Combined Effects of Binge Alcohol and Exercise on Intensity of Perineuronal Nets

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Introduction

- Excessive alcohol consumption has been shown to cause neurotoxicity within cortical regions among other areas of the brain (Barton et al., 2017).
- Exercise is a key component in the lives of many individuals who also consume alcohol and shows an ameliorating effect in the neurological changes induced by alcohol (Barton et al., 2017).
- PNNs are lattice-like protein structures that surround the soma and processes of neurons in the extracellular matrix.
- PNNs have been known to exist for over a century and seem to regulate two main functions: neuroplasticity and neuroprotection (Reichelt et al., 2019).
- Limited research indicates that the expression of PNNs increases after exposure to binge alcohol and decreases with physical exercise in several cortical regions (Reichelt et al., 2019; Smith et al., 2015).
- Given this connection, an experiment was conducted to determine the changes in perineuronal net intensity across subjects exposed to sedentary conditions, binge alcohol use, exercise, and a combination of the two.
- In this experiment, it was hypothesized that binge alcohol would increase cortical PNNs, exercise would result in decreased PNN intensity, but animals exposed to binge alcohol and exercise would not differ from the controls.

Results

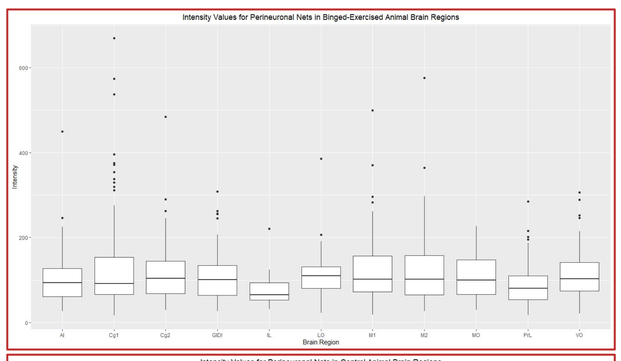

Figure 5: PNN Intensity variation in a binge-exercise animal (n=1) across brain regions of interest

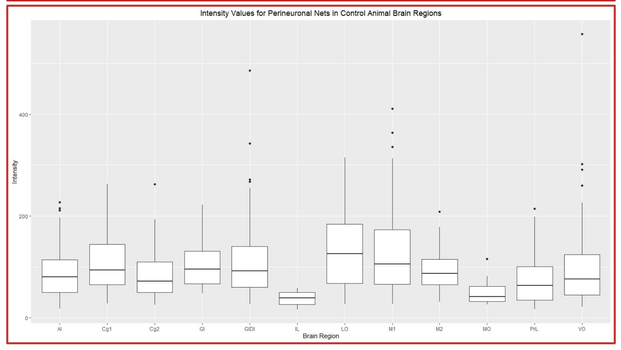

Figure 6: PNN Intensity variation in a control animal (n=1) across brain regions of interest

Methodology

Experimental Design

- Long-Evans Rat brain tissue (n=38) was acquired from previous work in the Leasure lab (West et al., 2019).
  - Weekly intragastric gavage ethanol exposure (5 g/kg) and control for 11 weeks
  - Exercise consisted of access to running wheels for 2 hours each for 3 days after the day of ethanol or control exposure
  - Experimental groups consisted of control, exercise, binge, and binge-exercise animals

Immunofluorescence

- Brain tissue was stained from 38 animals (bregma levels 4.20 mm to -9.12 mm) using fluorescein-conjugated *Wisteria Floribunda* Agglutinin (WFA; 10 ug/mL; Vector Labs) and Parvalbumin antibodies (1:2000; Novus Biologicals)

PNN Microscopy

- An Olympus BX51WI was used to capture Z-stacks (20X) for each region using the rat brain atlas (Paxinos & Watson, 2005); Agranular Insula (AI), Cg1 (Cingulate area 1), Cg2 (Cingulate area 2), GIDI (Granular/Dysgranular insula), IL (Infralimbic cortex), LO (Lateral orbital), M1 (Primary motor cortex), M2 (Secondary motor Cortex), MO (Medial orbital), PrL (Prelimbic cortex), and VO (Ventral orbital).
- Z-stacks were captured at 10 microns thick with a step size of 0.357 microns.

PNN Analysis

- ImageJ and the PIPSQUEAK AI plugin were used for Quantification.
- Z-stacks were converted to 8-bit format and summed to create the images for analysis.
- Methods were developed to ensure proper rating from each rater and ensure interrater reliability.
  - Background subtraction was standardized by ensuring ROIs were not placed around areas lacking tissue staining or containing PNNs (Figure 3).
  - In analysis where PIPSQUEAK AI did not properly select the PNNs within the image, selection parameters were standardized to ensure proper ROI designation around PNN structures (Figure 4).
  - PNN intensity is represented by the mean pixel values of the ROIs with the background pixel values subtracted to reduce noise to signal ratio.
  - The PNN intensity was then averaged for all PNNs within each image and compiled for each region. For each region with multiple bregma levels, the averages for each level (bregma 4.20 – 0.00 mm) were compared to determine if intensity varies for posterior and anterior location in addition to regional differences.

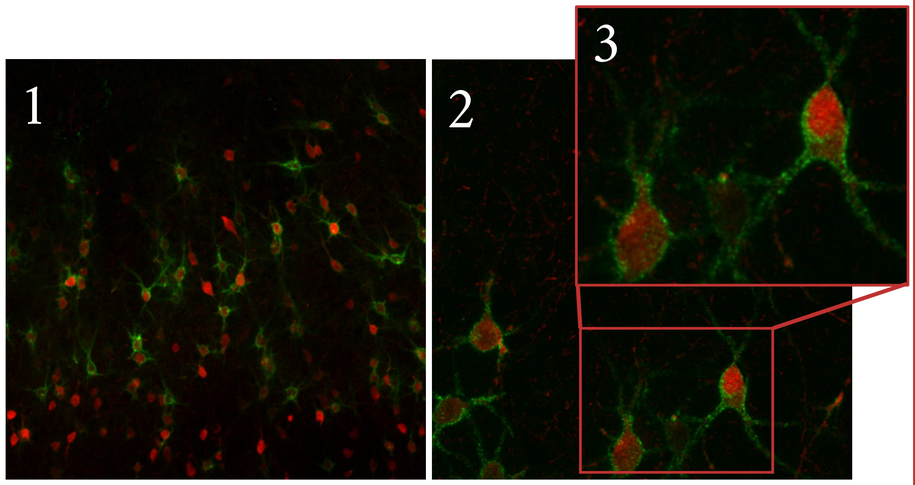

Figure 1: Brain tissue in Cg1 at 20x (1) and 63x (2) with immunofluorescent staining using fluorescein-conjugated WFA (10 µl/ml) to visualize PNNs (green) and Parvalbumin for PV+ inhibitory interneurons (red). A magnified portion of image 2 demonstrates the net like structure of PNNs (3).

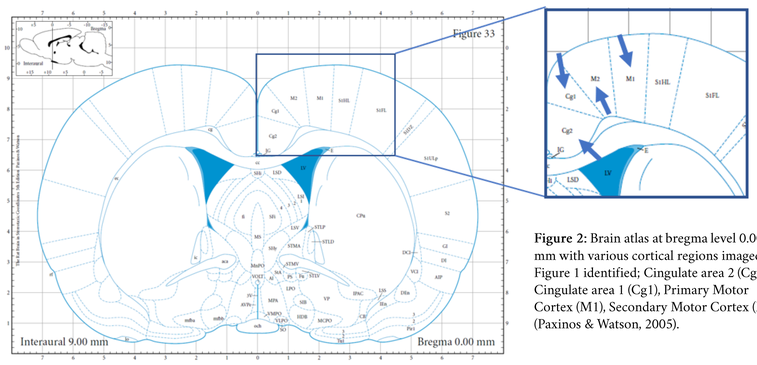

Figure 2: Brain atlas at bregma level 0.00 mm with various cortical regions imaged in Figure 1 identified; Cingulate area 2 (Cg2), Cingulate area 1 (Cg1), Primary Motor Cortex (M1), Secondary Motor Cortex (M2) (Paxinos & Watson, 2005).

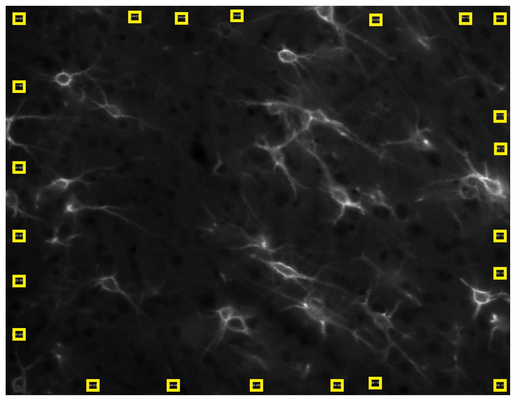

Figure 3: Background Subtraction was performed using ImageJ and PIPSQUEAK AI with 22 identically sized regions of interest (ROIs) to ensure valid interpretation of signal to noise ratio.

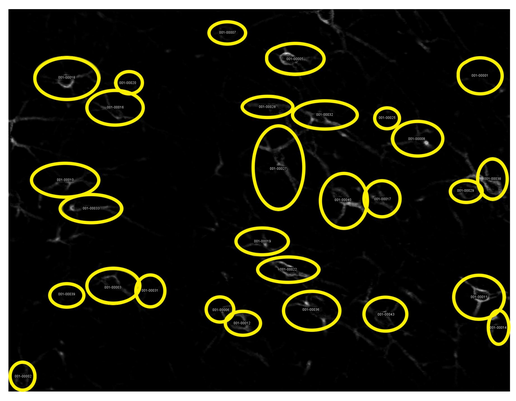

Figure 4: Fluorescent Intensity and PNN quantity were quantified using ImageJ and the PIPSQUEAK AI Plugin, followed by rater intervention to ensure accurate ROI selection.

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Discussion

- Regions in the binge-exercise animal that showed the largest differences from control include LO, Cg1, Cg2, M2, IL, and MO. An increase in the intensity of the PNNs was seen in Cg1, Cg2, M2, IL, and MO. LO contained a decrease in intensity.
- However, the sample size does not allow for a statistically significant evaluation of the interactions and effects of binge alcohol use and exercise.

Future Directions

- While the lack of data on the effects of these interventions restricts clear interpretation, this experiment serves to also further develop the methodological approaches within the field of research surrounding perineuronal nets.
- The use of PIPSQUEAK AI and ImageJ allows for a standardized replication of methods to create consistent findings across the work of others.
- The use of multiple bregma levels will allow for the understanding of distribution of PNNs throughout the brain moving from anterior to posterior.
- Further research will involve the further processing of the large set of Z-stacks captured from the 38 animal tissue samples used in this experiment.