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Roles of neuropeptides in *O*,*O*,*S*-trimethylphosphorothioate (OOS-TMP)-induced anorexia in mice ☆

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Abstract

O,*O*,*S*-Trimethylphosphorothioate (OOS-TMP), an impurity present in various organophosphorus insecticides, has previously been shown to induce hypophagia. The major goal of this study was to investigate its mechanism of action. Both intracerebroventricular (i.c.v.) and intraperitoneal (i.p.) injection transiently induced hypophagia at a dose of 5 mg/kg within 6 h, without causing lung injury. Hypophagia was accompanied by up-regulation of corticotropin releasing factor (CRF) (2.92 ± 0.45 *vs.* 1.7 ± 0.5, at 2 h after i.c.v., 3.40 ± 1.38 *vs.* 1.76 ± 0.41 at 6 h after i.p., $P < 0.05$) in the hypothalamus. After i.c.v. injection, hypophagia recovered by 6 h after dosing. At doses higher than 5 mg/kg, i.c.v. injection induced continuous hypophagia from 20 min to 72 h after dosing, accompanied by hypothermia and lung injury. OOS-TMP was considered to induce hypophagia through enhancing expression of CRF.

Keywords: Anorexia; Hypothalamus; Lung injury; Neuropeptides; OOS-TMP

O,*O*,*S*-Trimethylphosphorothioate (OOS-TMP) is an impurity found in a number of widely used organophosphorous insecticides [1–3]. It is suspected to have been a causative agent in the fatal accidents in malaria workers in Pakistan [4]. Toxicological investigations have unveiled the unique features of OOS-TMP: it causes lung injury and profound emaciation, a so-called wasting syndrome, without inhibition of acetylcholinesterase (AchE) activity [5–10].

Anorexia is one of the typical symptoms of the wasting syndrome caused by OOS-TMP, and is accompanied by hypothermia and lung injury. However, the underlying mechanism of this hypophagia remains an enigma. A previous study has shown that intracerebroventriucular (i.c.v.) administration in rats induced only hypophagia and slight weight loss without other symptoms [11], which suggests that the eating disorder caused by OOS-TMP is at least partly mediated by direct action on the central nervous system.

It is well established that the central nervous system plays a pivotal role in regulation of appetite and body weight. In the brain, the hypothalamus is a central feeding organ that mediates the regulation of short-term and long-term food intake and energy balance, via secretion and synthesis of various orexigenic and anorectic neuropeptides [12,13]. Among these orexigenic signals are orexin, neuropeptide Y (NPY), melanin-concentrating hormone (MCH), and agouti gene-related protein (AgRP), which potently stimulate appetite and decrease energy expenditure, whereas proopiomelanocortin (POMC), cocaine- and amphetamine-regulated transcript (CART), corticotropin releasing factor (CRF), and urocortin (UCN) 1–3 are anorexigenic peptide molecules that reduce

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food intake and increase energy expenditure. To date, no information has been available on the role of the hypothalamus in the inhibition of food consumption by OOS-TMP. The principle aim of the present study was to explore OOS-TMP-induced hypophagia. We thus performed both i.c.v. and intraperitoneal (i.p.) injection; monitored food consumption, body temperature, and body weight; measured the mRNA expression levels of eating-related orexigenic and anorexigenic neuropeptides in the hypothalamus; and examined lung histopathology.

Materials and methods

Animals. The study protocol was approved by the Animal Research Committee of Kyoto University. All mice were handled in accordance with the Animal Welfare Guidelines of Kyoto University. We used male mice throughout this study. We purchased 6- to 7-week-old ddY mice from Japan SLC (Shizuoka, Japan). They were housed individually and given free access to a palletized chow and water during the acclimatization period, except when otherwise indicated. All animals were maintained at an ambient temperature of 24 ± 2 °C and $50 \pm 10\%$ humidity, with a controlled light/dark cycle (12:12 h).

Chemicals and OOS-TMP. All chemicals were of the purest analytical grade. OOS-TMP was synthesized and purified as previously described by Hasegawa et al. [10]. The purity of the compound was found to be >99.8% as determined by NMR (JEOL JNM-EX400KS, JEOL, Akishima, Tokyo, Japan).

LD_{50}. Mice were allowed to acclimatize to laboratory conditions for at least 3 days prior to experiment. Oral LD_{50} of OOS-TMP at 120 h was determined by the method of Weil [14], using five doses of OOS-TMP and groups of four animals each. The selected doses were 0, 15, 30, 60, and 120 mg/kg per 2 ml corn oil. The animals which were fasted for 16 h prior to treatment received OOS-TMP by gavage between 9 and 10 a.m., and were observed for 120 h.

i.c.v. Cannulation and temperature-sensing chip. Surgical operation for i.c.v. cannulation was carried out as reported by Asakawa et al. [15]. Briefly, mice were anesthetized with sodium pentobarbital by i.p. injection (80–85 mg/kg) and fixed in a stereotaxic frame (SR-6; Narishige, Tokyo, Japan). A small hole was made in the skull using a needle inserted 0.9 mm lateral to the central suture and 0.9 mm posterior to the bregma. A 24-gauge cannula beveled at one end over a distance of 3 mm (Safelet-Cas; Nipro, Osaka, Japan) was inserted into the third cerebral ventricle for i.c.v. injection. The stainless-steel cannula was fixed to the skull with dental cement and capped with silicon, without an obtruder. Temperature-sensing microchips (Digital Angel Corporation, Paul, MN, USA) were implanted in the interscapular region. The animals were allowed to recover for 1 week before any experimental manipulation.

i.c.v. Injection and experimental process. Conscious animals were gently restrained by hand. Four microliters of various concentrations of OOS-TMP, dissolved in 5% dimethylsulfoxide (DMSO) in 0.9% saline, were injected intracerebroventricularly using a microsyringe via PE-20 tubing, fitted with a 27-gauge needle that was inserted through the guide cannula to a depth of 3 mm below the external surface of the skull. Control animals received an equivalent injection of vehicle. Before feeding tests, mice were fasted for 16 h with unlimited access to water. A standard diet (F-2, 3.73 kcal/g; Funahashi Farm Corp., Chiba, Japan) was used. OOS-TMP or vehicle was administered by a single i.c.v. injection to food-deprived mice at 10:00 a.m. Food intake and body temperature were recorded at 20 min and 1, 2, 4, 6, 24, 48, and 72 h, and body weight was monitored at 0, 6, 24, 48, and 72 h after i.c.v. injection. At the end of observation, blood samples were obtained from the orbital sinus under diethyl ether anesthesia. Mice were sacrificed by rapid decapitation. The hypothalamus was removed quickly and frozen at −70 °C. Whole brains were frozen in liquid nitrogen immediately and kept at −70 °C until use. Blood samples were centrifuged at 3000*g* for 15 min at 4 °C, and sera were separated and frozen at −70 °C until analyzed. Lungs were excised from the carcass and fixed in 10% (v/v) neutral-buffered formalin.

i.p. Injection and experimental process. After fasting for 16 h, conscious mice received i.p. injection of OOS-TMP 5 mg/kg. Control animals received an equivalent dose of 5% DMSO in 0.9% saline. Food intake was recorded at 20 min and 1, 2, 4, and 6 h following i.p. injection. At 6 h, mice were sacrificed by rapid decapitation. The hypothalamus was dissected out quickly and frozen at −70 °C until use. Lungs were excised from the carcass and fixed in 10% (v/v) neutral-buffered formalin.

Real-time RT-PCR analysis of gene expression in the hypothalamus. Hypothalamic gene expression was examined as previously reported [16]. Total RNA was extracted from the hypothalamus using an RNeasy Lipid Tissue Mini Kit (Qiagen, Tokyo, Japan). Aliquots (10 ng) were amplified using QuantiTect SYBR Green RT-PCR (Qiagen, Tokyo, Japan). Quantification of amplified products was performed on an ABI PRISM 7700 Sequence Detection System purchased from Applied Biosystems Japan (Tokyo, Japan). All expression data were normalized to the expression level of mouse glyceraldehyde 3-phosphate dehydrogenase (G3PDH) from the same individual samples.

Lung histopathology. Fixed lungs were processed routinely, embedded in paraffin wax, and their sections were stained with hematoxylin–eosin (HE).

Clinical chemistry. Serum CRP, LDH, and AchE were determined by conventional methods. In addition, we also determined brain AchE activity using brain homogenates in five volumes of phosphate buffer (pH 7.5) standardized to protein concentrations, as reported previously [10].

Statistical analysis. Data are presented as means ± SD. Statistical analyses of cumulative food intake and mRNA expression for neuropeptides were performed by the *t* test. As for the dose–response of cumulative food intake, body temperature change, and relative body weight, we performed one-way analysis of variance (ANOVA). When ANOVA was significant, the Duncan procedure was performed for multiple comparison. Statistical analysis used STATISTICA software (StatSoft, Japan). $P < 0.05$ was considered to be significant.

Results

LD_{50}

The 120 h oral LD_{50} of OOS-TMP for mice was found to be 60 mg/kg (95% CI 40–89 mg/kg). Death occurred at 3–4 days following treatment. At all doses, animals presented with severe hypophagia and body weight loss (data not shown).

Dose–response and time course of cumulative food intake after i.c.v. injection

A dose–response relationship between food intake and dose of OOS-TMP is shown in Fig. 1A and B. All the treated animals had inhibited food intake. Mice dosed with OOS-TMP at 5 mg/kg ate significantly less food during the first 2 h in comparison with control mice, but recovered by 4 h. By contrast, at doses >5 mg/kg, animals revealed hypophagia and continued up to 72 h.

Changes in body temperature and weight after i.c.v. injection

The change in body temperature is summarized in Fig. 2A and B. OOS-TMP-induced hypothermia at 20 min, except at a dose of 5 mg/kg. At doses between 5 and 15 mg/kg, body temperature recovered by 6 h, but again, declined irreversibly. At

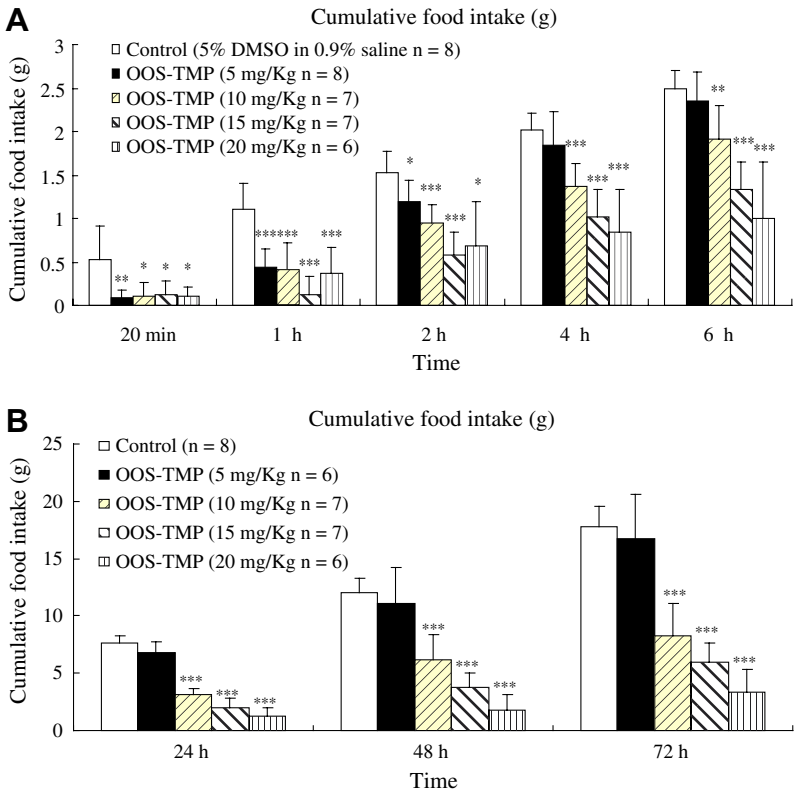

Fig. 1. Effects of single i.c.v. injection of OOS-TMP on cumulative food intake in mice. (A) Dose–response and time course. Cumulative food intake at 0 and 20 min, and 1, 2, 4, and 6 h after i.c.v. injection of fasted animals with OOS-TMP (5, 10, 15, 20 mg/kg). (B) Dose–response and time course. Cumulative food intake at 24, 48, and 72 h after i.c.v. injection of OOS-TMP (5, 10, 15, 20 mg/kg). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* control.

20 mg/kg, hypothermia continued consistently throughout the whole experimental period.

OOS-TMP decreased body weight after 24 h at doses >5 mg/kg, while at 5 mg/kg, it had no such effect (data not shown).

Effect of OOS-TMP on mRNA level for hypothalamic neuropeptides after i.c.v. injection

As shown in Fig. 3, real-time RT-PCR analysis at 2 h after i.c.v. injection of 5 mg/kg OOS-TMP revealed a significant reduction in the level of mRNA for orexigenic factor orexin, and an increase in mRNA for anorectic signals CRF and UCN1 in the hypothalamus, compared with control levels (0.57 ± 0.22 *vs.* 0.98 ± 0.07, $P < 0.01$ for orexin; 2.92 ± 0.45 *vs.* 1.70 ± 0.50, $P < 0.01$ for CRF; 3.24 ± 1.62 *vs.* 1.22 ± 0.28, $P < 0.01$ for UCN1).

Lung histopathology after i.c.v. injection

We examined lung histology by microscopic observations after i.c.v. administration of OOS-TMP. At 2 h, no detectable morphological changes were observed in mice given 5 mg/kg OOS-TMP compared with control animals (data not shown). However, at 24 h, desquamation of Clara cells into bronchi was observed to a mild degree at this dose, but no alveolar damage was detected (data not shown). At 10 mg/kg, extensive desquamation of Clara cells and alveolar damage were observed (data not shown) at 24 h. There were no morphological changes in either alveolar or bronchiolar region with OOS-TMP 5 mg/kg i.p. (data not shown).

Clinical chemistry after i.c.v. injection

AchE activity was not inhibited 24 h after i.c.v. dosing with OOS-TMP at 5 mg/kg: 31.2 ± 7.39 nmol/min, $n = 5$ for control mice, and 31.2 ± 7.39 nmol/min, $n = 5$ in the brain of treated mice; 6 ± 2.83 nmol/min for control mice and 8.15 ± 1.89 nmol/min in the serum of treated mice ($P > 0.05$). It was concluded that OOS-TMP did not inhibit AchE. The level of LDH and CRP in treated mice was not significantly different from that in control mice (data not shown).

Effects of OOS-TMP after i.p. administration

We also investigated the cumulative food intake after i.p. treatment with 5 mg/kg OOS-TMP from 20 min to 6 h. Anorexia began by 6 h, and animals displayed considerable hypophagia (Fig. 4A). We also examined mRNA expression in the hypothalamus for CRF, orexin, and UCN1, peptides which were changed after i.c.v. treatment. mRNA level for CRF was found to be up-regulated

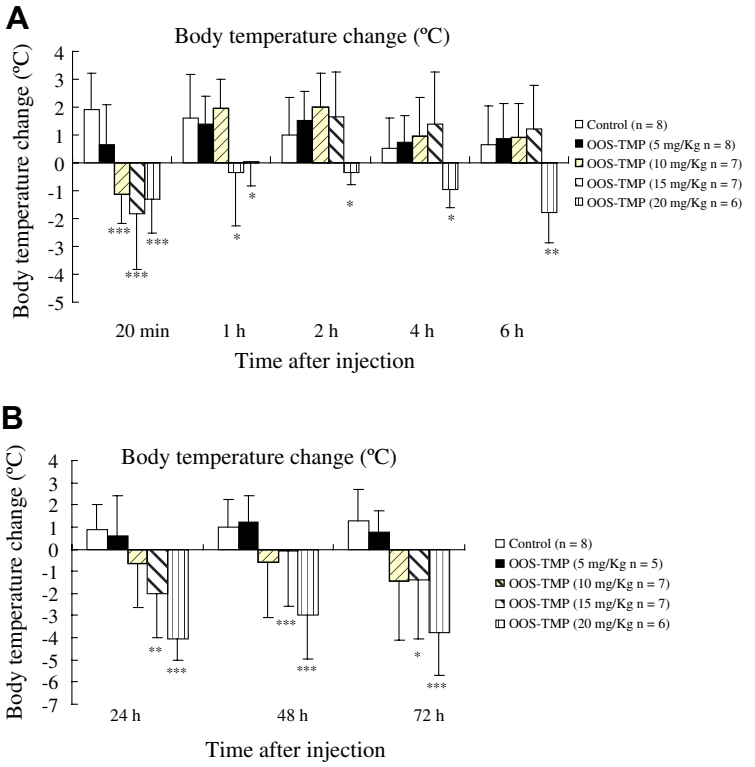

Fig. 2. Effects of single i.c.v. injection of OOS-TMP on body temperature in mice. (A) Body temperature changes at 0 and 20 min, and 1, 2, 4, and 6 h after i.c.v. injection of OOS-TMP (5, 10, 15, 20 mg/kg). Initial body temperature was 36.28 ± 1.19 °C in control mice; 36.44±0.98 °C in mice administered 5 mg/kg OOS-TMP; 35.78 ± 0.98 °C in mice administered 10 mg/kg OOS-TMP; 35.49±1.78 °C administered 15 mg/kg OOS-TMP; and 36.13 ± 0.86 °C in mice administered 20 mg/kg OOS-TMP; there were no significant differences among these values (ANOVA, $P > 0.05$). We set the average temperature for each dose at 0 °C, and then observed the relative body temperature changes after 20 min, and 1, 2, 4, 6, 24, 48, and 72 h. (B) Body temperature changes at 24, 48, and 72 h after i.c.v. treatment of fasted animals with OOS-TMP (5, 10, 15, 20 mg/kg). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* control.

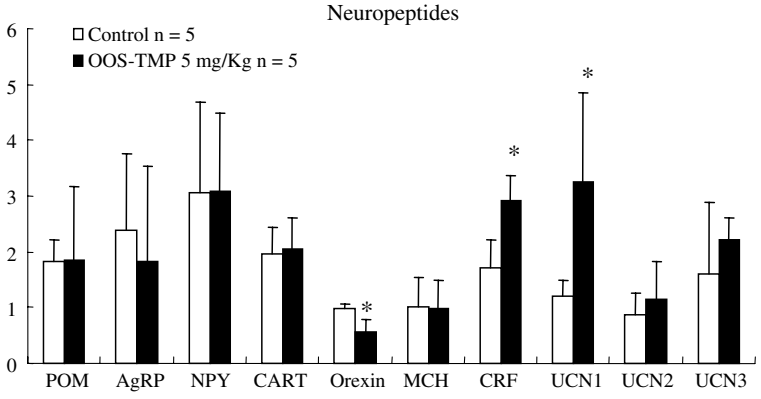

Fig. 3. Neuropeptide mRNA expression in the hypothalamus following i.c.v. injection. Expression of appetite-regulating neuropeptides (POMC, AgRP, NPY, CART, orexin, MCH, CRF, UCN1, UCN2, and UCN3) in the hypothalamus after 2 h in mice treated with i.c.v. OOS-TMP 5 mg/kg. All expression data were normalized to the expression level of mouse G3PDH from the same individual samples. $^{*}P < 0.05$ *vs.* control.

significantly (3.40 ± 1.38 *vs.* 1.76 ± 0.41, $P < 0.05$), while other peptides, such as UCN1 and orexin, were not (Fig. 4B). Lung injury was not found in mice dosed at 5 mg/kg i.p. (data not shown).

Discussion

We found that administration of OOS-TMP inhibited food intake irrespective of administration route, without causing lung injury, at a dose of 5 mg/kg (1/12 of LD_{50}). The inhibition after i.c.v. OOS-TMP at 5 mg/kg did, however, recover by 24 h, while at doses greater than 5 mg/kg, inhibition did not recover. In parallel, inhibitions of food intake following both i.p. and i.c.v. administration at 5 mg/kg were accompanied by up-regulation of CRF in the hypothalamus. As previously reported [9] and [10], we also confirmed that neither brain nor serum AchE was inhibited by OOS-TMP at 5 mg/kg.

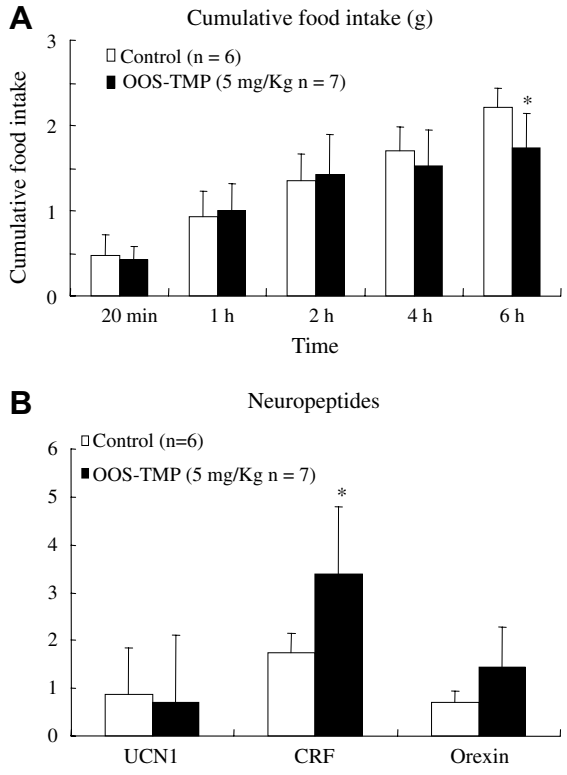

Fig. 4. Effect of OOS-TMP on cumulative food intake and neuropeptide mRNA expression in the hypothalamus following i.p. administration. (A) Cumulative food intake after 0 and 20 min, and 1, 2, 4, and 6 h after i.p. injection of OOS-TMP (5 mg/kg). (B) Expression of neuropeptides (orexin, CRF, and UCN1) in the hypothalamus at 6 h in mice treated with i.p. OOS-TMP 5 mg/kg, as assessed by real-time quantitative PCR. $^{*}P < 0.05$ *vs.* control.

The up-regulation of CRF in the hypothalamus, time course of hypophagia, and absence of pathological changes in the lung, collectively suggest that the hypophagia induced by 5 mg/kg OOS-TMP is mediated by up-regulation of CRF.

During our experiments, treated mice immediately showed sleep-like behavior with closed eyes and reduced locomotor activity; in addition, they stopped looking for food and showed a dramatic 3–4 °C loss of body temperature. These features are very similar to the behavior of mice treated with i.c.v. glucagon-like peptide-1 (GLP-1) [17]. The results with GLP-1 suggest that OOS-TMP acts on neurons in a similar manner to those sensitive to GLP-1. Alternatively, anorexia accompanied by hypothermia may be similar to the symptoms seen when bombesin (BN) and its structurally related peptides, gastrin-releasing peptide (GRP) and neuromedin B, are given intracerebroventricularly to mice. The mechanism of BN-induced hypophagia and hypothermia appears to mainly involve the GRP receptor and the NO–cGMP–PKG pathway [18]. Likewise, the effects of OOS-TMP may be mediated by the same system. Further study is needed to establish which neurons are involved in the hypophagia induced by OOS-TMP.

Appetite and energy homeostasis are regulated by various feeding-stimulatory (orexigenic) and feeding-inhibitory (anorexigenic) signaling pathways in the hypothalamus. CRF is a major anorexigenic signal in the hypothalamus. In addition to its role as a controller of the hypothalamus–pituitary–adrenal axis, CRF inhibits food intake, increases energy expenditure and produces sustained weight loss [19]. In the current study, the increment in mRNA expression for CRF following both i.c.v. and i.p. injection indicates that CRF is a key signaling molecule and highlights its importance in OOS-TMP-reduced anorexia. In contrast, in the long term, some unknown mechanisms other than this pathway compensate for its role and warrants further investigation.

A more complete understanding of the molecular mechanism of OOS-TMP-induced anorexia will be useful in designing anti-obesity drugs and understanding the wasting syndrome induced by OOS-TMP. The mechanism of up-regulation of CRF by OOS-TMP warrants further study.

References

[1] G. Pellegrini, R. Santi, Potentiation of toxicity of organophosphorus compounds containing carboxylic ester functions toward warm-blooded animals by some organophosphorus impurities, J. Agric. Food Chem. 20 (1972) 944–950.
[2] N. Umetsu, F. Grose, R. Allahyari, S. Abu-El-Haj, T. Fukuto, Effect of impurities on the mammalian toxicity of technical malathion and acephate, J. Agric. Food Chem. 25 (1977) 946–952.
[3] W. Aldridge, J. Miles, D. Mount, R. Verschoyle, The toxicological properties of impurities in malathion, Arch. Toxicol. 42 (1979) 95–106.
[4] E.J. Baker, M. Warren, M. Zack, R. Dobbin, J. Miles, S. Miller, L. Alderman, W. Teeters, Epidemic malathion poisoning in Pakistan malaria workers, Lancet 1 (1978) 31–34.
[5] R. Verschoyle, E. Reiner, E. Bailey, W. Aldridge, Dimethylphosphorothioates. Reaction with malathion and effect on malathion toxicity, Arch. Toxicol. 49 (1982) 293–301.
[6] T. Imamura, J. Gandy, T. Fukuto, Selective inhibition of rat pulmonary monooxygenase by *O*,*O*,*S*-trimethyl phosphorothioate treatment, Biochem. Pharmacol. 32 (1983) 3191–3195.
[7] W. Aldridge, D. Dinsdale, B. Nemery, R. Verschoyle, Some aspects of the toxicology of trimethyl and triethyl phosphorothioates, Fundam. Appl. Toxicol. 5 (1985) S47–S60.
[8] J. Gandy, T. Imamura, Cellular responses to *O*,*O*,*S*-trimethyl phosphorothioate-induced pulmonary injury in rats, Toxicol. Appl. Pharmacol. 80 (1985) 51–57.
[9] A. Koizumi, M. Montalbo, O. Nguyen, L. Hasegawa, T. Imamura, Neonatal death and lung injury in rats caused by intrauterine exposure to *O*,*O*,*S*-trimethylphosphorothioate, Arch. Toxicol. 61 (1988) 378–386.
[10] J. Hasegawa, Y. Wada, M. Sageshima, M. Suzuki, S. Kamiyama, N. Abe, A. Koizumi, Structure and pulmonary toxicity relationship on *O*,*O*-dimethyl *S*-alkyl phosphorothioate esters, Pharmacol. Toxicol. 66 (1990) 367–372.
[11] K. Ohtaka, N. Hamade, Y. Yamazaki, M. Suzuki, A. Koizumi, A direct involvement of the central nervous system in hypophagia and inhibition of respiratory rate in rats after treatment with *O*,*O*,*S*-trimethyl phosphorothioate, Arch. Toxicol. 69 (1995) 559–564.
[12] E. Gropp, M. Shanabrough, E. Borok, A. Xu, R. Janoschek, T. Buch, L. Plum, N. Balthasar, B. Hampel, A. Waisman, G. Barsh, T. Horvath, J. Brüning, Agouti-related peptide-expressing neurons are mandatory for feeding, Nat. Neurosci. 8 (2005) 1289–1291.

[13] S. Arora, Role of neuropeptides in appetite regulation and obesity—a review, Neuropeptides 40 (2006) 375–401.
[14] C.S. Weil, Tables for convenient calculation of median-effective does (LD50 or ED 50) and instructions in their use, Biometrics 8 (1952) 249–255.
[15] A. Asakawa, A. Inui, T. Kaga, H. Yuzuriha, T. Nagata, M. Fujimiya, G. Katsuura, S. Makino, M. Fujino, M. Kasuga, A role of ghrelin in neuroendocrine and behavioral responses to stress in mice, Neuroendocrinology 74 (2001) 143–147.
[16] M. Toyoshima, A. Asakawa, M. Fujimiya, K. Inoue, S. Inoue, M. Kinboshi, A. Koizumi, Dimorphic gene expression patterns of anorexigenic and orexigenic peptides in hypothalamus account male and female hyperphagia in Akita type 1 diabetic mice, Biochem. Biophys. Res. Commun. 352 (2007) 703–708.
[17] T. Bungo, M. Shimojo, Y. Masuda, T. Tachibanab, S. Tanaka, K. Sugahara, M. Furuse, Intracerebroventricular administration of mouse leptin does not reduce food intake in the chicken, Brain Res. 817 (1999) 196–198.
[18] H. Tsushima, M. Mori, Mechanisms underlying anorexia after microinjection of bombesin into the lateral cerebroventricle, Pharmacol. Biochem. Behav. 80 (2005) 289–296.
[19] A. Inui, Eating behavior in anorexia nervosa—an excess of both orexigenic and anorexigenic signalling? Mol. Psychiatry 6 (2001) 620–624.