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Mechanism for the acute effects of organophosphate pesticides on the adult 5-HT system

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ABSTRACT

The neurotransmitter serotonin (5-HT) is involved in mood disorder aetiology and it has been reported that (organophosphate) OP exposure affects 5-HT turnover. The aim of this study was to elucidate the mechanism underlying OP effects on the adult 5-HT system. First, acute *in vivo* administration of the OP diazinon (0, 1.3, 13 or 39 mg/kg i.p.) to male Hooded Lister rats inhibited the activity of the cholinergic enzyme acetylcholinesterase in blood and in the hippocampus, dorsal raphe nucleus (DRN), striatum and prefrontal cortex. Diazinon-induced cholinesterase inhibition was greatest in the DRN, the brain's major source of 5-HT neurones. Second, acute *in vivo* diazinon exposure (0 or 39 mg/kg i.p.) increased the basal firing rate of DRN neurones measured *ex vivo* in brain slices. The excitatory responses of DRN neurones to α_1-adrenoceptor or AMPA/kainate receptor activation were not affected by *in vivo* diazinon exposure but the inhibitory response to 5-HT was attenuated, indicating 5-HT_{1A} autoreceptor down-regulation. Finally, direct application of the diazinon metabolite diazinon oxon to naive rat brain slices increased the firing rate of DRN 5-HT neurones, as did chlorpyrifos-oxon, indicating the effect was not unique to diazinon. The oxon-induced augmentation of firing was blocked by the nicotinic acetylcholine receptor antagonist mecamylamine and the AMPA/kainate glutamate receptor antagonist DNQX. Together these data indicate that 1) acute OP exposure inhibits DRN cholinesterase, leading to acetylcholine accumulation, 2) the acetylcholine activates nicotinic receptors on 5-HT neurones and also on glutamatergic neurones, thus releasing glutamate and activating 5-HT neuronal AMPA/kainate receptors 3) the increase in 5-HT neuronal activity, and resulting 5-HT release, may lead to 5-HT_{1A} autoreceptor down-regulation. This mechanism may be involved in the reported increase in risk of developing anxiety and depression following occupational OP exposure.

1. Introduction

Organophosphate (OP) chemicals are commonly used as domestic and agricultural pesticides. For example, diazinon (Dimpylate) is used in sheep dip and in dog and cat flea collars, and chlorpyrifos is a very commonly used insecticide for crops. OPs are neurotoxic and some such as sarin are used as chemical weapons. High level OP exposure irreversibly inhibits acetylcholinesterase (AChE), the enzyme responsible for the breakdown of acetylcholine. The resulting acetylcholine accumulation causes hypercholinergic symptoms. Clinical symptoms are generally observed when brain AChE is inactivated to 20–50% of normal activity, less than 10% activity results in seizures, paralysis, and respiratory failure [20]. Regulations are in place to prevent poisoning but there is some evidence that lower OP levels that do not result in substantial toxicity can still have long term health consequences. Thus, some studies have reported occupational OP exposure increases the risk of developing symptoms related to anxiety and mood disorders [21,26,31,37]. However, other studies have reported no association

Abbreviations: AChE, acetylcholinesterase; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; DMSO, dimethyl sulfoxide; DNQX, 6,7-dinitroquinoxaline-2,3-dione; DRN, dorsal raphe nucleus; 5-HT, 5-hydroxytryptamine; OP, organophosphate.

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between occupational OP exposure and neuropsychology [9,30,34]. These inconsistencies may be due in part to the limitations of retrospective human exposure studies, in particular the lack of comprehensive exposure data and adequate control subjects [29]. Therefore, the potential association between OPs and psychiatric symptoms remains a contentious issue and, without a plausible mechanism of action, it is unlikely to be resolved.

The neurotransmitter 5-HT (serotonin) and associated proteins are heavily implicated in both the aetiology and treatment of mood and anxiety disorders. The majority of 5-HT neurones in the brain are located in the dorsal raphe nucleus (DRN) [39] and these neurones project to frontal brain regions including the prefrontal cortex, caudate putamen and hippocampus [14]. 5-HT release in the DRN and in the projection areas is largely dependent on the firing activity of the DRN neurones, which is regulated by excitatory receptors, including α_1-adrenoceptors and AMPA/kainate receptors, and inhibitory receptors, including the 5-HT_{1A} autoreceptor [1,13,15,35].

Previous studies examining the effects of acute OP exposure on the adult 5-HT system have focussed mainly on 5-HT levels and turnover in brain homogenates. They reported that acute OP exposure affects 5-HT turnover [8,10,27], indicating that the 5-HT system has been activated but the mechanism remains unresolved. The aim of this study was to make a detailed functional investigation of the effects of acute low level OP pesticide exposure on the adult rat 5-HT system. Firstly, the effect of acute *in vivo* exposure to the OP diazinon, at doses below the threshold to induce clinical symptoms (<50% cholinesterase inactivation), on cholinesterase activity was determined; brain regions associated with the 5-HT system were assessed. Secondly, the effect of acute low level *in vivo* diazinon exposure on neuronal activity in the DRN was determined *ex vivo* in brain slices. Finally, a neuropharmacological study was conducted in *in vitro* brain slices to further investigate the effects of the OP pesticides, diazinon and chlorpyrifos on 5-HT neuronal activity.

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). All efforts were made to minimise animal suffering, to reduce the number of animals used, and to utilise alternatives to *in vivo* techniques, if available. Male Hooded Lister rats (Charles River, Kent, UK) were housed in groups 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 and water.

2.2. In vivo treatments

After a minimum 5 day acclimatisation period, rats received a single intraperitoneal (i.p.) injection to allow accurate and efficient delivery of diazinon. In the cholinesterase activity study, rats received 0, 1.3, 13 or 39 mg/kg diazinon and in the electrophysiology study rats received 0 or 39 mg/kg diazinon. Doses were selected to cause <50% cholinesterase inactivation (below the threshold to induce overt hypercholinergic toxicity). Diazinon (Sigma–Aldrich, UK) was mixed with ethanol and Cremophor EL to make a suspension and diluted to the appropriate volume with 0.9% saline (final concentration 1% ethanol, 10% Cremophor EL) [4]. The diazinon mixture or vehicle mixture (1% ethanol, 10% Cremophor EL, 89% saline) was administered shortly after preparation (1 ml/kg).

2.3. Cholinesterase activity

At 4, 8 or 24 h after treatment animals were overdosed with isoflurane and decapitated. As we did not expect time of collection to significantly affect cholinesterase activity in rats administered with vehicle, all vehicle treated rats were killed at one time point (4 h after injection). Trunk blood was collected into heparinised tubes, diluted 1:25 in cold 0.1% saponin in phosphate buffered saline (PBS) and frozen at −20 °C. Brains were rapidly removed, cut into 3 mm coronal slices, rapidly frozen, and stored at −80 °C until dissection. The hippocampus, DRN, cerebellum, striatum and prefontal cortex were dissected, homogenised in ice cold Tris-buffered saline (pH 7.4), diluted 1:25 in 0.1% saponin, incubated on ice for 10 min and frozen at −20 °C.

Protein concentration in brain homogenates was quantified using a Bradford assay. Briefly, samples (and bovine serum albumin standards) were diluted 1:50 with Bradford reagent and incubated at room temperature for 10 min before absorbance was read at 595 nm. Cholinesterase activity in blood and brain homogenates was quantified using a modified version of Ellmans colorimetric assay [4,7,22]. Limits of quantification in blood were 519 and 564 nmol min^{-1} $ml^{-1}$for AChE and butrylcholinesterase, respectively, and in brain homogenate were 23 and 6 nmol min^{-1} mg^{-1} protein for AChE and butrylcholinesterase, respectively (SDs of repeated measurements * 5). AChE and butrylcholinesterase assay precision was <6% (mean coefficient of variance). Blood (diluted a further 1:5 with 0.1% saponin) or brain homogenate samples (10 μl) were added to the wells of a 96 well plate with PBS (110 μl, 0.1 M, pH 7.4). 5,5'-dithio-bis-2nitro-benzoate (99 μl, 0.25 mM, Sigma–Aldrich Company Ltd) as the chromagen and either acetyl(beta-methyl)thiocholine iodide (11 μl, 155 mM; Greyhound Chromatography, UK) or butyrylthiocholine iodide (11 μl, 218 Mm; Sigma–Aldrich Company Ltd) as the substrate were then added. Absorbance of 5'-dithio-bis-2nitro-benzoate was read at 412 nm for 30 min at 5 min intervals (blood 35 °C; brain homogenate 25 °C).

2.4. In vitro electrophysiology

For the *in vivo* OP exposure study, rats were killed the day after injection (18 ± 1 h) by isoflourane overdose and then decapitated. For the *in vitro* OP exposure study, treatment naïve rats ($n = 43$) were killed by decapitation without prior anaesthesia. Following decapitation, the brain was quickly removed and submerged in oxygenated (95% O_2/5% CO_2) sucrose slush (sucrose 200, HEPES 10, $MgSO_4$ 7, NaH_2PO_4 1.2, KCl 2.5, $NaHCO_3$ 25, $CaCl_2$ 0.5, D-glucose 10 mM, pH 7.4). Slice preparation and electrophysiology have previously been described in detail [17]. Briefly, coronal slices of the midbrain (350 μm thick) were cut and placed in an interface perfusion chamber in an atmosphere of humidified 95% O_2:5% CO_2. Slices were perfused with oxygenated aCSF (NaCl 124, $MgSO_4$ 2.4, KH_2PO_4 1.25, KCl 3.25, $NaHCO_3$ 26, $CaCl_2$ 2, D-glucose 10 mM, pH 7.4) containing the α_1-adrenoceptor agonist phenylephrine (3 μM) to evoke spontaneous firing. The aCSF, the 95% O_2:5% CO_2 and the bed of the chamber were warmed to 36–37 °C.

Extracellular recordings were made from neurones in the DRN (Bregma − 7.6 to − 8.3). For experiments using *ex vivo* brain slices from animals administered diazinon ($n = 5$) or vehicle ($n = 5$), all neurones encountered were recorded, as OP exposure may have altered the firing rate and regularity. Two to five neurones were recorded from slices from each animal. Following a period of recording of basal firing activity of each neurone, test compounds were applied via the perfusion medium. 5-HT hydrochloride (25 or 50 μM), phenylephrine (10 μM) and AMPA (3 μM) were applied for 2 min. In slices taken from treatment-naïve rats, putative 5-HT neurones ($n = 84$) were identified on their location in the DRN