Lipopolysaccharide induced neuroinflammation on Sprague Dawley hippocampi.

The subjects were given a seven-day habituation period prior to the start of the experimental period where the first dose of LPS and/or PBS was administered. The experimental period extended for 10 days. The 10 days was decided as it has been shown that neuroinflammation can be introduced and changes can be seen within seven days. Thus, the total housing period of the subjects was 19 days.

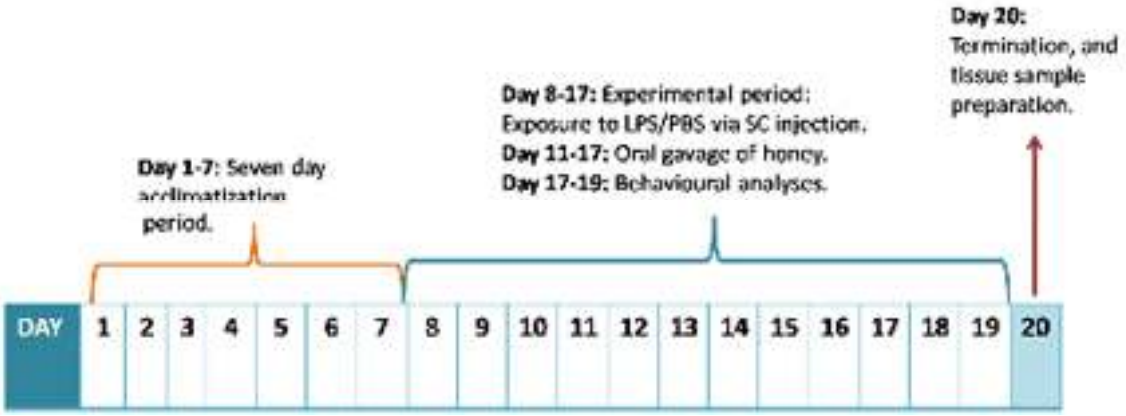


Figure 1: An overview of the procedures that were conducted throughout this study. During the seven-day acclimatization period, subjects were acclimatized to handling and the environment. During this 10-day experimental period, Manuka honey was introduced in the intervention groups (PBS+H group and LPS+H group) on day 11 via oral gavage Behavioural analyses were performed between day 17-19. Day 20 was reserved for termination and sample preparation.

The right hemisphere was used for biochemical analyses, while the left hemisphere was used for confocal microscopy. **Figure 2** summarizes the process of sample collection.

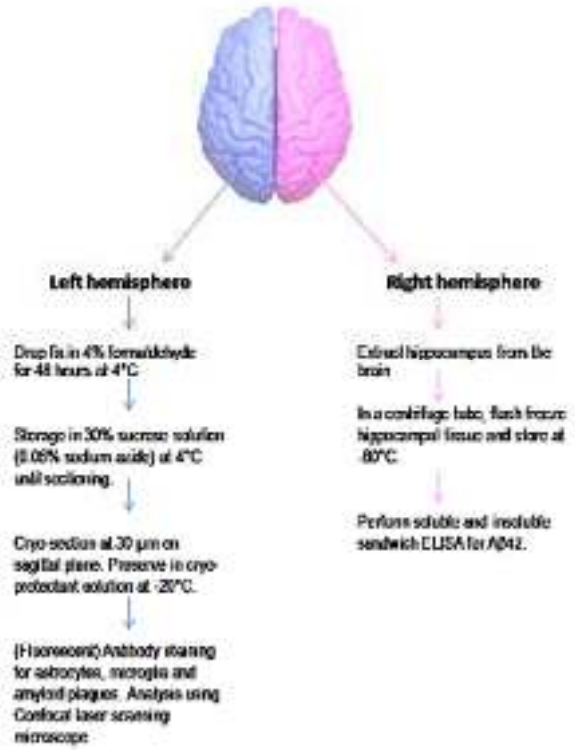


Figure 2: A summary showing the sequence of events followed to prepare hippocampal tissue samples for biochemical and histological assays after the subjects were terminated.

The subjects were randomly assigned into four sample groups, with each group containing ten subjects (n=10). The four groups and their treatment were assigned as indicated in **table 1**.

Table 1: Categorization of the experimental groups and the treatment received per group. SC=subcutaneous

Sample group name	Treatment type	Treatment received	Category
PBS	PBS only	Daily SC injection of 0.1M PBS at a volume of 0.1ml/kg for 10 days.	Control group

PBS + H	PBS and Honey	Daily SC injection of 0.1M PBS at a volume of 0.1ml/kg for 10 days + 0.5 ml honey* per kg of rat via oral gavage from day 4 until day 10.	Experimental group 1
LPS	LPS	Daily SC injection of 0.1M LPS dissolved in 0.1M PBS at a volume of 0.1ml/kg for 10 days.	Experimental group 2
LPS + H	LPS and Honey	Daily SC injection of 0.1M LPS dissolved in 0.1M PBS at a volume of 0.1ml/kg for 10 days. + 0.5 ml honey* per kg of rat via oral gavage from day 4 until day 10.	Experimental group 3

*The Manuka honey was mixed 50% v/v with distilled water to enable a comfortable consistency for the rats.

Behavioural analyses

Spatial recognition two-trial Y-maze test

Testing occurred in two trials inside a Y-shaped chamber of three arms distanced at an angle of 120° relative to each other. The subject was placed at the central mid-zone area where all three arms meet, and it is allowed to explore the maze freely.

Exploration trial**:** Plexiglas intercepted the novel arm of the maze, leaving only two arms available to explore. Subjects were brought in their housing cage into the laboratory testing

room and allowed to acclimatize for one and a half hours in the absence of individuals and the testing apparatus. The rat was then placed in the central mid-zone of the Y-maze and allowed to freely explore the two available arms for two minutes and 30 seconds. The subjects were given a four-hour inter-trial interval before the testing trial of the procedure was conducted.

Testing (recognition) trial**:** The Plexiglas intercepting the novel arm was removed, leaving all three arms available for exploration. Each rat was brought into the laboratory testing room and allowed to acclimatize for ten minutes. The rat was placed in the mid-zone of the Y-maze and allowed to freely explore all arms of the maze for two minutes and 30 seconds.

Given that this test relies on novelty seeking and the innate tendency of rodents to explore their surroundings, it is anticipated that they will spend more time in the previously inaccessible (novel) arm than the two familiar arms. Therefore, the ability to differentiate the novel arm from familiar ones is used as a marker of spatial recognition memory.[22]

Once the exploration and testing (recognition) trial were completed, the following parameters were considered for each arm of the maze:

i. Number of head entries
ii. Time spent within the arm
iii. The average speed
iv. The average number of visits
v. Time mobile
vi. Time immobile

Novel Object Recognition Test (NORT)

Apparatus**:** An open arena made from non-porous plastic with the following dimensions: a 65 cm (length) 60 cm (breadth) x 20 cm (height).

Training trial**:** This was conducted on day 17 of the experimental period. Each subject was brought in its housing cage into the laboratory testing room and allowed to acclimatize for ten minutes. The subject was placed at the centre of the arena and allowed to acclimatize for 30 seconds. Next, it was briefly removed from the arena while two identical objects (X + X), were placed inside the arena at opposite ends of each other (i.e., West and East). The subject was placed in between the two objects with its head facing the interior wall of the arena and

allowed to explore the identical objects for two minutes. Exploratory activity was assessed and used to evaluate memory retention and recall.

Testing trial**:** This was performed on day 18 of the experimental period. One of the familiar objects (X) used during the training trial together with a novel object (Y) was placed inside the open arena, at opposite ends of one other. The subject was brought in its holding cage into the laboratory testing room and immediately placed at the centre of the objects (X + Y) inside the arena with its head facing the interior wall of the arena. The rat was allowed to freely explore the environment and the familiar object (X) vs. novel object (Y) for two minutes, as seen in **figure 3**. Exploratory activity was assessed and used to determine memory recall. Exploration was defined as the sweeping or sniffing of the object with the rats' nose pointed up towards the object, within 3 cm or less from the object.

Figure 3: Testing trial of the novel object recognition test. The familiar object (A) and novel object (B) were placed at opposite ends. The test subject was allowed to explore each object for 2 minutes freely.

Open Field Analysis

Apparatus**:** A wall-enclosed Plexiglas chamber with the dimensions: 1 m (length) x 1 m (breadth) and a height of 0.5 m to prevent the rat from escaping the enclosure. Using ANY-maze Video Tracking System, the base was divided into 25 smaller squares of 20 cm x 20 cm, which comprised the outermost area. The outmost area was further divided into an innermost area of 85 cm x 85 cm. **Figure 4 (A)** illustrates the open-field arena during the assessment, whereas **Figure 4 (B)** is a visual representation of the apparatus as displayed on the video tracking software.

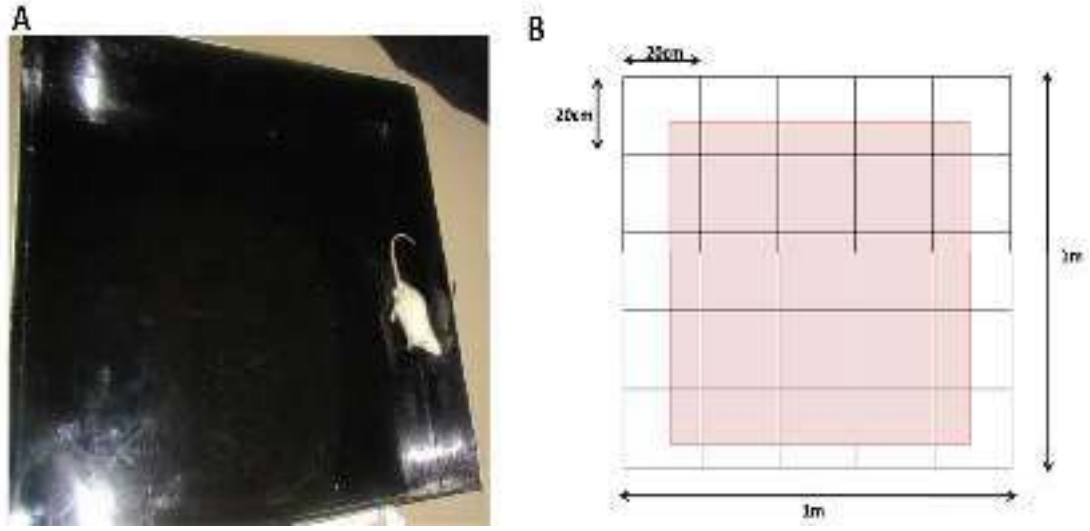


Figure 4: An illustration of the open-field arena with the subject inside (A). The surface of the open-field arena as depicted on Any-maze software (B). The outermost area comprises the entire 1 m x 1 m perimeter, and the innermost area includes the shaded region.

RESULTS

All statistical tests were completed using GraphPad™ Prism (version 9.2.0) for Windows (GraphPad™ Software, San Diego, California USA). The D'Agostino-Pearson normality test was used to establish that the dataset was normally distributed. Data was analysed using one-way analysis of variance (ANOVA). Post-hoc checks with Dunnett's multiple comparisons test were used to compare the three experimental groups to the control group. The results were represented as mean ± standard error of mean (SEM). Error bars indicate SEM. The p-value was set at $p < 0.05$, with a 95% confidence interval.

To characterize the effects of LPS-induced neuroinflammation on spatial working memory, learning capacity, and exploratory behaviour, the control group (PBS only) was compared to the three experimental groups (LPS only vs. LPS + H vs. PBS + H) using the Y-maze test, NORT and open-field test, respectively. Only significant results will be discussed.

Spatial recognition two-trial Y-maze test

Memory and learning impairments in the long arm of the y-maze were evaluated by analysing the number of head entries into the arm during the exploration and testing/recognition trial as shown in **table 1.** Data from the testing trials were compared to exploration trial. To determine the performance of subjects, the control group was compared against the experimental groups.

Table 1: A summary of the mean scores and p-values obtained when evaluating the average speed of the subject in the long arm of the Y-maze chamber during the exploration trial (Trial 1/2), testing/recognition trial (Trial 2/2), in comparison to the novel arm (Trial 2/2-Novel arm).

Summary of the descriptive statistics of the average speed-long arm				
Group	Trial	Mean	Adjusted P-value	Significant ?
PBS	Trial 1/2	0.07220	-	-
PBS vs. LPS		0.07110	0.9971	No
PBS vs. LPS+H		0.07000	0.9797	No
PBS vs. PBS+H		0.06610	0.7237	No
PBS	Trial 2/2	0.08440	-	-
PBS vs. LPS		0.07520	0.7589	No
PBS vs. LPS+H		0.06510	0.2314	No
PBS vs. PBS+H		0.07320	0.6408	No
PBS	Trial 2/2 (Novel arm)	0.06290	-	-
PBS vs. LPS		0.07030	0.3504	No
PBS vs. LPS+H		0.07520	0.0667	No
PBS vs. PBS+H		0.07630	0.0547	Yes *

Lastly, the time spent in a state of motion and the time spent immobile in the long and novel arm was evaluated for inter-trial and inter-group comparison. This is shown in **table 2** and **table 3. Table 2** shows significant differences ($p<0.0440$) and ($p<0.0237$) in the long arm during trial 2/2 was noted when the control group was compared to the LPS group and PBS + H group, respectively.

Table 2: A summary of the mean scores and p-values obtained when evaluating the time lapsed while the subject was mobile in the long arm of the Y-maze chamber during the exploration trial (Trial 1/2), testing/recognition trial (Trial 2/2) , in comparison to the novel arm (Trial 2/2-Novel arm).

Summary of the descriptive statistics of the time mobile-long arm				
Group	Trial	Mean	Adjusted P-value	Significant?
PBS	Trial 1/2	19.12	-	-
PBS vs. LPS		20.13	0.9403	No
PBS vs. LPS+H		21.05	0.7144	No
PBS vs. PBS+H		18.79	0.9976	No
PBS	Trial 2/2	13.18	-	-
PBS vs. LPS		8.780	0.0440	Yes *
PBS vs. LPS+H		10.88	0.4251	No
PBS vs. PBS+H		8.320	0.0237	Yes *
PBS	Trial 2/2 (Novel arm)	30.02	-	-
PBS vs. LPS		33.37	0.7187	No
PBS vs. LPS+H		29.81	>0.9999	No
PBS vs. PBS+H		31.19	0.9810	No

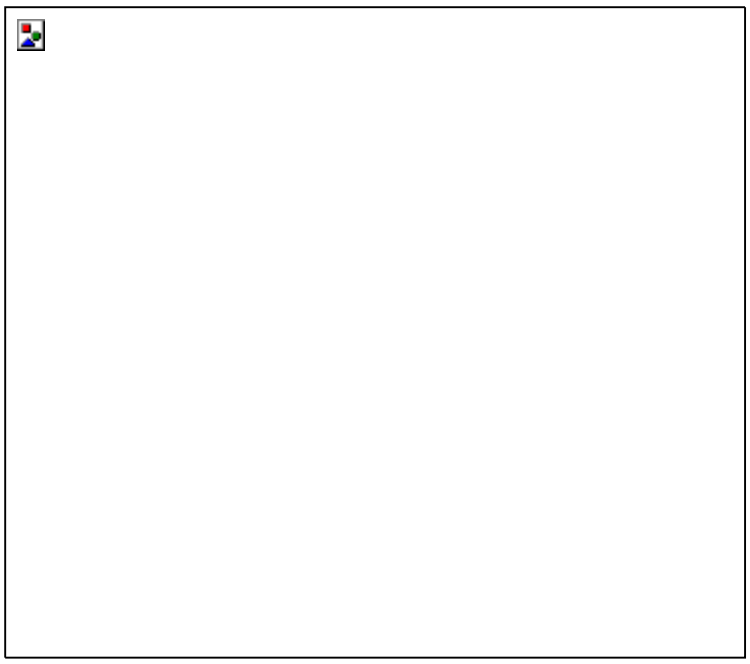
Table 3 shows that significant differences ($p<0.0183$) and ($p<0.0449$) in the novel arm during the testing trial were present when the control group was compared to the LPS + H group and PBS + H group, respectively.

Table 3: A summary of the mean scores and p-values obtained when evaluating the time lapsed while the subject was immobile in the long arm of the Y-maze chamber during the exploration trial (Trial 1/2), testing/recognition trial (Trial 2/2) , in comparison to the novel arm (Trial 2/2-Novel arm).

Summary of the descriptive statistics of the time immobile-long arm				
Group	Trial	Mean	Adjusted P-value	Significant ?
PBS	Trial 1/2	26.10	-	-
PBS vs. LPS		20.66	0.3107	No
PBS vs. LPS+H		22.08	0.5463	No
PBS vs. PBS+H		23.74	0.8487	No
PBS	Trial 2/2	14.97	-	-
PBS vs. LPS		15.76	0.9936	No
PBS vs. LPS+H		20.36	0.3636	No
PBS vs. PBS+H		13.05	0.9225	No
PBS	Trial 2/2 (Novel arm)	20.26	-	-
PBS vs. LPS		16.97	0.4828	No
PBS vs. LPS+H		12.50	0.0183	Yes *
PBS vs. PBS+H		13.51	0.0449	Yes *

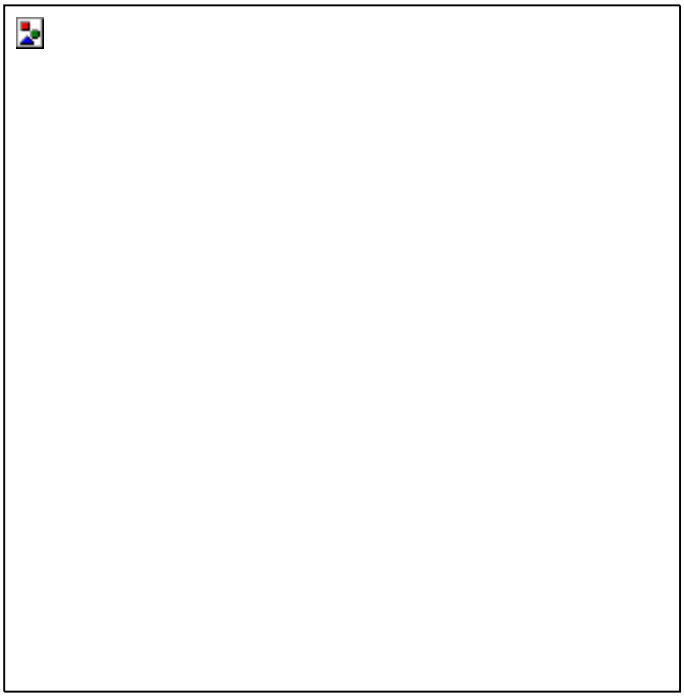
Next, the time spent in the familiar arm was analyzed and presented in **table 4**. A significant difference ($p<0.0072$) during the trial 2/2 in the familiar arm was noted when the PBS group was compared to the PBS + H group.

Table 4: A summary of the mean scores and p-values obtained when evaluating the average time spent in the familiar arm of the Y-maze chamber during the exploration trial (Trial 1/2), testing/recognition trial (Trial 2/2), in comparison to the novel arm (Trial 2/2-Novel arm).



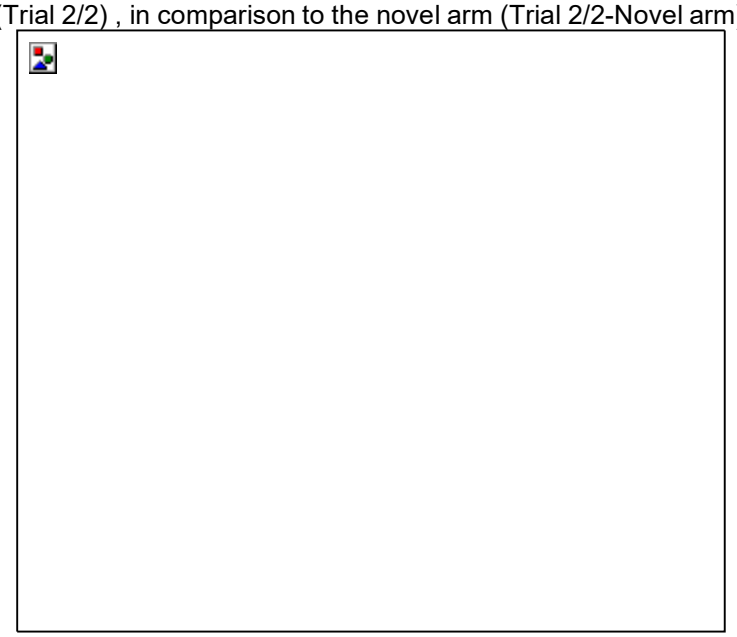
Then, the average speed scored within the familiar arm was assessed and presented in **table 5.** A significant difference ($p<0.0347$) during trial 2/2 in the novel arm was noted when the PBS group was compared to the PBS + H group.

Table 5: A summary of the mean scores and p-values obtained when evaluating the average speed of the subject in the familiar arm of the Y-maze chamber during the exploration trial (Trial 1/2), testing/recognition trial (Trial 2/2), in comparison to the novel arm (Trial 2/2-Novel arm).



However, when time immobile was analyzed in **table 6** Significant differences in the familiar arm was noted when the PBS group was compared to the PBS + H ($p<0.0132$) group during trial 2/2. Significant differences were also observed in the novel arm when the PBS group was compared to the LPS + H ($p<0.0183$) and PBS + H ($p<0.0449$) groups.

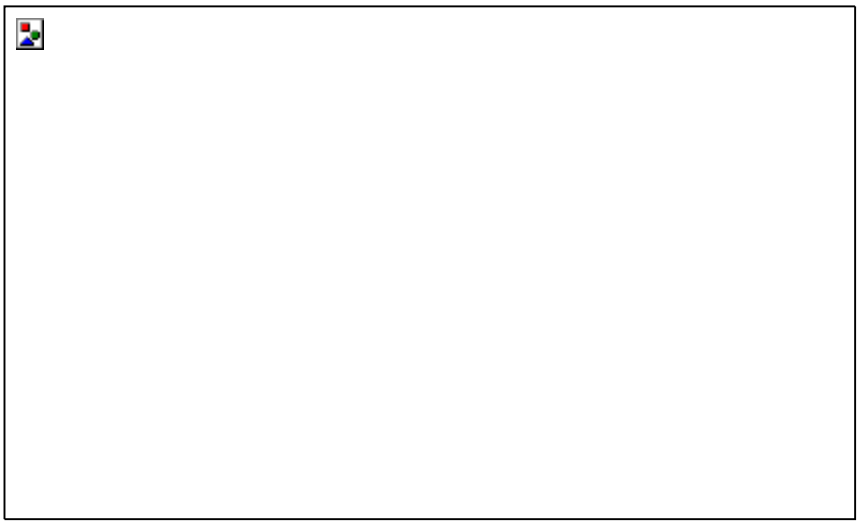
Table 6: A summary of the mean scores and p-values obtained when evaluating the time lapsed while the subject was immobile in the familiar arm of the Y-maze chamber during the exploration trial (Trial 1/2), testing/recognition trial (Trial 2/2) , in comparison to the novel arm (Trial 2/2-Novel arm).



Novel Object Recognition Test (NORT)

For the testing phase, a novel object was introduced into block B of the arena. To begin, the number of visits were analyzed during the testing/recognition phase of this assay. Data is presented in **table 7**. A significant difference ($p<0.0044$) in the number of entries in block B was noted when the control group was compared to the LPS group during the testing trial. This suggests that LPS treatment did not affect recognition memory in this trial since the LPS group entered the zone containing the novel object more often than the control group.

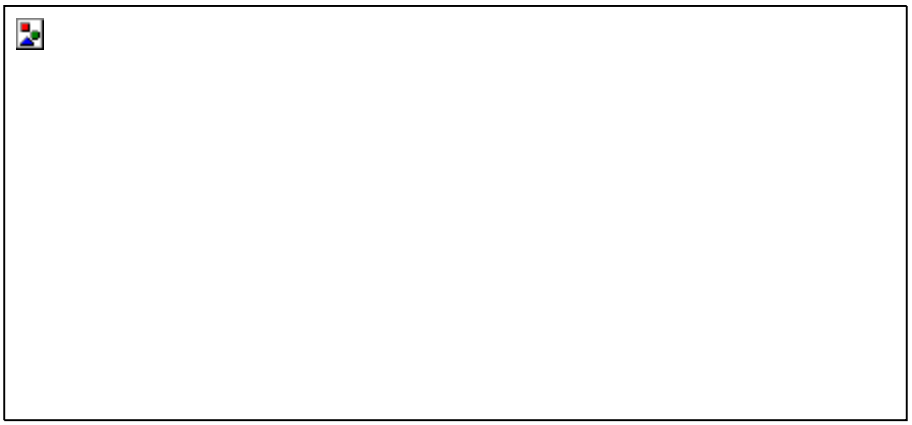
Table 7: A summary of the mean scores and p-values obtained when evaluating the number of entries made within the perimeter of Block A and Block B, during the testing trial



Open Field Analysis

Habituation was not required for this assay. To begin, the number of head within the outer zone during the training phase were analyzed and presented in **table 8.** As shown, the number of head entries was not significantly different across the groups.

Table 8: A summary of the mean scores and p-values obtained when evaluating the number of entries completed by the subject in the outer zone of the open-field arena during the testing trial.



For the central zone, the number of head entries made was analyzed and presented in **table 9.** Results show that the average was not significantly different between the groups**.**

Table 9: A summary of the mean scores and p-values obtained when evaluating the number of entries completed by the subject in the central zone of the open-field arena during the testing trial.

Altogether, the open-field analysis indicates that ten-day systemic exposure to 0.1 M LPS was not potent enough to induce anxiety-like behaviour and locomotor impairments.

Soluble $A\beta_{42}$ Assay

Biochemical analysis indicated that there are no significant differences between the four groups. **Figure 5** is a representative summary of the quantity of soluble $A\beta_{42}$ present.

The p-value indicates that the quantity of $A\beta_{42}$ detected in the hippocampi of the experimental group did not significantly differ from the amount detected in the control group after LPS-induced neuroinflammation.

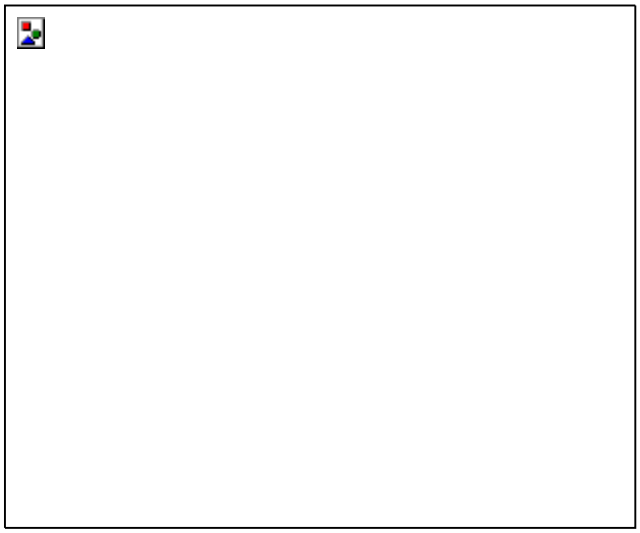


Figure 5: A dot plot (mean ± SEM) graph showing quantity of $A\beta_{42}$ peptide found in the hippocampal area using $A\beta_{42}$ sandwich ELISA . The p-value indicates that the of soluble Aβ present in the hippocampus did not signifantly differ between the groups.

Results reveal no statistical differences in $A\beta_{42}$ levels among the groups.

Immunofluorescence assay

To characterize the effects of LPS-induced neuroinflammation on hippocampal astrocytes, microglia, and amyloid presence, the control group was compared to the three experimental groups by immunostaining with GFAP, Iba1 antibodies (and CD68 for colabelling) and ThT stain respectively. Analysis of the mean scores, per group revealed non-significant differences between them, although notable differences in fluorescent intensity and quantity of cells was observed between the groups and thus discussed.

Sections stained with the GFAP antibody emitted a blue fluorescence that is observable on the microphotographs of **figure 6 (A-D)**.

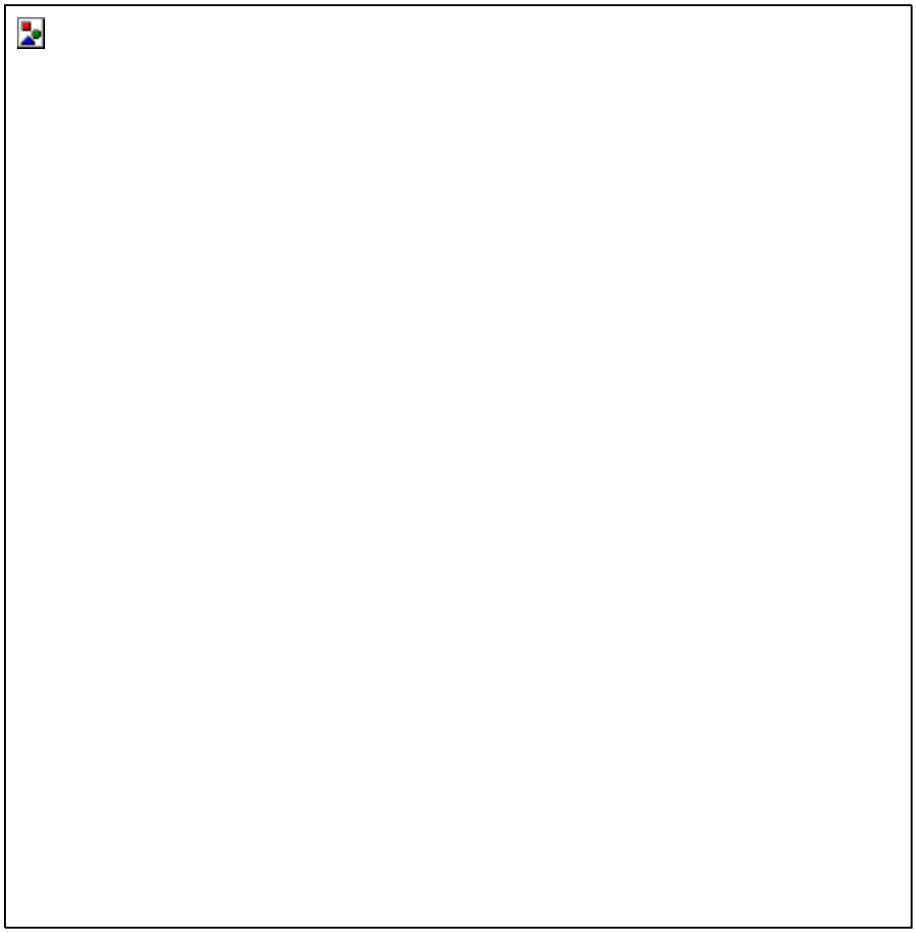


Figure 6 (A-D): Hippocampal microphotographs from the four groups stained with anti-GFAP. Scale bar was set at 10 µm. (A) Microphotograph from the control group. (Label 1) An astrocyte cell emitting slight fluorescence. (B) Microphotograph from the PBS + H group. (Label 2) An astrocyte cell emitting more fluorescence than the control group. (C) Microphotograph from the LPS group. (Label 3) Astrocytes showing a greater degree of fluorescence and cell quantity than the control group. (D) Microphotograph from the LPS + H group. (Label 4) Astrocytes emitting more fluorescence and cell quantity than the control group.

Sections stained with anti-Iba-1 antibody emitted a red fluorescence that is observable on the micrographs of **figure 6 (A-D)**. Here, activation was defined as an increase in fluorescent intensity, indicated by the CTCF value.

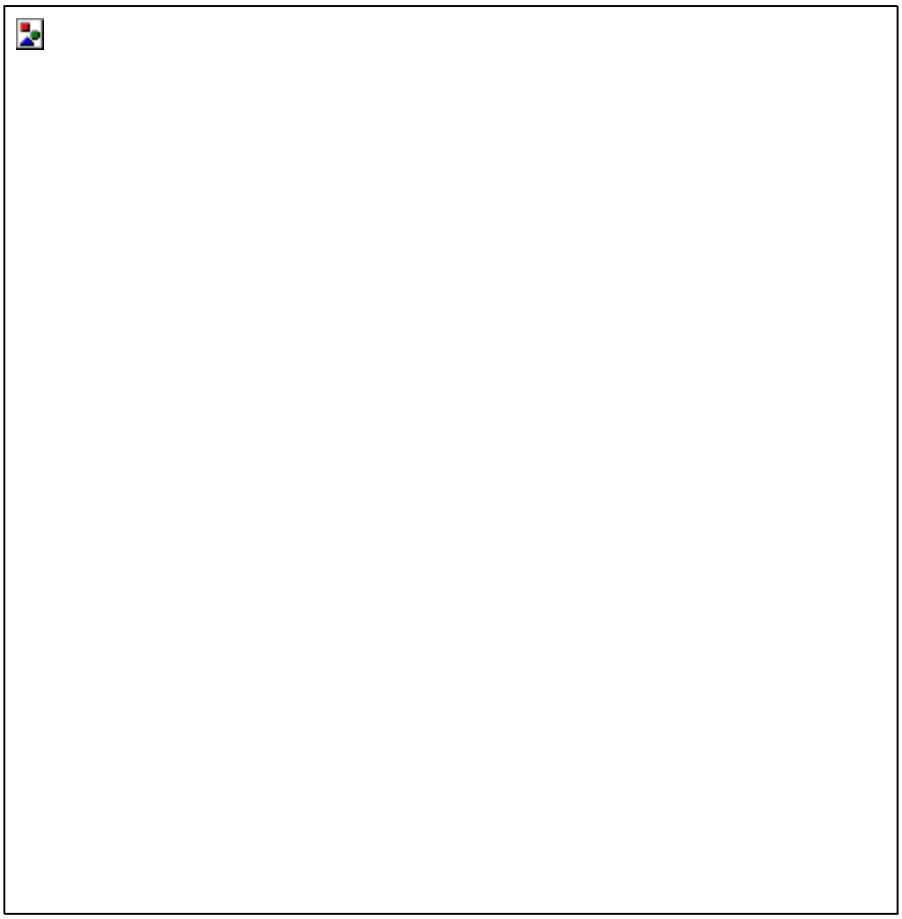


Figure 7 (A-D): Hippocampal microphotographs of the four groups stained with anti-Iba1. Scale bar was set at 10 µm. (A) Micrograph from the control group. (Label 1) Microglia showing slight activation (fluorescence). (B) Microphotograph from the PBS + H group. (Label 2) Microglia showing more activation than the control group. (C) Microphotograph from the LPS group. (Label 3) Microglia showing a significanty higher degree of fluorescence and cell quantity than the control group. (D) Microphotograph from the LPS + H group. (Label 4) Microglia showing a fair degree of fluorescence and cell quantity than the control group.

Sections co-labelled with Iba1 and CD68 antibody emitted a combination fluorescence that is observable as an orange on the micrographs of **Figure 7 (A-D)**. Here, microglial activation was defined as the co-localization of Iba-1-positive cells and CD68-positive cells.

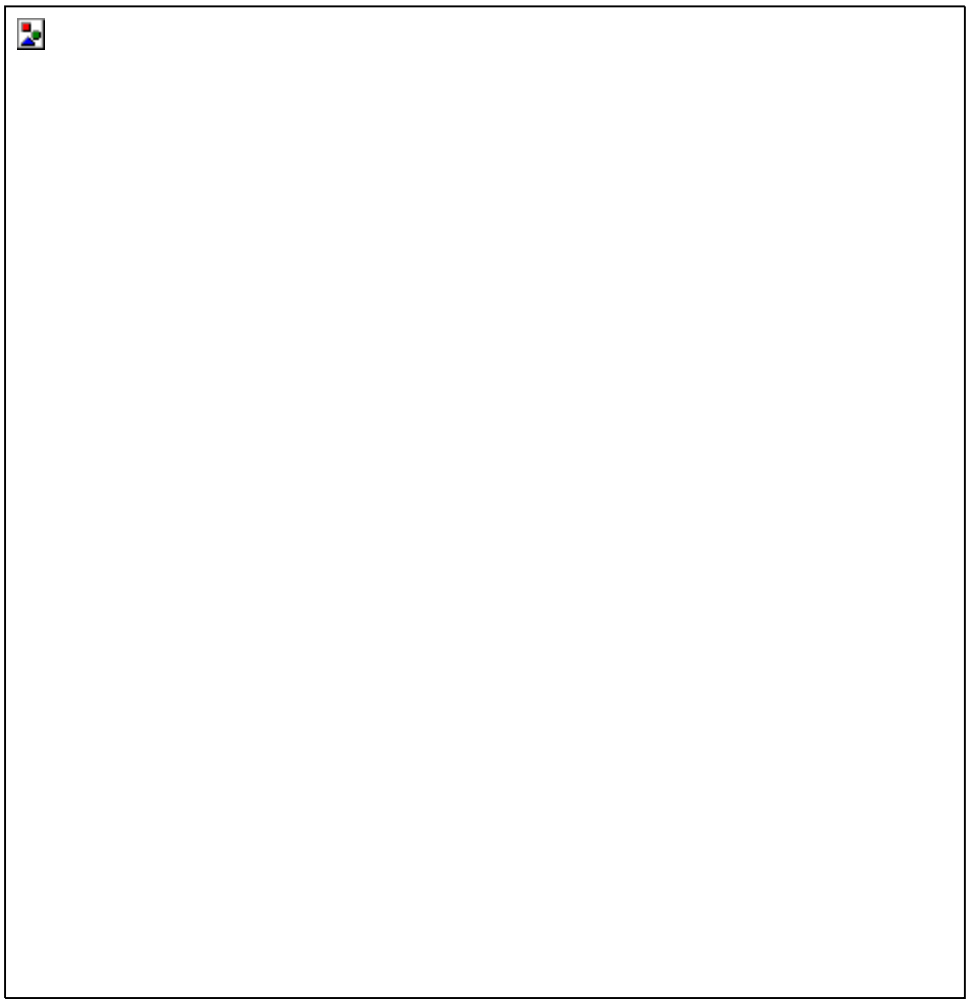


Figure 8 (A-D): Hippocampal micrographs from the four groups co-stained with anti-Iba1 and CD68. Scale bar was set at 10 µm. (A) Micrograph from the control group. (Label 1) Microglia showing minimal activation. (B) Micrograph from the PBS + H group. (Label 2) Microglia in close-proximity to CD68-labelled lysosomes. (C) Micrograph from the LPS group. (Label 3) CD68 positive microglia cells indicating a degree of microglial activation and cell quantity than the control group. (D) Micrograph from the LPS + H group. (Label 4) A CD68 positive microglia cell indicating a degree of microglial activation than the control group.

Sections stained with ThT stain emitted a green fluorescence observable on the representative Micrographs of **Figure 8 (A-D)**. Here, amyloid progression was determined by the comparing the CTCF values of the experimental groups to the control group.

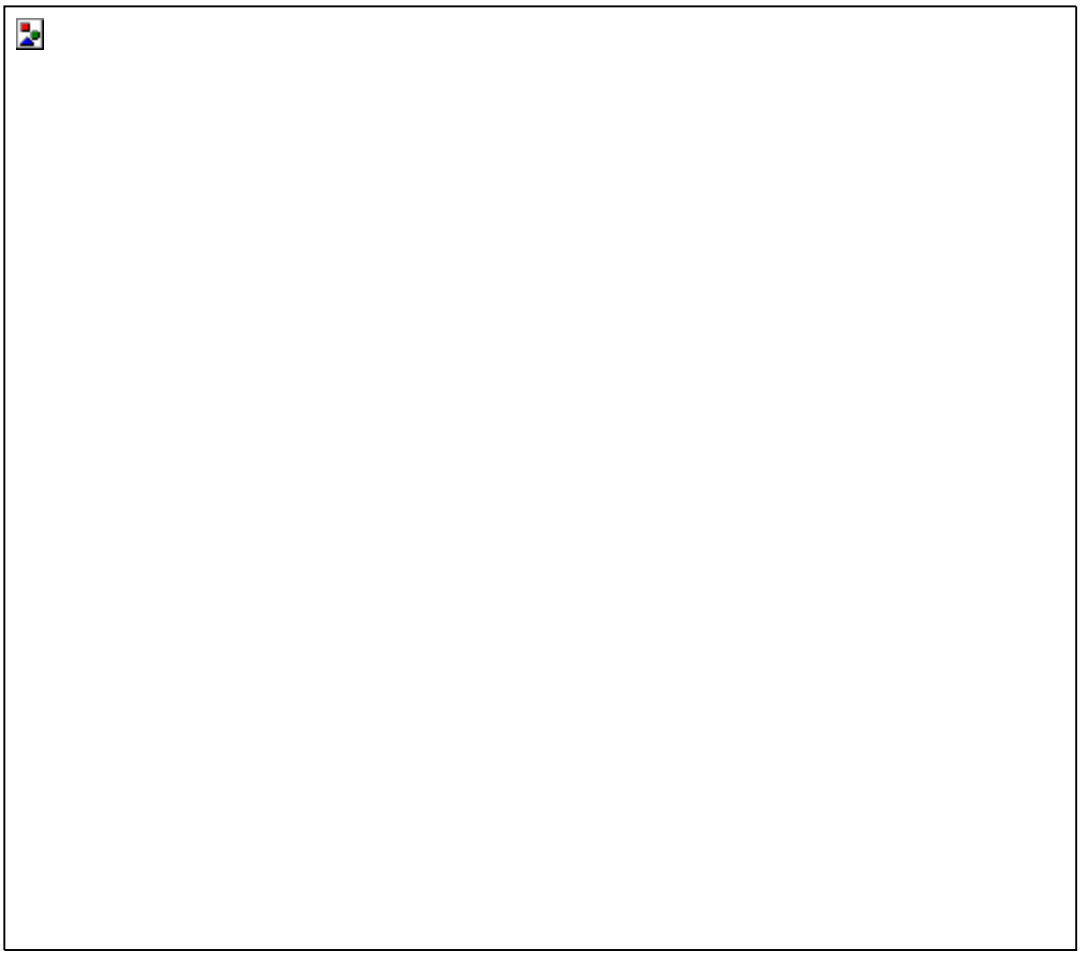


Figure 9 (A-D): Hippocampal Micrographs of the four groups stained with ThT. Scale bar was set at 10 µm. (A) Micrograph from the control group. (Label 1) Aβ fibrils staining positive with ThT. (B) Microphotograph from the PBS + H group. (Label 2) An amyloid fibril showing more fluorscent intensity than the control group. (C) Micrograph from the LPS group. (Label 3) Aβ fibrils appear to be increased than the control group. (D) Micrograph from the LPS + H group. (Label 4) Aβ fibrils showing less fluorescence and cell quantity than the LPS group.