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Reversal learning impairment and alterations in the prefrontal cortex and the hippocampus in a model of portosystemic hepatic encephalopathy

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Abstract

Patients with liver dysfunction often suffer from hepatic encephalopathy (HE), a neurological complication that affects attention and memory. Various experimental animal models have been used to study HE, the most frequently used being the portocaval shunt (PCS). In order to determine brain substrates of cognitive impairment in this model, we assessed reversal learning and c-Fos expression in a rat model of portosystemic derivation. PCS and sham-operated rats (SHAM) were tested for reversal learning. Brains were processed for c-Fos immunocytochemistry. The total number of c-Fos positive nuclei was quantified in the prefrontal cortex and hippocampus. The spatial reference memory task showed no differences between groups in escape latencies. The no-platform probe test showed that both the PCS and the SHAM learned the location of platform. However, the PCS group perseverated in the old target during reversal. The PCS group presented less c-Fos- positive cells in prelimbic cortex, CA1 and dentate gyrus of the dorsal hippocampus than SHAM. Overall, these results suggest that this specific model of portosystemic hepatic encephalopathy produces reversal learning impairment that could be linked to dysfunction in neuronal activity in the prefrontal cortex and hippocampus.

Key words: Hepatic encephalopathy, portacaval shunt, reversal learning, c-Fos, rat.

Introduction

Many patients with liver cirrhosis suffer from a neuropsychiatric syndrome called hepatic encephalopathy (HE). As HE progresses, motor function and intellectual abilities deteriorate and patients show neurophysiological[1,2] and neuropsychological disturbances that affect attention, memory and orientation[3-5].

The factor or factors that determine the development of HE are still unclear and there are still many unanswered questions about HE, in relation to its ethiopathogeny[6,7], diagnosis[8,9] or treatment[10]. Hence, it is necessary to recur to further experimentation with the purpose of clarifying the brain substrates of the cognitive deficits found in HE.

Various experimental models of hepatic disease have been developed. Perhaps the most frequently used is that of portocaval shunt, a good model of type B HE[11], which corresponds to encephalopathy associated with portosystemic shunt that does not necessarily involve any hepatocytic alteration. Although this model does not reproduce many characteristics of portosystemic encephalopathy[11], its validity to study the biochemical alterations found in human HE has been documented[12-15]. Although portacaval-shunted rats present difficulties to learn diverse types of tasks, such as avoidance or conditional discrimination task[16-18], little research has focused on the brain substrates of the spatial learning deficits found in the Morris Water Maze (MWM) in this model[19,20].

Spatial reversal learning in the MWM is assessed by moving the hidden platform to the opposite quadrant of the maze. Thus, the rats must replace the previous spatial map with a new one in order to find the platform in the new position. This kind of learning has been described as a form of extinction that is manifested as a new spatial preference[21]. The prefrontal cortex, prelimbic (PL) and infralimbic (IL) regions, and the hippocampus are regions involved in spatial reversal[22,23].

The study of c-Fos can provide information about neuronal plasticity required for memory processes[24]. The c-Fos or c-fos-encoded protein is the product of the c-fos oncogene, an immediate-early gene (IEG). The expression of c-fos is induced after learning and is indicative of a change in neuronal

activity[24-26].

The purpose of this work is to assess spatial reversal of a rat model of portosystemic derivation. We will also analyse brain activation of prefrontal cortex and hippocampus by c-Fos immunocytochemistry.

Methods ands materials

1. Subject

A total of 22 male Wistar rats were used from the animalarium of Oviedo University. The procedures used were carried out according to the Directive 86/609/EEC (The Council Directive of the European Community) concerning the protection of animals used for experimental purposes.

2. Procurement of experimental models

The animals were randomly distributed into two groups: sham-operated (n = 12) and end-to-side portacaval shunt (n = 10). 6 animals with end-to-side portacaval shunt operation (PCS) and 6 with sham operation (SHAM) were tested in reversal learning task. Two more groups composed of animals without any learning experience, PCS-basal (n = 4) and SHAM-basal (n = 6), were used to compare basal activity of c-Fos.

Surgery was carried out under induction of anaesthesia by i.m. injection of ketamine (100 mg/Kg) and xylacine (12 mg/Kg). With respect to post-surgical care, the rats were kept close to a source of heat until they recovered consciousness to avoid hypothermia. The postoperative period lasted 45 days.

2.1. Portacaval shunt

The end-to-side portacaval shunt operation was performed according to a modified Lee's technique[27,28]. The total time in which the portal vein and inferior vena cava were clamped for anastomosis was less than 15 min. The abdominal incision was closed on two layers with an absorbable suture (polyglycolic acid) and 3-0 silk.

2.2. Sham operation

A bilateral subcostal laparotomy with prolongation to the xyphoid apophysis, followed by isolation of the portal vein with later clamping for 5 min, was performed. The operative field was irrigated with saline solution during the intervention. Finally, the abdominal incision was closed on two layers with an absorbable suture (polyglycolic acid) and 3-0 silk.

3. Evaluation of reversal learning

Reversal learning was evaluated in the circular pool designed by Morris[29] also called the Morris Water Maze (MWM). The water maze consists of a circular pool (diameter=150 cm) filled with water (30 cm deep, 22 ± 1 ºC). Rats are trained to escape from the water by swimming to reach a hidden platform. The pool was divided into 4 imaginary quadrants (quadrants A, B, C and D). The behaviour of the animal in the MWM was assessed by the EthoVision Pro programme.

Rats were trained on the hidden platform test. The animals were given 4 acquisition trials per day, for up to 4 days, to learn the location of the submerged platform hidden in the centre of quadrant D. For each trial, the rat was placed in the pool at one of four possible locations and then given 60 s to find the platform. If the platform was not found in 60 s, the rat was placed on the platform. When trial ended, the animals were allowed to remain 15 s on the platform before the next trial began. The inter-trial interval lasted 30 s. Daily, at the end of the session, rats were given a 25 s probe trial in the maze with the platform removed. The latency to find the platform, distance covered and velocity were recorded. In the case of probe trial, quadrant search was evaluated by measuring the percent of time spent in each quadrant of the pool. Then, spatial learning was demonstrated by greater swim times in the quadrant where the platform had been located previously, in comparison to other quadrants of the pool.

The next day, following the hidden platform test, rats were tested for reversal learning. The animals were given 4 acquisition trials. The hidden platform was located in a different quadrant in the maze, opposite to its previous location. As before, rats were given a probe trial.

4. c-Fos immunocytochemical analysis

Ninety minutes after the end of the reversal learning task, the animals were decapitated, brains were removed, frozen in isopentane (Sigma-Aldrich, Germany) and stored at -40 ºC. Coronal sections (30 μm) of the brain were cut at -20 ºC in a cryostat (Leica CM1900, Germany). Gelatinized slides containing the sections were post-fixed in buffered 4% paraformaldehyde (0.1 M, pH 7.4) and rinsed in phosphate-buffered saline (0.01M, pH 7.4). They were incubated with 3% hydrogen peroxidase in PBS and were washed in PBS. After blocking with PBS-T solution containing 10% Triton X-100

(Sigma, USA) and 3% bovine serum albumin, the sections were incubated with a rabbit polyclonal anti-c-Fos antibody solution (1:10000) (Santa Cruz Biotech, USA) diluted in PBS-T for 24 h at 4ºC. Afterwards, slides were washed with PBS, and incubated in a goat anti-rabbit biotinylated IgG secondary antibody (Pierce, USA; diluted 1:200 in incubating solution). They were reacted with avidin-biotin peroxidase complex (Vectastain ABC Ultra-sensitive Elite Kit, Pierce). After washes in PBS, the reaction was visualized treating the sections in a commercial nickel-cobalt-intensified diaminobenzidine kit (Pierce). The reaction was terminated by washing the sections twice in PBS. Finally, the slides were dehydrated and cover-slipped.

The total number of c-Fos positive nuclei was quantified in two alternate sections containing the Prelimbic (PL) and Infralimbic (IL) cortex, and the dorsal hippocampus (CA1, CA3 and DG). Coronal sections of these brain regions were located using the stereotaxic atlas of Paxinos and Watson[30]. The distance of the brain regions counted from bregma was: +3.0 for PL and IL, and -3.72 for CA1, CA3 and DG.

The quantification was done by systematically sampling each of the regions selected using counting frames superimposed over the region. Counting of c-Fos positive nuclei was performed using a microscope (Olympus BH-2, Japan) coupled to an analogic camera (Sony XC-77, Japan) and a TV monitor. The c-Fos positive nuclei were defined based on homogenous grey-black stained elements with a well-defined border. Finally, cell counts from the two selected sections for a given brain region in each animal were averaged and the mean was used for statistical analysis.

5. Data Analysis

All data were analysed in the Sigma-Stat 3.2 program (Systat, Richmond, USA) and were expressed as mean ± SE.

For the initial hidden platform test, the latencies to reach the hidden platform for each day or session (average of four trials) were analysed with a two-way repeated measures ANOVA (between factor: Group; within factor: Session). When a significant session effect was found, a further repeated measures ANOVA was conducted for each group. Distance covered and velocity were analysed using a Student *t*-test. The time spent in each quadrant during the probe test was analysed separately for each group and session using a one-way ANOVA design (factor: Quadrants). Tukey's test was used as a post hoc test. For the reversal task, the latencies to reach the hidden platform in acquisition trials (average of four trials) were compared with a Student *t*-test. The time spent in each of the four quadrants during the probe test in reversal was analysed separately for each group using a one-way ANOVA.

The results obtained by c-Fos quantification were analysed by a one-way ANOVA.

Results

1. Reference-memory

There were differences in escape latencies between PCS and SHAM ($F(1,10) = 5.176$, $p = 0.046$). PCS presented longer escape latencies than SHAM on day 2 ($t_{10} = 3.648$, $p = 0.004$). The variable day also showed a significant effect ($F(3,30) = 46.06$, $p < 0.001$). The SHAM group showed an improvement over the days ($F(3,15) = 22.028$, $p < 0.001$), presenting longer latencies to target on Day 1 compared to the rest of training days ($p < 0.001$). Similarly, the PCS group showed a reduction in latencies over the days ($F(3,15) = 11.92$, $p < 0.001$). PCS presented shorter latencies on Days 4 and 3 compared to Day 1 ($p < 0.05$), and on Day 4 compared to Day 2 ($p = 0.036$) (Fig 1).

The distance covered (mean±SE) by SHAM (1349 ± 300 cm) and PCS (1282±204 cm) was similar ($t_{10} = 0.183$, $p = 0.859$). Also, the SHAM group (55 ± 8 cm/s) showed a similar swimming velocity to the PCS group (52 ± 6 cm/s) ($t_{10} = 0.251$,

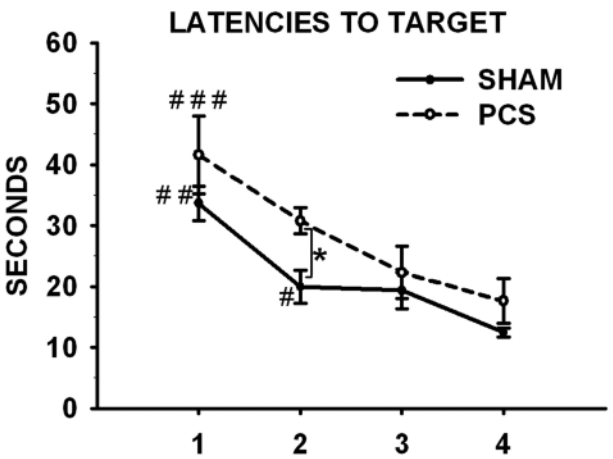

Fig. 1. — Escape latencies (mean ± SEM) of the portacaval shunt group (PCS) and the sham-operated group (SHAM) during the four training sessions of the reference memory task. Significance of differences between PCS and SHAM (* < 0.05) in escape latencies; significance of differences between sessions (# $p < 0.05$, more details in Results section).

p = 0.807).

Probe test analysis showed that the PCS group learned the location of the platform on Day 4. On this day, the PCS group spent more time in the quadrant where the platform was located compared to the rest of the days ($F_{(3,20)} = 6.53$, p = 0.003), D vs. B, A and C ($p < 0.05$). On Day 3, there were also differences in the percentage of permanence between quadrants ($F_{(3,20)} = 3.317$, p = 0.041), but the differences were exclusively found in the comparison between Quadrants D and B (p = 0.043). With respect to the SHAM group, spatial learning was shown since Day 3 ($F_{(3,20)} = 10.605$, $p < 0.001$), when the SHAM group spent more time in the reinforced quadrant with regard to the rest. There were differences between quadrant D and A, B, C ($p < 0.05$). On Day 4, this learning was also shown. There were differences between quadrants ($F_{(3,20)} = 9.217$, $p < 0.001$). Quadrant D was preferred by the SHAM rats compared to A, B and C ($p < 0.05$). Although on Day 2, there were differences in the time of permanence between quadrants ($F_{(3,20)} = 9.217$, $p < 0.001$), these differences were only found between D and A (p = 0.045) (Fig. 2).

2. Reversal learning

There were no significant differences between the PCS and SHAM groups in latencies to reach the hidden platform during the acquisition trials of the reversal test ($t_{10} = 1.123$, p = 0.288) (Fig. 3.a).

However, the groups differed in the transfer test. The SHAM group was able to learn the new location of platform in the reversal test. The SHAM rats presented differences in the time of permanence in the quadrants of the pool ($F_{(3,20)} = 10.401$, $p < 0.001$). They preferred the new platform position or quadrant C versus the remaining quadrants A, B and D ($p < 0.05$).

However, the PCS group did not learn the new location of platform. The PCS group presented differences in the time of permanence in the quadrants of the pool ($F_{(3,20)} = 9.487$, $p < 0.001$). The PCS group preferred Quadrants C and D equally,

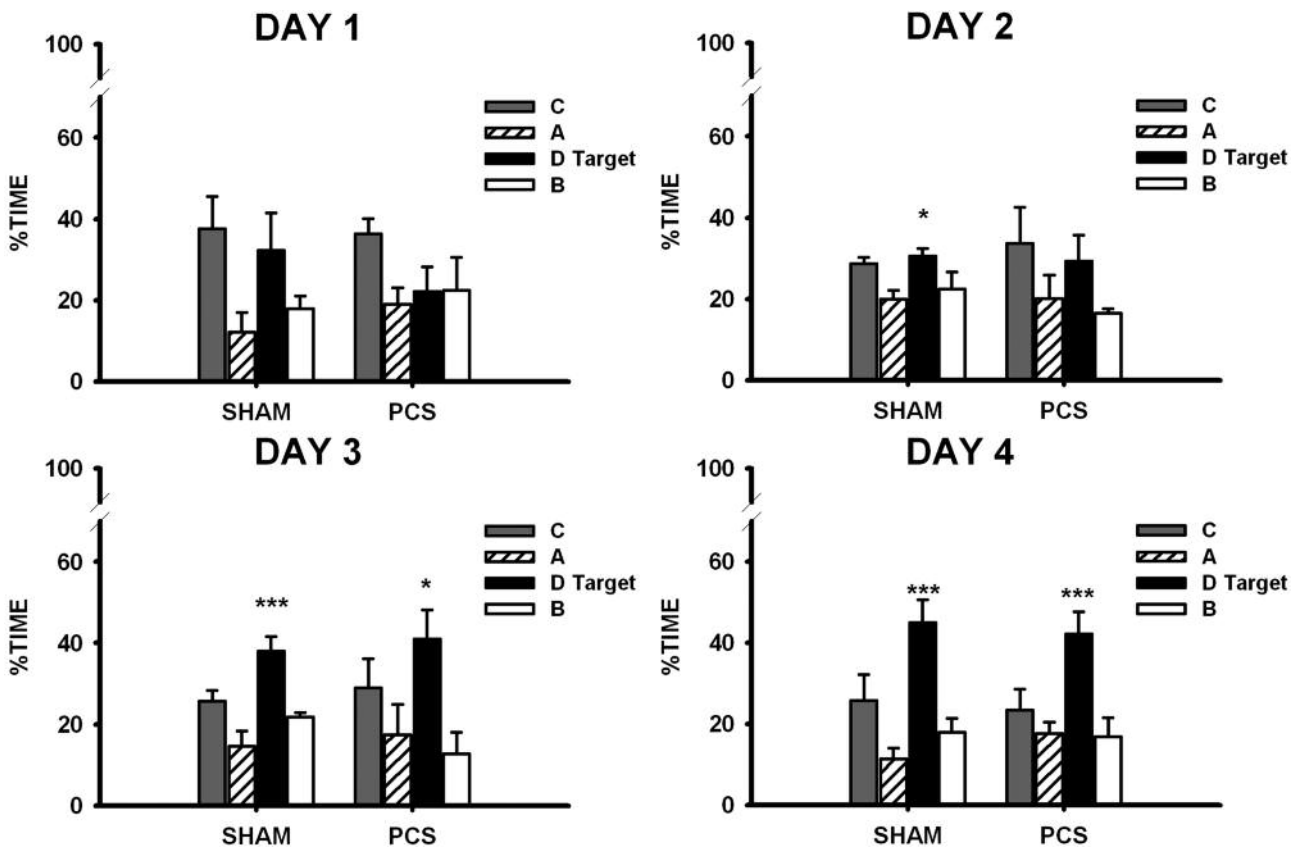

Fig. 2. — Probe tests in the spatial reference memory test during four training sessions (mean ± SEM). In the probe tests, three asterisks above the bar indicate a statistically significant difference in the time spent in Quadrant D (target) compared to time spent in any of the other quadrants (***$p < 0.05$). One asterisk above the bar indicates significant difference in the time spent in Quadrant D compared to the time spent in one of the other three quadrants (*$p < 0.05$).

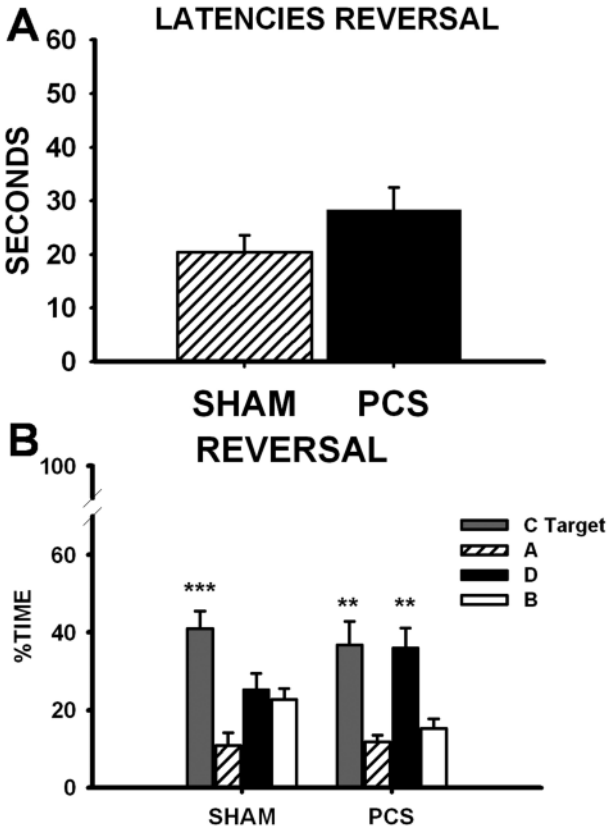

FIG. 3. — (A) Escape latencies (mean ± SEM) of the portacaval shunt group (PCS) and the sham-operated group (SHAM) during the acquisition trials of the reversal test. (B) Probe test in the spatial reversal task (mean ± SEM). Three asterisks above the bars indicate a statistically significant difference in the time spent in Quadrant C (Target) compared to time spent in any of the other three quadrants (*** $p < 0.05$). Two asterisks above the bar indicate a significant difference in the time spent in Quadrants C or D (previous target) compared to time spent in two of the other three quadrants (**$p < 0.05$).

as both quadrants differ from A and B ($p < 0.05$) (Fig. 3.b).

3. c-Fos quantification results

The results show differences between groups in PL cortex ($F_{(3,18)} = 46.071$, $p < 0.001$). The post hoc Tukey's test revealed that in PL cortex, the SHAM group had a greater number of c-Fos positive cells than the PCS ($p < 0.007$) and both SHAM-basal and PCS-basal presented lower c-Fos cells than the trained groups ($p < 0.001$). Differences between groups were also found in IL cortex ($F_{(3,18)} = 16.674$, $p < 0.001$), since the trained groups had more c-Fos cells compared with the basal groups ($p < 0.007$). Differences between groups were found in the hippocampal regions: DG ($F_{(3,18)} = 8.083$, $p < 0.001$), CA3 ($F_{(3,18)} = 27.242$, $p < 0.001$) and CA1 ($F_{(3,18)} = 22.362$, $p < 0.001$). Regarding DG, more c-Fos cells were found in SHAM group than in PCS group ($p = 0.006$) and basal groups ($p < 0.006$). With respect to CA3, both trained groups, presented more c-Fos positive cells than the basal groups ($p < 0.001$). Finally, SHAM group showed greater number of c-Fos positive cells than PCS ($p = 0.015$) in CA1. In this region, basal groups presented lower c-Fos cells than SHAM and PCS ($p < 0.001$ and $p < 0.02$, respectively) (Fig. 4. and Fig. 5.).

Discussion

Our work reveals the presence of cognitive alterations in the process of reversal learning in the MWM in a model of Type B hepatic encephalopathy by portacaval shunt. This learning impairment is accompanied by a low number of c-Fos positive cells in the PL cortex, CA1 and DG of the dorsal hippocampus.

A reversal task in the MWM revealed differences between SHAM and PCS, as the latter was unable to remember the new location of the platform during the probe test of the reversal task. These differences cannot be justified by motor problems. The PCS model presented abnormalities in structures and pathways of the motor system[31,32]. However, the PCS group presented similar displacement speed and distance covered to the SHAM group. Therefore, memory alterations were not due to the impairment of motor activity in the experimental model used, in accordance with other studies[20].

The PCS rats presented a learning delay in the MWM. Performance of the probe test allowed us to assess the learning ability over the days. Hence, we could observe that the PCS group was able to remember the location of the hidden platform on the final day of the task, which reveals a delay in spatial learning by allocentric information, supporting previous results[20]. However, studies aimed at evaluating spatial learning of the PCS group in the MWM present contradictory results. Some studies report an absence of differences between the SHAM and PCS groups[33], whereas other studies reveal memory deficits[19,20]. The disparity among data may be largely due to differences in the MWM procedure.

Once spatial learning was established, we moved the hidden platform to the opposite quadrant of the pool. Thus, we could observe that, in contrast to the SHAM group, which rapidly learned the new location, the PCS model showed preference for the previous platform position. The PCS model of HE shows perseveration in the previous reinforced quadrant, confirming that spatial reversal is affected.

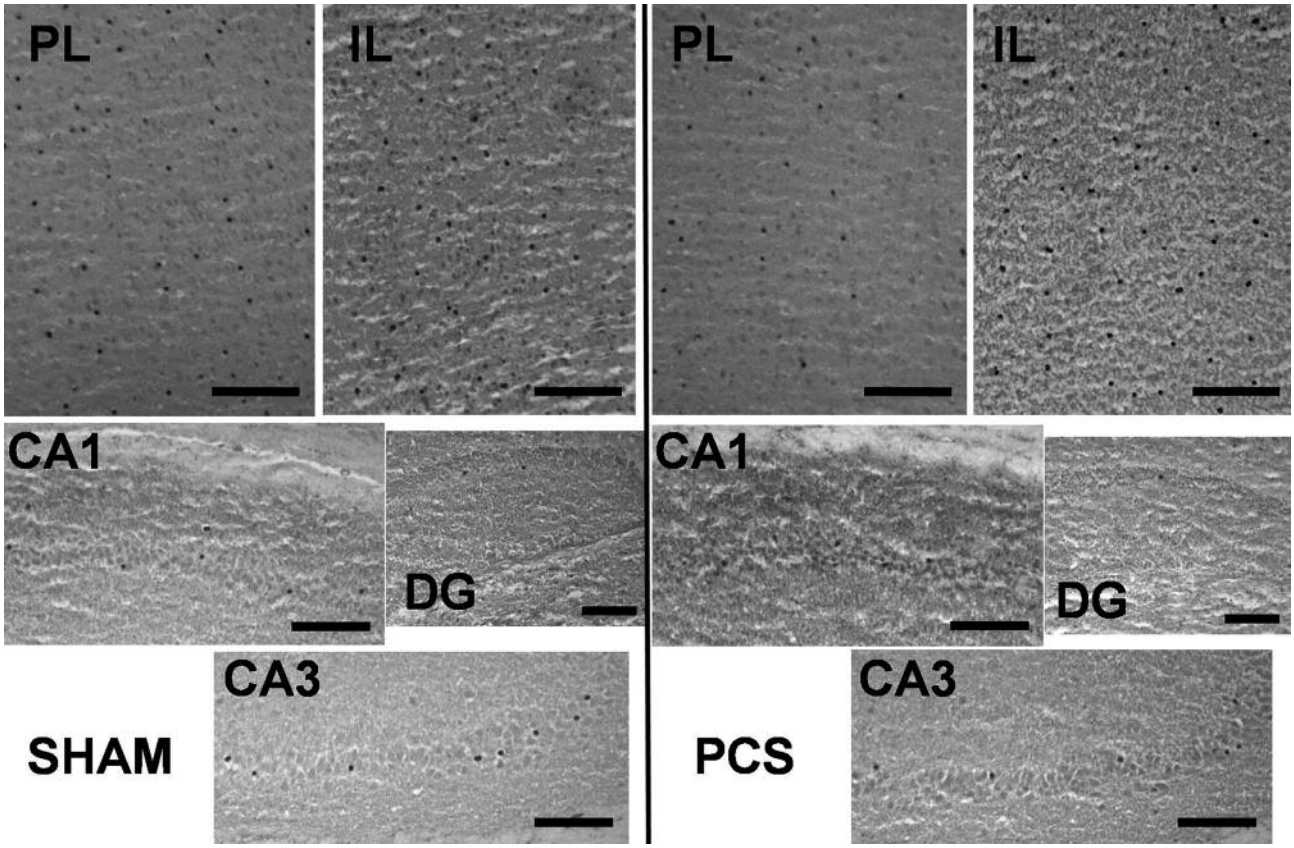

FIG. 4. — Representative photomicrographs showing sections of the prelimbic (PL) and infralimbic (IL) regions, CA1, CA3 and dentate gyrus (DG) hippocampal subfields that were immunostained for c-Fos protein. Scale bar is 150 m.

As far as we know, no studies to date have assessed reversal learning in a model of HE by PCS. It is important to know that the PCS model not only shows spatial learning delay, it also presents problems in the ability to adjust to changing environment. The PCS model presents absence of cognitive flexibility, showing inability to learn a new spatial position.

In animals, one study has found a deficit of cognitive flexibility in a model of toxic liver failure[34], revealing impairment of plasticity of memory. In humans, few studies have been carried out on memory alterations in patients with minimal or early HE and, although some authors argue that memory disturbances are not a major symptom of HE[3], others state that patients with cirrhosis present poorer performance in several memory tests, including the Wechsler Memory Scale-Revised test and an abbreviated version of Benton's Visual Retention Test[35]. Recently, Ortiz *et al*.[5], using the Auditory Verbal Learning Memory Test, showed a learning deficit and an impairment in long-term memory and recognition in these patients.

Regarding the cerebral substrates of the alterations accompanying HE, other studies have reported brain oxidative metabolism impairment associated with behavioural alterations in PCS rats[36,37]. With respect to immediate-early genes, no studies to date have assessed immediate-early gene expression in the PCS model. An increase in c-fos immunoreactivity was found in the nucleus accumbens of hyperammonemic rats following injection of glutamate receptors agonist[38], and a reduction in expression and dephosphorylation of c-fos and Sp1 mRNA in cultured rat astrogial cells was reported in the same animal model[39]. Recently, in a model of cirrhosis, impairment of spatial working memory and a decrease in c-Fos positive cells in the hippocampus and prefrontal cortex[40] have been shown. Interestingly and in accordance with our data, differences in the number of c-Fos positive cells appeared after performing the memory task and not when comparing basal activity in untested animals[40].

Our c-Fos results showed that c-Fos immunoreactivity in PL cortex and CA1 and DG of the dorsal hippocampus in the PCS model is lower than in the SHAM model after performing the spatial reversal task in the MWM. This could be due to the rise in

cerebral ammonia. It is well demonstrated that rat models with portacaval anastomosis present hyperammonemia[11]. Recently, it has been demonstrated that acute ammonia induces brain RNA oxidation[41]. This could affect gene expression and local protein synthesis, as well as the postsynaptic protein synthesis required for memory consolidation. Related to this, it is well known that PCS rats present alteration of long-term potentiation in the hippocampus[19], a process known to be involved in spatial reversal learning in the MWM[42]. The PCS learning deficit may be associated with increased ammonia levels that impair induction of NMDA receptor-dependent long-term potentiation in the hippocampus and also alter the neural glutamate-nitric oxide cyclic GMP pathway, a pathway involved in memory[16,43].

In addition to the hippocampus, several works have demonstrated the involvement of prefrontal cortex in spatial memory[23,44]. The hippocampus and PL cortex are connected, and CA1 is the mayor efferent[45,46]. Low c-Fos immunoreactivity both in CA1 and PL cortex could be an effect of hippocampal-prefrontal cortical circuit dysfunction. The correct functioning of this pathway is essential for memory[47]. Therefore, disrupted hippocampal-prefrontal cortex circuit would be responsible for the poor cognitive functioning of the PCS group.

In conclusion, the results obtained show that the model of Type B HE by portosystemic shunt presents alterations in spatial reversal and dysfunction of the neural activity of hippocampus and prefrontal cortex.

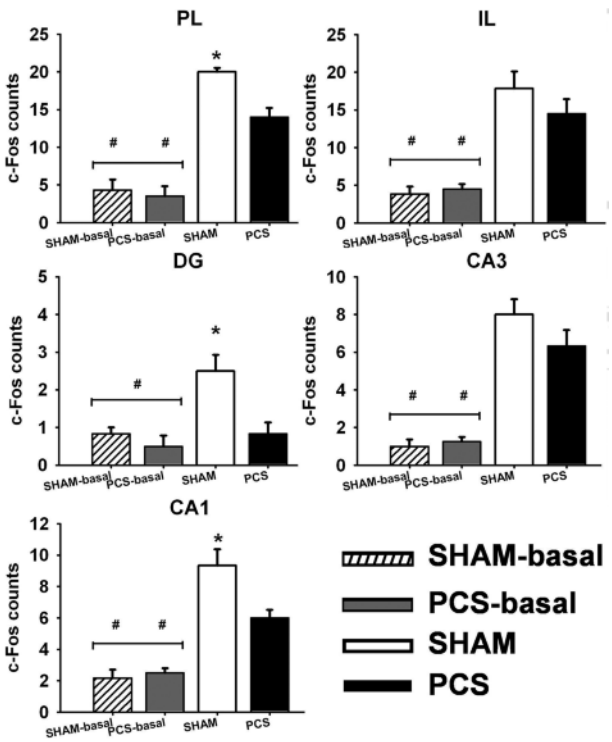

FIG. 5. — Number of c-Fos-immunoreactive cells present in prelimbic (PL) and infralimbic (IL) cortex, CA1, CA3 and dentate gyrus (DG) hippocampal subfields of sham-operated rats (SHAM-basal), rats with portacaval-shunt (PCS-basal), sham-operated rats (SHAM) and rats with portacaval-shunt (PCS) that were submited to the learning task are shown (mean±SEM). Significance of differences between trained groups, PCS vs SHAM (*$p < 0.05$); basal groups (SHAM-basal and PCS-basal) vs SHAM #($p < 0.006$); basal groups vs trained groups ## ($p < 0.05$).

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