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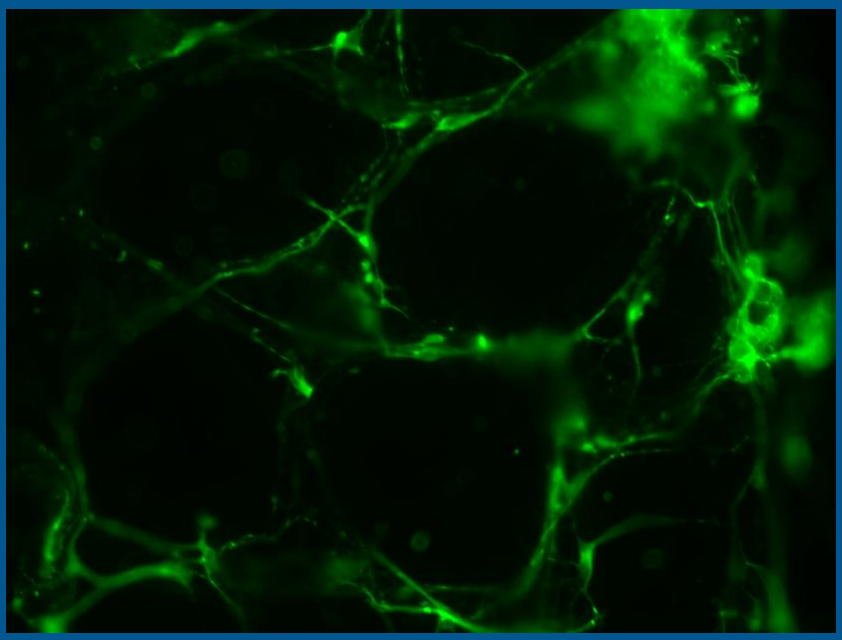

Preliminary Study of the Impact of Cortisol on Dendritic Formation on Human Mixed Cortical Neurons *in vitro*

Anna Lally and Natalie Fournier

BIO 324 / Neurobiology
Final Research Paper
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Introduction

Chronic stress is a well-defined risk factor for depression and anxiety disorders and is characterized by the increased levels of stress hormones such as glucocorticoids or the cortisol-inducing corticotropin-releasing hormone (Curran et al., 2017; Qiao et al., 2015). Glucocorticoids are necessary for neuronal development and survival (Abdanipour et al., 2014; McEwen, 2019). Involved in many processes beyond stress, glucocorticoid and cortisol levels fluctuate normally throughout not only short periods of time (ie from morning to evening) but long term, playing numerous roles throughout one's lifetime (McEwen, 2019).

However, glucocorticoids at stress-mediated levels can produce neuronal loss. It was found that cortisol levels higher than 5uM, as well as longer-term doses between 24 and 120 hours, trigger loss of viability *in vitro* (Abdanipour et al., 2014). Indeed, vastly fluctuating or dysregulated glucocorticoid release is linked to chronic stress and, via said dysregulation, continues to impact brain areas and pathways involved in mood and stress regulation (Abdanipour et al., 2014; Conrad, 2006; Koutmani & Karalis, 2015) .

Additionally, chronic stress and response to glucocorticoid levels have been linked to other neurochemical factors and molecules including BDNF and sex hormones such as estrogen, giving the issue of chronic stress increasing complexity and nuance in terms of potential sex differences and protective biological factors (McEwen et al., 2016). In relation to more short term fluctuations, individuals with chronic stress have enhanced cortisol secretion after awakening as compared to those with low stress, as well as women having a more pronounced

cortisol increase during the early morning period (Schulz et al., 1998). It was also found that those experiencing chronic stress have lower cortisol levels later in the day, demonstrating suppressed cortisol levels—and potentially increased dysregulation—in those under chronic stress (Schulz et al., 1998).

On a larger, and potentially more long-term scale, chronic stress appears to induce structural remodeling of the hippocampus and prefrontal cortex in particular (McEwen et al., 2016). Both areas share commonality in neuronal remodelling, primarily in dendrite retraction and reduction in arborization; however, there is evidence of potential reversal in restructuring upon the termination of chronic stress or introduction of medications such as antidepressants which assist in Hypothalamic-Pituitary-Adrenal (HPA) axis regulation (Lenze et al., 2011; McEwen et al., 2016; Oomen et al., 2010).

The hippocampus as a learning and memory center functions in relation to the development of depression and anxiety disorders. It has been found that patients with depression have a smaller hippocampus than healthy individuals, perhaps linked to the decrease of dendritic spine density, reduction in dendritic branching, and potentially neuronal atrophy seen in relation to chronic stress (Conrad, 2006; Curran et al., 2017; Qiao et al., 2015). The hippocampus has been found to be sensitive to glucocorticoid exposure, producing changes within dendritic arbors, specifically reduced branch number and length (Conrad, 2006; Oomen et al., 2010). The changes which occur in the hippocampus under chronic stress have the potential to disrupt the HPA axis, leading to dysregulation of glucocorticoid release and further hippocampal neuron damage, as the hippocampus functions as an inhibitory component of the HPA axis, capable of controlling the negative feedback loop which suppresses extended and unnecessary release of glucocorticoids (Abdanipour et al., 2014; Conrad, 2006).

However, there are studies which state that low levels of stress can, in fact, be beneficial for learning and memory, as well as data regarding other aspects of neuronal function, such as axonal transport, demonstrating that low levels of cortisol may improve function and speed at which axonal transport is conducted (Izeddin et al., 2011; Joels et al., 2009; Shors, 2004). Additionally, there is evidence to suggest that early life adversity and associated glucocorticoid changes may prime the organism for more optimal performance in high-stress situations later in life and that corticosterone *in vitro* can lead to enhanced synaptic plasticity (Oomen et al. 2010).

The aim of the present preliminary study is to investigate the effects of cortisol on the growth and maturation of neuronal cell processes in human-induced pluripotent stem cell (hiPSC)-derived mixed cortical neurons. Literature suggests that cortisol at a high level has a negative impact on the growth and formation of dendrites—vital components to synaptic connection and plasticity—and that there is a relationship between chronic stress and early adverse experience, decreased cortical volume, and mental illness. We utilized immunofluorescence and quantitative light microscopy to test the hypothesis that exposure to cortisol will decrease dendrite formation on hiPSC-derived mixed cortical neurons in culture.

Methods

Cell culture, fixation, and immunofluorescence procedures were conducted as described in Morris (2020). The neurons used were provided by EverCell Bio.

Cortisol Treatment

For the experimental condition, a cortisol treatment was prepared and allocated to the experimental wells beginning on day 07. A 10uM solution of hydrocortisone (Sigma-Aldrich, H0396) was created using culture media; the solution was further diluted to the defined treatment concentration of 5uM through a treatment of 250uL in an overlay on the existing 250ul in appropriate wells. The wells were incubated for 14 hours before being washed twice with NMM media. The control wells received 250uL of untreated media and were subject to the same incubation and washing process, so as to control for potential effects of introducing new media and washing. All conditions were fed every two days.

The process outlined above--including a complete buffer change, addition of cortisol treatments to the experimental wells, and feeding--occurred on days 09 and 13 as well, prior to fixation and treatment for immunofluorescence.

Immunofluorescence Staining and Visualization

Immunofluorescence staining was completed in accordance with the methodology outlined in Morris (2020). Primary antibodies used in the current study are MAP2 (BD, 309628) at 1:5000 dilution; secondary antibodies used were Alexafluor 488 (Goat Anti-Chicken) and DAPI (antifade reagent).

Cells in culture were visualized using fluorescence microscopy via a Nikon 80i upright microscope equipped with differential interference contrast optics and a Nikon Plan Fluor 40x/0.75 objective and a Diagnostic Instruments (DI) Spot RT3 Slider camera with a DI O.76x HRD076-NIK adapter driven by DI Spot software version 5.2.5 on an Apple iMac (21.5 inch, 2013) computer using OS X 10.13.6.

Data Collection

Fiji (ImageJ version 2.1.0/1.53c) was used for the data collection process, including measuring and evaluating brightness and creating composite immunofluorescence images. Data are derived from all visible dendrites of neurons in culture in four images captured from two wells of the control condition, totaling in n=26 visible dendrite formations; experimental data points are derived from all visible dendrites of neurons in culture in two images captured from one well of the cortisol-treated condition, totaling in n=33. Visible dendrites are defined in the present study as being in-focus, linear stretches of MAP2 fluorescence; measurements were taken along areas at least three pixels in width or 1.5um, and at most 16 pixels or 7.9um where extending from a cell body. Dendrites crossing other neuronal processes or cell bodies were discounted. All dendrites were confirmed using paired fluorescence and transmitted light microscopy images.

To obtain brightness values, the analyze-measure feature was utilized. The polygon selection tool was used to outline the dendrites in at least two consecutive areas, depending on length; if the dendrite was three to five pixels, or 1.5 to 2.5um, in width, the line tool was used with width set to two pixels. For each dendrite brightness value taken, an adjacent background brightness value was taken by moving the polygon or line tool to a background area parallel to the dendrite area measured. The mean brightness value from each measurement was recorded and averaged to get the mean brightness for each complete dendrite; the mean brightness values of the adjacent background were averaged, then subtracted from the average dendrite value, resulting in the final mean brightness value for individual dendrites. These final values were recorded in excel prior to use in R, as well as SD and SE.

Measurements of all visible cell bodies in each condition were taken; the control condition n=6 visible cell bodies, from one image captured from one well of the control condition, and the experimental condition n=6 cell bodies, from two images captured from one

well of the cortisol treatment condition. Visible cell bodies are defined as being in-focus, not overlapping with other cell bodies or processes, and attached to a previously quantified dendrite; all cell bodies were confirmed using DAPI stained fluorescent images as a composite (Figure 1B). The polygon tool was used to outline the cell body and take the mean brightness of the area. The mean background brightness value of the associated dendrite was then subtracted from the cell body brightness to determine the final mean brightness for specific cell bodies. Final values were recorded with their paired dendrites in excel prior to use in R.

Data Analysis

Programming software R (Version 1.3.1056, "Water Lily" (5a4dee98, 2020-07-07) for macOS) was used to clean and analyze data, conduct statistical tests, and produce graphs/figures.

Results

Dendrite Brightness Between Conditions

It was hypothesized that the presence of cortisol exposure will decrease dendrite formation of human neurons in culture. To test this hypothesis, the brightness of in-focus MAP2-stained dendrites from both the control and experimental conditions was measured and averaged for each condition.

The control condition was found to have a mean brightness of 22.43242 (Figure 1A & 1B, Figure 3 & Figure 4); the experimental condition was found to have a mean brightness of 10.20912 (Figure 2). Prior to conducting statistical tests, the data was used to produce a boxplot to gauge significance and equal variance (Figure 5). The data appear to have a promising difference between conditions. The samples have unequal variance, most likely due to the unequal sample sizes. Additionally, the R model function was used to test normality—the control and experimental data displayed a normal distribution, thereby allowing the use of a standard statistical test.

A Welch's T-Test was used to determine significance between the control and experimental group. The control group ($M=22.43242$, $SD=5.2878$; $n=26$) compared to the experimental group ($M=10.20912$, $SD=4.18$; $n=33$) showed a significant difference in dendrite brightness, $t(46.818)=9.648$, $p< 0.001$ (see Figure 6).

Due to the preliminary nature of this study, as well as small and varying sample sizes, our statistical result does not have much power; however, the result of the T-test does imply a trend that supports our hypothesis.

Soma-Dendrite Brightness Difference Between Conditions

To further determine trends related to the hypothesis that cortisol negatively impacts dendritic formation, data was collected for cell soma associated with dendrites within the above dataset. Measurements were taken of every clear cell body attached to an in-focus dendrite (quantification specifications described in methodology), resulting in n=6 cell bodies and n=6 dendrites for both the experimental and control groups. The mean brightness was taken for each cell body, and the background brightness value of its associated dendrite was subtracted to maintain consistency.

The mean brightness of soma in the control group was found to be M=32.62083, SD=5.65, as compared to the mean brightness of the experimental group, M=20.87917, SD=7.93. The mean brightness of the of the six soma-associated dendrites from the control data (M=22.2185) align closely with the mean of the complete control dendrite dataset (M=22.43242, n=26); however, the mean brightness of the 6 soma-associated dendrites from the experimental group (M=7.878333) is lower than the mean of the complete experimental sample, although within one sd (M=10.20912, n=33). This variation does not appear to have any significance, as brightness value collection was based on the cell bodies that were well-defined and on the focal plane of the images, which appears to be random.

To determine if there is a decrease in dendritic formation between conditions, the difference between cell body and dendrite brightness is the most pertinent. MAP2 expression, quantified by brightness, illustrates maturation of the dendritic formations, and brighter values associated with a dendrite in relation to its cell body demonstrates dendritic maturity; conversely, significantly greater brightness of cell bodies in relation to brightness values of their associated dendrites implies less developed and mature dendritic formations.

The difference between soma and dendrite brightness of the control group, 10.402, was a narrower margin than that of the experimental group, 13.001 (Figure 8), demonstrating a potential trend regarding dendrite maturation, specifically decreased maturation in the presence

of cortisol. These data may further support the hypothesis that cortisol exposure decreases dendrite formation in human neurons.

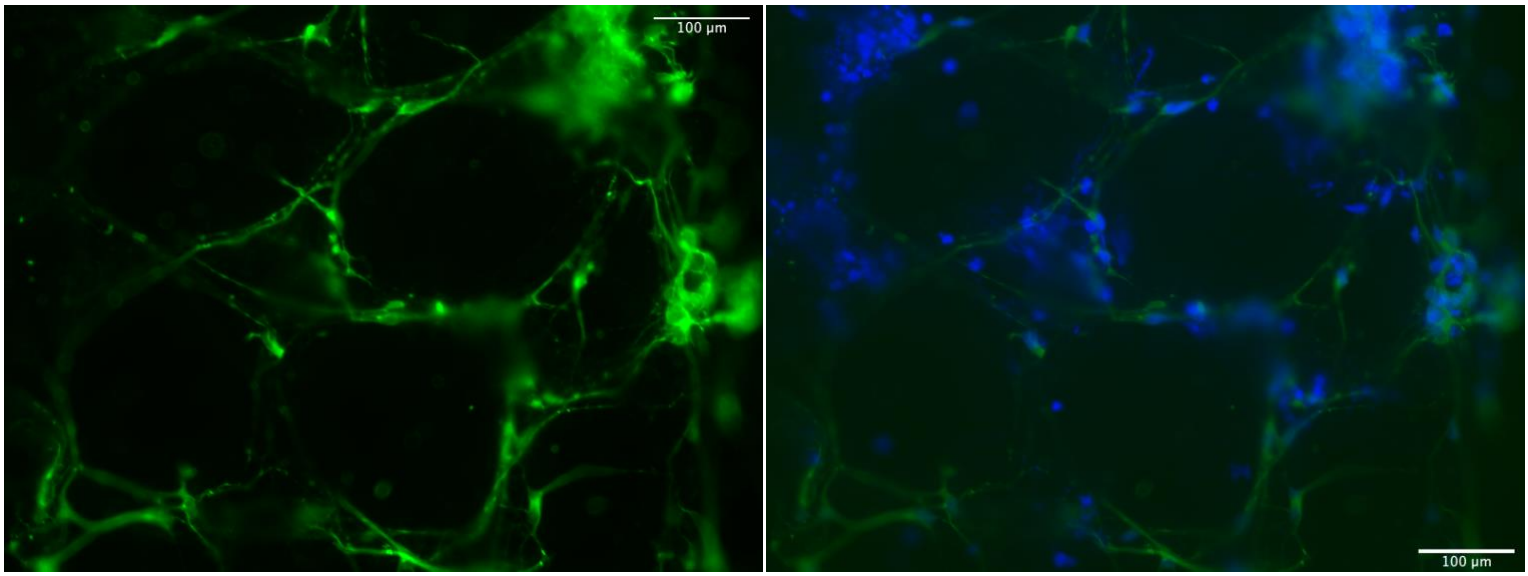

Figure 1: 1A (left) MAP2 Expression in Control Condition (Well 2); 1B (right) MAP2 and DAPI Composite Image for Control Condition (Well 2). MAP2 images were used for collecting dendrite and cell body quantification data. Labeled cultures were generated by Melanie Gardiner and images were generated by Robert Morris, used here with permission.

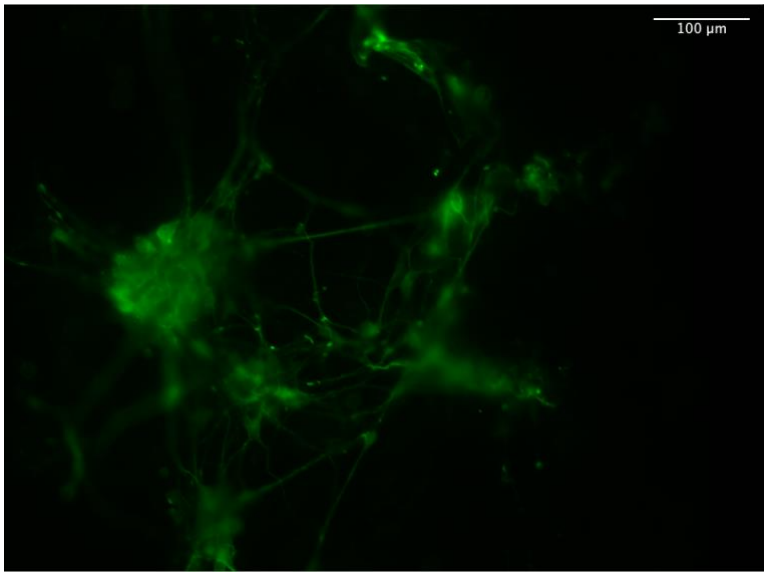

Figure 2: MAP2 Expression in Experimental Condition (Well 5, Focal Plane 1). Cell body and dendrite image used for quantification data. Labeled cultures were generated by Melanie Gardiner and images were generated by Robert Morris, used here with permission.

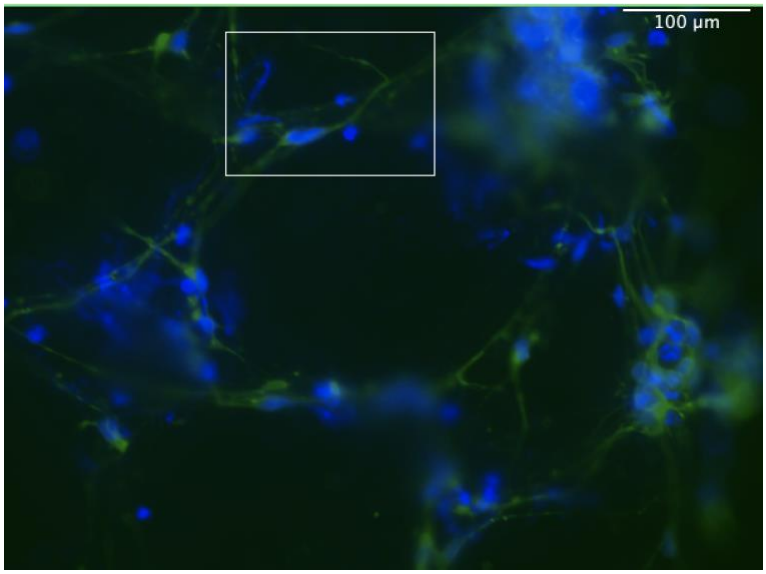

Figure 3: Magnified Composite DAPI/MAP2 Image of Control Condition (Figure 1B) Highlighting Specific Cell Body and Associated Dendrite. Cell Body and Dendrite within boxed area used in control dataset measuring difference in cell body/dendrite brightness. Composite images were used to qualify cell bodies for quantification. Labeled cultures were generated by Melanie Gardiner and images were generated by Robert Morris, used here with permission.

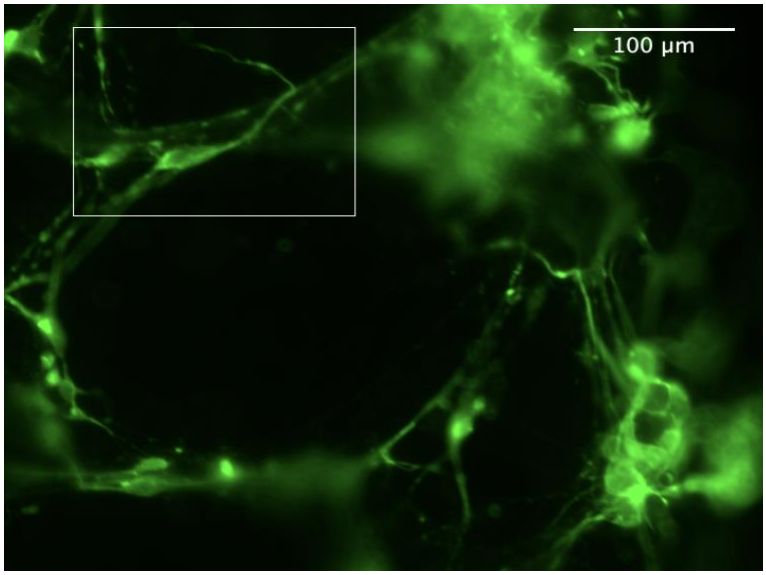

Figure 4: Magnified MAP2 Image of Control Condition (Figure 1A). Magnified MAP2 images were used for collecting dendrite and cell body quantification data. The soma and its associated dendrite seen within the boxed area was used in both datasets. Labeled cultures were generated by Melanie Gardiner and images were generated by Robert Morris, used here with permission.

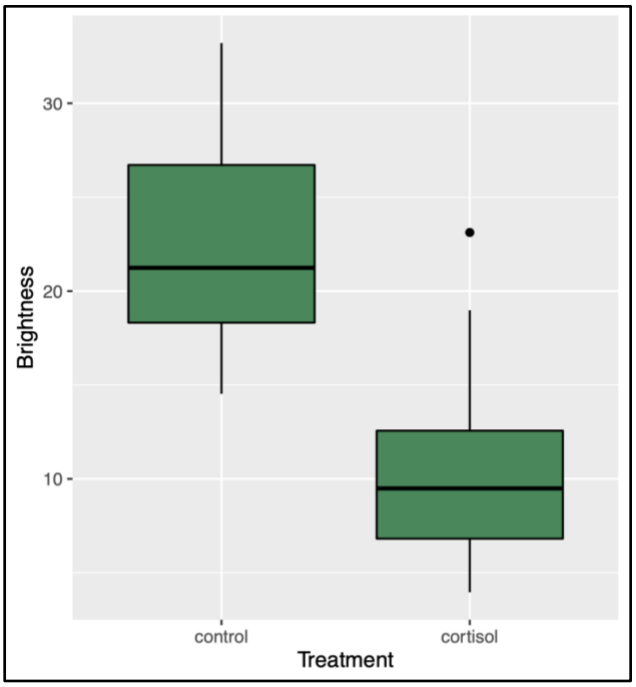

Figure 5: Boxplot depicting the distribution of dendrite brightness between the control and experimental conditions. The control condition has slightly more variance than the cortisol condition, as shown by the depth of the boxes, representing the interquartile range of each dataset. The cortisol condition has a seeming outlier, shown by the point above the experimental boxplot; the outlier was included in all following data visualizations and analysis. The boxplots demonstrate a difference in mean brightness, with the control group showing higher brightness values than that of the experimental group.

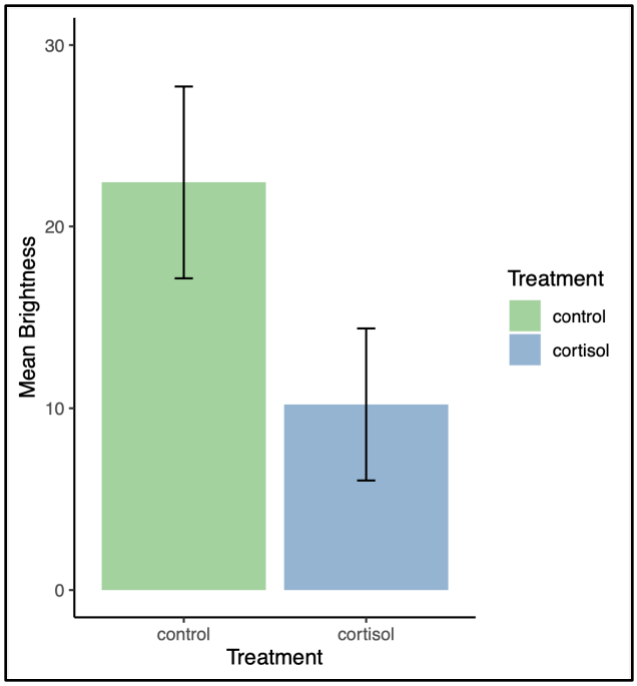

Figure 6: Mean dendrite brightness of the control versus experimental conditions with +/- sd error bars. The bar graph was constructed using all brightness values collected from visible dendrites of control and experimental conditions. There is a clear difference in brightness between conditions including variation +/- 1 SD, the mean brightness of MAP2 expression of quantified dendrites from the control condition being significantly higher than that of the cortisol-treated dendrites. The difference seen here is confirmed through statistical analysis.

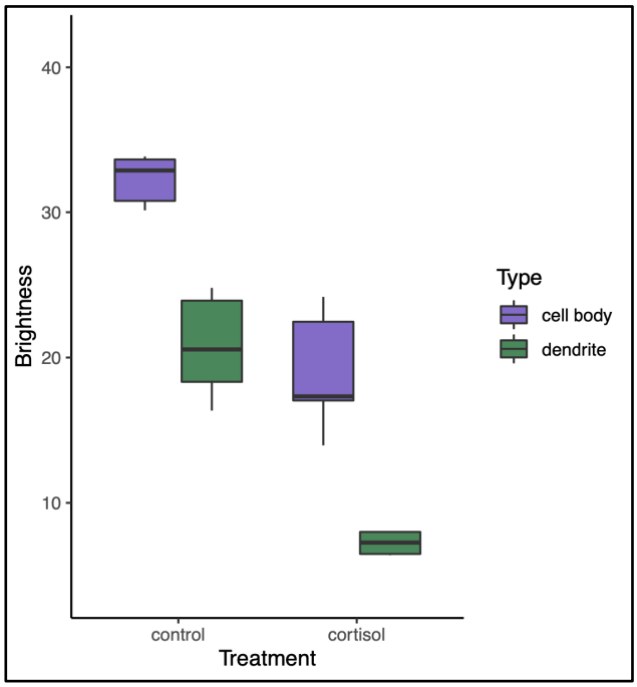

Figure 7: Boxplot depicting the distribution of dendrite and cell body brightness between the control and experimental conditions. For both conditions, the cell bodies were significantly brighter than the associated dendrites; the difference between cell body and dendrite brightness appears to be larger for the experimental group. The dataset displays unequal variance for all associated measurements, most likely due to low sample size (n=6 for all cell bodies and dendrites in both conditions). No statistical tests will be conducted from this dataset.

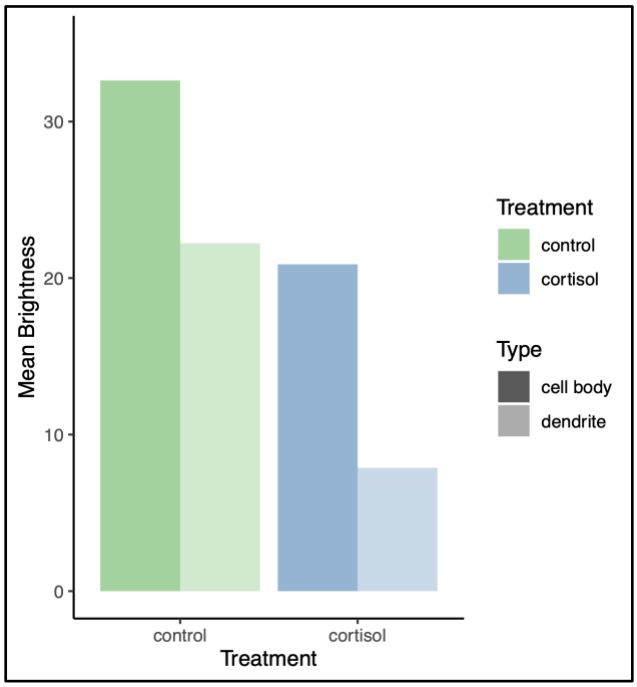

Figure 8: Comparison of mean brightness of dendrites versus associated cell bodies for control and experimental conditions. The mean brightness for both dendrites and cell bodies in the control group are greater than the mean brightness values of dendrites and cell bodies in the cortisol group. The difference between cell body and dendrite brightness in the cortisol group is larger than the difference in the control group; this variation in difference between control and experimental groups illustrates a potential trend of lessened dendritic maturation in the presence of cortisol.

Discussion

This preliminary study tested the hypothesis that cortisol exposure will decrease dendrite formation on human neurons in culture; the data gathered and analyzed within the scope of the present study support the hypothesis. The results suggest that the presence of cortisol in culture during the growth and development of human neurons negatively impacts dendrite formation and maturation, as seen by lessened MAP2 expression and, therefore, decreased brightness in the experimental condition.

Comparing mean brightness of the control versus the experimental condition via a t-test for unequal variance shows a significant difference in MAP2 expression within the scope of the present study; the clear difference is seen graphically as well (Figure 8). Similarly, the preliminary data regarding soma versus dendritic MAP2 expression may suggest cortisol negatively impacts differentiation of neurons in culture.

The presence of stress hormones such as cortisol have been linked to neurogenesis inhibition in numerous studies; the action of stress hormones during pre and early postnatal development creates long lasting effects on cellular behavior, including proliferation, differentiation, and synaptogenesis (Koutmani & Karalis, 2015; Perry, 2002).

The present study supports evidence in the literature that the stress hormone cortisol is detrimental to the development of neurons (Abdanipour et al., 2014; Koutmani & Karalis, 2015; Yan et al., 2010). The preliminary results demonstrate decreased MAP2 expression, related to cited functional and formational changes in neuron growth, specifically reduction in differentiation (Yan et al., 2010).

The present study and related literature has implications in areas regarding mental health research, particularly stress modeling as related to childhood trauma and development. Viewing the impacts of cortisol at varying concentrations and durations on a cellular level will assist in understanding the long lasting impacts that organismal stress has on neuronal development, as well as contribute to the growing body of literature regarding the impacts of early childhood stress and trauma on the individuals life beyond the instances of trauma itself.

Due to the small sample size and unequal variance, there is little statistical power in the preliminary data gathered in the present study. Results replicated in larger studies, as well as refinement of data collection techniques may improve upon the current data. In future studies, the expansion of cortisol treatment type, either via differing conditions (acute versus chronic) or

varying concentrations with a consistent administration condition, could provide insight on potential differences in stress hormone effects on neuronal development. Additionally, more focused studies on individual neuronal subtypes including dopaminergic, serotonergic, and noradrenergic cells, as well as defined pyramidal cells, would be of value to the body of literature, as further study is needed in order to understand the impact of organismal chronic stress on a cellular level, as well as to further define the effects of chronic stress and dysregulated glucocorticoid release in specific neuronal types and brain areas. Finally, within future studies there is room for expansion on preexisting *in vivo* work in the literature in relation to the present preliminary study, including discussion of sex differences and maternal protective factors related to prenatal and early postnatal stress.

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