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METHAMIDOPHOS, AN ORGANOPHOSPHORUS INSECTICIDE, INDUCES PRO-AGGRESSIVE BEHAVIOUR IN MICE

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Running Title: Methamidophos induces pro-aggressive behaviour in mice

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Abstract

Although evidence indicates that exposure to organophosphorus (OP) pesticides induces neurobehavioral disorders, little is known about the effects of OP on aggressive behaviour. Our study investigated the effects of repeated exposure to an OP pesticide, methamidophos, on the isolation-induced aggressive behaviour in mice. Forty seven male mice were individually housed for a month. Socially isolated animals were then confronted with a standard non-isolated opponent for 15 minutes (pre-treatment trial), and the latency and frequency of aggressive and general exploratory behaviours were recorded. Based on the presence of attack behaviour in the pre-treatment trial, mice were classified as isolation-induced aggressive and non-aggressive. All mice were then treated for seven days with methamidophos (3.5 mg/kg/day, n=22, ip) or saline (1 mL/kg/day, control group, n=25, ip) and a second trial performed. Repeated exposure to methamidophos induced attack behaviour in non-aggressive mice. The treatment with methamidophos also decreased plasma butyrylcholinesterase and brain acetylcholinesterase activity. These results suggest that methamidophos has a pro-aggressive effect on socially isolated mice.

1. Introduction

Methamidophos (O,S-dimethyl phosphoroamidothioate) is a highly toxic organophosphorus (OP) pesticide widely used worldwide (Caldas et al. 2011; Caldas et al., 2006; Gray, 1982; Lu 2010; Robinson and Beiergrohslein, 1982; Yu et al. 2015). Among the possible sources of human exposure, the diet route poses a substantial risk and recent reports show that methamidophos is the highest-ranking pesticide exceeding by 100% the acceptable daily intake (Melnyk et al. 2016). Occupational exposure is also widespread, particularly amongst farmers, where the combination of absence of personal protection equipment during handling and a high frequency of usage may lead to acute and long term poisoning (Recena et al. 2006b; Recena et al. 2006a). Worldwide around 350,000 cases of self-poisoning with pesticides are recorded per year, and it is estimated that nearly 30% of global suicides are due to pesticide self-poisoning, with most cases recorded in low and middle income countries (Gunnell et al., 2007; Hulse et al., 2014).

Acute exposure to this OP induces severe systemic disturbances, mostly associated with acetylcholine (ACh) accumulation on synaptic clefts due to inhibition of acetylcholinesterase (AChE) activity (Chowdhary et al. 2014; Ecobichon, 2000; Jeyaratnam, 1990). The symptoms observed after acute poisoning mainly result from overstimulation of cholinergic receptors present in synapses of the autonomic nervous system, central nervous system and neuromuscular junctions (O'Malley 1997; Eddleston et al. 2008b). Although acute symptoms are the most alarming because of their severity and even fatality, prolonged exposure to non-lethal doses of OP also presents with negative neurological effects [see Mackenzie Ross et al., 2013 for review].

Central nervous system exposure to OP is high due to their lipophilicity which confers a great ability to cross the blood brain barrier (Ferrer 2003). Measurements of brain AChE activity combined with anatomical and behavioural studies provide evidence of OP-induced neurological effects in animal models (Ali et al. 1980; Socko et al. 1999; Sánchez-Amate et al. 2001a; Pelegrino et al. 2006; López-Crespo et al. 2007a; Lima et al. 2009a). Pelegrino and colleagues (2006) showed that repeated administration of sublethal doses of methamidophos induces atrophy of the molecular layer of the parietal cortex of rats. Neurobehavioural changes such as reduction in locomotor and exploratory activity (Ali et al. 1980; Socko et al. 1999; López-Crespo et al. 2007b), as well as depressive (Lima et al. 2009b) and anxiety-like behaviour (Sánchez-Amate et al. 2001b) have also been described after experimental exposure to OP pesticides. Although a number of studies point to important effects on behaviour, the effects of these compounds on aggressive behaviour remains poorly explored.

Clinical reports also provide evidence for OP-induced neurological disorders. Humans exposed to OP acute toxicity present a higher risk of depression, suicide or cognitive impairment (Savage et al. 1988; Rosenstock et al. 1991; Stallones and Beseler 2002; Beseler et al. 2006). A higher percentage of anxiety and depression was observed in sheep farmers chronically exposed to OP pesticides when compared with non-exposed individuals (Stephens et al. 1995; Mackenzie Ross et al. 2010). Likewise, an increased risk of depression (Beseler et al. 2008) and suicide (Parrón et al. 1996; Wesseling et al. 2010) has also been described among individuals chronically exposed to OP when compared with subjects with similar socioeconomic and demographic characteristics.

A large number of the aforementioned psychiatric disorders show association with aggressive behaviour (Haller and Kruk 2006), and there are some reports of unprovoked aggressive behaviour, including two homicides, following short and long term exposure to cholinesterase inhibiting pesticides (Devinsky et al. 1992). Further, experimental studies show that rats chronically exposed to the OP dichlorvos exhibited marked aggression compared with control animals (Sarin and Gill 1998). Despite the evidence mentioned above, the effects of short term exposure to OPs on aggressive behaviour remain poorly understood. This has implications for the prevention of early psychiatric episodes, particularly among populations frequently exposed to OPs. Better understanding of this feature of OP exposure is highly relevant for socio-economic policy making in impoverished countries whose economies are dependent on agriculture. We hypothesized that short term repeated exposure to non-lethal doses of OP would promote aggressive behaviour in mice. In the present work, we tested the effects of subacute exposure to sublethal doses of methamidophos in a murine model of isolation-induced aggression (Malick 1979; Miczek 1983; Miczek et al. 2001; Haller and Kruk 2006). We also monitored locomotor activity, brain acetylcholinesterase (AChE) and plasma butyrylcholinesterase (BChE) activity.

2. Material and Methods

2.1. Animals

Experiments were performed in male Swiss mice weighing between 25 and 35 g bred in-house. All experiments were performed in accordance with the Biomedical Research Guidelines for Animal Welfare, as stated by the Federation of the Brazilian Society of Experimental Biology and the project was approved by the Ethics Commitee in Animal Experimentation (CEUA-UFES n° 058/2010). Mice were housed in cages in a temperature and humidity controlled room with a 12 h light/dark cycle. Standard mice chow and tap water were available *ad libitum*.

2.2. Dose and Treatment

Methamidophos (*O,S*-dimethyl phosphoramidothioate; 60 percent m/v, Tamaron, Bayer) was diluted in saline (0.9 %) for administration by means of intraperitoneal injections. The i.p route was chosen to allow accurate and efficient delivery of the chosen dose and to reduce exposure variability. A lethality curve to methamidophos was previously determined in mice (Maretto et al. 2012) and a sublethal dose was chosen for the treatment protocol. Mice were treated with repeated administration of methamidophos (MTP, 3.5 mg/kg/daily, i.p., n=22) for seven days and a similar regimen was adopted for a control group treated with saline (SAL, 0.9 %, 1 mL/kg/day, i.p., n=25). The seven day treatment period was based on studies from Amr *et al.*(1997) that investigated psychiatric disorders in a population of pesticides formulators exposed to OPs on a daily basis for at least 9 months per year in the last two years. Considering that the ratio between human and mouse lifespan is approximately 40:1 (Dutta and Sengupta 2016), we adopted a 7 day treatment period to produce an equivalent exposure to 9 months in humans. Assignment to methamidophos or saline was randomized, and the experimenter was blinded during treatment, data collection and analysis.

2.3.   Social isolation-induced aggression

Long-term social isolation in mice induces many behavioural changes that resemble those seen in depressive and anxiety disorders (Malick 1979; Fone and Porkess 2008). It also induces territorial and aggressive behaviour, resulting in attacks against an opponent (Malick 1979). Socially isolated mice were individually housed for 28 days in cages measuring 30 x 20 x 13 cm. The standard opponent mice were housed in groups of five in cages measuring 41 x 34 x 16 cm. After the isolation period, each isolated mouse was individually placed in a neutral cage (41 x 34 x 16 cm) together with a weight-matched non-isolated mouse (pre-treatment trial), i.e. a standard opponent. The latency and frequency of attacks, tail rattling and general exploratory behaviours (number of rearings and self grooming) were recorded over a 15 min period (Brain and Poole 1974; Brain 1980). Animals were classified as aggressive or non-aggressive based on the presence or absence of an attack in this first trial. A second trial (i.e. post-treatment trial) was conducted 24 hours after the last injection of methamidophos or saline, i.e. 8 days later. The same standard opponent was used in the pre-treatment and post-treatment trials for each isolated animal tested.

2.4.   Open field test

In order to evaluate whether methamidophos treatment could induce exploratory behaviour dysfunction that might interfere with the isolation-induced aggression test, we performed the open field test in a separate group of animals treated with saline or methamidophos under the same dose regimen (n=10/group). The open field apparatus consisted of a circular arena with a peripheral and a central area (total area= 1256 m², radius of the external circle = 20 cm, radius of the internal circle = 11.8 cm) divided in 12 spaces by a grid cross on the floor (4 central and 8 peripheral), each space corresponding to a 105 cm² dimension. Twenty-four hours after the last injection of saline or methamidophos, each mouse was positioned in the centre of the apparatus and monitored for a period of 10 minutes. The total number of peripheral and central squares crossed was recorded. In addition to providing a measurement of general locomotor activity, which could interfere with other behavioural measures, preference or avoidance of central squares may also provide an evaluation of the anxiety level (Prut and Belzung 2003).

2.5. Sample collection

After the open field test, animals were decapitated, and brain and blood were harvested. Blood samples were collected in heparinized plastic microtubes and centrifuged at 1792 G for 10 minutes at 4°C (SL-5AM® - Spinlab Scientific, South Korea) to obtain plasma. Plasma samples were stored at -20°C until dosage assays were perfomed. Brain tissue was quickly removed and stored in centrifuge tubes at -80°C until the day of the assay.

2.6. Plasma butyrylcholinesterase (BChE) activity

Plasma activity of BChE or AChE is frequently used for the diagnosis of OP exposure (Eddleston et al. 2002). BChE activity is more easily measured and is used in the clinical setting as a useful tool for

indicating likelihood of exposure and for monitoring OP elimination (Eddleston et al, 2008). We aimed to verify that the sublethal dose chosen exceeded the occupational exposure level as stated in the Brazilian National Regulatory Law (Brasil 1978) for occupational exposure to cholinesterase inhibitors. According to this law, cholinesterase inhibitors may produce a maximum of 50% inhibition of the BChE. Therefore, the BChE assay was adopted as a screening tool to verify the effectiveness of the dose chosen in exceeding the maximum allowed exposure level. Plasma BChE activity was measured using a commercial kit (Doles, Brazil), which uses propionylthiocholine as substrate, and follows a colorimetric method described by Ellman et al. (1961) and modified by Dietz et al. (1973). BChE activity was measured in International Units (I.U./mL) and expressed as percentage of control activity. One I.U. of cholinesterase is the amount of enzyme that hydrolyses one µmol of substrate/minute/mL of serum at 37°C.

2.7. Brain acetylcholinesterase (AChE) activity

Whole brain AChE activity was determined using Ellman's *et al.* (1961) method, modified by Lassiter *et al.* (2003) and Pires *et al.* (2005). Briefly, whole brains were weighed, homogenized in phosphate buffer with Triton-X 1% (proportion of 1 ml of phosphate buffer to 20 mg of tissue) and centrifuged at 7800 G for 5 minutes at 4°C. A volume of 135µl of supernatant was transferred to a cuvette containing the following reagents: 35µl of 5mM dithio-bisnitrobenzoic acid (DTNB), 10µl of 75mM acetylthiocholine (ATCh) and 820µl of 0.1M phosphate buffer (pH 8.0). The colour development was recorded at 412 nm, using a spectrophotometer (Evolution 300 PC, Thermo Scientific, USA). Protein concentration in brain homogenates was quantified using a Bradford assay. AChE activity was calculated in µmoles of ATCh hydrolyzed per hour per mg of protein. AChE activity was expressed as percentage of control activity and measured values in µmol/h/mg of protein.

2.8. Statistical Analysis

Results are reported as mean ± standard error of the mean (S.E.M.). Data from the social isolation-induced aggression test were analysed using a two-way ANOVA for repeated measures followed by Bonferroni's post test, with methamidophos or saline treatment as the between-subject factor, and pre- and post-treatment trials as the within-subject factor. Data from the open field test, brain AChE and plasma BChE activity were analysed using paired Student *t*-test. $P < 0.05$ was considered to be significant. All analyses were performed using Graphpad prism software (Graphpad prism 5.0, USA).

3. Results

3.1. Social isolation-induced aggression

In the pre-treatment trial, performed after the isolation period, it was observed that some mice became aggressive, which could be identified by the presence of attacks against the opponent mouse, and some mice did not. Based on this observation, mice were grouped as isolation-induced aggressive and non-aggressive, and mice within each group were randomly assigned to either methamidophos or saline. According to this classification, we compared data from: (i) all isolated mice grouped together,

(n=47); or (ii) as separate groups of pre-treatment aggressive (n=25) and non-aggressive (n=22) mice. Figure 1 shows summary data from the grouped mice. No statistical difference was observed for all parameters analysed either for the repeated measures or for the treatment, and there was no interaction between treatment and time (for statistical values see Table 1). When analysing aggressive mice separately, we found no statistical difference between saline and methamidophos treatment (Figure 2, Table 1). However, non-aggressive mice showed significantly higher number of attacks over time [$F_{(40,1)}=5.287$; $p=0.0268$], compared to saline controls [$F_{(40,1)}=4.328$; $p=0.0439$], and there was a significant interaction between factors [$F_{(40,1)}=4.328$; $p=0.0439$] (Figure 3). Non-aggressive mice treated with methamidophos did not differ in number of attacks from aggressive mice in the pre-treatment trial ($p>0.05$). All other parameters evaluated in the non-aggressive group after methamidophos treatment were not statistically different (Table 1).

3.2. Open field test

The results of the open field test are presented in Table 2. No statistical difference was observed between the different treatment groups for peripheral squares crossed ($t_{18}=-0.936$, $p=0.362$). However, there was a tendency to reduction in the number of central squares crossed in the methamidophos-treated group when compared to saline-treated animals ($t_{18}=2.08$, $p=0.052$).

3.3. Brain AChE and Plasma BChE activity

Repeated administration of a sublethal dose of methamidophos induced a significant reduction of 72% on BChE activity when compared to saline group ($t_{17}=6.97$, $p<0.001$; Figure 4A). BChE activity was 10.73 I.U./mL in the saline, and 3.07 I.U./mL in the methamidophos group. Methamidophos treatment also significantly decreased brain AChE activity when compared to saline group ($t_{18}=3.00$, $p<0.01$; Figure 4B). Mice treated with saline exhibited AChE activity of 4.04 µmol/h/mg of protein and those treated with methamidophos exhibited AChE activity of 3.16 µmol/h/mg of protein.

4. Discussion

In the present study, repeated administration of methamidophos induced a pro-aggressive behaviour in mice that did not present isolation-induced aggressive behaviour before OP treatment. This effect was observed in absence of any interference in the peripheral locomotor activity evaluated through the open field test. Mice treated with methamidophos also showed a trend towards reduction in locomotor activity in the central squares, suggesting that exposure to methamidophos had an anxiogenic-like effect. It is noteworthy that, although a sublethal dose of methamidophos was employed, final plasma BChE activity resembled that of acute OP poisoning. BChE is a sensitive marker of exposure to OPs (Eddleston et al. 2008a). Plasma levels of BChE achieved the diagnosis criteria for cholinesterase inhibitors exposure stated by the Brazilian National Regulatory Law (Brasil 1978), which sets 50% inhibition of BChE activity as a maximum biological exposure level. Although, BChE inhibition does not straight correlate with clinical features in OP-poisoned patients, the independent measurement of brain AChE confirms that methamidophos treatment significantly

decreased whole brain AChE activity in this study. In fact, Singh (1985) has demonstrated that brain samples are more sensitive to methamidophos than plasma samples.

It is important to consider, however, some methodological limitations of the dosage regimen employed in the present study. . The measurement of brain AChE activity by the Ellman's method without usage of a specific inhibitor of BChE does not allow us to exclude BChE activity in our brain samples. Notwithstanding, according to Vellom et al. (1993), AChE promotes rapid catalysis of the substrate used, acetylthiocholine, while BChE shows far less selectivity for the size of the acyl group. This is clearly demonstrated by the kinetic constants (Kcat/Km ratio) for the catalysis of acetylthiocholine by AChE (4.4×10^9) and by BChE (0.24×10^9). Additionally, Lassiter and colleagues (2003) showed that only 12% of overall cholinesterase activity was reduced in rodent brain samples after inhibition of BChE with iso-OMPA. This indicates that almost 90% of cholinesterase activity measured in rodent brains relates to AChE.

Methamidophos, like other OP pesticides, is a cholinesterase inhibitor. Evidence suggests that cholinesterase inhibitors may increase aggression in animals and humans. Studies performed by Allon *et al.* (2005) reported that rats acutely exposed to sarin vapour, a potent AChE inhibitor, exhibited signs of aggression and weight loss. Although Allon *et al.* (2005) did not use a specific test for evaluation of aggressive behaviour. Mice treated with another OP compound, chlorpyrifos, during gestational and postnatal phases, showed enhanced agonistic behaviour (Ricceri et al. 2006). In rats, 10 days exposure to diisopropylfluorophosphate, an OP pesticide, increased shock-induced aggression (Ray et al. 1989). Moreover, chronic exposure to the OP dichlorvos increased aggressive behaviour in rats while reducing brain AChE activity (Sarin and Gill 1998). Further, physiostigmine, a reversible AChE inhibitor, has been reported to enhance intermale mouse aggression at low doses (Charpentier 1969). In fact, increase in aggression in mice and rats has been previously attributed to cholinesterase inhibitors and muscarinic receptor agonists (Bell et al. 1985). Moreover, there are some case reports of patients exhibiting aggressive behaviour while using donepezil, a reversible cholinesterase inhibitor used for the treatment of dementia (Bouman and Pinner 1998; Bianchetti et al. 2003). Although, the above mentioned reversible cholinesterase inhibitors differ from OP compounds in their kinetics and dynamic properties, there is a common feature between them which is the consequent ACh accumulation in synapses and interference with cholinergic transmission. Devinsky *et al.* (1992) reported episodes of unprovoked aggressive behaviour, including two homicides after OP exposure. Among the cases reported, one occurred after one month of exposure while the other two cases involved more than 3 years of OP exposure. Amr *et al.* (1997) investigated psychiatric disorders in a population of pesticide formulators exposed daily for at least 9 months of the year, and found that irritability was amongst the most frequent symptoms observed. Although no previous studies have investigated the effects of short term repeated OP exposure in the isolation-induced aggression test, the above mentioned evidence are in accordance with our results, in which a pro-aggressive behaviour induced by repeated methamidophos exposure could be observed.

Given the evidence for cholinergic involvement in aggressive behaviour, it is possible that the pro-aggressive behaviour induced by methamidophos treatment can be attributed to cholinergic hyper stimulation in the central nervous system (CNS). This hypothesis is supported by the finding that

Flinders Sensitive Line hypercholinergic rats are significantly more aggressive (Pucilowski et al. 1990). Moreover, cholinomimetic drugs administered into cerebral ventricles of cats elicited affective type of aggression (Beleslin and Samardzić 1979). Likewise, injections of acetylcholine into the hypothalamus and periaqueductal grey matter, brain areas involved in mediating aggressive behaviour, led to defensive rage in cats (Allikmets 1974). Additionally, carbachol injection into the lateral hypothalamus elicited killing behaviour in rats (Smith et al. 1970), while scopolamine, a muscarinic antagonist, reduced aggressive behaviour in mice (Winslow and Camacho 1995). Although we did not measure AChE activity in specific brain structures, the reduction in whole brain AChE activity after methamidophos treatment could generate cholinergic hyper stimulation in brain areas mediating aggressive behaviour. Nevertheless the central cholinergic hyper stimulation seems to be an important mechanism involved in the effects observed after the methamidophos treatment, recent finds on the ghrelin peptide and plasma BChE activity points alternative mechanisms for the aggression effects mediated by the OP exposure (Chen et al. 2015). Knockout mice with plasma BChE gene deletion exhibited increased levels of ghrelin as well as increased levels of aggression (Chen et al. 2015). In fact, previous *in vitro* studies had shown that purified plasma BChE promoted hydrolysis of the ghrelin peptide (De Vriese et al. 2004). Interestingly, overexpression of BChE led to low ghrelin levels in the blood stream and reduced aggression and social stress in mice (Brimijoin et al. 2015). Although caution should be exercised when drawing comparisons between our study and genetically modified mice, it is possible that the large inhibition of plasma BChE activity induced by methamidophos treatment could increase ghrelin levels, which could account for the increase in aggressive behaviour in the present study. Indeed, a recent study using chronic administration of another OP, chlorpiryfos, showed an increase in plasma levels of ghrelin in mice (Peris-Sampedro et al. 2015).

Methamidophos exposure only increased aggression in non-aggressive socially isolated mice. This is in line with previous studies showing that drug-induced changes in aggressive behaviour can be different in mice depending on the aggression level exhibited in the pre-screening test (Felip et al. 2001; Miczek et al. 2002; Lumley et al. 2004; Redolat et al. 2005). Additionally, the anxiogenic-like effect seems to be more obviously detectable in animals with low levels of emotional reactivity (Lisboa et al. 2010). It is possible that this selective effect induced by methamidophos exposure might be associated with a reduction in GABA levels in certain brain areas, which could differently affect sociable and unsociable mice. Sustková-Fišerová et al. (2009) showed that socially isolated non-aggressive mice had almost three times higher levels of GABA in the pre-frontal cortex when compared with aggressive mice. Additionally, chronic exposure to methamidophos was shown to decrease GABA release in cerebral cortex and hippocampal slices (Noriega-Ortega et al. 2011).

Exposure to some OP pesticides, at low doses, also interferes with monoaminergic neurotransmission, particularly via serotonin modulation (Ali et al. 1980; Aldridge et al. 2005; Slotkin et al. 2006). Accordingly, Lima et al. (2011) reported that exposure to low doses of methamidophos affects synaptic transmission by reducing serotoninergic biomarkers in regions such as cerebral cortex and midbrain. Vergnes et al. (1986) also showed that depletion of 5-HT increases aggression in a variety of species in several different social situations. In fact, decreased 5-HT levels in the pre-frontal cortical area are detectable when a resident rat is attacking an intruder (Van Erp and Miczek, 2000). In

the same way, pharmacological manipulation using agonists or reuptake inhibitors aiming to increase 5-HT neurotransmission reduced aggressive behaviour in isolated male mice (Olivier et al. 1989; Sánchez et al. 1993; Sánchez and Hyttel 1994). However, it was beyond the scope of the present study to identify the neurotransmitter systems involved in increased aggression by OP exposure, and further studies will be required to reveal the mechanism.

Our data showed that approximately 50 % of the socially isolated mice did not exhibit aggressive behaviour, i.e. absence of at least one attack episode. Previous studies also described similar proportion of non-aggressive mice in singly housed Swiss mice (Krsiak 1975; D'Amato and Castellano 1989). It is noteworthy, however, that aggressive and non-aggressive mice were confronted with the same standard opponent in the trials tested, which could have interacted with the processing of novelty by the isolated mice. Indeed, conflicting results have been shown after repeated exposure to the same opponent either in the social interaction or in the resident intruder test. Parmigiani and Brain (1983) observed that familiar intruders were less attacked by the resident mouse in the second trial. However, other studies (Brain and Poole 1974; Winslow and Camacho 1995; Koike et al. 2009) observed that repeated exposure to the same intruder raises the aggressiveness of the resident against it. In these studies, the inter-trial time is within minutes to hours after the first trial. In our study, the re-exposure only occurred 8 days after the first trial, which could account for the differences observed. Additionally, (Krsiak 1975) suggest that isolation-induced timidity is stable in repeated interactions. Recently, Hsieh and colleagues (2017) conducted a study in which they showed that the natural social interaction in CD1 mice does not only happen due to novelty. They also showed that testing the mice in one single compartment, similarly to our study, favours aggressive instead of sociopositive behaviours. Finally, if re-exposure alone led to increase in aggressive behaviour in the present study, the same effect would have been observed in the saline control group.

5. Conclusions

Our results show for the first time that non-aggressive socially-isolated mice became aggressive after exposure to sublethal doses of methamidophos. Therefore, alongside many other behavioural changes associated with OP exposure, OP compounds also induce aggressive behaviour that seems to be associated, at least partially, with changes in brain AChE and plasma BChE activity.

Perspectives

While developing countries are the largest buyers of pesticides worldwide, they often do not have effective regulatory policies and enforcement procedures on occupational exposure and environmental contamination. The literature suggests that, amongst other factors, higher suicide rates in rural regions seem to be associated with higher levels of impulsive behaviour and ready access to highly toxic pesticides (Jiang et al. 2013). The evidence from our study, that short term exposure to the OP methamidophos produces a pro-aggressive behaviour, is corroborated by clinical reports on aggressive behaviour related to pesticides exposure (Devinsky et al. 1992; Amr et al. 1997). Our study highlights the importance of potentially implementing psychiatric screening tools such as the General Health Questionnaire (GHQ-28) or the Buss-Perry Aggression Questionnaire (BPAD) (Goldberg et al.

1997; Zivari-Rahman et al. 2012; Alcorn, Joseph et al. 2013) in the populations occupationally exposed to OPs, particularly agricultural workers. This should be done alongside physical exams and measurements of cholinesterase activity. Psychiatric evaluation might be a useful tool to improve early symptom detection and improve vigilance in this working population.

Conflict of interest

The authors declare that they have no competing financial interests, including grant support, employments, patents, payments for expert witness or testimony, personal financial interests and forms of compensation.

References

Alcorn, Joseph L, B.S., Gowin JL, et al (2013) Combined Antisocial Substance Use Disorder. J neuropsychiatry Clin Neurosci 25:229–232.

Aldridge JE, Levin ED, Seidler FJ, Slotkin TA (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 113:527–531. doi: 10.1289/ehp.7867

Ali SF, Chandra O, Hasan M (1980) Effects of an Organo-Phosphate (Dichlorvos) on Open-Field Behavior and Locomotor-Activity - Correlation with Regional Brain Monoamine Levels. Psychopharmacology (Berl) 68:37–42.

Allikmets L (1974) Cholinergic mechanisms in aggressive behaviour. Med Biol 51:19–30.

Allon N, Rabinovitz I, Manistersky E, et al (2005) Acute and long-lasting cardiac changes following a single whole-body exposure to sarin vapor in rats. Toxicol Sci 87:385–90.

Amr MM, Halim ZS, Moussa SS (1997) Psychiatric disorders among Egyptian pesticide applicators and formulators. Environ Res 73:193–199. doi: 10.1006/enrs.1997.3744

Beleslin DB, Samardzić R (1979) Evidence of central cholinergic mechanisms in the appearance of affective aggressive behaviour: dissociation of aggression from autonomic and motor phenomena. Psychopharmacology (Berl) 62:163–167. doi: 10.1007/BF00427131

Bell R, Warburton DM, Brown K (1985) Drugs as research tools in psychology: cholinergic drugs and aggression. Neuropsychobiology 14:181–92.

Beseler C, Stallones L, Hoppin JA, et al (2006) Depression and pesticide exposures in female spouses of licensed pesticide applicators in the agricultural health study cohort. J Occup Environ Med 48:1005–13. doi: 10.1097/01.jom.0000235938.70212.dd

Beseler CL, Stallones L, Hoppin JA, et al (2008) Depression and pesticide exposures among private pesticide Applicators enrolled in the agricultural health study. Environ Health Perspect 116:1713–1719. doi: 10.1289/ehp.11091

Bianchetti A, Trabucchi M, Cipriani G (2003) Aggressive behaviour associated with donepezil treatment: A case report. Int J Geriatr Psychiatry 18:657–658. doi: 10.1002/gps.824

Bouman WP, Pinner G (1998) Violent behavior-associated with donepezil. Am J Psychiatry 155:1626–7.

Brain P and, Poole A (1974) Some studies on the use of "standard opponents"in intermale aggression testing in TT albino mice. Behaviour 53:539–548.

Brain PF (1980) Adaptive Aspects of Hormonal Correlates of Attack and Defence in Laboratory Mice: A Study in Ethobiology. Prog Brain Res 53:391–413. doi: 10.1016/S0079-6123(08)60078-3

Brasil (1978) Portaria GM n° 3.214, de 08 de junho de 1978. NR 7 -Programa de controle médico de saúde ocupacional. Diário Of da República Fed do Bras 1–16.

Brimijoin S, Chen VP, Pang YP, et al (2015) Physiological roles for butyrylcholinesterase: A BChE-ghrelin axis. Chem Biol Interact 1–5. doi: 10.1016/j.cbi.2016.02.013

Caldas ED, Boon PE, Tressou J (2006) Probabilistic assessment of the cumulative acute exposure to organophosphorus and carbamate insecticides in the Brazilian diet. Toxicology 222:132–142. doi: 10.1016/j.tox.2006.02.006

Caldas ED, De Souza M V, Jardim ANO (2011) Dietary risk assessment of organophosphorus and dithiocarbamate pesticides in a total diet study at a Brazilian university restaurant. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 28:71–9. doi: 10.1080/19440049.2010.538935

Charpentier (1969) Analysis and measurement of aggression in mice. In: Aggressive behavior. pp 86–

99

Chen VP, Gao Y, Geng L, et al (2015) Plasma butyrylcholinesterase regulates ghrelin to control aggression. Proc Natl Acad Sci 112:201421536.

Chowdhary S, Bhattacharyya R, Banerjee D (2014) Acute organophosphorus poisoning. Clin Chim Acta 431:66–76. doi: 10.1016/j.cca.2014.01.024

D'Amato FR, Castellano C (1989) Behavioral effects of morphine in mice: Role of experimental housing. Pharmacol Biochem Behav 34:361–365. doi: 10.1016/0091-3057(89)90327-4

De Vriese C, Gregoire F, Lema-Kisoka R, et al (2004) Ghrelin degradation by serum and tissue homogenates: Identification of the cleavage sites. Endocrinology 145:4997–5005. doi: 10.1210/en.2004-0569

Devinsky O, Kernan J, Bear DM (1992) Aggressive behavior following exposure to cholinesterase inhibitors. J Neuropsychiatry Clin Neurosci 4:189–94.

Dietz AA, Rubinstein HM, Lubrano T (1973) Colorimetric determination of serum cholinesterase and its genetic variants by the propionylthiocholine-dithiobis(nitrobenzoic acid)procedure. Clin Chem 19:1309–13.

Dutta S, Sengupta P (2016) Men and mice: Relating their ages. Life Sci 152:244–248. doi: 10.1016/j.lfs.2015.10.025

Ecobichon DJ (2000) Our changing perspectives on benefits and risks of pesticides: a historical overview. Neurotoxicology 21:211–218.

Eddleston M, Buckley NA, Eyer P, et al (2008a) Management of acute organophosphorus pesticide poisoning. Lancet (London, England) 371:597–607. doi: 10.1016/S0140-6736(07)61202-1

Eddleston M, Buckley NA, Eyer P, Dawson AH (2008b) Management of acute organophosphorus pesticide poisoning. 371:597–607. doi: 10.1016/S0140-6736(07)61202-1

Eddleston M, Roberts D, Buckley N (2002) Management of severe organophosphorus pesticide poisoning. Crit care 6:259.

Ellman GL, Courtney KD, Andres VJ, Featherstone RM (1961) a New and Rapid Colorimetric of Acetylcholinesterase Determination of Acetylcholinesterase Activity. Biochem Pharmacol 7:88–95.

Felip CM, Rodriguez-Arias M, Aguilar MA, Minarro J (2001) Antiaggressive and motor effects of the DA release inhibitor CGS 10746B. Aggress Behav 27:382–390. doi: 10.1002/ab.1023

Ferrer A (2003) Pesticide poisoning. An Sist Sanit Navar 26:1–2.

Fone KCF, Porkess MV (2008) Behavioural and neurochemical effects of post-weaning social isolation in rodents-Relevance to developmental neuropsychiatric disorders. Neurosci Biobehav Rev 32:1087–1102. doi: 10.1016/j.neubiorev.2008.03.003

Goldberg DP, Gater R, Sartorius N, et al (1997) The validity of two versions of the GHQ in the WHO study of mental illness in general health care. Psychol Med 27:191–197. doi: 10.1017/S0033291796004242

Gray AJ (1982) Distribution and Excretion of [14CH3S] Methamidophos after Intravenous Administration of a Toxic Dose and the Relationship with Anticholinesterase Activity. Pestic Biochem Physiol 18:28–37.

Gunnell D, Eddleston M, Phillips MR, Konradsen F (2007) The global distribution of fatal pesticide self-poisoning: systematic review. BMC Public Health 7:357.

Haller J, Kruk MR (2006) Normal and abnormal aggression: Human disorders and novel laboratory models. Neurosci Biobehav Rev 30:292–303. doi: 10.1016/j.neubiorev.2005.01.005

Hulse EJ, Davies JOJ, Simpson AJ, et al (2014) Respiratory complications of organophosphorus nerve agent and insecticide poisoning. Implications for respiratory and critical care. Am J Respir Crit Care Med 190:1342–54. doi: 10.1164/rccm.201406-1150CI

Jeyaratnam J (1990) Acute pesticide poisoning: a major global health problem. World Health Stat Q 43:139–44.

Jiang C, Li X, Phillips MR, Xu Y (2013) Matched case-control study of medically serious attempted suicides in rural China. Shanghai Arch psychiatry 25:22–31. doi: 10.3969/j.issn.1002-0829.2013.01.006

Koike H, Ibi D, Mizoguchi H, et al (2009) Behavioral abnormality and pharmacologic response in social isolation-reared mice. Behav Brain Res 202:114–21. doi: 10.1016/j.bbr.2009.03.028

Krsiak M (1975) Timid Singly-Housed Mice: Their Value in Prediction of Psychotropic Activity of Drugs. Br J Pharmacol 55:141–150. doi: 10.1111/j.1476-5381.1975.tb07622.x

Lassiter TL, Marshall RS, Jackson LC, et al (2003) Automated measurement of acetylcholinesterase activity in rat peripheral tissues. Toxicology 186:241–253. doi: 10.1016/S0300-483X(02)00752-7

Lima CS, Nunes-Freitas AL, Ribeiro-Carvalho A, et al (2011) Exposure to methamidophos at adulthood adversely affects serotonergic biomarkers in the mouse brain. Neurotoxicology

32:718–724. doi: 10.1016/j.neuro.2011.08.002

Lima CS, Ribeiro-Carvalho A, Filgueiras CC, et al (2009a) Exposure to methamidophos at adulthood elicits depressive-like behavior in mice. Neurotoxicology 30:471–8. doi: 10.1016/j.neuro.2009.01.009

Lima CS, Ribeiro-Carvalho A, Filgueiras CC, et al (2009b) Exposure to methamidophos at adulthood elicits depressive-like behavior in mice. Neurotoxicology 30:471–8. doi: 10.1016/j.neuro.2009.01.009

Lisboa SF, Reis DG, da Silva AL, et al (2010) Cannabinoid CB1 receptors in the medial prefrontal cortex modulate the expression of contextual fear conditioning. Int J Neuropsychopharmacol 13:1163–1173. doi: 10.1017/S1461145710000684

López-Crespo G a, Carvajal F, Flores P, et al (2007a) Time course of biochemical and behavioural effects of a single high dose of chlorpyrifos. Neurotoxicology 28:541–7. doi: 10.1016/j.neuro.2007.01.013

López-Crespo G a, Carvajal F, Flores P, et al (2007b) Time course of biochemical and behavioural effects of a single high dose of chlorpyrifos. Neurotoxicology 28:541–7. doi: 10.1016/j.neuro.2007.01.013

Lu JL (2010) Analysis of Trends of the Types of Pesticide Used, Residues and Related Factors among Farmers in the Largest Vegetable Producing Area in the Philippines. J Rural Med JRM / Japanese Assoc Rural Med 5:184–9. doi: 10.2185/jrm.5.184

Lumley L a., Robison CL, Slusher BS, et al (2004) Reduced isolation-induced aggressiveness in mice following NAALADase inhibition. Psychopharmacology (Berl) 171:375–381. doi: 10.1007/s00213-003-1610-z

Mackenzie Ross S, McManus IC, Harrison V, Mason O (2013) Neurobehavioral problems following low-level exposure to organophosphate pesticides: a systematic and meta-analytic review. Crit Rev Toxicol 43:21–44. doi: 10.3109/10408444.2012.738645

Mackenzie Ross SJ, Brewin CR, Curran H V, et al (2010) Neuropsychological and psychiatric functioning in sheep farmers exposed to low levels of organophosphate pesticides. Neurotoxicol Teratol 32:452–459. doi: 10.1016/j.ntt.2010.03.004

Malick JB (1979) The pharmacology of isolation-induced aggressive behavior in mice. Curr Dev Psychopharmacol 5:1–27.

Maretto GX, do Nascimento CP, Passamani LM, et al (2012) Acute exposure to the insecticide O,S-dimethyl phosphoramidothioate (methamidophos) leads to impairment of cardiovascular reflexes in rats. Ecotoxicol Environ Saf 80:203–207. doi: 10.1016/j.ecoenv.2012.03.001

Melnyk LJ, Wang Z, Li Z, Xue J (2016) Prioritization of pesticides based on daily dietary exposure potential as determined from the SHEDS model. Food Chem Toxicol 96:167–173. doi: 10.1016/j.fct.2016.07.025

Miczek K (1983) Ethological analysis of drug action on aggression and defense. Prog Neuropsychopharmacol Biol Psychiatry 7:519–524.

Miczek KA, Fish EW, De Bold JF, De Almeida RM (2002) Social and neural determinants of aggressive behavior: Pharmacotherapeutic targets at serotonin, dopamine and gama-aminobutyric acid systems. Psychopharmacology (Berl) 163:434–458. doi: 10.1007/s00213-002-1139-6

Miczek KA, Maxson SC, Fish EW, Faccidomo S (2001) Aggressive behavioral phenotypes in mice. Behav Brain Res 125:167–181. doi: 10.1016/S0166-4328(01)00298-4

Noriega-Ortega BR, Armienta-Aldana E, Cervantes-Pompa JÁ, et al (2011) GABA and Dopamine Release from Different Brain Regions in Mice with Chronic Exposure to Organophosphate Methamidophos. J Toxicol Pathol 24:163–8. doi: 10.1293/tox.24.163

O'Malley M (1997) Clinical evaluation of pesticide exposure and poisonings. Lancet 349:1161–1166.

Olivier B, Mos J, van der Heyden J, Hartog J (1989) Serotonergic modulation of social interactions in isolated male mice. Psychopharmacology (Berl) 97:154–156. doi: 10.1007/BF00442239

Parmigiani, Stefano; Brain PF (1983) Effects of residence, aggressive experience and intruder familiarity on attack shown by male mice. Behav Process 8:45–57.

Parrón T, Hernández AF, Villanueva E (1996) Increased risk of suicide with exposure to pesticides in an intensive agricultural area. A 12-year retrospective study. Forensic Sci Int 79:53–63. doi: 10.1016/0379-0738(96)01895-6

Pelegrino JR, Calore EE, Saldiva PHN, et al (2006) Morphometric studies of specific brain regions of rats chronically intoxicated with the organophosphate methamidophos. Ecotoxicol Environ Saf 64:251–5. doi: 10.1016/j.ecoenv.2005.05.002

Peris-Sampedro F, Cabré M, Basaure P, et al (2015) Adulthood dietary exposure to a common pesticide leads to an obese-like phenotype and a diabetic profile in apoE3 mice. Environ Res

142:169–176.

Pires RGW, Pereira SRC, Oliveira-Silva IF, et al (2005) Cholinergic parameters and the retrieval of learned and re-learned spatial information: a study using a model of Wernicke-Korsakoff Syndrome. Behav Brain Res 162:11–21. doi: 10.1016/j.bbr.2005.02.032

Prut L, Belzung C (2003) The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur J Pharmacol 463:3–33. doi: 10.1016/S0014-2999(03)01272-X

Pucilowski O, Eichelman B, Overstreet D, et al (1990) Enhanced Affective Aggression in Genetically Bred Hypercholinergic Rats. Neuropsychobiology 91:37–41.

Ray A, Sen P, Alkondon M (1989) Biochemical and pharmacological evidence for central cholinergic regulation of shock-induced aggression in rats. Pharmacol Biochem Behav 32:867–871.

Recena MCP, Caldas ED, Pires DX, Pontes ERJC (2006a) Pesticides exposure in Culturama, Brazil--knowledge, attitudes, and practices. Environ Res 102:230–6. doi: 10.1016/j.envres.2006.01.007

Recena MCP, Pires DX, Caldas ED (2006b) Acute poisoning with pesticides in the state of Mato Grosso do Sul, Brazil. Sci Total Environ 357:88–95. doi: 10.1016/j.scitotenv.2005.04.029

Redolat R, Gómez MC, Vicens P, Carrasco MC (2005) Bupropion effects on aggressiveness and anxiety in OF1 male mice. Psychopharmacology (Berl) 177:418–427. doi: 10.1007/s00213-004-1965-9

Ricceri L, Venerosi A, Capone F, et al (2006) Developmental neurotoxicity of organophosphorous pesticides: fetal and neonatal exposure to chlorpyrifos alters sex-specific behaviors at adulthood in mice. Toxicol Sci 93:105–13. doi: 10.1093/toxsci/kfl032

Robinson CP, Beiergrohslein D (1982) Inhibition of human erythrocyte and plasma cholinesterases by methamidophos. J Appl Toxicol 2:217–218.

Rosenstock L, Keifer M, Daniell WE, et al (1991) Chronic central nervous system effects of acute organophosphate pesticide intoxication. The Pesticide Health Effects Study Group. Lancet 338:223–7.

Sánchez-Amate MC, Flores P, Sánchez-Santed F (2001a) Effects of chlorpyrifos in the plus-maze model of anxiety. Behav Pharmacol 12:285–92.

Sánchez-Amate MC, Flores P, Sánchez-Santed F (2001b) Effects of chlorpyrifos in the plus-maze model of anxiety. Behav Pharmacol 12:285–92. doi: 10.1097/00008877-200107000-00007

Sánchez C, Arnt J, Hyttel J, Moltzen EK (1993) The role of serotonergic mechanisms in inhibition of isolation-induced aggression in male mice. Psychopharmacology (Berl) 110:53–59. doi: 10.1007/BF02246950

Sánchez C, Hyttel J (1994) Isolation-induced aggression in mice: effects of 5-hydroxytryptamine uptake inhibitors and involvement of postsynaptic 5-HT1A receptors. Eur J Pharmacol 264:241–7. doi: 10.1016/0014-2999(94)00470-6

Sarin S, Gill KD (1998) Biochemical and behavioral deficits in adult rat following chronic dichlorvos exposure. Pharmacol Biochem Behav 59:1081–1086. doi: 10.1016/S0091-3057(97)00517-0

Savage EP, Keefe TJ, Mounce LM, et al (1988) Chronic Neurological Sequelae of Acute Organophosphate Pesticide Poisoning. Arch. Environ. Heal. An Int. J. 43:38–45.

Singh AK (1985) Kinetic analysis of inhibition of brain and red blood cell acetylcholinesterase and plasma cholinesterase by acephate or methamidophos. Toxicol Appl Pharmacol 81:302–309. doi: 10.1016/0041-008X(85)90167-X

Slotkin TA, Tate CA, Ryde IT, et al (2006) Organophosphate Insecticides Target the Serotonergic System in Developing Rat Brain Regions: Disparate Effects of Diazinon and Parathion at Doses Spanning the Threshold for Cholinesterase Inhibition. Environ Health Perspect 114:1542–1546. doi: 10.1289/ehp.9337

Smith DE, King MB, Hoebel BG (1970) Lateral hypothalamic control of killing: evidence for a cholinoceptive mechanism. Science (80-) 167:900–901. doi: 10.2307/1728444

Socko R, Gralewicz S, Gorny R (1999) Long-term behavioural effects of a repeated exposure to chlorphenvinphos in rats. Int J Occup Med Env Heal 12:105–117.

Stallones L, Beseler C (2002) Pesticide poisoning and depressive symptoms among farm residents. Ann Epidemiol 12:389–94.

Stephens R, Spurgeon A, Calvert IA, et al (1995) Neuropsychological effects of long-term exposure to organophosphates in sheep dip. Lancet 345:1135–1139. doi: 10.1016/S0140-6736(95)90976-1

Sustková-Fiserová M, Vávrová J, Krsiak M (2009) Brain levels of GABA, glutamate and aspartate in sociable, aggressive and timid mice: an in vivo microdialysis study. Neuro Endocrinol Lett 30:79–84.

van Erp AM, Miczek KA (2000) Aggressive behavior, increased accumbal dopamine, and decreased cortical serotonin in rats. J Neurosci 20:9320–5.

Vellom DC, Radić Z, Li Y, et al (1993) Amino acid residues controlling acetylcholinesterase and

butyrylcholinesterase specificity. Biochemistry 32:12–17. doi: 10.1021/bi00052a003

Vergnes M, Depaulis A, Boehrer A (1986) Parachlorophenylalanine-induced serotonin depletion increases offensive but not defensive aggression in male rats. Physiol Behav 36:653–658. doi: 10.1016/0031-9384(86)90349-5

Wesseling C, Joode BVW De, Keifer M, et al (2010) Symptoms of psychological distress and suicidal ideation among banana workers with a history of poisoning by organophosphate or n-methyl carbamate pesticides. 778–785. doi: 10.1136/oem.2009.047266

Winslow JT, Camacho F (1995) Cholinergic modulation of a decrement in social investigation following repeated contacts between mice. Psychopharmacology (Berl) 121:164–172. doi: 10.1007/BF02245626

Yu B, Ding B, Shen H, et al (2015) [Analysis of reports of cases of pesticide poisoning in Jiangsu Province, China, from 2006 to 2013]. Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi 33:194–8.

Zivari-Rahman M, Lesani M, Shokouhi-Moqaddam S (2012) Comparison of Mental Health, Aggression and Hopefulness between Student Drug-Users and Healthy Students (A Study in Iran). Addict Heal 4:36–42.

Figures Captions

Figure 1. Grouped data of aggressive and non-aggressive isolated mice in the isolation-induced aggression test, before and after repeated administration of methamidophos (3.5 mg/kg/day, ip, n=22) or saline (Control, n=25). Two-way ANOVA for repeated measures. Data represent mean ± SEM.

Figure 2. Data presented by aggressive animals in the isolation-induced aggression test, before and after repeated administration of methamidophos (3.5 mg/kg/day, ip, n=12) or saline (Control, n=13). Repeated treatment with methamidophos did not change aggressive behaviour in mice that previously exhibited aggressive behaviour induced by social isolation. Two-way ANOVA for repeated measures. Data represent mean ± SEM.

Figure 3. Data presented by non-aggressive animals in the isolation-induced aggression test, before and after repeated administration of methamidophos (3.5 mg/kg/day, ip, n=10) or saline (Control n=12). Repeated treatment with methamidophos increased aggressive behaviour in mice that previously did not exhibited aggressive behaviour induced by social isolation. *p<0.05 indicates statistical difference from the methamidophos compared with the control group (Two-way ANOVA for repeated measures followed by Bonferroni's post test). Data represent mean ± SEM.

Figure 4. Plasma butyrylcholinesterase (BChE, panel A) or brain acetylcholinesterase (AChE, panel B) activity of mice after repeated administration of methamidophos (hatched bar, n=10 for BChE and AChE) or saline (control, open bar, n=9 for BChE; n=10 for AChE). BChE and AChE activity are expressed as percentage of control. Comparisons with Student *t* test. **p<0.001 and *p<0.01 indicates statistical difference compared with the control group.

Table Caption

Table 1. Statistical analysis (Two-way ANOVA for repeated measures) of grouped data, aggressive animals and non-aggressive animals.

Table 2. Parameters evaluated in the open field test in mice treated with repeated administration of methamidophos (3.5 mg/kg/day, ip, n=10) or saline (Control, n=10).
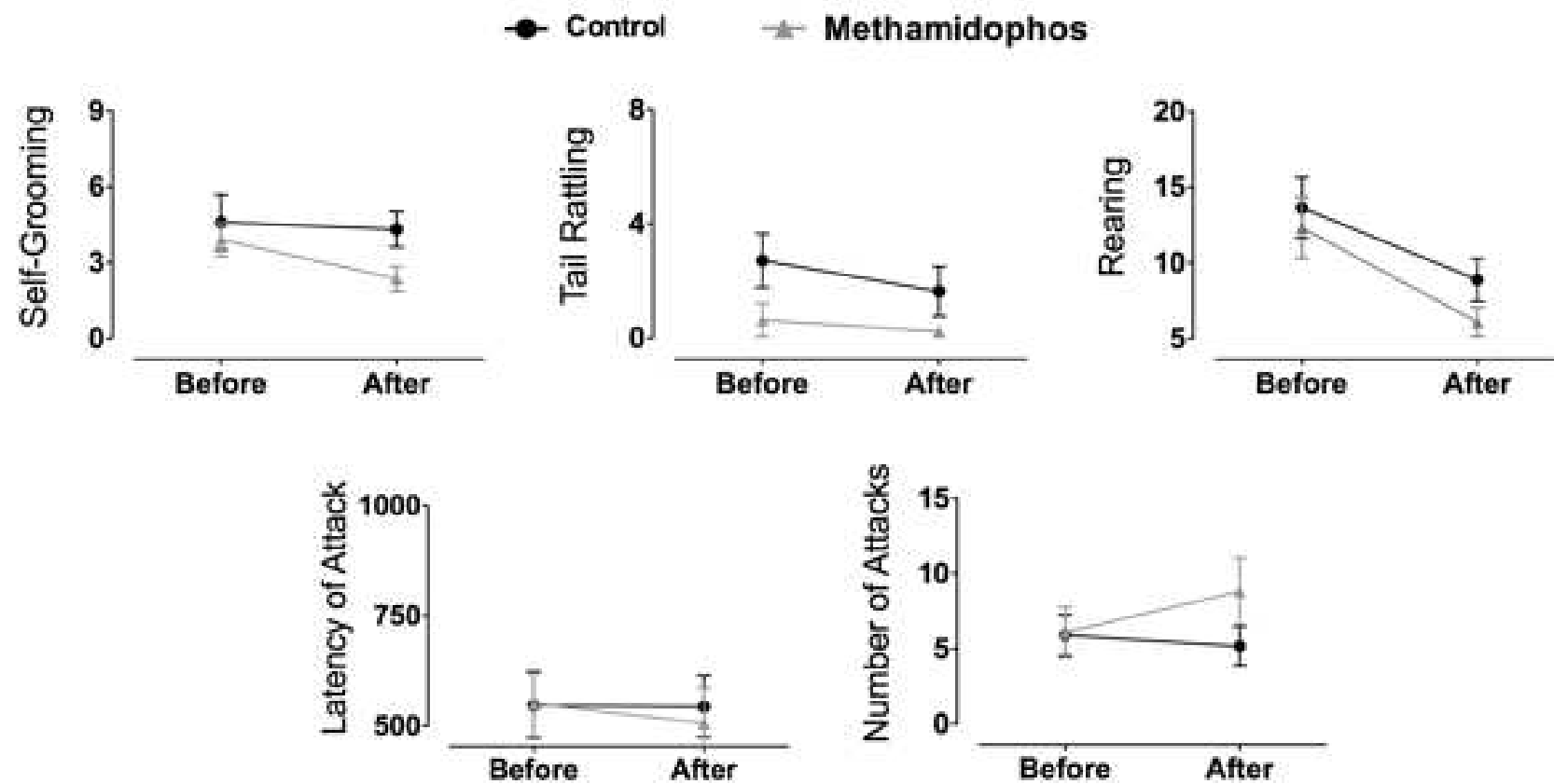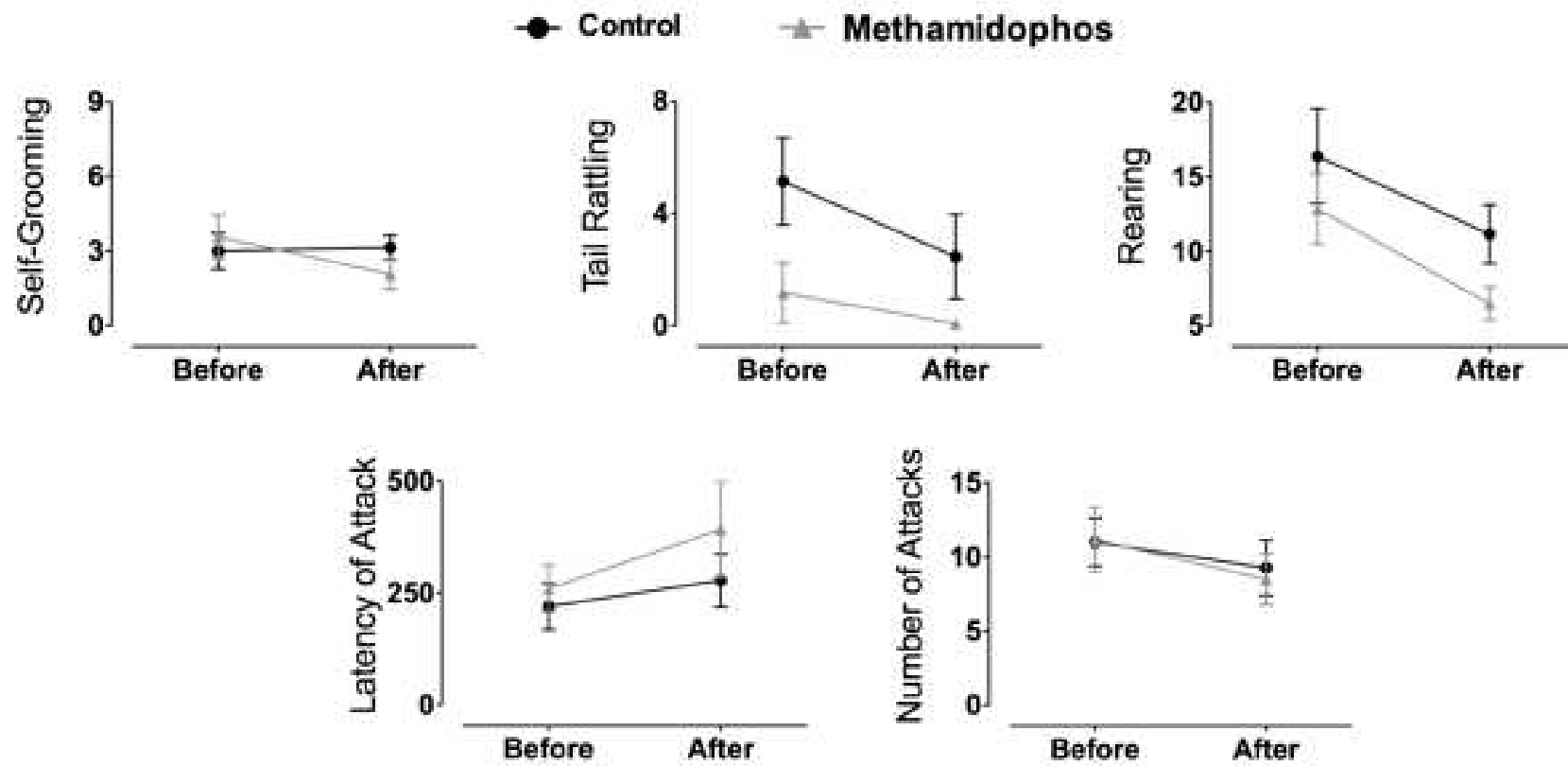
Figure 1

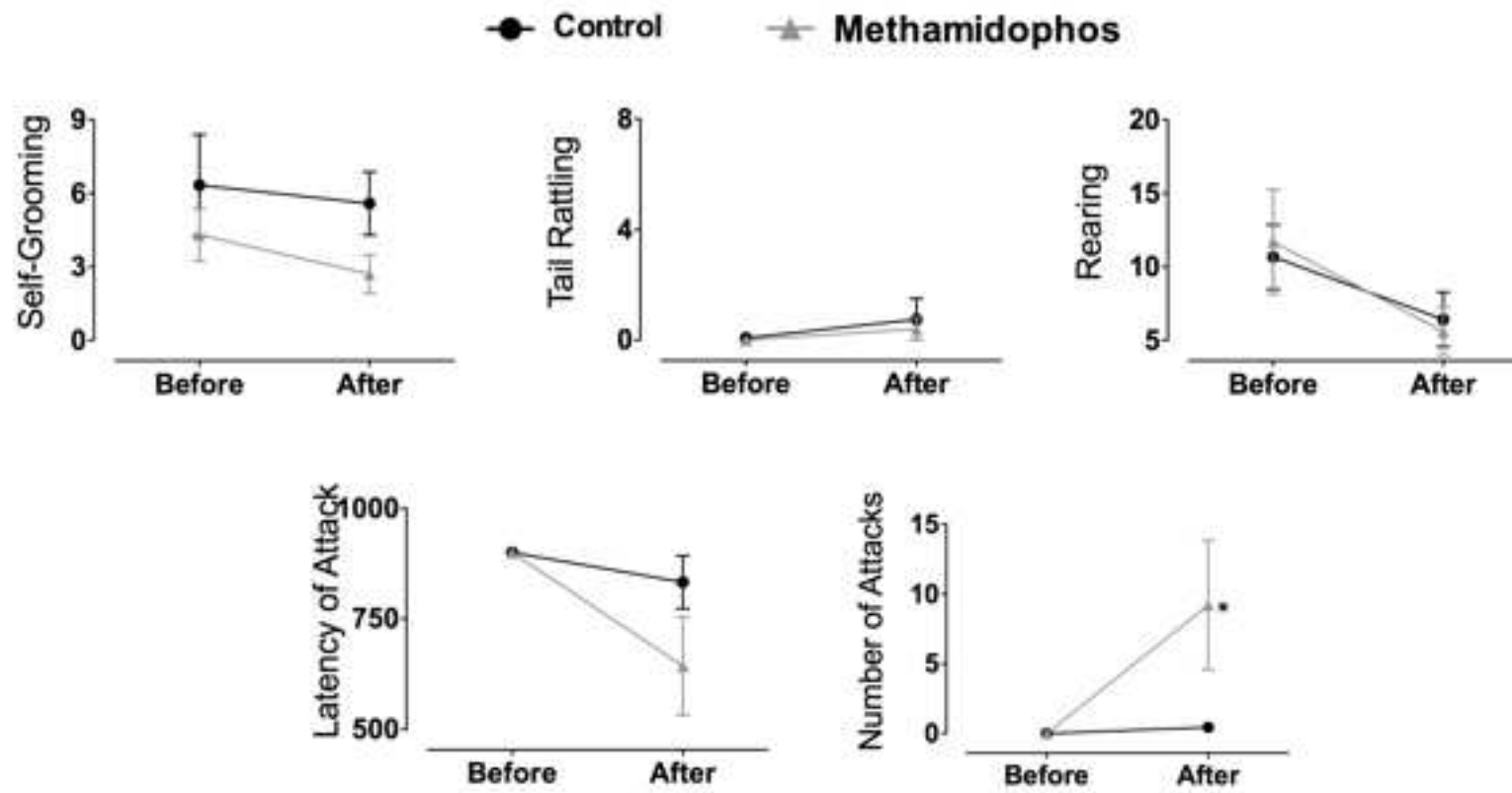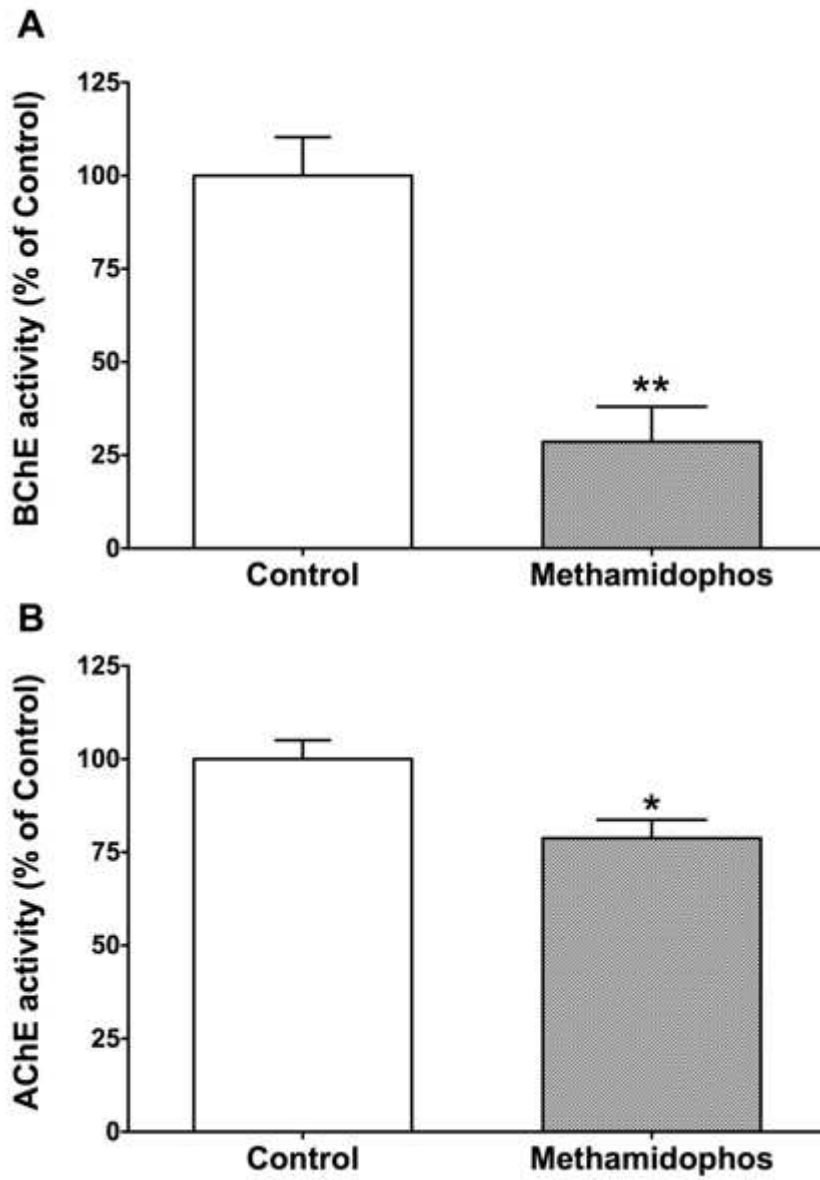**Figure 2**

Figure 3

Figure 4

Table 1

	Behaviour	Treatment Factor	Time Factor	Interaction
	Latency to attack	$F_{(1,90)}=0.04$, $p>0.05$	$F_{(1,90)}=0.09$, $p>0.05$	$F_{(1,90)}=0.08$, $p>0.05$
	Number of attacks	$F_{(1,90)}=1.35$, $p>0.05$	$F_{(1,90)}=0.37$, $p>0.05$	$F_{(1,90)}=1.08$, $p>0.05$
Grouped data	Tail rattling	$F_{(1,90)}=2.52$, $p>0.05$	$F_{(1,90)}=1.00$, $p>0.05$	$F_{(1,90)}=0.200$, $p>0.05$
	Self-grooming	$F_{(1,90)}=2.81$, $p>0.05$	$F_{(1,90)}=1.34$, $p>0.05$	$F_{(1,90)}=0.64$, $p>0.05$
	Rearing	$F_{(1,90)}=1.50$, $p>0.05$	$F_{(1,90)}=2.50$, $p>0.05$	$F_{(1,90)}=0.19$, $p>0.05$
	Latency to attack	$F_{(1,46)}=1.20$, $p>0.05$	$F_{(1,46)}=1.83$, $p>0.05$	$F_{(1,46)}=0.27$, $p>0.05$
	Number of attacks	$F_{(1,46)}=0.02$, $p>0.05$	$F_{(1,46)}=1.40$, $p>0.05$	$F_{(1,46)}=0.06$, $p>0.05$
Aggressive animals	Tail rattling	$F_{(1,46)}=4.55$, $p>0.05$	$F_{(1,46)}=2.31$, $p>0.05$	$F_{(1,46)}=0.41$, $p>0.05$
	Self-grooming	$F_{(1,46)}=0.12$, $p>0.05$	$F_{(1,46)}=0.95$, $p>0.05$	$F_{(1,46)}=1.43$, $p>0.05$
	Rearing	$F_{(1,46)}=3.14$, $p>0.05$	$F_{(1,46)}=3.25$, $p>0.05$	$F_{(1,46)}=0.05$, $p>0.05$
	Latency to attack	$F_{(1,40)}=2.47$, $p>0.05$	$F_{(1,40)}=7.24$, $p<0.05$*	$F_{(1,40)}=2.47$, $p>0.05$
	Number of attacks	$F_{(1,40)}=4.32$, $p<0.05$*	$F_{(1,40)}=5.28$, $p<0.05$*	$F_{(1,40)}=0.08$, $p<0.05$*
Non-aggressive animals	Tail rattling	$F_{(1,40)}=0.22$, $p>0.05$	$F_{(1,40)}=1.39$, $p>0.05$	$F_{(1,40)}=0.08$, $p>0.05$
	Self-grooming	$F_{(1,40)}=2.80$, $p>0.05$	$F_{(1,40)}=0.64$, $p>0.05$	$F_{(1,40)}=0.08$, $p>0.05$
	Rearing	$F_{(1,40)}=0.001$, $p>0.05$	$F_{(1,40)}=3.72$, $p>0.05$	$F_{(1,40)}=0.15$, $p>0.05$

Table 2

Treatment	Crossed Squares	
	Central	Peripheral
CTRL-Sal	21.2 ± 4.8	60.3 ± 5.0
MTD 3.5 mg/Kg	10.5 ± 1.9 [#]	68.8 ± 7.6

Notes: Data represent the mean ± S.E.M. # p=0.052 from the MTP 3.5mg/Kg compared with saline group.