

## Oxidative Stress in Autism: Elevated Cerebellar 3-nitrotyrosine Levels

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**Abstract:** It has been suggested that oxidative stress and/or mercury compounds play an important role in the pathophysiology of autism. This study compared for the first time the cerebellar levels of the oxidative stress marker 3-nitrotyrosine (3-NT), mercury (Hg) and the antioxidant selenium (Se) levels between control and autistic subjects. Tissue homogenates were prepared in the presence of protease inhibitors from the frozen cerebellar tissue of control (n=10; mean age, 15.5 years; mean PMI, 15.5 hours) and autistic (n=9; mean age 12.1 years; mean PMI, 19.3 hours) subjects. The concentration of cerebellar 3-NT, determined by ELISA, in controls ranged from 13.69 to 49.04 pmol g<sup>-1</sup> of tissue; the concentration of 3-NT in autistic cases ranged from 3.91 to 333.03 pmol g<sup>-1</sup> of tissue. Mean cerebellar 3-NT was elevated in autism by 68.9% and the increase was statistically significant (p=0.045). Cerebellar Hg, measured by atomic absorption spectrometry ranged from 0.9 to 35 pmol g<sup>-1</sup> tissue in controls (n=10) and from 3.2 to 80.7 pmol g<sup>-1</sup> tissue in autistic cases (n=9); the 68.2% increase in cerebellar Hg was not statistically significant. However, there was a positive correlation between cerebellar 3-NT and Hg levels (r=0.7961, p=0.0001). A small decrease in cerebellar Se levels in autism, measured by atomic absorption spectroscopy, was not statistically significant but was accompanied by a 42.9% reduction in the molar ratio of Se to Hg in the autistic cerebellum. While preliminary, the results of the present study add elevated oxidative stress markers in brain to the growing body of data reflecting greater oxidative stress in autism.

**Key words:** Protein nitration, oxidative stress markers, antioxidants, autistic-pathology, nitrotyrosine, mercury, selenium

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### INTRODUCTION

**Role of oxidative stress in the pathology of neuropsychiatric disorders:** There is growing evidence supporting the role of oxidative stress in the pathophysiology of a number of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD) and Alzheimer's disease (AD)<sup>[1]</sup>. There is also emerging evidence supporting the role of oxidative stress involvement in autism<sup>[2-8]</sup>. The support comes from observations of increased lipoxidation markers in blood<sup>[2, 4]</sup> and in urine<sup>[5, 6]</sup> and increased nitric oxide (NO)<sup>[2, 7, 8]</sup> and thiobarbituric acid-reacting substances<sup>[2]</sup> in autism. There is also evidence for the disruption of antioxidant defense mechanisms in autism manifested by lower than control levels of glutathione peroxidase (GSPHx)<sup>[9, 10]</sup>, by lower

levels of plasma glutathione levels and higher ratios of oxidized glutathione to reduced glutathione<sup>[11, 12]</sup>, lower levels of two major serum antioxidant metalloproteins ceruloplasmin (copper-binding protein) and transferrin (iron-binding protein)<sup>[4, 13]</sup> and lower levels of naturally occurring free radical scavengers<sup>[14]</sup>. Impaired methionine metabolism is reported in autism and is associated with altered plasma glutathione levels<sup>[11, 12]</sup>. As stressed by Kern and Jones<sup>[3]</sup>, there is a correlation between antioxidant proteins and loss of previously acquired skills in a subset of children with autism<sup>[13]</sup>.

Higher than control levels of circulating pro-oxidant organic toxins, heavy metals, xanthine oxidase and cytokines have also been observed in autism<sup>[14]</sup>. A strong oxidant, homocysteine, is increased in plasma from children with autism<sup>[15]</sup>. An increase in pro-oxidative inflammatory cytokines has been reported in

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autistic brain tissue<sup>[16]</sup>. Hypoperfusion, promoting oxidative stress, has been documented in several regions of autistic brains by both SPECT and PET scans<sup>[17]</sup>.

**Oxidative stress markers in autism:** Little is known about oxidative stress markers in autism. While data on oxidative lipid modification has been derived from plasma and urine analysis, modification to proteins and DNA has not been explored in autism. Further, there are no published data reflecting modification of brain protein or DNA by excess free radicals, nor there is prior measurement of substances known to modulate levels of oxidative stress, other than elevated parenchymal and cerebrospinal inflammatory cytokines<sup>[16]</sup>. The approach used in this study is a logical extension of the proposed role of oxidative stress in other neuropsychiatric disorders. In AD, for instance, examination of postmortem brain demonstrated elevated nitrotyrosine, as well as elevated carbonyls, oxidized DNA bases and lipid peroxidation products<sup>[18]</sup>.

The present study examined cerebellar levels of 3-nitrotyrosine (3-NT), a relatively specific marker of oxidative damage mediated by peroxynitrite, which is formed by the reaction of superoxide with nitric oxide (NO) on tyrosine residues in proteins<sup>[19]</sup> or by reaction of nitrite and hydrogen peroxide<sup>[20]</sup>. Transition metal homeostasis, redox activity and levels of metalloenzymes, such as transferrin, ceruloplasmin and superoxide dismutase (SOD), all are involved in this process<sup>[97]</sup>. It has been shown that peroxynitrite formation is downregulated by metalloenzyme such as SOD<sup>[98]</sup>. Increased levels of NO metabolites (nitrite and nitrate) in red blood cells<sup>[7]</sup> and plasma<sup>[8]</sup> combined with decreased levels of metalloenzymes<sup>[4, 10, 13]</sup> reported in autism could favor formation of nitrotyrosine. Increased brain nitrotyrosine in Alzheimer's disease<sup>[21, 99, 100]</sup> is associated with increased levels of NO in platelets<sup>[22]</sup>.

**Implication of environmental factors in autism: heavy metals:** Many environmental factors have been implicated in autism, including pesticides<sup>[23, 24]</sup>, mercury (Hg)<sup>[25, 26]</sup>, lead<sup>[27]</sup> and perhaps polychlorinated biphenyls (PCBs)<sup>[28]</sup>. These environmental factors share the ability to induce oxidative stress. Pesticides stimulate free radical production, induce lipid peroxidation and disturb the body's antioxidant status<sup>[23]</sup>. Lead induces oxidative stress and increases DNA damage<sup>[29]</sup>. PCBs induce lipid peroxidation<sup>[30]</sup>. Urinary porphyrin profiles strongly suggest heavy metal toxicity in autism<sup>[31]</sup>.

Major sources of human exposure to Hg are primarily from eating high levels of marine fish or inland fish raised in waters contaminated by the past industrial activities, such as chlorine production<sup>[32, 33]</sup> and chronic inhalation of Hg gas associated with metal ore mining and processing (e.g. gold). More insidious environmental contamination such as in southern New Jersey, where industrial waste disposal has led to the contamination of potable water<sup>[34]</sup> that eventually resulted in direct human exposure to Hg gas, may be responsible for additional exposure. Local environmental release of Hg resulting in increased air concentrations of Hg has been associated with higher incidence of autism in Texas<sup>[35]</sup> and California<sup>[36]</sup>.

Mercury strongly associates with oxidative stress<sup>[25, 26]</sup>. Methyl mercury (MeHg) increases oxidative stress in primary neuronal culture<sup>[37]</sup> and inhibits brain cytochrome<sup>[38]</sup>. Inorganic Hg induces oxidative stress in *in vitro* synaptosome preparations<sup>[39]</sup> and its administration *in vivo* enhanced lipid peroxidation in the rat cerebral cortex and cerebellum<sup>[40]</sup>. Hg-induced injury to human glioma cells can be prevented by specific hydroxyl radical scavengers<sup>[41]</sup>. Brain Hg levels have not been measured previously in autism. The studies discussed here do not consider the source of Hg exposure and uptake but rather aim to define the relationship between brain oxidative stress markers and brain Hg levels. Hg-induced oxidative stress results in oxidative modification of DNA, protein and lipids, as well as inhibition of the enzymes crucial for the brain development<sup>[42]</sup>. Thus, elevated levels of mercury in brain potentially interfere with normal brain development.

**Antioxidant status in autism:** This study also examined brain levels of antioxidant, selenium (Se). Antioxidant deficiency has been implicated in the pathophysiology of autism as reviewed by McGinnis<sup>[14]</sup>. It has been observed that levels of antioxidants are lower in autistic individuals; for example, decreased Se levels in the red blood cells have been reported in autism<sup>[43]</sup>. Se is an essential trace element for animals and humans, which is taken up from the soil in plants. Due to geographical and regional variations of Se concentration in water and in soil, its uptake differs greatly depending on location and dietary habits. It has been suggested that supra-nutritional levels of Se may be needed to prevent degenerative disease<sup>[44]</sup>. The biological significance of Se and selenocompounds has been recently reviewed by Whanger<sup>[45]</sup>. Se is an essential component of various enzymes, such as glutathione peroxidase (GSHPx). Lower levels of Se

and GSPHx in autistic children<sup>[10]</sup> may favor lipid peroxidation. Se is also a component of enzymes involved in conversion of T4 to T3<sup>[46]</sup>, which is critical for normal brain development. It is thus of interest that hypothyroidism has been reported in autistic children<sup>[102]</sup>. Another, less known function of Se is its ability to counteract the neurotoxicity of heavy metals, such as Hg<sup>[47]</sup>. Since Se<sup>[48]</sup> has been shown to accumulate preferentially in brain tissue, direct measures of this trace element in the brain are critical to our understanding of the role of Se deficiency in the pathology of autism.

The present study compares the concentrations of 3-NT, Hg and Se in cerebellar tissue from control and autistic cases. The data should be viewed as preliminary, owing to small sample size and heterogeneity within both control and autistic cohorts. The relationship of the measured parameters to abnormal neurodevelopment or expression of symptoms in autism remains, at this time, hypothetical pending more extensive studies.

## MATERIALS AND METHODS

**Human brain samples:** Human postmortem frozen cerebellar tissue samples were obtained from the NICHD Brain and Tissue bank for Developmental Disorders at the University of Maryland, with the exception of one sample obtained from the Harvard Brain Bank. The use of the postmortem tissue has been approved by Protocol# 2000-P-001686/4. Donor profile is presented in Table 1. Analyses of tissue levels of 3-NT, Hg and Se were performed blindly.

**Determination of cerebella 3-NT:** Cerebellar 3-NT was measured according to the procedure previously described<sup>[49]</sup>. In brief, cerebellar homogenates were prepared in phosphate buffered saline (PBS) containing protease inhibitors (Complete, EDTA-free protease inhibitor cocktail, Boehringer Mannheim, Indianapolis, IN), 0.5% Nonident P40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate (SDS; Sigma, St Louis, MO). The homogenates were centrifuged at 10,000 x g for 10 min. The supernatants were assayed for 3-NT content using Nitrotyrosine ELISA Kit (Oxis International Inc., Portland, OR) according to manufacturer's instructions. Briefly, the samples were incubated in microtiter wells coated with antibody to nitrotyrosine. A biotinylated secondary antibody was used and the product of the reaction of streptavidin-peroxidase with tetramethyl benzidine as a substrate was measured by absorbancy at 450nm. Data is

expressed as 3-NT levels in picomoles per gram of tissue.

**Analysis of cerebellar Hg levels:** Approximately 0.5 grams of homogenate used for 3-NT analysis was preincubated for one hour with trace metal-free HNO<sub>3</sub> and then digested using a CEM MS-2100 microwave digestion system. An aliquot of digested homogenate was then incubated with Hg-free water, HCL and 0.1N BrO<sub>3</sub>Br solution overnight in dark. Following the incubation, a hydroxylamine hydrochloride solution was added to reduce the BrO<sub>3</sub>Br in the sample. Different Hg standards were processed in the same manner. Samples were mixed with a stannous chloride solution using a four channel peristaltic pump and delivered into a closed gas/liquid Tekran separator which volatilizes the Hg which was then collected on a gold sand trap. The trap was dried for approximately 5 minutes with UHP argon (Ar) gas and was then heated and purged with Ar to sweep the Hg vapor into a Tekran cold vapor atomic fluorescence spectrophotometer (CVAFS) Hg Detector 2500. Using a HP Integrator, the peak area of the response was determined and compared to a standard curve of peak area versus Hg concentration.

**Analysis of cerebellar Se levels:** Cerebellar Se levels were measured in the same cerebellar homogenates used for 3-NT and Hg analysis. The analysis, performed by Vitamin Diagnostics Laboratory (Cliffwood Beach, NJ), was based on the atomic absorption spectrometry (hydride method) described earlier<sup>[50, 51]</sup>. Briefly, cerebellar tissue homogenates were subjected to a two-step digestion process, first with HNO<sub>3</sub> at 135<sup>o</sup> C for 15 min. followed by a digestion with H<sub>2</sub>SO<sub>4</sub> and HClO<sub>4</sub> at 220<sup>o</sup> C for 10 min. The solution was diluted with HCl, reduced with NaBH<sub>4</sub> and the Se was measured using Perkin Elmer Spectrophotometer (FIAS-100/400; cell temperature 900<sup>o</sup>) at 196nm with a seronorm blood and Biorad urine used as controls.

**Cell culture:** Glial (C6, ATCC) cells initially were propagated in F12 medium (ATCC) containing 15% horse serum and 2.5% fetal bovine serum and gradually adapted to serum- free medium-Stemline Neural Stem Cell Expansion Medium (Sigma). Prior to the analysis, they were plated in a 96-well microtiter plate at 5,000 cells/well. Following 24 hours preincubation, MeHg-hydroxide (Alfa Aesar, Ward Hill, MA) was added to a final concentration of 2-4 μM; to some wells, sodium selenite (Sigma) was added to a final concentration of 100 μM.

All cells were incubated for an additional 24 hours. The number of viable cells at the end of incubation was determined using non-radioactive cell proliferation assay (Promega) based on the ability of viable cells to convert tetrazolium salt into formazan that can be detected by spectrophotometry. Following incubation overnight, the optimized dye solution (containing tetrazolium salts) was added to each well and incubated for an additional 4 hours. At this time, a stop/digestion solution was added to each well, followed by additional incubation for 1 hour. The absorbancy was measured at 570 nm.

**Statistical analysis:** Data was analyzed by ANOVA. The relationship between cerebellar 3-NT and Hg was subjected to regression analysis. All values are reported as Mean  $\pm$  SE. For all statistical tests, the 0.05 level of confidence was accepted for statistical significance.

## RESULTS

**3-NT in human postmortem cerebellar tissue:** The data on cerebellar 3-NT is presented in Fig. 1. The levels of 3-NT were significantly increased in cerebellar tissue from autistic subjects ( $n=6$ ;  $p=0.045$ ), as compared to control subjects ( $n=9$ ). There was a 68.9% increase in 3-NT levels in autism ( $48.67 \pm 9.61 \text{ pmol g}^{-1}$ ) vs controls ( $28.82 \pm 3.75 \text{ pmol g}^{-1}$ ). These are the first reported quantitative levels of 3-NT in brain tissue. Prior reports on an increased 3NT in brain of Alzheimer's patients were based on immunohistochemical methods<sup>[21, 99, 100]</sup>; the most recent study reported relative 3-NT brain content<sup>[101]</sup>. Plasma concentration of 3-NT, in healthy controls, ranges from  $1.63 \pm 0.15 \text{ ng mg}^{-1} \text{ protein}$ <sup>[52]</sup> to  $35.21 \pm 4.87 \text{ ng mL}^{-1}$  ( $135.4 \pm 18.7 \text{ nM}$ ). The urinary 3-NT levels in healthy human volunteers range from 1.6 to  $33.2 \text{ nM}$ <sup>[53]</sup>. In nasal lavage fluid of rhinitis patients, 3-NT concentration of  $41.40 \pm 20.96 \text{ ng mL}^{-1}$  ( $159.02 \pm 80.6 \text{ nM}$ ) has been reported<sup>[54]</sup>.

Analysis of cerebellar Hg levels. Hg levels were measured in the same homogenates used for 3-NT analysis. The data reported in Table 2, suggest a 68.2% increase in cerebellar Hg in autism but the increase at this point is not statistically significant.

**Relationship between cerebellar 3-NT and Hg levels:** Initial analysis of cerebellar 3-NT and Hg levels suggest a correlation with  $r=0.7961$  ( $p=0.0113$ ).

**Analysis of cerebellar Se levels:** Se levels were measured in the same cerebellar homogenates used for

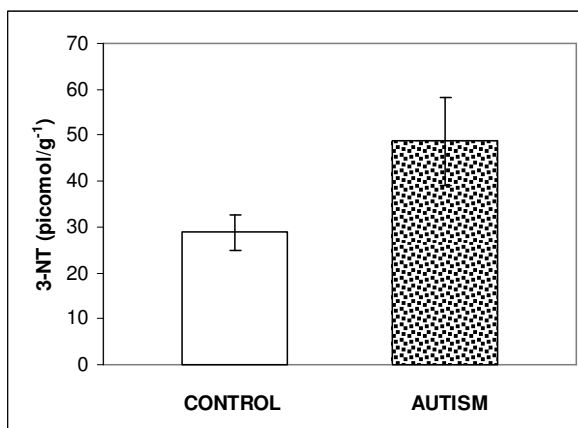


Fig. 1: 3-NT levels in cerebella from control and autistic subjects. Cerebellar 3-NT was measured with ELISA and recorded as absorbance at 450 nm. Data are converted to picomole per gram of tissue ( $\text{pmol g}^{-1}$ ) and presented as Mean  $\pm$  SEM of control ( $n=9$ ) and autistic ( $n=6$ ) cases;  $p < 0.05$

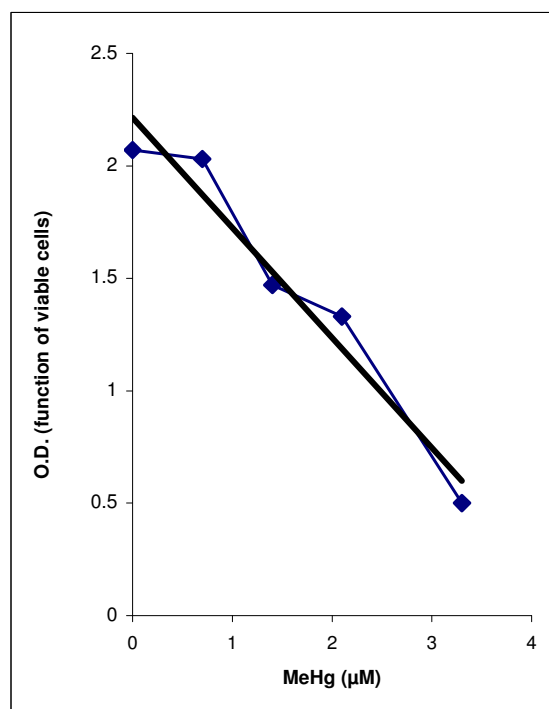


Fig. 2: Effect of MeHg cytotoxicity in human glioma (C6) cells. Cells were exposed to 2-4  $\mu\text{M}$  MeHg for 24 hrs. The cell viability was measured by a non-radioactive cell proliferation assay. Data are plotted as O.D. units, related to the number of viable cells vs. MeHg concentration in micromoles ( $\mu\text{M}$ )

3-NT and Hg analysis. The recovery of Se was over 90 percent and the limit of detection was 0.1 ng. The data shown in Table 3 indicates a small (5.7%) decrease in cerebellar Se level in autism, but the decrease was not statistically significant. It is, however, of interest that

Table 1: Distribution of control and autistic donors of cerebellar samples

CASE #	DIAGN.	AGE (Years)	SEX	PMI (Hours)	CASE #	DIAGN	AGE (Years)	SEX	PMI (Hours)
1376	C	37	M	12	4321	A	9	M	12
738	C	9	F	12	967	A	32	M	30
1080	C	16.5	M	21	797	A	9	M	13
1670	C	13	M	5	4849	A-ADI-R	7	M	20
4722	C	14.5	M	16	732	A	15	M	28
4898	C	7.5	M	12	1349	A	5.5	M	39
1708	C	8	F	20	4925	A	9	M	27
1500	C	7	M	18	4899	A	14	M	9
1136	C	34	F	19	4721	A	8	M	16
1706	C	8.5	F	20					

C, control cases (n=10); A, autistic cases (n=9); All samples were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland with the exception of sample 4925 obtained from the Harvard Brain Bank. The ages are rounded to the nearest year, and postmortem interval (PMI) is recorded in hours.

Table 2: Hg levels in cerebella from autistic and control subjects.

Cerebellar Hg was measured by atomic fluorescence spectrometry. Data are converted to picomole per gram of tissue ( $\text{pmol g}^{-1}$ ) and presented as Mean $\pm$ SEM of control (n=9) and autistic (n=6) cases; the increase was not statistically significant

Diagnosis	Hg ( $\text{pmol g}^{-1}$ tissue)
Control	14.93 $\pm$ 3.26
Autism	25.11 $\pm$ 8.25

Table 3: Se levels in cerebella from autistic and control subjects.

Cerebellar Se was measured by atomic absorption spectrometry. Data are converted to picomole per gram of tissue ( $\text{pmol g}^{-1}$ ) and presented as Mean $\pm$ SEM of control (n=9) and autistic (n=6) cases; the difference was not statistically significant

Diagnosis	Se ( $\text{pmol g}^{-1}$ tissue) X 10 <sup>3</sup>
Control	2.52 $\pm$ 0.12
Autism	2.38 $\pm$ 0.12

Table 4: Effect of MeHg cytotoxicity in human glioma (C6) cells.

Cells were exposed to 2-4  $\mu\text{M}$  MeHg for 24 hrs in the absence or presence of 100( $\mu\text{M}$ ) Se. The cell viability was measured by a non-radioactive cell proliferation assay. Data are presented as O.D. units, related to the number of viable cells. Data are mean of three determinations

Treatment	O.D. (Units)	Inhibition (%)
Control	1.85	0
MeHg (2.0 $\mu\text{M}$ )	1.16	37.3
MeHg (2.0 $\mu\text{M}$ ) + Se (100 $\mu\text{M}$ )	1.41	23.8

the highest cerebellar Se concentration (3,190  $\text{pmol g}^{-1}$ ) was observed in the controls.

#### Relationship between cerebellar Se and Hg levels:

While, the decrease in cerebellar Se in autism was not significant, it was accompanied by a 42.9% reduction in the molar ratio of Se to Hg from 407.4 in control to 232.9 in the autistic cerebellum. Since Se is involved in binding of Hg in the brain, a decrease in Se/Hg molar ratio in autism may reflect a relative deficiency in this antioxidant that could further exacerbate Hg toxicity.

#### Effect of methyl Hg and Se on glioma (C6) cells in culture:

The effect of MeHg, known to induce oxidative stress in glioma (C6) cells is shown in Fig. 2 and Table 4. The addition of MeHg to C6 cell cultures resulted in decreased O.D. readings indicating reduced number of viable cells; this reduction was dose-dependent as shown in Fig. 2. The simultaneous addition of Se (100  $\mu\text{M}$ ) partially counteracted cytotoxicity of MeHg (Table 4).

## DISCUSSION

#### Implications of increased cerebellar 3-NT in autism:

Although a large body of evidence implicates oxidative stress in the pathogenesis of neurodegenerative and neuropsychiatric disorder, relatively few systematic studies document changes in oxidative stress markers, especially those related to protein modification. The 3-NT is a specific marker for oxidative damage to proteins. Increased brain levels of 3-NT has been documented in Alzheimer's disease and Parkinson's disease by immunohistochemical methods [21, 99, 100]. While the most recent study reported relative increase in 3-NT brain content in Alzheimer's disease [101], there are no reports of quantitative brain levels of this oxidative stress marker in humans. The 3-NT in mice liver is reported at concentration ranging from 0.17 to 0.3  $\text{pmol per mg protein}$  [60]. Thus, our report contains novel data on brain 3-NT levels and the evidence of an increase in brain levels of 3-NT in autism.

Increased nitration of proteins has been documented in multiple pathologies. The proteomic approach identified specific target proteins in the Alzheimer's hippocampus [61]. A more recent study has identified 32 unique nitrotyrosine sites within 29 different proteins with more than half of these proteins

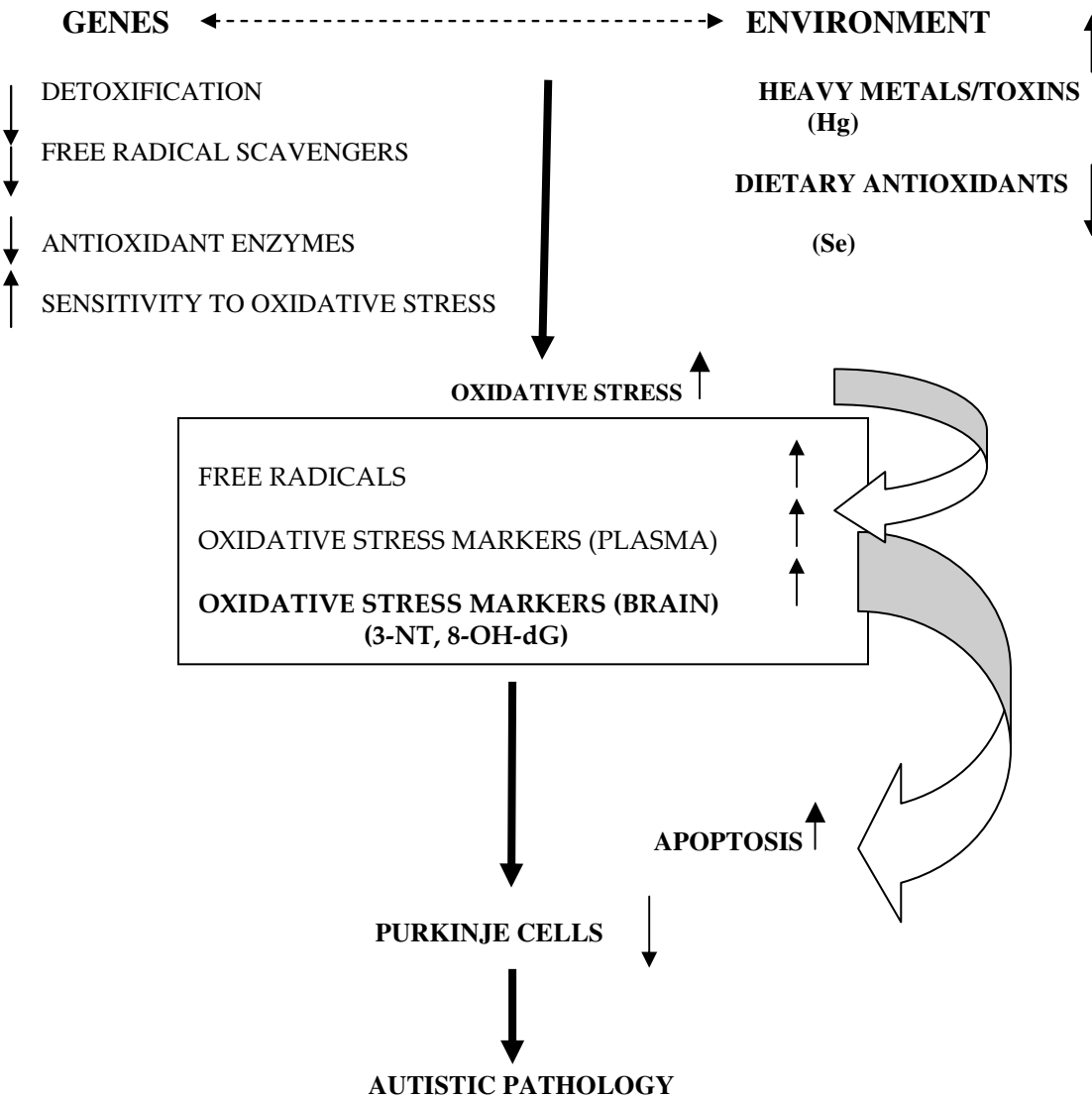


Fig. 3: A potential model of oxidative-stress-mediated gene-environment interactions in autism

being involved in Parkinson's disease, Alzheimer's disease and other degenerative diseases<sup>[62]</sup>. With regard to nitration of specific brain proteins, three - glial fibrillary acidic protein (GFAP), synaptophysin and superoxide dismutase (SOD) - may be particularly relevant to autism. GFAP is elevated in the CSF of children with autism<sup>[63, 64]</sup> and in autistic brain tissue<sup>[65, 66]</sup>. Nitration of GFAP in the rat brain has been identified by proteomics<sup>[1]</sup>. A reduced density of synaptophysin-expressing synaptic vesicles has been observed in autism<sup>[67, 68]</sup>. Our own observations suggest a decrease in synaptophysin levels in the cerebella of subjects with autism<sup>[65]</sup>. The reduction in synaptophysin expression may contribute to altered behavior in autism, since animals with a spatial learning deficit display a

significant reduction in synaptophysin levels<sup>[69]</sup>. Synaptophysin has been shown to be the main target of nitration in the rat hippocampus following the infusion of amyloid beta-protein<sup>[70]</sup>. SOD is an endogenous scavenger of superoxide anions<sup>[71]</sup>. Decreased plasma levels of SOD have been observed in autism<sup>[10, 13]</sup>. Nitration of SOD has been observed in the aging heart<sup>[72]</sup> and under conditions of oxidative stress in mice<sup>[73]</sup>. Thus, the increase in the overall nitration level in the cerebella of subjects with autism predicts oxidative stress-related modification of proteins relevant to autistic pathology. Altered levels of proteins combined with their oxidative modification could affect significantly cerebellar development, resulting in autistic pathology.

**Increased cerebellar Hg in autism?** Hg is a developmental neurotoxicant. CDC reports that between 316,588 and 637,233 children each year have umbilical cord blood Hg levels  $>5.8$  micrograms per liter, which is associated with loss of IQ<sup>[75]</sup>. The studies on the role of mercury in the pathogenesis of autism have yielded conflicting results. Some studies suggest a potential link between environmental Hg exposure and autism<sup>[25, 26, 31, 35]</sup>. The evidence implicating Hg in oxidative stress is even more abundant<sup>[37-41]</sup>.

Few studies to date report brain Hg levels. Interestingly our values are within the range of reported ones. Studies of inhabitants of Southern Poland, an area of concentrated mining industry, reports brain levels of Hg ranging from 0-14.2 ng g<sup>-1</sup> with an average of  $2.3 \pm 1.9$  ng g<sup>-1</sup><sup>[55]</sup>. Studies from areas in the vicinity of Hg mines in Slovenia reported values ranging from 0-281 ng g<sup>-1</sup><sup>[56]</sup>. Hair Hg levels in Japan ranged from 1.12 to 54.5 ng g<sup>-1</sup>. The brain levels of Hg in suckling rats exposed to mercury compounds increased from 33 ng g<sup>-1</sup> in controls to 73-108 ng g<sup>-1</sup> in Hg-treated animals<sup>[57]</sup>. While we found increased Hg levels in cerebella from subjects with autism, the increase was not statistically significant. However, the correlation between cerebellar Hg and 3-NT levels suggests that increased oxidative stress in brains of autistic subjects may be related in part to the increased mercury. It is possible that increased levels of Hg in the autistic brain may be caused by defects in the detoxification mechanisms as suggested by studies of Geier and Geier<sup>[75]</sup>. Irrespective of the sources of Hg contamination, exposure and the mechanism involved in its accumulation in the brain, increased brain Hg concentration is likely to result in oxidative stress and damage to cells as indicated by the results of our glial cultures. Japanese studies have shown that Hg exposure, indicated by hair Hg levels above 6.9 ng g<sup>-1</sup> (34.5 pmol g<sup>-1</sup>), significantly affected brain stem auditory evoked potential (BAEP)<sup>[76]</sup>. It is interesting that BAEP abnormalities have been observed in autism<sup>[77]</sup>, although reported changes have not been consistent<sup>[78]</sup>.

**Cerebellar Se in autism:** It has been observed that levels of antioxidants including Se are lower in autistic individuals<sup>[43]</sup>. Se is essential component of glutathione peroxidase (GSPHx) and a decrease in GSPHx has been observed in children with autism<sup>[10]</sup>. Another, less known function of Se is its ability to counteract the neurotoxicity of heavy metals, such as Hg<sup>[47]</sup>.

Se values range from 57.3 to 117.9  $\mu\text{g L}^{-1}$  in maternal blood, from 51.1 to 104.2  $\mu\text{g L}^{-1}$  in umbilical

cord blood and from 0.56-1.06  $\mu\text{g g}^{-1}$  in placenta<sup>[58]</sup>. Nail Se values ranging from 0.6 to 0.9  $\mu\text{g g}^{-1}$ <sup>[58]</sup> and from 0.89 to 1.03  $\mu\text{g g}^{-1}$  have been reported. Human brain Se concentrations of  $\sim 1.4$  nmol g<sup>-1</sup> is also reported<sup>[56]</sup>.

Since Se<sup>[48]</sup> has been shown to accumulate preferentially in brain tissue, direct measures of these trace elements in the brain are critical to our understanding of the role of Se deficiency in the pathology of autism. Brain Se levels reported here are in agreement with previously reported brain Se concentrations<sup>[56]</sup>. Furthermore, since Se binds Hg in the brain<sup>[56]</sup>, direct measurement of brain Se levels is important in assessing heavy metal toxicity. A decrease in Se to Hg ratio in autism observed in our study may reflect a relative deficiency in this antioxidant.

Our tissue culture studies suggest a potential effect of Se in counteracting heavy metal toxicity. These data are in agreement with observations that Hg-induced injury to human glioma cells can be prevented by specific hydroxyl radical scavengers<sup>[41]</sup>. A greater number of samples should be tested before the deficiency of this antioxidant in autism is confirmed.

**Oxidative-stress model of autistic pathology:** Our finding of increased nitrotyrosine levels in autistic cerebella lends further support to the hypothesis of oxidative stress involvement in autistic pathology. While it would be important to extend this study to a greater number of cases and to verify the geographical distribution of cases to link Hg level to the environmental exposure, a more likely explanation for heavy metal cytotoxicity in autism is the reduced ability by autistic patients to eliminate heavy metals from their system as suggested by a decreased capacity of urinary metal excretion<sup>[75]</sup>; defective metal elimination in conjunction with the deficiency of the intrinsic antioxidant system may thus lead to oxidative brain damage. Although the level of Se is not significantly altered in autistic brain, the molar ratio of Se to Hg is significantly decreased. It has been shown that Se binds Hg in brain and the reduced Se/Hg ratio may thus further enhance oxidative stress.

One of the outcomes of oxidative stress is increased apoptosis. Purkinje cells are especially sensitive to ischemia<sup>[79, 80]</sup> and hypoxia<sup>[79, 81]</sup>. Oxidative stress-induced apoptosis may contribute to Purkinje cell hypoplasia observed in a subset of autistic cases, which in turn, contributes to the stereotypic behavior observed in autism. Systematic studies indicate the anatomic abnormalities in autistic brains that include reduced numbers of Purkinje cells in the cerebellum<sup>[82-88]</sup>. The

decrease has been most consistently observed in the cerebellar hemispheres primarily in the posterior inferior region<sup>[88]</sup>. The posterior cerebellar lobes are associated with motor execution<sup>[89]</sup> that may be affected in autism<sup>[90]</sup>. The decrease in Purkinje cell number is likely to affect the neuronal communication and could contribute to autistic behavior in light of the cerebellar involvement in motor functions and planning, learning, cognitive flexibility, sensory processing, exploratory activity and other cognitive<sup>[91]</sup> and emotional<sup>[92]</sup> processes. Individuals with cerebellar abnormalities exhibit greater attention-orienting deficits and visuospatial attention<sup>[85]</sup>. Early vermal cerebellar lesions, such as tumor removals, lead to behavioral disturbances similar to those found in autism<sup>[93]</sup>. The rates of stereotyped behavior in autism were positively correlated with the magnitude of cerebellar hypoplasia<sup>[94]</sup>.

Based on the cumulative evidence of aforementioned data and the findings derived from our study, we propose a tentative oxidative-stress-mediated model of autistic pathology (Fig. 3). This model incorporates possible environmental impacts and is also consistent with recent genetic analysis that identified several genes linked to autism. Interestingly enough, some of these genes code for factors involved in the antioxidant defense mechanism such as glutathione-S-transferase (GST M1)<sup>[12, 95]</sup> an allele involved in the metabolism of glutathione, which is an intrinsic antioxidant, as well as differences in allele frequency for genes encoding reduced folate carrier<sup>[12]</sup>. Polymorphism for metal-responsive transcription factor (MTF-1) in autism<sup>[96]</sup>, potentially alters expression of metallothionein and tissue levels of heavy metals such as mercury.

In conclusion, this study examined an oxidative stress marker 3-NT, related to protein modification and two potential modulators of oxidative stress, Hg and Se, in control and autistic brains. Our data indicate an increase in 3-NT levels in the autistic cerebellum, along with a positive correlation between elevated cerebellar 3-NT and high Hg levels, a trend toward decreased Se levels and a reduction in the molar ratio of Se/Hg. Caution should be applied to over interpreting these data due to the rather small sample size and heterogeneity within both control and autistic populations. The preliminary data suggest a need for more extensive studies of oxidative stress, its relationship to the environmental factors and its possible attenuation by antioxidants in autism.

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