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Chronic inorganic mercury exposure induces sex-specific changes in central TNFα expression: Importance in autism?

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Abstract

Mercury is neurotoxic and increasing evidence suggests that environmental exposure to mercury may contribute to neuropathologies including Alzheimer's disease and autism spectrum disorders. Mercury is known to disrupt immunocompetence in the periphery, however, little is known about the effects of mercury on neuroimmune signaling. Mercury-induced effects on central immune function are potentially very important given that mercury exposure and neuroinflammation both are implicated in certain neuropathologies (i.e., autism). Furthermore, mounting evidence points to the involvement of glial activation in autism. Therefore, we utilized an in vivo model to assess the effects of mercury exposure on neuroimmune signaling. In prairie voles, 10 week mercury exposure (60 ppm $HgCl_2$ in drinking water) resulted in a male-specific increase in TNFα protein expression in the cerebellum and hippocampus. These findings are consistent with our previously reported male-specific mercury-induced deficits in social behavior and further support a role for heavy metals exposure in neuropathologies such as autism. Subsequent studies should further evaluate the mechanism of action and biological consequences of heavy metals exposure. Additionally, these observations highlight the potential of neuroimmune markers in male voles as biomarkers of environmental mercury toxicity.

Keywords

heavy metals; environmental toxins; voles; cytokines; chemokines; autism

Introduction

Environmental exposure to heavy metals is a significant risk to human health [12]. Mercury, for example, certainly is neurotoxic and accumulation of mercury in the brain is accompanied by abnormal neuronal function in several brain regions, including in the cerebellum and the hippocampus [5, 17, 54]. Increasing evidence suggests that environmental mercury exposure may contribute to neuropathologies such as Alzheimer's disease (AD) and the autism spectrum disorders (ASD) [20-22, 28, 38-40].

Among the mechanisms implicated in mercury-induced neurotoxicity are mitochondrial dysfunction and oxidative stress [33, 45]. However, sub-lethal exposure to mercury also disrupts immunocompetence [29, 30] suggesting that changes in neuroimmune function may

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provide a link between metals exposure and the development of disorders such as autism. Consistent with this idea, recent studies have shown that glial activation and neuroinflammation may contribute to the neuropathology of autism [3, 11, 35, 47, 53]. For example, levels of proinflammatory cytokines, including tumor necrosis factor alpha (TNFα), consistently are elevated in the central nervous system (CNS) of autism patients [9, 35, 53].

Unfortunately, relatively little is known about the effects of mercury exposure on neuroimmune function [18, 46, 56] and how such effects may contribute to the development of disorders such as autism. Prairie voles have been used extensively in studies of the physiological and neurochemical underpinnings of social behaviors [2, 26, 57]. These animals are highly social: individual prairie voles appear to actively seek contact with conspecifics, and, in fact, display evidence of significant stress when isolated [16]. We recently have been able to produce behaviors in voles that model two of the major characteristics of autism: prairie voles that ingest heavy metals in their drinking water subsequently exhibit sex-specific deficits in social behavior [13]. Male, but not female, prairie voles that receive metals treatment significantly reduce social contact when confronted with an unfamiliar individual, apparently via a dopamine-mediated mechanism. Importantly, the social avoidance is not displayed when a familiar sibling rather than a stranger is encountered. In the present investigation, we used the prairie vole model to examine the effects of chronic mercury exposure on neuroimmune signaling.

Methods

We first wanted to test whether prairie voles display a typical central response to an immune challenge, in this case, systemic administration of bacterial lipopolysaccharide (LPS). Twenty male voles were injected with 3 mg/kg of LPS. Four animals were sacrificed immediately after LPS administration to provide a baseline value against which longer LPS exposures could be compared. Additional groups of 4 animals each were sacrificed at 6, 12, 24, and 48 hours after LPS administration. At each time point, brains were removed at termination and the cerebellum and hippocampus were quickly frozen and subsequently assayed for TNFα protein expression. Brain tissue was prepared using a modified version of the protocol described by Vargas et al, [53]. Briefly, tissue was sonicated on ice for 10 sec. in 0.5 ml buffer [50nM Tris-HCl, pH 7.4, 150nM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate, 1.0% IGEPAL, 0.5% sodium deoxycholate, 10ug/mL aprotinin and 0.5mM phenylmethylsulfonyl fluoride. A standard dual-antibody solid phase immunoassay (ELISA Development Kits, Peprotech) was used for quantitation of TNFα protein in brain tissue homogenates, according to the manufacturer's instructions and as previously described [14]. Brain samples and standards (100 μl for each) were added to antibody-coated wells of 96 well plates. Next, 100 μl of antigen-specific biotinylated detection antibody was added to each well followed by a 2 h incubation at room temperature. Liquid contents then were aspirated and the wells were washed 3× with wash buffer. Avidin-peroxidase conjugate (100 μl) was added to each well and incubated for 30 min at room temperature. Wells again were aspirated and washed 3×, and 100 μl of ABTS liquid substrate solution (Sigma cat. # A3219) was added to each well followed by 25 min incubation at room temperature. Absorbance was measured at 450 nm (with wavelength correction set at 650 nm) on a BIO-TEK HT spectrophotometer. Total protein/well was determined using the bicinchoninic acid (BCA) protein assay as previously described [15] in order to normalize data when appropriate (pg chemokine protein/mg total tissue protein). One-way Analysis of Variance (ANOVA) was used to test for significant effects of time after LPS administration. Student-Neuman-Kuels (SNK) pair-wise comparisons were used to further probe significant ($p < 0.05$) main effects. Data are presented as means ± standard errors.

We then examined vole brain tissue after chronic exposure to low-dose mercury ingestion. Tissue for this experiment was collected at termination of subjects used in a previously reported study of the effects of chronic metals exposure on vole social behavior [13]. Details of animal husbandry are provided in that report and all experimental manipulations, animal handling procedures, and behavioral testing were approved by the Oklahoma State University Center for Health Sciences Institutional Animal Care and Use Committee. Same-sex pairs were randomly assigned to treatment groups that received a dilute (60 ppm) mercuric chloride ($HgCl_2$) solution as their sole drinking water source for 10 weeks. This concentration is near the lower end of the range of concentrations found during a survey of the toxicology literature. Control pairs received normal drinking water for the same period. Groups otherwise were treated identically. Fresh metals solutions were prepared weekly and solutions were replaced as part of normal cage maintenance, but drinking bottles were replaced if they became contaminated with bedding outside of the normal cage maintenance regimen. Since animals required multiple housing, the amounts of mercury ingested were assumed to be equal between the two individuals in each pair. Mercury exposure was followed by measurement of TNFα, and chemokine (CCL2 and CXCL10) levels in the cerebellum and hippocampus using ELISA as described above with kits specific for each analyte (Peprotech). Two-way ANOVA was used to assess group differences in the neurochemical measures with sex and treatment as factors. Student-Neuman-Kuels pair-wise comparisons were used to further probe significant ($p < 0.05$) main effects or interactions. Data are presented as means ± standard errors.

Results

Neuroimmune response to systemic LPS challenge

Neuroimmune responses in males after peripheral administration of LPS were compared at five time points after administration. Values at time 0 (for animals terminated immediately after LPS administration) served as the baseline against which values from subsequent time points were compared. During the experiment, there were no group differences in the animals' ages ($F_{4,15} = 0.29$, $p = 0.88$) or body masses ($F_{4,15} = 0.10$, $p = 0.98$). There were significant effects of LPS administration on TNFα in both the cerebellum ($F_{4,15} = 4.56$, $p < 0.02$) and the hippocampus ($F_{4,15} = 4.71$, $p < 0.02$). In both brain regions, TNFα was significantly elevated over baseline at six hours after LPS administration ($p < 0.01$), but not at 12, 24, or 48 hours after treatment.

TNFα expression in vole brain after chronic mercury ingestion

Two-way ANOVA for TNFα in the cerebellum revealed significant main effects of treatment ($F_{1,56} = 4.13$, $p < 0.001$), sex ($F_{1,56} = 13.35$, $P < 0.05$), and a significant interaction ($F_{1,56} = 8.07$, $P < 0.01$). SNK pair-wise comparisons established that cerebellum TNFα expression (Fig 1A) was greater in mercury-treated males than in water-treated males ($p < 0.002$) or in either group of females (both p-values < 0.001). No other group differences were found (all p-values > 0.49). In general, the same pattern was seen for TNFα in the hippocampus (Fig 1B). There was a significant effect of sex ($F_{1,57} = 8.22$, $p < 0.01$) and a significant treatment × sex interaction ($F_{1,57} = 8.44$, $p < 0.01$), however, the main effect of treatment failed to reach statistical significance ($F_{1,57} = 3.26$, $p = 0.076$). Pair-wise comparisons again established mercury-treated males as having significantly greater TNFα expression than that for any other group (all p-values < 0.005). No other pair-wise comparisons reached statistical significance (all p-values > 0.46).

CCL2 expression in vole brain after chronic mercury ingestion

Two-way ANOVA for CCL2 in the cerebellum (Fig 1C) did not reveal any significant main effects (sex: $F_{1,36} = 0.0005$, $p = 0.98$; treatment: $F_{1,36} = 1.28$, $p = 0.27$), or interaction ($F_{1,36}$

= 1.41, p = 0.24). Similarly, there were no significant main effects of sex ($F_{1,35}$ = 2.13, p = 0.15) or treatment ($F_{1,35}$ = 3.20, p =0.08), and no interaction ($F_{1,35}$ = 0.27, p = 0.61) for CCL2 in the hippocampus (Fig 1D).

CXCL10 expression in vole brain after chronic mercury ingestion

Two-way ANOVA for CCL2 in the cerebellum (Fig 1 E) and the hippocampus (Fig 1F) also did not reveal any significant main effects of sex (cerebellum: $F_{1,87}$ = 1.16, p = 0.29; hippocampus: $F_{1,23}$ = 0.88, p = 0.36) or treatment: (cerebellum: $F_{1,87}$ = 0.19, p = 0.67; hippocampus: $F_{1,23}$ = 1.45, p = 0.24), and there were no significant sex × treatment interactions (cerebellum: $F_{1,87}$ = 0.69, p = 0.41; hippocampus: $F_{1,23}$ = 0.70, p = 0.41).

Discussion

We examined neuroimmune responses in prairie voles as part of an on-going series of studies into the potential for heavy metals exposure being a risk factor in the development of autism. We found that the expression of at least some neuroimmune factors is altered after chronic metals exposure and that the effects are consistent with previously reported metals-induced changes in social behavior.

As a first step in this process we needed to establish that vole neuroimmune responses were similar to those in other species. This was found to be the case for TNFα, which was elevated in at least two brain regions by six hours after administration of an LPS challenge. For purposes of this study, the elevation of TNFα in a temporally appropriate timeframe suggests that vole neuroimmune responses are not fundamentally different from those of the other species.

There is substantial evidence that mercury affects function in peripheral immune cells, including, lymphocyte adhesion [50], mast cell activation [27] and cytokine expression in numerous cell types [19, 27, 29, 42]. In addition, there is mounting evidence suggesting that immune signaling is instrumental in the neuropathology of autism. For instance, glial activation and several markers of neuroinflammation are present in autism [11, 35, 47, 53]. Among the neuroinflammatory markers often associated with autism is TNFα [9, 35], but it remains unclear exactly how elevated TNFα contributes to the symptoms or development of ASD. However, the importance of TNFα likely extends beyond its classic, proinflammatory and pro-apoptotic roles [8, 48]. Indeed, TNFα is increasingly recognized as instrumental in complex physiologic processes including neuroprotection, ionic homeostasis and synaptic plasticity [8, 23, 48, 49]. For example, overexpression of TNFα in response to methamphetamine (METH) treatment appears to activate vesicular dopamine uptake, thereby attenuating the METH-induced increase in extracellular dopamine in the striatum [41]. The ability of TNFα to modulate dopamine metabolism is particularly intriguing in an autism context given the apparent role for central dopamine functioning in the switching from social to asocial behavior in voles [13] and other CNS processes [51]. Taken together, as postulated by Chertoff et al., [8], the detrimental (or beneficial) effects of TNFα likely involve numerous factors including the level and site of TNFα expression, as well as the time at which this cytokine is expressed relative to other stimuli.

To our knowledge, this is the first report on the effects of mercury exposure on cytokine/chemokine expression in the brain: chronic exposure to low levels of inorganic mercury enhanced TNFα expression in both the cerebellum and hippocampus. Of particular interest is that TNFα levels were elevated after mercury exposure in male voles only. This finding is important for two reasons. First, this is another example of sexual dimorphism in a response to an immune challenge (see also Klein et al., [31, 32]). Second, the brain tissue used in this study was from animals whose social behavior previously had been carefully characterized.

In that study, we reported that prairie voles' preference for social proximity is quite sensitive to exposure to environmental toxins. After 10 weeks of chronic, low-level exposure to metals (mercury or cadmium) in their drinking water, these normally social animals began to avoid strangers and displayed social withdrawal quite similar to the regressive aspects of autism that are seen in human autistic patients. Further, the metals-induced changes in social behavior occurred only in male voles [13], which parallels the well-established higher incidence of autism in males. Thus, these results are consistent with a number of studies in other animal models linking mercury in various forms to the neuropathology of autism [24, 25, 34, 44].

Chemokines also can be elevated in the CNS of those with autism: CXCL10 is elevated in the cerebrospinal fluid (CSF) and CCL2 is elevated in the CSF and brain (particularly the cerebellum) of autistic individuals [53]. Chemokines are instrumental in physiological and pathological process in the brain. Within the brain, CCL2 expression is predominately in the astrocytes [53]. CCL2 is expressed constitutively in astrocytes and neurons and is transcriptionally induced by proinflammatory cytokines, particularly in astrocytes [4, 7, 43]. Both CXCL10 and CCL2 are well characterized with respect to their roles in the recruitment of inflammatory cells to sites of insult [6]. Increasing data also indicate that these chemokines have neuromodulatory [1, 4, 37], neuroprotective [36, 55] and neurotoxic actions [52]. CCL2 reportedly has more of a neuromodulatory role, particularly on dopaminergic neurons, whereas, CXCL10 appears to be more of a neurotoxin. Thus, dysregulation of these cytokines/chemokines can impact multiple aspects of neurophysiology and neuropathology. We did not observe any mercury effect on chemokines in the specific brain regions we examined. However, this does not preclude changes in chemokine expression in other brain regions after mercury exposure. Further, we examined chemokine expression only at a single time-point after 10 weeks of chronic mercury exposure. It is possible that chemokine expression in these brain regions is transient, or that the length of exposure was not of sufficient duration to elicit a chemokine response. Interestingly, and of potential importance, is that our initial *in vitro* studies showed that chronic mercury exposure inhibited TNFα-induced chemokine (CCL2 and CXCL10) expression in human astroglial cells (data not shown). Further studies assessing the temporal effects of mercury are therefore warranted, as is the assessment of chemokine expression in other brain regions.

Together, these data demonstrate that neuroimmune signaling is altered by mercury exposure *in vivo* and that further study is needed to identify the mechanism of action and biologic consequences. Gaining a better understanding of mercury's effects on TNFα, CCL2, and CXCL10 is important given the physiological and pathological importance of these mediators. Furthermore, these observations in voles are particularly important given the potential for these neuroimmune factors to serve as biomarkers of mercury toxicity. Voles already are recognized as potentially useful sentinel animals for monitoring environmental levels of various toxins such as metals [10]. The present results suggest that inflammatory signaling in the vole brain, especially that in males, may be a useful biomarker of mercury contamination in the environment.

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Highlights

- Peripheral LPS induces increased TNFα in vole brain
- Chronic mercury induces increased TNFα in vole brain
- Mercury induced TNFα in vole brain is male specific
- Mercury induced TNFα in brain parallels previously reported social deficits in males

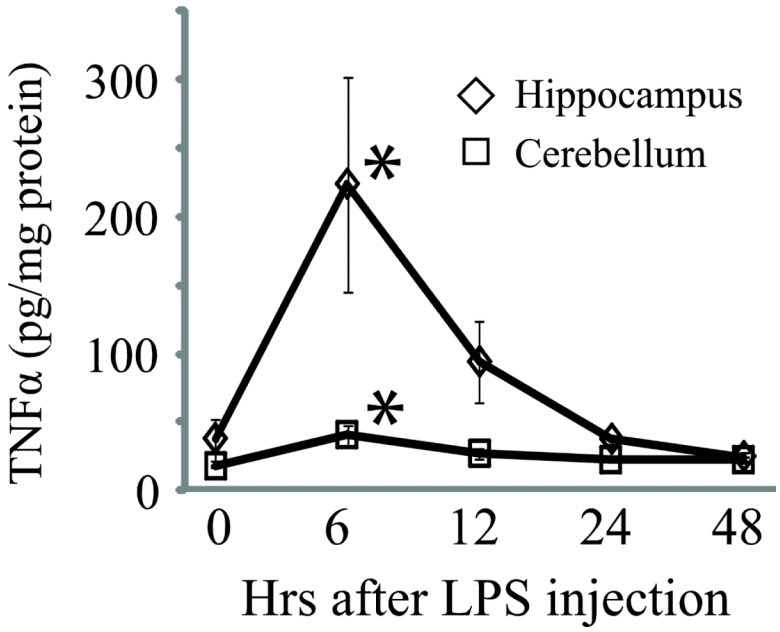

Fig. 1.

TNFα expression in male vole brain was elevated in both the hippocampus and cerebellum 6 h after peripheral administration of 3 mg/kg LPS. * $P < 0.05$ vs. 0 hr.

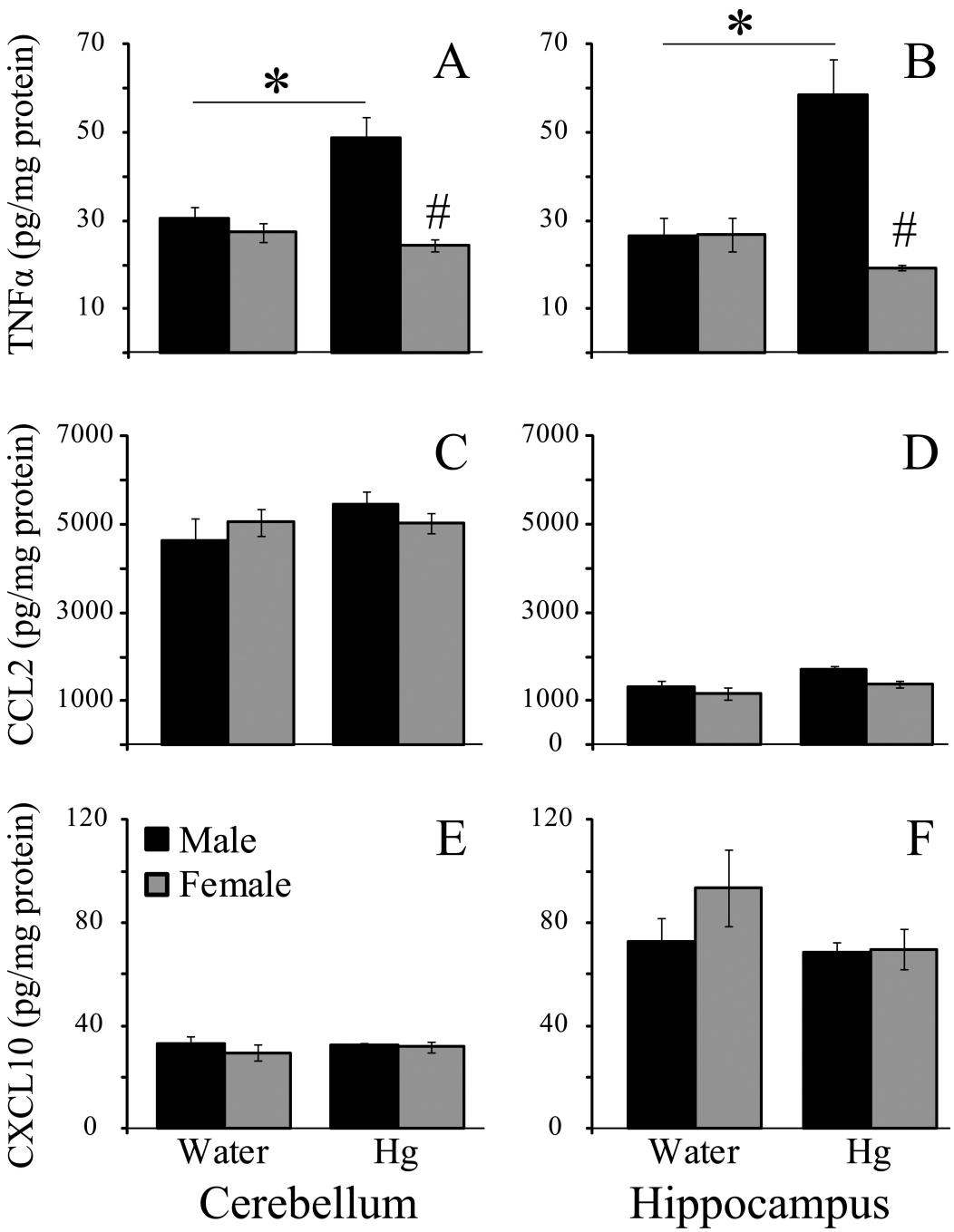

Fig 2.

Neuroimmune factor responses in vole brain after chronic mercury exposure. Ten weeks of chronic exposure to mercury in drinking water altered TNFα expression in a sex-specific fashion in the cerebellum and hippocampus of prairie voles (A, B). In both brain regions, exposure to mercury increased TNFα in male voles but not in females. This mercury exposure paradigm did not alter the expression of CCL2 (C, D) or CXCL10 (E, F). * - significantly greater than water-treated voles of either sex. # - significantly different from within-treatment opposite sex animals.