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Research Article

Suppression of Thyroid Hormone Receptor-Mediated Transcription by Methamidophos

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ABSTRACT: Methamidophos is a cholinesterase inhibitor organophosphate (OP) used commonly as a pesticide. Its use is currently a global concern due to widespread occurrence, persistence, bioaccumulation and neurotoxic potential. We therefore examined the effect of methamidophos on thyroid hormone receptor (TR)-mediated gene expression using transient transfection-based reporter gene assay. Our results shows that methamidophos (10^{-6} M) suppressed thyroid hormone (TH)-induced TR-mediated transcription. We further examined the effects of methamidophos on TR-thyroid hormone response element (TRE) binding using the liquid chemiluminescent DNA pull-down assay (LCDPA), and found no dissociation of TR from TRE. Using mammalian two hybrid assay, we showed that methamidophos did not recruit co-activator (steroid receptor co-activator 1; SRC-1) to TR in the presence of TH. Also, it did not recruit co-repressors (nuclear co-repressor; NCoR) to TR in the absence of TH at all concentrations examined. The effects of methamidophos on cerebellar Purkinje cell dendritogenesis, granule cell neurite morphology and synaptic plasticity are currently under investigation. Taken together, our results show that methamidophos can potentially disrupt TR–mediated gene expression, suggesting that methamidophos may interfere with thyroid hormone-mediated activities in various target organs including the developing brain.

Keywords: Methamidophos, Thyroid hormones, Purkinje cell,

INTRODUCTION

Cholinesterase inhibitor organophosphates (OPs) are among the most commonly used pesticides worldwide for domestic and agricultural purposes, and they account for nearly half of the insecticide world usage (Casida and Quistad 2004). Methamidophos is one of most commonly used OP. The health implications of their use are currently a global concern especially in developing countries where agro-based industries are still a major occupational source. Although largely beneficial to improve overall crop yield, its use is also largely associated with potential risk and adverse health implications in humans. 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 resistance to 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 health. (Zhang et al, 2002, Azmi et al, 2006). Acute toxic effect of OP poisoning is caused by inhibition of acetycholinesterase (AChE) in both the central and peripheral nervous system. This leads to accumulation of acetylcholine in the synaptic cleft, thereby causing cholinergic hyperstimulation which is often referred to as “cholinergic storm” (Sultatos, 1994, Mearns et al, 1994). Equally worrisome, is chronic low dose exposure to methamidophos. Studies in animal models have shown that exposure to low dose methamidophos resulted in inhibition of other neurotransmitters beside cholinesterase (Slotkin and Seidler 2007, Shahroukhi et al, 2007). Especially, the

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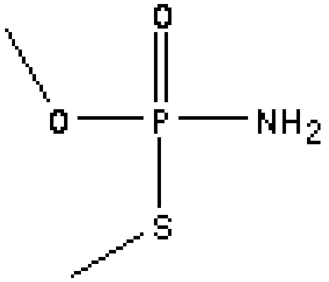
serotonergic system was greatly inhibited during the perinatal developmental phase in animal models (Slotkin et al, 2008, 2009). Studies have also shown that exposure to OP resulted in neurochemical and neurobehavioral abnormalities (Aldridge et al, 2003, Raines et al, 2001), induced depression-like behavior in mice (Lima et al, 2009). Limited epidermiological studies suggest an association between long term exposure to OP and neurological disturbance (Rolden-Tapia, 2006), especially an association between OP and psychiatric disorders (London et al, 2005), But very little is still known about the mechanisms of methamidophos action. Also, there is paucity of information on the effect of methamidophos on TR-mediated gene expression, possible mechanisms, and consequent effects on neuronal development and function.

Metamidophos



Triiodothyronine (T_3)

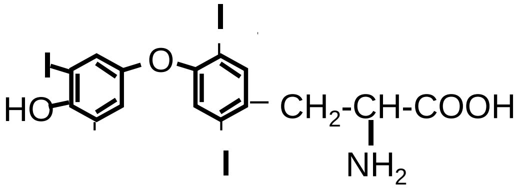


Figure 1
Structure of metamidophos and T_3

TH is essential for normal brain development and function in animals and humans. During the perinatal period, TH controls and mediates numerous neuronal activities in different brain regions. (Potterfield and Hendrich, 1993). Hypothyroidism especially during the period of brain growth spurt (Dobbings and Sands, 1979) which in humans begins from the third trimester of pregnancy throughout the first two years of life and first three to four weeks in rats and mice after birth, causes abnormal brain development (cretinism) with severe physical and / or mental retardation in the offsprings (Koibuchi and Chin, 2000; Yen, 2001), as well as abnormal behavioral patterns (Haddow et al, 1999).

TH functions are biologically mediated by TRs. TR are ligand-regulated transcription factors that are widely expressed (Bradley et al, 1992). TR is bound to specific DNA sequence known as TH response element (TRE) which are located upstream of the target gene. When TR binds to a TRE, it in turn interacts with retinoid X receptor (RXR) to form heterodimers, which then binds to a number of coregulators such as corepressors and coactivators in a ligand-dependant manner to repress or activate transcription (Koibuchi N, 2008). Disruption of TR-TRE binding, recruitment of corepressors to TR, or dissociation of coactivators from TR by methamidophos may lead to suppression of TR-mediated gene expression and consequently could impair normal neuronal development and function.

This study was designed to examine the effect of methamidophos on TR-mediated transactivation and elucidate possible mechanisms involved.

MATERIALS AND METHODS

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

Plasmids: Expression vectors of TRβ1, and glucocorticoid receptor (GR) have been previously described (Iwasaki et al, 2001; Koibuchi et al, 1999). The luciferase (LUC) reporter constructs, the chick lysozyme (F2)-thymidine kinase (TK)-LUC (F2-TRE), is described elsewhere (Koibuchi et al, 1999). 5x upstream activating sequence (UAS)-TK-LUC in the PT109 vector and mouse mammary tumor virus (MMTV) promoter that is fused to luciferase promoter (MMTV-LUC), which contain glucocorticoid response element (GRE), were described previously (Iwasaki et al, 2001). Expression vector for Human SRC-1 have been described elsewhere (Takeshita et al, 1998). Expression vector of Gal4-DNA-binding domain (DBD)-fused SRC-1-nuclear receptor binding domain (NBD)-1 (aa 595-780) (otherwise described as nuclear receptor-interacting domain) was described previously (Takeshita et al, 2002). VP16-TRB1-ligand binding domain (LBD) was constructed by inserting PCR-generated fragments inflame downstream of the VP16 activation domain in AASV-VP16. The Gal4-blank and

GAL4-N-CoR (aa 1579-2454) were described previously (Takeshita et al, 2002)

Cell culture: CV-1 cells were cultured in Dulbecco's modified eagle's medium supplemented with 5 µg/mL penicillin/streptomycin and 10% fetal bovine serum deprived of small lipophilic hormone at 37ºC under a 5%Co_2 atmosphere as previously described (Iwasaki et al, 2002).

Transient transfection-based reporter gene assays: Cells were plated in 24-well plates 48 hours before transfection using calcium-phosphate precipitation method (Iwasaki et al, 2002). The internal control was cytomegalovirus-β-galactosidase plasmid. Sixteen to 24 hours after transfection, wells were refilled with fresh medium containing the indicated concentration of ligand and/or methamidophos for 24 hours. Cells were then harvested to measure the luciferase activities as described elsewhere (Iwasaki et al, 2002). Total amounts of DNA per well was balanced by adding pcDNA3 plasmids (Invitrogen, San Diego, CA). The LUC activities were normalized to β-galactosidase activity and then calculated as relative LUC activities. All transfection studies were repeated at least three times in triplicate. Data shown represent mean ± S.E.M. of one representative experiment performed in triplicate.

Trypan blue exclusion: Trypan blue exclusion was previously described (Jiang et al, 2001). Briefly, CV-1 cells were plated in 12-well plates two days before adding methamidophos. Each cell was incubated in the presence or absence of 10^{-9} M to 10^{-6} M methamidophos. After twenty-four hours, the medium was changed and the cells incubated for 2-5 minutes in a solution of 0.2% trypan blue in phosphate-buffered saline. Number of total cells and Trypan Blue-stained cells were counted using a heamocytometer.

Liquid Chemiluminescent DNA pull-down Assay (LCDPA): This assay to examine nuclear receptor-DNA binding in solution was previously described (Iwasaki et al, 2008). Briefly, a GST-fused TH receptor (GST-TR) bound to glutathione-sepharose beads was incubated with a digoxigenin (DIG)-labeled double–stranded DNA fragment containing a TH response element (TRE) in protein-DNA binding buffer. After extensive washing, protein-DNA binding on beads is detected using anti-DIG antibody conjugated to alkaline phosphatase. Protein-DNA binding is then measured by a chemiluminescent reaction using a luminometer. We perform LCDPA at least three times and data shown represent means ± S.E.M. of one experiment.

Statistical Analysis:
Statistical significance was determined using ANOVA and post-hoc comparison was made using Bonferroni's test. The *p*-values <0.05 were considered significant and marked with asterisk in the figures.

RESULTS

Effect of methamidophos on TR-mediated transcription

We examined the effect of methamidophos on TR-mediated transcription using the transient transfection-gene expression studies in CV-1 cell (Figure 2). Suppression of TR-mediated expression was seen on the F2-TRE-LUC at 10^{-6} M. The effect of methamidophos suppression on TR-mediated transcription was not as a result of cell death as confirmed by Trypan blue exclusion (data not shown). Also, methamidophos did not suppress GR-mediated transcription (Figure 3) indicating that the suppression was TR-specific.

Methamidophos did not prevent SRC-1 binding to TRβ1 in the presence of TH

We examined the effect of methamidophos on binding between TRβ1 and SRC-1 in CV-1 cells using mammalian two hybrid assay. In this assay, the interaction between SRC-1-NBD-1 and TRβ1-LBD with or without T_3 and or metamidophos was examined. The NBD-1 of SRC-1 was fused to the Gal-4-DNA binding domain, and the LBD of TRβ1 was fused to VP16 transactivation domain. Transactivation mediated by Gal4- SRC-1-NBD-1 and VP16-TRβ1-LBD proceeded in the presence of T_3 (Figure 4; column 4).

Transcriptional activation caused by SRC-1-NBD-1 and VP16-TRβ1-LBD interaction with T_3 was not affected by methamidophos at concentrations of 10^{-9} M and 10^{-8} M (Figure 4; columns 5-6), nor at10^{-7} M and 10^{-6} M (Figure 4; columns 7-8). These results indicate that methamidophos did not affect the binding between SRC-1 and TRβ1-LBD in the presence of TH.

Methamidophos did not recruit N-CoR to TRβ1 in the presence of TH.

We examined the effect of methamidophos on binding between N-CoR and TRβ1 in CV-1 cells using mammalian two hybrid assay. Gal4-N-CoR or VP16-TRβ1 and 5x UAS-TK-LUC were co-transfected into CV-1 cells. Gal4-N-CoR and VP16-TRβ1 precedes the transcriptional activity (Figure 5, column 4) in the absence of T_3, while no activation was observed with T_3. Transcriptional activities were not markedly altered in the presence of 10^{-9} M and 10^{-6} M methamidophos regardless of T_3 10^{-7} M (Figure 5; column 5-8), neither was there any significant dissociation of N-CoR from TR in the absence of T_3 (Figure 4) suggesting that methamidophos may not recruit N-CoR to TR in the presence of T_3.

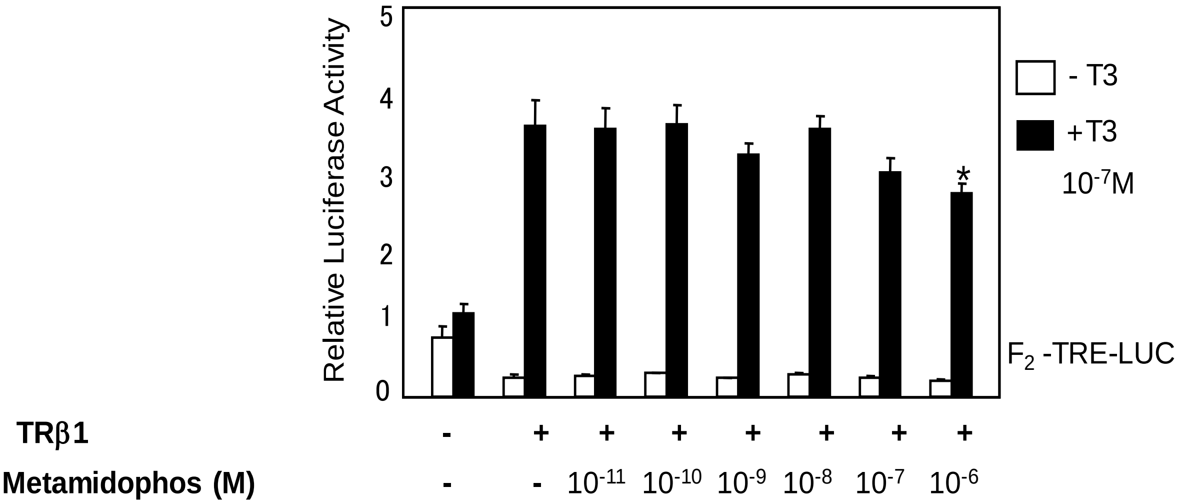


Figure 2
Methamidophos suppressed TR-mediated transcription in the presence of TH. Expression plasmids encoding TRβ1 (10 ng) were transfected together with F2-TK-LUC (100 ng) into CV-1 cells. Cells were cultured with or without 10^{-7} M T_3 and indicated amount of methamidophos. Total amounts of DNA for each well were balanced by adding vector pcDNA3. Data represent mean ± S.E.M. of experiments performed in triplicate. *, statistically significant $p<0.05$ by ANOVA) versus TRβ1 (+), T_3 (+), and methamidophos (-).

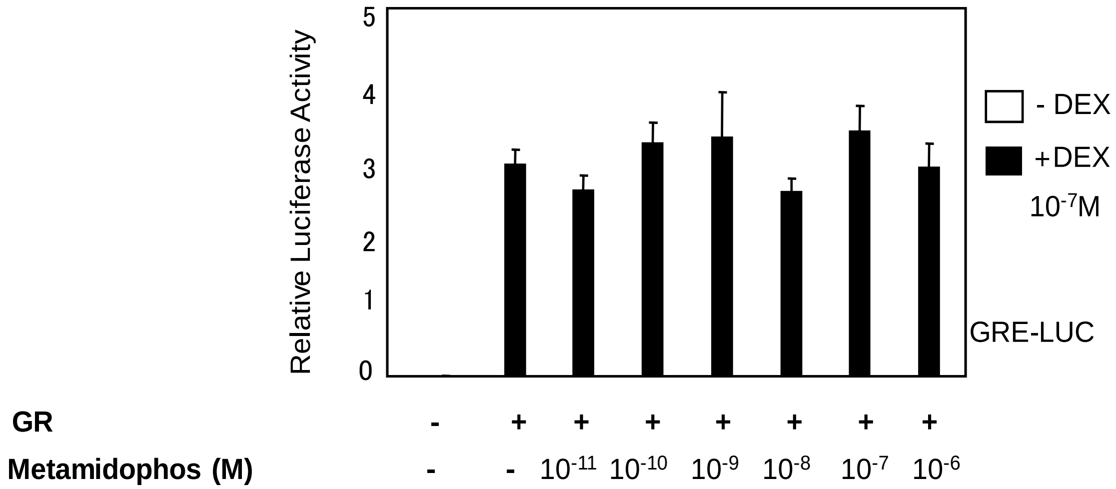


Figure 3
Methamidophos did not suppress GR-mediated transcription in the presence of Dexamethasone. Expression plasmids encoding GR (10 ng) were transfected together with glucocorticoid response element (GRE)-LUC reporter plasmids (100 ng) into CV-1 cells. Cells were cultured in the absence or presence of dexamethasone (DEX) (10^{-7} M) and indicated concentrations of methamidophos. Total amounts of DNA for each well were balanced by adding vector pcDNA3. Data represent mean ± S.E.M. of experiments performed in triplicate. No statistical significance was uncovered by ANOVA.

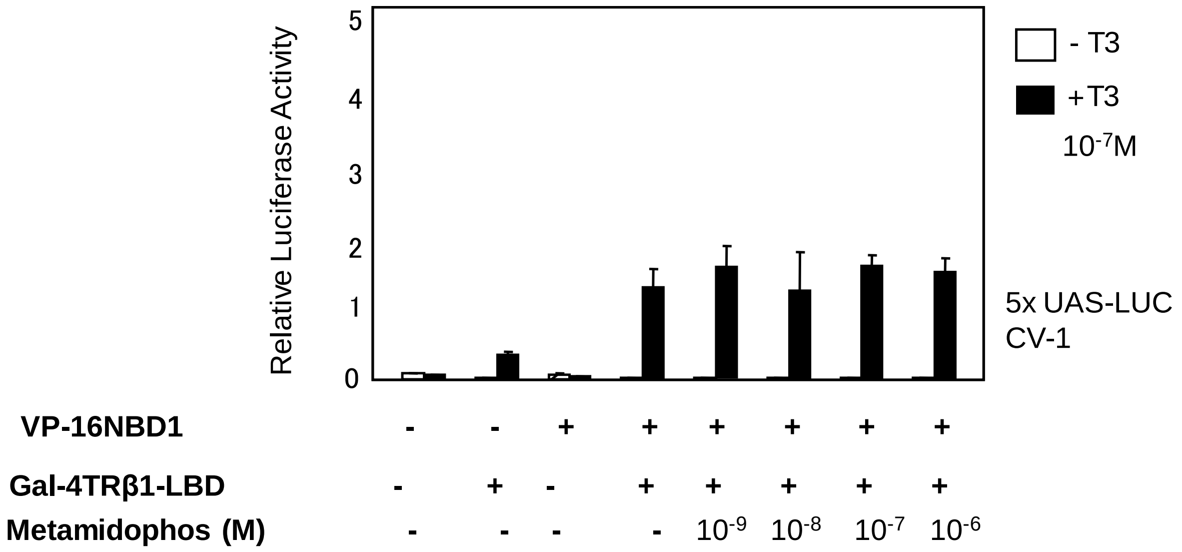


Figure 4
Methamidophos did not prevent SRC-1 binding to TRβ1 in the presence of TH.
Expression plasmids encoding Gal4-DBD-fused SRC-1-NBD-1 (10 ng) were transfected with VP16-constructs (50 ng) and 5xUAS-TK-LUC-reporter plasmids (170 ng) into CV-1 cells. Cells were incubated with or without T_3 (10^{-7} M) and indicated concentrations of methamidophos. Total amounts of DNA for each well were balanced by adding vector pcDNA3. Data represent mean ± S.E.M. of experiments performed in triplicate. No statistical significance was uncovered by ANOVA.

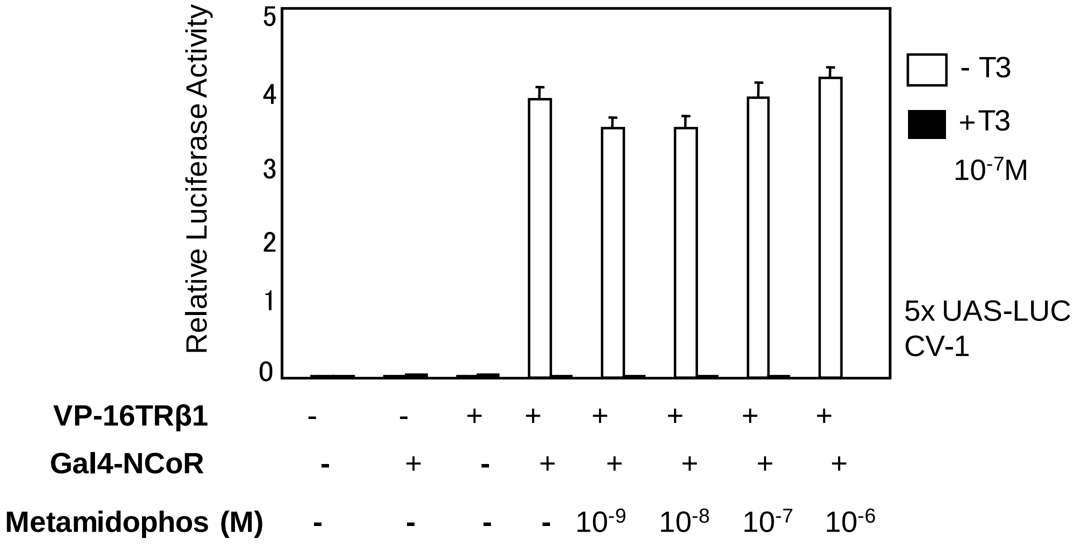


Figure 5
Methamidophos did not recruit N-CoR to TRβ1 in the presence of TH
Expression plasmids harboring Gal4-DBD-fused N-CoR (100 ng) were transfected with VP16- TRβ1-LBD (50 ng) and 5x UAS-TK-LUC (100 ng) into CV-1 cells with or without T_3 (10^{-7} M) and/or indicated amount of methamidophos. Total amounts of DNA for each well were balanced by adding vector pcDNA3. No statistical significance was determined by ANOVA.

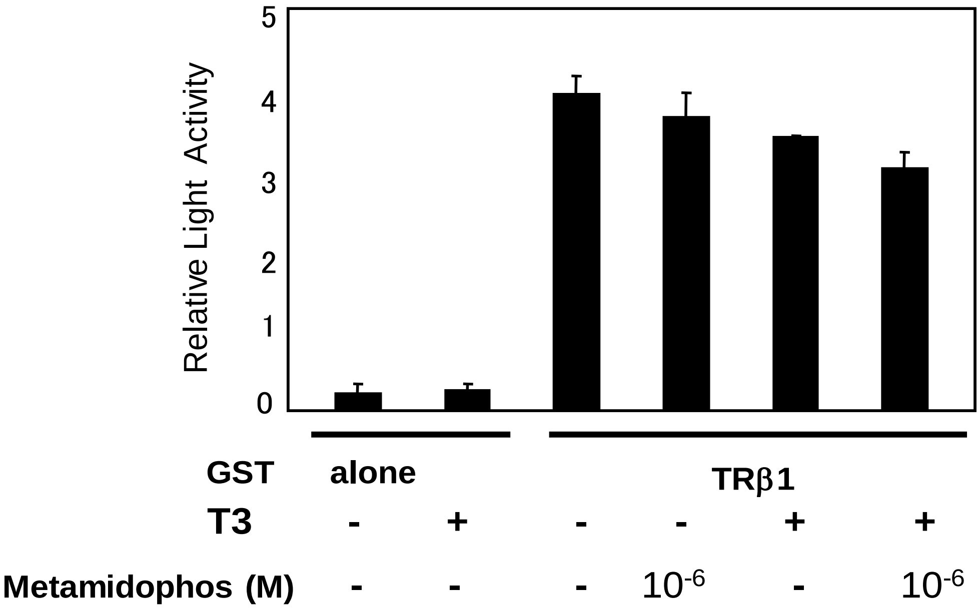


Figure 6
Methamidophos have no effect on TR-TRE binding in the presence of TH
GST-TRβ1 bound to sepharose bead is incubated with DIG-F2 containing TRE in protein-DNA binding buffer with or without T_3 (10^{-6} M) and 10^{-6} M methamidophos. Data represent mean ± S.E.M. of experiments performed in triplicate. No statistical significance was uncovered by ANOVA.

Methamidophos did not dissociate TR-TRE binding in the presence of TH.

Finally, we then performed liquid chemiluminescent DNA pull down assay to examine the effect of methamidophos on TR binding to TRE. We have confirmed previously that the results of this assay were compatible to those of electrophoretic mobility shift assay (Iwasaki et al, 2008).

10^{-6} M methamidophos did not dissociate TR from TRE in the presence of T_3 10^{-6}M (Figure 6, column 6), indicating that the suppression of TR-mediated transcription by methamidophos was not due to partial dissociation of TR from TRE.

DISCUSSION

In this study, we show that methamidophos caused suppression of TR-mediated transcription in the presence of T_3 (Figure 2). This suppression could potentially interfere with normal neuronal development and function especially during the critical perinatal developmental phase of the brain in neonates.

Suppression of TR-mediated gene expression by methamidophos as shown in our studies was not as a result of cell death because trypan blue exclusion showed that methamidophos did not affect cell viability under our experimental conditions (data not shown). Also, the suppression of TR-mediated transcription by methamidophos was TR-specific because it did not suppress GR-mediated gene expression in the presence of TH (Figure 3)

We initially hypothesized that methamidophos effect on TR-mediated transcription could be by dissociation of the coactivator complex from TR or through the recruitment of a corepressor complex to TR. However, methamidophos did not dissociate SRC-1 from TR at all concentrations examined (Figure 4), neither did it recruit complexes containing N-CoR to TR (Figure 5) indicating mechanisms other than interaction with nuclear cofactors located upstream of the target gene could be involved in its suppression of TR-mediated action.

Liquid chemiluminescent DNA pull down assay which examines effect on various compounds on protein-DNA binding also did not show any dissociation of TR from TRE in the presence of methamidophos and T_3 (Figure 6). This indicates that DNA-protein interaction involving response elements may not be primarily involved in methamidophos effect on TR-mediated transactivation, suggesting that methamidophos may act via other mechanisms on TR to suppress gene expression.

TH is essential for normal brain development and growth. Hypothyroid conditions, especially during the perinatal period have been known to induce cretinism with severe cognitive and/or mental disorders in the offsprings (Koibuchi et al. 2000). Since TH also tightly regulate fundamental gene expression both directly and indirectly in vast brain regions including the cerebellum (Koibuchi and Iwasaki, 2006), the suppresive effects of methamidophos on TH homeostasis may disrupt normal brain development via TH-dependent gene regulations. More studies are however required to further elucidate in details the mechanism by which methamidophos inhibits TR-mediated gene expression.

In conclusion, our study shows that methamidophos suppressed TR-mediated transcription and could thereby inhibit normal TH homeostasis. This in turn may disrupt normal neuronal development and function. Studies to further examine the effect of metamidophos on cerebellar neuronal cells are currently underway. Given the widespread use of methamidophos as pesticides, there is urgent need to carefully regulate its use to avoid future specter of complications.

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***Competing Interest**: All authors declare no conflict of interest.*

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