Published in final edited form as:

Neurotoxicol Teratol. 2012 July ; 34(4): 387–394. doi:10.1016/j.ntt.2012.04.007.

Methylmercury tolerance is associated with the humoral stress factor gene Turandot A

Cecon T. Mahapatra¹ and Matthew D. Rand*

Department of Anatomy and Neurobiology, College of Medicine, University of Vermont, Burlington, VT 05405, United States

Abstract

Methylmercury (MeHg) is an environmental neurotoxicant that targets the developing nervous system. In an effort to understand mechanisms of MeHg toxicity we have identified candidate genes that confer tolerance to MeHg using a *Drosophila* model. Whole genome transcript profiling of developing larval brains of MeHg-tolerant and non-tolerant flies has identified Turandot A (TotA) as a potential MeHg tolerance gene. TotA is a secreted humoral stress response factor in *Drosophila* that is a direct target of conserved innate immunity signaling pathways. Here we characterize TotA expression in newly generated isogenic lines (isolines) of flies derived from our previously established MeHg-tolerant and non-tolerant populations. TotA mRNA transcript and protein expression is seen to be higher in the tolerant isolines than the non-tolerant lines. Elevated TotA expression in the tolerant lines was seen to span all the larval developmental stages pointing toward a difference in the TotA gene regulation between the MeHg tolerant and non-tolerant strains. We show that TotA is most highly expressed in the fat body (liver equivalent) and is selectively upregulated in the fat body of tolerant flies relative to brain and gut tissues. Fat body-specific transgenic expression of TotA invokes MeHg tolerance as seen by enhanced development of flies reared on MeHg food. In addition, cell based assays show that high TotA expressing C6 cells are more tolerant to MeHg than the low TotA expressing S2 cells. Knockdown of TotA in the C6 cells trends toward a reduction in MeHg tolerance. Identification of TotA as a MeHg tolerance gene suggests a role for conserved cytokine/immune signaling pathways in modulating MeHg toxicity.

Keywords

Methylmercury; Turandot A; Neurotoxicity; Mercury resistance; Innate immunity; *Drosophila*

1. Introduction

Methylmercury (MeHg) is a persistent environmental neurotoxicant that poses a risk to humans due to its accumulation in dietary fish and seafood. In pregnant women, the fetus is considered to be at a high risk of MeHg toxicity due to transplacental exposure (Watanabe and Satoh, 1996). The fetal brain is exceptionally sensitive to MeHg. Prenatal MeHg exposure in humans, and in laboratory animals, can result in permanent neurological deficits in children despite negligible neurological effects on their mothers (Grandjean et al., 1997; Murata et al., 2004). Adults can be affected by chronic exposure to MeHg, which gives

neurological signs such as paresthesia and ataxia in severe cases (Hunter and Russell, 1954). Studies based in cell models indicate adverse effects of MeHg in calcium homeostasis (Sirois and Atchison, 1996), apoptosis (Lu et al., 2011; Sakaue et al., 2005; Wilke et al., 2003), ROS generation (Sarafian, 1999) and protein synthesis (Sarafian et al., 1984; Syversen, 1981, 1982). Despite knowledge of a number of mechanisms of MeHg toxicity, little is known about factors that can protect the individual against MeHg insult.

Several population studies looking at effects of prenatal exposure to MeHg show a large variation in outcomes of neurological endpoints. These results suggest that tolerance and susceptibility to MeHg toxicity vary widely among individuals within a population and, furthermore, indicate the potential existence of mechanisms that protect individuals from MeHg. Studies in vitro show that glutathione (GSH) serves as a first line of cellular defense for MeHg by conjugating MeHg and facilitating its excretion (Aschner et al., 2007). Additional studies on GSH-related genes that aid in MeHg metabolism have elucidated polymorphisms that affect the MeHg retention in erythrocytes in humans (Custodio et al., 2004). Such studies provide evidence for a genetic basis for variation in MeHg tolerance. Apart from the GSH-related genes, little is known about other protective genes or pathways that respond favorably to MeHg stress.

Drosophila melanogaster is a highly effective molecular genetic model system for examining fundamental processes in toxicology (Rand, 2010). In an effort to identify candidate genes responsible for MeHg tolerance, we performed a whole genome transcript profiling of third instar *Drosophila* larval brains of laboratory selected MeHg-tolerant and non-tolerant strains of flies. (Mahapatra et al., 2010). The candidate most strongly expressed due to selection and MeHg exposure was *Turandot A* (TotA; CG31509). TotA is a member of a family of *Drosophila* humoral stress response genes for which mammalian homologs have yet to be described. TotA is a 129 amino acid secreted protein that is known to respond to environmental stressors such as heat shock, chemical insult, UV exposure and bacterial infection (Ekengren et al., 2001). Results of our screen for tolerance genes have lead to the hypothesis that elevated TotA expression contributes to MeHg tolerance.

In this study we establish a role for TotA in MeHg tolerance behavior in *Drosophila*. Using isogenic MeHg-tolerant and non-tolerant lines (isolines) we find that high basal TotA expression correlates with tolerance to MeHg during development spanning the larval to adult stages. The fat body of the fly, which is functionally comparable to the liver of higher organisms, is the site of highest TotA expression. TotA over-expression using fat body-specific transgenic expression yields higher MeHg tolerance in developing larvae. *Drosophila* C6 cells that express high levels of TotA in culture show greater resistance to MeHg than the S2 cell line, which expresses little endogenous TotA. Knockdown of TotA expression using dsRNAi in C6 cells leaves the cells less tolerant to MeHg. The data suggests that TotA acts in a protective pathway that invokes a MeHg tolerant state.

2. Materials and method

2.1. *Drosophila* strains and culturing

Wild type Canton S and c754 (Fat body Gal4 driver, Bloomington stock #6984) fly lines were obtained from the Bloomington *Drosophila* Stock Center, Indiana University. Several isogenic MeHg-tolerant and non-tolerant lines were generated in the lab. These lines were derived from parent populations reported previously (Mahapatra et al., 2010). Twenty individual lines from single parent mating were established from each of the MeHg tolerant (E20, F20, H20) and non-tolerant (E0, F0, H0) populations. Each isolate was established through inbreeding for greater than 11 generations of sibling matings.

The transgenic flies used in the experiments (UAS-TotA60b) and UAS-TotA41a) were a gift of Dr. Dan Hultmark (Umea, Sweden). All flies were reared at 25 °C on standard yeast/agar media with a 12-hour light dark cycle.

2.2. Eclosion assays

Eclosion is the emersion of adult flies from the pupal case. Eclosion assays were performed as previously reported (Mahapatra et al., 2010). Methylmercury chloride (MeHg; Aldrich #442534) stock solutions (50 mM) were prepared in dimethyl sulfoxide (DMSO) and used such that the final concentration of DMSO never exceeded 0.1%. Fly food consisting of cornmeal, molasses and agar (Jazz mix AS153; Fisher Scientific) was prepared with concentrations of MeHg ranging from 0 to 25 μ M, a previously established effective concentration range (Mahapatra et al., 2010). Control experiments were done by using equivalent amount of DMSO as compared to the experimental. First instar larvae were transferred to food vials containing MeHg (0–25 μ M) or DMSO control in batches of 50 larvae per vial. The number of adult flies that successfully develop to dark pupal stage or to eclosion was scored on day 13. Three replicates were performed for each experiment (n = 150 flies for each data point). MeHg tolerance was determined from comparative plots of the rate of development (dark pupae and/or eclosion) versus concentrations of MeHg in the food.

2.3. Quantitative PCR

Real time quantitative PCR (qPCR) was performed on cDNA reverse transcribed from total RNA prepared from whole larva or larval tissue extracts. qPCR was done using the iTaq SYBR Green Super mix with ROX (Bio-Rad #172-5850) to determine the level of transcript expression for TotA and the RP49 ribosomal protein housekeeping gene. Primer information for TotA and RP49 is available in the supplementary material of our previous paper (Mahapatra et al., 2010). The target gene expression was normalized to the housekeeping gene expression, to determine the relative expression level of TotA. Gene expression levels were determined by the comparative C_T method (Livak and Schmittgen, 2001).

2.4. Western blotting

Cell lysates were prepared by using a lysis buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 1% Nonidet P-40) and separated by SDS-PAGE and transferred to PVDF membranes for Western blot analysis by standard protocols using an Odyssey infrared imager (Li-Cor, Lincoln, NE). Antibodies used: polyclonal anti-TotA (a gift from Dr. Dan Hultmark, Umea, Sweden) and IRDYE 800 secondary antibodies (Rockland, Gilbertsville, PA). Quantification of the bands was done using the LiCor software. Intensity of the TotA immunoreactivity was normalized to the level of actin in the sample as determined on the same membrane (anti-actin antibody JLA20 from the Developmental Studies Hybridoma Bank, University of Iowa).

2.5. Double stranded RNA interference (dsRNAi) of TotA expression

dsRNAi treatment used to knockdown TotA expression in the C6 cells was performed as described (Clemens et al., 2000). A DNA fragment of coding sequence of TotA (500 bp) was amplified by PCR and used for the RNA synthesis template. A control dsRNA was generated from DNA template containing coding sequence for green fluorescent protein (GFP). Primers for RNA synthesis contained a 5' T7 primer binding site followed by the sequence directed to the target gene (primer sequences are available upon request). In vitro RNA synthesis was done using the Mega script T7 transcription kit (Ambion, Austin, TX). Double stranded RNA was formed by heating to 65 °C and cooling to anneal complimentary strands.

dsRNAi was added to 10^5 cells at a concentration of 30 $\mu\text{g}/100$ mm culture dish and incubated for 3 days. RNAi-treated cells were then re-suspended and plated at 75% confluence in a six well plate for subsequent MeHg and control treatment for 18 h followed by viability assays (see below). Media from dsRNAi treated and control (GFP-dsRNAi) cells were collected for Western blotting to confirm the knockdown of TotA protein level.

2.6. Cell viability assay

The *Drosophila* embryonic S2 cell line and the C6 cell line derived from the *Drosophila* central nervous system (ML-DmBG2-C6, Ui et al., 1994) available at Drosophila Genomic Resource Center, Indiana University) were used. Cells were routinely maintained at 25 °C in Sang's M3 insect culture medium (Sigma, St. Louis, MO) containing 12.5% fetal bovine serum (Serum Source International, Charlotte, NC), 10 $\mu\text{g}/\text{mL}$ insulin, bactopeptone (2.5 g/L), yeastolate (1 g/L)(1XBPYE) supplement (Difco, Sparks, MD), 100 units/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin (Invitrogen, Carlsbad, CA).

Cell viability was determined using the MTT assay as described (Aras et al., 2008). Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. The cells were treated with various concentrations of MeHg ranging from 0 to 50 μM for 18 h before adding the reagent and then incubated for 4 h at 25 °C and the reaction was stopped by adding a solution containing 10% Triton-X in isopropanol and allowing it to shake for 30 min at room temperature. Absorbance was quantified by measuring each sample in triplicate at 570 nm and corrected for background at 650 nm using a Synergy H4 microplate reader (BioTek, Winooski, VT, USA).

2.7. Statistical analysis of the results

Results of eclosion assays and cell viability assays are expressed as the mean $\pm$ S.D. (standard deviation) of three independent determinations. Data were evaluated statistically using either one-way analysis of variance (ANOVA) or by mean contrast tests performed using pairwise Student's *t*-test for determinations at each MeHg concentration (SPSS statistical analysis software). In all cases, $p < 0.05$ was considered statistically significant level.

3. Results

3.1. Identification of TotA as a MeHg tolerance gene

In our previous study, whole-genome transcript profiling of larval brains of lab-selected MeHg tolerant and non-tolerant flies in the presence and absence of MeHg stress helped us to identify several MeHg tolerance candidate genes (Mahapatra et al., 2010). We found that Turandot A (TotA) is the most strongly expressed candidate upon selection and MeHg exposure (Mahapatra et al., 2010). To begin characterization of the role of TotA with respect to MeHg resistance, we created several isolines by single parent mating derived from each of the three tolerant and non-tolerant populations we had established previously (Fig. 1). Several of the lines derived from MeHg non-tolerant (E0) and tolerant (E20) populations were tested for developmental tolerance of MeHg via our previously described eclosion assay (Mahapatra et al., 2010). Isolines derived from the tolerant population (E20-1, E20-2 and E20-3) show greater tolerance to MeHg than the non-tolerant isolines with more than 75% eclosion rate seen at 10 μM MeHg (Fig. 2). At this concentration, the non-tolerant isolines (E0-1, E0-2 and E0-3) show less than 30% eclosion rate (Fig. 2). Furthermore, at 15 μM MeHg, the E20 isolines maintain greater than 60% eclosion where as the three non-tolerant E0 isolines showed less than 5% eclosion rate (Fig. 2).

We next examined the correlation of MeHg tolerance among the strains with TotA mRNA and protein expression. Using RNA derived from 3rd instar whole larvae extracts and quantitative PCR, we find higher basal TotA expression in the tolerant E20-1, E20-2 and E20-3 isolines, with TotA expression in the E20 lines approximately six to 18-fold higher than the E0-1 non-tolerant line (Fig. 3A).

TotA protein levels among the MeHg-tolerant and non-tolerant isolines, as determined by Western blot analysis of larval extracts, appear to correspond with TotA transcript levels. The TotA protein is a 129 amino acid polypeptide with an N-terminal signal sequence such that the mature TotA protein is 12 kDa (Ekengren et al., 2001). Consistent with the mRNA levels, all of the E20 isolines showed higher levels of TotA protein relative to the E0 non-tolerant lines (Fig. 3A, B).

We then examined the correlation of TotA protein expression and MeHg tolerance in additional isolines derived from the parent populations from our previous selection paradigm (i.e., the F0/F20 and H0/H20 lines, see Mahapatra et al., 2010). A plot of the percent eclosion (on 10 μ M MeHg food) versus the TotA protein level (determined by quantitative Western blotting) in 18 isolines confirms the overall association of high TotA expression in larvae with higher eclosion rates (Fig. 4). Overall, these results support the hypothesis that elevated TotA expression protects larval development from MeHg exposure.

3.2. TotA expression across developmental stages of tolerant and non-tolerant isolines

In our eclosion assay MeHg exposure occurs across all stages of larval development. We therefore asked whether TotA expression is constitutively higher in the tolerant lines during the 1st, 2nd and 3rd larval instar stages, as well as in the early pupal stage. Previous studies have demonstrated developmentally regulated expression of TotA with maximal TotA expression seen at the 3rd instar larval stage, which persists through the pupal stages (Ekengren et al., 2001). Comparing TotA transcript levels between the E20-3 and E0-3 isolines we found that TotA expression is consistently higher in E20-3 line across all the larval stages (L1-3) and the early pupal (EP) stage (Fig. 5). These data support the notion that constitutive high expression of TotA across all the developmental stages contributes to MeHg tolerance. In addition, the fundamental differences in TotA gene regulation between these two lines could serve as a critical inroad to resolving the genotypic basis of the MeHg tolerance trait.

3.3. Tissue specific expression of TotA

In our initial transcriptome analyses of potential MeHg tolerance genes we chose to analyze transcripts exclusively in the developing central nervous system (brain), since MeHg is known to target this tissue (Mahapatra et al., 2010). To begin to determine more accurately where TotA might be acting at a tissue level we examined expression in various larval organs in addition to the brain, including the fat body and gut. Analyses of RNA derived from brain, fat body and gut from both the tolerant and non-tolerant lines localized the major TotA transcription site to the fat body (Fig. 6). *Drosophila* fat body is functionally equivalent to the mammalian liver (Lemaitre and Hoffmann, 2007). TotA expression in the fat body and whole larvae of the E20-3 line is approximately nine-fold and 26-fold higher than in the non-tolerant E0-3, respectively (Fig. 6). TotA is very highly expressed in the fat body as compared to the other tissues such as brain and gut. Unlike the fat body, TotA expression in the gut of E20-3 line showed lower expression than in the E0-3 line. However, TotA expression in the brain of E20-3 line showed higher expression than in the E0-3 line, consistent with observations in our initial screen (Mahapatra et al., 2010). These data highlight the highly regulated transcriptional regulation of TotA expression in the fat body,

and are consistent with previous determinations showing TotA is synthesized in the fat body (Ekengren et al., 2001).

3.4. TotA over-expression in the fat body enhances MeHg tolerance during development

Since TotA is predominantly expressed in the fat body we attempted to create MeHg tolerant larvae via transgenic over-expression of TotA in the fat body. Using the GAL4-UAS system (Brand and Perrimon, 1993) we first validated the expression pattern of c754 GAL4 driver in the fat body (Hrdlicka et al., 2002). Crossing c754 to UAS-GFP we observed strong green fluorescence localized predominantly to fat body tissues in the 3rd instar larvae (Fig. 7A). Expression was also observed in abdominal histoblasts, a small population of precursor cells of the adult abdomen (Fig. 7A). We hence refer to the c754 driver line as the FBG4 line. We next examined expression of TotA using two independent UAS-TotA lines (UAS-TotA41a and UAS-TotA60b) crossed to FBG4. These two lines carry the UAS-TotA gene insertion at different second chromosome locations. The offspring from the FBG4×UAS-TotA flies (FBG4>TotA) showed highly elevated levels of TotA transcripts in larval extracts with FBG4>TotA41a and FBG4>TotA60b producing more than 95-fold and 74-fold higher TotA expression, respectively, as compared to the FBG4>wt. (Fig. 7B). While expression of TotA (FBG4>TotA) did not substantially increase overall eclosion rates compared to control larvae (FBG4>wt), at 10 μ M MeHg, there is a significant increase in eclosion of FBG4>TotA41a and FBG4>TotA60b as compared to FBG4>wt (Fig. 7C, dashed lines). In addition, development to a more advanced stage was apparent with FBG4>TotA larvae as seen by a significantly greater number of larvae reaching the dark pupal stage when exposed to 10–20 μ M MeHg (Fig. 7C, solid lines). These data indicate that TotA over-expressing flies are enhanced for MeHg tolerance.

3.5. MeHg protective effects in *Drosophila* cell lines

To investigate a more explicit activity of TotA, we examined MeHg protective effects of TotA expressed in two *Drosophila* cell lines: the S2 line, which exhibits low endogenous TotA expression and the C6 line, which shows high TotA expression, as seen by Western blotting conditioned cell culture media (Fig. 8A). We conducted MTT cytotoxicity assays, which showed a concentration dependent decrease in cell viability with MeHg (Fig. 8B). C6 cells showed an overall higher tolerance to MeHg, giving a 60% loss in cell viability at 20 μ M MeHg compared to more than 90% loss in viability of S2 cells at this concentration (Fig. 8B).

We next attempted to knockdown TotA expression in the C6 cells to see if it rendered effects on MeHg tolerance. The C6 control cells were incubated with double stranded inhibitory RNA (dsRNAi) directed toward TotA or toward a GFP sequence as a control. dsRNAi knockdown of TotA proved effective in lowering protein expression in the C6 cells (Fig. 9A). TotA knockdown resulted in more reduction in cell viability with MeHg treatments relative to control cells, which, despite not reaching statistical significance, was consistent across the 5–20 μ M MeHg treatments (Fig. 9B). In sum, these observations are consistent with the hypothesis that elevated TotA expression contributes to resistance to MeHg.

4. Discussion

Investigations into the genetic basis of MeHg toxicity are few in number. In our previous study, whole genome transcript profiling of developing larval brains of MeHg tolerant and non-tolerant flies identified candidate genes for MeHg tolerance (Mahapatra et al., 2010). In our current study, we have extended our analysis of one of these genes, TotA, which is highly expressed in MeHg tolerant flies.

Previous work, predominantly in the Hultmark Laboratory, has shown that TotA encodes a stress-induced humoral factor, which is turned on in response to several stressors such as high temperature, bacterial infection and UV exposure (Ekengren and Hultmark, 2001). These investigators have shown increased resistance to heat stress in TotA over-expressing flies. In a similar experimental design, we over-express TotA and show an induced tolerance to MeHg during larval to pupal development. In newly generated isolines of flies we find higher constitutive expression of TotA across the larval to adult developmental stages in the tolerant isolines as compared to the non-tolerant isolines. Furthermore, in a cell culture system we observe that cells expressing more TotA are more MeHg tolerant. Together these data support the hypothesis that TotA is integrally linked to mechanisms that defend against MeHg toxicity.

4.1. Regulation of TotA expression and MeHg tolerance

In MeHg-tolerant isogenic flies TotA is constitutively expressed at an elevated level across several stages of development. Notably, TotA is highly expressed even in the absence of the stressor in these tolerant isolines. This profile suggests that constitutive expression, and not “stress response”, is the favorable trait for this TotA allele. One possibility for this constitutive expression is that the promoter region of the TotA gene in the tolerant allele carries sequences that up-regulate its activity. We may have isolated this allele through the artificial selection process we performed previously. A precedent for this is seen in case of the pesticide resistance DDT-R allele, which is reliant upon Cyp6g1-mediated insecticide resistance in *Drosophila* (Daborn et al., 2002). DDT-R gives constitutive elevated expression of Cyp6g1, leading to the resistance to a variety of insecticides including DDT. This expression occurs as a result of insertion of a long terminal repeat (LTR) of an Accord retro transposon inserted 291 bp upstream of the Cyp6g1 transcription start site (Chung et al., 2007). It is therefore possible that elevated expression of TotA in MeHg tolerant lines has arisen via selection for an allele affecting the promoter region of the gene. The fact that we observe TotA upregulation in fat body alone, and not in all the tissues, suggests that regulation is occurring specifically at the level of the fat body. It is of note that immune signaling and environmental stress pathways, such as JAK-STAT or MAPK kinase, are known to induce TotA expression in the fat body during response to bacterial challenge (Agaisse et al., 2003; Brun et al., 2006). The potential relevance of these pathways is discussed below. Future experiments will probe the sequence variation in regulatory elements of the TotA gene in an effort to identify the mechanism behind elevated TotA expression.

4.2. Homology search for TotA

TotA is a 129 amino acid protein, which is synthesized in the fat body and is secreted to the hemolymph. The Turandot family consists of eight members, TotA, B, C, Z, X, E, F, M (Ekengren and Hultmark, 2001). All Tot family members have an N-terminal signal peptide sequence and are thus predicted to be secreted proteins. While several of the Tot family members are known stress responders, TotA has been best characterized for its expression in response to conserved cellular stress pathways (Agaisse and Perrimon, 2004). Attempts to identify a TotA homolog in mammalian species have not yielded an obvious candidate. This is despite searches based on primary sequence as well as predicted secondary and tertiary structures. It is of note, however that there are several examples of functionally conserved pathways shared in flies, mice and humans, that have one or more core component proteins that exhibit little sequence homology. For example, in *Drosophila*, the unpaired (upd) gene shares no sequence similarity to any of the vertebrate cytokines, but has proven to be the functional ligand for the JAK/STAT pathway in *Drosophila*. Similarly, amino acid sequence conservation between *Drosophila* Dome and the vertebrate cytokine receptor is low, with the intracellular region of Dome having little similarity to any known protein. Nonetheless,

Dome has five extracellular fibronectin III modules, of which the first and second show homology to the cy-tokine binding modules of the interleukin 6 receptor (IL-6R) family. In spite of low similarity, biochemical analyses confirm that Dome is the receptor for Upd and activator of STAT. By analogy, we predict that TotA could be a cytokine-like protein that is able to induce signaling in a pathway that counters the effects of MeHg and other stressors. Such an activity for TotA can now be explored in future experiments in this model system, for example, by probing gene expression changes in the fat body of transgenic flies overexpressing TotA. In addition, purification of recombinant TotA protein will facilitate biochemical and cell culture approaches to identifying a specific activity for this putative ligand.

4.3. TotA and signaling pathways operating during stress

Several pathways are linked to turning on TotA expression. In *Drosophila*, Mekk1 (Mitogen-activated protein kinase kinase), a component of the MAPK pathway, is shown to regulate TotA gene expression upon septic injury (Brun et al., 2006). Imd and JAK-STAT pathways also regulate TotA expression upon septic injury in *Drosophila* (Agaisse et al., 2003). It is not clear which of these pathways, if any, might be operating in the case of MeHg stress. One possibility is that TotA is a stress inducible gene that has little inherent activity, but rather acts as a biomarker for the activity of a pathway that is operating in combating MeHg stress. In this instance, high constitutive TotA expression seen in tolerant flies may simply reflect a basal activation of conventional stress associated pathways that then poises the fly to resist MeHg toxicity. It is therefore possible that TotA expression in this insect model may serve as an invaluable tool to validating the effect of JAK-STAT on MeHg tolerance. The JAK-STAT pathway has been implicated as a neuroprotective pathway (Ji et al., 2004; Ostrowski et al., 2011) and thus its activation may serve a role in alleviating MeHg insults. In this regard, elevated JAK-STAT signaling may be favorable in the context of MeHg toxicity since MeHg is known to have inhibitory activity toward JAK-STAT (Monroe and Halvorsen, 2006). As the JAK-STAT pathway can be perturbed by other environmental toxicants, such as cadmium (Monroe and Halvorsen, 2009), future analyses utilizing TotA as an indicator will have broader implications for resolving the mechanisms of environmental chemical insults.

4.4. TotA working in cooperation with other genes to protect from MeHg

We find that TotA transgenic over-expression results in greater pupal development yet no effect on increasing eclosion. This “partial” enhancement of MeHg tolerance suggests that “full” tolerance to MeHg requires combined activity of a number of genes, consistent with predictions that the MeHg tolerance trait is polygenic (Magnusson and Ramel, 1986). In this regard, investigation of additional candidate genes that we have identified is warranted. Exploring the effect of up-regulating TotA together with one or more additional candidate genes will be highly informative. We have recently identified that over expression of CYP6g1, another candidate identified in our transcriptome analysis, confers MeHg tolerance as seen by higher rates of eclosion (Rand, 2012). CYP6g1 is a member of the larger family of cytochrome p450 Phase I xenobiotic metabolizing enzymes, which show a high degree of structural and functional conservation across all metazoans. We therefore anticipate extending these studies to investigate the activity of mammalian homologs of this family of genes in MeHg toxicity. Furthermore, future experiments investigating the co-expression of TotA and CYP6g1 in our *Drosophila* model will shed light on the possibility that summed activity of two gene candidates is sufficient to invoke substantial MeHg tolerance.

Acknowledgments

We wish to thank Felix Eckenstein and members of the Rand lab: Nate Jebbett, Greg Engel and Amanda Burton for review and discussion of the data and the manuscript. We thank Greg Engel for assistance in Statistical analyses. We are grateful to Dan Hultmark (Umeå University, Sweden) for the generous sharing of UAS-TotA flies and anti-TotA antibody. We thank the UVM Center of Biomedical Excellence (COBRE) Molecular Core facility for use of qPCR instrumentation (supported by the National Center for Research Resources (5 P30 RR 032135) and the National Institute of General Medical Sciences (8 P30 GM 103498)). The anti-actin monoclonal antibody developed by J.J.-C. Lin was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242.

Funding information: This work was supported by NIEHS R01-ES015550 awarded to M.D.R.

References

- Agaisse H, Perrimon N. The roles of JAK/STAT signaling in *Drosophila* immune responses. *Immunol Rev.* 2004; 198:72–82. [PubMed: 15199955]
- Agaisse H, Petersen UM, Boutros M, Mathey-Prevot B, Perrimon N. Signaling role of hemocytes in *Drosophila* JAK/STAT-dependent response to septic injury. *Dev Cell.* 2003; 5(3):441–50. [PubMed: 12967563]
- Aras, MA.; Hartnett, KA.; Aizenman, E. *Curr Protoc Neurosci.* John Wiley and Sons; 2008. Assessment of cell viability in primary neuronal cultures. Chapter 7: Unit 7.18
- Aschner M, Syversen T, Souza DO, Rocha JB, Farina M. Involvement of glutamate and reactive oxygen species in methylmercury neurotoxicity. *Braz J Med Biol Res.* 2007; 40(3):285–91. [PubMed: 17334523]
- Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development.* 1993; 118(2):401–15. [PubMed: 8223268]
- Brun S, Vidal S, Spellman P, Takahashi K, Tricoire H, Lemaitre B. The MAPKKK Mekk1 regulates the expression of Turandot stress genes in response to septic injury in *Drosophila*. *Genes Cells.* 2006; 11(4):397–407. [PubMed: 16611243]
- Chung H, Bogwitz MR, McCart C, Andrianopoulos A, Ffrench-Constant RH, Batterham P, et al. Cis-regulatory elements in the Accord retrotransposon result in tissue-specific expression of the *Drosophila melanogaster* insecticide resistance gene Cyp6g1. *Genetics.* 2007; 175(3):1071–7. [PubMed: 17179088]
- Clemens JC, Worby CA, Simonson-Leff N, Muda M, Maehama T, Hemmings BA, et al. Use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways. *Proc Natl Acad Sci U S A.* 2000; 97(12):6499–503. [PubMed: 10823906]
- Custodio HM, Broberg K, Wennberg M, Jansson JH, Vessby B, Hallmans G, et al. Polymorphisms in glutathione-related genes affect methylmercury retention. *Arch Environ Health.* 2004; 59(11):588–95. [PubMed: 16599007]
- Daborn PJ, Yen JL, Bogwitz MR, Le Goff G, Feil E, Jeffers S, et al. A single p450 allele associated with insecticide resistance in *Drosophila*. *Science.* 2002; 297(5590):2253–6. [PubMed: 12351787]
- Ekengren S, Hultmark D. A family of Turandot-related genes in the humoral stress response of *Drosophila*. *Biochem Biophys Res Commun.* 2001; 284(4):998–1003. [PubMed: 11409894]
- Ekengren S, Tryselius Y, Dushay MS, Liu G, Steiner H, Hultmark D. A humoral stress response in *Drosophila*. *Curr Biol.* 2001; 11(18):1479. [PubMed: 11566110]
- Grandjean P, Weihe P, White RF, Debes F, Araki S, Yokoyama K, et al. Cognitive deficit in 7-year-old children with prenatal exposure to methylmercury. *Neurotoxicol Teratol.* 1997; 19(6):417–28. [PubMed: 9392777]
- Hrdlicka L, Gibson M, Kiger A, Micchelli C, Schober M, Schock F, et al. Analysis of twenty-four Gal4 lines in *Drosophila melanogaster*. *Genesis.* 2002; 34(1–2):51–7. [PubMed: 12324947]
- Hunter D, Russell DS. Focal cerebellar and cerebellar atrophy in a human subject due to organic mercury compounds. *J Neurol Neurosurg Psychiatry.* 1954; 17(4):235–41. [PubMed: 13212411]
- Ji JZ, Elyaman W, Yip HK, Lee VW, Yick LW, Hugon J, et al. CNTF promotes survival of retinal ganglion cells after induction of ocular hypertension in rats: the possible involvement of STAT3 pathway. *Eur J Neurosci.* 2004 Jan; 19(2):265–72. [PubMed: 14725620]

- Lemaitre B, Hoffmann J. The host defense of *Drosophila melanogaster*. *Annu Rev Immunol*. 2007; 25:697–743. [PubMed: 17201680]
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{−(ΔΔC_T)} Method. *Methods*. 2001; 25(4):402–8. [PubMed: 11846609]
- Lu TH, Hsieh SY, Yen CC, Wu HC, Chen KL, Hung DZ, et al. Involvement of oxidative stress-mediated ERK1/2 and p38 activation regulated mitochondria-dependent apoptotic signals in methylmercury-induced neuronal cell injury. *Toxicol Lett*. 2011; 204(1):71–80. [PubMed: 21549813]
- Magnusson J, Ramel C. Genetic variation in the susceptibility to mercury and other metal compounds in *Drosophila melanogaster*. *Teratog Carcinog Mutagen*. 1986; 6(4):289–305. [PubMed: 2875540]
- Mahapatra CT, Bond J, Rand DM, Rand MD. Identification of methylmercury tolerance gene candidates in *Drosophila*. *Toxicol Sci*. 2010; 116(1):225–38. [PubMed: 20375079]
- Monroe RK, Halvorsen SW. Mercury abolishes neurotrophic factor-stimulated Jak–STAT signaling in nerve cells by oxidative stress. *Toxicol Sci*. 2006 Nov 8; 94(1):129–38. [PubMed: 16896058]
- Monroe RK, Halvorsen SW. Environmental toxicants inhibit neuronal Jak tyrosine kinase by mitochondrial disruption. *Neurotoxicology*. 2009 Jul; 30(4):589–98. [PubMed: 19635391]
- Murata K, Weihe P, Budtz-Jorgensen E, Jorgensen PJ, Grandjean P. Delayed brainstem auditory evoked potential latencies in 14-year-old children exposed to methyl-mercury. *J Pediatr*. 2004; 144(2):177–83. [PubMed: 14760257]
- Ostrowski D, Ehrenreich H, Heinrich R. Erythropoietin promotes survival and regeneration of insect neurons in vivo and in vitro. *Neuroscience*. 2011 Aug 11; 188:95–108. [PubMed: 21600963]
- Rand MD. Drosophotoxicology: the growing potential for *Drosophila* in neurotoxicology. *Neurotoxicol Teratol*. 2010; 32(1):74–83. [PubMed: 19559084]
- Rand, MD. Contributions of the *Drosophila* model to understanding methylmercury effects on development. In: Ceccetelli, S.; Aschner, M., editors. *Current topics in neurotoxicity: methylmercury and neurotoxicity* Springer Science. 2012. Ch. 17
- Sakaue M, Okazaki M, Hara S. Very low levels of methylmercury induce cell death of cultured rat cerebellar neurons via calpain activation. *Toxicology*. 2005; 213(1–2):97–106. [PubMed: 15982794]
- Sarafian TA. Methylmercury-induced generation of free radicals: biological implications. *Met Ions Biol Syst*. 1999; 36:415–44. [PubMed: 10093932]
- Sarafian TA, Cheung MK, Verity MA. In vitro methyl mercury inhibition of protein synthesis in neonatal cerebellar perikarya. *Neuropathol Appl Neurobiol*. 1984; 10(2):85–100. [PubMed: 6203047]
- Sirois JE, Atchison WD. Effects of mercurials on ligand- and voltage-gated ion channels: a review. *Neurotoxicology*. 1996; 17(1):63–84. [PubMed: 8784819]
- Syversen TL. Effects of methyl mercury on protein synthesis in vitro. *Acta Pharmacol Toxicol*. 1981; 49(5):422–6.
- Syversen TL. Effects of repeated dosing of methyl mercury on in vivo protein synthesis in isolated neurones. *Acta Pharmacol Toxicol*. 1982; 50(5):391–7.
- Ui K, Nishihara S, Sakuma M, Togashi S, Ueda R, Miyata Y, et al. Newly established cell lines from *Drosophila* larval CNS express neural specific characteristics. *In Vitro Cell Dev Biol Anim*. 1994; 30A(4):209–16. [PubMed: 8069443]
- Watanabe C, Satoh H. Evolution of our understanding of methylmercury as a health threat. *Environ Health Perspect*. 1996; 104(Suppl. 2):367–79. [PubMed: 9182044]
- Wilke RA, Kolbert CP, Rahimi RA, Windebank AJ. Methylmercury induces apoptosis in cultured rat dorsal root ganglion neurons. *Neurotoxicology*. 2003; 24(3):369–78. [PubMed: 12782102]

Fig. 1.

Generation of MeHg-tolerant and non-tolerant isolines: Three MeHg-tolerant (E20, F20 and H20) and three non-tolerant (E0, F0 and H0) populations were previously derived from a mass population (Mahapatra et al., 2010). Isogenic lines (isolines) were generated for this study by performing single female–male mating from each of the above populations. Progeny from single female matings was inbred for more than 11 generations to establish the isogenic lines.

Fig. 2.

Eclosion assay of MeHg-tolerant and non-tolerant isolines. Eclosion of non-tolerant (E0-1, E0-2, E0-3, dashed lines) isolines are compared with the tolerant lines (E20-1, E20-2 and E20-3, solid lines) by determining eclosion rates on various concentrations of MeHg in the food medium (see Materials and method). Each data point represents the mean and standard deviation of three trials ($n=150$ larvae per data point). Asterisks indicate significant differences between E20 and E0 samples at each concentration determined by ANOVA (*, $p < 0.01$; **, $p < 0.001$).

Fig. 3. TotA mRNA expression in MeHg-tolerant and non-tolerant isolines. (A) TotA expression was determined by qPCR of RNA extracted from 3rd instar larvae of the tolerant (E20-1, E20-2, E20-3) and non-tolerant (E0-1, E0-2, E0-3) isolines. Expression levels are expressed relative to the level in the non-tolerant E0-1 larvae. Determinations are done on pooled RNA from 10 3rd instar larvae. (B) Whole animal lysates of 3rd instar larvae of the tolerant (E20-1, E20-2, E20-3) and non-tolerant (E0-1, E0-2, E0-3) isolines were separated by SDS-PAGE and Western blotting was performed with a polyclonal antibody to TotA. Actin protein levels were determined by immunoblotting the same membrane sequentially with

anti-actin monoclonal antibody. Relative Molecular weights of standard protein ladder are indicated.

Fig. 4.

Association of TotA expression and MeHg tolerance across isolines. A plot of eclosion rate versus TotA protein expression for 18 isogenic lines derived from E0, F0, H0, E20, F20 and H20 parent populations is shown. Western blot analyses were done on pooled lysates of 10 3rd instar larva using a polyclonal antibody to TotA and signal intensity quantified (see Materials and method). Eclosion rates were measured on food containing 10 μ M MeHg and performed in triplicate (n=150 larva).

Fig. 5. Relative TotA mRNA expression across developmental stages in MeHg-tolerant and non-tolerant strains: The fold-change (log scale) of TotA transcript expression at various developmental stages relative to the non-tolerant E0-3 L1-larvae is shown. Expression levels are determined by qRT PCR of RNA derived from whole animal extracts of first instar (L1), second instar (L2), third instar (L3) larvae and early pupae (EP) of the MeHg-tolerant (E20-3) and non-tolerant (E0-3) lines.

Fig. 6.

TotA expression in various tissues of MeHg-tolerant and non-tolerant larva. TotA transcript levels were determined by qPCR of RNA from whole larvae (WL), gut, fat body (FB) and brain samples of non-tolerant (E0-3) and tolerant (E20-3). Expression levels are relative to the level in non-tolerant E0-3 whole larvae.

Fig. 7.

Fat body expression of TotA induces MeHg tolerance. A) Fat body GAL4 (FBG4) crossed to UAS-GFP gives strong green fluorescence localized predominantly to fat body tissues (FB) in the 3rd instar larvae. B) Relative levels of TotA under expression of the FBG4>TotA41a and FBG4>TotA60b combinations compared to the control (FBG4>wt) were determined by qRT PCR of RNA from larval extract ($n = 10$ larvae per genotype). C) MeHg tolerance was determined the FBG4>TotA41a, FBG4>TotA60b and FBG4>wt combinations by eclosion assays. Overall development was scored either by eclosion (dotted lines) or progression to the dark pupal stage (solid lines). Results are expressed as means $\pm$ S.D. of three independent determinations. Asterisks indicate significant differences of FBG4>TotA41a or FBG4>TotA60b from FBG4>wt control (Student's t -test; *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$).

Fig. 8.

TotA expression and MeHg tolerance in S2 and C6 cells. A) TotA protein expression was determined by Western blotting of culture media collected from S2 and C6 cells. B) Cell viability after exposure to indicated concentration of MeHg for 18 h was assayed by MTT assay (see Materials and method). Results are expressed as means±S.D. of minimum three independent determinations. Asterisks indicate significant differences between viability of the cell types at each MeHg concentration (Student's *t*-test; *, $p < 0.05$; **, $p < 0.01$).

Fig. 9.

Effect of TotA knockdown on MeHg tolerance in C6 cells. A) TotA protein expression was analyzed by Western blotting of culture media of TotA dsRNAi-treated and control dsRNAi-treated C6 cells. TotA protein expression shows a substantial decrease with TotA dsRNAi treatment as compared to the control (GFP) dsRNAi. B) Control and TotA dsRNAi pre-treated cells were treated with indicated concentration of MeHg for 18 h followed by MTT cell viability assays. TotA dsRNAi treatment of C6 cells shows lower viability at all MeHg concentrations compared to cells treated with control dsRNAi. Results are expressed as means±S.D. of minimum three independent determinations.