Resilience to acute sleep deprivation is associated with attenuation of hippocampal mediated learning impairment

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Abstract

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Sleep deprivation is a universal issue that affects individuals in differing ways. While some individuals experience a deficit in daily performance, others experience resiliency as they can maintain high levels of physical and mental functionality. Although we know that a loss of sleep causes cognitive dysfunction in areas such as learning and memory, we do not understand which neural mechanisms contribute to the attenuation of learning impairment. Recently, our laboratory produced a cognitive assay known as the Box Maze that can assess learning impairment in sleep-deprived mice. Based on data accumulated from the box maze, we hypothesized that a grading platform could separate fast and slow learners in mice that have or have not been exposed to sleep deprivation. This grade could then be further explored with biomarkers, providing insight to the attenuation of learning impairment in the hippocampi of sleep deprived, fast learning mice. This study utilized an existing database of box maze escape times across 16-18 month old, male, C57BL/6 mice that were or were not sleep deprived with all other conditions standardized. After data mining, a total of 40 mice fit the criteria for the study. The grading platform utilizes a logarithmic trend line of the box maze trials to ultimately separate fast and slow learners. The separation was based on the R^2 value which represented the learning curve of each individual mouse. The results showed that sleep deprived mice had more slow learners than fast whereas control mice showed the opposite. The hippocampus of these mice then underwent immunohistochemistry to explore biomarker levels that would be insightful of the attenuated learning impairment in sleep deprived mice that were graded as fast learners. The results showed that fast learners in the sleep deprived groups~~,~~ expressed similar levels of biomarkers as that of the control fast learning groups. The data provide~~s~~ evidence that sleep-deprived mice that performed well in a cognitive assay show less hippocampal mediated learning impairment. These findings provide the rationale for clinical investigations into neurobiological resilience with increasing age.

Keywords: Sleep deprivation, Resiliency, Learning paradigm, Aging

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Dedication.

My work is dedicated to my mom, dad, sister, and boyfriend who supported me tirelessly throughout all the ups and downs of my entire academic pursuit.

Introduction.

Resilience and Aging: Environmental adversity can often lead to stress as a biological reaction. Additionally, dysregulation and chronic exposure to stress response can then lead to adverse health outcomes. However, not all those who experience stress will develop diseases. Individuals in the population who have enhanced stress resilience mechanisms can adapt successfully to stress without the burden of illnesses. Increased resiliency to stress also contributes to a slower aging process and improved overall health and quality of life. It is apparent that individuals perceive stressful events differently, and some individuals are less vulnerable to stress than others and are, therefore, deemed as resilient [1].

However, the study of resilience has been more common among younger study populations, but sparse when it comes to aging people [2].

The aging process itself is one of the most challenging public health issues faced by developed countries. With the growth in aged populations, there has been an expansion in initiatives and interventions to promote successful aging [3]. The topic of resilience is a rapidly growing field of interest in research, especially in older adults and the impact it has on successful aging. Aging well has many components, such as being free from chronic disease and maintaining high level of physical and mental functionality [4].

Sleep Deprivation: Sleep is an essential function for energy conservation, as well as the homeostasis of multiple physiological and behavioral processes [5]. The American Academy of Sleep Medicine and the National Sleep Foundation recommend that adults sleep at least 7 hours per day [6,7]; however, the increasing demand for long work shifts and "around-the-clock" work has led to a marked reduction in the average sleep duration in developed countries [8,9]. The issue of sleep loss has become so pervasive in society that the Center for Disease Control has recently elevated it to public health epidemic status [10]. However, research has also shown that some individuals are vulnerable, and others are resistant to sleep deprivation [11,12]; notably, this phenotypic stability is maintained across months and years [13]. Approximately a third of healthy adults show high levels of deficit in performance when moderately sleep deprived. Another third display moderate deficits while the final third shows little to no performance deficit even with severe sleep loss. The underlying reasons for such differential neurobehavioral vulnerability to sleep loss are mostly unknown and unexplained by demographic and other factors [14-16]. To date, there have been studies looking at different genes and "omics" approaches to relate biomarkers to sleep deprivation responses [12]. Ultimately, more work needs to be done, including the investigation of molecular mechanisms and markers.

*Box Maze***:** The box maze is a spatial navigation task designed as a single day assay that focuses on assessing learning behavior in a time-effective and relatable manner. The maze serves as a behavioral paradigm for learning impairment in short-term sleep deprivation studies in mice [20]. The design of the box maze intends for the mouse to be placed into the plastic box where it is then presented with multiple identical PVC caps that serve as a false escape. The mouse is then run through the maze for a total of 4 trials each being 120 seconds long [21,22]. Overall, the box maze is a quick cognitive test in which one mouse can be tested within 15 minutes in a single day. Additionally, utilizing the partial sleep deprivation mouse model as a stressor to exploit the cognitive impairments that arise with sleep loss facilitates the study of innate resiliency in individual mice. Although there have been many studies that have focused on the cognitive impairments that occur with human sleep deprivation, far less attention has been given to establishing an experimental animal model to describe similar accounts [23]. Therefore, the box maze provides an opportunity to explore the deficits in cognitive performance that occur with experimental sleep deprivation in an animal model. Thus, preclinical studies aimed at improving cognitive performance can be measured.

Materials and Methods.

Box maze database. Box maze data was collected for the past two years after our laboratory developed the box maze protocol. The raw data was then collected into a database on a shared Google drive that is used communally by all lab members. Through laboratory efforts, the drive has been compiled with a large collection of box maze data from many different studies. The mice that were specifically used for this study were 16-18 months of age, male, C57BL/6 (B6) mice provided by the Jackson Laboratory. After extensive mining of all available data from the drive, a total of n=40 mice were suitable for the hypothesis being tested. Half of the mice underwent the learning assessment after being exposed to a partial sleep deprivation protocol while the remaining half were those that were kept on a standard sleeping cycle. Additionally, the mice were housed in groups no larger than 5 and provided with standard chow, water, and bedding provided by the animal facility. Cage changes were done during weeks that mice were not undergoing sleep deprivation and assessment in the learning paradigm to avoid any other forms of stress. For animals undergoing partial sleep deprivation, the protocol started shortly after lights on based on a 12:12 dark to light cycle. The mice were kept awake with cage tapping with intervals of 10-15 minutes between taps. This protocol was followed for a total of four days, with the learning assessment occurring right after the final day of sleep deprivation.

All experimental procedures were done on an approved University of Washington Animal Care and Use Committee (IACUC) protocol.

Grading platform. The box maze produces data in the form of escape latencies with four trials per mouse. This raw data can then be plotted on a graph through programs such as Excel, Graphpad Prism, etc. The grading platform further defines the escape latencies with a single value known as the coefficient of determination (R^2). After plotting the raw data, a logarithmic trend line can be inserted to the data points in which the R^2 value and slope will be observed for each mouse. If the slope is positive, the mouse is graded as a slow learner whereas a negative slope calls for further analysis. The logarithmic trend line was chosen as the most appropriate to describe the data points because a learning paradigm should be one in which the data quickly levels off [24]. The R^2 ranges from a value of 0 to 1 with 1 being that the data points lie closely to the logarithmic trend line.

Immunohistochemistry. Immunohistochemistry was performed on 5 μm thick, paraffin embedded mouse brain tissue mounted onto slides. Slides were rehydrated with xylene, decreasing concentrations of ethanol, and deionized water. Antigen retrieval was performed by immersing the slides in a hot water bath at 98 degrees C incubated in a 1:10 Citrate Antigen Retrieval solution in autoclaved deionized water and cooled down to room temperature for 20 minutes. Slides were then stained using an avidin-biotin HRP kit (anti-rabbit HRP-DAB Cell & Tissue Staining Kit, R&D Systems Minneapolis, MN) with manufacturer instructions slightly modified for best staining outcome. Slides were applied with a 3% peroxidase blocking reagent for 15 minutes to quench endogeneous peroxidase activity which reduces background noise on the final stain result. Slides were washed in a TBST solution for 5 minutes. To reduce non-specific hydrophobic interactions between the primary antibody and the tissue, serum blocking reagent was placed onto each section for 15 minutes. After draining the serum off of the slides, avidin blocking reagent was placed onto them for 15 minutes followed by a rinse with TBST for 5 minutes. To prevent the binding of previously applied avidin, biotin blocking reagent was placed onto the slides for 15 minutes. Primary antibody in TBST at the following concentrations: HDAC2 1/500 (ab7029, Abcam, Cambridge UK), BDNF 1/500 (ab108319, Abcam, Cambridge UK), MCP-1 1/200 (ab25124, Abcam, Cambridge UK), Synaptophysin 1/500 (ab32127, Abcam, Cambridge UK) was applied overnight in a humidified chamber.

Slides were rinsed 3 times in TBST for 5 minutes each, then incubated with a biotinylated secondary antibody for 30 minutes, and rinsed 3 times in TBST for 5 minutes each. Slides were incubated in HSS-HRP for thirty minutes and rinsed in TBST 3 time for 2 minutes each. DAB Chromogen was applied to slides and incubated in the solution for 5 minutes each before rinsing with deionized water for 5 minutes. Slides were dehydrated in an increasing concentration of ethanol and xylene then mounted with a coverslip.

Imaging and uploading to Qupath. IHC slides were photographed under a Nikon Eclipse E400 microscope with a Nikon D7100 camera through a microscope camera adaptor. All photos were taken under a magnification of 4x so that the entire hippocampus could be captured within each photo field. Photos were then uploaded onto a Google drive where slides were separated into project files organized by staining group.

QuPath analysis. QuPath version v0.2.0-m11 was downloaded from Github (https://QuPath.github.io/) [28]. When downloading, QuPath allows the user to determine how much RAM it will take up; so we determined 6 GB would be sufficient for the analysis of the project. A new project was created on QuPath per each staining group examined. This step was done to avoid having to redo the steps of the project workflow for each image. First, the image type was set to H-DAB in order for QuPath to recognize the images as a DAB Chromogen stain.

The RGB values for DAB were then calibrated to better represent the project by selecting and ROI that is representative of positive stains along the hippocampus. Smaller ROIs were used that selected only the smaller areas of positive staining to reduce any potential background noise. These ROIs were then averaged to come up with a new RGB DAB value for the project code. The hippocampus was then annotated with the polygon wand to only measure staining at the desired region of the tissue. However, if there were any folds or staining irregularities through a manual check, the slide was omitted from analysis. To quantify the staining, superpixels were created to analyze the hippocampus [29]. Within the annotated hippocampus, QuPath groups similar pixels into a cluster called a superpixel based on the RGB values set for DAB. Pixel based analysis was chosen as the desired method of quantification because this study is looking at multiple stains, and pixel analysis allows us to follow an almost identical protocol between each group [29]. Superpixel size was set to 25 μm^2 in order to balance capturing positively stained sections at a high resolution and processing speed. QuPath then applies a DAB intensity to each of the superpixels previously set by the initial DAB RGB calibrating. Not only does it process as positive or negative, but it also separates the staining at three levels of thresholds: 0.2, 0.4 and 0.6 or a positive at all three levels. This therefore allowed the capture of staining intensity across all positively stained cells in the annotated region. In order to visualize the DAB staining thresholds, a “heat map” was generated for each image [29]. QuPath allows users to apply a gradient of color according to the quantifications of DAB staining generated previously. The heat map allows a qualitative complement of the previous analyses and serves useful in identifying certain regions that have higher levels of staining. QuPath generates the heat map by assigning a color to each superpixel which indicates the different levels of DAB staining based on the previously described thresholds. However, the upper and lower bounds of the color spectrum must be set by the user and made equal for each new project. This way, the results within each project can be compared to one another relative to staining distribution and intensity across the hippocampus. It should be noted, however, that the color is not indicative of positivity, but rather captures the differences in intensity of stain.

Statistics. Welch's *t*-tests, Pearson Correlations, and graph creation were performed using Prism statistical software (Graphpad Software, La Jolla, CA, USA). The *p*-value was set for a statistical significance of $p<0.05$. All the data were presented as mean ± SEM.

Results.

The box maze grading platform is able to separate slow learners from fast learners.

Upon analyzing the 20 sleep deprived and 20 control mice that were selected from the database of box maze results, we found n=6 fast learners in the sleep deprived group and n=15 fast learners in the control group (Figure 3). Plotting the raw escape latencies for each mouse produced a graph that allowed further distinction of fast and slow learners through utilizing the coefficient of determination (Figure 2). It was observed that those who learned fast had an R^2 value that was close to 1 while those that learned slow were close to 0. However, there were also mice that ranged somewhere in the middle. After finding the average R^2 value across n=80 control mice, we set the fast learner threshold value at greater than 0.74. The results from grading each mouse showed that among the sleep deprived group, fewer mice are able to perform well on the learning assessment due to some sort of synaptic impairment that is known to occur with the loss of sleep [31,32,41]. However, as hypothesized, there is a smaller group of fast learners within the sleep deprived that are able to maintain the higher levels of cognitive functioning despite being exposed to the effects of sleep loss. The R^2 obtained from the grading platform also proved itself to be of value when comparing the fast from slow learners when comparing the box maze data to other variables such as sleep deprivation or staining intensity. Because raw box maze data that is plotted has many factors to account for, such as escape latency and the 4 trials per mouse, it is difficult to compare to other factors. Therefore, the grading platform also provides additional value in that it is able to give a single number to describe the learning curve across the four trials per mouse (Figure 1).

Heat map generation and positive superpixel data on QuPath showed that staining thresholds in sleep deprived fast learning mice were similar to that of control fast learning mice.

Slides that were stained through immunohistochemistry and imaged were processed through the whole-image analyzing software program, QuPath [28]. The results, expressed in superpixels, showed that HDAC2, MCP-1, Synaptophysin, and BDNF levels in sleep deprived, fast learning mice were similar to that of the control, fast learning mice. The HDAC2 is a histone deacetylase complex which indicates that some level of epigenetic alterations is occurring. HDAC2 functions to represses transcriptional activity and therefore decreases expression of DNA products in the brain [33]. There are many types of HDAC, but HDAC2 was of interest to our results because it negatively regulates memory formation and synaptic plasticity [34]. Sleep deprived, fast learning mice had significantly reduced levels of HDAC2 when compared to that of slow learning mice (Figure 5B and C). MCP-1 levels were also significantly reduced in the sleep deprived fast learning group which suggests that there was less neuroinflammation occurring in the hippocampus of mice that were graded as fast in their learning when exposed to short term sleep loss (Figure 5B and C) [35,36]. On the other side of things, Synaptophysin and BDNF were of interest to observe synaptic function and plasticity, respectively [37,38,39]. Results showed similar patterns as described previously, because levels of positive superpixels among the sleep deprived fast learners and control fasting learners were similar to one another (Figure 5B and C). The independent t-tests performed also show that the control group (Figure 5A) is relevant because the statistics are similar to the experimental group. We expected that a portion of the control group should also be slow learners to show the efficacy of the box maze and grading platform. It also shows that the resiliency of the sleep deprived fast learners were significantly different from the slow learners. Overall, the control group in our study showed similarity to the experimental group in light of the sleep deprivation that was given to the experimental group. Additionally, the heat map was able to visualize these results in a qualitative way to show that there were higher levels of super positive pixels as indicated by a color gradient (Figure 4) of red being high and blue being low. The heat map also provided additional insight on the distribution of staining at different areas of the hippocampus. Overall, the heat map served as a qualitative understanding of all four stains that were tested through immunohistochemistry. It complemented the superpixel positivity with a quick visual understanding of what was being expressed by the superpixel data.

Learning capability in sleep deprived fast learners showed a correlation suggestive of a decrease in hippocampal mediated molecular neuropathology.

When comparing individual box maze escape latency data of each mouse to the respective superpixel positivity generated from QuPath, a correlation can be observed in each group. Using the Pearson Correlation coefficient and p-value, each staining group was compared to the total percent of positive superpixels. MCP-1 had a negative Pearson Coefficient of -0.90 and a p value of <0.0001 (Figure 6A) which indicates that there is a strong negative correlation superpixel positivity decreases as R^2 value increases. This is suggestive of lower inflammation levels in mice that were faster in learning the maze. HDAC2 also had a negative Pearson Coefficient value of -0.84 and a p value of <0.0001 (Figure 6B) which also indicates a strong negative correlation of superpixel positivity with decreasing R^2 value. Since HDAC2 is a deacetylase complex, this association is suggestive of higher levels of transcriptional repression in DNA products that ultimately impaired learning in mice. Synaptic markers BDNF and Synaptophysin have opposite results in the both showed a strong statistically significant positive correlation in superpixel positivity and R^2 value (Figure 6C and D). BDNF has a Pearson coefficient of 0.91 and a p value of <0.0001 which shows that synaptic plasticity according to positive super pixel was increased in fast learning mice. Synaptophysin also showed similar results as it had a Pearson coefficient of 0.97 and was statistically significant with a p-value of <0.0001. This is indicative of increased synaptic plasticity according to positive superpixel values in mice that are faster learners. The correlations for all listed stains were statistically significant as indicated by their p-values. Directly comparing individual escape latency values with the individual QuPath superpixel positivity percentage shows that fast learners in the maze generally exhibit lower levels of inflammation and histone deacetylation, while synaptic plasticity and function are increased. These results therefore offer a statistically significant and novel way to measure levels of neural molecules in mice after being run through the box maze. These escape latencies can then easily be compared with QuPath positive superpixels as a quick way of investigating molecular pathways of learning impairment in the hippocampus.

Discussion.

The grading platform for learning in mice is an effective way to separate slow from fast learners in the box maze. With the R^2 threshold set at 0.74 through the means of averaging the escape latencies of n=80 control mice, the results of the grading platform produce a single value that can easily be compared to numbers of other variables of interest through the box maze. Ultimately, the grading gives a R^2 value that captures the totality of the box maze results (escape latency for four trials) for each individual mouse. This platform is also effective in separating fast and slow learning mice in both short term sleep deprived and control populations that were run through the box maze. Through this analysis, we found that n=6 mice out of n=20 that were sleep deprived were graded as fast in the platform. Control mice, as expected, had results that showed a higher proportion of fast learners n=15 to slow learners n=5.

The results from the grading platform indicate that the separation process yields expected results that can then be used for further investigation of different processes. Through utilizing immunohistochemistry, the results have shown that the grading platform in combination with the short term sleep deprivation box maze protocol produces results that are suggestive of a hippocampal learning impairment in slow learning mice. These results can be quickly visualized through a QuPath heat map that shows a difference in superpixel positivity among the different staining groups generated through the grading platform. Although the exact neural mechanisms of sleep deprivation were not investigated in this project, the heat map of the immunohistochemistry gives a better idea of positive staining in regards to location and intensity based on the novel idea of super pixels through QuPath and provides a glimpse of the molecular pathway involved.

QuPath is a new software so there is still much to be explored and refined with analyzing protocols. Currently, a PubMed search only brings up a total of 22 papers in total that have used this program to analyze whole image slides. Additionally, because QuPath is only an analyzing software, it is critical that immunohistochemistry protocols be performed in a uniform and consistent manner. This would require careful planning of tissue harvesting, sectioning, and imaging that would allow for the best results. Further molecular analysis should be performed to confirm the QuPath data so that conclusions can be drawn on the molecular pathway associated with hippocampal learning impairment observed in sleep deprived mice. QuPath was able to show a significant correlation between positive super pixels and box maze learning times through the R^2 value. These observations are therefore a valuable insight into utilizing the box maze learning classification of slow or fast in future studies looking into resilience to environmental stressors of sleep deprivation. QuPath can also be used as a novel application to study correlations between positive super pixels in immunohistochemistry slides from a variety of different protocols and tissues.

The model of short-term sleep deprivation in middle age mice is translationally relevant in that middle age men are observed to have reduced sleep according to EEG data in comparison to young men [47]. Mice, 16-18 months old, also represent the general population that are in their late 50's and early 60's [48]. This is an important age group to study resilience, as cognitive decline is often seen shortly after midlife and occurs noticeably at ages 70 or higher [49]. The data generated in this study offers valuable insight on the importance of intervention in individuals that are or are not resilient to stressors such as sleep deprivation to attenuate the impacts of cognitive decline that is often observed in the older population.

Figures with Figure legends.

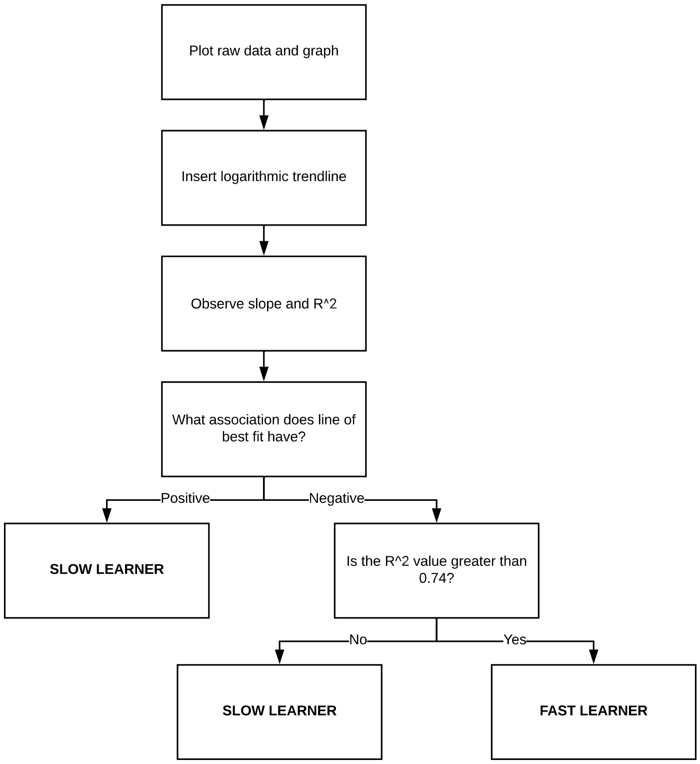

Figure 1. Visualization of the grading platform formatted as a flow chart. By starting from the square at the top and working down the flow chart each mouse was categorized as a slow or fast learner based on learning graph data. The total study population was n=40, and they were sorted as directed by the grading platform.

A. **B.**

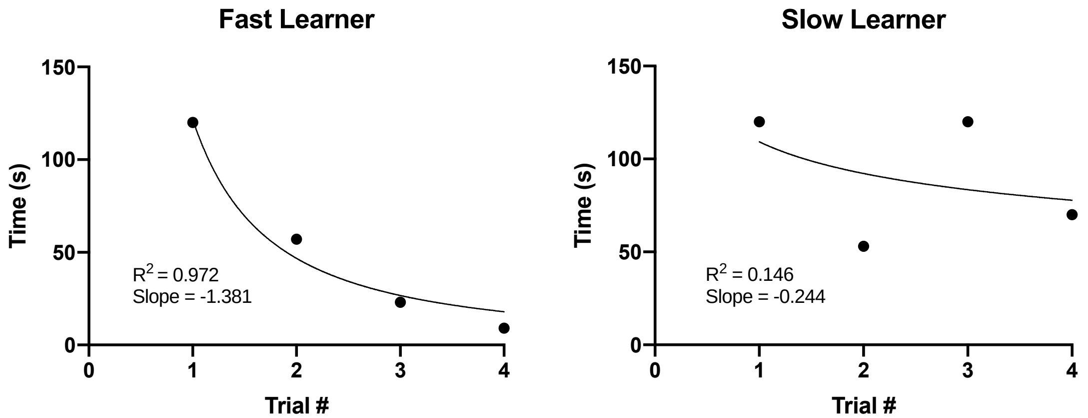

Figure 2. Example of a slow learner versus a fast learner. Data points were plotted onto a graph with a logarithmic trend line as directed by the grading platform. **(A)** shows a R^2 value of 0.97 and the individual data points do lie close to the trend line which ultimately indicates fast learning. **(B)** shows an example of a slow learner with a negative slope, and an R^2 value of 0.15.

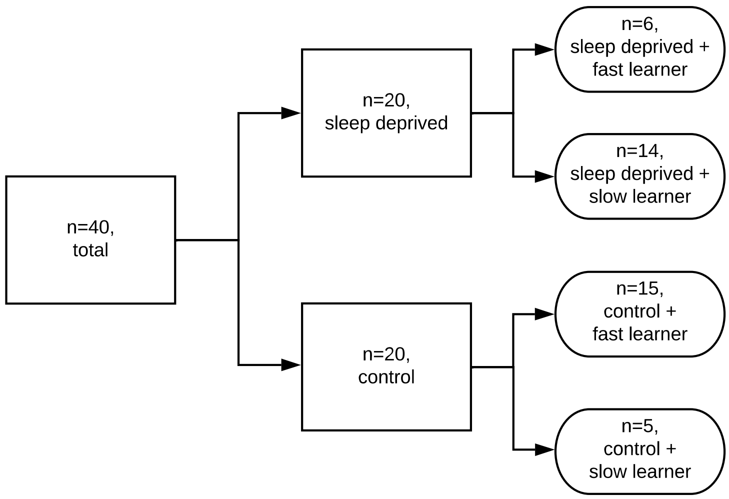

Figure 3. **Grading platform separation data**. Of a total of n=40 middle aged, C57BL/6 mice, n=20 were exposed to short term sleep deprivation whereas n=20 were a control group (non-sleep deprived). All n=40 were analyzed through the grading platform to classify each mouse as either a fast learner or a slow learner based on the R^2 value. In the n=20 sleep deprived mice, n=6 were classified as sleep deprived, fast learners whereas n=14 were sleep deprived, slow learners. In the n=20 control mice, n=15 were classified as control fast learners whereas n=5 were control, slow learners.

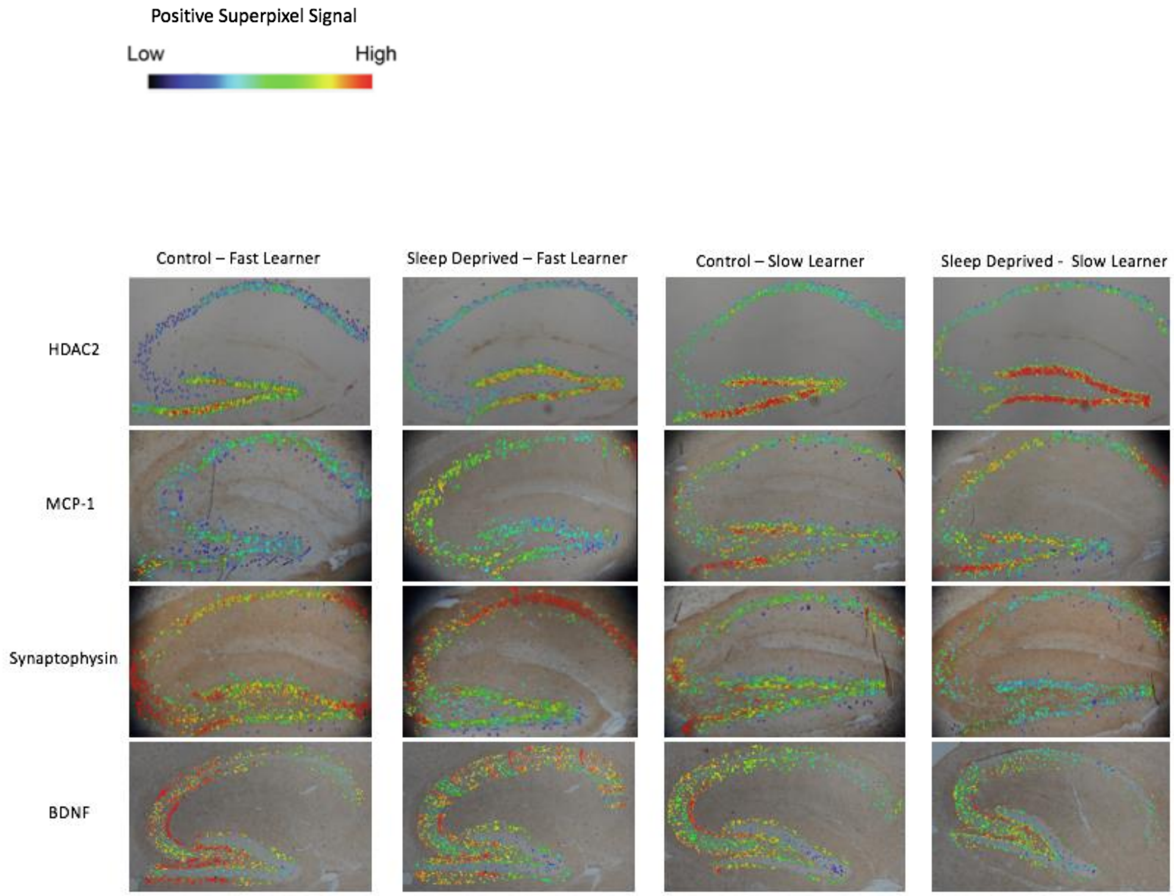

Figure 4. Sagittal hippocampal sections were imaged at 40x and analyzed using QuPath. Visual representation of positive superpixels using a heat map. Rows represent staining groups while columns show graded groups for control or sleep deprived mice. Staining intensity is based on positive superpixel signal with dark blue suggesting lower levels, green being moderate, and red as high. The rows with HDAC2 and MCP-1 show that fast learners had higher amounts of blue signal whereas slow learners showed more red. The rows with Synaptophysin and BDNF show that fast learners had higher amounts of red signal whereas slow learners showed much more blue and green.

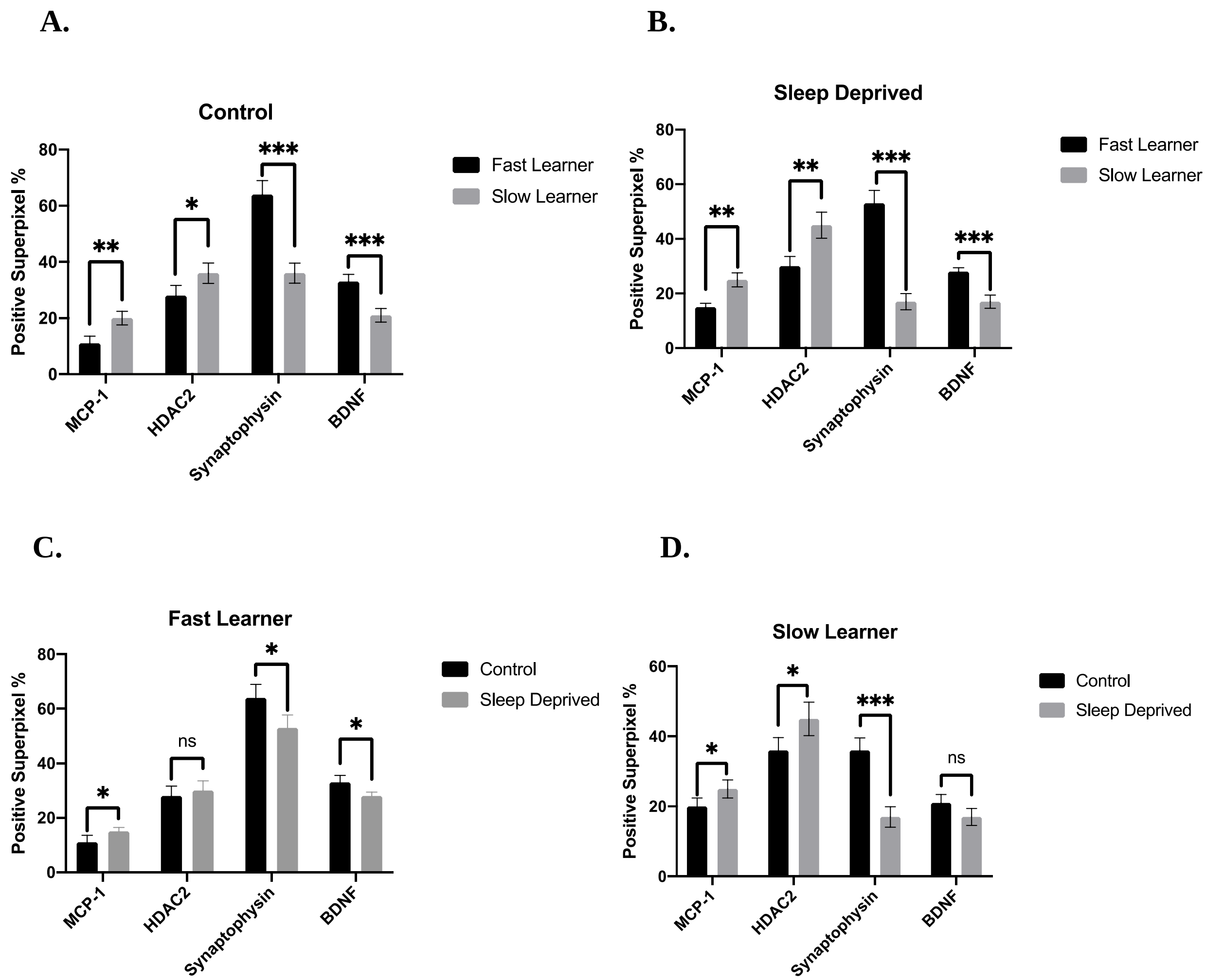

Figure 5. Positive Superpixel percentage in the hippocampus when stained with MCP-1, HDAC2, **synaptophysin, and BDNF**. (A) control mice, (B) sleep deprived mice, (C) fast learners, (D) slow learners. *Significant by Welch's T-Test of control to sleep deprived groups in light of fast and slow learners (A,B) then fast to slow learners in regards to control and sleep deprived mice (C,D).

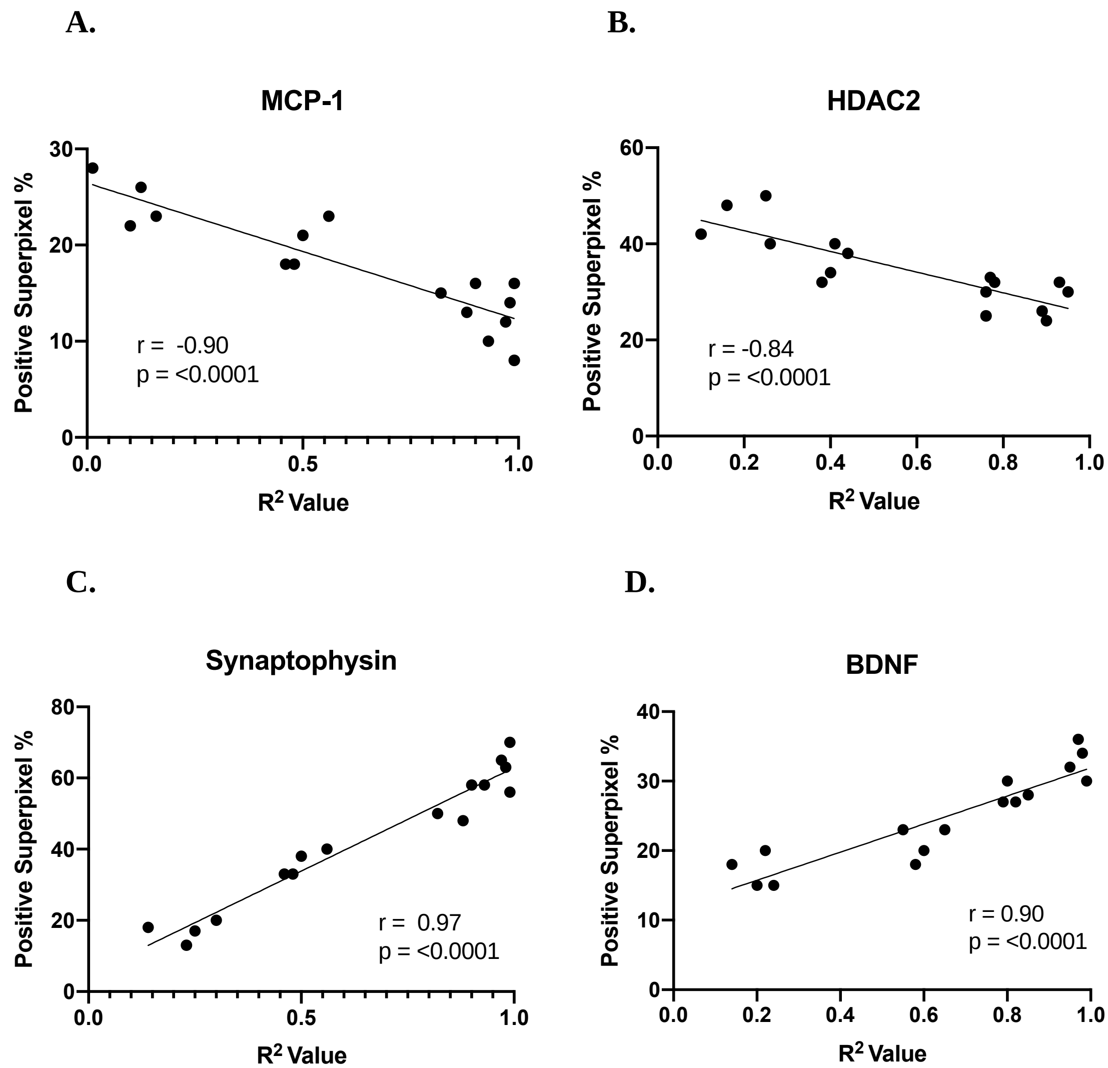

Figure 6. Positive superpixel percentage in each stain compared to the learning curve expressed by the R^2 value in each mouse. Pearson's correlation on **(A)** MCP-1 and **(B)** HDAC2 show a statistically significant negative correlation while **(C)** Synaptophysin and **(D)** BDNF show a statistically significant positive correlation.

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