Savy CS, Fitchett AE, Blain PG, Morris CM, Judge SJ.

Gene expression analysis reveals chronic low level exposure to the pesticide diazinon affects Psychological Disorders gene sets in the adult rat.

***Toxicology* 2018, 393, 90-101.**

Gene expression analysis reveals chronic low level exposure to the pesticide diazinon affects psychological disorders gene sets in the adult rat

Claire Y. Savy[a,b], Ann E. Fitchett[a,b], Peter G. Blain[a,b], Christopher M. Morris[a,b] and Sarah J. Judge[a,b1]

[a]Medical Toxicology Centre, Newcastle University, Newcastle upon Tyne, NE2 4AA, UK
[b]Institute of Neuroscience, Newcastle University, Newcastle upon Tyne, NE2 4HH, UK

[1]Corresponding Author:
E-mail: s.j.judge@ncl.ac.uk

https://doi.org/10.1016/j.tox.2017.11.006

Abstract

Chronic low level exposure to organophosphate (OPs) pesticides in adulthood has been linked to adverse neurobehavioural deficits and psychological disorder symptoms, although this remains a contentious issue. The OP-induced biological changes that could underlie these effects are unclear. We assessed gene expression changes following chronic low level exposure to diazinon, a pesticide with a high dietary exposure risk. Adult male rats were orally exposed to diazinon (0, 1, 2 mg/kg, 5 days a week for 12 weeks). After 4 weeks, there was a difference in anxiety-like behaviour in the marble burying test between diazinon and vehicle exposed rats; this difference persisted for 8 weeks. Chronic diazinon exposure did not significantly inhibit acetylcholinesterase activity, the primary mechanism of action of high level OPs. Affymetrix GeneChip® HT RG-230 PM Arrays were used for gene profiling followed by Ingenuity Pathway analysis. In the hippocampus, the most significant gene expression changes caused by OP exposure were associated with Psychological Disorders, and Cell-To-Cell Signalling and Interaction functions. Genes encoding the AMPA3 glutamate receptor, glutaminase, dopamine transporter and tyrosine hydroxylase were up-regulated, whereas the gene encoding the GABAB1 receptor was down-regulated. In the dorsal raphe nucleus, genes associated with development and the Psychological Disorders function were significantly affected, including the up-regulation of the gene encoding the α1b-adrenoceptor, the major driver of serotoninergic (5-HT) neuronal activity. These data indicate that chronic exposure to diazinon in adulthood, below the threshold to inhibit acetylcholinesterase, stimulates glutamatergic, dopaminergic and serotonergic synaptic transmission which may underlie adverse neurological outcomes.

Keywords: serotonin, glutamate, dopamine, organophosphate, pesticide, anxiety

Abbreviations; AChE, acetylcholinesterase; BChE, butrylcholinesterase; DRN, dorsal raphe nucleus; DTNB, 5,5'-dithio-bis-2nitro-benzoate; 5-HT, 5-hydroxytryptamine, serotonin; OP, organophosphate

1. Introduction

Organophosphate (OPs) chemicals are commonly used worldwide to kill or repel pests in agriculture and horticulture and are also used in the home. Acute exposure results in hypercholinergic toxicity characterised by miosis, lacrimation, salivation, diarrhoea, bradycardia, bronchospasm, confusion, nausea and dizziness (Morris et al., 2014). The effects can develop further to paralysis, seizures, coma and cardiorespiratory failure. One to three days after the acute cholinergic crisis, an intermediate syndrome occurs, characterised by limb weakness and respiratory muscle paralysis.

Whilst acute OP poisoning is well characterised, the health effects of low level OP exposure remain contentious. Some studies report that low level OP exposure is associated with neurobehavioural impairments; workers exposed to quinalphos during manufacturing processes demonstrated a reduction in memory, learning and vigilance (Srivastava et al., 2000) and sheep farmers exposed to OPs for an average of 23 years, showed reduced performance in mental flexibility, visual working and auditory memory (Mackenzie Ross et al., 2010). Other studies have reported no association between chronic low level OP use and neurobehavioural performance (Daniell et al., 1992; Rodnitzky et al., 1975; Steenland et al., 2000) although a recent meta-analysis on 22 occupational OP exposure studies found that memory and attention scores were consistently lower in chronically exposed workers than in unexposed workers (Meyer-Baron et al., 2015). Chronic low level OP exposure has also been associated with psychological disorder symptoms; OP exposed greenhouse workers (Bazylewicz-Walczak et al., 1999), tobacco workers (Salvi et al., 2003) and termiticide applicators (Steenland et al., 2000) showed elevated levels of anxiety and depression. In a 1995 study, sheep farmers had 50% greater susceptibility to psychiatric disorders than controls (Stephens et al., 1995). This was supported by a further study in which over 40% of the exposed sheep farmers had clinically significant levels of anxiety and depression compared to fewer than 23% of controls (Mackenzie Ross et al., 2010). In contrast, depression was elevated but not significantly in UK farmers chronically exposed to low levels of pesticides in a different study (Povey et al., 2014). Fiedler *et al*. (Fiedler et al., 1997) also reported no significant association between levels of anxiety and depression and low level pesticide use in fruit tree workers. Inconsistencies between studies means there is uncertainty about attributing adverse neurological outcomes to low level OP exposure. The lack of a plausible mechanism of action adds to the uncertainty.

The mechanism of action of OPs in acute poisoning is well established. The enzyme acetylcholinesterase (AChE) is inhibited, leading to acetylcholine accumulation in the central and autonomic nervous systems, and at neuromuscular junctions. Activation and then subsequent desensitisation of muscarinic and nicotinic cholinergic receptors by the excess

acetylcholine leads to the characteristic hypercholinergic symptoms. In contrast to acute OP poisoning, the diversity of adverse neurological outcomes associated with low level OP exposure suggests that more than one specific neurotransmitter system is disrupted.

Investigations to understand the changes in the brain following low level exposure have mainly focused on developmental exposure rather than adulthood exposure. Developmental OP exposure alters the expression of genes involved in neural cell development, signalling and transcription control, cytotoxicity and apoptosis, potentially causing neural cell loss (Mankame et al., 2006; Slotkin and Seidler, 2007a; Slotkin and Seidler, 2010; Slotkin and Seidler, 2012). It also alters the expression of genes related to synthesis, storage and degradation (tph, slc18a2, β-hydroxylase, slc6a2, chga and chgb) and neurotransmission (htr2a, htr6, htr3b, htr5a, drd4) of the monoamine neurotransmitters 5-HT and dopamine (Aldridge et al., 2003; Slotkin and Seidler, 2007a; Slotkin and Seidler, 2007b). This is perhaps the result of OPs inducing cells to differentiate into monoaminergic neurones (Jameson et al., 2006). Neural cell loss and changes in monoaminergic transmission probably underlie the memory deficits, anhedonia and anxiety-like behaviour observed in animals following neonatal exposure to the OPs chlorpyrifos (Aldridge et al., 2005; Levin et al., 2001) and diazinon (Roegge et al., 2008; Timofeeva et al., 2008). Whilst these adverse outcomes are similar to those associated with chronic low level OP exposure in adults, the mechanisms underlying such outcomes may not necessarily be similar. During the prenatal and neonatal period, the developing brain is vulnerable to chemical exposure causing a generalised disruption of cellular replication, migration and differentiation. As there is relatively little neural proliferation in the mature adult brain in comparison to the developing brain, we hypothesised that other mechanisms would underlie OP-induced neurological effects in adults.

The aim of this study was to investigate the effects of chronic low level OP exposure in adults. Brain regions associated with psychiatric and neurobehavioural outcomes were selected: the dorsal raphe nucleus (DRN), the major source of 5-HT monoaminergic neurones in the brain and heavily implicated in the aetiology of anxiety and depression, and the hippocampus, which plays a key role in learning and memory. Adult male rats were orally exposed to the OP diazinon for 12 weeks (5 days a week). Diazinon is used in sheep dip and in some pet flea treatments but also has one of the highest dietary exposure risk factors for pesticides, mainly through fruit and vegetable ingestion (Melnyk et al., 2016). Given that many brain proteins could underlie psychological disorder symptoms and neurobehavioural deficits, we measured changes in gene expression using microarray to make a comprehensive assessment. In addition, we assessed AChE and butrylcholinesterase (BChE) activity in blood and brain to determine if OP exposure was below the threshold to inhibit ChE activity, and also marble-burying behaviour during the chronic exposure period. Marble burying behaviour is an ethological measure of anxiety in rodents, and we have found that it is a sensitive behavioural measure of low level OP exposure (Savy et al., 2015).

2. Materials and methods

2.1. Animals

All animal procedures were carried out in adherence to the UK Home Office guidelines laid out in the Animals (Scientific Procedures) Act 1986 and the European Union Directive 2010/63/EU. Adult male Lister Hooded rats (Charles River, UK) (250-340g on arrival) were housed in groups of 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. Animals and their body weights were monitored throughout the study in order to calculate dosing volumes and monitor health.

2.2. Treatments

Diazinon (Greyhound Chromatography and Allied Chemicals, UK) was suspended in olive oil (1ml/kg) shortly before administration and administered orally. To avoid the stress of oral gavage, animals were trained to voluntarily ingest olive oil by licking the end of a syringe. Voluntary ingestion training was conducted for several days prior to treatment. For the study we wanted to administer very low diazinon levels so we conducted a pilot dose-ranging study (0, 1, 2, 4 mg / kg for 5 days; $n = 6$ per dose) to determine if repeated oral diazinon doses inhibited ChE activity. AChE activity was not affected but 4 mg / kg diazinon significantly inhibited BChE (Bonferonni post hoc comparisons, $p < 0.05$; Supplementary Figure 1), so for the main study animals received diazinon doses of 0, 1 or 2 mg / kg ($n = 12$ per dose) for 12 weeks, 5 consecutive days per week.

2.3. Behaviour

Marble burying-behaviour was assessed before treatment began and then every 4 weeks during treatment. Tests were conducted 72 hours after a 5 day treatment period, between 09:00 and 11:00 under 40W white light. Briefly, rats were placed in individual test cages containing a 5 cm layer of sawdust and 9 glass marbles arranged evenly at one end of the cage for 10 mins. The number of marbles buried, the latency to first dig, latency to bury the first marble, time spent in the marble half of the cage and frequency of rears were recorded (Savy et al., 2015).

2.4. Tissue collection

Six weeks after exposure, tail vein blood was collected in heparinised tubes, diluted 1:25 with 0.1% saponin solution and stored in aliquots at -20°C until analysis for ChE activity. Seventy-four hours after the final diazinion dose animals were overdosed with isoflurane. Trunk blood was collected in heparinised tubes, diluted 1:25 with 0.1% saponin solution and stored in aliquots at -20°C. Brains were rapidly removed and cut into 3 mm coronal slices using a brain block. Slices were placed on microscope slides, rapidly frozen on dry ice and stored at -80°C until dissection.

2.5. Cholinesterase activity

The prefrontal cortex, caudate putamen, hippocampus, substantia nigra and cerebellum from the left hemisphere ($n = 12$ per dose) and whole dorsal raphe nucleus ($n = 6$ per dose) were dissected on ice, homogenised in ice-cold Tris buffered

saline (pH 7.4) and diluted 1:25 in 0.1% saponin solution 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. AChE and BChE in tissue samples were quantified using a modified version of Ellmans colorimetric assay (de Blaquiere et al., 2000; Ellman et al., 1961). Briefly, brain homogenate or blood (diluted a further 1:5 with 0.1% saponin) (10 µl) was added to wells with phosphate buffered saline (110 µl, 0.1M, pH 7.4), the chromatogen 5,5'-dithio-bis-2nitro-benzoate (DTNB; 99 µl, 0.25 mM, Sigma-Aldrich Company Ltd) and either acetyl-β-(methyl)thiocholine iodide (11 µl, 155 mM; Greyhound Chromatography, UK) or butyrylthiocholine iodide (11 µl, 218 mM; Greyhound Chromatography, UK) as the substrate. Absorbance of DTNB was read at 412 nm for 30 minutes (blood 35°C; brain homogenate 25°C). AChE and BChE activity was expressed as nmol min-1 ml-1 for blood and nmol min-1 µg-1 protein for brain homogenate.

2.6. Brain gene expression

The hippocampus from the right hemisphere (0, 2 mg / kg diazinon, $n = 12$ per dose) and whole dorsal raphe nucleus (0, 2 mg / kg diazinon, $n = 6$ per dose) were dissected on dry ice, homogenised in Tri Reagent (Sigma-Aldrich) and stored at -80ºC until further preparation. RNA was extracted from homogenate using the RNeasy kit for RNA extraction (Qiagen, Valencia, CA, USA). Homogenate samples were incubated for 5 mins at room temperature with bromochloropropane (100µl) and then centrifuged (12,000 x g, 10 mins, 4ºC). The aqueous phase (400µl) was vortexed with 100% ethanol (200µl), transferred to a filter cartridge-collection tube assembly and centrifuged (12,000 x g, 30 secs, room temperature). The flow-through was discarded and the filter cartridge-collection tube assembly washed and centrifuged twice. The samples in the filter cartridges were incubated at room temperature for 2 mins with elution buffer (100µl) and centrifuged (30 secs, room temperature) to elute the RNA from the filter. Samples were stored at -20ºC and shipped on dry ice to AROS Applied Biotechnology (Denmark), who used Affymetrix GeneChip® HT RG-230 PM 24-Array Plate on the Gene Titan platform to conduct expression profiling according to the manufacturer's instructions. The HT RG-230 PM Array Plate (Affymetrix) enables the measurement of gene expression of more than 30,000 transcripts and variants from more than 28,000 rat genes on a single array.

2.7. Statistical analysis

Data were analysed using SPSS. Body weight comparisons were made using a repeated measures ANOVA (time as within subject factor; treatment as the between subject factor). ChE activity comparisons were made using one way ANOVAs (6 week blood ChE activity, vehicle brain ChE activity) or repeated measures ANOVAs (blood/brain region as the within subject factor; treatment as the between subject factor), followed by Bonferonni post hoc comparisons. Marble burying behavioural comparisons were made using a repeated measures ANOVA (time as within subject factor; treatment as the between subject factor) followed by one way ANOVAs for each 4 week period or treatment group, and Bonferonni post hoc comparisons. Data are represented as the mean ± SEM.

Statistical analysis for the gene expression data was conducted by the Bioinformatics Service (Newcastle University). Briefly, after hybridisation of expression chips, raw data were exported to the Bioconductor package, RankProd (Hong et al., 2006), log-scale transformed (log, basis 2) and normalised (non-linear transformation employing the loess smoother). Probe sets (78,963) were detected in all samples (20% and 90% bound of the fluorescence intensity of the chip). For the detection of gene expression changes, an ANOVA was applied with a cut off at a false discovery rate $p < 0.05$ (Benjamini and Hochberg, 1995). Ingenuity pathway analysis was conducted on genes that were significantly altered to generate functions and canonical pathways using the Ingenuity Systems reference set (Ingenuity Knowledge Base) that were most significant to the data set. The p value was determined by the probability that the association between the genes in the dataset and the function or canonical pathway is explained by chance alone. The significance of the association between the data set and the function or canonical pathway was calculated using Fischer's exact test. Finally network analysis was used to identify networks of altered genes. Networks were generated using the IPA Network Generation Algorithm and scored based on the number of network eligible molecules they contained from the dataset (Elstner et al., 2011).

3. Results

3.1. Body weight is unaffected by chronic low level exposure to diazinon

Body weights increased during treatment but there was no effect of diazinon exposure on weight (main effect of time; $F_{(1.4,46)} = 1086.3$, $p < 0.001$, effect of treatment; $F_{(2,32)} = 0.4$, $p = 0.666$).

3.2. Cholinesterase activity is unaffected by chronic low level exposure to diazinon

Six weeks into the chronic diazinon exposure period tail blood samples were taken. AChE activity and BChE activity in the blood samples were not affected by oral exposure to diazinon (0, 1, 2 mg / kg, $n = 12$ per dose; AChE $F_{(2,33)} = 1.3$, $p = 0.280$; BChE $F_{(2,33)} = 2.0$, $p = 0.146$; Supplementary Figure 2). After 12 weeks of diazinon exposure trunk blood and brain samples were collected. AChE activity and BChE activity in samples collected from animals administered vehicle (0 mg / kg diazinon) varied between different brain regions (Table 1). Blood and brain AChE or BChE activity in samples collected from diazinon-exposed animals were not significantly different to activity in the vehicle group (Table 1).

3.3. Anxiety-like behaviour in the marble burying test is affected by low level exposure to diazinon

Marble burying behaviour was assessed before treatment began (0 weeks) and after 4, 8 and 12 weeks of diazinon exposure (0, 1, 2 mg / kg; n = 11-12 per dose). Before treatment began, there was no difference between treatment groups but after 4, 8 and 12 weeks of exposure the number of marbles buried by diazinon-exposed groups was significantly lower in comparison to the vehicle group (Figure 1a, Table 2). The number of marbles buried by animals administered vehicle (0 mg /kg diazinon) did not change significantly over the 12 week test period (Figure 1a, Table 2). The latency to bury the first marble

in diazinon-exposed groups was also significantly higher in comparison to the vehicle group, (Figure 1b), although further analysis revealed this was only significant after 12 weeks of exposure (Table 2). The latency to first dig (Figure 1c), the frequency of rears (Figure 1d), and the time spent in the marble half of the cage (Figure 1d) increased over the course of the 12 week test period but were not affected by diazinon exposure (Table 2).

Table 1. Acetylcholinesterase (AChE) and butrylcholinesterase (BChE) activity following chronic oral exposure to the pesticide diazinon in the adult rat. ChE activity in samples collected from rats treated with 0 mg / kg diazinon (olive oil) varied significantly between brain regions (One way ANOVA; AChE $F_{(5,65)} = 596.8$, $p < 0.001$; BChE $F_{(5,65)} = 89.9$, $p < 0.001$). Diazinon exposure (0, 1, 2 mg / kg) for twelve weeks had no significant effect on blood and brain AChE or BChE activity (repeated measures ANOVA; AChE main effect of treatment $F_{(2,14)} = 0.2$, $p = 0.98$, main effect of region $F_{(6,84)} = 3.06$, $p < 0.01$, treatment*region interaction $F_{(12,84)} = 0.45$, $p = 0.68$; main effect of treatment BChE $F_{(2,14)} = 0.7$, $p = 0.94$, main effect of region $F_{(6,84)} = 1.75$, $p = 0.12$, treatment*region interaction $F_{(12,84)} = 1.17$, $p = 0.32$).

Acetylcholinesterase activity

	nmol/ min	**% of 0 mg /kg activity**					
Diazinon dose	**0 mg /kg**	**0 mg /kg**		**1 mg / kg**		**2 mg /kg**	
		Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	*n*
Prefrontal cortex	98.0 ± 1.4	100.0 ± 1.3	12	97.8 ± 1.7	11	98.7 ± 2.1	12
Caudate putamen	689.0 ± 20.0	100.0 ± 2.8	12	91.8 ± 2.6	10	96.7 ± 3.1	11
Hippocampus	108.2 ± 5.6	100.0 ± 5.0	12	87.4 ± 3.4	11	99.2 ± 4.8	12
Substantia nigra	160.9 ± 5.2	100.0 ± 3.1	12	117.7 ± 4.1	11	112.6 ± 4.0	12
Dorsal raphe nucleus	244.2 ± 12.7	100.0 ± 4.8	6	101.8 ± 16.3	5	111.6 ± 8.0	6
Cerebellum	61.0 ± 2.9	100.0 ± 4.6	12	93.5 ± 3.5	11	92.2 ± 1.6	12
Blood	4.1 ± 0.3	100.0 ± 4.5	12	103.8 ± 4.9	11	94.6 ± 2.8	12

Butrylcholinesterase activity

	nmol/ min	**% of 0 mg /kg activity**					
Diazinon dose	**0 mg /kg**	**0 mg /kg**		**1 mg / kg**		**2 mg /kg**	
		Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	*n*
Prefrontal cortex	15.7 ± 0.7	100.0 ± 4.2	12	96.8 ± 1.7	11	94.4 ± 2.5	12
Caudate putamen	20.0 ± 0.6	100.0 ± 2.9	12	94.9 ± 5.5	11	90.3 ± 2.5	11
Hippocampus	16.5 ± 0.4	100.0 ± 2.3	12	92.6 ± 3.2	11	92.8 ± 2.4	12
Substantia nigra	30.6 ± 1.1	100.0 ± 3.5	12	111.2 ± 6.1	11	101.2 ± 4.4	12
Dorsal raphe nucleus	37.7 ± 1.8	100.0 ± 4.3	6	97.3 ± 18.3	5	101.9 ± 5.8	6
Cerebellum	22.6 ± 0.6	100.0 ± 2.5	12	101.5 ± 2.0	11	99.8 ± 2.1	12
Blood	2.5 ± 0.2	100.0 ± 4.7	12	103.7 ± 3.5	11	100.8 ± 2.0	12

3.4. Gene expression in the hippocampus is affected by chronic low level exposure to diazinon

Of the 31,842 probe sets significantly detected in hippocampal samples (7796 up-regulated, 24046 down-regulated), the signal intensity of 430 were significantly different between samples from vehicle and diazinon treated animals (Table 3, full list available at Judge et al. (2017).

Many of the hippocampal genes affected by diazinon exposure were involved in synaptic transmission; diazinon exposure up-regulated the gene encoding unc13 (*unc13c*; 1.89 fold change), which primes synaptic vesicles for release (Lee et al., 2013) and down-regulated the gene encoding synapsin II (*syn2*; 0.66 fold change), which clusters vesicles in the synaptic reserve pool (Vasileva et al., 2012). Neurones lacking synapsin may release neurotransmitters faster as vesicles are not confined to the reserve pool. Several genes associated with glutamatergic neurotransmission were up-regulated by diazinon exposure: glutaminase (*gls*; 1.92 fold change), a glutamate synthesis enzyme, mitogen-activated protein kinase 8 (*mapk8*; 2.12 fold change), which regulates many cellular processes including apoptosis, proliferation and AMPA receptor trafficking (Adler et al., 2011), Glu3 (*gria3*; 1.71 fold change), one of the AMPA receptor subunits, neurexin 3 (*nrxn3*; 1.71 fold change), which stabilises AMPA receptors in the neuronal membrane (Aoto et al., 2015), and versican (*vcan*; 1.73 fold change), an extracellular matrix protein which may form a complex with hippocampal AMPA receptors (Saroja et al., 2014). Diazinon exposure also altered the expression of genes involved in dopaminergic transmission; genes encoding tyrosine hydroxylase, a dopamine synthesis enzyme (*th*; 1.09 fold change) and the dopamine transporter (*slc6a3*;1.59 fold change) were up-regulated whereas the gene encoding neurotensin (*nts*; 0.57 fold change), a neuropeptide involved in modulating the effects of other neurotransmitters on dopaminergic neurotransmission (Binder et al., 2001), was down-regulated. Diazinon exposure also down-regulated the genes encoding the $GABA_{B1}$ receptor (*gabbr1*; 0.64 fold change) and the $5\text{-}HT_{5B}$ receptor (*htr5b*; 0.58 fold change). Apart from the down-regulation of the gene encoding choline O-acetyltransferase (*chat*; 0.68 fold change), an acetylcholine synthesis enzyme, diazinon exposure did not significantly affect any other genes specifically related to cholinergic transmission. Some of the largest fold changes were in genes involved in neural cell development; the genes

encoding neurogenic differentiation factor 1 (*neurod1*; 2.89 fold change) and kit ligand also known as stem cell factor (*kitgl*; 2.06 fold change) were up-regulated.

Ingenuity pathway analysis generated functions and canonical pathways that were associated with the hippocampal genes most significantly affected by diazinion exposure. The most over represented biological functions were Psychological Disorders, and Cell-To-Cell Signalling and Interaction (Table 4, full list available at Judge et al. (2017)). The most over represented canonical pathways were Nitrogen metabolism, D-glutamine and D-glutamate metabolism, Epidermal growth factor signalling, Huntington's disease signalling, and Glutamate Receptor signalling (Table 5).

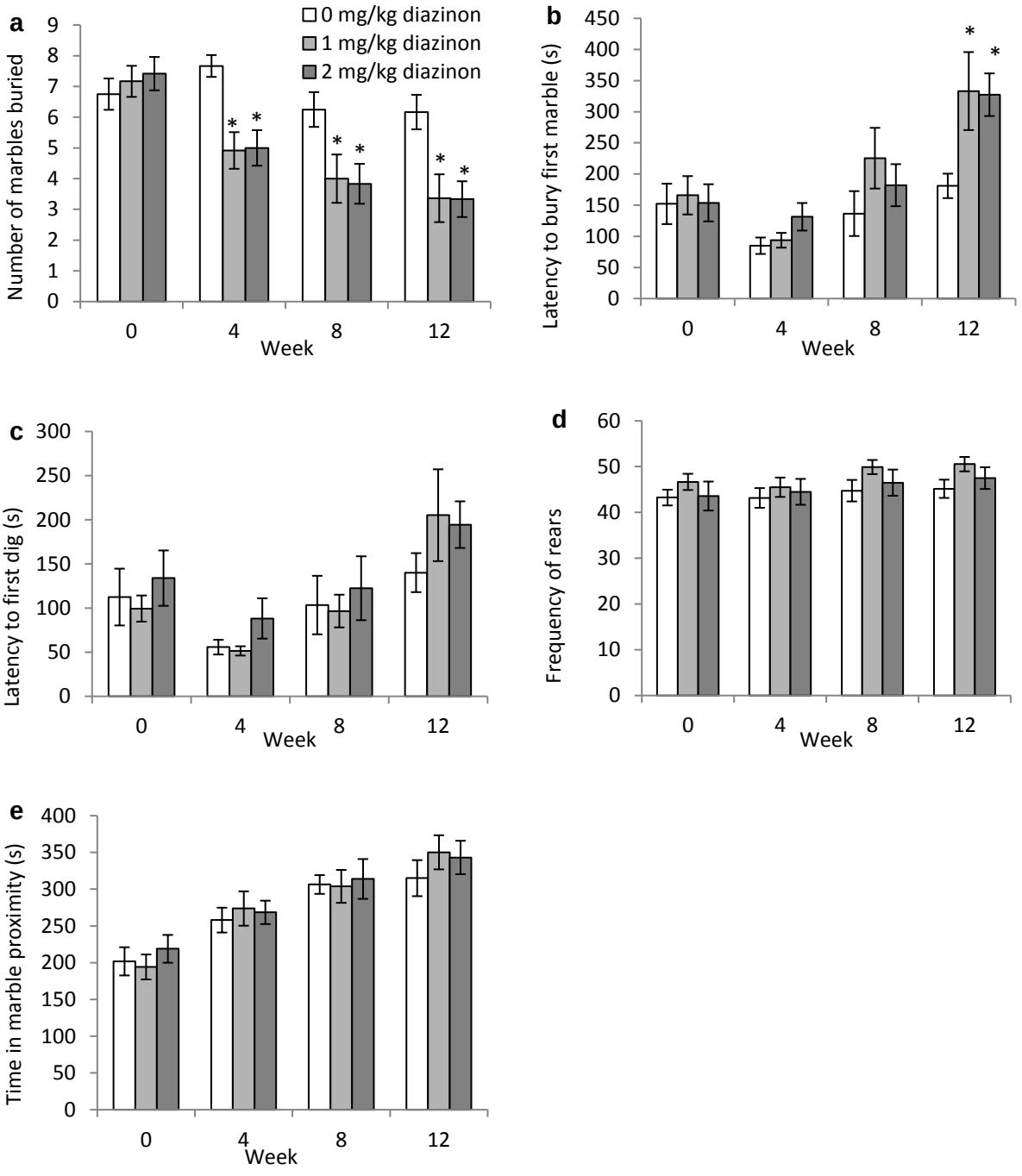


Figure 1 Chronic oral exposure to the pesticide diazinon significantly altered marble burying behaviour in the rat. (**a)** The number of marbles buried was significantly lower and (**b**) the latency to bury the first marble was significantly higher in diazinon-exposed groups in comparison to the 0 mg/ kg group (vehicle). (**c**) The latency to first dig, (**d**) the frequency of rears and (**e**) the time spent in the marble half of the cage, were not affected by diazinon exposure but these measures did increase over the course of twelve week test period. Mean ± SEM. * $p < 0.05$ in comparison to vehicle group

Table 2 Statistical test results showing the effect of oral diazinon exposure (0, 1 , 2 mg /kg) or weeks of treatment (0, 4, 8, 12) on adult rat behaviour in the marble burying test.

Repeated measures ANOVA				**One way ANOVA**							
	Behaviours	**All data**		**0 weeks**		**4 weeks**		**8 weeks**		**12 weeks**	
		F (df)	***P***	**F (df)**	***P***	**F (df)**	***P***	**F (df)**	***P***	**F (df)**	***P***
Dose	Number of marbles buried	**7.2$_{(2,32)}$**	**0.003**	0.4$_{(2,32)}$	0.66	**9.0$_{(2,32)}$**	**0.001**	**4.2$_{(2,32)}$**	**0.025**	**6.6$_{(2,32)}$**	**0.004**
	Latency to bury first marble	**9.5$_{(2,32)}$**	**0.036**	0.1$_{(2,32)}$	0.94	2.3$_{(2,32)}$	0.118	1.2$_{(2,32)}$	0.301	4.4$_{(2,32)}$	0.020
	Latency to first dig	1.0$_{(2,32)}$	0.362								
	Frequency of rears	1.5$_{(2,32)}$	0.247								
	Time in marble half of cage	0.4$_{(2,32)}$	0.704								
				0 mg/kg		**1 mg/kg**		**2 mg/kg**			
				F (df)	***P***	**F (df)**	***P***	**F (df)**	***P***		
Week	Number of marbles buried	**18.2$_{(3,96)}$**	**0.000**	1.9$_{(3,44)}$	0.15	**6.3$_{(3,42)}$**	**0.001**	**9.5$_{(3,44)}$**	**0.000**		
	Latency to bury first marble	**16.2$_{(3,96)}$**	**0.000**	2.2$_{(3,44)}$	0.10	**5.9$_{(3,42)}$**	**0.002**	**8.5$_{(3,44)}$**	**0.000**		
	Latency to first dig	**9.3$_{(3,96)}$**	**0.000**	1.8$_{(3,44)}$	0.16	**5.5$_{(3,42)}$**	**0.003**	2.2$_{(3,44)}$	0.097		
	Frequency of rears	**8.4$_{(3,96)}$**	**0.000**	0.2$_{(3,44)}$	0.86	1.9$_{(3,42)}$	0.147	0.4$_{(3,44)}$	0.750		
	Time in marble half of cage	**28.8$_{(3,96)}$**	**0.000**	**7.7$_{(3,44)}$**	**0.000**	**9.3$_{(3,42)}$**	**0.000**	**6.3$_{(3,44)}$**	**0.001**		
Dose x week	Number of marbles buried	**3.2$_{(6,96)}$**	**0.006**								
	Latency to bury first marble	1.5$_{(6,96)}$	0.179								
	Latency to first dig	0.5$_{(6,96)}$	0.844								
	Frequency of rears	0.3$_{(6,96)}$	0.939								
	Time in marble half of cage	0.4$_{(6,96)}$	0.902								

In order to visualise connections between the hippocampal genes most significantly affected by diazinon exposure and identify other genes in the network, affected genes were mapped into Ingenuity pathway analysis networks (Judge et al., 2017). The two highest scoring networks (networks containing the most genes affected by diazinon exposure) were the Cell Death, Genetic Disorder and Cellular Assembly and Organisation network (Figure 2), and the Cell-To-Cell Signalling and Interaction, Nervous system development and function and Molecular transport network (Figure 3). The Cell Death, Genetic Disorder and Cellular Assembly and Organisation network is enriched with genes encoding proteins involved with synaptic plasticity and proliferation: Akt (*akt*), a serine/threonine-specific protein kinase involved in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation (Alessi et al., 1996), fragile X mental retardation protein (*fmr1*), a binding protein involved in synaptic plasticity, platelet derived growth factor (*Pdgfbb*), which is involved in cell proliferation and differentiation, stem cell factor (*kitlg*) and C-Jun N-terminal kinase 1 (*mapk8*) mentioned previously. The Cell-To-Cell Signalling and Interaction, Nervous system development and function and Molecular transport network is enriched with genes encoding proteins associated with synaptic transmission: the N-type voltage dependent calcium channel subunit (*Cacna1b)*, which is involved neurotransmitter release from the presynaptic terminals, the inhibitory receptors $GABA_{B1}$ (*gabbr1*) and $GABA_{B2}$ receptors (*gabbr2*), the excitatory 5-HT_{2C} receptor (*htr2c)*, the post-synaptic density protein 95 (*Dlg4*), which is necessary for NMDA receptor signalling (Stephenson, 2006) and calcium/calmodulin-dependent serine protein kinase (*Cask*), a scaffolding protein involved in synaptic transmembrane protein anchoring and ion channel trafficking (Hsueh, 2006). In addition, the gene encoding huntingtin (htt), which is involved in many cellular processes including vesicle trafficking, postsynaptic signalling and apoptosis (Cattaneo et al., 2001; Gutekunst et al., 1995; Nasir et al., 1995) is central to this network.

Table 3 List of genes with most significant differences in expression between hippocampal samples from vehicle and diazinon treated animals. Fold changes above 1 are up-regulated, fold changes below 1 are down-regulated.

Gene name	Protein name	Fold change	P Value
Ogn	Osteoglycin	2.35	0.001
Coch	Coagulation factor C homolog	2.18	0.001
Mapk8	Mitogen-activated protein kinase 8	2.12	0.001
Neurod1	Neurogenic differentiation factor 1	2.89	0.002
Nars2	Asparaginyl-tRNA synthetase 2	2.23	0.002
Usp33	Protein Usp33	2.16	0.002
Ptprd	Receptor-type tyrosine-protein phosphatase delta isoform	2.17	0.003
Slc6a3	Sodium-dependent dopamine transporter (DA transporter)	1.59	0.003
Tubb2c	Tubulin beta-4B chain	2.19	0.003
Etv1	Ets variant 1	2.17	0.003
Atp2b1	Calcium-transporting ATPase	2.07	0.003
Sfrs18	PNN-interacting serine/arginine-rich protein	2.05	0.003
Col1a2	Collagen alpha2 type I	1.71	0.004
Kitlg	Kit ligand	2.06	0.004
slc13a4	Solute carrier family 13, member 4	1.63	0.004
Sema6a	Semaphorin 6A	1.99	0.005
UBE3A	Ubiquitin protein ligase E3A	1.89	0.007
Col1a2	Collagen alpha2 type I	1.58	0.007
sltm	Similar to modulator of estrogen induced transcription	1.94	0.008
Ano3	Anoctamin 3	1.85	0.008
rnpc3	RNA-binding region (RNP1, RRM) containing 3	1.91	0.008
Klhl4	Kelch-like family member 4	1.93	0.008
Gls	Glutaminase	1.92	0.009
Fat2	Protocadherin Fat 2	1.86	0.009
Ca8	Carbonic anhydrase-related protein	1.86	0.010
atrx	ATRX, chromatin remodeler	1.89	0.010
Lum	Lumican	1.83	0.010
hnrnpa2b1	Heterogeneous nuclear ribonucleoprotein A2/B1	1.85	0.011
Unc13c	Protein unc-13 homolog C (Munc13-3)	1.89	0.011
Slc6a20	Sodium- and chloride-dependent transporter XTRP3	1.60	0.011
Uba6	Protein Uba6	1.90	0.011
Zcchc7	Zinc finger CCHC domain-containing protein 7	1.91	0.011
Eif3a	Eukaryotic translation initiation factor 3, subunit A	1.87	0.013
Cp	Ceruloplasmin	1.85	0.013
Colec12	Collectin-12	1.82	0.013
Sfrs18	PNN-interacting serine/arginine-rich protein	1.79	0.013
Zeb2	Zinc finger E-box binding homeobox 2	1.88	0.013
col8a2	Collagen, type VIII, alpha 2	1.56	0.014
Mysm1	histone H2A deubiquitinase	1.84	0.014
Sass6	Spindle assembly abnormal protein 6 homolog	1.84	0.014
BMP6	bone morphogenetic protein 6	1.59	0.014
Chat	Choline O-acetyltransferase	0.68	0.002
Crlf1	Cytokine receptor-like factor 1	0.58	0.003
Lancl1	LanC-like protein 1	0.61	0.003
Nts	Neurotensin/neuromedin N	0.57	0.003
Htr5b	5-hydroxytryptamine receptor 5B	0.58	0.005
Crabp1	Cellular retinoic acid-binding protein 1	0.58	0.012
Fzr1	Fizzy/cell division cycle 20 related 1	0.63	0.013
Klhl22	Kelch-like protein 22	0.63	0.013
Nisch	Nischarin (Imidazoline receptor 1)	0.62	0.014
Ttr	Transthyretin	0.68	0.014
Cryba2	BetaA2-crystallin	0.56	0.015

Table 4 Biological Functions most relevant to hippocampal genes significantly altered by chronic diazinon exposure in the adult rat identified using Ingenuity Pathway Analysis.

Biological Functions	P value	Genes
Psychological Disorders	9.07E-06-3.3E-02	PBRM1,SLC24A2,NRXN3,TUBB2C,DDX42,MITF,FMR1,GABBR1,SFPQ,CP,HTR5B,ITPR1,FZR1,SLC6A3,KITLG,ZEB2,CALB1,BMP6,SYN2,PPARGC1A,GRIA3
Cell-To-Cell Signalling and Interaction	1.24E-05-3.64E-02	NEUROD1,GCHFR,UNC13C,NRXN3,FMR1,GLS,NTS,GABBR1,Accn1,ITPR1,MAPK8IP1,VCAN,SLC6A3,KITLG,CALB1,BMP6,SYN2,NISCH,LIN7C,GRIA3
Cell Death	3.33E-05-3.64E-02	NEUROD1,CRLF1,RBM5,ATP2B1,CDKN2D,ATRX,MAPK8,CCAR1,BOK,ITPR1,UBE3A,MAPK8IP1,VCAN,SLC6A3,IQUB,KITLG,IMMT,TFRC,CAST,CALB1,BMP6,SLC47A1,PPARGC1A
Molecular Transport	6.6E-05-3.64E-02	MRC1,AP3S1,SLC24A2,UNC13C,NRXN3,ATP2B1,FMR1,HNRNPA2B1,NTS,CP,Accn1,SLC6A3,HOOK3,HNRNPA3,SLC6A20,CALB1,HOOK1,NEUROD1,SLC13A4,GABBR1,ITPR1,MAPK8IP1,KITLG,TFRC,CPNE7,SYN2,SLC47A1,PPARGC1A,GRIA3,LIN7C,NISCH
Small Molecule Biochemistry	6.6E-05-3.64E-02	SLC24A2,UNC13C,GCHFR,NRXN3,MITF,GLS,MAPK8,NTS,GABBR1,CP,MAPK8IP1,SLC6A3,KITLG,CLK1,IMMT,TFRC,EFEMP1,BMP6,CALB1,SYN2,SLC47A1,PPARGC1A,NISCH,LIN7C
Cellular Function and Maintenance	7.83E-05-3.13E-02	MRC1,NEUROD1,SLC24A2,UNC13C,FMR1,CDKN2D,SEMA6A,MAPK8,CP,ITPR1,UBE3A,KITLG,HOOK3,MPP5,TFRC,CAST,BMP6,CALB1,HOOK1,SYN2,PPARGC1A
Nervous System Development and Function	9.75E-05-3.64E-02	COL8A2,UNC13C,NRXN3,FMR1,NTS,Accn1,SPAG9,VCAN,IQUB,SLC6A3,CAST,CALB1,NEUROD1,MITF,GLS,ATRX,CDKN2D,MAPK8,GABBR1,ITPR1,MAPK8IP1,UBE3A,ZEB2,BMP6,SYN2,PPARGC1A,GRIA3
Cell Morphology	2.04E-04-3.64E-02	NEUROD1,KITLG,FMR1,LUM,MAPK8,ITPR1,CAST,BMP6,IQUB,LIN7C,PPARGC1A
Cell Signalling	3.7E-04-2.44E-02	SLC24A2,ATP2B1,MAPK8,ITPR1,SPAG9,MAPK8IP1,MDFIC

Table 5 Cell signalling and metabolic pathways most relevant to hippocampal genes in the adult rat significantly altered by chronic low level diazinon exposure, identified using Ingenuity Pathway Analysis.

Ingenuity Canonical Pathways	P value	Genes	Protein
Nitrogen Metabolism	0.020	GLS	glutaminase
		CA8	carbonic anhydrase VIII
D-glutamine and D-glutamate Metabolism	0.025	GLS	glutaminase
Epidermal growth factor signalling	0.032	MAPK8	mitogen-activated protein kinase 8
		ITPR	inositol 1,4,5-trisphosphate receptor, type 1
Huntington's Disease Signalling	0.046	NEUROD1	neurogenic differentiation 1
		GLS	glutaminase
		MAPK8	mitogen-activated protein kinase 8
		ITPR1	inositol 1,4,5-trisphosphate receptor, type 1
Glutamate Receptor signalling	0.048	GLS	glutaminase
		GRIA3	AMPA3 receptor subunit

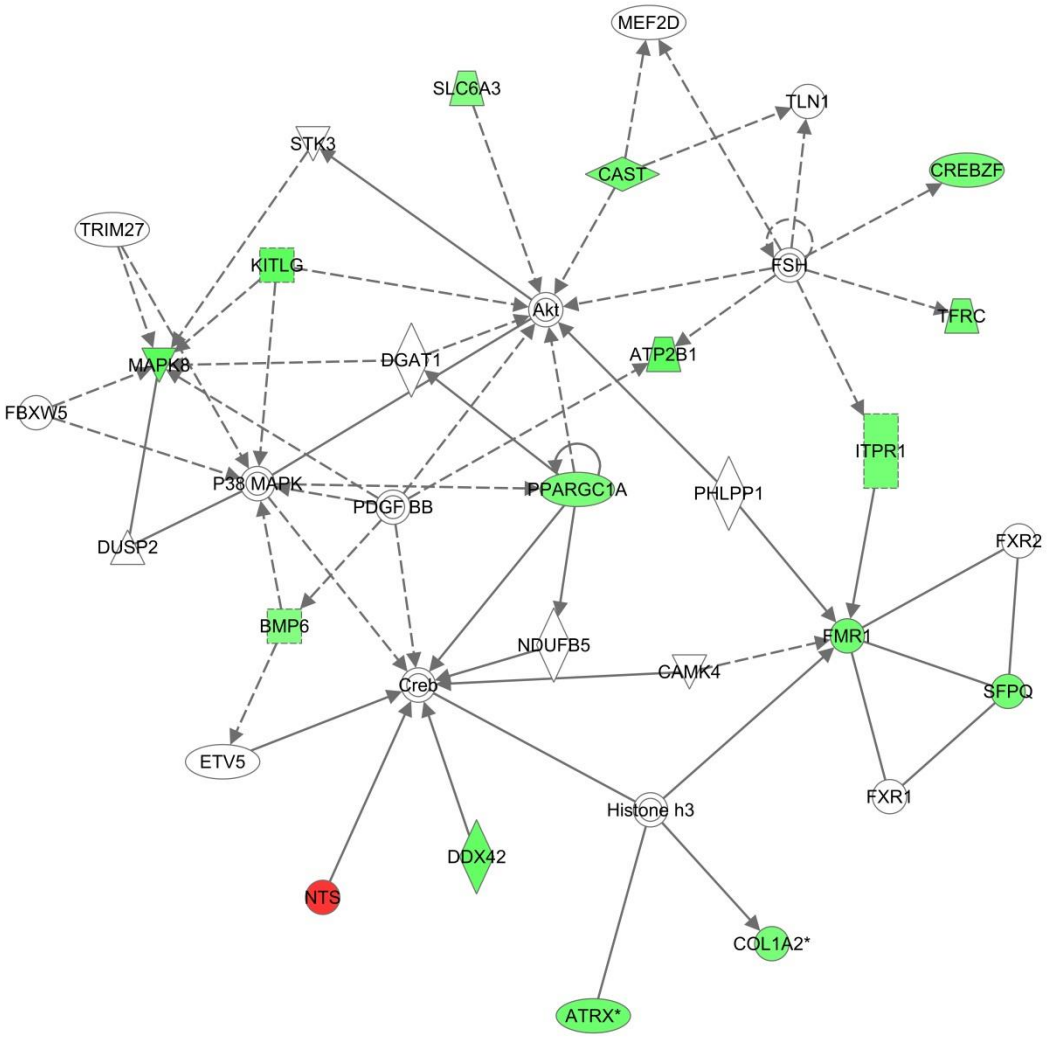


Figure 2 Primary network of differentially expressed genes identified in the adult rat hippocampus after chronic low level exposure to diazinon using Ingenuity pathway analysis. The main functions of this network are Cell death, Genetic disorders and Cellular assembly and organisation. Up-regulated and down-regulated genes are highlighted in green and red respectively.

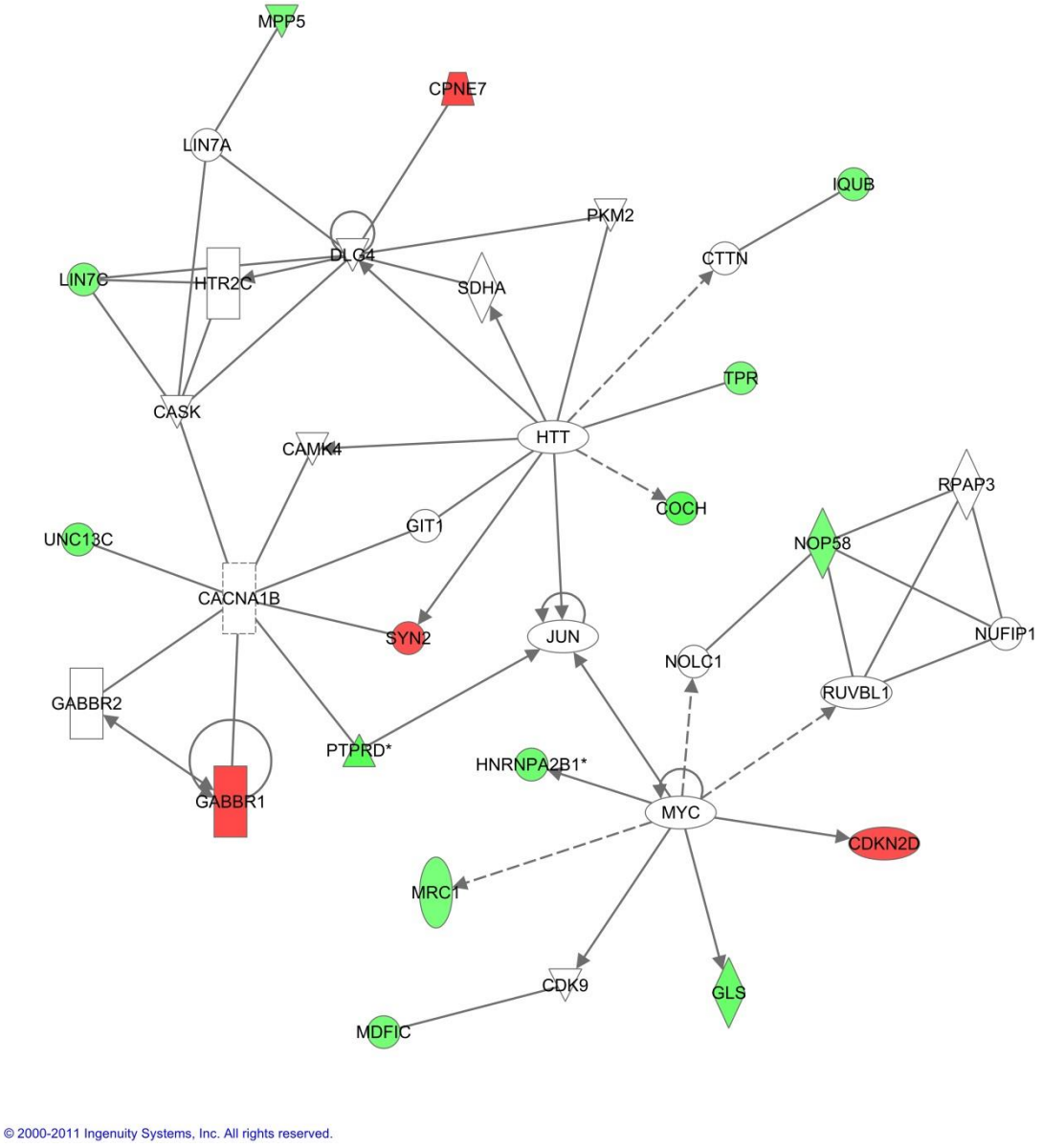


Figure 3 Secondary network of differentially expressed genes identified in the adult rat hippocampus after chronic low level exposure to diazinon using Ingenuity pathway analysis. The main functions of this network are Cell-To-Cell signalling, Nervous system development and function, and Molecular transport. Up-regulated and down-regulated genes are highlighted in green and red respectively.

3.5. Gene expression in the dorsal raphe nucleus is affected by chronic low level exposure to diazinon

Of the 47,121 probe sets significantly detected in dorsal raphe samples (23,558 up-regulated, 23,563 down-regulated), the signal intensity of 70 was significantly different between samples from vehicle and diazinon treated animals (Table 6, full list available at Judge et al. (2017)).

Diazinon exposure up-regulated several dorsal raphe nucleus genes encoding proteins associated with synaptic transmission: the α_{1b}-adrenoceptor (*adra 1b*; 1.50 fold change), the main driver of 5-HT neuronal excitation (Baraban and Aghajanian, 1980), the regulator of G protein signalling 4 (*rgs4*; 1.88 fold change), which dampens the effects of G-protein coupled receptors including the inhibitory 5-HT_{1A} autoreceptor in the dorsal raphe nucleus (Beyer et al., 2004), gastrin-releasing peptide (*Grp*; 1.89 fold change), which depolarises 5-HT neurones (Pinnock and Woodruff, 1991) and complexin 3 (*cplx3*; 1.77 fold change), which facilitates neurotransmitter release (Yang et al., 2013). In contrast, genes encoding proteins associated with xenobiotic uptake and metabolism were down-regulated: the cytochrome P450, family 1, subfamily B, polypeptide (*cyp1b1*; 0.52 fold change), which metabolises xenobiotics and the solute carrier organic anion transporter family 1A2 (*Slco1a5*; 0.71 fold change), involved in cellular uptake.

Ingenuity pathway analysis revealed that the genes in the dorsal raphe nucleus significantly affected by diazinon exposure were most strongly associated with development. The most over represented functions were Embryonic Development and Nervous System Development and Function (Table 7, full list available at Judge et al. (2017)). The

Psychological Disorders function was also over represented. Canonical pathways were identified but none were over represented. The highest scoring network (network containing the most genes affected by diazinon exposure) was the Endocrine system development, Lipid metabolism and Small molecule biochemistry network (Figure 4; full list available at Judge et al. (2017)). Central to this network are genes encoding proteins associated with synaptic structure and synaptic signalling: extracellular regulated kinase 1/2 (*erk1/2*), involved in many processes including proliferation and synaptic plasticity, and huntingtin (*htt)*, the α_{1b}-adrenoceptor (*adra1b*) and regulator of G protein signalling 4 (*rgs4*), mentioned previously. In addition the network is enriched with genes encoding endocrine system proteins: transthyretin (*ttr*), which transports thyroid hormone, prolactin receptor (*prlr*) and follicle-stimulating hormone (*fsh*).

Table 6 List of dorsal raphe nucleus genes with most significant differences in expression between rats treated with vehicle and diazinon. Fold changes above 1 are up-regulated, fold changes below 1 are down-regulated.

Gene name	Protein name	Fold change	P Value
Hs3st2	Heparan sulfate glucosamine 3-O-sulfotransferase 2	2.08	0.000
IPCEF1	Interaction protein for cytohesin exchange factors 1	2.38	0.000
Nxph3	Neurexophilin-3	1.88	0.000
Rprm	Reprimo, TP53 dependent G2 arrest mediator candidate	2.16	0.000
Ldb2	LIM domain binding 2	1.93	0.002
Cbln2	Cerebellin-2	1.87	0.003
Grp	Gastrin-releasing peptide	1.89	0.003
Bmper	BMP-binding endothelial regulator	1.84	0.003
Rgs4	Regulator of G-protein signaling 4	1.88	0.003
Gfra2	Glial cell line derived neurotrophic factor family receptor alpha 2	1.86	0.003
Sstr1	Somatostatin receptor	1.84	0.003
Cplx3	Complexin III	1.77	0.005
Foxp2	Forkhead box protein P2	2.01	0.010
Igfbp6	Insulin-like growth factor-binding protein 6	1.68	0.010
Homer1	Homer protein homolog 1	1.63	0.011
P4ha3	Prolyl 4-hydroxylase subunit alpha 3	1.69	0.012
Rgs 4	Regulator of G-protein signaling 4	1.71	0.016
GARNL3	GTPase activating Rap/RanGAP domain-like 3	1.63	0.021
Pbld	Phenazine biosynthesis-like domain-containing protein	1.51	0.025
Myh2 /// Myh3	Myosin heavy chain	1.39	0.034
Inpp5d	Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 1	1.54	0.038
adra1b	α-1B adrenergic receptor	1.50	0.042
Mfrp	Membrane frizzled-related protein	0.48	0.000
Sostdc1	Sclerostin domain-containing protein 1	0.52	0.000
Cyp1b1	Cytochrome P450 1B1	0.52	0.002
F5	Coagulation factor V	0.68	0.002
Tmem27	Transmembrane protein 27	0.52	0.004
Otx2	Homeobox protein OTX2	0.56	0.004
Prlr	Prolactin receptor, isoform CRA_a	0.53	0.008
col8a2	Collagen, type VIII, alpha 2	0.65	0.013
Slco1a5	Sodium-independent organic anion transporter 1	0.71	0.014
Folr1	Folate receptor 1	0.70	0.014
Kcne2	Potassium voltage-gated channel subfamily E member 2	0.68	0.026
Rbm47	RNA-binding protein 47	0.66	0.027
Ttr	Transthyretin	0.50	0.027
Krt18	Keratin, type I cytoskeletal 18	0.68	0.037
Col8a1	Collagen, type VIII, alpha 1	0.60	0.038
slc13a4	Solute carrier family 13 member 4	0.72	0.040
Scgb1c1	Secretoglobin family 1C member 1	0.75	0.050

4. Discussion

Chronic oral exposure to the pesticide diazinon during adulthood, below the threshold to significantly affect AChE or BChE activity, significantly altered anxiety-like behaviour. Furthermore, chronic exposure to diazinon altered the expression of genes associated with synaptic transmission and plasticity, and neuronal development in brain regions associated with psychological disorders and neurobehavioural deficits.

4.1. Length and level of exposure

In this study adult animals were exposed to diazinon for 12 weeks. Whilst we accept this does not mimic exposure to diazinon through the use of sheep dip a few times a year, it does relate to workers that regularly use diazinon (for example

sheep dipping contractors), people consuming fruit and vegetables contaminated with diazinion (Melnyk et al., 2016), and people living with pets coated with diazinon; flea collars containing as much as 3.6 g of diazinon slowly release the product to spread over the pet's coat. Animals were orally exposed to 1 - 2 mg / kg diazinon which we consider to be low level exposure. Diazinon exposure did not significantly affect AChE and BChE activity and though choline O-acetyltransferase gene expression (*chat*) was down-regulated, indicating a decrease in hippocampal acetylcholine, no other cholinergic genes were affected. Taken together this would suggest that diazinon exposure was insufficient to inhibit AChE activity and that the down-regulation of *chat* was due to another mechanism (see Gene expression changes subsection).

Table 7 Biological Functions most relevant to dorsal raphe nucleus genes significantly altered by chronic diazinon exposure in the adult rat identified using Ingenuity Pathway Analysis.

Biological Functions	**P value**	**Genes**
Embryonic Development	1.11E-05-4.95E-02	COL8A2,FOXP2,SOSTDC1,RGS4,COL8A1,BMPER,GFRA2,PRLR,CYP1B1,ADRA1B,OTX2,LDB2
Nervous System Development and Function	1.11E-05-4.95E-02	CPLX3,IGFBP6,FOXP2,COL8A2,TTR,COL8A1,RGS4,GFRA2,GRP,HOMER1,CYP1B1,OTX2
Organ Development	1.11E-05-4.95E-02	COL8A2,FOXP2,SOSTDC1,COL8A1,GFRA2,PRLR,HOMER1,OTX2,CYP1B1,ADRA1B,LDB2
Organismal Development	1.11E-05-4.95E-02	COL8A2,FOXP2,SOSTDC1,RGS4,COL8A1,BMPER,GFRA2,PRLR,OTX2,CYP1B1,ADRA1B,LDB2
Tissue Development	1.11E-05-4.95E-02	COL8A2,FOXP2,COL8A1,RGS4,GRP,GFRA2,PRLR,CYP1B1,ADRA1B,INPP5D,OTX2,LDB2
Visual System Development and Function	1.11E-05-3.49E-02	COL8A2,FOXP2,MFRP,COL8A1,CYP1B1,OTX2
Developmental Disorder	1.42E-05-4.18E-02	COL8A2,FOXP2,MFRP,TTR,RGS4,SSTR1,GRP,GFRA2,CYP1B1,OTX2,INPP5D,HOMER1,ADRA1B
Psychological Disorders	2.16E-05-4.99E-02	SHOC2,FOXP2,TTR,GARNL3,IPCEF1,SSTR1,COL8A1,RGS4,P4HA3,HOMER1,ADRA1B,LDB2
Cancer	3.73E-05-4.95E-02	IGFBP6,TTR,SSTR1,RGS4,GRP,CYP1B1,INPP5D,LDB2,SOSTDC1,HS3ST2,PRLR,TMEM27,ADRA1B

4.2. Behaviour

We have previously reported that marble burying behaviour is a sensitive behavioural measure of low level OP exposure (Savy et al., 2015), and the current study supports this. Marble burying behaviour is frequently described as an ethological measure of anxiety in rodents and has been used to screen anxiolytic drugs in rodents (Broekkamp et al., 1986; Njung'e and Handley, 1991). However, it has been suggested that the marble burying test is instead a measure of repetitive behaviour (digging) and the marbles are buried as a by-product of digging (Thomas et al., 2009). If the marble burying test quantified anxiety-like behaviour, repeated testing would induce habituation and reduce anxiety-like behaviour. In vehicle-treated rats in this study none of the measures associated with digging (e.g. number of marbles buried, latency to first dig etc) were affected by repeated testing, indicating that these measures are probably not a measure of anxiety. Nevertheless, the number of marbles buried and the latency to first dig were sensitive to chronic diazinon exposure and indicates that exposure reduced repetitive behaviour.

The biological changes underlying the diazinon-induced reductions in marble-burying are still unclear Lesioning studies have demonstrated the involvement of the hippocampus, nucleus accumbens and dorsal raphe nucleus in defensive burying behaviour (De Boer and Koolhaas, 2003) and reduced marble burying behaviour is correlated with increased 5-HT neurotransmission (Kobayashi et al., 2008; Njung'e and Handley, 1991). We have previously shown that acute diazinon exposure can increase 5-HT neuronal firing in the dorsal raphe nucleus (Judge et al., 2016), which would lead to an increase in 5-HT release in forebrain areas. In this study the up-regulation of the genes encoding the α_{1b}-adrenoceptor, the main driver of 5-HT neuronal excitation (Baraban and Aghajanian, 1980) and the regulator of G protein signalling 4, which dampens the effects of G-protein coupled receptors including the inhibitory 5-HT_{1A} autoreceptor in the dorsal raphe nucleus (Beyer et al., 2004), would suggest that chronic low level diazinon exposure also stimulates 5-HT neuronal firing. Taken together we conclude that chronic diazinon exposure reduces marble-burying behaviour through an increase in 5-HT neurotransmission.

4.3. Gene expression changes

We used microarrays to make a comprehensive assessment of the effects of chronic diazinon exposure on the hippocampus and dorsal raphe nucleus. This approach allowed us to detect 31,842 probe sets in hippocampal samples and 47,121 probe sets in dorsal raphe nucleus samples. Detecting thousands of genes will lead to false positives which we controlled for statistically.

Genes associated with the Psychological Disorders function were significantly affected by diazinon exposure in both the hippocampus and dorsal raphe nucleus. Whilst this adds weight to the studies reporting low level OP exposure is associated with anxiety and depression (Bazylewicz-Walczak et al., 1999; Mackenzie Ross et al., 2010; Salvi et al., 2003; Steenland et al., 2000; Stephens et al., 1995), the following need to be considered. The significant gene expression changes identified in this study were not confirmed using real-time polymerase chain reaction (RT-PCR), and the corresponding protein products were not quantified and so we cannot state with certainty that the individual changes detected resulted in changes in protein levels. Nevertheless, gene expression changes are frequently indicative of protein level changes, multiple genes affected by diazinon exposure were associated with a particular function or network or pathway, and some of the significant gene expression changes are consistent with previously published studies (see below), all of which increases the likelihood the gene expression changes are relevant.

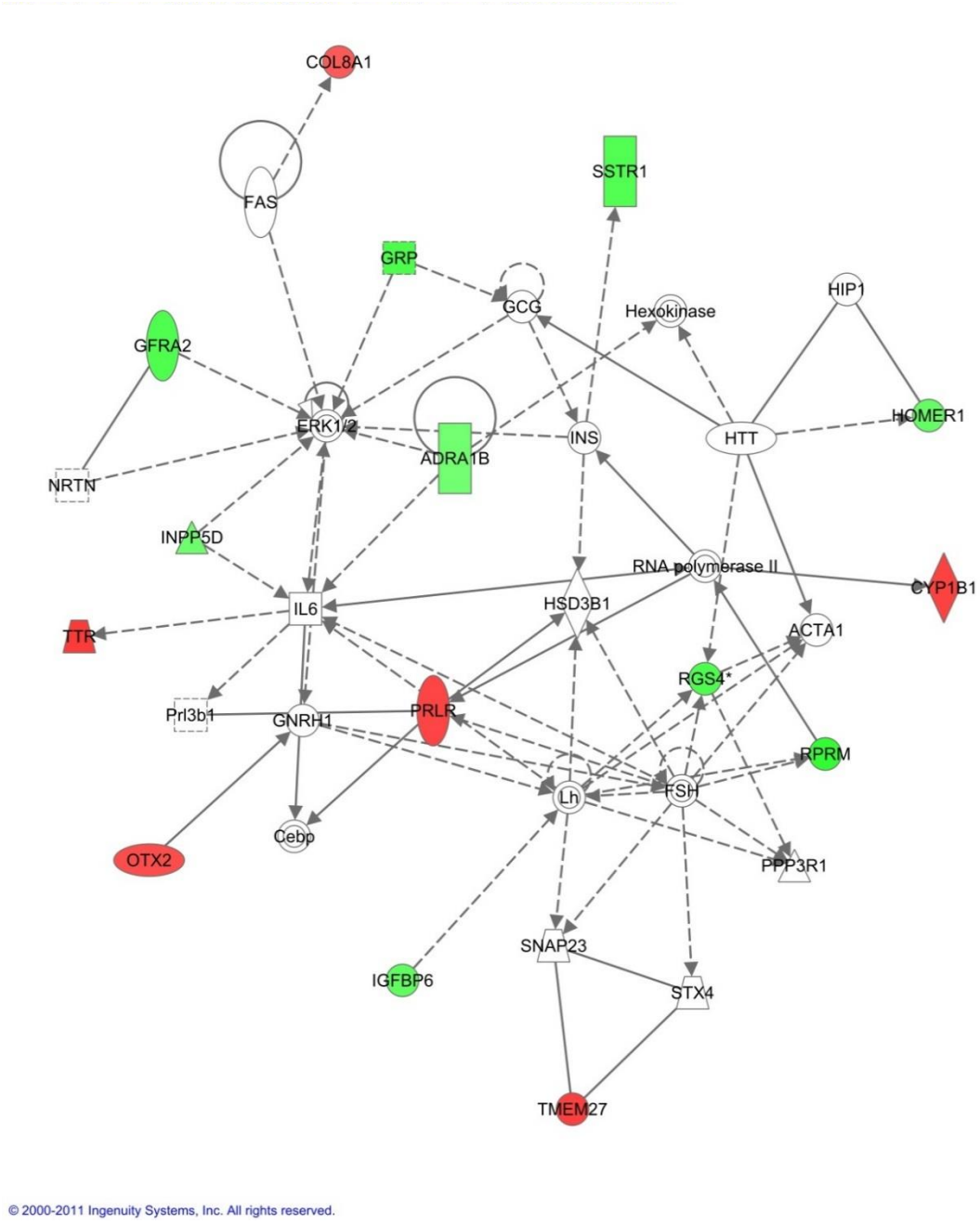


Figure 4 Primary network of differentially expressed genes identified in the adult rat dorsal raphe nucleus after chronic low level exposure to diazinon using Ingenuity Pathway Analysis. The main functions of this network are Endocrine system development, Lipid metabolism and Small molecule biochemistry. Up-regulated and down-regulated genes are highlighted in green and red respectively.

Many of the genes affected by diazinon exposure in both the hippocampus and dorsal raphe nucleus were associated with synaptic transmission and, in particular, monoaminergic neurotransmission. As mentioned above gene expression changes indicate chronic diazinon exposure stimulates 5-HT neurotransmission (see Behaviour subsection). Likewise, diazinon exposure up-regulated genes associated with dopaminergic neurotransmission. These data are consistent with the gene expression changes induced by neonatal diazinon exposure (Slotkin and Seidler, 2007a), and, although *th* (tyrosine hydroxylase) was the only monoaminergic gene common to both studies, the gene expression changes overall indicate the same stimulatory effect on monoaminergic neurotransmission. In addition, chronic diazinon exposure in adults appears to stimulate glutamatergic neurotransmission, demonstrated by the up-regulation of genes encoding glutaminase, mitogen-activated protein kinase 8, Glu3, one of the AMPA receptor subunits, neurexin 3, which stabilises AMPA receptors in the neuronal membrane (Aoto et al., 2015) and Homer protein homolog 1 which has been implicated in the regulation of metabotropic glutamate receptors (mGluR1a and mGluR5) (Bottai et al., 2002; Reim et al., 2001). Exposure of PC12 cells, a developmental model, and neonatal rats to diazinon altered the expression of glutamate transporter and receptor genes (Slotkin and Seidler, 2007a; Slotkin and Seidler, 2009). Again, although dissimilar genes are affected, it appears that diazinon stimulates glutamatergic neurotransmission in neonates and adults.

What we had not predicted was that the most significantly affected genes, particularly in the dorsal raphe nucleus, would be associated with development. The increased expression of genes strongly associated with proliferation, such as *neurod1* and *kitlg*, and the Cell Death, Genetic Disorder and Cellular Assembly and Organisation network being the highest scoring network in the hippocampus, may indicate chronic diazinon exposure causes neuronal death followed by neurogenesis. Alternatively, the increased expression of genes associated with cellular assembly and organisation may indicate that chronic diazinon exposure causes neuronal restructuring. Reorganisation and strengthening synapses to support increased neurotransmission is consistent with the other gene expression changes reported here. Previous reports on neonatal exposure indicate that diazinon exposure alters the expression of genes involved in neural cell development (Mankame, Hokanson et al. 2006; Slotkin and Seidler 2007; Slotkin and Seidler 2010; Slotkin and Seidler 2012). Given the overall similarities between the gene expression changes reported in neonates and here in adults, we postulate that the mechanism of action underlying diazinon-induced neurological effects during development may be similar to the mechanism of action in adulthood.

To date this is the first study to report on genome wide changes following low level diazinon exposure in adulthood. Adulthood exposure for 21 days to another organophosphate pesticide, chlorpyrifos, has previously been reported to alter hippocampal gene expression but only at levels which induce ~ 90% cholinesterase inhibition, lower doses which induce ~50% ChE inhibition did not significantly alter gene expression, using microarray analysis (Lee et al., 2016). Furthermore, the vast majority of genes altered by diazinon ($n = 70$) and chlorpyrifos ($n = 50$) were different, with chlorpyrifos significantly affecting genes encoding neurotrophins and neuropeptides such as brain-derived neurotrophic factor (Lee et al., 2016). As neurotrophins and neuropeptides are involved in neural development and psychological disorders it could be argued that that there may be a shared mechanism of action, but given the differences in gene expression and that low level exposure to diazinon and chlorpyrifos can have disparate neurochemical effects (Savy et al., 2015), diazinon may have neurological effects below the threshold for cholinesterase inhibition that chlorpyrifos does not.

4.4. Conclusion

Chronic diazinon exposure levels below the threshold to inhibit cholinesterase, can alter gene expression. Some of the genes most significantly affected are associated with the Psychological Disorders gene set, and glutamatergic and monoaminergic neurotransmission, which plays a key role in neurobehaviour and psychological disorders. Genes associated with development were also significantly affected by diazinon exposure in adulthood. Whilst the results should be considered in view that corresponding protein products were not quantified, the gene expression changes are sufficient to cause behavioural changes. Taken together the data are consistent with chronic low level diazinon exposure causing adverse neurological effects.

Acknowledgements

This work was supported by the Health Protection Agency (UK), a Department of Health Policy Research Programme (UK) and Newcastle University.

References

Adler, S., et al., 2011. Alternative (non-animal) methods for cosmetics testing: current status and future prospects-2010. Arch Toxicol. 85**,** 367-485. 10.1007/s00204-011-0693-2.

Aldridge, J. E., et al., 2005. Developmental exposure of rats to chlorpyrifos leads to behavioral alterations in adulthood, involving serotonergic mechanisms and resembling animal models of depression. Environ Health Perspect. 113**,** 527-31.

Aldridge, J. E., et al., 2003. Serotonergic systems targeted by developmental exposure to chlorpyrifos: effects during different critical periods. Environ Health Perspect. 111**,** 1736-43.

Alessi, D. R., et al., 1996. Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO Journal. 15**,** 6541-6551.

Aoto, J., et al., 2015. Distinct circuit-dependent functions of presynaptic neurexin-3 at GABAergic and glutamatergic synapses. Nat Neurosci. 18**,** 997-1007. 10.1038/nn.4037.

Baraban, J. M., Aghajanian, G. K., 1980. Suppression of firing activity of 5-HT neurons in the dorsal raphe by alpha-adrenoceptor antagonists. Neuropharmacology. 19**,** 355-63.

Bazylewicz-Walczak, B., et al., 1999. Behavioral effects of occupational exposure to organophosphorous pesticides in female greenhouse planting workers. Neurotoxicology. 20**,** 819-826.

Benjamini, Y., Hochberg, Y., 1995. Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society Series B-Methodological. 57**,** 289-300.

Beyer, C. E., et al., 2004. Regulators of G-protein signaling 4: modulation of 5-HT1A-mediated neurotransmitter release in vivo. Brain Res. 1022**,** 214-20. 10.1016/j.brainres.2004.06.073.

Binder, E. B., et al., 2001. Neurotensin and dopamine interactions. Pharmacol Rev. 53**,** 453-86.

Bottai, D., et al., 2002. Synaptic activity-induced conversion of intronic to exonic sequence in Homer 1 immediate early gene expression. Journal of Neuroscience. 22**,** 167-175.

Broekkamp, C. L., et al., 1986. Major tranquillizers can be distinguished from minor tranquillizers on the basis of effects on marble burying and swim-induced grooming in mice. Eur J Pharmacol. 126**,** 223-9.

Cattaneo, E., et al., 2001. Loss of normal huntingtin function: new developments in Huntington's disease research. Trends Neurosci. 24**,** 182-8.

Daniell, W., et al., 1992. Neuropsychological performance among agricultural pesticide applicators. Environ Res. 59**,** 217-28.

de Blaquiere, G. E., et al., 2000. Electrophysiological and biochemical effects of single and multiple doses of the organophosphate diazinon in the mouse. Toxicol Appl Pharmacol. 166**,** 81-91. 10.1006/taap.2000.8960.

De Boer, S. F., Koolhaas, J. M., 2003. Defensive burying in rodents: ethology, neurobiology and psychopharmacology. Eur J Pharmacol. 463, 145-61.
Ellman, G. L., et al., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 7, 88-95.
Elstner, M., et al., 2011. Expression analysis of dopaminergic neurons in Parkinson's disease and aging links transcriptional dysregulation of energy metabolism to cell death. Acta Neuropathol. 122, 75-86. 10.1007/s00401-011-0828-9.
Fiedler, N., et al., 1997. Long-term use of organophosphates and neuropsychological performance. Am J Ind Med. 32, 487-496. Doi 10.1002/(Sici)1097-0274(199711)32:5<487::Aid-Ajim8>3.0.Co;2-P.
Gutekunst, C. A., et al., 1995. Identification and localization of huntingtin in brain and human lymphoblastoid cell lines with anti-fusion protein antibodies. Proc Natl Acad Sci U S A. 92, 8710-4.
Hong, F., et al., 2006. RankProd: a bioconductor package for detecting differentially expressed genes in meta-analysis. Bioinformatics. 22, 2825-7. 10.1093/bioinformatics/btl476.
Hsueh, Y. P., 2006. The role of the MAGUK protein CASK in neural development and synaptic function. Current Medicinal Chemistry. 13, 1915-1927.
Jameson, R. R., et al., 2006. Chlorpyrifos affects phenotypic outcomes in a model of mammalian neurodevelopment: critical stages targeting differentiation in PC12 cells. Environ Health Perspect. 114, 667-72.
Judge, S. J., et al., 2016. Mechanism for the acute effects of organophosphate pesticides on the adult 5-HT system. Chem Biol Interact. 245, 82-9. 10.1016/j.cbi.2015.12.014.
Judge, S. J., et al., 2017. Differential gene expression in the rat hippocampus and dorsal raphe nucleus following chronic low level exposure to the pesticide diazinon. Mendeley Data, v1. http://dx.doi.org/10.17632/wxwbd88724.1
Kobayashi, T., et al., 2008. Neurochemical responses to antidepressants in the prefrontal cortex of mice and their efficacy in preclinical models of anxiety-like and depression-like behavior: a comparative and correlational study. Psychopharmacology (Berl). 197, 567-80. 10.1007/s00213-008-1070-6.
Lee, J. S., et al., 2013. Superpriming of synaptic vesicles after their recruitment to the readily releasable pool. Proc Natl Acad Sci U S A. 110, 15079-84. 10.1073/pnas.1314427110.
Lee, Y. S., et al., 2016. Repeated exposure to neurotoxic levels of chlorpyrifos alters hippocampal expression of neurotrophins and neuropeptides. Toxicology. 340, 53-62. 10.1016/j.tox.2016.01.001.
Levin, E. D., et al., 2001. Persistent behavioral consequences of neonatal chlorpyrifos exposure in rats. Brain Res Dev Brain Res. 130, 83-9.
Mackenzie Ross, S. J., et al., 2010. Neuropsychological and psychiatric functioning in sheep farmers exposed to low levels of organophosphate pesticides. Neurotoxicol Teratol. 32, 452-9. 10.1016/j.ntt.2010.03.004.
Mankame, T., et al., 2006. Alteration of gene expression in human cells treated with the agricultural chemical diazinon: possible interaction in fetal development. Hum Exp Toxicol. 25, 225-33.
Melnyk, L. J., et al., 2016. Prioritization of pesticides based on daily dietary exposure potential as determined from the SHEDS model. Food Chem Toxicol. 96, 167-73. 10.1016/j.fct.2016.07.025.
Meyer-Baron, M., et al., 2015. Meta-analysis on occupational exposure to pesticides - Neurobehavioral impact and dose-response relationships. Environmental Research. 136, 234-245. 10.1016/j.envres.2014.09.030.
Morris, C. M., et al., Acute Toxicity of Organophosphorus Compounds. In: M. Balali-Mood, M. Abdollahi, (Eds.), Basic and Clinical Toxicology of Organophosphorus Compounds. Springer-Verlag London, 2014, pp. 45-78.
Nasir, J., et al., 1995. Targeted disruption of the Huntington's disease gene results in embryonic lethality and behavioral and morphological changes in heterozygotes. Cell. 81, 811-23.
Njung'e, K., Handley, S. L., 1991. Evaluation of marble-burying behavior as a model of anxiety. Pharmacol Biochem Behav. 38, 63-7.
Pinnock, R. D., Woodruff, G. N., 1991. Bombesin excites a subpopulation of 5-hydroxytryptamine-sensitive neurones in the rat dorsal raphe nucleus in vitro. Journal of Physiology. 440, 55-65.
Povey, A. C., et al., 2014. Pesticide exposure and screen-positive neuropsychiatric disease in British sheep farmers. Environ Res. 135, 262-70. 10.1016/j.envres.2014.09.008.
Reim, K., et al., 2001. Complexins Regulate a Late Step in Ca2+-Dependent Neurotransmitter Release. Cell. 104, 71-81. 10.1016/s0092-8674(01)00192-1.
Rodnitzky, R. L., et al., 1975. Occupational Exposure to Organophosphate Pesticides - Neurobehavioral Study. Archives of Environmental Health. 30, 98-103.
Roegge, C. S., et al., 2008. Developmental diazinon neurotoxicity in rats: later effects on emotional response. Brain Res Bull. 75, 166-72. 10.1016/j.brainresbull.2007.08.008.
Salvi, R. M., et al., 2003. Neuropsychiatric evaluation in subjects chronically exposed to organophosphate pesticides. Toxicol Sci. 72, 267-71. 10.1093/toxsci/kfg034.
Saroja, S. R., et al., 2014. Hippocampal proteoglycans brevican and versican are linked to spatial memory of Sprague-Dawley rats in the morris water maze. J Neurochem. 130, 797-804. 10.1111/jnc.12783.
Savy, C. Y., et al., 2015. Low-level repeated exposure to diazinon and chlorpyrifos decrease anxiety-like behaviour in adult male rats as assessed by marble burying behaviour. Neurotoxicology. 50, 149-156. 10.1016/j.neuro.2015.08.010.
Slotkin, T. A., Seidler, F. J., 2007a. Comparative developmental neurotoxicity of organophosphates in vivo: transcriptional responses of pathways for brain cell development, cell signaling, cytotoxicity and neurotransmitter systems. Brain Res Bull. 72, 232-74. 10.1016/j.brainresbull.2007.01.005.
Slotkin, T. A., Seidler, F. J., 2007b. Prenatal chlorpyrifos exposure elicits presynaptic serotonergic and dopaminergic hyperactivity at adolescence: critical periods for regional and sex-selective effects. Reprod Toxicol. 23, 421-7. 10.1016/j.reprotox.2006.07.010.

Slotkin, T. A., Seidler, F. J., 2009. Oxidative and excitatory mechanisms of developmental neurotoxicity: transcriptional profiles for chlorpyrifos, diazinon, dieldrin, and divalent nickel in PC12 cells. Environ Health Perspect. 117, 587-96. 10.1289/ehp.0800251.

Slotkin, T. A., Seidler, F. J., 2010. Diverse neurotoxicants converge on gene expression for neuropeptides and their receptors in an in vitro model of neurodifferentiation: effects of chlorpyrifos, diazinon, dieldrin and divalent nickel in PC12 cells. Brain Res. 1353, 36-52. 10.1016/j.brainres.2010.07.073.

Slotkin, T. A., Seidler, F. J., 2012. Does mechanism matter? Unrelated neurotoxicants converge on cell cycle and apoptosis during neurodifferentiation. Neurotoxicol Teratol. 34, 395-402. 10.1016/j.ntt.2012.04.008.

Srivastava, A. K., et al., 2000. Clinical, biochemical and neurobehavioural studies of workers engaged in the manufacture of quinalphos. Food and Chemical Toxicology. 38, 65-69. Doi 10.1016/S0278-6915(99)00123-4.

Steenland, K., et al., 2000. Neurologic function among termiticide applicators exposed to chlorpyrifos. Environmental Health Perspectives. 108, 293-300. Doi 10.2307/3454346.

Stephens, R., et al., 1995. Neuropsychological effects of long-term exposure to organophosphates in sheep dip. Lancet. 345, 1135-9.

Stephenson, F. A., 2006. Structure and trafficking of NMDA and GABAA receptors. Biochemical Society Transactions. 34, 877-881.

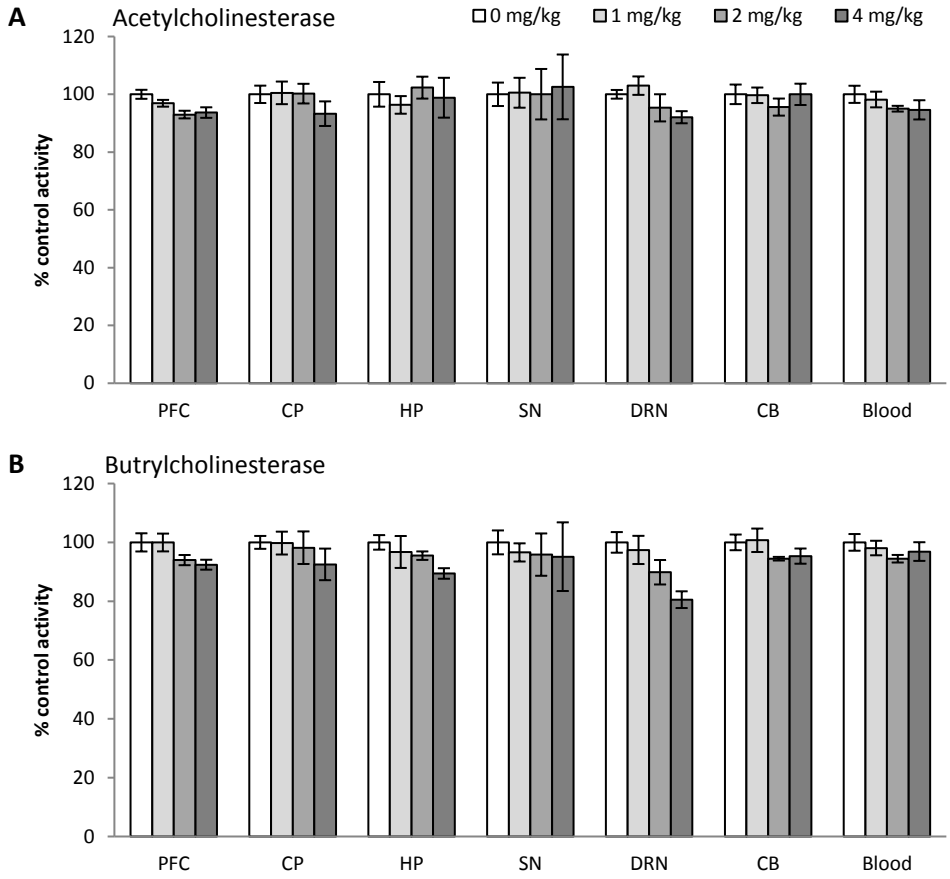
Thomas, A., et al., 2009. Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. Psychopharmacology (Berl). 204, 361-73. 10.1007/s00213-009-1466-y.

Timofeeva, O. A., et al., 2008. Persistent cognitive alterations in rats after early postnatal exposure to low doses of the organophosphate pesticide, diazinon. Neurotoxicol Teratol. 30, 38-45. 10.1016/j.ntt.2007.10.002.

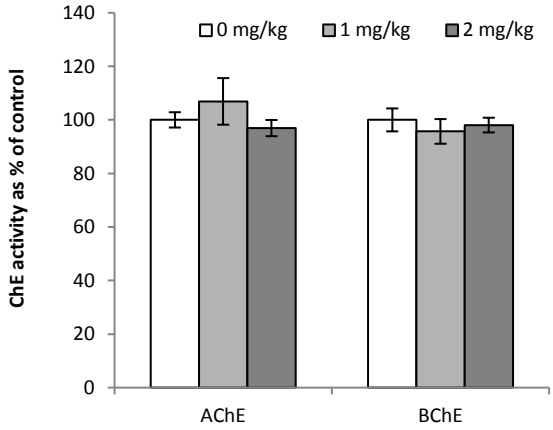
Vasileva, M., et al., 2012. Synapsin-dependent reserve pool of synaptic vesicles supports replenishment of the readily releasable pool under intense synaptic transmission. Eur J Neurosci. 36, 3005-20. 10.1111/j.1460-9568.2012.08225.x.

Yang, X., et al., 2013. Deconstructing complexin function in activating and clamping Ca2+-triggered exocytosis by comparing knockout and knockdown phenotypes. Proc Natl Acad Sci U S A. 110, 20777-82. 10.1073/pnas.1321367110.

SUPPLEMENTARY DATA



Supplementary Figure 1. Acetylcholinesterase (AChE) and butrylcholinesterase (BChE) activity following 5 day repeated oral exposure to the pesticide diazinon in the adult rat. **A.** AChE activity was not affected by diazinon exposure (0, 1, 2, 4 mg /kg, $n = 6$ per dose; main effect of treatment $F_{(3,17)} = 0.90$, $p = 0.46$, main effect of region $F_{(6,102)} = 0.51$, $p = 0.80$, treatment*region interaction $F_{(18,102)} = 0.45$, $p = 0.97$). **B**. BChE activity was affected by diazinon exposure (main effect of treatment $F_{(3,18)} = 3.94$, $p < 0.05$, main effect of region $F_{(6,108)} = 0.55$, $p = 0.77$, treatment*region interaction $F_{(18,108)} = 0.45$, $p = 0.97$) but only 4 mg / kg diazinon was significantly different to 0 mg / kg (Bonferonni post hoc comparisons, $p < 0.05$). Mean ± SEM. Prefrontal cortex (PFC), caudate putamen (CP), hippocampus (HP), substantia nigra (SN), cerebellum (CB), dorsal raphe nucleus (DRN).



Supplementary Figure 2. Blood acetylcholinesterase (AChE) and butrylcholinesterase (BChE) activity is not inhibited by six weeks oral exposure to the pesticide diazinon. Diazinon (0, 1, 2 mg /kg, $n = 12$ per dose) had no effect on blood AChE ($F_{(2,33)} = 1.3$, $p = 0.28$; 0 mg / kg = 3.9 ± 0.1 nmol / min µl blood) or BChE activity ($F_{(2,33)} = 2.0$, $p = 0.15$; 0 mg / kg = 3.2 ± 0.1 nmol / min µl blood). Mean ± SEM.