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Effect of Methamidophos on cerebellar neuronal cells

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Summary: Methamidophos is a toxic organophosphorus compound that inhibits acetlycholinesterase activity, and induces neurotoxicity. It is a synthetic chemical commonly used as pesticides to limit pest damages to cultivated plants. Currently, there is serious public health concern over its safety and use due to its global nature, persistence and bioaccumulations. We have previously reported that methamidophos suppressed thyroid hormone receptor (TR)-mediated transcription, but did not dissociate the interaction between TR and its response element (thyroid hormone response element; TRE), neither did it interact with nuclear cofactors. In the present study, we investigated the effects of methamidophos on cerebellar neuronal cells. Using primary cerebellar culture from new born rats, We observed that Purkinje cell dendrite arborization were greatly impaired in the absence of thyroid hormone (TH), However, low dose methamidophos 10^{-6} M did not significantly impair dendrite arborization of cerebellar Purkinje cells in the presence of thyroid hormone (TH). However, using granule cell reaggregate culture, we observed that low dose methamidophos 10^{-6} M remarkably suppressed granule cell neurite extension in the presence of TH. Taken together, our study shows that low dose methamidophos may negatively impact TH-mediated cerebellar neuronal cell development and function, and consequently could interfere with TH-regulated neuronal events.

Keywords*:* Methamidophos, Thyroid hormone, Purkinje cells, Granule cell, Neuronal development

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INTRODUCTION

Thyroid hormone (TH) is vital for normal neuronal development and function in animals and humans. During the perinatal critical period of brain development, TH regulates numerous neuronal activities in different brain regions, and mediate neuronal migration and differentiation (Potterfield, 2000). TH deficiency especially during the period of brain growth spurt results in aberrant brain development (cretinism) with severe physical and / or mental retardation in the offsprings (Koibuchi and Chin, 2000). Specifically, perinatal hypothyroidism causes reduction in the growth and branching of Purkinje cell dendrites (Nicholson and Altman, 1972), decreased number of synapses between the Purkinje cells and granule cell axons (Nicholson and Altman, 1972), delayed disappearance of the external granule cell layer (EGL) and delayed migration of granule cells into the internal granule cell layer (IGL) (Nicholson and Altman, 1972). TH homeostasis is controlled by sensitive feedback mechanism within the hypothalamic-pituitary-thyroid axis (Capen, 1997). Thyroid stimulating hormone (TSH) induces the thyroid to synthesize thyroxine (T4), which is then deiodinated to the more active form tri-iodothyronine (T3). TH functions are biologically regulated by TRs. TR are ligand-dependant transcription factors that are widely expressed (Bradley et al, 1992). TR is bound to specific DNA sequence known as TH response element (TRE) upstream of the target gene. Alterations in TH homeostasis may lead to suppression of TR-mediated gene expression which could impair normal neuronal developmental events.

The world pesticide market is currently experiencing a dramatic demand, growing by 11.2 % annually between 1960 and 1992 (Mansour et al, 2004). While many pesticides have been withdrawn or banned in the developed worlds, these banned pesticides are still been produced and sold in the developing world by domestic companies or by some multinationals acting through subsidiaries or joint ventures (Szmedra, 1994). These include DDT and organophosphorus (OPs).

Globally, OPs account for approximately 40% of total pesticide sales by volume (WRI, 1996). Cholinesterase inhibitor OPs are among the most commonly used pesticides for domestic and agricultural purposes, and they account for

approximately 50% of world usage (Casida and Quistad 2004). Methamidophos is one of most frequently used OP pesticide. There is currently great health concern over their usage especially in developing countries where agro-based industries are still a major occupational source and about 95% of the world's 1.1 billion farmers currently resides (WRI, 1994). Although pesticides are largely beneficial to improve overall crop yield, its continous use is also largely associated with potential risk and adverse health implications in humans and wildlife. Due to poor and un-enforced regulations of its use, careless handling, improper disposal and accidental spills, many potentially but avoidable fatal incidences have been recorded both in humans and on the environment. (Azmi et al, 2006).

Due to their ability to resist bio-degradation and stability in the environment, there is increasing evidence of their bioaccumulation in humans and in the environment, and consequently may impact negatively on human and environmental health. (Zhang et al, 2002; Azmi et al, 2006).

Occupational or environmental OP poisoning is characterized by miosis, altered level of consciousness, respiratory failure, vomiting, salivating and breathing difficulty (Lotti, 2000, 2001). In severe cases, convulsive seizures and respiratory arrest culminating in death can occur (FAO/WHO, 1992). These symptoms are due to inhibition of acetylcholinesterase which hydrolyses acetylcholine (Edwards and Tchounwou, 2005). The neurological effects of OP is due to their ability to cross the blood brain barrier (Ferrer, 2003). This can lead to accumulation of acetylcholine in the synaptic junction thereby precipitating a cholinergic storm, characterized by continuous stimulation of glands, muscles and the CNS (Drexler et al, 2010). Other neurotoxic effect ascribed to OP is OP-induced delayed neurotoxicity, which is characterized by paralysis of the lower limbs, partial sensory loss, and degeneration of long axons in the spinal cord and peripheral nerves (Gubert et al, 2011).

OP has been shown to directly affect some neurotransmitter systems. OP causes remarkable decreses in the gamma aminobutyric acid (GABA) levels, while increasing dopamine, norepinephrine, serotonin, and glutamate levels in the brain (Kar and Matin, 1972: Glisson et al, 1974:, Ali et al, 1980:, Giacobini et al, 1996: Solberg and Belkin, 1997). However, very little is still known about the effects of methamidophos on TH-mediated cerebellar development.

This study was therefore designed to investigate the effect of methamidophos on Purkinje cell dendrite arborization and on granule cell neurite extension in the developing cerebellum.

MATERIALS AND METHODS

Chemicals: Thyroxine (T4) and tri-iodothyronine (T3) was purchased from Sigma Chemical Co. (St. Louis USA). Methamidophos was purchased from WAKO Chemicals (Tokyo, Japan) and was >98% pure.

Primary cerebellar cultures

Wistar rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Newborn rats were euthanised under diethylether aneasthesia on the first day of birth. The animal experimentation protocol in the present study was approved by the Animal Care and Experimentation Committee, Gunma University and all efforts were made to minimize numbers of animals used and their sufferings. Details of the culture methods are described elsewhere (Kimura-kuroda et al. 2002). Briefly, the cerebella were digested with papain and dissociated cells were suspended in a serum-free medium without TH and plated in wells of chamber slides (8-mm-diameter wells, Nunc Lab-Tek, IL) at a density of $2x10^5$ cells /0.2 ml. The slides were precoated with 0.1mg/ml poly-L-lysine (SIGMA). Next day after cell plating, T4 and /or methamidophos were added to the culture medium and half of the culture medium was replaced with fresh medium every 3-4 days for 17 days. To exclude the effects of dimethy sulfoxide (DMSO), all experimental media used had a final concentration of 0.01% DMSO.

Immunohistochemistry to analyze Purkinje cell dendritc development

Immunocytochemistry of the cultured cells are described elsewhere (Kimura-Kuroda et al. 2002; 2007). Briefly, Purkinje cells were immunostained with mouse-monoclonal (1:1000; McAB 300, Swant, Bellinzona, Switzerland) anti-calbindin-28 K antibody and fluorescein isothiocyanate (FITC)-labeled donkey anti-mouse antibody (1:200; Molecular probes, Oregon, USA) and observed under a laser conforcal scanning microscope (FV1000D spectral type inverted microscope IX81, Olympus, Tokyo, Japan). To quantify dendritic arborization, the total area covered by the dendritic tree on ten randomly selected Purkinje cells in each experiment was determined by tracing the outline of the cell and dendritic branches and computing the area using NIH image software. Data shown represent mean ± SE.M. Results from one

set of experiment are shown graphically. The relative dendritic area is shown.

Preparation of granule cell precursors (GCPs)

Wistar rats with postnatal day 4 (P4) pups were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and housed in standard cages in our animal facility. All efforts were made to minimize numbers of animals used and their sufferings. Detailed protocol of the culture with modifications is described elsewhere (Okano-Uchida et al., 2003). Briefly, cerebella from P7 pups were taken under diethylether aneasthesia, cut into small piece and incubated at 37ºC for 30 minutes in papain solution (16.5 units/ml papain/200 µg/ml L-cysteine/0.008% DNase).Tissue was rinsed in Dulbecco's phosphate buffered solution (PBS) containing 1.5 mg/ml ovomucoid, 1.5 mg/ml bovine serum albumin (BSA), and 0.008%DNase and triturated in same solution to obtain a single cell suspension. Cells were centrifuged at 1,000rpm for 10 min at room temperature and suspended in Dulbecco's PBS containing 10 mg/ml ovomucoid and 10 mg/ml BSA and centrifuged again. Cells were resuspended in panning buffer (PBS containing 0.002% BSA and 5 µg/ml insulin) and passed through a cell strainer (Falcon). To obtain a fraction enriched in GCPs, the cell suspension was loaded onto a step gradient of 35% and 60% Percoll (Amersham Biosciences) and centrifuged at 3,000 rpm for 25 minutes at room temperature. GCPs were recovered from the 35%/60% interface, washed twice in panning buffer and suspended in neurobasal medium (Invitrogen) containing 100 units/ml of penicillin, 100 µg/ml of streptomycin, 1 mM sodium pyruvate, 2 nmM L-glutamine, 2% B-27 (all obtained from Invitrogen), 5 µg/ml insulin, 100 µg/ml apotransferrin, 100 µg/ml BSA, 16 µg/ml putrescine, 40 ng/ml sodium selenite, and 30 µM N-acetly cysteine (all obtained from Sigma) and plated in a 100mm tissue culture dish precoated with 25µg/ml poly-L-lysine and incubated for 20 min at room temperature. The dish was shaken vigorously and the nonadherent cells were seeded onto 12-well plates (Falcon) at a density of 10^6 cells/ml to form aggregates. Next day, GCP aggregates were plated on 8-well slide glasses (Matsunami Osaka) precoated with 100 µg/ml poly-L-lysine and treated with T_3 and or methamidophos and incubated for 48 hours at 37ºC, 5% CO_2.

Analysis of Granule cell neurite extension

Granule cell neurite extension was observed real time using light microscope (Axiovert 10, Zeiss, Germany) with a CCD video camara (XC-77, Sony, Tokyo, Japan) and Meiji Infinity 1 software (DK300, Meiji,Ontario, Canada). The area of neurite extension was analyzed using Image J software (NIH). The area of aggregate was excluded from measurement. Data represent mean ± S.E.M., and results from one experiment are shown graphically. At least two independent experiments were performed and results were consistent for each experiment. The relative neurite areas of granule cells are shown.

Statistical analysis

Statistical significance was determined using ANOVA and *p*-values <0.05 were considered significant and marked with asterisk in the figures.

RESULTS

Methamidophos did not suppress cerebellar Purkinje cell dendritic arborization

To examine the effect of TH on Purkinje cell development, we performed primary cerebellar culture. Seventeen days after onset of culture, cells were fixed and immunostained with anti-calbindin antibody to visualize Purkinje cells. The addition of T_4 (10nM) greatly increased dendrite arborization of Purkinje cells (Fig 2) Purkinje cells cultured in the presence of TH showed elaborate dendrites with main primary shafts, secondary branches and birfurcations (Fig 1). Addition of 1 µM methamidophos to the culture in presence of 10 nM T_4 did not significantly reduce the dendritic arborization of the Purkinje cells (Fig 2). Such Purkinje Cells still elaborated dendrites

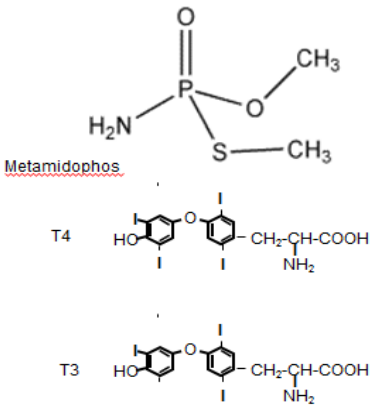

Fig 1. Structure of metamidophos, T_3, and T_4.

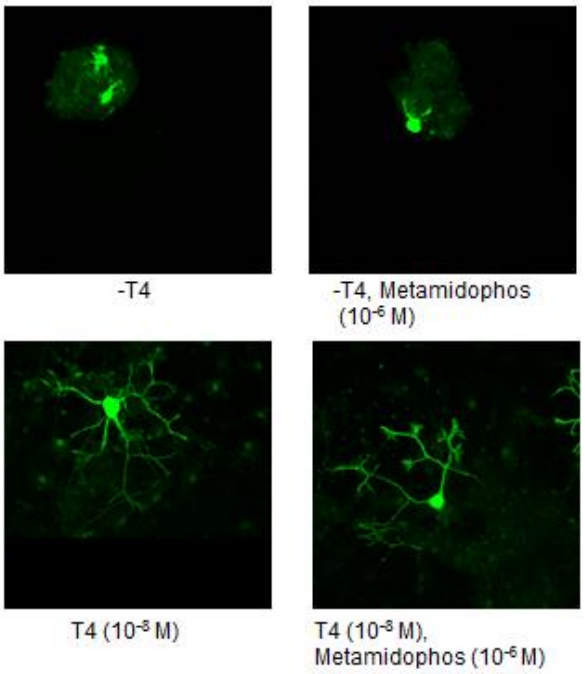

Fig 2. 10^{-10} M methamidophos did not suppress TH-induced dendrite arborization of Purkinje cell (17 DIV). Photomicrographs showing the effect of 10^{-6} M methamidophos on Purkinje cell dendrite arborization in the absence or presence of T_4 (10 nM). Scale bars indicate 50 μM

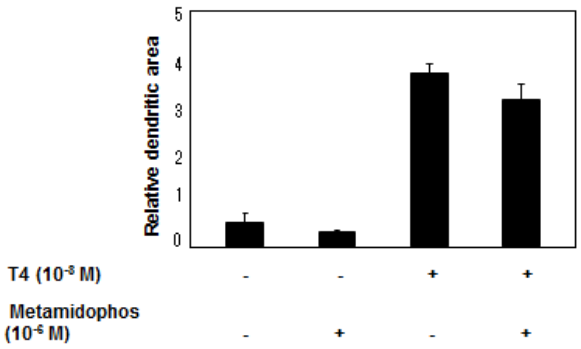

Fig 3A. Effect of methamidophos on Purkinje cell dendrite development (17DIV) Data are expressed as mean ± S.E.M. (n = 10 determinations). No significance was uncovered by ANOVA.

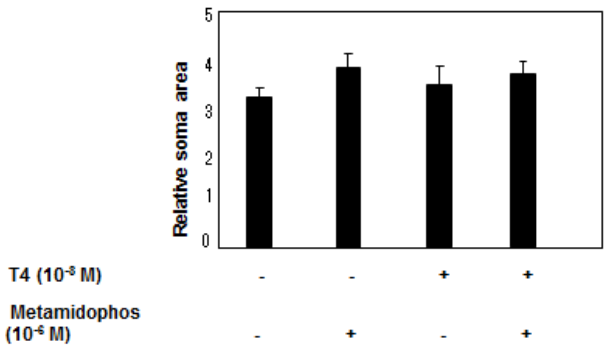

Fig 3B. Effect of methamidophos on Purkinje cell body area (17DIV) Data are expressed as mean ± S.E.M. (n = 10 determinations). No significance was uncovered by ANOVA.

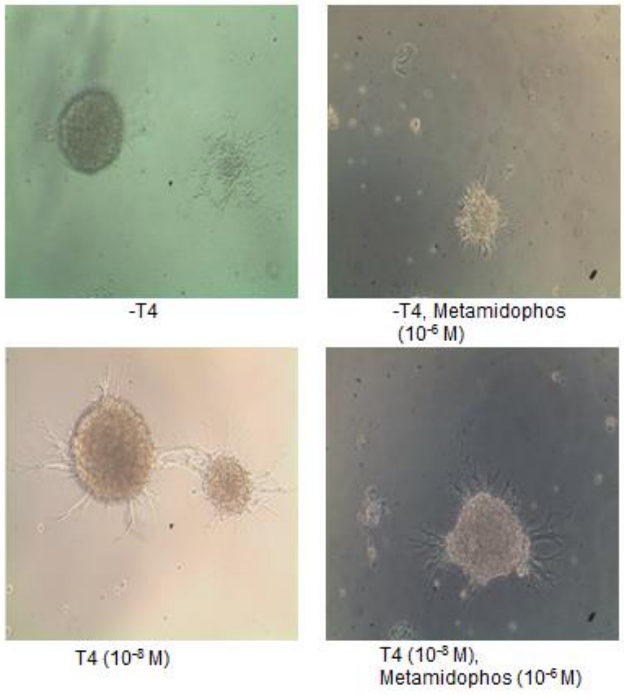

Fig 4. Methamidophos suppress TH-induced neurite extension of granule cells. Photomicrographs showing the effect of 10^{-6} M methamidophos on granule cell neurite extensions in the absence or presence of T_3 (10 nM). Scale bars indicates 50 μM.

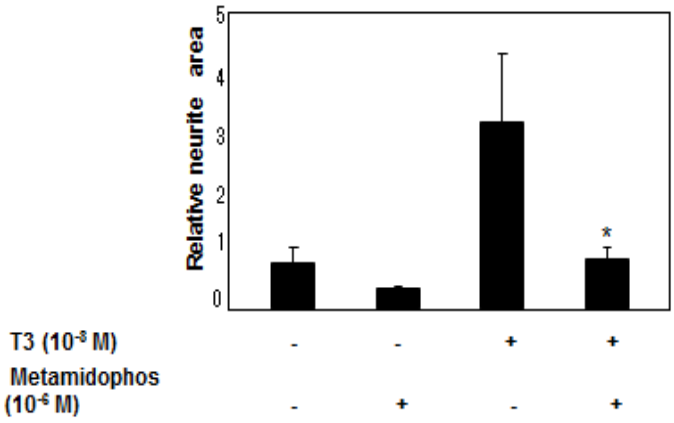

Fig 5. Effect of methamidophos on granule cell aggregate neurite extension. Data are expressed as mean ± S.E.M (n = 3 determinations). #, statistically significant $p<0.05$ by ANOVA) for T_3 (+), methamidophos (-) vs. T_3 (+), methamidophos (+). Data shown are representative of at least two independent experiments.

characterized by main shaft, secondary branches and birfurcations (Fig 2). Also, the area of dendrite was not remarkably altered in the presence or absence of methamidophos as confirmed by quantitative analysis (Fig 3A), neither was the area of Purkinje cell body remarkably altered in the presence of methamidophos (Fig 3B). Taken together, these data indicate that methamidophos did not impair Purkinje cell dendrite arborization at 1 μM concentration.

Methamidophos suppresses TH-induced neurite extension of granule cells.

We further investigated the effects of methamidophos on TH-mediated action using purified granule cell reaggregate culture. Forty-eight hours after onset of culture, granule cell were observed using CCD camera. TH (T_3) greatly stimulated neurite growth and extension compared to control culture without T_3 (Fig 4). Granule cell aggregate in the presence of T_3 form elaborate tree like neurites with several secondary shafts and bifurcating branches while those in the control medium showed fewer neurites that were thinner, had less bifurcating branches and extended shorter distance (Fig 4). Addition of 10^{-6} M methamidophos to the aggregate culture together with T_3, greatly impaired neurite growth and extension of the granule cell aggregate (Fig 4). Granule cell cultured in the presence of methamidophos and T_3 developed fewer neurite with poor growth, markedly reduced length and the secondary branches and bifurcations were poorly developed (Fig 4). The area of neurite extension of granule cells was also reduced as confirmed by quantitative analysis (Fig 5). These data clearly indicate that methamidophos effectively suppressed TH-dependent growth and extension of granule cell neurite at low dose.

DISCUSSION

In the present study, we showed that methamidophos suppressed TH-mediated granule cell neurite extension, and may consequently impact negatively on the developing brain. Thyroid hormone receptor (TR) is ubiquitously expressed in the Purkinje cell (Koibuchi N, 2008), our initial hypothesis was that methamidophos could affect TR-mediated developmental events in the Purkinje cell and thereby disrupting normal brain development and functions especially in the brain.

Our study clearly reveals that dendrite arborization of cerebellar Purkinje cell was clearly dependent on TH presence. The length of the primary shaft, secondary branches and the number of fine bifurcation of Purkinje cell dendrites were dramatically increased in the presence of TH compared to those cultured in the absence of TH (Fig 2). Addition of methamidophos however did not significantly reduce Purkinje cell dendrite arborization. Purkinje cells cultured in the presence of methamidophos and T_4 did not differ both in dendritic branching and cell body area from those cultured in the presence of T_4 only (Fig 3A & B). This clearly indicates that methamidophos did not affect Purkinje cell growth and development at 10^{-6} M.

On the other hand, T_3 remarkably enhanced the extension of neurites by granule cell aggregates. Granule cell aggregates cultured in the presence of T_3 formed elaborate neurites with several secondary shafts and bifurcating branches and extended longer distance compared with those cultured in the absence of T_3 (Fig 4). Addition of 10^{-6} M methamidophos to the aggregate culture together with T_3, significantly impaired neurite growth and extension of the granule cell aggregate (Fig 4). The length, diameter of neurites, number of branches and birfurcations were significantly reduced in the presence of methamidophos (Fig 4). Also, the area of neurite extension was significantly reduced with methamidophos treatment (Fig 5). Taken together, these data clearly show that methamidophos impaired granule cell neurite extension and may consequently impact negatively on brain development.

TH is essential for normal brain growth and function and conditions of hypothyroidism especially during the perinatal period have been known to induce cretinism with severe cognitive and/or mental disorders in the off-springs (Koibuchi et al. 2000). Previous studies have shown that TR is abundantly expressed in the developing Purkinje and granule cells (Bradley et al 1992; Strait et al. 1991). Also, the cerebellum is vital for learning, memory and vestibular function. The interaction between granule and Purkinje cells are essential for higher normal neuronal functions in the cerebellum. Therefore disruption of normal granule cell development and close interactive network with the Purkinje cell by methamidophos possibly via interaction with TR could markedly impair normal cerebellar function. In conclusion, methamidophos impairment of granule cell neurite extension may disrupt normal TH-mediated growth and development of the cerebellum and consequently, the whole brain. Caution is therefore advised in the proper handling and usage of methamidophos and regulatory laws should be implemented and strictly enforced. Also, enlightenment campaign should be embarked upon especially in the rural populace, to highlight the proper use and hazards associated with methamidophos usage as pesticides.

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