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Effects of Early Experience and Plasticity on Neuronal Morphology Within the Prefrontal Cortex in a Rodent Model of Hypoxia-Ischemia

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EFFECTS OF EARLY EXPERIENCE AND PLASTICITY ON NEURONAL MORPHOLOGY WITHIN THE PREFRONTAL CORTEX IN A RODENT MODEL OF HYPOXIA-ISCHEMIA

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A Thesis Summited in Partial Fulfillment of the Requirements for Departmental Honors in Biology for the Bachelors in Science in the Department of Biology

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Abstract

Hypoxia-ischemia (HI) is low oxygenation to the brain paired with low blood supply that can disrupt normal patterns of brain development. HI injury is characterized by many long-term cognitive and behavioral deficits including working memory. Neuronal plasticity due to early sensory or learning experience has been suggested to facilitate recovery of function after neonatal brain injury. Plasticity is the ability for the nervous system, more specifically neurons, and their synapses to modify their function and morphology due to experiences, which in turn correlate with changes in behavior. The objective of the present study was to investigate the effects of neonatal hypoxia-ischemia on the morphology of layer five pyramidal neurons within the prefrontal cortex (Cg3) of rats with or without early life working memory experience (postnatal day 36-61). We hypothesized that both HI and sham subjects exposed to 20 days of working memory training, using an 8-arm radial water maze, early in life would show distinct morphological changes in Cg3 pyramidal neurons. Findings suggest that early life working memory training regulates shifts in neuronal morphology following neonatal brain injury.

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Introduction

Hypoxia-ischemia (HI) is low oxygenation to the brain paired with low blood supply that can disrupt normal patterns of brain development. HI most commonly results from umbilical cord occlusion, placental disruptions, or prolonged labor; this injury is a major cause of infant mortality in preterm and very low weight infants (Vannucci, 2004). HI injury is characterized by many long-term cognitive and behavioral deficits including working memory (Volpe, 2001). Rodent models are commonly used for the investigation of the effects of HI and can help with the understanding of memory impairments resulting from this injury (Delcour *et al.*, 2012). Working memory is crucial for retaining information that is only needed for a short period of time. For example remembering which arm of a radial maze contained food in the case of rodents (Hyde, 1998). A process known as plasticity may facilitate the recovery of function after such injury. Plasticity is the ability for the nervous system, more specifically neurons, and their synapses to modify their function and morphology due to experiences, which in turn correlate with changes in behavior (Kolb, 2003).

Exposure to environmentally enriching experiences (8-arm radial water maze) is a commonly used approach in the attempt for remediation of brain injury by promoting synaptic plasticity in rodent models (Nithianantharajah & Hannan, 2006). Daily exposure to environmental enrichment has been shown to improve working memory (Pereira *et al.*, 2008; Rojas *et al.*, 2013; Maiti et al, 2008). Introducing environmentally enriching experiences at an early age has been found to promote plasticity in the developing brain (Als, 2004). Als and associates (2004) evaluated the effects of early experience on brain morphology and function in

preterm infants, which suggested that early experience improves brain development (Als, 2004). HI subjects with standard living conditions exhibited significant reduction in dendritic spine density in the right hippocampus (ipsilateral to the HI insult) compared to the controls; however, this difference was not seen in HI animals exposed to environmental enrichment (Rojas *et al.*, 2013). Therefore, the conjunction of environmentally enriching experiences presented by the 8-arm radial water maze and the exposure of the experience early in life could have the potential to be a powerful approach in the attempt for the remediation of brain injury caused by HI.

The Prefrontal cortex is of great interest due to its executive functions, along with learning, decision-making, working memory, and reorganization in response to injury (Comeau, 2010). Central features of the prefrontal cortex also include its behavioral flexibility ranging from strategy switching, attentional set shifting, and inhibition of pre-potent responses as well as resolving conflicting response tendencies, thus suggesting a general role of the prefrontal cortex in resolving interferences (Peters, 2013). In a study by Peters and associates (2013), inactivation of the prefrontal cortex of rats was performed, After this procedure rats were tested in an olfactory discrimination-learning task. Results suggest that the prefrontal cortex plays an important role in promoting the long-term retrieval of memories (Peters, 2013). By maintaining a working memory buffer, neurons within the prefrontal cortex may contribute to learning associations (Gilmartin, 2013). In a study by Gilmartin and associates (2013) memory formation was inhibited by silencing neurons within the prefrontal cortex. Results from this study suggest that the prefrontal cortex has a working memory component and that associations may require activity in this area (Gilmartin, 2013).

Few studies have looked into learning induced changes in morphology of pyramidal cells within the Cg3 layer V area of the prefrontal cortex (Comeau, 2010). The prefrontal cortex has

not been studied in detail in relation to 8-arm radial water maze experience, which has been widely used for working memory assessment (Hyde, 1998). Considering the role of the prefrontal cortex in working memory, specifically looking at the Cg3 layer V region of the prefrontal cortex, it is hypothesized that significant differences in dendritic length and complexity will be present. These differences are expected due to early enriching experiences induced by the 8-arm radial water maze.

Methods

Subjects

Forty male Wistar rats (Charles River Laboratories, Wilmington, MA) previously assessed on an 8-arm radial water maze working memory task (as described by Penley *et al.*, in press) were the source of the tissue utilized in the present study. In this study histological assessment of brain sections from Wistar rats having four different treatments was performed. The treatment groups were hypoxia-ischemia induced injury to the right hemisphere of the brain of subjects that were either exposed to early experience (8-arm radial water maze) or deprived of early experience (HI+ experience, N= 13, HI-experience, N=12). Sham operated subjects that were either exposed to early experience or deprived of early experience (Sham+ experience, N=8, Sham-experience, N=7).

Surgeries

On postnatal day seven (P7) surgeries were performed in the Fogarty Life Sciences Vivarium at Rhode Island College and were as follows. Rats were anesthetized using 4% isoflurane administered through a nose cone. The animals were stabilized on a surgical surface and warmed with an isothermal heating pad (Braintree Scientific, Braintree, MA). Betadine was

used to sanitize the incision area before making a longitudinal midline incision in the neck. The right common carotid (RCC) artery was completely cauterized. Sham subjects followed the same procedure except the RCC cauterization. The incision was sutured and each pup was labeled with a footpad ink injection. Post-surgery, all pups were returned to their mothers for adequate feeding and recovery. HI subjects were placed in a hypoxia chamber with 8% humidified oxygen (balanced in nitrogen) for 120 minutes. Sham subjects were exposed to room air for the 120 minutes. Following the 120 minutes, all pups were returned to their dam. The pups remained with their mother until weaning on P21. On P21, the subjects were paired housed (see Gaudet, 2013).

Early enrichment

Early enrichment induced by an 8-arm radial water maze task was at postnatal day 36 to 61 and 88 to 113 for the experienced group and for the inexperienced group 8-arm radial water maze testing was initiated at postnatal day 88 to 113.

Histology

After enrichment subjects were transcardially perfused as described by Gaudet (2013) and brains collected to later be stained with a Golgi-cox solution (1% potassium dichromate, 1% mercuric chloride, 0.8% potassium chromate dissolved in water), which impregnates nerve cells making their processes stand out, then sectioned with a vibrating microtome (Leica VT1000S, Leica Microsystems, Bannockburn, IL) (see Gibb 1998). All of the procedures mentioned above were performed by the Developmental Behavioral Neuroscience lab under the direction of Dr. Threlkeld and assistance of lab members at Rhode Island College. All of these procedures were

approved by the Rhode Island College Institutional Animal Care and Use Committee (RIC IACUC, protocol F44-2).

Microscopy

After tissue was sectioned and stained an Olympus BX53 light microscope was used in conjunction with Neurolucida 11.0 (MBF, Burlington, VT), a type of three-dimensional neuronal reconstruction software, in order to collect all of the morphological data. This was done by tracing the areas of interest, these areas were all within the prefrontal cortex mainly Cg3 layer V area from most anterior section to about 0.0mm Bregma (Paxinos & Watson, 2009). After tracing these areas at 2x magnification candidate cells were marked with a triangle icon at 10x then further inspected. Some exclusion happened at this point due to cells not meeting the criteria used by Rojas and associates (2013). At 40x magnification a closer look was taken, which determined if the cells were going to be traced or not. Neuron tracing took place at 100x oil magnification. The criteria for choosing the individual pyramidal neurons was as follows: cell bodies located within the Cg3 layer V area of the prefrontal cortex, well impregnated, relatively isolated from other neurons and tapering appearance towards the ending (Rojas 2013). Although this criterion was followed very closely some adjustments were made including that neurons must have at least three dendrites of which at least one must bifurcate and having a well defined apical dendrite. Three neurons per subject were randomly chosen and traced, after all tracings were done (120 tracings) all data was exported to Neurolucida explorer 11.0 (MBF, Burlington, VT), where further analysis was performed consisting of the Sholl analysis, which provided with dendritic length values for both apical and basal dendrites and neuron summary, which provided with complexity, number of nodes, number of dendrites, number of intersections, number of ends and dendritic order values for both apical and basal dendrites.

Statistical analysis

The Sholl analysis is a widely used method to indirectly measure dendritic length in microns (Sholl, 1953). Neurolucida Explorer (MBF, Burlington, VT) was used to create concentric rings centered at the cell body of the neuron, which increase in size at 10μm intervals. The program then calculated the number of intersections with each concentric ring in tridimensional space for both apical and basal dendrites of pyramidal neurons. Neuron summary analysis provided with complexity, number of nodes, number of dendrites, number of intersections, number of ends and dendritic order values for both apical and basal dendrites of pyramidal neurons. This data was then exported to SPSS (Armonk, NY) statistical analysis software in which an analysis of variance (ANOVA) was performed in order to determine if there was a significant difference between the treatments.

Results

Number of basal dendrites (Pyramidal neurons only have one apical dendrite thus only basal number of dendrites was assessed)

A 2 (treatment) x 2 (experience) univariate ANOVA was computed to assess the number of basal dendrite per neuron. A main effect of experience [$F(1,36)=0.914$, $p=0.345$; Figure A] and a main effect of treatment [$F(1,36)=0.667$, $p=0.419$; Figure A] were not significant.

Sholl analysis (dendritic length)

Apical dendrite length

A 20 (ring) x 2 (experience) x 2(treatment) repeated measures ANOVA was computed to assess apical dendrite legnth. A main effect of experience [$F(1,36)=2.002$, $p<0.166$; Figure B] and a main effect of treatment [$F(1,36)=0.000$, $p<0.996$; Figure B] were not significant.

Basal dendrite length

A 20 (ring) x 2 (experience) x 2(treatment) repeated measures ANOVA was computed to assess basal dendrite legnth. A main effect of experience [$F(1,36)=0.769$, $p<0.387$; Figure B] and a main effect of treatement [$F(1,36)=0.808$, $p<0.375$; Figure B] were not significant.

Number of nodes

Apical dendrite nodes

A 2 (treatment) x 2 (experience) univariate ANOVA was computed to assess the number of basal dendrite nodes per neuron. A main effect of experience [$F(1,36)=1.854$, $p=0.182$; Figure C] and a main effect of treatment [$F(1,36)=0.050$, $p=0.825$; Figure C] were not significant.

Basal dendrite nodes

A 2 (treatment) x 2 (experience) univariate ANOVA was computed to assess the number of basal dendrite nodes per neuron. A main effect of experience [$F(1,36)=1.885$, $p=0.178$; Figure C] and a main effect of treatment [$F(1,36)=0.390$, $p=0.536$; Figure C] were not significant.

Number of ends

Apical dendrite ends

A 2 (treatment) x 2 (experience) univariate ANOVA was computed to assess the number of apical dendrite ends per neuron. A main effect of experience [F (1,36)=1.887, p=0.178; Figure D] and a main effect of treatment [F (1,36)=0.037, p=0.848; Figure D] were not significant.

Basal dendrite ends

A 2 (treatment) x 2 (experience) univariate ANOVA was computed to assess the number of basal dendrite ends per neuron. A main effect of experience [F (1,36)=0.285, p=0.597; Figure D] and a main effect of treatment [F (1,36)=0.466, p=0.499; Figure D] were not significant.

Dendrite complexity

Apical dendrite complexity

A 2 (treatment) x 2 (experience) univariate ANOVA was computed to assess apical dendrite complexity per neuron. A main effect of experience [F (1,36)=1.021, p=0.319; Figure E] and a main effect of treatment [F (1,36)= .444, p=0.509; Figure E] were not significant.

Basal dendrite complexity

A 2 (treatment) x 2 (experience) univariate ANOVA was computed to assess basal dendrite complexity per neuron. A main effect of experience [F (1,36)=2.402, p=0.130; Figure E] and a main effect of treatment [F (1,36)=0.010, p=0.921; Figure E] were not significant.

Dendrite order

Apical dendrite order

A 20 (ring) x 2 (experience) x 2(treatment) repeated measures ANOVA was computed to assess apical dendrite order per neuron. A main effect of experience [$F(1,36)=0.383$, $p<0.540$; Figure F] and a main effect of treatment [$F(1,36)=0.344$, $p<0.561$; Figure F] were not significant.

Basal dendrite order

A 20 (ring) x 2 (experience) x 2(treatment) repeated measures ANOVA was computed to assess basal dendritic order per neuron. A main effect of experience [$F(1,35)=0.221$, $p<0.642$; Figure F] and a main effect of treatment [$F(1,35)=0.232$, $p<0.633$; Figure F] were not significant.

Dendritic intersection

Apical dendrite intersection

A 20 (ring) x 2 (experience) x 2(treatment) repeated measures ANOVA was computed to assess apical dendrite intersecion per nueron. A main effect of experience [$F(1,36)=2.951$, $p<0.094$; Figure G] and a main effect of treatment [$F(1,36)=0.078$, $p<0.782$; Figure G] were not significant.

Basal dendrite intersection

A 20 (ring) x 2 (experience) x 2(treatment) repeated measures ANOVA was computed to assess apical dendrite intersection per neuron. A main effect of experience [$F(1,35)=0.353$, $p<0.556$; Figure G] and a main effect of treatment [$F(1,35)=0.089$, $p<0.767$; Figure G].

Discussion

The objective of the present study was to investigate the effects of neonatal hypoxia-ischemia, which is characterized by long-term cognitive and behavioral deficits including working memory, on neuron morphology. Pyramidal neurons within the prefrontal cortex (Cg3) of rats with or without early life working memory experience (postnatal day 36-61) were targeted for analysis. Early life working experiences have been found to promote synaptic plasticity and therefore help with the remediation after brain injury. Early life experience was induced in a previous study, which is the source of the tissue utilized in the present study, by an 8-arm radial water maze task. We hypothesized that both HI and sham subjects exposed to 20 days of working memory training, using an 8-arm radial water maze, early in life would show distinct morphological changes in Cg3 pyramidal neurons.

Statistical analysis revealed no overall significant effects of treatment for neither apical nor basal dendrite variables. Variables are as follow: apical and basal dendritic length measured by the Sholl analysis. Also dendritic order, dendritic intersection, number of nodes, number of dendrites, number of ends and complexity values which were revealed by the neuron summary analysis. For all variables the same trend was seen where in the experienced group (including those subjected to HI and those that were not) had larger values than that of the inexperienced group. This suggests that even though early experience did not significantly affect the morphology of pyramidal neurons within the Cg3 layer V of the prefrontal cortex, early life working memory training could be regulating shifts in neuronal morphology following neonatal brain injury in a more subtle way not detected by our analysis.

Tissue utilized in this study was obtained from a previous work looking at the effects of IAIP enzyme on behavior and morphology after HI injury in conjunction with early experience. The anatomical feature of interest was the hippocampus, which is the main player in learning and memory. When tissue was processed for histological assessment the anterior region of the brains had to be removed in order to facilitate tissue sectioning. Areas affected by this removal of tissue were the olfactory bulb and some frontal cortex. Because an examination of the medial prefrontal cortex was not a goal in that study, the place at which the olfactory bulb was removed was not the same for all subjects, which did not affect that study but it introduced a great deal of variability to the present study. We hypothesizes that if placement of olfactory bulb removal was more consistent and more anterior leaving more of the frontal cortex, which houses the prefrontal cortex, intact we might have observed significant trends. Also, we think that the inclusion of more neurons per subject and increasing the number of subjects could have been beneficial in order to see results that agreed with our hypothesis. Even though the medial prefrontal cortex is involved in working memory, which is the reason why we thought the 8-arm radial water maze experience would impact neuron morphology in this region, the utilization of a more medial prefrontal cortex-targeted experience could have resulted in changes in morphology. Also more sensitive statistical analysis could have picked up on significant differences not seen in the analysis used for this study.

After going back and criticizing our own methods and hypotheses, I would suggest that future studies should include the development of a more medial prefrontal cortex-targeted early life experience as well as the utilization of tissue specifically processed in order to keep the prefrontal cortex intact. Increasing the number of neurons utilized per subject or instead of

averaging all tree neurons per subject treating each neuron as an individual would also be beneficial in order to have a stronger statistical output.

Acknowledgements

I could not imagine the culmination of my undergraduate carrier at Rhode Island College in a better way than with this honors thesis. About two years ago I joined the Threlkeld lab and that moment marked the beginning of an amazing journey. Not only have I been challenged as a student, young scientist, and woman in science, but also as an individual. Quickly after joining the lab I realized that in order to be successful I had to raise the bar of what I once thought was enough in order to go beyond. The Threlkeld lab was the right place for me, and is what I needed in order to challenge myself. Learning, learning, learning that is all we did at the beginning, we learned techniques and procedures, different types of behavioral testing, histology work, lab management, ethics and the list goes on and on. After learning I was able to share my knowledge with new incoming lab members, teaching them what I once learned from Dr. Threlkeld, Cynthia, and previous lab members which gave me a great deal of satisfaction.

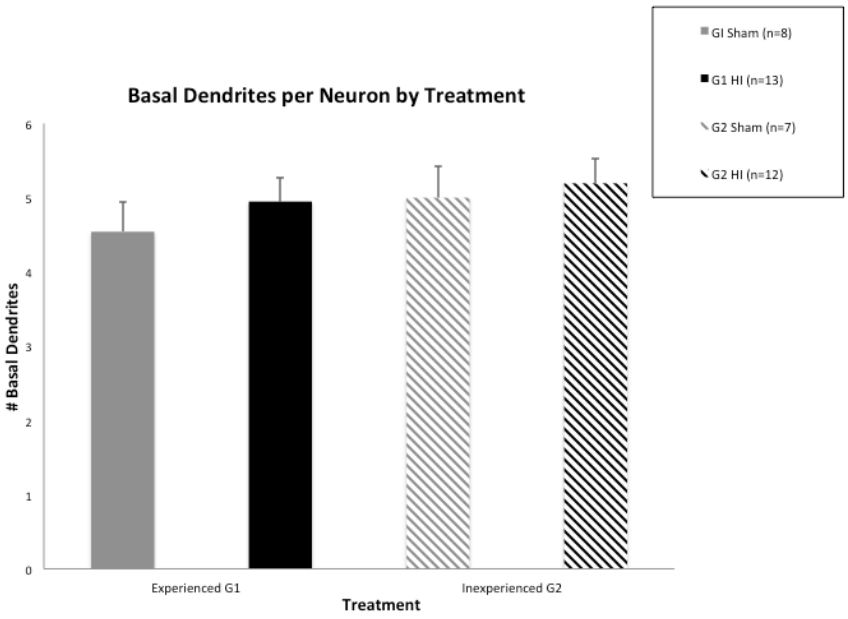
I am very grateful to Dr. Threlkeld for the opportunity to be part of his lab, as my PI, professor and mentor he has been there every step of the way. His words of wisdom, encouragement and expertise have been crucial in the development of this project as well as my personal development as a scientist. I also want to thank Cynthia Gaudet, she has inspired me to

work harder and push myself out of the comfort zone. She has been a mentor, teacher and a great friend and I am eternally grateful to have her in my life. My twin sister Keyshla Melendez who is also a member of the Threlkeld lab has been my greatest supporter, always there in moments of doubt. Always knowing what to say and encouraging me like no other, we are sisters, best friends, partners in crime and each others best competitors. Last but certainly not least I want to thank my mother, Grisella, without her hard work I would not be standing where I am. She thought me that nothing in life comes easy but those who work hard will achieve success. She is my driving force; my idol and I aspire to become half the women she is.

I stand here now with only a few weeks left of my undergraduate carrier feeling joyful but at the same time a bit sad. Four years went by fast but I am excited for what is next and I look forward to expand the education that Rhode Island College provided to me.

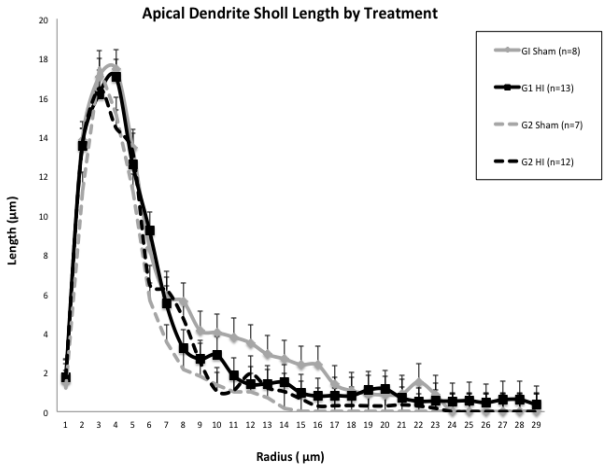
Graphs

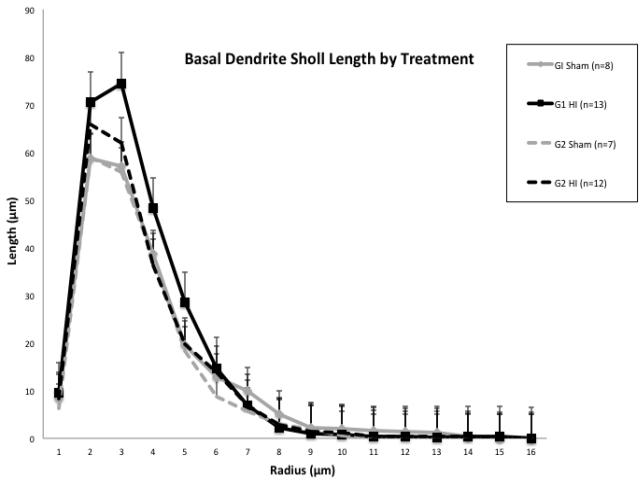
A)



Basal dendrite per neuron, a main effect of experience [F (1,36)=0.914, p=0.345] and a main effect of treatment [F (1,36)=0.667, p=0.419] were not significant.

B)





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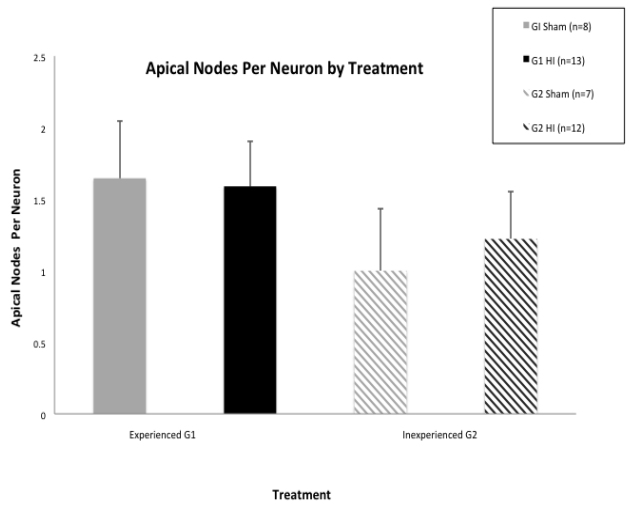

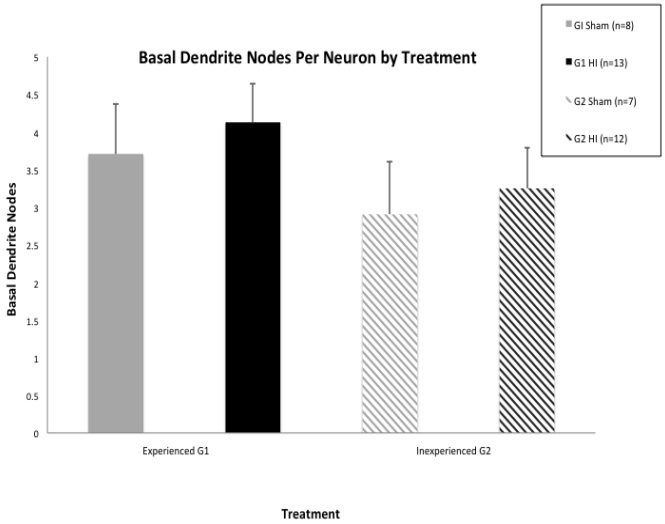

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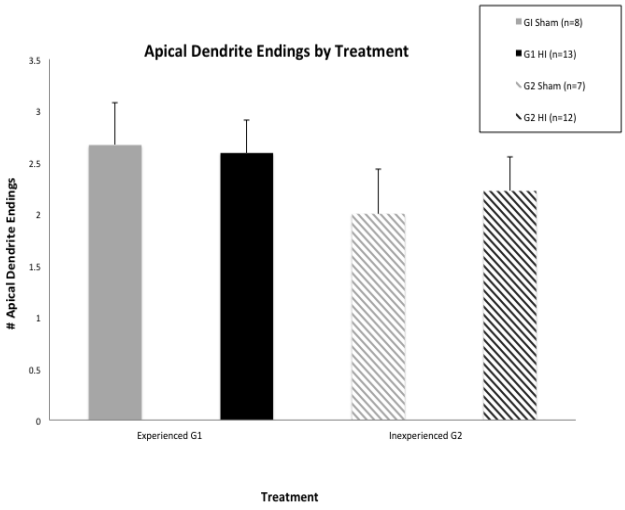
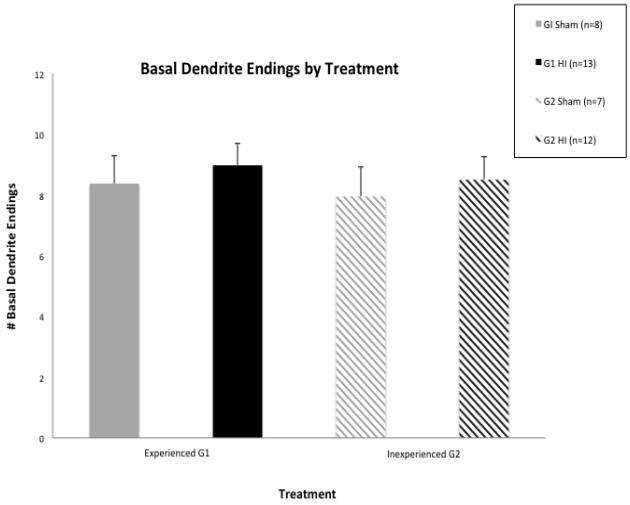
D)





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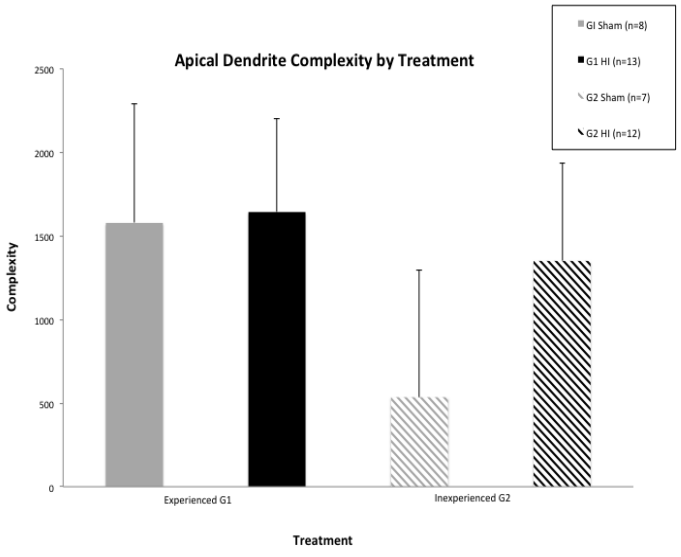
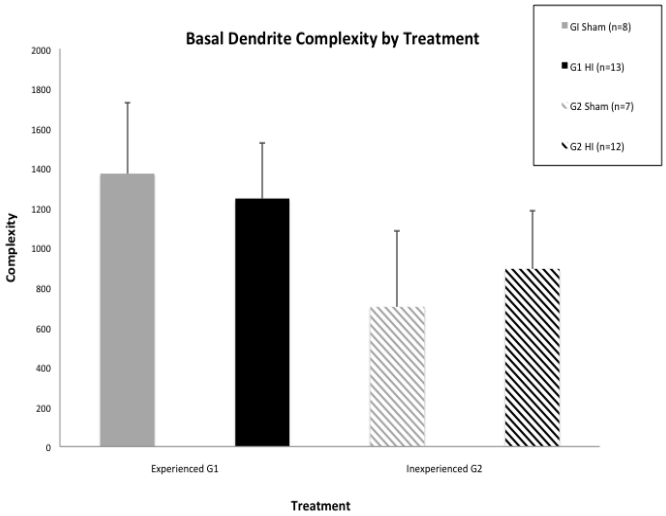
E)





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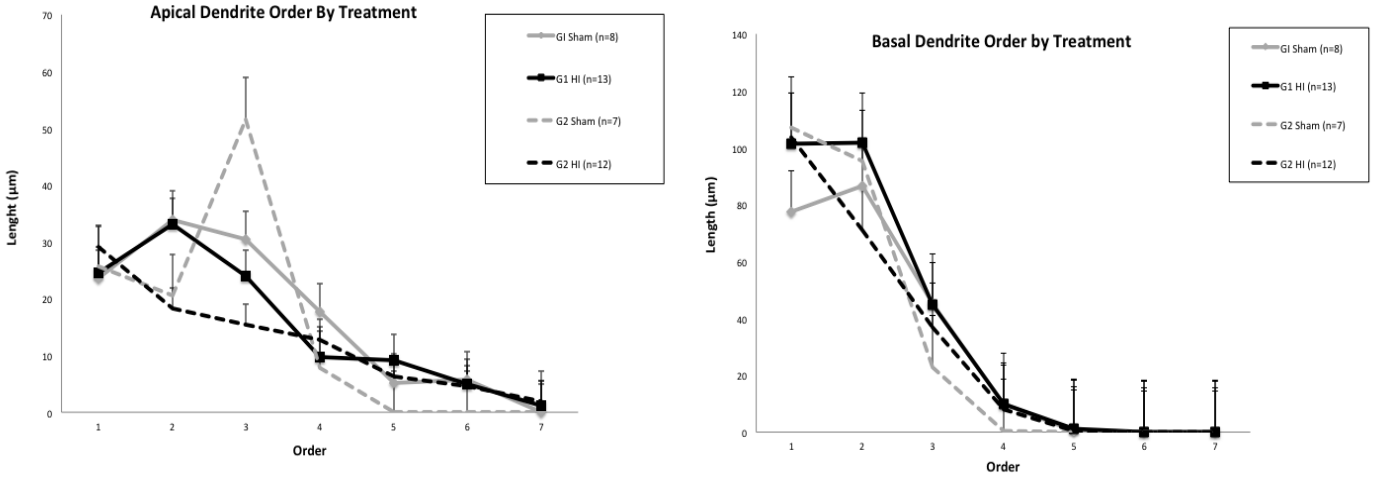
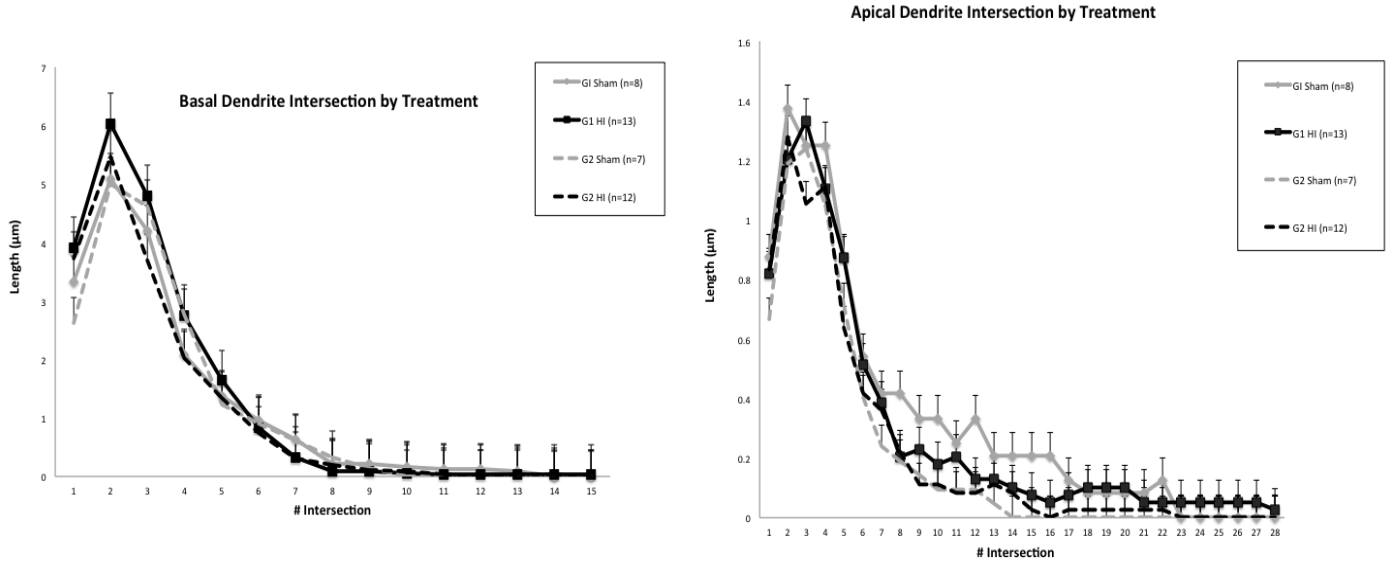
F)



Apical dendrite order per neuron, a main effect of experience [F(1,36)=0.383, p<0.540] and a main effect of treatment [F(1,36)=0.344, p<0.561] were not significant. Basal dendrite order per neuron, a main effect of experience [F(1,35)=0.221, p<0.642] and a main effect of treatment [F(1,35)=0.232, p<0.633] were not significant.

G)



Basal dendrite intersection per neuron, a main effect of experience [$F(1,36)=2.951$, $p<0.094$] and a main effect of treatment [$F(1,36)=0.078$, $p<0.782$] were not significant. Apical dendrite intersection per neruon, a main effect of experience [$F(1,35)=0.353$, $p<0.556$] and a main effect of treatment [$F(1,35)=0.089$, $p<0.767$] werer not significant.

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