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Low-level repeated exposure to diazinon and chlorpyrifos decrease anxiety-like behaviour in adult male rats as assessed by marble burying behaviour.

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Abstract

Occupational exposure to organophosphate (OPs) pesticides is reported to increase in the risk of developing anxiety and depression. Preclinical studies using OP levels, which inhibit acetylcholinesterase activity, support the clinical observations, but little is known of the effects of exposure below this threshold. We examined the effects of low level OP exposure on behaviours and neurochemistry associated with affective disorders. Adult rats were administered either diazinon (1 mg/kg i.p.) which is present in sheep dip and flea collars, chlorpyrifos (1 mg/kg i.p.) which is present in crop sprays, or vehicle for 5 days. OP exposure did not affect acetylcholinesterase activity (blood, cerebellum, caudate putamen, hippocampus, prefrontal cortex), anhedonia-like behaviour (sucrose preference), working memory (novel object recognition), locomotor activity or anxiety-like behaviour in the open field arena. In contrast OP exposure attenuated marble burying behaviour, an ethological measure of anxiety. The diazinon-induced reduction in marble burying persisted after exposure cessation. In comparison to vehicle, dopamine levels were lowered by chlorpyrifos, but not diazinon. 5-HT levels and turnover were unaffected by OP exposure. However, 5-HT transporter expression was reduced by diazinon suggesting subtle changes in 5-HT transmission. These data indicate exposure to occupational and domestic OPs, below the threshold to inhibit acetylcholinesterase, can subtly alter behaviour and neurochemistry.

Keywords: Serotonin, 5-HT, organophosphate, pesticide, depression, anxiety

1. Introduction

Organophosphate (OPs) chemicals are commonly used worldwide. They are mainly used to kill or repel pests in agriculture and horticulture but are also used in the home, in products such as flea treatments. In animals and man, the primary mode of action of OPs, is to inhibit the enzyme acetylcholinesterase (AChE), leading to high levels of acetylcholine in the peripheral and central nervous system and hypercholinergic symptoms. Whilst the effects of high level OP poisoning are relatively well understood, the effects of low level OP exposure remain contentious. Some epidemiological research indicates that low level OP exposure in an occupational setting is associated with mood changes and cognitive deficits, which are evident in affective disorders. Thus, increased levels of anxiety and depression, and deficits in memory and attention have been reported in sheep farmers and crop sprayers (Stephens, Spurgeon et al. 1995; Salvi, Lara et al. 2003; Mackenzie Ross, Brewin et al. 2010). However, other studies have reported no association between low level OP exposure and cognition and/or emotional status (Daniell, Barnhart et al. 1992; Fiedler, Kipen et al. 1997; Roldan-Tapia, Parron et al. 2005; Solomon, Poole et al. 2007). The difference may be due in part to the limitations in human exposure studies, such as the lack of comprehensive exposure data. Whilst assumptions can be made about which OPs may have been involved (for example, diazinon for sheep farmers and chlorpyrifos for crop sprayers) exposure level data are limited. Serum AChE activity levels are sometimes provided as evidence of low level exposure but this measure is difficult to interpret without a baseline measurement and even then only indicative of exposure in recent days (Cocker, Mason et al. 2002; Garfitt, Jones et al. 2002).

Whilst rodent studies have demonstrated that OP exposure in adulthood can cause cognitive deficits in attention and memory and emotional changes in anxiety-like behaviour and impulsivity (Moser, Phillips et al. 2005; Lopez-Crespo, Carvajal et al. 2007; Valvassori, Fortunato et al. 2007; Lima, Ribeiro-Carvalho et al. 2009; Lopez-Crespo, Flores et al. 2009; Yan, Jiao et al. 2012; Lopez-Granero, Cardona et al. 2013) few have investigated the effects of OP exposure below the threshold to significantly inhibit AChE activity. Likewise, the majority of investigations on OP-induced changes to the monoaminergic neurotransmitters, which play an important role in the behaviours altered, have focused on moderate to high level exposures (Ali, Chandra et al. 1980; Aldous, Farr et al. 1982; Prioux-Guyonneau, Coudray-Lucas et al. 1982; Sachana, Flaskos et al. 2001; Moreno, Canadas et al. 2008; Masoud, Kiran et al. 2011). It has been suggested that some OP-induced behavioural and/or monoaminergic effects are independent of AChE inhibition, the shared mechanism of action, because different OPs can sometimes have disparate effects (Prioux-Guyonneau, Coudray-Lucas et al. 1982; Ray and Richards 2001). OPs investigated are mainly those used in crop sprays or as chemical weapons, such as soman, so it is unclear if diazinon, the OP used in sheep dip and also the active ingredient in some commercially available cat and dog flea collars, causes behavioural and/or monoaminergic effects.

The main aim of this study was to determine if exposure to diazinon, below the threshold to induce significant AChE inhibition, affected behaviour and neurochemistry in the adult rat. As sheep farmers use sheep dip for relatively short periods (several days) a 5 day exposure period was used. Chlorpyrifos was included in the study to determine if the OP effects were disparate. The behavioural test battery included behaviours altered in affective disorders (anxiety-like behaviour, anhedonia, memory) in addition to locomotor activity, and the neurochemical assessments focussed on the 5-HT (serotonin) and dopaminergic systems, which are implicated in the aetiology of anxiety and depression. In some experiments assessments were made at set intervals after exposure cessation as previous studies have demonstrated that OP-induced behavioural and neurochemical changes may persist/increase after this point.

2. Materials and methods

2.1. Animals

All experiments were carried out in accordance with the UK Animals (Scientific procedures) Act of 1986 and the European Community Council Directive of 24 November 1986 (86/609/EEC). Male Hooded-Lister rats (Charles River, Kent, UK) were housed in groups of 3-4 in RC1 cages (56 x 38 x 20 cm) in a temperature controlled room (21–24°C) with 12:12h light/dark cycle (lights on at 07:00) with *ad libitum* access to food (RM03 rat chow, Charles River, UK) and water. After being delivered animals acclimatised for a minimum of 5 days before studies began when animals were 9-10 weeks old. Animals were observed and weighed daily to monitor their health and calculate dosing volumes.

2.2. Treatments

Diazinon or chlorpyrifos (Greyhound Chromatography and Allied Chemicals, UK) were mixed with ethanol and Cremophor EL to make a stock suspension (de Blaquiere, Waters et al. 2000). Organophosphate stocks and vehicle stock (1:10 ethanol and Cremophor EL) were diluted with 0.9% saline shortly before administration (final concentration 1% ethanol, 10% Cremophor EL). OPs were administered through intraperitoneal (i.p.) injections to allow accurate and efficient delivery and reduce variability. Although the majority of chronic OP exposure studies administer OPs orally, the majority of studies with shorter periods of OP exposure use either subcutaneous (Lopez-Crespo, Carvajal et al. 2007; Moreno, Canadas et al. 2008; Lopez-Crespo, Flores et al. 2009) or i.p. injection as the route of administration (Ali, Chandra et al. 1980; Prioux-Guyonneau, Coudray-Lucas et al. 1982; Coudray-Lucas, Le Guen et al. 1987; Sachana, Flaskos et al. 2001). An i.p. injection of 40 mg/kg diazinon to adult male wistar rats (340-370g) and 100 mg/kg chlorpyrifos to adult male albino rats (250-350g) (Tomokuni, Hasegawa et al. 1985; Sachana, Flaskos et al. 2001) is reported to substantially inhibit brain AChE, but we wanted to administer a five day exposure dose which was below the threshold to inhibit AChE. Therefore pilot dose ranging studies were conducted with the second highest dose in each range 100 times less than the previously reported acute AChE inhibiting dose (0, 0.1, 0.4, 1 mg/kg i.p. diazinon; 0, 0.3, 1, 3 mg/kg i.p. chlorpyrifos, Supplementary Figure 1). Exposure to 1 mg/kg diazinon and 1 mg/kg chlorpyrifos (i.p.) for five consecutive days was below the threshold to induce significant AChE inhibition. For study 1 (behavioural test battery, cholinesterase and dopamine levels) rats received daily i.p. injections of 0 (1 ml/kg, $n = 12$), 1 mg/kg diazinon ($n = 12$) or 1 mg/kg chlorpyrifos ($n = 12$) for 5 consecutive days. For study 2 (marble burying only) rats received 1 mg/kg diazinon ($n = 12$) for 5 days. For study 3 (5-HT levels) 5 groups ($n = 12$) received injections for 10 days (10 days vehicle, 5 days vehicle/5 days diazinon, 5 days vehicle/5 days chlorpyrifos, 5 days diazinon/5 days vehicle, 5 days chlorpyrifos/5 days vehicle). This was to ensure differences observed in the study were due to time since cessation of exposure (1 day and 6 days) and not due to time since final injection. For study 4 (5-HT transporter) rats received 0 (1 ml/kg, $n = 9$) or 1 mg/kg diazinon ($n = 9$) for 5 days.

2.3. Behaviour

In study 1, locomotor activity and anxiety-like behaviour were assessed each day during the treatment period. Treatment effects on anhedonia-like behaviour, working memory, and marble burying behaviour were assessed the day after the end of the treatment period. **Locomotor activity and anxiety-like behaviour**: At least four hours after dosing (4 − 5.5 hours) individual rats were placed in the open field arena (elevated grey PVC box 80 × 80 × 50 cm) for 10 minutes. For analysis, the arena was divided into 3 x 3 squares (27 x 27 cm^2) giving 1 centre, 4 corner and 4 transition squares. Distance travelled, latency to enter the centre square and frequency of entries into and time spent in the centre square were recorded and analysed using Ethovision XT v 5.0 (Noldus, Netherlands). In addition, the number of faecal boli produced was recorded. Daily exposure to the arena also ensured rats were habituated to the arena before the novel object test. **Anhedonia-like behaviour (sucrose preference test)**: To ensure rats had tasted sucrose prior to testing they were provided with 1% sucrose for 3 hours in their homecage 2 days prior to treatment. Sucrose preference was assessed 1 day prior to treatment (to confirm there was not a significant difference between groups) and 1 day after the end of treatment. Briefly, rats were placed in test cages for 2 hours with 1 bottle of tap water and 1 bottle of 1% sucrose (sucrose intake (ml)/water intake + sucrose intake (ml)*100). **Working memory (novel object recognition test)**: During the 3 minute sample phase rats were placed in the arena containing two identical objects (non-porous ceramic, metal or glass objects similar in size to an adult rat) positioned in adjacent corners ~ 15 cm away from the walls of the arena. After a 15 minute interval in a clean empty cage rats were placed back into the arena containing a familiar object (from sample phase) and a novel object for the 3 minute testing phase. Exploratory behaviour of the objects was recorded and preference for the novel object calculated (novel object exploration time/total exploration time)-(familiar object exploration time/total exploration time) (Ennaceur, Michalikova et al. 2005). **Marble burying-behaviour**: Behaviour was assessed 1 day prior to treatment (to confirm there was not a significant difference between groups) and 1 day after the end of treatment. Briefly, rats were placed in test cages containing a 5 cm layer of sawdust and 9 glass marbles arranged evenly at one end of the cage for 10 minutes. The number

of marbles buried by each rat was counted. In study 2, marble burying behaviour was assessed 1 day prior to treatment and then 1, 4, 8, 11 and 15 days after treatment. All behavioural experiments except sucrose preference were conducted under 40W white light.

2.4. Tissue collection

After behavioural testing in study 1, rats were overdosed with isoflurane and decapitated. Trunk blood was collected in heparinised tubes, diluted 1:25 in cold 0.1% saponin and frozen at -20°C for later cholinesterase assay. In studies 3 and 4 rats were overdosed with isoflurane and decapitated 1 day after treatment. Brains from all rats were rapidly removed, cut into 3 mm coronal slices, rapidly frozen and stored at -80°C in the freezer until dissection.

2.5. Acetylcholinesterase activity

The hippocampus, cerebellum, caudate putamen and prefrontal cortex from the left hemisphere in study 1 rats were dissected, homogenised in ice cold Tris-buffered saline (pH 7.4), diluted 1:25 in 0.1% saponin, incubated on ice for 10 minutes and frozen at -20°C. Protein concentration in brain homogenate was quantified using a bicinchoninic acid assay (Sigma Aldrich). Samples (and bovine serum albumin standards) were incubated 1:20 with the bicinchoninic acid reagents at 37°C for 30 minutes before absorbance was read at 562 nm. Acetylcholinesterase activity (AChE) in blood and brain homogenate was quantified using a modified version of Ellmans colorimetric assay (Ellman, Courtney et al. 1961; de Blaquiere, Waters et al. 2000). Brain homogenate and blood samples (diluted a further 1:5 with 0.1% saponin) (10 µl) were added to wells with phosphate buffered saline (110 µl, 0.1M, pH 7.4). 5,5'-dithio-bis-2nitro-benzoate (DTNB; 99 µl, 0.25 mM, Sigma-Aldrich Company Ltd) as the chromatogen and acetylthiocholine iodide (11 µl, 155 mM; Greyhound Chromatography, UK) as the substrate were then added. Absorbance of DTNB was read at 412 nm for 30 minutes (blood 35°C; brain homogenate 25°C). AChE activity was expressed as nmol min^{-1} ml^{-1} for blood and nmol min^{-1} µg^{-1} protein for brain homogenate.

2.6. Neurochemistry

Brain regions that are the source of or receive projections from serotonergic and dopaminergic neurones were selected for neurochemical analysis. For dopamine and dopamine metabolite assessment the caudate putamen and prefrontal cortex from the right hemisphere of study 1 rats were dissected, homogenised in 0.1M perchloric acid and centrifuged at 13,000 rpm for 5 minutes. For 5-HT and 5-HT metabolite assessment the whole dorsal raphe nucleus, and the hippocampus and prefrontal cortex from the right hemisphere of study 3 rats were dissected, homogenised in 0.1M perchloric acid and centrifuged at 14,000 rpm for 15 minutes at 4°C. The supernatants were collected and stored at -20°C until analysis. Samples were assayed by reverse-phase high-pressure liquid chromatography (HPLC) with electrochemical detection. Briefly, samples (and the standards 3-hydroxytyramine, 3,4-dihydroxyphenylacetic acid and homovanillic acid) for the dopamine assay were diluted in mobile phase 1 (88 mM NaH$_2$PO$_4$, 1.2 mM octane sulfonic acid, 999 mM ethylenediaminetetraacetic acid, 12% methanol, pH 4.0). Samples (and the standards serotonin creatinine sulfate and 5-Hydroxyindole-3-acetic acid) for the 5-HT assay were diluted in mobile phase 2 (127 mM NaH$_2$PO$_4$, 1.2 mM octane sulfonic acid, 74 mM ethylenediaminetetraacetic acid, 15% methanol, pH 4.0). They were separated using a 150×4.6 mm 5 µm reverse phase C18-column (Varian Inc., USA) maintained at 40°C and detected using a Coulochem III (ESA Inc., USA) electrochemical detector fitted with a 5020 guard cell (E +440 mV for dopamine, +350 mV for 5-HT) and a 5011 microdialysis cell ($E1$ -300 mV and $E2$ +400 mV for dopamine, $E1$ +120 mV and $E2$ +220 mV for 5-HT). The resulting peak heights were measured and quantified with reference to external standards. Assay limits (3*baseline noise) were 4, 5, 11, 11, 12 fmol for 5-HT, 5-HIAA, dopamine, DOPAC and HVA respectively. Lowest sample values recorded were 79, 60, 192, 44, 20 fmol for 5-HT, 5-HIAA, dopamine, DOPAC and HVA respectively. Dopamine levels are expressed as fmol/µg protein. Protein concentration in brain homogenate was quantified using a bicinchoninic acid assay described above.

2.7. Western Blot Analysis

The hippocampus and prefrontal cortex from the left hemisphere in study 4 rats were dissected, homogenised in ice cold RIPA buffer (50 mM Tris pH 7.6, 1% IGEPAL CA-630, 0.25% sodium deoxycholate, 150 mM NaCl, 1.7 mM NaF, 5 mM β-glycerophosphate, 1 mM Na$_3$VO$_4$ and 1 mM phenylmethanesulfonyl fluoride) and frozen at -20°C. Protein concentration was quantified using a Bradford assay. For western blot analysis, homogenate (20 µg protein) was prepared with NuPAGE LDS Sample loading buffer and Sample reducing agent (Life Technologies, Paisley) and loaded onto NuPAGE 10% Bis-Tris gels (Life Technologies, Paisley), along with molecular weight markers and an antigen standard for the 5-HT transporter (Origene, MD). Using an Owl HEP Series Semidry Transfer system (Thermoscientific) proteins were transferred to nitrocellulose membrane (GE Healthcare, Amersham). Membranes were blocked with Tris Buffered Saline, with 0.2% TWEEN 20 and 5% dried skimmed milk powder (TBST-M) and then incubated with a goat polyclonal anti-5-HT transporter antibody (Santa Cruz Biotechnology Inc.) at a 1:200 dilution in TBST-M, and with rabbit anti-GAPDH-HRP (Abcam, Cambridge) at 1:1000 dilution in TBST-M, as a loading control. After incubation with an anti-goat IgG-HRP antibody (Abcam, Cambridge) at a dilution of 1:6000 in TBST-M, the membranes were exposed to ECL detection reagent (GE Healthcare, Amersham), and the chemiluminescences detected with Kodak X-ray film (Sigma, Poole). Image J was used to determine the band densities and are expressed relative to GAPDH density.

2.8. Data Analysis

Data were analysed using SPSS. Values > 3*interquartile range of a data set were removed from the analysis and data sets were tested for normality (Shapiro–Wilk test). Comparisons between treatments were made using: a) independent t-tests (5-HTT expression); b) univariate ANOVAs with treatment as a fixed factor (marble burying, sucrose preference and novel object discrimination index); or c) repeated-measures ANOVAs with either session / day as a within subject factor and treatment as a between subject factor (body weight, locomotor activity and anxiety-like behaviour), or blood/brain region as a within subject factor and treatment and time since exposure (5-HT only) as between subject factors (acetylcholinesterase activity, neurotransmitter levels). For non-normally distributed data sets (persistence in marble burying) comparisons were made using the Wilcoxon signed ranks test.

3. Results

Body weight was not affected by OP exposure (repeated measures ANOVAs) but was affected by day (Study 1, $F_{1,34} = 4.5$, $p < 0.05$; Study 2, $F_{4,44} = 98.8$, $p < 0.001$; Study 3, $F_{4,241} = 600$, $p < 0.001$; Study 4, $F_{2,32} = 13.6$, $p < 0.001$).

3.1. Behaviour

Locomotor activity (distance travelled, Figure 1A) and anxiety-like behaviour, which included latency to enter the centre (Figure 1B), the frequency of entries into and time spent in the centre of the open field arena and the number of faecal boli produced per session (data not shown) were not affected by OP exposure (repeated measures ANOVA). Anxiety-like behaviour was affected by session (latency to enter centre, $F_{2,60} = 6.7$, $p < 0.01$; frequency of centre entries, $F_{4,132} = 3.1$, $p < 0.05$; time spent in centre, $F_{4,132} = 5.1$, $p < 0.01$; faecal boli number, $F_{4,132} = 3.4$, $p < 0.05$). Anhedonia-like behaviour (sucrose preference test) and working memory (novel object recognition test), assessed the day after exposure, were also not significantly different affected by OP (one way ANOVA, Figure 1C,D). In contrast, both diazinon and chlorpyrifos exposure attenuated marble burying behaviour (Figure 2A). The reduction in behaviour following diazinon exposure persisted for more than 1 week after exposure cessation (Figure 2B).
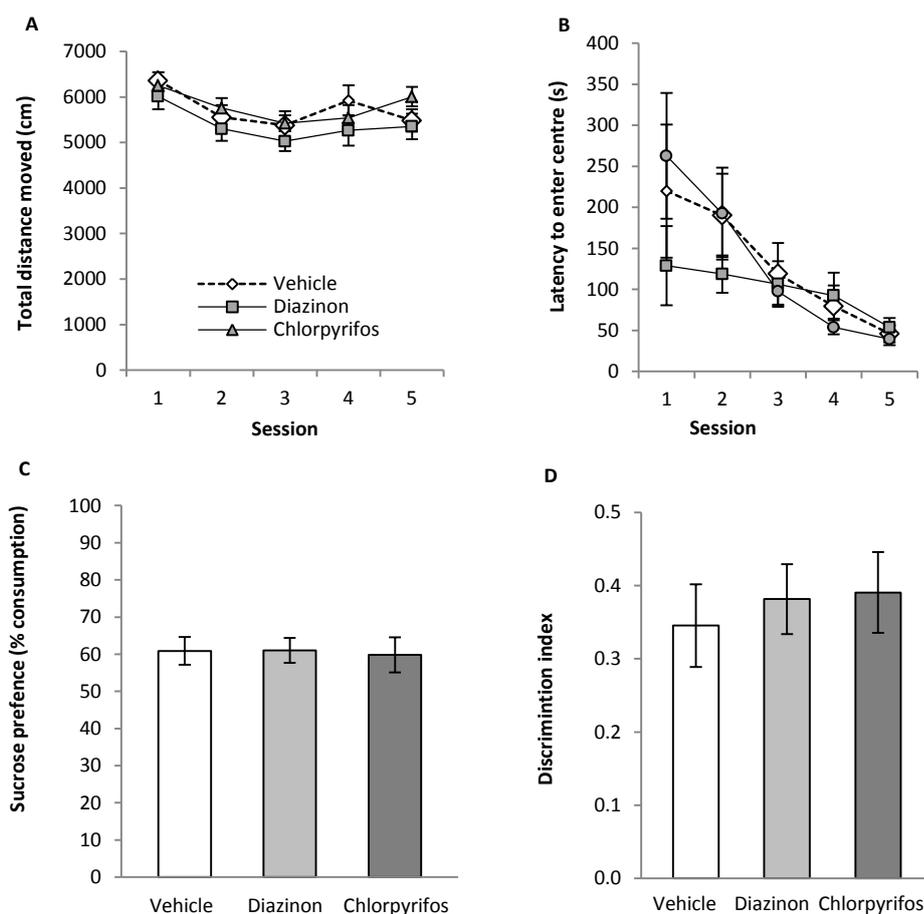

Figure 1. Behaviour in rats during and following 5 day organophosphate exposure. (A) Locomotor activity (distance travelled) and (B) anxiety-like behaviour (latency to enter the centre), which were assessed 4 hours after each exposure in an open field arena, were not significantly different between groups exposed to vehicle (1 ml/kg i.p., $n = 12$), diazinon (1 mg/kg i.p., $n = 12$) and chlorpyrifos (1 ml/kg i.p., $n = 12$) (repeated measures ANOVA). Anxiety-like behaviour was affected by session ($F_{2,60} = 6.7$ $p < 0.01$). (C) Anhedonia-like behaviour (sucrose preference test) and memory (novel object recognition test), assessed the day after exposure, were also not significantly different between groups (one way ANOVA). Mean ± SEM.
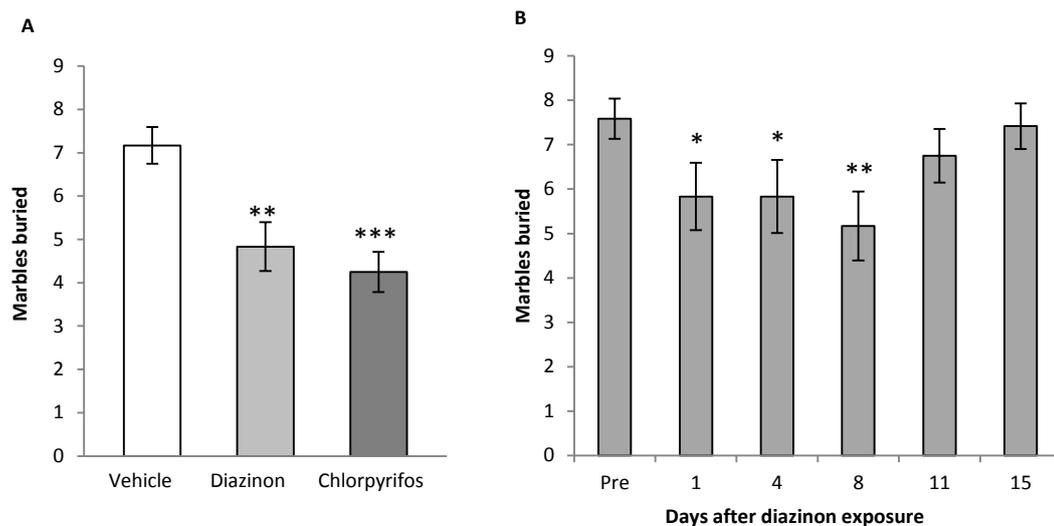
Figure 2. Behavioural changes following 5 day organophosphate exposure. (A) Exposure to either diazinon (1 mg/kg i.p., $n = 12$) or chlorpyrifos (1 mg/kg i.p., $n = 12$) reduced marble burying behaviour (assessed 1 day after exposure) in comparison to vehicle (1 ml/kg i.p., $n = 12$) (One way ANOVA $F_{2,35} = 10.1$ p < 0.001, Bonferoni *post hoc* tests ** p < 0.01, *** p < 0.001 in comparison to vehicle). (B) The diazinon-induced reduction in marble burying behaviour (1 mg/kg, $n = 12$) persisted for at least 8 days after of exposure cessation (Wilcoxon signed ranks test, * p < 0.05, ** p < 0.01 in comparison to pre exposure). Mean ± SEM.

3.2. Neurochemistry

Exposure to diazinon (1 mg/kg i.p.) or to chlorpyrifos (1 mg/kg i.p.) for 5 days had no effect on AChE in any brain region examined (cerebellum, caudate putamen, hippocampus, prefrontal cortex) or in blood (repeated-measures ANOVA, Supplementary Figure 2). Exposure to chlorpyrifos but not diazinon lowered dopamine levels in comparison to vehicle (Table 1). Dopamine metabolite levels and turnover were unaffected by OP exposure (Table 1). 5-HT and 5-HT metabolite levels were unaffected by OP exposure. However, 5-HT transporter expression in the prefrontal cortex and hippocampus was reduced following diazinon exposure (Figure 3). An increase in 5-HT turnover was observed 6 days after treatment (in comparison to 1 day) which approached significance (Table 2).

Table 1
Dopamine and dopamine metabolite levels in brain homogenate 1 day after exposure (5 days) to diazinon (1 mg/kg i.p.), chlorpyrifos (1 mg/kg i.p.) or vehicle (1 ml/kg).

Brain region	Treatment	Dopamine (fmol/µg)	DOPAC (fmol/µg)	HVA (fmol/µg)	DOPAC/dopamine	HVA/dopamine
Prefrontal	Vehicle	2.04 ± 0.12	0.85 ± 0.12	0.63 ± 0.08	0.39 ± 0.05	0.30 ± 0.04
cortex	Diazinon	2.07 ± 0.20	0.66 ± 0.06	0.50 ± 0.06	0.36 ± 0.03	0.25 ± 0.01
	Chlorpyrifos	1.86 ± 0.28	0.64 ± 0.09	0.52 ± 0.06	0.35 ± 0.05	0.30 ± 0.05
Caudate	Vehicle	10.17 ± 1.00	1.13 ± 0.09	0.59 ± 0.05	0.11 ± 0.01	0.06 ± 0.01
Putamen	Diazinon	10.38 ± 0.74	1.28 ± 0.11	0.60 ± 0.04	0.13 ± 0.01	0.06 ± 0.01
	Chlorpyrifos	7.95 ± 0.73	1.13 ± 0.15	0.54 ± 0.07	0.12 ± 0.01	0.06 ± 0.01

Results are expressed as mean ± SEM fmol/µg protein ($n = 10$–12). Dopamine levels were affected by treatment ($F_{2,30} = 3.9$ p < 0.05, repeated-measures ANOVA) with a treatment x region interaction ($F_{2,30} = 3.4$ p < 0.05). Chlorpyrifos but not diazinon lowered dopamine levels in comparison to vehicle when both areas were analysed together (*post hoc* multiple comparisons, p < 0.05). There was no treatment effect when prefrontal cortex and caudate putamen were analysed individually (univariate ANOVA). 3,4-Dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA).

4. Discussion

4.1. Exposure route and levels

We used a 5 day exposure period reflecting the practice of sheep dipping for several days each year. However, the route of pesticide administration we used (i.p. injection) differs from the predicted route of exposure from sheep dipping, which is dermal (Buchanan et al. , 2001). Consequently the rate of pesticide absorption in this study will have been more rapid than it would be through occupational exposure (Garfitt, Jones, 2002, Tomokuni, Hasegawa, 1985). Levels of exposure

experienced by sheep dippers are difficult to quantify and so we cannot state that the levels of exposure in this study accurately reflect potential occupational exposures. Nevertheless the levels of exposure in this study were low and did not result in overt hypercholinergic symptoms or changes in locomotor activity, which is altered by higher OP levels(Lopez-Crespo, Carvajal, 2007, Nieminen et al. , 1990, Pope et al. , 1992). AChE activity in the blood and the brain regions examined was not significantly inhibited indicating that the behavioural and neurochemical changes observed following 5 days of exposure were not a consequence of cholinesterase inhibition. Although we cannot exclude the possibility that AChE may have been inhibited briefly after each daily dose in this study, this may not be the case as AChE activity in all brain regions examined in a previous study (Judge et al, in preparation) was not inhibited at 4, 8 or 24 hours after exposure to 1 mg/kg i.p. diazinon (chlorpyrifos was not examined). In addition, we cannot dismiss the possibility there may have been localised AChE inhibition in unexamined brain regions. OPs can have brain region specific effects (Santhoshkumar et al. , 1996, Srivastava and Shivanandappa, 2011) and we have demonstrated that acute diazinon exposure albeit at higher OP levels, can inhibit AChE in the dorsal raphe nucleus, the major source of 5-HT in the brain, without affecting other brain regions (Judge et al, in preparation).
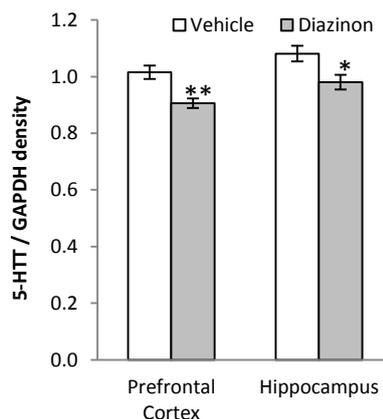

Figure 3. 5-HT transporter expression in rat brain homogenate following 5 day exposure to vehicle (1 ml/kg i.p., $n = 9$) or diazinon (1 mg/kg i.p., $n = 9$). Exposure to diazinon decreased 5-HT transporter expression in comparison to vehicle (independent t-tests, * $p < 0.05$, ** $p < 0.01$). Mean ± SEM.

Table 2
5-HT and 5-HT metabolite levels in rat brain homogenate after the cessation of exposure (5 days) to diazinon (1 mg/kg i.p.), chlorpyrifos (1 mg/kg i.p.) or vehicle (1 ml/kg).

Brain region	Time after treatment (days)	Treatment	5-HT (fmol/mg)	5-HIAA (fmol/mg)	5-HIAA/5-HT
Prefrontal	1	Vehicle	894 ± 56	550 ± 28	0.65 ± 0.06
cortex	1	Diazinon	904 ± 36	554 ± 30	0.62 ± 0.04
	1	Chlorpyrifos	862 ± 52	535 ± 19	0.60 ± 0.04
	6	Diazinon	832 ± 47	558 ± 30	0.69 ± 0.05
	6	Chlorpyrifos	779 ± 48	627 ± 33	0.84 ± 0.07
Hippocampus	1	Vehicle	1135 ± 60	1231 ± 73	1.10 ± 0.07
	1	Diazinon	1125 ± 44	1211 ± 53	1.09 ± 0.07
	1	Chlorpyrifos	1178 ± 53	1351 ± 102	1.14 ± 0.05
	6	Diazinon	1108 ± 63	1212 ± 61	1.13 ± 0.08
	6	Chlorpyrifos	1066 ± 46	1241 ± 60	1.17 ± 0.05
Dorsal raphe	1	Vehicle	6407 ± 165	7800 ± 270	1.22 ± 0.04
nucleus	1	Diazinon	6764 ± 232	8130 ± 369	1.21 ± 0.05
	1	Chlorpyrifos	5804 ± 284	7240 ± 277	1.27 ± 0.05
	6	Diazinon	6113 ± 509	7915 ± 366	1.35 ± 0.08
	6	Chlorpyrifos	6126 ± 465	7538 ± 353	1.28 ± 0.07

Results are expressed as mean ± SEM fmol/mg wet tissue weight ($n = 11–12$). The increase in 5-HT turnover 6 days after treatment, in comparison to 1 day, approached significance ($F_{1,55} = 3.8$, $p = 0.056$, repeated measures ANOVA). There was no treatment x time effect indicating that this trend was not due to one organophosphate alone. 5-Hydroxyindoleacetic acid (5-HIAA).

4.2. Behaviour

Anhedonia (decreased ability to experience pleasure) is a core symptom in depression. Developmental OP exposure is reported to reduce anhedonia in adulthood (chocolate milk preference) (Roegge, Timofeeva et al. 2008) but this is the first study to specifically examine the effects of adult OP exposure on anhedonia. Sucrose preference in all three treatment groups was quite low (~ 60%) in comparison to some previous reports (D'Aquila, Newton et al. 1997; Tonissaar, Herm et al. 2006) but preference (for chocolate milk) in the aforementioned developmental study was also quite low, although sensitive enough to detect OP-induced changes. Therefore our results indicate that the low level OP exposure regime used in this study does not alter anhedonia in adults. Likewise, memory in the novel object recognition test was not affected by OP exposure. There have been previous studies examining the effects of adulthood OP exposure on memory (Moser, Phillips et al. 2005; Terry, Gearhart et al. 2007; Terry, Buccafusco et al. 2011; Yan, Jiao et al. 2012) but at levels sufficient to significantly inhibit AChE activity. In this study, all treatment groups demonstrated a significant preference for the novel object indicating that the test was sensitive but there was not a significant difference between groups. We may conclude therefore that memory processes do not appear to be sensitive to adulthood OP exposure below the threshold to induce significant AChE inhibition. This is in contrast to the well-reported effects of developmental OP exposure on memory (Levin, Addy et al. 2001; Aldridge, Levin et al. 2005; Timofeeva, Roegge et al. 2008).

Previous studies investigating OP-induced changes in anxiety in adults have focused on high level or chronic exposure to chlorpyrifos. Acute high level exposure decreases thigmotaxis (preference for outer edge of arena) (Lopez-Crespo, Carvajal et al. 2007), whereas chronic low level exposure (11 months) increases thigmotaxis (Moser, Phillips et al. 2005) indicating that the length/level of exposure is important. OP exposure in this study did not affect behaviour in the open field arena indicating that low level exposure is below the threshold to affect anxiety-like behaviour characterised by thigmotaxis. In contrast, "anxiety-like behaviour" in the marble burying test (increased burying) was attenuated by exposure to chlorpyrifos and diazinon. A number of conclusions can be drawn from these results. Firstly, the marble burying test appears to be sensitive enough to detect more subtle OP-induced changes in behaviour. This is the first time the effect of OP exposure on marble burying behaviour has been investigated. Secondly, chlorpyrifos and diazinon both affected marble burying which could indicate a shared mechanism of action, although it should be noted that this is a complex behaviour and so it is possible each OP affected a different aspect of marble burying behaviour through different mechanisms. Finally, low level OP exposure appears to reduce anxiety. Chlorpyrifos-induced anxiolysis has been reported previously (Lopez-Crespo, Carvajal et al. 2007) as has chlorpyrifos-induced anxiogenesis (Lopez-Crespo, Carvajal et al. 2007; Lopez-Crespo, Flores et al. 2009). Sánchez-Amate and colleagues concluded that chlorpyrifos-induced effects on anxiety are dependent on the anxiety test selected. Although the marble burying test is often described as an ethological test of anxiety in the literature, marble burying does not adapt with repeated testing as with other anxiety-like behaviours (see thigmotaxis behaviour above). Furthermore, it is sensitive to many psychoactive compounds, not just anxiolytics — which enhance serotonergic (5-HT) transmission such as acute SSRIs and tricyclic antidepressants (Kobayashi, Hayashi et al. 2008). We should therefore be cautious about concluding that the OP exposure in this study resulted in anxiolysis or that the change in behaviour is attributable to the subtle neurochemical alterations observed (see below). However, this does not diminish the potential of the marble burying test being a sensitive tool to assess OP exposure.

4.3. Neurochemistry

Dopamine levels in brain tissue have previously been reported to be either unaffected or else elevated shortly after high level chlorpyrifos exposure (Karen, Li et al. 2001; Pung, Klein et al. 2006), possibly as a consequence of monoamine oxidase downregulation (Xu, Chang et al. 2012). We observed a decrease in dopamine levels in this low level exposure study, indicating that the effect of chlorpyrifos is dependent on exposure level. Dopamine levels appear to follow a similar bell-shaped dose-response curve in response to other cholinergic compounds, such as dichlorvos or epibatidine, with low level exposure decreasing and high level exposure increasing dopamine levels (Ali, Chandra et al. 1980; Choudhary, Raheja et al. 2002; Janhunen and Ahtee 2004). Although the mechanism of action was not investigated in this study, it is unlikely to be one common to all OPs as dopamine levels were unaffected by diazinon. The disparate effects of chlorpyrifos and diazinon on dopamine levels also indicate that a decrease in dopamine levels does not underlie the OP-induced decrease in marble burying. Indeed, reductions in marble-burying correlate poorly with dopamine levels (Kobayashi, Hayashi et al. 2008).

Although most studies have reported that 5-HT levels in brain tissue are unaffected shortly after exposure to a range of OPs, even at high levels (Prioux-Guyonneau, Coudray-Lucas et al. 1982; Fernando, Hoskins et al. 1984; Coudray-Lucas, Le Guen et al. 1987), some OPs do affect 5-HT turnover (Prioux-Guyonneau, Coudray-Lucas et al. 1982; Fernando, Hoskins et al. 1984) suggesting 5-HT system activation. In this study, 5-HT and 5-HT metabolite content in brain tissue was unaffected shortly after exposure, but 5-HT transporter expression was reduced after diazinon exposure. These data indicate that low level exposure is below the threshold to deplete 5-HT content or disrupt metabolism but still affects 5-HT transmission subtlety. Interestingly, 5-HT transporter expression is strongly implicated in anxiety aetiology, with increased 5-HT transporter expression associated with anxiolysis and decreased 5-HT transporter expression associated with anxiogenesis (Line, Barkus et al. 2011). Therefore, a reduction in 5-HT transporter expression conflicts with the decrease in "anxiety-like behaviour" we observed in the marble burying test. This adds further weight to our caution about concluding OP exposure resulted in anxiolysis and attributing the behavioural changes to the subtle neurochemical changes we observed. These data are however consistent with a previous report that OP exposure during adulthood decreases 5-HT transporter expression (Lima, Nunes-Freitas et al. 2011) and also the increased levels of anxiety and depression reported in sheep farmers and crop sprayers (Mackenzie Ross et al. , 2010, Salvi et al. , 2003, Stephens et al. , 1995).

There is evidence that OP-induced effects on the 5-HT system components progress with time, albeit at much higher exposure levels (el-Etri, Nickell et al. 1992; Pung, Klein et al. 2006; Moreno, Canadas et al. 2008; Oswal, Garrett et al. 2013). Consistent with this, we observed that 5-HT turnover was elevated 6 days after cessation of exposure in comparison to 1 day with a trend towards significance. One explanation for this change with time could be that the 5-HT system adapts to the initial OP-induced alterations in 5-HT transmission such as reduced 5-HT transporter expression (Jennings, Licht et al. 2012). Further work is needed to determine if the behaviours unaffected by low level exposure in this study, when assessed 1 day after exposure, may alter over time.

In summary, exposure to diazinon or chlorpyrifos in adulthood below the threshold to significantly inhibit AChE does not alter anhedonia and memory deficits, which are associated with affective disorders. However, "anxiety like behaviour" is reduced in the marble burying test. Thus, the marble burying test may be a useful tool of assessment in future OP studies. Further work is needed to determine if these effects of low level exposure to diazinon are the beginning of a chain of progressive damage to the 5-HT system resulting in more significant effects over time.

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Supplementary Figures

A. 5 day diazinon exposure

☐ 0   mg / kg
☐ 0.1 mg / kg
☐ 0.4 mg / kg
■ 1.3 mg / kg

B. 5 day chlorpyrifos exposure

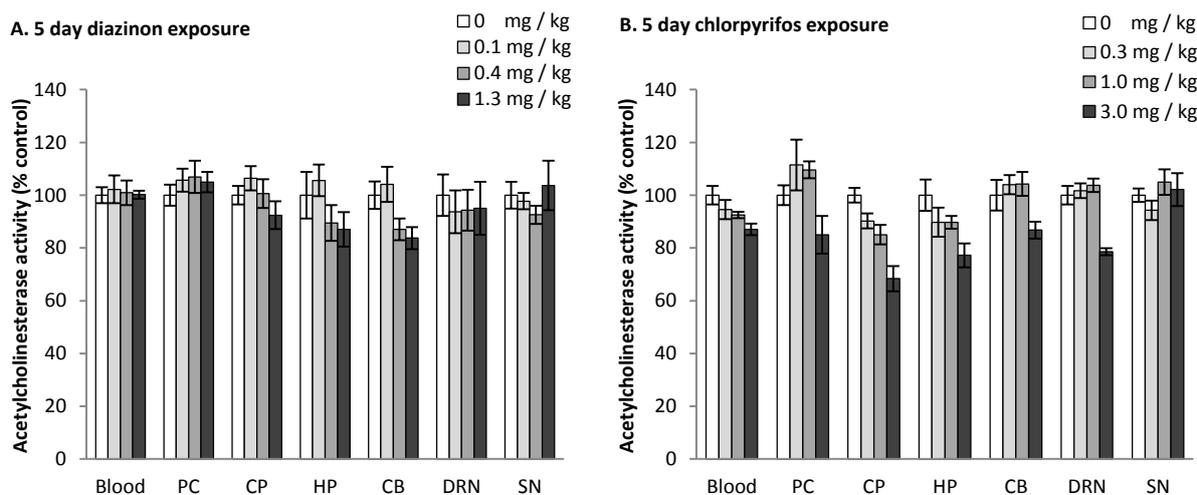☐ 0   mg / kg
☐ 0.3 mg / kg
☐ 1.0 mg / kg
■ 3.0 mg / kg

Figure 1. Acetylcholinesterase activity following 5 day exposure to low-level diazinon (A) or to chlorpyrifos (B). **A.** Acetylcholinesterase activity was not affected by repeated exposure to diazinon (0, 0.1, 0.4, 1.3 mg / kg i.p.; n = 10 per dose; repeated-measures General Linear Model). **B.** Acetylcholinesterase activity was affected by repeated exposure to chlorpyrifos (0, 0.3, 1.0, 3.0 mg / kg i.p.; n = 6 per dose; $F_{3,17}$ = 10.5, p < 0.001; repeated-measures General Linear Model). Exposure to 3 mg / kg significantly inhibited acetylcholinesterase activity in comparison the vehicle group (Bonferoni *post hoc* tests, p < 0.005). Results are expressed as mean ± SEM activity as a percentage of activity in vehicle group. Prefrontal cortex (PC), caudate putamen (CP), hippocampus (HP), cerebellum (CB), dorsal raphe nucleus (DRN, substantia nigra (SN)
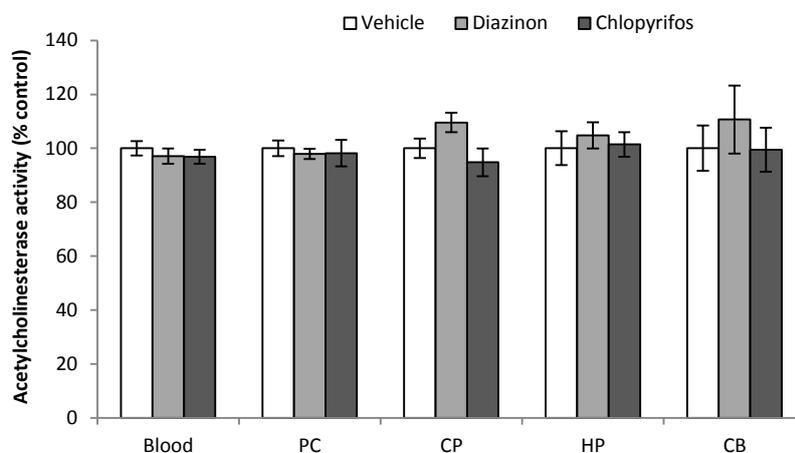

☐ Vehicle   ■ Diazinon   ■ Chlopyrifos

Figure 2. Acetylcholinesterase activity following 5 day exposure to diazinon (1 mg/kg i.p.), chlorpyrifos (1 mg/kg i.p.) or vehicle (0 mg/ml i.p.). Acetylcholinesterase activity was not affected by exposure (repeated-measures General Linear Model). Results are expressed as mean ± SEM activity as a percentage of control activity (n = 10–12). Absolute acetylcholinesterase activity in vehicle samples was 3.8 ± 0.1 nmol / min / µl in blood, 93.0 ± 2.7 nmol / min / mg protein in prefrontal cortex (PC), 627.2 ± 22.6 nmol / min / mg protein in caudate putamen (CP), 93.9 ± 5.9 nmol / min / mg protein in hippocampus (HP) and 49.8 ± 2.0 nmol / min / mg protein in the cerebellum (CB, Mean ± SEM).