STEREOLOGICAL ASSESSMENT OF THE THALAMUS IN A RAT MODEL OF PERIVENTRICULAR NODULAR HETEROTOPIA

An Undergraduate Honors Project Presented

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To

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Abstract

Malformations of neocortical development such as microgyria (MG) and periventricular nodular heterotopia (PNH) have been observed in the brains of language learning impaired (LLI) humans. Rats with MG have shown rapid auditory processing (RAP) deficits similar to acoustic deficits observed in some human LLI populations. Threlkeld et al., (2009) previously reported RAP and other learning impairments in rats with PNH resulting from disruption to embryonic neuronal cell division by way of Methylazoxymethanol (MAM) treatment on embryonic day 15 (E15). The thalamus and its subnuclei may be vulnerable to neurodevelopmental disruptions. Studies of MG rats have shown changes in cell size within the auditory thalamus, medial geniculate nucleus (MGN). Similar findings have also been observed in human dyslexic brains, providing further evidence that thalamic disruption may play a major role in behavioral pathology associated with neurodevelopmental disorders. In this study, a stereological assessment was performed on 11 MAM and 5 control brains of rats from a previous behavioral study showing RAP deficits in MAM treated subjects, specifically looking for effects of treatment on nuclear volume, and estimated cell number, area, and volume within the dorsal lateral geniculate nucleus (dLGN) and the MGN. Results showed no significant treatment effects on the MGN in any measure, although a trend was seen in nuclear volume. Significant treatment effects were seen, however, in the dLGN in all measures except for a trend found in cell area. This study further supports E15 MAM treatment in rats as a model for teratogen mediated pervasive developmental disorders. Future studies are suggested to examine the effects of E15 MAM exposure on dLGN dependent behaviors in rats and explore the relationship between pathological severity and general sensory processing deficits.

Keywords: Methylazoxymethanol, periventricular nodular heterotopia

Stereological Assessment of the Thalamus in a Rat Model of Periventricular Nodular Heterotopia

Developmental abnormalities within the brain, specifically the neocortex, such as microgyria (MG) and periventricular nodular heterotopia (PNH) have been linked to language learning conditions in humans, including reading impairment and specific language impairment (Chang et al., 2005; Galaburda, Sherman, Rosen, Aboitiz, & Geschwind, 1985). MG is represented by focal abnormal layering and folding of the cerebral cortex (Dvofák & Feit, 1977). PNH is characterized by clusters of abnormally displaced neurons. In humans, both MG and PNH are the result of disruptions in the migratory process of neurons beginning as early as the second month of embryonic nervous system development (National Institute of Neurological Disorders and Stroke, 2007). Gestation in a rat is approximately 21 days. Disruption to the developing rat neocortex from embryonic day 12 to the fifth postnatal day produces similar malformations to those observed in the brains of learning-impaired humans (Chang et al., 2005; Galaburda et al., 1985; Paredes, Pleasure, & Baraban, 2006; Threlkeld, McClure, Rosen, & Fitch, 2006). During normal embryonic brain development progenitor cells give rise through asymmetrical division to neurons which then migrate to their respective final resting places from the ventricular zone towards the pia mater along radial glial cells (Hatten, 1999). Disruption caused by focal lesion to the cortex or teratogen exposure at any point during this process can lead to brain malformations (Herman, Galaburda, Fitch, Carter, & Rosen 1997; Threlkeld et al., 2009).

Galaburda, Menard, and Rosen (1994) found that five dyslexic brains assessed post mortem exhibited MG and other developmental malformations in the left cerebral cortex. The observed cortical abnormalities were associated with cell size differences within the medial

geniculate nucleus (MGN) of the thalamus, compared to control brains. They also found neurons located within the magnocellular layer of the lateral geniculate nucleus to be an average of 27% smaller when compared to controls. Their findings reflected the notion that language-processing deficits present in dyslexics may accompany abnormalities found in different levels of the auditory sensory processing and integration system, as well as other sensory systems.

In a later study, Herman, Galaburda, Fitch, Carter, and Rosen (1997) speculated that early damage to the cortex could consequently detrimentally affect other areas of the brain involved in auditory processing such as the MGN. In this study, MG was induced through a focal freezing lesion, an effective, known model to induce neuronal migration disorders. Using this model, the researchers discovered that in male MG rats, there existed cell size differences within the MGN compared to sham counterparts. They further found that MG males had significant deficits in auditory temporal processing as compared to controls. Peiffer, Rosen, and Fitch (2002) also confirmed similar findings in number and size discrepancies of cells within the MGN having employed an MG rat model. Additionally, Rosen, Mesples, Hendriks, and Galaburda (2006) produced congruent findings within the MGN.

Another well accepted method for inducing neuronal migration disorders (NMD) and other anomalies within a rat model is through the use of the antimitotic teratogen Methyl-azoxymethanol (MAM) given to adult dams (pregnant rats) on embryonic day 15 (E15), which corresponds to the peak of neurogenesis within the neocortex of the developing rodent brain (Colacitti et al., 1998; Gourevitch, Rocher, Le Pen, Krebs, & Jay, 2004; Paredes, Pleasure, & Baraban, 2006; Sancini et al., 1998). A teratogen is any substance that has the capability of interfering with fetal development. Threlkeld et al., (2009) also used an (E15) MAM rat model

showing PNH, hippocampal heterotopia, and cortical dysplasia in the treatment subjects. Additionally, they found that rats with PNH had auditory processing deficits similar to those observed in rats with cerebral cortical MG.

The current study sought to determine the effects of such MAM induced cortical malformations (found in the brains of treatment subjects from the Threlkeld et al. (2009) study) on over-all regional volumes, as well as cell number and size within both the dorsal lateral geniculate nucleus (dLGN) and MGN of the thalamus compared to sham counterparts. Furthermore, this study set out to investigate additional pathology underlying NMD that may be critical for understanding human developmental disabilities.

Method

Subjects and Treatment

All surgical procedures from Threlkeld et al., (2009) were conducted at the University of Connecticut. Purchased-time-mated Wistar dams (Charles River, Wilmington, MA) received a single intraperitoneal injection of 25 mg/kg MAM (Midwest Research Institute, USA) diluted to 1 mL in .9% NaCl, on E15. Subjects were injected under light isoflurane anesthesia. Control dams received a single saline injection (also under light isoflurane). At birth, subjects were culled into litters of 10 (eight males and two females), to control for litter size and sex ratio effects. At P21, subjects were right or left ear marked and housed into like-treated pairs. At P60, animals were single housed prior to adult behavioral testing. All subjects were maintained on a 12:12 light/dark cycle with food and water available *ad libitum*. After weaning, a total 59 male rats (MAM $N = 36$, control $N = 23$) were utilized for behavioral testing. Subjects' prior

behavioral experience was uniform across all groups. All procedures were conducted in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, including adequate measures to minimize pain and discomfort. The Institutional Animal Care and Use committee (IACUC) at the University of Connecticut approved all procedures.

Histology

All tissue extraction procedures from Threlkeld et al., (2009) were completed at the University of Connecticut. On postnatal day 100, subjects were weighed, anesthetized with ketamine/xylazine (100/15 mg/kg), and transcardially perfused with saline followed by 10% phosphate buffered formalin. Brains were extracted, placed in formalin, and shipped to Glenn D. Rosen at Beth Israel Deaconess Medical Center for anatomical processing. Brains were embedded in celloidin, and serially sectioned in the coronal plane at 30 μm. A series of every tenth section was stained with cresyl violet for Nissl substance. A screener identified the distribution and relative severity of the malformations without knowledge of treatment group or litter of origin. The most common anatomical anomalies consisted of disrupted cortical lamination, hippocampal dysplasia (see *Figure 1*-A), periventricular nodular heterotopia (PNH) (see *Figure 1*-B), and hippocampal heterotopia.

Stereology

All stereological assessment in cell size and cell number counts were performed within the present study using Stereo Investigator (MBF Bioscience, Williston, VT, USA) and a zeiss axio-imager M1 microscope at Women and Infants Hospital of Rhode Island and the Warren Alpert

Medical School of Brown University. The borders of the thalamic nuclei of the subject were determined using a standard stereotaxic atlas as a guide (Paxinos & Watson, 1986). Each subject's nuclei were then traced according to those determinations by using the software (see *Figure 2*). Images of each section's tracings were then exported for volume measurement. Volumes of the thalamic nuclei were then determined through point counting using Cavalieri's estimator of volume and ImageJ software at Rhode Island College (see *Figure 3*). The optical fractionator workflow tool within Stereo Investigator was then used to place random counting frames within each tracing. Only the cells visible within the counting frame's active borders and throughout the depth of the tissue within that area were marked and subsequently counted (see *Figure 4*-A and **Table 1**). The program would then extrapolate an overall estimated cell number within each nucleus. After selecting each individual cell within a frame, the nucleator probe tool within the program would then randomly place four arrays extending from the initial cell marker. The borders of each cell were then marked at each of the four intersections with the arrays, allowing the program to estimate cell size and volume (see *Figure 4*-B). Section thickness was measured every section, and this value was used in all computations. The MGN measurements included the ventral, dorsal, and medial subnuclei. Those performing microscopy were unaware of subject treatment at all times.

Results

All statistical analyses were performed using PASW software along with the extrapolated data from the Stereo Investigator program. A multivariate analysis of variance (MANOVA) comparing Treatment, MAM (n = 11) and control (n = 5), for MGN and dLGN volumes revealed a significant effect of treatment for dLGN, $F(1,14) = 28.3$, $p < .001$, and near significance for

MGN, $F(1,14) = 4.48$, $p = .053$(see *Figure 5*). MANOVA also showed a significant main effect of treatment on estimated cell number within the dLGN, $F(1,14) = 13.7$, $p = .002$, indicating significantly fewer cells in dLGN of MAM treated subjects. No effect was seen between the groups in the MGN for cell number, $F(1,14) = .036$, $p = .852$ (see *Figure 6*). Comparing treatment for MGN and the dLGN average cell area, MANOVA revealed no effect of treatment in the MGN, $F(1,14) = 1.21$, $p = .29$, however a trend was seen in the dLGN, $F(1,14) = 4.05$, $p = .064$, which parallels the significant reduction seen in cell number and overall volume of the nucleus (see *Figure 7*). Finally, a MANOVA comparing Treatment against MGN and dLGN average cell volume showed a significant effect of treatment for dLGN, $F(1,14) = 5.83$, $p = .030$, indicating significantly smaller cell volume found within the dLGN of MAM subjects as compared to controls. No effect of treatment was seen in the MGN, $F(1,14) = 1.50$, $p = .24$ (see *Figure 8*). Assumptions of MANOVA were evaluated and found to be adequate.

Discussion

In this study, embryonic day E15 MAM treatment did not result in any significant treatment effects on any MGN measure. However, there was a near significant effect of MAM treatment on MGN regional volumes as compared to controls. These results do not reflect the findings of cell size differences within the MGN as the previous literature presented. One reason for this might be that the parameters used for counting grids and frame sizes may have been too confined. To explain, it is possible that because of the size of the larger cells found within the MGN of controls and because of the protocol defined by the program for cell counting within the frames, the probability of the larger cells making contact with the red borders of the counting frame may have been too high. This may have allowed for some of the large cells in the MGN to be omitted from the data. Future stereological assessment could include adjustment of these

parameters so as to increase statistical power. Moreover, it is important to note that additional stereology involving more subjects may reveal subtle differences in the MGN not found in this assessment. Furthermore, in that the auditory system is not limited to the MGN, but rather is comprised of many neural bodies such as the superior olivary complex, the lateral leminiscus, the inferior colliculi, and the primary auditory cortex, it is possible that the auditory deficits found in these subjects during previous behavioral testing, may be the result of insult to one or more of those other bodies within the auditory system (Baldeweg, Richardson, Watkins, Foale, & Gruzelier, 1999; Threlkeld, Penley, Rosen, & Fitch, 2008).

Interestingly, this study showed significant reduction in dLGN regional volumes, cell number, and cell volume, as well as a trend toward reduced cell area within the dLGN of MAM treated subjects when compared to controls. These novel findings parallel data presented by Ashwell (1987), promoting an alternate analytical approach, used Methalozoxymethanol Acetate treatment on E15 rat dams, revealing an 87% defecit in neuronal number within the dLGN of 5 week post-exposed animals. Similar findings were shown in a E14 hamster model of MAM employed by Woo, Niederer, and Finlay (1996), showing that accompanying severe depletion of layers IV and V of the cortex, there was detectable cell loss within the dLGN respective to severity of depletion.

Given that the dLGN is the visual system subnucleus of the thalamus, this study's findings suggest that previously observed auditory deficits in E15 MAM treated subjects may also be accompanied by visual processing deficits. The present results may also reflect the fact that gross malformations such as PNH, hippocampal heterotopia, and cortical dysplasia were more proximally located to the dLGN than the MGN in MAM subjects, thereby having a greater effect on that region. Regardless, this study further supports E15 MAM treatment in rats as a model for

teratogen mediated pervasive developmental disorders. Future studies are suggested in examining the effects of E15 MAM exposure on dLGN dependent behaviors in rats, and in exploring the relationship between pathological severity and general sensory processing deficits.

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Table 1

Stereological Probe Parameters

Thalamic Nuclei	Optical Fractionator and Nucleator			Point Counting, Sampling Frequency (*µm*)	Section Periodicity
	Counting Frame (*µm*)	Disector Height	Sampling Frequency (*µm*)		
MGN	25 x 25	20	200 x 200	200 x 200	Every 10th
dLGN	25 x 25	20	200 x 200	200 x 200	Every 10th

Note. Parameters were determined based on the number of sections per subject, considering similar parameters used in previous studies.

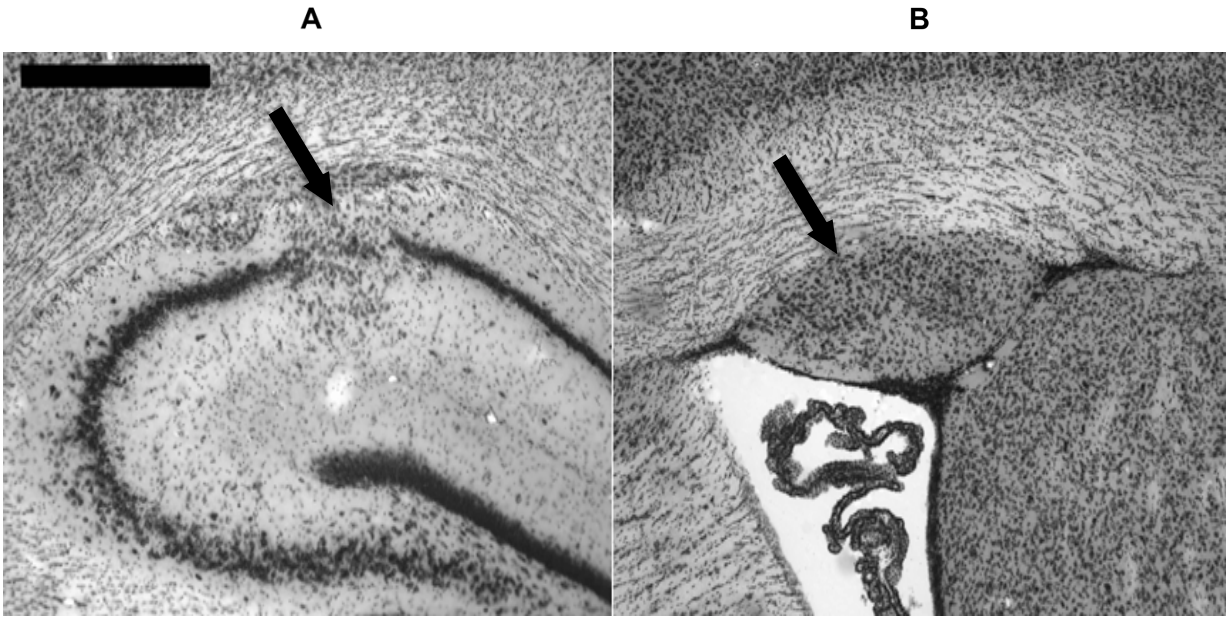

Figure 1. **Examples of Anatomical Anomalies in MAM Subject**. Photomicrographs show (A) hippocampal dysplasia and (B) periventricular nodular heterotopia (PNH) in E15MAM treated subjects.

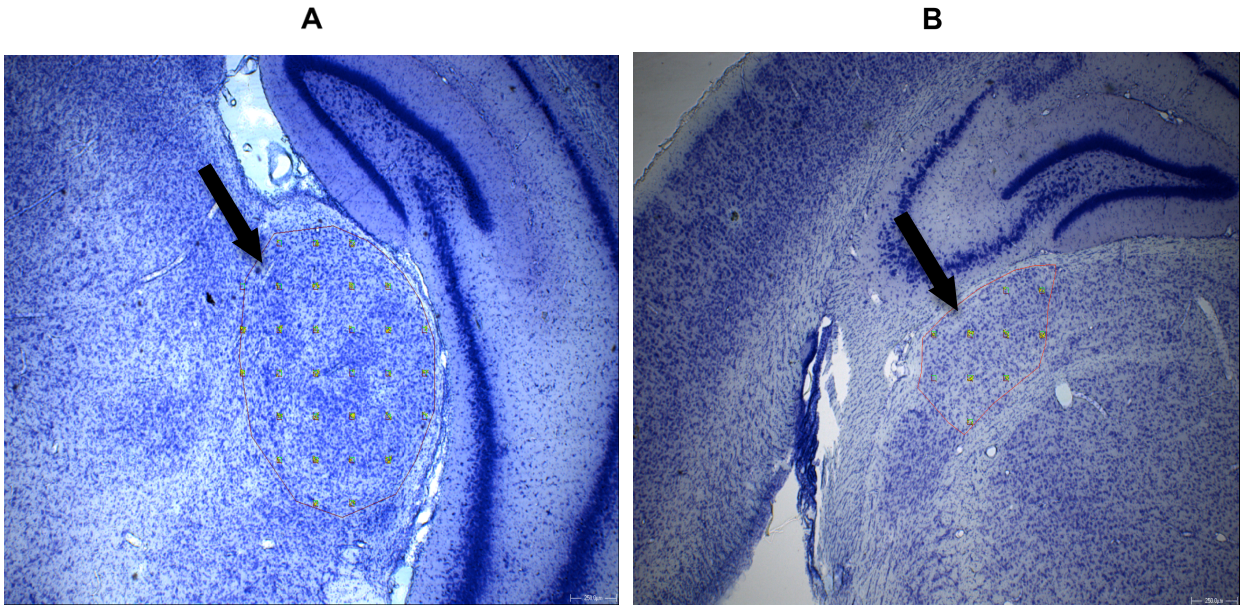

Figure 2. **Tracings and Counting Grid**. Photomicrographs show randomly placed counting frames at 2.5x magnification within (A) left hemisphere MGN and (B) right hemisphere dLGN coronal rat brain section tracings.

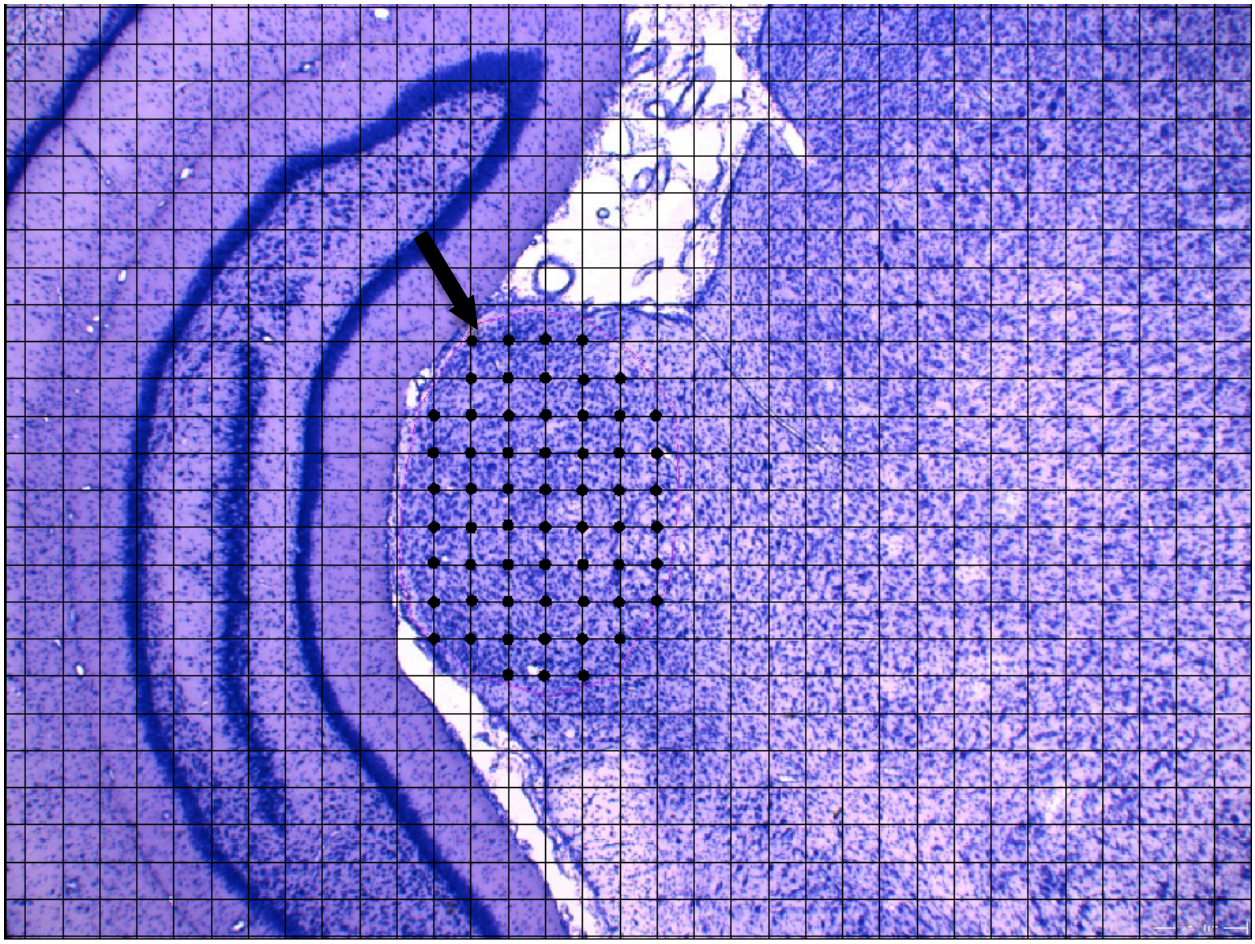

Figure 3. **ImageJ Point Counting for Cavalieri's Estimator of Volume.** Photomicrograph shows randomly placed grid on tracing of MGN of E15MAM subject. Dots were placed wherever lines from the grid intersected within the borders of the tracing. The dots were then counted, the sum of which was used to determine volume using Cavalieri's Estimator.

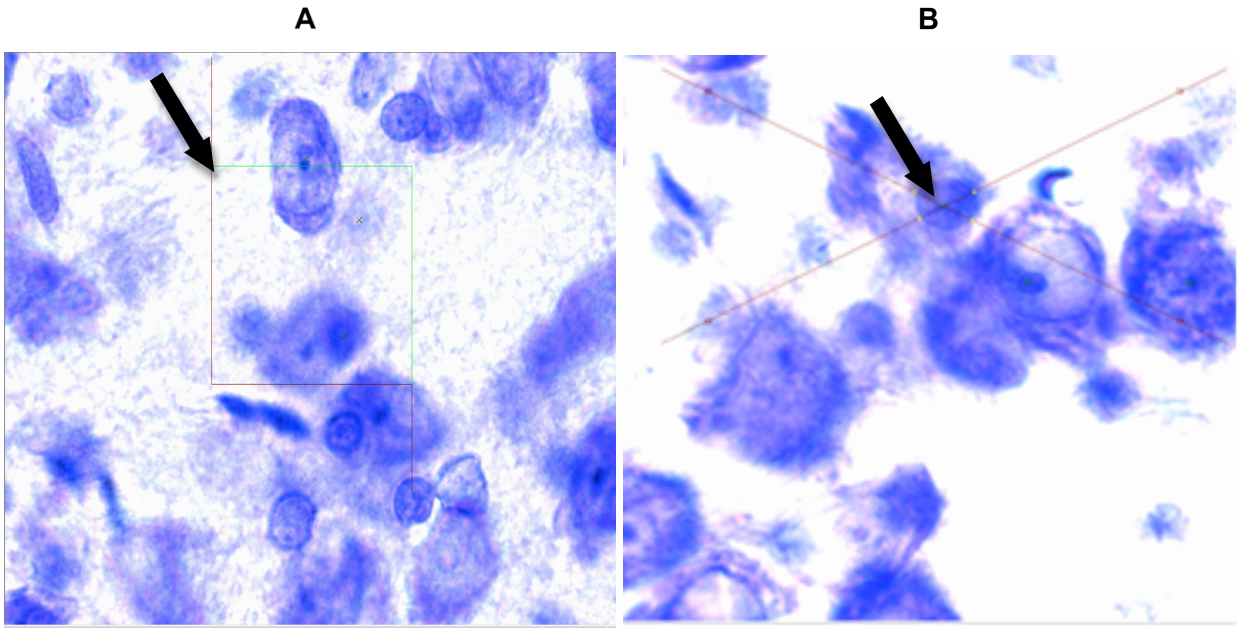

Figure 4. **Counting Grid and Nucleator Probe**. Photomicrographs show (A) counting grid frame and cell markers at 100x magnification. Cells were counted if they were either completely within the counting frame or at least touching the green borders of the frame. However, cells were not counted if they were at all making contact with either red border of the frame. (B) Nucleator arrays at 100x magnification extending from the marker that were placed on the visible nucleolus of the cell. The four yellow markers indicate the intersection points of the arrays with the cell border. Both volume and area of cells were determined using these measures.

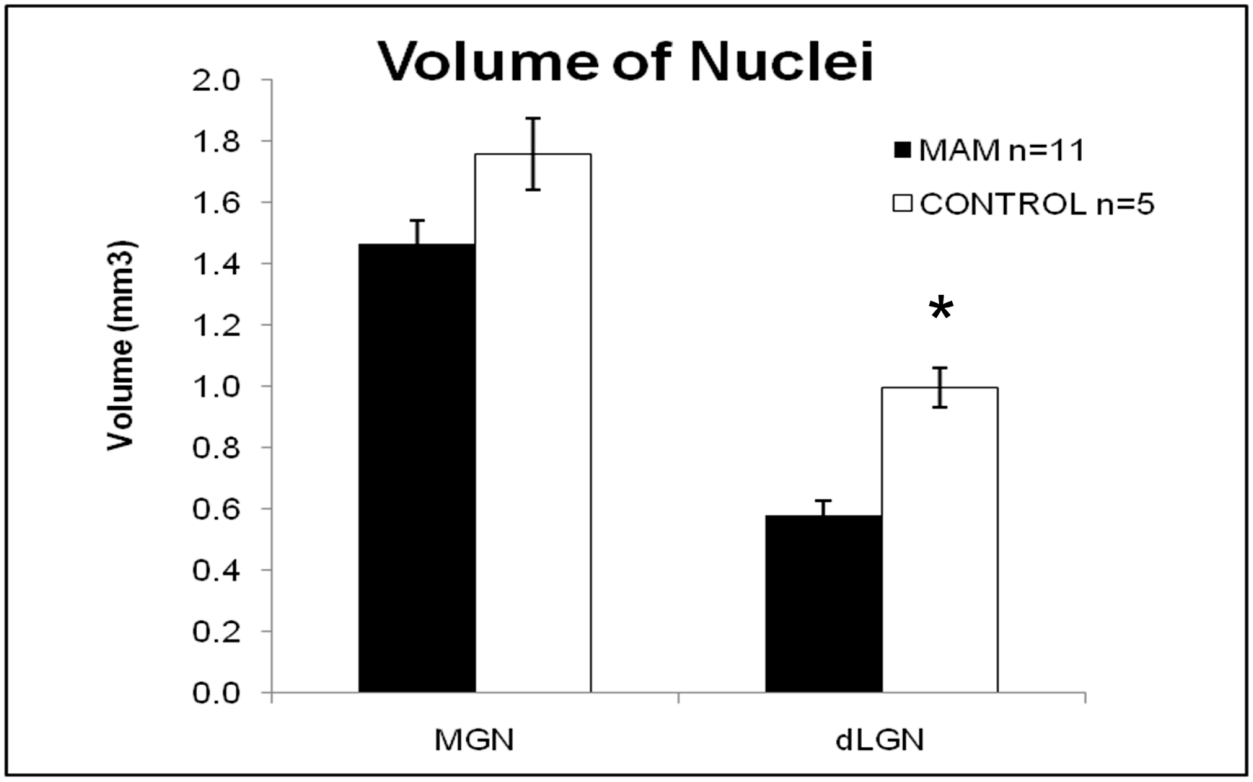

Figure 5. **Bar Graph Showing Overall Nucleic Volume**. Graph shows multivariate ANOVA comparing Treatment (control and MAM) for MGN and dLGN volumes. Results revealed a significant effect of treatment for dLGN, $F(1,14) = 28.3$, $p < .001$. Comparison between MAM and control subjects for MGN volume shows a near significant effect, $F(1,14) = 4.48$, $p = .053$.

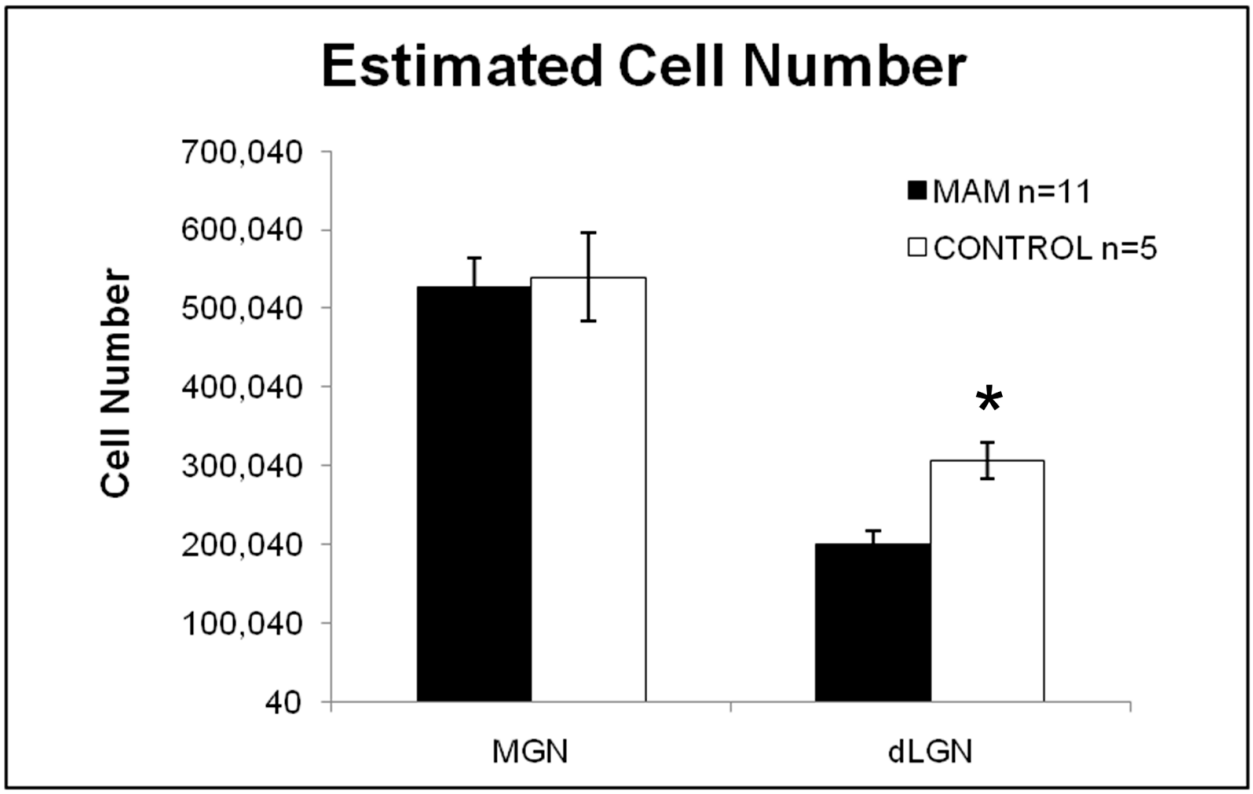

Figure 6. **Bar Graph Showing Estimated Cell Number**. Graph shows multivariate ANOVA comparing Treatment (control and MAM) for MGN and dLGN estimated cell number. Results revealed a significant effect of treatment for dLGN, $F(1,14)=13.7$, $p = .002$, indicating significantly fewer cells in dLGN of MAM treated subjects. No effect was seen between the groups in the MGN for cell number, $F(1,14) = .036$, $p = .852$.

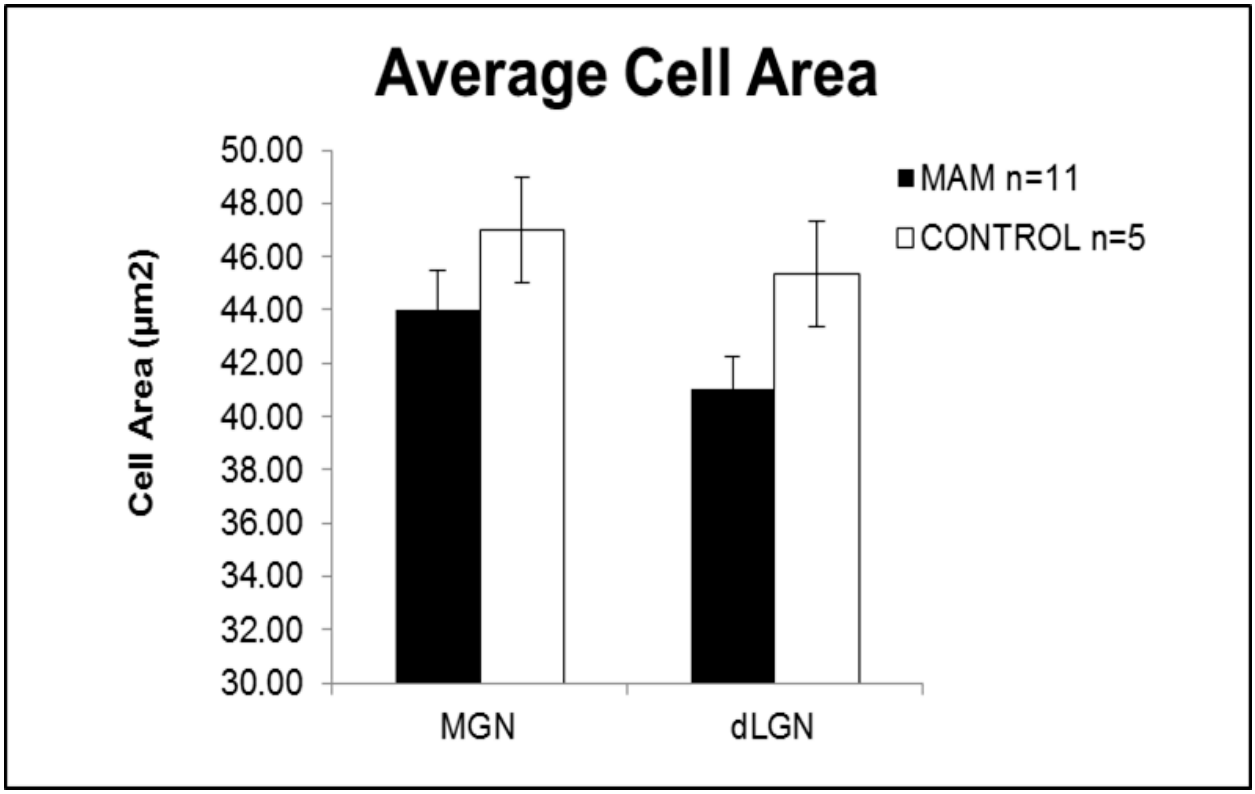

Figure 7. **Bar Graph Showing Average Cell Area**. Graph shows multivariate ANOVA comparing Treatment (control and MAM) for MGN and dLGN average cell area. No effect of treatment was seen in the MGN, $F(1,14) = 1.21$, $p = .29$, however a trend was seen in the dLGN, $F(1,14) = 4.05$, $p = .064$, which parallels the significant reduction seen in cell number and overall volume of the nucleus.

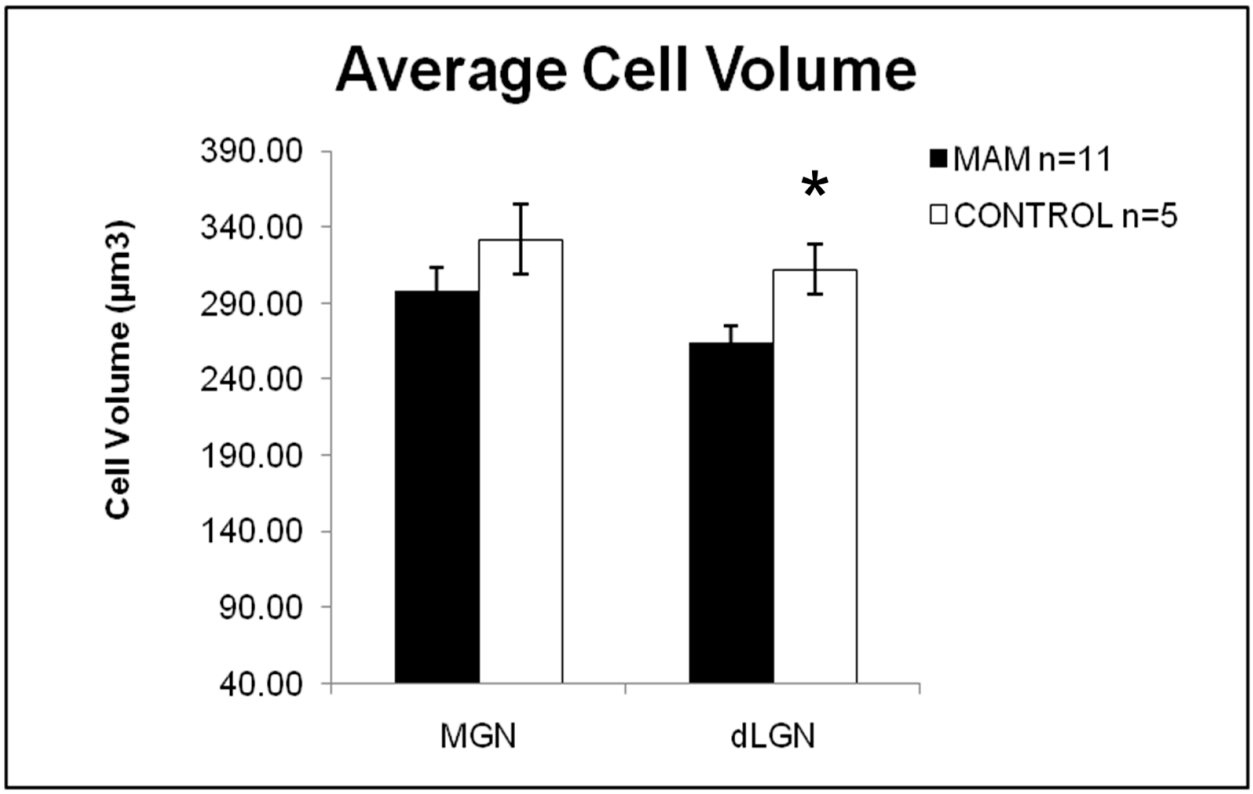

Figure 8. **Bar Graph Showing Average Cell Volume.** Graph shows multivariate ANOVA comparing Treatment (control and MAM) for MGN and dLGN average cell volume. Results revealed a significant effect of treatment for dLGN, $F(1,14) = 5.83$, $p < .030$, indicating significantly greater cell volume found within the dLGN of control subjects as compared to MAM subjects. No effect of treatment was seen in the MGN, $F(1,14) = 1.50$, $p = .24$.