extracellular AEA levels are more extensively affected than 2AG by high-dose exposure to either PS or CPF. In contrast, Nomura and coworkers (2008) reported that CPO (4 mg/kg, ip) inhibited both MAGL and FAAH activity in whole mouse brain 4 hours after dosing, but only 2AG tissue levels were significantly elevated. It must also be noted that we previously reported elevated extracellular hippocampal 2AG levels following CPF (280 mg/kg) but not parathion (27 mg/kg) at 4 days after dosing, and no change in extracellular AEA levels (Pope *et al.*, 2010). Our earlier studies involved a somewhat more limited sample size (n=4/treatment group vs n=5–6 for controls and n=6–9 for OP treatment groups in the present study) and the control AEA levels in that study were quite variable. At the time, we did not feel justified in considering outliers in such a small data set. Based on our current findings, we reanalyzed AEA levels from that study (Pope et al., 2010) and noted that one of the data points from the control group was a statistical outlier (Grubb's test; Z value = 2.635, p<0.01). When that one data point was removed, one-way ANOVA indicated a significant treatment effect (F=6.396, p=0.02, 2 df), with a 113% and 43% increase in hippocampal AEA by CPF and PS, respectively. That reanalysis of our previous findings provides results that are in general agreement with the observations in the present study (Figure 3). Our overall conclusion from these studies is that CPF and PS have a more robust effect on both the enzyme that hydrolyzes AEA (FAAH) and the extracellular levels of AEA, compared to the enzyme primarily responsible for metabolism of 2AG (MAGL) and extracellular 2AG levels.

It is difficult to predict how tissue and extracellular levels of eCBs may relatively change in response to anticholinesterase exposure. Several studies suggest that tissue eCB levels can markedly change in a time-dependent manner following sacrifice. Sugiura and coworkers (2001) reported a five-fold increase in 2AG levels in rat brain within one minute after decapitation. FAAH activity appears important in post-mortem accumulation of AEA (Patel *et al.*, 2005). Bazinet and coworkers (2005) reported about a 4-fold increase in AEA in rat brain within 5 min of decapitation. Thus the time between sacrifice and freezing of the tissue, along with the relative activity of FAAH and MAGL, could be critical determinants in the estimation of tissue eCB levels. In contrast, extracellular eCBs collected by microdialysis would likely be less sensitive to modification during sampling as they are transferred immediately into a refrigerated fraction collector and thereby removed from any *in situ* synthetic and/or degradation pathways.

Endocannabinoids were discovered as retrograde neuromodulators (i.e., a signal released from a postsynaptic neuron acting on the presynaptic terminal) released by depolarized neurons (Kreitzer and Regehr, 2001; Ohno-Shosaku *et al.*, 2001; Wilson and Nicoll, 2001). Several investigators have used reverse dialysis of perfusion buffer (aCSF) with a high concentration of potassium (60–90 mM) to depolarize neurons near the dialysis probe and thereby evaluate depolarization-induced eCB release (Giuffrida *et al.*, 1999; Bequet *et al.*, 2007; Wiskerke *et al.*, 2012). In our study, we perfused the hippocampus with higher potassium (100 mM) and calcium (10 mM) to see if local depolarization would lead to differential changes in AEA or 2AG release due to prior OP exposure. While depolarizing conditions led to moderate increases in both AEA and 2AG (Figure 5A–D), there was relatively little apparent effect of OP exposure, in particular with CPF. With the extensive degree of FAAH inhibition noted with both OPs (Figure 2B), we anticipated that local depolarization would lead to more robust increases in AEA in the OP-treated rats because of lesser FAAH-mediated clearance.

We postulated that the greater elevation of extracellular AEA levels in CPF-treated rats (Figure 3) played a role in the limited expression of toxicity following exposure to this OP, and that pharmacological blockade of eCB signaling would increase the incidence of signs. We therefore treated additional rats with either vehicle, PS (27 mg/kg) or CPF (280 mg/kg) as before, and then administered the CB1 receptor antagonist/inverse agonist AM251 (3 mg/kg, ip) once or every day for three days, beginning 24 hours after OP exposure. Surprisingly, AM251 had no apparent effect on the toxicity of CPF but dramatically decreased signs of toxicity following PS (Figure 6). This finding was completely unexpected as we previously reported that the synthetic cannabinoid receptor agonist WIN 55,212-2 (1.5 mg/kg, ip) reduced functional signs of toxicity following paraoxon exposure (Nallapaneni *et al.*, 2006). While CPF appeared more effective at increasing extracellular AEA levels, blocking the CB1 receptor only affected signs of toxicity following PS exposure. It must be noted that our earliest evaluation of extracellular eCBs was not until 2 days after OP exposure, thus earlier changes in extracellular eCB levels (from 0–2 days after CPF dosing) could have modulated the expression of toxicity prior to challenge with AM251 (starting 24 hours after dosing). One could also argue that the substantial elevation of AEA following CPF led to CB1 receptor desensitization and thus a lack of response to CB1-selective agents. This possibility seems remote, however, as previous studies have reported that while MAGL inhibition sufficient to increase 2AG levels leads to CB1 desensitization and tolerance, FAAH inhibition and increased AEA levels do not affect CB1 receptor signaling or elicit tolerance (Schlosburg *et al.*, 2010). While eCBs and CB1 appear to play a role in the expression of cholinergic toxicity following PS exposure, the role of eCB signaling in acute toxic responses to CPF remains unclear.

We recently reported that CB1^{-/-} and CB1^{+/+} mice showed very similar toxic responses following acute CPF exposure (Baireddy *et al.*, 2011). Along with the data presented above, these findings suggest that CB1-mediated signaling has little role in toxic outcome following exposure to CPF. It is becoming clear however that eCBs, in particular AEA, can act at other molecular targets in addition to CB1 (Micale *et al.*, 2013). The cannabinoid CB2 receptor has been typically associated with immune cell function, but more recent studies suggest that CB2-mediated signaling may play a role in the CNS. Oddi and coworkers (2012) reported that partial lesioning of the cerebellum (hemi-cerebellectomy) in mice led to induced expression of CB2 receptors in distant neurons, with CB2 (but not CB1) agonists showing neuroprotective effects in this model. Moreover, a CB2 agonist (JWH-015) increased neuronal nitric oxide synthase expression, reduced neuroinflammation and decreased oxidative/nitrosative stress in response to hemi-cerebellectomy. AM251 is a selective blocker of CB1 receptors, thus CB2-mediated signaling could potentially be increased following CPF exposure, influencing the toxic outcome with CPF.

Non CB1/non CB2 receptors have also been reported to mediate some effects of eCBs. AEA, but not 2AG, is an agonist for transient receptor potential vanilloid 1 (TRPV1, Zygmunt *et al.*, 1999; van der Stelt *et al.*, 2005). While TRPV1 receptors have been historically associated with sensory neuron and pain/inflammatory responses, TRPV1 receptors are now known to be located throughout the CNS (Mezey *et al.*, 2000; Roberts *et al.*, 2004; Fernandes *et al.*, 2012). AEA acts as an extracellular signal at cannabinoid (CB1 and CB2) receptors to decrease calcium influx into the presynaptic terminal, but acts intracellularly on TRPV1 receptors to increase calcium influx (van der Stelt, 2005). Some evidence suggests that AEA can work in a dual fashion, activating CB1 receptors at lower concentrations but leading to more TRPV1 activation at higher concentrations (Ahluwalia *et al.*, 2003). If AM251 blocks AEA signaling in either CPF- or PS-treated rats to shift the balance towards greater TRPV1 activation, this would suggest that more and not less toxicity might occur.

The orphan G protein-coupled receptor GPR55 has also been proposed as a molecular target for eCBs (Gaspero *et al.*, 2013). GPR55 is a plasma membrane receptor as with other members of the GPCR superfamily. Like TRPV1, GPR55 activation is coupled to influx of calcium. Interestingly, the CB1 antagonist AM251 acts as an agonist at GPR55. Thus, if AEA influences cholinergic toxicity through GPR55 in either PS- or CPF-treated rats, AM251 (as an agonist at GPR55) would again likely lead to more and not less cholinergic signs.

Finally, FAAH is the primary enzyme in brain that inactivates the fatty acid amides palmitoylethanolamide (PEA) and oleoylethanolamide (OEA). These acylethanolamides have been shown to have anti-inflammatory activity, and changes in their levels are associated with a number of conditions including multiple sclerosis, schizophrenia and eating disorders (Hansen, 2010). Similar to AEA, PEA and OEA have been reported to activate TRPV1, but unlike AEA they can also activate the nuclear PPAR receptors (Fu *et al.*, 2003; Lo Verme *et al.*, 2005; Movahed *et al.*, 2005). FAAH inhibitors have been shown to increase brain PEA and OEA levels (Ahn *et al.*, 2008). OP inhibitors of FAAH including PS and CPF may therefore increase the levels of PEA or OEA to modulate TRPV1 and/or PPAR signaling. The effects of PS and CPF on these acylethanolamides are unknown.

Together, these studies show that acute exposure to PS and CPF can lead to marked increases in extracellular AEA levels in rat hippocampus. Increased levels of AEA could in turn influence the release of acetylcholine and/or non-cholinergic neurotransmitters, contributing to the ultimate expression of toxicity following anticholinesterase exposures. Pharmacological blockade of cannabinoid CB1 receptors by AM251 had relatively little

influence on signs of toxicity following CPF but markedly reduced signs of toxicity following PS exposure. The molecular basis for the differential toxic consequences of acute PS and CPF exposure and the complex role that eCBs may play remain unclear.

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Highlights

- Acute chlorpyrifos and parathion exposure markedly inhibited hippocampal cholinesterase and fatty acid amide hydrolase
- Chlorpyrifos and parathion both increased hippocampal extracellular anandamide levels
- Monoacylglycerol lipase and 2-arachidonoylglycerol levels were lesser affected
- The CB1 cannabinoid receptor antagonist AM251 had no effect on chlorpyrifos but markedly reduced toxicity following parathion

Figure 1. Functional signs of toxicity after parathion or chlorpyrifos exposure in rats

Adult, male Sprague Dawley rats were treated with either vehicle (peanut oil, 2 mg/kg, sc, n=9–18), parathion (27 mg/kg, sc, n=15–24) or chlorpyrifos (280 mg/kg, sc, n=15–23) and evaluated for involuntary movements 2 and 4 days later as described in Methods. Scores are reported as median ($\pm$ quartile). An asterisk indicates a significant difference in the parathion-treated rats compared to controls.

Figure 2. Effects of parathion and chlorpyrifos on A) cholinesterase, B) fatty acid amide hydrolase and C) monoacylglycerol lipase activity in rat hippocampus 2 and 4 days after exposure

Rats were treated as in Methods (controls, n=9; PS, n=11–12; CPF, n=11–12) and sacrificed 2 or 4 days later. Hippocampus was rapidly dissected and frozen at -70°C until assay. Tissues were homogenized in PBS and assayed for enzyme activity as described in Methods. Data were calculated as nmol substrate hydrolyzed $\text{min}^{-1} \text{mg protein}^{-1}$ and plotted as percent of control values. Values are reported as mean $\pm$ SEM. Control values for cholinesterase activity were: 2 days = $69.6 \pm 2.0 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$; 4 days = $71.7 \pm 2.0 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$. Control values for FAAH activity were: 2 days = $805 \pm 20 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$; 4 days = $662 \pm 7 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$. Control values for MAGL activity were: 2 days = $26.6 \pm 1.0 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$; 4 days = $22.0 \pm 0.4 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$. An asterisk indicates a significant difference compared to controls.

Figure 3. In vivo effects of parathion and chlorpyrifos on extracellular levels of anandamide (AEA) in rat hippocampus

A microdialysis cannula was surgically implanted into hippocampus 6–7 days prior to exposure to vehicle (n=5–6), parathion (n=6–9) or chlorpyrifos (n=7–9) as described in the Methods section. At day 2 (Figure 3A) or day 4 (Figure 3B) after dosing, a dialysis probe was inserted into the cannula and tissue perfused (0.8 μ l/min) with artificial cerebrospinal fluid (aCSF) containing 30% hydroxypropyl- β -cyclodextrin. After 5 hours, twelve 15-min fractions were collected into a refrigerated fraction collector. At the beginning of fraction 5, the perfusion buffer was switched to aCSF containing 30% hydroxypropyl- β -cyclodextrin but with elevated potassium (100 mM KCl) and calcium (10 mM CaCl_2). At the beginning of fraction 9, the perfusion buffer was switched back to aCSF containing 30% hydroxypropyl- β -cyclodextrin. Dialysates were frozen until analysis by LC/MS-MS as described in Methods. Values are reported as mean $\pm$ SEM. Control values for AEA were: 2 days = $0.42 \text{ nM} \pm 0.15 \text{ nM}$, n=6; 4 days = $0.33 \pm 0.06 \text{ nM}$, n=5. Both parathion and chlorpyrifos led to significant increases in extracellular AEA levels, with statistically greater elevation in chlorpyrifos- vs parathion-treated rats. Symbols represent: circle, controls; square, PS-treated; triangle, CPF-treated.

Figure 4. In vivo effects of parathion and chlorpyrifos on extracellular levels of 2-arachidonoylglycerol (2AG) in rat hippocampus

Rats were treated and microdialysis and eCB analysis conducted as described in Figure 3 and the Methods section. Values are reported as mean $\pm$ SEM. Control values for 2AG were: 2 days = 2.1 ± 0.25 nM; 4 days = 2.77 ± 0.64 nM. Chlorpyrifos exposure was associated with a significant increase in 2AG levels at 2 days after dosing (Figure 4A), but no significant treatment related effects were noted at 4 days after exposure (Figure 4B) with either parathion or chlorpyrifos. Symbols represent: circle, controls; square, PS-treated; triangle, CPF-treated.

Figure 5. Depolarization-induced release of anandamide (AEA) and 2-arachidonoylglycerol (2AG)

Rats were treated and microdialysis and eCB analysis conducted as described in Figure 3 and the Methods section. Depolarization-induced release was estimated by the area under the curve for eCB levels in fractions 7–11 for A) AEA at 2 days, B) AEA at 4 days, C) 2AG at 2 days and D) 2AG at 4 days after dosing. Values are reported as mean percent of baseline (fraction 7) $\pm$ SEM. Depolarization led to slight-moderate increases in the release of AEA and 2AG. AEA release 2 days after dosing was significantly affected by treatment, but no group-wise comparisons were significant. No treatment-related effects were noted with 2AG at 2 days or with either eCB at 4 days after exposure. Symbols represent: circle, controls; square, PS-treated; triangle, CPF-treated.

Figure 6. The cannabinoid CB1 receptor antagonist AM251 reduces parathion toxicity

Rats were treated with either vehicle (n=34), parathion (27 mg/kg, sc, n=59) or chlorpyrifos (280 mg/kg, sc, n=13) as described in the Methods section. Subsets of vehicle controls were then given either the vehicle for AM251 (ethanol: Alkamuls EL-620: saline; 1:1:18, n=23) or AM251 (3 mg/kg, ip) three times (n=11), daily beginning 24 hours later. Parathion-treated rats were given either vehicle (n=26) or AM251 (3 mg/kg, ip) three times (n=27), daily beginning 24 hours later, or AM251 only once at 24 hours after dosing (n=6). Chlorpyrifos-treated rats were given either vehicle (n=6) or AM251 three times, daily beginning 24 hours after exposure (n=7). Rats were evaluated for involuntary movements daily as described in Methods. Values are reported as median. AM251 (AM) had no influence on involuntary movements in controls or rats treated with chlorpyrifos, but significantly reduced signs in parathion-treated rats with either one or three daily doses. Symbols represent: open square, AM only; filled circle, PS only; inverted filled triangle, PS + AM (1x); open triangle, PS + AM (3x). Vehicle controls and all chlorpyrifos-treated rats had median scores of 2 and are not shown.