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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.

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 (Mackenzie Ross et al., 2010; Salvi et al., 2003; Stephens et al., 1995). However, other studies have reported no association between low level OP exposure and cognition and/or emotional status (Daniell et al., 1992; Fiedler et al., 1997; Roldan-Tapia et al., 2005; Solomon 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 et al., 2002; Garfitt 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

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(Lima et al., 2009; Lopez-Crespo et al., 2007, 2009; Lopez-Granero et al., 2013; Moser et al., 2005; Valvassori et al., 2007; Yan et al., 2012) 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 (Aldous et al., 1982; Ali et al., 1980; Masoud et al., 2011; Moreno et al., 2008; Prioux-Guyonneau et al., 1982; Sachana et al., 2001). 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 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 × 38 × 20 cm) in a temperature controlled room (21–24 °C) with 12:12 h 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 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 et al., 2007, 2009; Moreno et al., 2008) or i.p. injection as the route of administration (Ali et al., 1980; Coudray-Lucas et al., 1987; Prioux-Guyonneau et al., 1982; Sachana et al., 2001). An i.p. injection of 40 mg/kg diazinon to adult male wistar rats (340–370 g) and 100 mg/kg chlorpyrifos to adult male albino rats (250–350 g) (Sachana et al., 2001; Tomokuni et al., 1985) 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 h) individual rats were placed in the open field arena (elevated grey PVC box 80 × 80 × 50 cm) for 10 min. For analysis, the arena was divided into 3 × 3 squares (27 × 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 h 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 h 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 min 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 min 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 min 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 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 min. The number of marbles buried by each rat was counted. In study 2, marble burying