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Research article

Neuroinflammation in the prefrontal-amygdala-hippocampus network is associated with maladaptive avoidance behaviour

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ABSTRACT

Maladaptive avoidance behaviour is often observed in patients suffering from anxiety and trauma- and stressor-related disorders. The prefrontal-amygdala-hippocampus network is implicated in learning and memory consolidation. Neuroinflammation in this circuitry alters network dynamics, resulting in maladaptive avoidance behaviour. The two-way active avoidance test is a well-established translational model for assessing avoidance responses to stressful situations. While some animals learn the task and show adaptive avoidance (AA), others show strong fear responses to the test environment and maladaptive avoidance (MA). Here, we investigated if a distinct neuroinflammation pattern in the prefrontal-amygdala-hippocampus network underlies the behavioural difference observed in these animals. Wistar rats were tested 8 times and categorized as AA or MA based on behaviour. Brain recovery followed for the analysis of neuroinflammatory markers in this network. AA and MA presented distinct patterns of neuroinflammation, with MA showing increased astrocyte, EAAT-2, IL-1β, IL-17 and TNF-α in the amygdala. This neuroinflammatory pattern may underlie these animals' fear response and maladaptive avoidance. Further studies are warranted to determine the specific contributions of each inflammatory factor, as well as the possibility of treating maladaptive avoidance behaviour in patients with psychiatric disorders with anti-inflammatory drugs targeting the amygdala.

1. Introduction

Avoidance behaviour is performing a specific motor response to prevent or protect the individual from an upcoming aversive event [1]. Maladaptive avoidance behaviour is often observed in patients suffering from anxiety and trauma- and stressor-related disorders [2], being a serious burden to public health and resulting in an impoverished quality of life. Maladaptive avoidance behaviour leads to impaired control of fear responses and is associated with altered network dynamics within the prefrontal-amygdala-hippocampus network (PFC-AMG-HC), a critical circuitry involved in fear-related behaviour and learning [1,3] [–] [5].

Furthermore, distinct levels of neuroinflammation in these areas could result in locally impaired cellular activity, leading to

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negative plastic changes in the PFC-AMG-HC network, resulting in maladaptive avoidance behaviour [6]. Neuroinflammatory responses are mainly regulated by glial cells (i.e., microglia and astrocytes) via the release of pro and anti-inflammatory mediators [7], being essential for the polarization of surrounding glial cells through autocrine and paracrine stimulation, which induces the classical activation of M1 macrophages [8]. In physiological conditions, glial cells participate in the modulation of the microenvironment by pruning synapses and regulating extracellular concentrations of metabolites and neurotransmitters [9,10], especially glutamate clearance via excitatory amino acid transporters (EAAT)-2 found in astrocytes [11]. However, pathological activation of glial cells could result in the release of an array of pro-inflammatory mediators [9] that disrupt physiological functions, such as neurotransmitter metabolization and synapse control [10], leading to edema and increased blood-brain barrier permeability [12,13]. Despite the advances in understanding the role of neuroinflammation in learning, memory and neuroplasticity, there is a need for studies describing the inflammatory pattern in the amygdala, HC and PFC associated with maladaptive avoidance behaviour.

The two-way active avoidance test is a well-established translational model to study avoidance behaviour [14]. In this paradigm, some animals will learn the task and display appropriate and adaptive avoidance behaviour, while others will exhibit an increased fear response to context and maladaptive avoidance behaviour [3,15] [–] [17]. Considering that neuroinflammation among the PFC-AMG-HC network could be responsible for this behavioural distinction, here we investigated the neuroinflammatory signature of this network by assessing pro- and anti-inflammatory mediators, astrocytes and microglia density, and the expression of EAAT-2.

2. Methods

2.1. Animals and husbandry

A total of 36 male Wistar rats (weighing 200–250 g) from the University of Sao Paulo animal facility were used in this study. The rats were housed in standard rat cages (max of three rats per cage) for one week before the experimental procedures for habituation to the animal facility. The animals were maintained in appropriate housing conditions with controlled light/dark cycle (12/12 h) and temperature (22 ± 2 °C), wood shavings covering the cage floor and animals had free access to water and food. All experiments were conducted and reported in accordance with the ARRIVE guidelines. The Ethics Committee on the Use of Animals at Hospital Sirio Libanes approved the protocols used in this project (CEUA#2013/12).

2.2. Experimental design

Fig. 1 illustrates the study design. Animals were randomly assigned to experimental or control groups. Animals in the experimental group were tested for 8 consecutive days (25 min/day) in the two-way active avoidance test, and animals in the control group were exposed to the test apparatus for the same time/days as the experimental group; however, the footshock was turned off (Controls, n = 12). The experimental animals that learnt the test and, therefore, showed adaptive avoidance behaviour were included in the Adaptive Avoidance group (AA; n = 12), while the ones that showed a high fear response and maladaptive avoidance behaviour were included in the Maladaptive Avoidance group (MA; n = 12). Immediately after the behavioural experiment on the 8th day, animals were euthanized by transcardiac perfusion or euthanized for fresh tissue collection for the evaluation of astrocytes, microglia, pro- and anti-inflammatory mediators and EAAT-2 in the PFC, AMG, and HC.

2.3. Two-way active avoidance test

The test was performed as previously described [15,18]. Briefly, animals were tested 8 consecutive days (one 25-min session per day) in a two-way shuttle box placed inside a soundproof isolation cubicle (Insight Equipment, Brazil). The two-way shuttle box comprises two identical chambers connected through an open door in the middle of a partition wall. The grid floor delivers the scrambled foot shocks, and infrared beam sensors detect the animals' movement and transition between compartments. Moving between compartments (shuttling) delayed the delivery of the footshock (0.6 mA; 0.5 s) by 30 s; however, in the absence of shuttling, the footshock delivery occurred every 5 s. Adaptive avoidance responses were defined as shuttling before the delivery of the footshock (i.

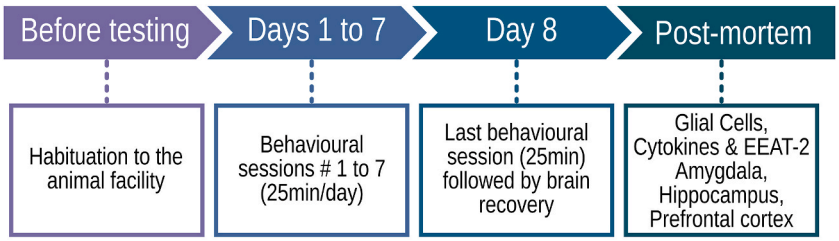

Fig. 1. Study design. Following habituation to the animal facility, rats were tested in the two-way active avoidance test. Brain recovery was performed to investigate the neuroinflammatory signature of the avoidance network by assessing pro- and anti-inflammatory mediators, astrocytes and microglia density, and the expression of excitatory amino acid transporter 2 (EAAT-2) in the amygdala, hippocampus and prefrontal cortex. Full blots can be found in Supplementary Fig. 2.

e., response to stimulus interval shuttles). In contrast, escape responses were considered a shuttle after the footshock delivery (i.e., stimulus-to-stimulus interval shuttles). All shuttles produced 0.3 s feedback stimuli (apparatus light blink). Controls animals were exposed to the two-way shuttle box for the same time/days as described above, but the footshock was turned off. The experimental animals that showed adaptive avoidance behaviour were included in the Adaptive Avoidance group (AA; >20 adaptive avoidance responses in two consecutive days), while the ones that showed a high fear response and maladaptive avoidance behaviour were included in the Maladaptive Avoidance group (MA; <20 adaptive avoidance responses) [15,18]. Freezing was defined as the absence of movement except that required for breathing, and it was assessed during the first 5 min of the test [15,19]. The behavioural evaluation was performed by an observer blinded to group allocation. Group allocation into MA or AA was determined at the end of behavioural experiments.

2.4. Euthanasia and tissue collection

After the last behavioural test (i.e. 8th day), animals were randomly divided into two brain recovery types; fixated tissue was collected via transcardial perfusion, and fresh tissue was recovered following decapitation. Perfusion was performed with deeply anesthetized rats (ketamine/xylazine, 0.5/2.3 mg/kg, i.p.; morphine 10 mg/kg, i.p.) initially with 0.9 % phosphate-buffered solution, followed by 4 % paraformaldehyde solution (PFA) dissolved in 0.1 M phosphate buffer (PB, pH 7.4). After perfusion, the brains were collected and stored in 4 % paraformaldehyde (PFA) dissolved in 0.1 M sodium phosphate buffer for 4 h. Then, the brains were transferred to a 30 % sucrose solution in PB and, 48h after, cut in a freezing sliding microtome (40 μm thickness). Fixed tissue was used for immunohistochemical assays to detect glial cells (i.e., astrocytes and microglia) in the PFC, AMG, and HC. Fresh brain tissue was dissected on an ice-cold metal matrix (1.0 mm gap, coronal plane) and then placed on a Petri dish on ice. The PFC, AMG, and HC were recovered and stored at −80 °C until analysis aimed at evaluating the expression of pro- and anti-inflammatory markers and EAAT-2.

2.5. Immunohistochemistry

Immunohistochemistry was performed as previously described (Antunes et al., 2020). Immunoreactivity to GFAP (IR-GFAP - a marker for astrocytes) and Iba-1 (IR-Iba1 - a marker for microglia) was evaluated in the PFC (from bregma 3.72 mm–2.76 mm; infralimbic and prelimbic regions), AMG (from bregma −2,28 mm to −2,76 mm; Medial, Basolateral, Basomedial, Central and Lateral subnuclei), and HC (from bregma −2,76 mm to −3,48 mm; CA1, CA2 and CA3 fields) [20]. Briefly, selected tissue was incubated overnight at room temperature with mouse anti-GFAP (1:1000, Sigma-Aldrich) or rabbit anti-Iba-1 (1:1000, Wako). After washing, the tissue was incubated in appropriate biotinylated secondary antibody (1:200, Jackson ImmunoResearch) at room temperature for 2h, followed by incubation in avidin-biotin-peroxidase complex (ABC, Vector Laboratories) for 90 min at room temperature. Labelling was visualized with diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich). Finally, images were captured using a light microscope (Eclipse E1000, Nikon, NY, USA). Additionally, a separate slide was used for negative reagent controls, where the primary antibody was omitted to guarantee that the label observed was specific to the protein targeted. Adjoining Nissl-stained sections provided histological landmarks for accurately identifying and delineating the different nuclei. Analysis was performed using ImageJ software (National Institutes of Health, MD, USA; http://rsbweb.nih.gov/ij/) to cover the entire region of interest. Following the determination of the threshold, the "analyze particles" plugin in the ImageJ software was used (median size of 2–3 mm^2 and circularity of 0.25–1.0).

2.6. Enzyme-linked immunosorbent assay - ELISA

Brain tissue was processed using a radioimmunoprecipitation buffer (RIPA, 50 mM Tris, 150 % mM, 1 mM ED TA, 0.5 % deoxycholate Cl, NP-1%) with protease inhibitors (Sigma). Total protein levels were quantified in each sample using Bradford assay, normalized by the amount of protein, and stored at −80 °C until analysis. Samples from the PFC, AMG, and HC were evaluated to determine the levels of the cytokines interleukins IL-1β, IL-6, IL-10, IL-17, TNF-α, and the chemokine CX3CL1. Duplicate samples were processed using ELISA commercial kits following the manufacturer's recommendations, and readings were performed on the Infinite M200 PRO® spectrophotometer equipment at the target wavelength (450 nM) and the correction wavelength (620 nM). After normalization with correction wavelength, duplicates were averaged, and the blank control was subtracted. A four-parameter logistic standard curve was generated, and the concentration of each sample was calculated.

2.7. Western blotting

Tissue samples from the PFC, AMG, and HC were diluted in Laemmli buffer for separation by SDS-PAGE. After electrophoretic separation, the proteins were transferred to a nitrocellulose membrane (0.2 μm in diameter, Millipore). After blocking with 5 % BSA in Tris-Saline buffer for 1 h, membranes were incubated overnight (12–16 h) with the rabbit anti-EAAT2 antibody (1:2000, Millipore) followed by the appropriate secondary antibody (1:2000, Amersham Biosciences). The excess conjugate was removed with an additional wash cycle, and membranes were developed using the ECL chemiluminescence kit (Amersham Biosciences). β-actin labelling (1:1000, Millipore) was used as a loading control. The signals for EAAT2 and β-actin were quantified in each lane, and the background signal was subtracted from the signal of each individual band. Then, a lane normalization factor was calculated by dividing the β-actin signal in each lane by the highest observed β-actin signal on the blot. The normalized signal of EAAT2 was calculated by dividing the observed signal intensity by the lane normalization factor. Then, the average count of the control group was normalized to 100 %, and the percentage of change in each group was calculated.

2.8. Statistical analysis

The sample size of this study was established by considering the optimal number of animals for each experimental group [21]. Results are expressed as mean ± standard deviation. Data were analyzed using GraphPad Prism (CA, USA), and statistical significance was assessed using two-way ANOVA repetitive measures for behavioural measures (i.e., avoidance and freezing scores) and one-way ANOVA for neurochemical evaluation (i.e., Histology, ELISA and Western-blotting) followed by Newman-Keuls multiple comparison post hoc test. For all analyses, statistical significance was set at $p < 0.05$.

3. RESULTS

3.1. Behavioural characterization

During the behavioural sessions, the animals were divided into those who showed adaptive avoidance behaviour (AA group) and those who showed high fear response to the test environment and maladaptive avoidance behaviour (MA group). While significant adaptive avoidance behaviour differences between groups were observed from the 3rd training section until the end of testing ($F_{(1,190)} = 13.42$; $p < 0.001$; Fig. 2A), significant differences between groups in freezing behaviour (i.e. fear response to the environment) was detected as early as the first day of training ($F_{(2,105)} = 4.81$; $p = 0.001$; Fig. 2B).

3.2. Glial cells

Astrocyte and microglia density, as well as expression of EAAT-2 (amino acid transporter involved in glutamatergic regulation), were investigated in the AMG, HC, and PFC of animals in the AA, MA and control groups (Table 1, Supplementary Figures 1-3). Differences between AA and MA groups were found in the AMG, with MA animals showing increased astrocyte density in the AMG (Total AMG: $p < 0.001$, Fig. 3A; Central nucleus: $p < 0.01$, Fig. 3B; Lateral nucleus: $p < 0.001$, Fig. 3C) along with increased EAAT-2 expression in the same nucleus ($p < 0.05$, Fig. 3D). Control animals showed increased astrocyte density and reduced microglia density in the HC compared to both MA and AA groups (Table 1, Supplementary Figures 1-3). No further differences were observed.

3.3. Pro- and anti-inflammatory mediators

Pro- and anti-inflammatory mediators were investigated in the AMG, HC, and PFC of animals in the AA, MA and control groups (Table 2, Supplementary Figure 4). While animals in the MA group showed increased pro- and anti-inflammatory mediators in the amygdala (IL-1β: $p < 0.001$, Il-17: $p < 0.001$, and TNF-α: $p < 0.001$, Fig. 4A), animals in the AA group presented changes in the PFC (Increase: IL-6: $p < 0.001$, IL-10: $p < 0.05$ and IL-17: $p < 0.001$, Fig. 4B; Reduction: CX3CL1: $p < 0.05$, IL-1beta: $p < 0.001$ and TNF-α: $p < 0.001$, Fig. 4C). No further differences were observed.

4. Discussion

The present study aimed to investigate a neuroinflammatory signature in the PFC-AMG-HC network that could underlie the maladaptive avoidance behaviour and the high fear reaction to context observed in a portion of animals tested in the two-way active avoidance test. In this test, the number of adaptive avoidance responses is scored along with the pattern of fear response exhibited to

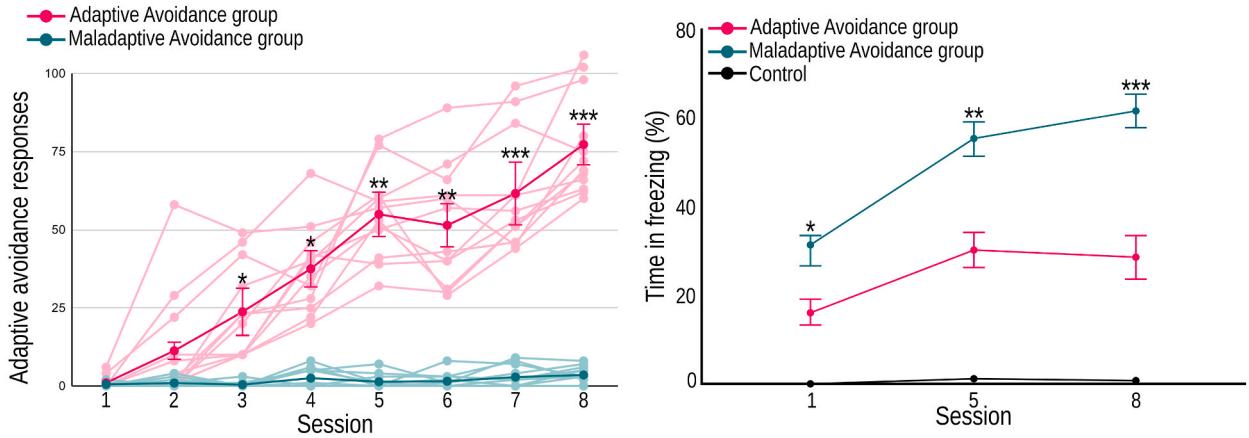

Fig. 2. Behavioural characterization. A. Number of adaptive avoidance responses exhibited by rats throughout the testing sessions. B. Percentage of time spent in freezing behaviour during the testing sessions 1, 5 and 7. Animals showing maladaptive avoidance behaviour spent significantly more time in freezing (fear response) than in other groups. Data are shown as average ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table 1
Astrocyte and microglia density and EAAT-2 expression in the Amygdala, Hippocampus and Prefrontal Cortex.

Astrocyte Density			
Amygdala	Basolateral	$F_{(2,15)} = 0.54$, $p > 0.05$	CTRL (37 ± 13); AA (42 ± 9); MA (43 ± 8)
	Basomedial	$F_{(2,15)} = 2.20$, $p > 0.05$	CTRL (54 ± 5); AA (58 ± 4); MA (66 ± 16)
	Central	$F_{(2,15)} = 7.82$, $p < 0.01$	CTRL (57 ± 18); AA (52 ± 17); **MA (86 ± 12)****
	Lateral	$F_{(2,15)} = 16.28$, $p < 0.001$	CTRL (9 ± 3); AA (13 ± 4); **MA (30 ± 10)*****
	Medial	$F_{(2,15)} = 0.25$, $p > 0.05$	CTRL (113 ± 10); AA (111 ± 14); MA (115 ± 6)
	Total	$F_{(2.15)} = 18.49$, $p < 0.001$	CTRL (54 ± 4); AA (55 ± 6); **MA (68 ± 3)*****
Hippocampus	CA1	$F_{(2,15)} = 33.71$, $p < 0.001$	**CTRL (167 ± 16)***; AA (44 ± 23); MA (66 ± 39)
	CA2	$F_{(2,15)} = 5.89$, $p < 0.05$	**CTRL (88 ± 36)***; AA (24 ± 18); MA (56 ± 38)
	CA3	$F_{(2,15)} = 8.27$, $p < 0.01$	**CTRL (119 ± 24)****; AA (70 ± 20); MA (77 ± 23)
	Total	$F_{(2,15)} = 39.93$, $p < 0.001$	**CTRL (124 ± 14)***; AA (46 ± 14); MA (66 ± 18)
Prefrontal cortex	Infralimbic	$F_{(2,15)} = 0.08$, $p > 0.05$	CTRL (43 ± 12); AA (45 ± 12); MA (44 ± 3)
	Prelimbic	$F_{(2,15)} = 0.17$, $p > 0.05$	CTRL (46 ± 3); AA (44 ± 15); MA (43 ± 6)
	Total	$F_{(2,15)} = 0.03$, $p > 0.05$	CTRL (44 ± 7); AA (44 ± 8); MA (44 ± 2)
EAAT-2 Expression			
Amygdala		$F_{(2,15)} = 6.28$, $p < 0.05$	CTRL (100 %); AA (93 ± 18 %); **MA (122 ± 18 %)***
Hippocampus		$F_{(2,15)} = 0.14$, $p > 0.05$	CTRL (100 %); AA (96 ± 19 %); MA (98 ± 17 %)
Prefrontal cortex		$F_{(2,15)} = 1.39$, $p > 0.05$	CTRL (100 %); AA (99 ± 4 %); MA (105 ± 10 %)
Microglia Density			
Amygdala	Basolateral	$F_{(2,15)} = 1.63$, $p > 0.05$	CTRL (24 ± 2); AA (16 ± 11); MA (23 ± 9)
	Basomedial	$F_{(2,15)} = 2.90$, $p > 0.05$	CTRL (16 ± 7); AA (29 ± 15); MA (19 ± 4)
	Central	$F_{(2,15)} = 1.30$, $p > 0.05$	CTRL (22 ± 12); AA (30 ± 9); MA (21 ± 7)
	Lateral	$F_{(2,15)} = 2.69$, $p > 0.05$	CTRL (24 ± 5); AA (17 ± 4); MA (27 ± 12)
	Medial	$F_{(2,15)} = 2.15$, $p > 0.05$	CTRL (15 ± 7); AA (17 ± 6); MA (28 ± 18)
	Total	$F_{(2,15)} = 1.20$, $p > 0.05$	CTRL (20 ± 3); AA (22 ± 5); MA (24 ± 4)
Hippocampus	CA1	$F_{(2,15)} = 4.70$, $p < 0.05$	**CTRL (8 ± 3)***; AA (16 ± 7); MA (13 ± 2)
	CA2	$F_{(2,15)} = 8.09$, $p < 0.01$	**CTRL (6 ± 1)****; AA (10 ± 1); MA (9 ± 2)
	CA3	$F_{(2,15)} = 13.38$, $p < 0.001$	**CTRL (8 ± 1)***; AA (17 ± 4); MA (13 ± 3)
	Total	$F_{(2,15)} = 24.07$, $p < 0.001$	**CTRL (7 ± 1)***; AA (14 ± 3); MA (12 ± 1)
Prefrontal cortex	Infralimbic	$F_{(2,15)} = 0.89$, $p > 0.05$	CTRL (22 ± 14); AA (29 ± 8); MA (22 ± 8)
	Prelimbic	$F_{(2,15)} = 1.85$, $p > 0.05$	CTRL (17 ± 6); AA (27 ± 13); MA (19 ± 9)
	Total	$F_{(2,15)} = 2.00$, $p > 0.05$	CTRL (20 ± 8); AA (28 ± 10); MA (20 ± 5)

AA: Adaptive Avoidance group, CTR: Control group, EAAT-2:Excitatory amino acid transporter-2, MA: Maladaptive Avoidance group. Data is presented as mean ± standard error.

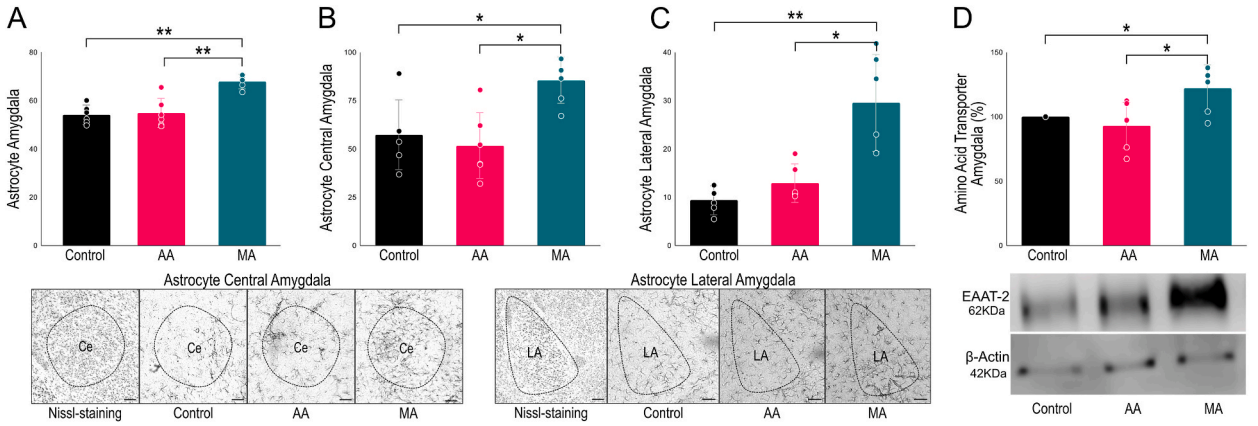

Fig. 3. Astrocyte density and excitatory amino acid transporter 2 (EAAT-2) expression in the amygdala of animals showing maladaptive avoidance (MA) behaviour. Animals in the MA group showed increased astrocyte density in the amygdala (A), especially in the Central (B) and Lateral (C) subnuclei, and increased expression of EAAT-2 (D) in this same nucleus, as compared to animals with adaptive avoidance (AA) behaviour and controls. Calibration bar: 10 μm. β-Actin was used as the loading control. Data are shown as average ± SEM. *$p < 0.05$; **$p < 0.01$.

classify animals as presenting adaptive (AA) or maladaptive avoidance (MA) behaviour. Although this classification is commonly only established in the last two consecutive trials [3,4,18], behavioural differences between groups were observed since the first test and continued until the end of the experiment, suggesting that intrinsic differences between animals could determine a predisposition to develop maladaptive avoidance behaviour and exacerbated fear responses.

An interesting feature observed in our results is the distinct anatomical pattern of changes between MA and AA animals. While MA showed differences in the AMG, AA presented changes in the PFC. This is in line with previous work showing that the dysfunctional activity of the AMG results in exacerbated fear responses and maladaptive avoidance behaviour and is involved in the pathophysiology

Table 2
Pro- and anti-inflammatory mediators in the Amygdala, Hippocampus and Prefrontal Cortex.

Pro- and anti-inflammatory mediators			
Amygdala	CX3CL1	$F_{(2,15)} = 3.33$, $p > 0.05$	CTRL (38 ± 7); AA (32 ± 6); MA (30 ± 3)
	IL-1beta	$F_{(2,15)} = 34.69$, $p < 0.001$	CTRL (72 ± 6); AA (70 ± 10); **MA (101 ± 4)*****
	IL-6	$F_{(2,15)} = 1.45$, $p > 0.05$	CTRL (637 ± 77); AA (576 ± 74); MA (572 ± 69)
	IL-10	$F_{(2,15)} = 0.01$, $p > 0.05$	CTRL (252 ± 39); AA (253 ± 35); MA (251 ± 17)
	IL-17	$F_{(2,15)} = 12.69$, $p < 0.001$	CTRL (88 ± 9); AA (71 ± 21); **MA (109 ± 3)*****
	TNF-alpha	$F_{(2,15)} = 26.69$, $p < 0.001$	CTRL (76 ± 18); AA (57 ± 7); **MA (109 ± 10)*****
Hippocampus	CX3CL1	$F_{(2,15)} = 0.01$, $p > 0.05$	CTRL (67 ± 12); AA (68 ± 14); MA (67 ± 12)
	IL-1beta	$F_{(2,15)} = 0.03$, $p > 0.05$	CTRL (137 ± 24); AA (138 ± 13); MA (135 ± 14)
	IL-6	$F_{(2,15)} = 0.14$, $p > 0.05$	CTRL (1297 ± 141); AA (1266 ± 266); MA (1241 ± 125)
	IL-10	$F_{(2,15)} = 0.38$, $p > 0.05$	CTRL (220 ± 27); AA (223 ± 14); MA (238 ± 57)
	IL-17	$F_{(2,15)} = 0.09$, $p > 0.05$	CTRL (179 ± 36); AA (189 ± 37); MA (184 ± 51)
	TNF-alpha	$F_{(2,15)} = 0.12$, $p > 0.05$	CTRL (176 ± 42); AA (166 ± 25); MA (167 ± 41)
Prefrontal cortex	CX3CL1	$F_{(2,15)} = 4.80$, $p < 0.05$	CTRL (53 ± 9); **AA (42 ± 3)***; MA (50 ± 4)
	IL-1beta	$F_{(2,15)} = 33.75$, $p < 0.001$	CTRL (147 ± 13); **AA (99 ± 10)*****; MA (133 ± 7)
	IL-6	$F_{(2,15)} = 12.98$, $p < 0.001$	CTRL (865 ± 40); **AA (1067 ± 100)*****; MA (834 ± 103)
	IL-10	$F_{(2,15)} = 5.07$, $p < 0.05$	CTRL (216 ± 28); **AA (244 ± 20)***; MA (192 ± 34)
	IL-17	$F_{(2,15)} = 54.39$, $p < 0.001$	CTRL (117 ± 19); **AA (193 ± 10)*****; MA (122 ± 11)
	TNF-alpha	$F_{(2,15)} = 13.36$, $p < 0.001$	CTRL (149 ± 22); **AA (86 ± 12)*****; MA (136 ± 30)

AA: Adaptive Avoidance group, CTR: Control group, EAAT-2:Excitatory amino acid transporter-2, MA: Maladaptive Avoidance group. Data is presented as mean ± standard error.

of anxiety disorders and trauma- and stressor-related disorders (i.e. post-traumatic stress disorder, PTSD) [5,22,23]. Furthermore, while lesions of the AMG in animals showing MA are sufficient to abolish freezing and rescue AA [3,24], pre-training lesions of the PFC impair adaptive avoidance responses and facilitate freezing [1]. Interestingly, similarly to our results, it has been shown that animals exposed to an acute high-stressful event (such as the footshock used in our test) show increased cytokine expression in the PFC [25], suggesting the exposure to stressful environments to be sufficient to induce changes in neuroinflammation within the PFC-AMG-HC network, without necessarily resulting in behavioural impairment. Along this line, here we observed reduced TNF-α and CX3CL-1 in the PFC of experimental animals (i.e., MA and AA), a feature that has been previously reported in other models of stress [26,27], and is associated with glutamatergic tone, microglial cell activation and neuronal plasticity [28–30].

An ever-growing body of evidence indicates that neuroinflammation in the AMG is central to the development of stress-induced disorders (for a review, see Ref. [31]). In fact, stress has been shown to increase permeability and reduce the integrity of the blood-brain barrier in the AMG leading to neuroinflammation [32]. Similar to our results, increased IL-1β, IL-17, and TNF-α levels in the AMG have been described in several animal models of stress, including restraint stress [32], chronic unpredictable mild stress [33,34] and cumulative mild stress [35]. In the latter, suppression of IL-17 effectively improved symptoms in animals exposed to cumulative mild stress early in life [35]. Acute stressors, such as the footshock used in the two-way active avoidance task, also increase the production of IL-1b and TNF-α and activate the hypothalamus-pituitary-adrenal (HPA) axis resulting in the release of glucocorticoids contributing to an exacerbated stress response [36,37]. Interestingly, increased TNF-α in the AMG is associated with altered levels of glutamate in this same nucleus, disturbances in learning and increased fear responses, which can contribute to the impaired fear extinction seen in psychiatric disorders [23,38]. Indeed, increased EAAT-2 expression (a major glutamate transporter expressed predominantly in astrocytes) has been reported in association with increased fear responses and is relevant to psychiatric and neurological disorders [39,40]. In line with these findings, our study observed increased astrocyte and EAAT-2 expression in the amygdala of MA animals, suggesting altered these to be involved in the presentation of maladaptive avoidance behaviour and expression of exacerbated fear responses.

Accordingly, it has been suggested that anti-inflammatory therapies could improve symptoms of psychiatric disorders in selected individuals [41,42]. In the context of PTSD, a double-blind, randomized clinical trial showed that administration of oral hydrocortisone prior to prolonged exposure therapy leads to greater treatment retention and PTSD symptom improvement [43]. A secondary analysis of another trial investigating the use of eicosapentaenoic acid (i.e., omega 3 supplement) showed that individuals who presented increased blood levels of omega 3 during the trial had lower PTSD symptom severity [44]. For the treatment of anxiety disorders, several anti-inflammatory compounds have been suggested to be associated with symptom improvement, including vitamin D [45], nano-curcumin [46], Carotenoids [47] and hydrogen gas [48]. The use of glutamatergic modulators has also been reported to improve PTSD symptoms, especially those reexperiencing arousal [49], and anxiety disorders [50]. A recent RCT showed preliminary evidence that Riluzole (i.e., a glutamatergic modulator used for treating amyotrophic lateral sclerosis) may selectively improve PTSD hyperarousal symptoms [51]. In patients with anxiety disorders, this same drug led to significant symptom improvement in the majority of treated patients [52]. These results are encouraging, as several anti-inflammatory drugs and glutamatergic modulators have a good safety profile and are well tolerated by patients.

A few limitations of this study and opportunities for future investigations are determining the interaction between these cytokines in the PFC and amygdala and their specific contributions to adaptive avoidance behaviour and investigating the use of anti-inflammatory drugs to modulate the network and possible impact on behaviour.

In conclusion, in this work, we provided evidence of a neuroinflammatory signature in the amygdala, along with increased levels of glutamatergic transporter, associated with the expression of maladaptive avoidance behaviour and high fear response. We suggest

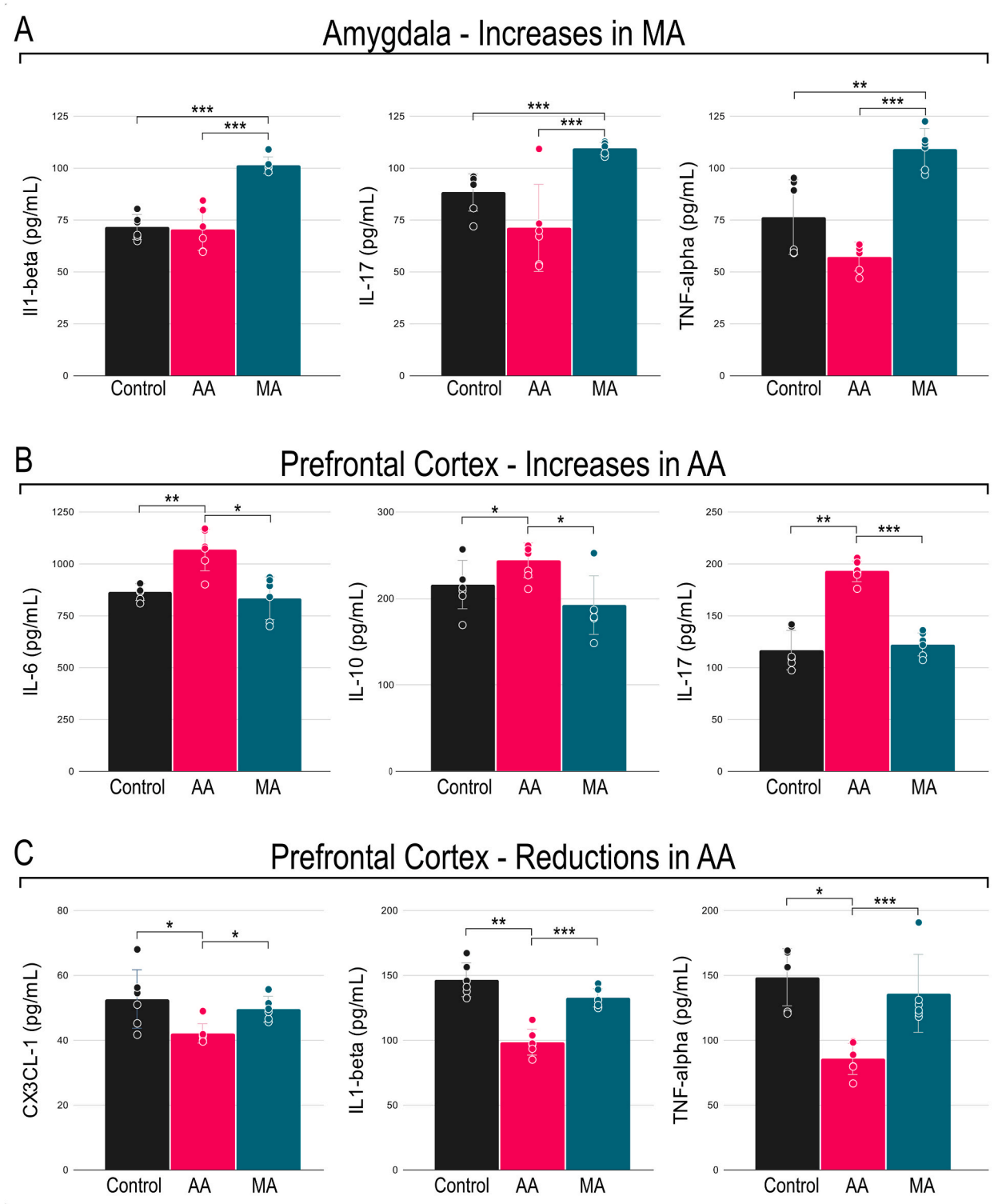

Fig. 4. Pro- and anti-inflammatory mediators in the Amygdala and Prefrontal Cortex. While IL-1β, IL-17 and TNF-α were found to be increased in the amygdala of animals showing maladaptive avoidance (MA) behaviour (A), animals in the adaptive avoidance (AA) group showed increased IL-6, IL-10 and IL-17 (B) and reduced CX3CL-1, IL-1β and TNF-α in the Prefrontal cortex. Data are shown as average ± SEMs. *$p < 0.05$; ***$p < 0.001$.

anti-inflammatory treatments or glutamatergic modulators may be useful for anxiety and trauma-stressor-related disorders. However, further investigations on the specific contributions of astrocytes and each cytokine are warranted, as well as studies on new technologies for amygdala-targeted delivery of drugs to improve psychiatric symptoms.

Data availability statement

Data will be made available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Geiza Fernanda Antunes: Writing – original draft, Methodology, Formal analysis, Data curation. **Flavia Venetucci Gouveia:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Formal analysis, Data curation, Conceptualization. **Mayra Akemi Kuroki:** Writing – original draft, Methodology, Formal analysis, Data curation. **Daniel Oliveira Martins:** Writing – original draft, Methodology, Formal analysis, Data curation. **Rosana de Lima Pagano:** Writing – review & editing, Supervision. **Ana Carolina Pinheiro Campos:** Writing – original draft, Methodology, Formal analysis, Data curation. **Raquel Chacon Ruiz Martinez:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e30427.

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