

MT-3 AND METAL CONCENTRATIONS IN MOUSE BRAIN TISSUE THAT EXHIBITS ALZHEIMER'S DISEASE

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Summary

The connection between the protein MT-3 and selected metals in brain tissue of mice which exhibit Alzheimer's Disease (AD) symptoms was studied. The data showed that these mice had significantly less aluminum (Al) in the brain tissue than normal mice, but that all the mice had similar levels for the other metals analyzed. This project also found evidence consistent with the presence of MT-3 in all mouse brain tissue.

Introduction

The question addressed in this project was whether there were significant variations in levels of metallothionein-3 (MT-3) and concentrations of the metals Zn, Cu, Al, Ca, Fe, Mg, and Mn present in brain tissue of mice (*Mus musculus*) genetically altered for another study. Transgenic positive mice were defined as mice that exhibited AD. Mice that did not have the AD gene, but which had been derived from embryos presented with the gene, were defined as transgenic negative. Both transgenic genotypes were compared to control mice of the same species.

Previous studies conflict in their analysis of MT-3 in AD mouse brains. One study suggests MT-3 is reduced while another suggests it is increased in AD mouse brains (1, 2). In either case, these studies suggest that MT-3 levels are affected by the presence of AD. The hypothesis that MT-3 plays a role in AD prompted the study reported in this paper.

MT-3 is a protein found specifically in the brains of both mice and humans (3). Research has shown that the congruity between human and mouse MT-3 is 87%, which is the highest for any human and animal comparison (4).

MT-3 belongs to a recently discovered family of proteins known as metallothioneins (MTs). MTs are so named because of their metal-binding properties, which are due to their having 20 cysteines, an unusually large number of cysteines for proteins that have only 61 to 68 amino acids (5). Since cysteines bind to metals, MT-3 levels are related to metal concentrations in the brain (6). Metal-ion homeostasis in the brains of mice and humans is thought to maintain healthy neurotransmissions. Therefore, MT-3 is thought to be the protein that manages this process and helps maintain the central nervous system (7).

MT-3 exhibits a growth-inhibitory factor that is believed to limit the size of neurons in the central nervous system (8). If this growth-inhibitory factor is not present, or if insufficient MT-3 exists in the brain in order for the growth-inhibitory factor to function properly, a neuron could grow beyond its usual size limit, overwork itself, and become dysfunctional (9). This may be a cause of AD.

The hypotheses of this project were first, that transgenic positive mice would have significantly less MT-3 in their brains than the transgenic negative mice. Second, because studies have shown that MT-3 levels show a direct positive correlation to metal levels in mice brains, the concentrations of those metals would be significantly less in the brains of transgenic positive

mice than in the brains of transgenic negative mice (10). Previous studies have suggested that Zn and Cu are the only metals that affect AD, but this project examined Al, Ca, Fe, Mg, and Mn as well as Zn and Cu (11).

Procedure

Step I: Tissue Sample Preparation

Brain tissue samples of genetically engineered mice were obtained from a previous study completed on March 26, 2002 in the Karen Hsaio laboratory. These samples were thawed and weighed. Then, 3 mL of dH₂O were added. The samples were homogenized for 30 seconds and put through a 0.45 micron filter in a syringe. The filtrate was centrifuged with a 30 kiloDalton + filter at 3,000 rpms for four hours and frozen at -80°C for use in gel electrophoresis, Western blotting, and atomic absorption as outlined below.

Step II: Gel Electrophoresis

A 15% acrylamide by volume resolving gel solution was prepared by mixing the contents shown in Table 1. The resolving gel solution was placed in between two glass plates, which had been cleaned with 70% ethanol, and allowed to polymerize.

Table 1: Resolving Gel Solution Contents

<u>Substance</u>	<u>Volume (mL)</u>
30% acrylamide	7.5
dH ₂ O	3.4
1.5 M Tris (pH 8.8)	3.8
10% sodium dodecyl sulfate	0.15
10% ammonium persulfate	0.15
tetramethylethylenediamine (TEMED)	0.006

Then, a 5% acrylamide by volume stacking gel solution was prepared with contents as shown in Table 2. The stacking gel solution was placed onto the resolving gel. A comb was placed into the stacking gel solution to form wells, and the solution was allowed to polymerize. Tissue samples, which had been prepared in Step I, were boiled in 100°C water and spun in a microfuge at 13,000 rpms for one minute to denature the protein. Then, from 15 to 30 µL of the tissue sample were placed into each of the wells and electrophoresed for one hour at 130 v. The existence of a specific protein was determined by staining the finished resolving gel in coomassie brilliant blue overnight and then destaining in 35% methanol and 10% acetic acid the next morning. The relative concentration of protein was determined by measuring the thickness of the band.

Table 2: Stacking Gel Solution Contents

<u>Substance</u>	<u>Volume (mL)</u>
30% acrylamide	0.67
dH ₂ O	2.7
1.5 M Tris (pH 8.8)	0.5
10% sodium dodecyl sulfate	0.04
10% ammonium persulfate	0.04
tetramethylethylenediamine (TEMED)	0.004

Step III: Western Blot

To prepare Western blots, a completed resolving gel, which had been prepared as described in Step II, was placed under a membrane slip with filter paper around the gel and membrane. This underwent electrotransfer overnight at 30 v. The membrane slip was then soaked in a 5% powdered-milk blocking solution, primary antibody, secondary antibody, and 25x color development for one hour each and washed three times with 0.05% Tween-20 TBS for five minutes between each soaking.

Step IV: Atomic Absorption Spectroscopy and Inductively Coupled Plasma (ICP)

Tissue samples, which had been prepared as described in Step I, were placed in 1 mL of 1% by volume nitric acid solution and analyzed by atomic absorption using a Varian SpectraAA-100 for levels of Zn and Cu. Samples were submitted to the University of Minnesota Research Analysis Laboratory for ICP analysis of Zn, Cu, Al, Ca, Fe, Mg, and Mn after being homogenized in metal free dH₂O.

Step V: Statistical Analysis

Data for metal concentrations from the transgenic positive and transgenic negative mice were compared to control mice to find p-values. The null hypothesis was that the transgenic types would have the same concentrations as the control type. The p-values were calculated using a two-sample test with a significance level of 0.05.

Results

Analysis of MT-3 in Figure 1 shows the electrophoresed gel of mouse brain tissue of one of the control mice seen in the lower column with molecular weight markers in the upper column. The thick band labeled MT-3 in the lower column of the gel is at 7000 Daltans, which corresponds to the molecular weight for MT-3. The thickness of the band shows a relative representation of MT-3 concentration. Gel electrophoresis and Western blots for transgenic positive and transgenic negative mice brain tissue showed identical results.

Figure 1: Control Mouse Brain Tissue and Molecular Weight Marker Gel
Molecular Weight Markers Control Mouse Brain Tissue

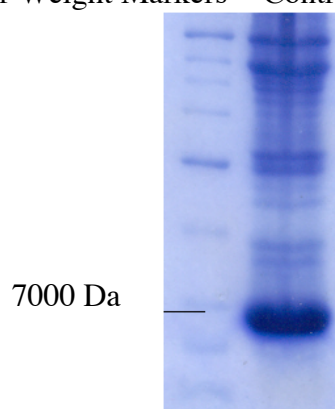


Table 3 shows average metal concentration in brain tissue of five transgenic positive mice, five transgenic negative mice, and two control mice. Metal concentrations are given in $\mu\text{g/gm}$ wet

weight tissue. The statistical analysis adjusted raw data to the wet weight of tissue. The average value is the mean numerical value of metal concentrations for the identified n-value.

Table 3: Metal concentrations in $\mu\text{g}/\text{gm}$ wet weight

Metal	Control (n=2) <u>Average</u>	Transgenic Negative (n=5) <u>Average</u>	Transgenic Positive (n=5) <u>Average</u>
Al	0.69	0.09	0.14
Ca	8.5	8.1	8.4
Cu	1.3	1.2	1.1
Fe	5.1	6.1	5.4
Mg	23.8	23.9	23.3
Mn	0.12	0.07	0.08
Zn	2.8	3.0	2.9

Table 4 shows p-values for metal concentrations in transgenic mice compared to the control mice based on data from Table 3. Aluminum was the only metal to show a significant difference within the transgenic genotypes compared to the control type ($p = 0.0055$ and $p = 0.0185$ for transgenic positive and negative, respectively). No other metal showed significant differences to the controls nor were there significant differences for any metal when comparing transgenic positive to transgenic negative mice, including aluminum.

Table 4: P-Values of Transgenic Types versus Control Type

<u>Metal</u>	<u>Positive</u>	<u>Negative</u>
Al	0.0055	0.0185
Ca	0.4730	0.3889
Cu	0.3692	0.4495
Fe	0.5974	0.7623
Mg	0.4730	0.5060
Mn	0.0797	0.0817
Zn	0.5519	0.5791

Conclusion

The results of this project showed that MT-3 exists in mouse brains. However, neither the gels nor the Western blots were quantifiable, so no conclusion could be drawn about the relationship between MT-3 levels and AD. The gels do strongly suggest the presence of MT-3 in transgenic positive, transgenic negative, and control mice.

Atomic absorption spectroscopy and ICP showed no significant differences in levels of Zn and Cu between the two transgenic genotypes. However, levels of Al in the transgenic positive and transgenic negative types were significant ($p < 0.05$) when compared to control mice ($p = 0.0055$ and $p = 0.0185$). It can be concluded that although recent studies indicated Zn and/or Cu as the metal(s) that affect AD, Al is likely responsible. The data suggest that mice, which were genetically altered to atavistically inherit AD even if they did not exhibit properties of AD, have

reduced Al concentrations compared to those of control mice, which were not genetically altered. Transgenic positive mice did exhibit AD and had reduced Al concentrations. Transgenic negative mice did not exhibit AD, although the genetic strain was present, but they still had reduced Al concentrations. Whether this reduction of Al is an anomaly, a cause, or a symptom of AD cannot be determined, because the transgenic mice were born with AD, and all experiments were performed posthumously.

The first hypothesis of this project, which was that transgenic positive mouse brain tissue samples would have significantly less MT-3 than that of transgenic negative samples, could not be verified, because there were no quantifiable data obtained.

The second hypothesis, which was that transgenic positive mouse brain tissue samples would have significantly less concentrated levels of Zn and Cu than those of transgenic negative samples, is inaccurate. The data showed that there was, in fact, no significant difference between the two transgenic genotypes for any metal, but when compared to control mice, Al concentrations proved significantly less.

The next step in this project would be to quantify MT-3 in order to more firmly establish a relationship between MT-3 and AD. A thorough investigation of the significance and source of difference in Al in the environment would also be very informative. Another avenue of interest that would benefit this project would be to obtain data concerning the offspring of transgenic negative mice: to quantify MT-3 and Al in brain tissue in order to determine if it is the process of genetic manipulation that is responsible for the changed levels in transgenic negative mice brain tissue.

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