

Assessing the Brain through the Eye: Hepatic Encephalopathy

**N. Arias^{1,2*}, M. Méndez^{2,3}, I. Alcalde^{3,4}, A. Íñigo-Portugués⁴, J. Merayo-Llodes^{3,4,5}, J. Arias⁶,
JL. Arias^{2,3}**

¹ Department of Experimental Psychology, University of Cambridge, Downing Street, Cambridge CB2 3EB, UK

² INEUROPA, Instituto de Neurociencias del Principado de Asturias

³ Laboratorio de Neurociencias, Departamento de Psicología, Universidad de Oviedo, Plaza Feijoo s/n, 33003 Oviedo, Spain

⁴ Fundación de Investigación Oftalmológica (FIO), Instituto Oftalmológico Fernández-Vega, Avda. Dres Fernández-Vega, 34, 33012, Oviedo, Spain

⁵ Surgery Department, School of Medicine, University of Oviedo, Avda. Julián Clavería s/n, 33006, Oviedo, Spain

⁶ Surgery Department, School of Medicine, Complutense University of Madrid, 28040 Madrid, Spain

Correspondence:

Natalia Arias. Department of Experimental Psychology, University of Cambridge, Downing Street, Cambridge CB2 3EB, UK. e-mail:na423@cam.ac.uk

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List of Abbreviations:

HE: hepatic encephalopathy

mHE: minimal hepatic encephalopathy

PP: portal pressure

HT: portal hypertension

SHAM: sham-operated

MWM: morris water maze

GFAP-IR: glial fibrillary acidic protein-immunoreactive astrocytes

PBS: phosphate buffer saline

TBS: tris buffer saline

BSA: bovine albumin serum

RT: room temperature

LYVE-1 lymphatic vessel endothelial hyaluronic acid receptor 1

TNF- α : tumor necrotic factor alpha

α -SMA: alpha-smooth muscle actin

DAPI: 4',6-diamidino-2-phenylindole

U: Mann-Whitney U test

ADS: anterodorsal striatum

AMS: anteromedial striatum

ALS: anterolateral striatum

ACC: accumbens nucleus

ACS: accumbens shell

CA1: cornu ammonis 1

CA3: cornu ammonis 3

DG: dentate gyrus

RE: right eye

LE: left eye

Abstract

Background & Aims: Minimal hepatic encephalopathy (mHE) has been shown to affect daily functioning, quality of life, driving and overall mortality. However, little is known about treating or diagnosing early impairments in mHE. We studied one of its precipitating factors, portal hypertension. The purpose was to evaluate the presence of histological changes in the eye in mHE and whether these changes could be related to deficits in behavioral task acquisition.

Methods: Rats were trained on a stimulus-response task and a spatial working memory task using the Morris water maze. Two groups of animals were used: a SHAM (sham-operated) group (n = 15) and a portal hypertension (HT) group (n = 15). The triple portal vein ligation method was used to create an animal model of mHE. Latencies to reach the platform, number of glial fibrillary acidic protein-immunoreactive astrocytes (GFAP-IR), mast cell expression and presence/absence of blood and lymphatic vessels were examined. **Results:** There were differences in stimulus-response behavioral performance, with a deficit in the acquisition in the HT group. However, no differences between groups were found on the spatial working memory task. At the same time, differences between groups were found in the GFAP-IR density, which was lower in the HT group, and in the number of mast cells and the presence of vessels, which were higher in the HT group. **Conclusions:** In this study, we provide the first preliminary insight into the validity of exploring the eye as a possible tool to assess the diagnosis of mHE conditions.

Keywords: minimal hepatic encephalopathy; stimulus-response task; striatum; GFAP-IR; mast cells; LYVE-1; TNF- α ; α -SMA.

Key points:

- Portal hypertension as precipitant factor of mHE was studied.
- Behavioral deficits in stimulus-response task but not in working memory were found.

- Brain changes through GFAP-IR showed lower expression in HT group.
- Mast cells and vessels in cornea were higher in HT group.

1. INTRODUCTION

Portal hypertension is a clinical syndrome defined by a pathological elevation of blood pressure in the portal system [1]. In humans, portal hypertension is characterized by prolonged elevation of portal venous pressure greater than 10 mmHg [2] which leads to hyper-dynamic circulation [3] and the creation of a portosystemic collateral circulation [4], due not only to the opening of preexisting vessels, but also to the formation of new vessels.

Portal hypertension and excess ammonia due to liver failure seem to be the main contributing factors leading to different cerebral and neurological alterations and to the occurrence of hepatic encephalopathy (HE) [5]. However, the role of ammonium is still under debate. Shawcross et al. [6] showed that systemic inflammation exacerbated psychological impairment in hyperammonemic cirrhosis patients, and after treatment for this inflammatory response, this impairment was reversed, even in the presence of similar hyperammonemia [6]. This result suggests that the behavioral impairments often associated with the onset of hyperammonemia in liver disease may be reliant on the interaction of ammonia with other metabolic and systemic perturbations. Moreover, recent findings highlighted the absence of task performance impairments due to hyperammonemia, which questions the importance of ammonia alone in HE symptom development [7]. So, how can portal hypertension trigger HE?

First, this increased blood flow is understood as stress stimuli by the vascular endothelium, producing an inflammatory response [8], which, by extension, affects the organs into which the blood drains. It has been shown that mast cells are the first responders to this

mechanical stimuli and are able to initiate inflammation due to their strategic location close to blood vessels [9] in the skin, airways and gut [10]. In fact, highlighting this function, mast cell infiltration has been found in great density in the duodenum, jejunum, ileum and superior mesenteric lymph node complex [11,12] in portal hypertensive rats at six weeks of evolution. Moreover, mast cells can migrate through intestinal lymph vessels to the systemic circulation and nervous system [13].

Regarding the nervous system, it has been shown that portal hypertensive-rats present increased SDF-1 alpha levels in the hippocampus and cerebellum, associated with increased TNF- α and CXCR4 levels in the hippocampus [14], which could be related to a structural remodeling process to adapt cerebral areas to the new metabolic state created by the portal hypertension [15]. Moreover, inflammation in the context of portal hypertension can arise directly in the brain, resulting in astrocytic, microglial and neuronal dysfunction, and taking part in the development of minimal hepatic encephalopathy (mHE) [16].

Second, the increase in the diameter and number of blood vessels in the submucosa has already been shown in the duodenum of portal hypertensive rats at six weeks of evolution. Furthermore, this process has been correlated with mast cell infiltration [11].

Both blood and lymph cells penetrate most tissues in the body, and dysfunction in their circulation is involved in a broad spectrum of diseases [17]. The cornea is the most well-studied and well-characterized eye tissue in blood and lymphatic vessel research [18]. As the forefront medium in the passage of light to the retina, it maintains a natural transparency and is devoid of any vasculatures. In addition, light-microscopic evidence for the invasion of lymphatics into heavily vascularized rabbit corneas has been reported [19], and vessels with thickened basement

membranes have also occurred in aging [20] and in various pathologic conditions [21,22], including diabetes mellitus [17].

In light of these data, the aims of this work were: (i) to reproduce the portal hypertension clinical symptoms that lead to the development of mHE; (ii) to highlight the existence of asymmetric cognitive differences in the mHE animals through the performance of a stimulus-response task and a working memory task; (iii) to explore the existence of earlier and accessible biomarkers to determine the contribution of portal hypertension to the development of mHE through cornea exploration.

2. MATERIAL AND METHODS

2.1. Animals

We used 30 male Wistar rats from the vivarium of the University of Oviedo, weighing 250-270 g at the beginning of the study. Rats were housed in groups of three to five, three weeks prior to the beginning of the experiments and maintained under standard laboratory conditions (20-22 °C, 65-70% relative humidity and a 12h light/dark cycle (08.00-20.00/20.00-08.00)). Food and water were available ad libitum throughout the course of all the experiments, and sessions were performed during the light phase, between 9:00 a.m. and 13:00 p.m. All procedures were carried out according to the European Parliament, Council of the European Union 2010/63/UE and ARRIVE guidelines. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Oviedo (Permit Code: PSI2010-19348 and PSI2013-45924-P); all efforts were made to minimize suffering. The animals were

distributed into portal hypertension and sham-operated animals (HT group, n = 15 and SHAM group, n = 15).

2.2. Procurement of experimental models

The experimental models that required surgery were anesthetized by i.p. injection of ketamine (100 mg/kg) and xylazine (12 mg/kg). With regard to postsurgical care, the rats were maintained close to a source of heat until they recovered consciousness (10 -15 min) to avoid postoperative hypothermia. They were then introduced into individual polycarbonate cages for 15 days and subsequently grouped in cages of four animals each until their behavioral evaluation.

2.2.1. Portal hypertension

A midline abdominal incision was performed, and a section of the intestinal loop was gently shifted to the left and covered with saline-moistened gauze. The portal vein was isolated, and three ligatures, fixed on a sylactic guide, were performed in its superior, middle and inferior portions. The stenoses were calibrated by simultaneous ligation (4-0 silk) around the portal vein and a 20-gauge blunt-tipped needle. The abdominal incision was closed in two layers with 2-0 silk. The postoperative period started immediately after the intervention and lasted until the behavioral evaluation 45 days later.

2.2.2. Sham-operated

A midline abdominal laparotomy with prolongation to the xyphoid apophysis was performed, followed by isolation of the portal vein. The operative field was irrigated with saline solution during the intervention, as in the portal hypertension interventions. Finally, the

laparotomy was closed by continuous suture on the two layers with 2-0 silk. The postoperative period started immediately after the intervention and lasted until the behavioral evaluation 45 days later.

2.3. Portal vein pressure measurement

Splenic pulp pressure, an indirect measurement of portal pressure (PP), was measured using the method described by Aller et al. [23].

2.4. Stimulus-response “habit” task

The water maze was made of fiberglass and measured 150 cm in diameter, with a 40 cm high wall. The water level was 30 cm, and its temperature was $22\pm 2^{\circ}\text{C}$. The platform used corresponded to a cylinder that was 10 cm in diameter and 28 cm high, of which 2 cm was below the surface of the water. The water maze was in the center of a 16 m² lit room (two halogen lamps of 4000 lx) and divided into four starting positions (north, south, east and west). A white plastic glass was attached to the top of the submerged platform and protruded above the water surface. The platform could be used as a step to mount the glass to escape the water. The behavior of the animal in the MWM was recorded by a video camera (Sony V88E) connected to a computer equipped with an EthoVision Pro program.

After the postoperative period of 45 days, rats were trained in one session (which took an average of 10 min) using a pre-set criterion where sham-operated rats had to achieve two consecutive trials with retention latencies under 8 s, and it could take them no more than 10 trials to reach this criterion, as in previous studies [24]. Thus, to ensure that an animal had reached the behavioral criterion, the presence of two consecutive trials with retention latencies under 8 s

should be required. For each cued training trial, the animal was placed in the tank facing the wall at one of four designated starting points (N, S, E and W) and allowed to escape onto the visibly cued platform. The visible escape platform was located in a different quadrant in each trial. A different starting point was used in each trial. Each trial ended once the animal had found the platform, or when 60 s had elapsed. If the animal had not reached the hidden platform after this time, it was placed on the platform for 15 s. During the inter-trial interval, the animals were placed in a black bucket for 30 s. Latencies to reach the escape platform were recorded and used as a measure of task acquisition.

2.5.Spatial working memory task

The spatial working memory was evaluated in the MWM as described by Méndez-López et al., [25]. Briefly, a habituation session was performed one day prior to the training schedule. The WM training involved a paired sample task. Each daily session consisted of two identical trials (sample and retention). During both trials, the platform was hidden 2 cm below the level of the water. The sample consisted of releasing the animal from one of the four starting points in the pool and letting it swim until it reached the hidden platform or 60 sec had elapsed. If the animal had not reached the hidden platform in this time, it was placed on the platform and kept there for 15 sec. The intertrial interval was 5 s. The task requires the animal to recall the position of the hidden platform during the sample. The locations of the platform and the starting positions varied on the different days in a pseudorandom order. Training finished when the group achieved the learning criteria, established as statistically significant lower retention latency compared to sample latency within one session, and also compared to the retention latency of the first session.

2.6.GFAP Immunohistochemistry

Sections of five animals from the SHAM group and five from the PH group were used to quantify the density of GFAP-IR in the dorsal hippocampus, dorsal and ventral striatum. The rats were anesthetized with sodium pentobarbital (100 mg/Kg) (Sigma-Aldrich) and intracardially perfused with phosphate buffer saline at 0.9% (PBS) (0.1 M; pH 7.4), followed by paraformaldehyde at 4% in PBS (0.1 M; pH 7.4). The brains were extracted and introduced in paraffin. Systematic cuts were made on both regions with a microtome (Leica, Germany) in 30- μ m thick serial sections at a known interval from the beginning to the end.

We performed GFAP-IR immunocytochemistry according to the method described by Blanco et al [26], which briefly consists of washing the previously deparaffinized sections in Tris Buffer saline (TBS) with Triton X-100 at 0.1%, and blocking them with human serum. Afterwards, these sections were incubated for one day with rabbit polyclonal primary antibody anti GFAP (1:800 in bovine albumin, BSA). The next day, after rinsing them in TBS with Triton X-100, we incubated them with a biotinylated secondary antibody (goat anti-rabbit IgG, 1:30 in BSA) and applied the avidin-biotin horseradish peroxidase complex (Vectastain Ultra-Sensitive ABC Staining Kit), developed with diaminobenzidine tetrahydrochloride, and cover-slipped the sections.

The entire quantification process was carried out using a Leica LAS Live Image Builder (Leica, Microsystems). To estimate the density of GFAP-IR, we used at least three equidistant sections in the selected structures from each animal. Quantification was done by placing four equidistant counting frames (frame size 0.150 mm \times 0.150 mm) on the screen. Only the cell bodies within each quadrant or dissector selected in the section were quantified. The total

thickness measured on each slide was 30 μm , leaving out 5 μm of thickness above and below the Z-axis to avoid possible overestimations.

2.7.Mast cells and vessels techniques

2.7.1 Macroscopic evaluation of the ocular surface

Rats' ocular surface was examined macroscopically under a Leica S6D stereoscopic microscope equipped with a Leica EC3 digital camera (Leica Microsystems, Wetzlar, Germany) to register the presence of blood and lymphatic vessels and leucomas in the cornea of the eye. Images were taken under a fixed 12.5X magnification.

Blood and lymphatic vessels were first registered as present or not present (assigned 1 and 0 values, respectively) in macroscopic images of the ocular surface of portal hypertension and sham-operated rats. The presence of lymphatic vessels in the corneal tissue was assessed by studying the LYVE1 immunohistochemical slides. Samples with LYVE1 positive vessels were registered as 1, and negative samples were assigned a 0 value.

2.7.2 Tissue Preparation

Once the behavioral studies and macroscopical examination were finished, animals were killed by decapitation, and their eye globes were enucleated and immersed in 4% paraformaldehyde fixative for 2 hours. Samples were then cryoprotected in 30% buffered sucrose solution, embedded in OCT compound (Sakura Finetek, Torrance, CA) and snap frozen in liquid Nitrogen. Serial 5 μm thick transverse sections containing the cornea and conjunctiva of the eye were obtained on a Microm HM 550 cryostat (Microm International GmbH, Walldorf,

Germany) and collected on microscope slides (Superfrost Plus, Menzel-Gläser GmbH, Braunschweig, Germany).

2.7.3 Immunocytochemistry

For fluorescent immunocytochemical preparations (both flat mounts and cryosections), the samples were blocked at room temperature (RT) with 10% goat normal serum and 1% of bovine serum albumin in phosphate buffer saline (PBS) 0.1 M pH 7.4 for two hours and incubated overnight with rabbit polyclonal antibodies to LYVE1 and TNF- α or mouse monoclonal antibody to α -SMA (α -smooth muscle actin) (all from Abcam, Cambridge UK). After washing three times in PBS, samples were incubated for 2 hours at RT with the corresponding secondary antibody (anti-rabbit IgG Alexa Fluor 488 or anti-mouse IgG Alexa Fluor 594, from Molecular Probes), rinsed again in PBS, and incubated for 10 minutes in 4',6-diamidino-2-phenylindole (0.2 μ g/mL DAPI). Finally, the tissue sections were washed in PBS and mounted in DAKO fluorescence mounting medium (DAKO).

To study the coexistence of lymphatic vessels and mastocytes in the same samples, immunohistochemical techniques were performed. After blocking endogenous peroxidase activity (3% H₂O₂ in methanol for 5 min), tissue sections were placed in a humidity chamber, blocked at room temperature (RT) with 10% goat normal serum for 2 hours, and incubated overnight in 10 μ g/mL anti-LYVE1 primary antibody (rabbit polyclonal to LYVE1-Lymphatic vessel marker; Abcam, Cambridge, UK). After washing three times in PBS, the sections were incubated for 2 hours at RT in the secondary antibody (biotinylated goat anti-rabbit IgG, 1:200; Vector Laboratories, Burlingame, CA), rinsed again in PBS, and incubated for 2 hours at RT in avidin-biotin-horseradish peroxidase complex (ABC reagent kit; Vector Laboratories). Then the

tissues were incubated for 5 min at RT in 0.02% diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA) and 0.003% H₂O₂, and then rinsed three times in PBS and twice in distilled water. Sections were then counterstained with toluidine blue.

2.7.4 Mast cell staining

To stain mast cells present in the connective tissue, sections previously immunostained to LYVE1 were counterstained with 0.1% toluidine blue (Sigma-Aldrich) in 1% NaCl for 2 min at RT, washed three times in distilled water, dehydrated in graded alcohols, cleared in xylene, and cover-slipped with Entellan (Merck, Darmstadt, Germany).

All images were obtained using a Leica DM 6000B microscope equipped with a Leica DFC310 FX camera (Leica Microsystems) and using a partial Nomarski illumination method. To quantify the number of mast cells present in the conjunctiva surrounding the cornea in portal hypertension and sham-operated animals, 3 histological sections through the central region of the cornea of each eye globe were used. Sections were spaced 50 µm from each other. Mast cells were counted manually in the conjunctiva on both sides of the cornea in the histological preparation. Only cells contained in a 40X magnification objective field (under a Leica DM 6000B microscope), starting at the corneoscleral limbus, were considered.

2.8. Statistical analysis

All data were analyzed in the Sigma-Stat 3.2 program (Systat, Richmond, USA) and expressed as the mean ± SEM. The results are considered statistically significant if $p < 0.05$.

A t-test for independent samples was used for the statistical comparison of portal pressures, mast cell expression and vessel presence. The latencies to reach the visibly cued platform during the training phase of the stimulus-response “habit” task were analyzed with a t-

test for independent samples. Moreover, a comparison between trial-performance in each group was analyzed by one-way repeated-measures ANOVA. Post hoc multiple comparison analyses were carried out when allowed, using pair-wise Tukey tests. Moreover, a non-parametric Mann–Whitney U test (U) for independent samples was conducted when normality or equal group variances failed.

A one-way ANOVA was used to compare distance covered and velocity between groups in the sample and retention trials during spatial working memory performance. Furthermore, a comparison of latencies between trial-performance in each group during sample and retention trials was analyzed by two-way repeated-measures ANOVA.

A t-test was used to analyze differences in GFAP-IR, mast cells, lymphatic and blood vessels between portal hypertension and sham-operated animals. Moreover, a non-parametric Mann–Whitney U test (U) for independent samples was carried out when normality or equal group variances failed.

3. RESULTS

3.1. Portal Pressure

Portal pressure increased in all the animals with portal hypertension (15.998 ± 0.663) ($U = 55.000$, $n_1 = 10$, $n_2 = 10$, $P < 0.001$), compared to that of the sham-operated animals (6.705 ± 0.267). This piece of data is important because it ensures the presence of portal hypertension in the HT group, which could explain differences in learning and the early development of mHE in the model.

3.2. Stimulus-response “habit” task

The t-test for independent samples showed differences in escape latencies between the HT and SHAM groups ($t_8 = -2.328$, $P = 0.048$), revealing the inability of HT animals to reach the behavioral criterion compared to the SHAM group. When a one-way repeated-measures ANOVA was carried out in both groups, the HT group did not show differences in their escape latencies across trials ($F_{(4,36)} = 1.000$, $P = 0.458$). However, there was a significant effect of the variable trial ($F_{(4,36)} = 3.747$, $P = 0.002$) in the SHAM group, with a progressive latency reduction being found across trials 7 ($P = 0.029$), 9 ($P = 0.003$) and 10 ($P = 0.003$), compared to trial one (Figure 1).

3.3.Spatial working memory task

The one-way ANOVA revealed no differences between the HT and SHAM groups in distance covered during the sample ($F_{(1,9)} = 0.534$, $P = 0.486$) and retention phases ($F_{(1,9)} = 0.235$, $P = 0.641$). In addition, no differences were found in velocity during the sample ($F_{(1,9)} = 0.305$, $P = 0.596$) and retention ($F_{(1,9)} = 0.924$, $P = 0.365$). Finally, the two-way repeated-measures ANOVA showed no differences in latencies during the sample between groups ($F_{(1,59)} = 1.189$, $P = 0.307$) or between days ($F_{(5,59)} = 0.92$, $P = 0.483$), and no interactions between group and day were found ($F_{(5,59)} = 0.429$, $P = 0.826$). Moreover, no differences in latencies during retention were found, respectively ($F_{(1,59)} = 0.137$, $P = 0.721$; $F_{(5,59)} = 0.441$, $P = 0.817$; $F_{(5,59)} = 2.469$, $P = 0.05$; Figure 2).

3.4.GFAP-IR results

GFAP represents the most specific astrocytic marker under normal and pathological conditions [27]. Differences in the GFAP-IR astrocyte number were found between groups in ADS ($t_8 = 4.765$, $P = 0.001$) and AMS ($t_8 = 5.317$, $P < 0.001$), where the number was

significantly lower in the HT group than in the SHAM group. However, no differences were found in ALS ($t_8 = 2.138$, $P = 0.065$), ACC ($U = 19.000$, $n_1 = 5$, $n_2 = 5$, $P = 0.095$), ACS ($U = 28.000$, $n_1 = 5$, $n_2 = 5$, $P = 1.000$), CA1 ($t_6 = 0.302$, $P = 0.773$), CA3 ($t_6 = 0.424$, $P = 0.687$) or DG ($t_6 = 0.0989$, $P = 0.924$). (Figure 3). Our results showed a decrease in GFAP-immunoreactive astrocytes in the striatum in early stages of HE.

3.5. Macroscopic assessment of the ocular surface

The ocular surface revealed differences between the SHAM and HT groups, where no pathological signs were found in the SHAM group, whereas the HT group showed the presence of whorls of blood vessels and leucomas (Figure 4).

3.6. Mast cell results

Differences were found between the HT and SHAM groups in the RE ($t_8 = 3.036$, $P = 0.016$) and in the LE ($t_8 = 4.000$, $P = 0.004$). However, no differences in the number of mast cells were found between the eyes in each group: HT ($t_8 = 0.000$, $P = 1.000$) and SHAM ($t_8 = 0.431$, $P = 0.678$; Figures 5).

3.7. Lymphatic and Blood vessel results

Differences were found in the HT and SHAM groups for both types of vessels, with the HT group showing a greater presence of these vessels in both eyes ($U = 37.500$, $n_1 = 5$, $n_2 = 5$, $P = 0.032$). Otherwise, no differences were found between RE and LE in each group: HT ($U = 30.000$, $n_1 = 5$, $n_2 = 5$, $P = 0.690$) and SHAM ($U = 27.500$, $n_1 = 5$, $n_2 = 5$, $P = 1.000$; Figures 5 and 6). We found lymphatic vessels in every cornea containing blood vessels.

Moreover, an additional staining of blood vessels and lymphatic vessels was done to support our data (Figure 7 A and B). Finally, the occurrence of lymphatic vessels in corneas from the HT group was accompanied by the expression of TNF- α in cornea (Figure 7 C and D). The TNF- α was highly expressed in the epithelium in terms of immunofluorescence intensity, compared to SHAM rats. In addition, we observed TNF- α positive elements in the upper stroma and profiling vessels coinciding with LYVE1 positive vessels (Figure 7 D and F).

4. DISCUSSION

Our work revealed that one of the main contributing factors to HE, portal hypertension, reproduced by a model of triple portal vein ligation [28], was related to cognitive deficits due to the development of mHE, detected early due to the presence of histological changes in the cornea.

When one of the main contributing factors to HE, portal hypertension, is reproduced by a model of triple portal vein ligation [5], differences in portal pressure values between the HT and SHAM groups are found, confirming the great development of the model. Moreover, the inability of HT animals to reach the behavioral criterion, compared to the SHAM group, during their performance of a stimulus-response task in which the visible platform was pseudo-randomly repositioned across trials to render its relation with distal cues trivial [28], highlighted the existence of a cognitive impairment in early stages of the disorder, referred to as minimal hepatic encephalopathy (mHE).

It is well known that at chronic stages of the HE disorder (bile duct ligation model) alterations in motor activity and mnemonic functions along with decreases in dopamine and

serotonin levels in the cortex, hippocampus, striatum and cerebellum have been reported [29]. However, in this study we are evaluating a model of one of the precipitating factors of minimal hepatic encephalopathy in the early stages of the disorder's development. Under these conditions, previous studies in our group have reported no motor alterations, slight deficits in spatial learning tasks [30], and different alterations in several brain regions on object-place recognition and reversal tasks [31].

Based on these previous results, we decided to explore the possibility of early impairments in the hippocampal system and striatum through the performance on two different behavioral tasks guided by a double dissociation between them, with the acquisition of the visible platform task being more striatum-dependent [32] and the spatial working memory task being more hippocampal-dependent [25]. Our results could suggest impairment in the striatum as one of the first targets in early stages of HE, due to the inability of these animals to perform the stimulus-response task, but no hippocampal damage, due to the lack of differences in the performance on the spatial working memory task, highlighting an asymmetric deficit at early stages of HE.

This asymmetry in the performance of both tasks could be related to a differential damage at early stages of HE. Arias et al. [33] have suggested the striatum as the brain structure primarily affected by inflammatory factors such as TNF- α highlighting the inflammatory origin of this disorder. Based on these data and taking into account that pro-inflammatory mediators (TNF- α) even lead to decreased GFAP expression [37], we attempted to assess glial damage through the study of GFAP-IR density in the dorsal hippocampus and striatum.

We found decreased GFAP-IR in the dorsal striatum in the HT group compared to the SHAM group, while no changes were found in the hippocampus. GFAP represents the most

specific astrocytic marker under normal and pathological conditions [27], and GFAP-immunoreactive astrocytes were found to be decreased in number in the cerebral cortex of rats with chronic liver failure resulting from portocaval anastomosis [34]. Similarly, decreased GFAP-IR was reported in human chronic liver failure in the basal ganglia, cerebral cortex and thalamic structures [35]. These results could support the inability of HT animals to perform the stimulus-response task compared to the working memory task. But, how could we assess this brain damage at clinical level?

A growing evidence supporting the role of inflammation in exacerbating the neurological manifestations in HE has been shown. Previous studies have reported that inflammation is perhaps the fundamental factor in which more metabolic alterations progressively develop [36], however, little is known about the evolution of lymphatic vessels along the development of this disorder.

It is widely accepted that blood and lymph cells penetrate most tissues in the body, and a dysfunction in their circulation is involved in a broad spectrum of diseases. In fact, angiogenesis and lymphangiogenesis, the formation of new blood and lymphatic vessels, are important aspects of tumor growth and inflammatory diseases [37]. However, unlike blood vessels, lymphatic vessels were not easily visible until the lymphatic vessel endothelial hyaluronic acid receptor-1 (LYVE-1) was identified [38].

In recent years, it has become clear that lymphangiogenesis can occur along with hemangiogenesis [39]. The cornea has been shown to be a great place to examine the formation of these new vessels because a normal human cornea has no blood or lymphatic vessels, but secondary to a disease, the cornea can become vascularized, a phenomenon that has been described both clinically and histopathologically [40]. It is known that vascularization in the cornea disturbs visual acuity, whereas corneal lymphangiogenesis modulates corneal immunity or

inflammation. However, in both cases, cornea neovascularization represents a major public health burden, almost always impairing visual function and sometimes ultimately leading to blindness. But what is the basis for these processes?

As we mentioned above, a normal cornea is avascular [45]; however, under certain conditions, such as inflammatory disorders or portal hypertensive encephalopathy, capillaries invade from the limbal vascular plexus, causing corneal neovascularization, as our results showed. As a consequence of an inflammatory process, the expression of chemokines and adhesion molecules is implied [46]. In fact, our results revealed that TNF-alpha is highly expressed in the HT group cornea compared to the SHAM group. These factors initiate the recruitment of inflammatory cells in the conjunctival mucosa where the mast cells are. Activated mast cells can release several cytokines, such as VEGF A, which plays a crucial role in inducing inflammatory neovascularization by supplying signals essential for pathological hemangiogenesis and lymphangiogenesis [47], and VEGF C and D, which are required for lymphangiogenesis [37,48].

Moreover, the appearance of an increased number of activated mast cells in HT group compared to SHAM group could be showing a bidirectional crosstalk between mucosal mast cells, the enteric system and brain [44], and it suggest that these inflammatory cells are potential integrators of the systemic inflammatory response that induces portal hypertension, leading to mHE.

Our study provides the first analysis of the pathologic distributions of blood and lymphatic vessels, as well as the presence of pro-inflammatory factors, such as TNF-alpha in the cornea and striatal decreased in GFAP-IR, along with cognitive deficits at early stages of HE trigger by the development of portal hypertension. These results reveal that the cornea is an important tool for the early diagnosis of portal hypertension's contribution to the development of

mHE. Finally, we presented behavioral and histological results that provide fresh insights into the development of new tools for the assessment of portal hypertension as a marker of mHE. Our research opens a new door to future tools for the early diagnosis of dysfunction in patients with mHE.

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References

- [1] Treiber G, Cseppregi A, Malfertheiner P. The pathophysiology of portal hypertension. *Dig Dis.* 2005, 23: 6-10.
- [2] Kumar A, Sharma P, Sarin SK. (2008) Hepatic venous pressure gradient measurement: time to learn!. *Indian J Gastroenterol.* 2008, 27: 74-80.
- [3] Iwakiri Y, Groszmann RJ. The hyperdynamic circulation of chronic liver diseases: from the patient to the molecule. *Hepatology.* 2006; 43: S121-S131.
- [4] Rodriguez G, Monerde G, Dieguez B, Aller MA, Arias J. Long-term portal hypertension in the rat by triple stenosing ligation of the portal vein. *An Med Int.* 2000; 17: 137-141.
- [5] Arias N, Méndez M, Fidalgo C, Aller MA, Arias J et al. Mapping metabolic brain activity in three models of hepatic encephalopathy. *Int J Hypertens.* 2013, 390872.
- [6] Shawcross DL, Davies NA, Williams R, Jalan R. Systemic inflammatory response exacerbates the neuropsychological effects of induced hyperammonemia in cirrhosis. *J Hepatol.* 2004 ; 40:247-254.
- [7] Wilkinson DJ, Smeeton NJ, Castle PC, Watt PW. Absence of neuropsychological impairment in hyperammonaemia in healthy young adults; possible synergism in development of hepatic encephalopathy (HE) symptoms?. *Metab Brain Dis.* 2011; 26:203-12.
- [8] Chien S, Li S, Shyy JY-J. Effects of mechanical forces on signal transduction and gene expression in endothelial cells. *Hypertension.* 1998; 31: 162-169.

- [9] Galli SJ, Kalesnikoff J, Grimbaldston MA, Piliponsky AM, Williams CMM, Tsai M. Mast cells as "tunable" effector and immunoregulatory cells: Recent advances. *Ann Rev Immunol.* 2005; 23: 749-786.
- [10] Metz M, Maurer M. Mast cells- key effector cells in immune responses. *TRENDS Immunol.* 2007; 28: 234-241.
- [11] Diez-Arias JA, Aller MA, Palma MD, Arias JL, Muñiz E, Sanchez M, Arias J. Increased duodenal mucosa infiltration by mast cells in rats with portal hypertension. *Dig Surg.* 2001; 18: 34-40.
- [12] Prieto I, Aller MA, Santamaria L, Nava MP, Madero R, Perez-Robledo JP, Arias J. Prehepatic portal hypertension produces increased mast cell density in the small bowel and in mesenteric lymph nodes in the rat. *J Gastroenterol Hepatol.* 2005; 20: 1025-1031.
- [13] Silverman AJ, sutherland AK, Wilhelm M, Silver R. Mast cells migrate from blood to brain. *J Neurosci.* 2000; 20: 401-408.
- [14] Merino JJ, Aller MA, Sanchez-Patan F, Rubio S, Loscertales M, Arias JL, Arias J. Regulation of chemokines levels upon liver encephalopathy induction through portal stenosis in hypertensive rats. Is SDF1 alpha underlie in repair mechanisms in the hippocampus of hypertensive rats? 2nd International Congress on Regenerative Biology. Stuttgart. Germany 2006.
- [15] Arias JL, Aller MA, Sanchez-Patan F, Arias J. The inflammatory bases of hepatic encephalopathy. *Eur J Gastroenterol Hepatol.* 2006; 18: 1297-1310.

- [16] Tranah TH, Vijay GK, Ryan JM, Shawcross DL (2013) Systemic inflammation and ammonia in hepatic encephalopathy. *Metab Brain Dis* 28: 1-5.
- [17] Ecoiffier T, Yuen D, Chen L. Differential distribution of blood and lymphatic vessels in the murine cornea. *Invest Ophthalmol Vis Sci*. 2010; 51: 2436-2440.
- [18] Chen L. Ocular lymphatics: state-of-the-art review. *Lymphology*. 2009; 42: 66 -76.
- [19] Collin, H. B. Endothelial cell lined lymphatics in the vascularized rabbit cornea. *Invest Ophthalmol*. 1966; 5: 337.
- [20] Cursiefen C, Cao J, Chen L, et al. Inhibition of hemangiogenesis and lymphangiogenesis after normal-risk corneal transplantation by neutralizing VEGF promotes graft survival. *Invest Ophthalmol Vis Sci*. 2004; 45: 2666-2673.
- [21] Hill JC. The relative importance of risk factors used to define high-risk keratoplasty. *Ger J Ophthalmol*. 1996; 5: 36-41.
- [22] Chen L, Huq S, Gardner H, de Fougères AR, Barabino S, Dana MR. Very late antigen 1 blockade markedly promotes survival of corneal allografts. *Arch Ophthalmol*. 2007; 125: 783-788.
- [23] Aller MA, Vara E, García C, Nava MP, Angulo A et al. Hepatic lipid metabolism changes in short- and long-term prehepatic portal hypertensive rats. *World J Gastroenterol*. 2006; 12: 6828-6834.
- [24] Teather LA, Packard MG, Smith DE, Ellis-Behnke RG, Bazan NG. Differential induction of c-Jun and Fos-like proteins in rat hippocampus and dorsal striatum after training in two water maze tasks. *Neurobiol Learn Mem*. 2005; 84: 75-84.

- [25] Méndez-López M, Méndez M, López L, Arias JL. Spatial working memory in Wistar rats: Brain sex differences in metabolic activity. *Brain Res Bull.* 2009; 79: 187-192.
- [26] Blanco E, Picón IM, Miranda R, Begega A, Conejo NM et al. (2006) Astroglial distribution and sexual differences in neural metabolism in mammillary bodies. *Neurosci Lett* 395: 82-86.
- [27] Bélanger M, Desjardins P, Chatauret N, Butterworth RF (2002) Loss of expression of glial fibrillary acidic protein in acute hyperammonemia. *Neurochem Int* 41: 155-160.
- [28] Morris RGM. Spatial localization does not require the presence of local cues. *Learn Motiv.* 1981; 12: 239-260.
- [29] Dhanda S, Sandhir R. Role of dopaminergic and serotonergic neurotransmitters in behavioral alterations observed in rodent model of hepatic encephalopathy. *Behav Brain Res.* 2015 Jan 29;286:222-235.
- [30] Arias N, Méndez M, Arias J, Arias JL. Brain metabolism and spatial memory are affected by portal hypertension. *Metab Brain Dis.* 2012; 27: 183-191.
- [31] Arias N, Méndez M, Arias JL. Differential contribution of the hippocampus in two different demanding tasks at early stages of hepatic encephalopathy. *Neuroscience.* 2015; 284: 1-10.
- [32] McDonald RJ, White NM. Parallel information processing in the water maze: evidence for independent memory systems involving dorsal striatum and hippocampus. *Behav Neural Biol.* 1994; 61: 260-270.
- [33] Guyon A, Nahon J-L. Multiple actions of the chemokine stromal cell-derived factor-1a on neuronal activity. *J Mol Endocrinol.* 2007; 38: 365–376.

- [34] Norenberg MD (1977) A light and electron microscopic study of experimental portal-systemic (ammonia) encephalopathy. *Lab Investigat* 36: 618–627.
- [35] Sobel RA, DeArmond SJ, Forno LS, Eng LF (1981) Glial fibrillary acid protein in hepatic encephalopathy. An immunohistochemical study. *J Neuropathol Exp Neurol* 40: 625–632.
- [36] Aller, M.A., Arias, J.L., Cruz, A., Arias, J. Inflammation: a way to understanding the evolution of portal hypertension. *Theor. Biol. Med. Model.* 2007; 13: 4–44.
- [37] Alitalo K, Tammela T, Petrova TV. Lymphangiogenesis in development and human disease. *Nature.* 2005; 438: 946-953.
- [38] Tammela T, Alitalo K. Lymphangiogenesis: molecular mechanisms and future promise. *Cell.* 2010; 140: 460-476.
- [39] Skobe M, Hamberg LM, Hawighorst T, et al. Concurrent induction of lymphangiogenesis, angiogenesis, and macrophage recruitment by vascular endothelial growth factor-C in melanoma. *Am J Pathol.* 2001; 159: 893-903.
- [40] Cursiefen C, Küchle M, Naumann GOH. Angiogenesis in corneal diseases: histopathology of 254 human corneal buttons with neovascularization. *Cornea.* 1998; 17: 611-613.
- [41] Detoraki A, Staiano RI, Granata F, Giannattasio G, Prevete N, de Paulis A, Ribatti D, Genovese A, Triggiani M, Marone G. Vascular endothelial growth factors synthesized by human lung mast cells exert angiogenic effects. *J Allergy Clin Immunol.* 2009; 123: 1142-1149.
- [42] Köszegi Z, Kovacs P, Wilhelm M, Atlasz T, Babai N, Kalli V, Hernadi I. The application of in vivo microiontophoresis for the investigation of mast cell-neuron interactions in the rat brain. *J Biochem Biophys Methods.* 2006; 69: 227-231.

- [43] Wilhelm M, Silver R, Silverman AJ. Central nervous system neurons acquire mast cell products via transgranulation. *Eur J Neurosci.* 2005; 22: 2238-2248.
- [44] Nassauw LV, Adriaensen D, Timmermans JP. The bidirectional communication between neurons and mast cells within the gastrointestinal tract. *Auton Neurosci.* 2007; 133: 91-103.
- [45] Ellenberg D1, Azar DT, Hallak JA, Tobaigy F, Han KY, Jain S, Zhou Z, Chang JH. Novel aspects of corneal angiogenic and lymphangiogenic privilege. *Prog Retin Eye Res.* 2010; 29: 208-248.
- [46] Leonardi A1, Motterle L, Bortolotti M. Allergy and the eye. *Clin Exp Immunol.* 2008; 153:17-21.
- [47] Cursiefen, C., Cao, J., Chen, L., Liu, Y., Maruyama, K., Jackson, D., Kruse, F.E., Wiegand, S.J., Dana, M.R., Streilein, J.W., 2004. Inhibition of hemangiogenesis and lymphangiogenesis after normal-risk corneal transplantation by neutralizing VEGF promotes graft survival. *Invest. Ophthalmol. Vis. Sci.* 2004; 45: 2666-2673.
- [48] Karkkainen, M.J., Haiko, P., Sainio, K., Partanen, J., Taipale, J., Petrova, T.V., Jeltsch, M., Jackson, D.G., Talikka, M., Rauvala, H., Betsholtz, C., Alitalo, K. Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nat. Immunol.* 2004; 5: 74-80.

Figure Captions

Figure 1. Latencies to reach the platform were calculated. Differences in escape latencies between the HT and SHAM groups were shown. * represents shorter latencies during trials 7, 9 and 10 compared to trial 1 ($P < 0.05$). Data represent mean \pm SEM.

Figure 2. Latencies to reach the platform during sample and retention in the spatial working memory test. No differences were found between groups. Data represent mean \pm SEM.

Figure 3. GFAP immunoreactivity results. Differences in the astrocyte number were found between groups in ADS and AMS. Data represent mean \pm SEM.

Figure 4. Macroscopic aspect of the ocular surface of the sham-operated group (**A**) and portal hypertension group (**B** and **C**). There were no pathological signs in the sham-operated cornea group (**A**). Vessels from the iris can be observed by transparency. In image **B**, whorls of blood vessels are present in the central cornea (arrowheads). Some white deposits (asterisk; leucomas) could be seen in the vicinity of vessels. In image **C**, dense corneal leucomas (asterisks) could be seen, associated with blood vessels (arrowheads). (Scale bars 50 μ m).

Figure 5. A. Number of mast cells (mean \pm SEM) in the HT and SHAM groups. # $P < 0.05$ was significantly higher in the HT group compared to the SHAM group. **B.** Represents the absence or presence of blood and lymphatic vessels (mean \pm SEM) in the HT and SHAM groups. # $P < 0.05$ was significantly higher in the HT group compared to the SHAM group.

Figure 6. Corneas from sham-operated animals showed a normal epithelial and stromal morphology (**A**). In **B**, mast cells (arrows) were found in low numbers in the vicinity of blood (asterisks) and LYVE1 positive lymphatic vessels (arrowheads) in sham-operated animals. A majority of the portal hypertensive rats presented large blood and LYVE1 positive lymphatic

vessels (arrowheads) invading the corneal tissue (C). Epithelial morphology was also altered. Hypertensive animals presented a significantly higher number of mast cells in the conjunctiva (D). (Scale bars 25 μm).

Figure 7. Immunofluorescent assay to demonstrate lymphatic vessels in cornea and TNF- α inflammatory marker. Coexistence of LYVE1 positive lymphatic vessels (arrowheads) and α -SMA positive blood vessels (asterisks) in the same region of the cornea in a flat mounted preparation of a hypertensive rat (B) Epithelium presented severe alterations in vascularized regions. Sham group corneas showed no alterations in their surface (A). In transversal sections, LYVE1 is overexpressed in the HT group in the epithelium and surrounding a lymphatic vessel (D). SHAM corneas showed a faint staining for LYVE1 in the epithelium (C). In a consecutive section from the same sample (F), we can observe the presence of TNF- α in the same vessel demonstrated previously with LYVE1 (D). TNF- α fluorescence is increased in the epithelium and upper stroma in HT corneas (F) compared to the sham group, where no expression of TNF- α is visible (E). (Scale bars 50 μm in A and B; 20 μm in C - F).