

Reviewing Section Editor: Prof. Jeffrey Mogil

Learning impairments and hippocampal dopaminergic changes in monoarthritic rats

Miguel Pais-Vieira ^{1,3}, Maria Manuela Mendes-Pinto ³, Deolinda Lima^{2,3}, Vasco Galhardo ^{1,3*}

1. Instituto de Histologia e Embriologia, Faculdade de Medicina, Universidade do Porto, Porto, Portugal
2. Laboratório de Biologia Molecular e Celular, Faculdade de Medicina, Universidade do Porto, Porto, Portugal
3. IBMC – Instituto de Biologia Molecular e Celular, Universidade do Porto, Portugal

Number of text pages: 25

Number of figures: 4

Number of words: 7077

Running header: learning impairments in chronic pain rats

Acknowledgements:

Supported by: FCT (SFRH/BD/24383/2005), Bial Foundation (grant 84/04)

Keywords: monoarthritis, Complete Freund's Adjuvant, dopamine, hippocampus, orbitofrontal cortex, learning, working memory, reference memory,

**Corresponding author:*

Vasco Galhardo, PhD

IBMC – Instituto de Biologia Molecular e Celular

Rua do Campo Alegre, 823

4150-180 Porto

PORTUGAL

Tel: +351 22 6074900

Fax: +351 22 6099157

e-mail: galhardo @ med.up.pt

ABSTRACT

Chronic pain is known to cause memory impairments in human subjects. Causes for these deficits are not clear, but previous studies have found abnormalities in anatomical structures and neurochemical functioning in several memory and pain related structures. It has previously been shown that monoarthritic animals present decreased tonic levels of dopamine and serotonin in the orbitofrontal cortex and increased dopamine and serotonin in the amygdala, but it is not known how other areas related to chronic pain and memory are affected. In this study we analyzed tonic levels of monoamines in the hippocampus (HPC) and compared learning and memory in control and monoarthritic animals in behavioural tasks known to be dependent on the integrity of the PFC/OFC and the Hippocampus (the water maze and radial arm maze). Persistent pain was induced by intra-articular injection of Complete Freund's Adjuvant and the development of monoarthritis was assessed by sensitivity to von Frey filaments. Different groups of animals were tested in two different stages to compare the evolution of learning and memory during the chronic pain process. Four experiments were made: in the first experiment, levels of monoamines in the HPC after 21 days of monoarthritis were compared between monoarthritic and control groups; in the second experiment, four groups of animals were tested for reference memory in the water maze; in the third experiment, two groups of animals were tested for executive function (known to be dependent of the hippocampus) in the radial arm maze; and in the fourth experiment two groups of animals were tested for short-term memory (known to be dependent of the prefrontal cortex) in the radial arm maze. Results show that monoarthritic animals presented reduced tonic levels of dopamine in the HPC and that, when tested 5 days after chronic pain, monoarthritic animals presented degraded learning curves in the water maze and decreased executive function in the radial arm maze. When animals were tested after 30 days of chronic pain, training did not lead to similar performances between groups in the radial arm maze showing a deficit in the short term memory component tested, but no deficits in the win-shift task. These results suggest that monoaminergic changes in the Hippocampus and the Prefrontal cortex might be the basis of learning and spatial working memory deficits found.

INTRODUCTION

Chronic pain is known to be associated with cognitive impairments in humans. These deficits are present in a wide range of cognitive abilities such as memory (Grace et al., 1999; Brown et al., 2002), attention (Dufton, 1989); (Crombez et al., 2000); (Harris et al., 2003); (McCracken and Eccleston, 2003); (Dehghani et al., 2003); (Grisart and Van Der, 2001)), decision-making (Apkarian et al., 2004a), and learning (Kewman et al., 1991); (Iezzi et al., 1999). The cause for the cognitive deficits in chronic pain is not clear, but anatomical changes (Apkarian et al., 2004b), abnormal brain chemistry (Grachev et al., 2000); Pais-Vieira et al., 2008), increased levels of stress and anxiety that correlate with depression (Brown et al., 2002) chronic use of antidepressants (McCracken and Iverson, 2001) as well as reduced sleep quality (Grace et al., 1999) are present in these patients.

One of the most common complaints in chronic pain patients is the presence of memory deficits (McCracken and Iverson, 2001); (Glass, 2006). Imaging studies have shown that chronic pain patients present specific brain activation patterns with the involvement of memory associated regions such as the thalamus, the prefrontal cortex and the hippocampus (see (Brooks and Tracey, 2005) for a review of imaging studies in pain conditions). The simultaneous use of brain regions by cognitive processing and chronic pain has led to the proposal that chronic pain can be considered as a 'cognitive state' that is competing with cognitive abilities for several tasks (Apkarian et al., 2004a). Despite these findings, a detailed understanding of the neurobiological basis of these impairments remains elusive and memory tests in chronic pain patients have shown both normal (Apkarian et al., 2004a) and impaired performances (Glass, 2006). Differences reported are not clear, but might be related to tests used, patient populations and the variables used to measure cognitive function.

An adequate description of the effects of chronic pain in learning and memory requires the detailed study of the cognitive abilities in animal models of chronic pain, but literature in this subject is still scarce. Previous works have studied the behavior of chronic pain rats in tasks with cognitive components, but always with the aim of developing tests for the study of pain assessment and drug effects. So far, no animal work has systematically studied memory in validated behavioural tasks in order to detect specific deficits. Despite the reports of deficits in the non-selective non-sustained attention (Millecamps et al., 2004), decreased global

behavioural function (Cain et al., 1997) and decreased learning in a discrimination task (Messaoudi et al., 1999) these tasks involve several cognitive abilities and thus, do not allow a clear understanding of whether there are specific impairments in memory in chronic pain animals or its neurobiological basis.

We have previously studied two important regions of the pain matrix (see (Brooks and Tracey, 2005) for a review of pain matrix regions) the OFC and the amygdala and found reductions of dopamine in the orbitofrontal cortex (Pais-Vieira et al., 2008). Despite the role of the dopaminergic system has been extensively studied in the last years (see (rias-Carrion and Poppel, 2007) for a review of the role of dopamine in learning and reward) and a clear role for it has been established both in chronic pain and in memory, an extensive description of dopaminergic changes in chronic pain has not yet been done. Here, we specifically hypothesized that animals with chronic pain would present learning and memory deficits in tasks with high spatial component accompanied by altered tonic levels of monoamines in the HPC. In this study, we looked for reference and working memory deficits in two different tasks dependent on the prefrontal cortex and hippocampus integrity. We analyzed groups of animals at 5 or 30 days of chronic pain. Reference memory was considered as memory of specific facts of actions that remains constant across sessions and was studied in the water maze, which is known to be dependent on the integrity of hippocampus (Morris et al., 1982). Working memory has been traditionally studied in two main sets: executive function as the ability to perform a sequence of behaviors (Baddeley, 1996) which is known to be dependent on the hippocampus, but not amygdala or prefrontal cortex, (McDonald and White, 1993);(Floresco et al., 1997); and short term memory, as the ability to recall trial unique information (Goldman-Rakic, 1995) which is know to be dependent on the prefrontal cortex. Previous works have shown a critical role for dopaminergic modulation and hippocampal-prefrontal cortical activity in reference memory, short-term memory and in executive function (Seamans and Phillips, 1994); (Zahrt et al., 1997;Floresco et al., 1997;Seamans et al., 1998), (Wang and Cai, 2006).

In order to evaluate if the presence of deficits would be due to general learning and memory deficits or due to a specific cognitive component (i.e. spatial reference memory, short term memory or executive function), learning abilities were studied in animals injected with Complete Freund's Adjuvant and control animals in different tasks. Four experiments were

performed: in the first experiment, levels of monoamines in the HPC were studied in control and monoarthritic animals; in the second experiment, four groups of animals were tested in the water maze for general learning abilities and reference memory; in the third experiment, four groups of animals were tested for executive memory in an eight arm baited procedure (win-shift) in the radial arm maze; and in the fourth experiment, four groups of animals were tested for both learning and short term memory in a one arm baited procedure (win-stay) in the radial arm maze. In the water maze experiment four groups of animals were tested 5 or 15 days after the injection of CFA or saline and in each of the radial arm maze experiments groups were tested at 5 or 30 days after the injection of CFA or saline.

MATERIALS AND METHODS

All the work was done in accordance to EU ethics committee on animal research as well as IASP guidelines for work in wake animals. Wistar rats (n=107) with weights between 300-320g at the beginning of the experiments were used (Charles River, Barcelona). During the experimental period, animals that performed the radial arm maze task received water *ad libitum*, but were food deprived to 85% of baseline body weight by limiting their access to food to a single daily meal. All the experiments were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Chronic inflammatory pain model

Chronic inflammatory pain was induced by injection in the tibio-tarsal joint under anesthesia with 50µg of *Mycobacterium butiricum* (Complete Freund's Adjuvant - CFA). Anesthesia was induced with an i.m. injection of xylazine and ketamine (10 and 60 mg/kg mixture, respectively, to create a model of persistent monoarthritic inflammatory pain (as described by Buttler et al., 1992). Control animals were only anesthetized.

Mechanosensory threshold

Sensory threshold as measured by von Frey filaments was studied as previously described (Chaplan et al., 1994). Briefly, the von Frey filaments (Somedic, Sweden) were a series of filaments with different diameters applied perpendicular to the plantar surface until slight

buckling was caused, and maintained for 6-8 seconds. Each filament was applied 10 times with an interval of 5 seconds between stimuli. A positive response was considered if the paw was sharply withdrawn or flinching occurred. Sensory threshold was considered has a minimum of 5 positive responses. Force values are presented in grams.

HPLC analysis of biogenic amines

Neurochemical analysis of tonic content of monoamines and monoamine metabolites in prefrontal areas was performed in controls (n=6) and in animals with 21 (n=6) days of pain. HPLC was applied according to a modified method of (Ali et al., 1993). Animals were decapitated with a small animal guillotine, and bilateral Hippocampal formation was freshly dissected and in 2-methylbutane over dried ice and stored at -80°C. Before HPLC analysis, samples were left overnight with 200µL of perchloric acid 0.2N at -20°C. The homogenate was centrifuged (5000 rpm, 3 min) and the supernatant filtered through a 0.2 µm Nylon microfilter (COSTAR) by centrifugation (10000 rpm, 5 min) for HPLC analysis. The resulting pellet was kept at -20°C until protein quantification.

The high performance liquid chromatography with electrochemical detection (ECD) was applied according to a modified method previously described (Ali et al., 1993). Analyses were carried out in a Gilson Medical Eletronics HPLC system (Middleton, WI) with a LC-234 auto-injector, equipped with a LC307 delivery pump and with a LC142 electrochemical detector, under reversed phase conditions with a Supelcosil LC 7.5 cm x 4.6 cm, 3 µm column. The software used was a 712 HPLC system controller data version 1.30 management. Compounds were eluted isocratically over 18 minutes runtime at a flow rate of 1mL/min. The mobile phase consisted of 70mM potassium dihydrogen phosphate buffer (pH adjusted to 3.0 with phosphoric acid), 1mM 1-hepatosulfonic acid, 107.5µM sodium EDTA and 10% methanol. Sample injection was 20µL and electrochemical detector was recorded with a glassy carbon working electrode set at + 0.75 V. Identification was performed by comparison with standard retention times determined by injections of standard mixture run at given intervals between samples analysis. Quantification was made using the calibration curves standards and with the protein content. Amount of each compound are expressed in ng of catecholamine per mg of protein. The r-values of each catecholamine were the following: dopamine=0.9998, DOPAC=0.9999, serotonin= 0.9998, 5-HIAA=0.9998, norepinephrine=0.9994, and

epinephrine=0.9998. Protein content was determined by the Bradford BIO-RAD protein assay reagent using BSA as a standard and a Sunrise Absorbance Microplate Reader (TECAN). To enhance the proteins extractability, pellets in phosphate buffer were previously sonicated in ice for 14 seconds with an ultrasonic homogenizer (SONOPLUS HD 2200) adjusted to 40% pulse/second, with a minimum output of 10%).

HPLC grade methanol was purchased from Panreac. 2-methyl butane and heptosulfonic acid were obtained from Fluka. Perchloric acid, sodium EDTA, potassium dihydrogen phosphate, sodium dihydrogen phosphate monohydrate and di-sodium hydrogen phosphate dihydrate all from Merck, and phosphoric acid from Aldrich. BIO-RAD protein assay from BIO-RAD. Ultrapure water (18.2 MΩ/cm) (MAXIMA ultra pure water) was used for all the analysis. The commercial standards: Dopamine [(3,4-Hydroxyphenethylamine)], DOPAC [(3,4-Hydroxyphenyl-acetic acid)], 5-HT [(5-Hydroxytryptamine)], 5-HIAA [(5-Hhydroxyindole-3-acetic acid)], NE [(-)- Norepinephrine], E [(-)-Epinephrine], and Bovine Serum Albumine (BSA) were all purchased from Sigma.

Learning and Memory Testing

To study the effect of chronic pain in learning and memory processes, animals injected with Complete Freund's Adjuvant or control animals were tested in two different tasks with a high spatial component: the water maze (Morris et al., 1982), the radial arm maze (Olton and Samuelson, 1976). Two different protocols were used in the radial arm maze.

Water maze

A five-day water maze protocol was performed with three trials per day separated by 30 minutes interval. Briefly, the water maze apparatus consisted of a circular pool of 150cm diameter and 60cm height, filled with water (30cm deep, 24-26 °C) made opaque by the addition of non-toxic white paint (EcoDesign). A wire mesh covered platform was placed in one of the quadrants of the pool. Animals were released from eight pre determined, but not sequential symmetrical positions. Swim speeds and latency to reach platform were recorded and analyzed. On the last day, after the third trial, the platform was removed and animals were allowed to swim for 60 seconds. If animals did not find the platform within the 60 seconds of

the trial they were gently guided to the platform and allowed to remain there for 5 seconds. Swimming paths were recorded in video and computer analyzed by Wintrack (version 2.4.) (Wolfers and Lipp, 1992). The amount of time spent in the platform quadrant, the distance traveled in the platform quadrant and the number of crossings of the exact platform location were compared between groups.

In the first water maze experiment twenty three animals (n=11 control and n=12 experimental animals) were tested 5 days after the injection of Complete Freund's Adjuvant. In the second water maze experiment (n=6 control and n=6 experimental animals) a similar protocol was used, but the two groups were tested 15 days after the injection of Complete Freund's Adjuvant or saline.

Radial Arm Maze with a win-shift protocol (executive function)

The radial arm maze was conducted in an eight-arm maze made of clear Plexiglas with a stainless steel grid floor with a central octagonal hub (Coulbourn Instruments, Allentown, PA, USA). At 2 cm from the end of each arm a small circular cup with 2 cm wide and 1 cm height would contain one food pellet per trial (20 mg chocolate flavor sucrose pellets, Research Diets Inc., New Brunswick, NJ, USA) in the first protocol and 2 pellets per trial in the second protocol. No arm was rebaited during each trial. The maze was located on the center of a room with several geometric cues in the walls. At the beginning of each trial the animal was placed in the center of a circular ring that was removed after 10 seconds. During a trial all doors were opened and animals were allowed to explore the maze for 6 minutes. Thirteen sessions were performed previous to experimental testing and 6 sessions after, each with one daily trial. The number of reentries in previously visited arms and the mean total number of errors across all sessions were analyzed. In the first experiment 19 animals were studied (n=10 CFA group and n=9 Control group) 5 days after the injection of CFA or saline. In the second experiment 12 animals were studied (n=6 CFA group and n=6 Control group) 30 days after the injection of CFA or saline. Both groups were previously introduced to the rewards while still in their home cages.

Radial Arm maze with a win-stay protocol (short term memory)

In the win-stay protocol each trial consisted in two phases: in the first phase all doors except one were closed and animals were allowed 30 seconds to enter the arm and eat two pellets previously placed in the food magazine in the end of the arm. In the second phase the same arm as in phase 1 was rebaited with two pellets, but all doors were opened and the animals were allowed to enter every arm. The second phase lasted for 4 minutes. 6 sessions were performed each with 5 trials spaced by two minutes each. The number of arms and the sequence of visits were recorded and analyzed. The total number of errors across sessions was also compared between groups. At the end of the trial the animal was returned to its home cage where it waited for the next trial. In the first experiment 12 animals were studied (n=6 CFA and n=6 Control group). In the second experiment 12 animals were studied (n=6 CFA group and n=6 Control group) 30 days after the injection of CFA or saline. Both groups of animals were previously introduced to the rewards while still in their home cages.

Statistical Analysis

In the radial arm the number of errors was transformed and analyzed as the square root of the original value. Repeated measures two way ANOVA (with time as the within subjects factor and treatment as between subjects factor) followed by Bonferroni's analysis was used to study water maze test latencies between groups and errors across sessions in both radial arm maze protocols. In the win-shift procedure the last 3 sessions previous to the injection of CFA were also studied and compared. Significant differences were considered if $p < 0.05$. Comparison between swim speeds, percentage of time spent in the platform quadrant, precise platform crossings, distance traveled in the platform quadrant, total average number of errors, and values of sensory threshold were studied with student's t test or Mann-Whitney U test.

RESULTS

Five rats were excluded from experiments: 3 rats were extremely anxious and did not perform the radial arm maze, remaining in a single arm for more than 3 consecutive sessions. Two rats from the chronic inflammation groups developed poliartthritis and results were excluded from the analysis.

Results from experiment 1: HPLC analysis

Sensory threshold 21 days after CFA or saline injection

CFA injection induced lower values of sensory threshold in all the animals while control animals remained with values similar to the beginning of the experiment (mean control 8.38 ± 1.90 ; mean monoarthritic 0.03 ± 0.01 ; Mann-Whitney $U = 0.00$, $P < 0.01$)

Comparison of Biogenic monoamines in the hippocampus

CFA injection induced lower values of dopamine in the HPC after 21 days sensory threshold in monoarthritic animals when compared to control animals (mean control 12.74 ± 3.22 ; mean monoarthritic 5.06 ± 1.02 ; $t_{10} = 2.27$, $P < 0.05$), but not of DOPAC (mean control 3.88 ± 0.75 ; mean monoarthritic 1.98 ± 0.84 ; $t_{10} = 1.69$, $P = 0.12$), 5HIAA (mean control 24.24 ± 8.15 ; mean monoarthritic 8.14 ± 2.17 ; $t_5 = 1.91$, $P = 0.11$) or 5HT (mean control 188.4 ± 75.88 ; mean monoarthritic 89.72 ± 35.38 ; $t_{10} = 1.18$, $P = 0.27$). (see figure 1) Levels of HVA, epinephrine and norepinephrine were too low to be detected.

Results from Experiment 2: Reference memory

Sensory threshold 5 days after CFA or saline injection

CFA injection induced lower values of sensory threshold in all the animals while control animals remained with values similar to the beginning of the experiment (mean control 8.06 ± 0.51 ; mean monoarthritic 2.72 ± 0.50 ; Mann-Whitney $U = 0.00$, $P < 0.01$)

Water Maze -5 days after CFA or saline injection

The first parameter analyzed in the water maze test was the swim speed between groups. Swim speed between monoarthritic and control animals was not different (mean control 0.338 ± 0.01 , mean monoarthritic 0.339 ± 0.01 ; $t_{22} = 0.03$, $P = 0.97$ n.s.).

The analysis of the learning curves of monoarthritic animals reveals differences essentially in the first five trials of the learning curve (these trials correspond to the first two days of

testing). The largest difference is in the first trial of the second day (as shown in figure 2) ($F_{14,420} = 2.65$, $P = 0.001$, for interaction trial \times group; and $F_{1,420} = 7.64$, $P < 0.01$ for groups).

The probe trial paths reveal that although monoarthritic animals have different learning curves, they can learn the water maze task. In the probe trial both groups spend similar amounts of time in the goal quadrant (mean control 43.63 ± 3.31 , mean monoarthritic 42.29 ± 1.97 ; $t_{17} = 0.35$, $P = 0.73$ n.s.). The distance travelled in the platform quadrant was similar (mean control 0.41 ± 0.02 , mean monoarthritic 0.43 ± 0.01 ; $t_{22} = 0.84$, $P = 0.41$, n.s.). The number of platform crossings was also similar between the two groups (mean control 2.5 ± 0.48 , mean monoarthritic 2.75 ± 0.49 ; $t_{22} = 0.36$, $P = 0.72$, n.s.).

As the values of sensory threshold were very similar in the monoarthritic animals, a floor effect was observed and we were not able to compare values of sensory threshold with the performance in the probe trial.

Sensory threshold 15 days after CFA or saline injection

CFA injection induced lower values of sensory threshold in all the animals while control animals remained with values similar to the beginning of the experiment (mean control 8.64 ± 1.78 ; mean monoarthritic 0.05 ± 0.01 ; Mann Whitney U = 0.00, $P < 0.01$)

Water Maze - 15 days after CFA or saline injection

As the swim speed between monoarthritic and control animals was different (mean control 0.38 ± 0.01 , mean monoarthritic 0.31 ± 0.01 ; $t_{10} = 4.90$, $P = 0.0006$) the results from this experiment were not further analyzed.

Results from Experiment 3: Executive function

Sensory threshold 5 days after CFA or saline injection

CFA injection induced lower values of sensory threshold in all the animals while control animals remained with values similar to the beginning of the experiment (mean control 8.77 ± 2.62 ; mean monoarthritic 0.03 ± 0.01 ; Mann-Whitney U = 0.00, $P < 0.0001$)

Radial Arm Maze - win-shift procedure 5 days after CFA or saline injection

Previous to CFA injection both groups learned to perform the task and presented similar values of reentries in previously visited arms ($F_{14,238} = 0.83$, $P = 0.63$ for interaction trial \times group, n.s.; $F_{14,238} = 18.53$, $P < 0.0001$ for time and $F_{1,238} = 0.09$, $P = 0.73$ for group, n.s.).

After the injection of CFA the monoarthritic group developed transient deficits ($F_{5,85} = 2.30$, $P = 0.05$ for interaction trial \times group, and $F_{1,85} = 0.04$, $P = 0.84$, n.s. for group effect). There was no time effect ($F_{5,85} = 1.32$, $P = 0.26$, n.s.). All the animals were able to perform the task.

Total number of errors was similar previous to CFA injection and animals presented a significant reduction of errors during training ($F_{1,17} = 0.10$; $P = 0.76$, n.s. for interaction time \times group, $F_{1,17} = 11.85$, $P < 0.01$ for time. $F_{1,17} = 0.07$ $P = 0.79$, n.s.-for group). The rank of the first error was also not different between groups, and there was no time effect (time; $F_{5,85} = 1.04$; $P = 0.40$); group; $F_{1,85} = 0.0005$; $P = 0.98$ interaction time \times group; $F_{5,85} = 1.30$; $P = 0.27$).

During the last three sessions previous to injection of CFA and in all the sessions after the injection of CFA both groups were able to collect all the rewards within the 6 minutes allowed.

Sensory threshold 30 days after CFA or saline injection

CFA injection induced lower values of sensory threshold in the all experimental animals when compared to the control group (mean control 9.23 ± 2.85 ; mean monoarthritic 0.07 ± 0.03 , Mann-Whitney $U = 0.00$, $P < 0.01$).

Radial Arm Maze - win-shift procedure 30 days after CFA or saline injection

In this procedure animals were trained only after the injection of CFA, and thus no prior knowledge of the task existed. A significant time effect was found ($F_{19,114} = 1.993$, $P = 0.01$). There were no differences between the two groups ($F_{19,114} = 0.71$, $P = 0.80$ for interaction session \times group, and $F_{1,114} = 0.19$, $P = 0.68$, n.s. for group effect). All the animals were able to perform the task. The average total number of errors was also similar between groups (mean control 1.74 ± 0.21 ; mean monoarthritic 1.90 ± 0.24 , $t_{165} = 0.50$, $P = 0.62$, n.s.).

Results from Experiment 4: Short term memory

Sensory threshold 5 days after CFA or saline injection

CFA injection induced lower values of sensory threshold in all the animals while control animals remained with values similar to the beginning of the experiment (mean control 10.77 ± 2.04 ; mean monoarthritic 0.03 ± 0.001 , Mann-Whitney $U = 0.00$, $P < 0.01$).

Radial Arm Maze - win-stay procedure 5 days after CFA or saline injection

In this procedure animals were trained only after the injection of CFA, and thus no prior knowledge of the task existed. A significant time effect was found ($F_{5,290} = 7.23$, $P < 0.0001$). There was no group effect in the number of errors per session ($F_{1,290} = 2.84$, $P = 0.14$, n.s.) and there was no significant interaction session \times group effect ($F_{5,290} = 1.44$, $P = 0.65$, n.s.). The total average number of errors was also similar between the two groups (control 4.89 ± 0.25 , monoarthritic 5.27 ± 0.22 ; $t_{358} = 1.11$, $P = 0.27$).

Sensory threshold 30 days after CFA or saline injection

CFA injection induced lower values of sensory threshold in all the animals when compared to the control group (mean control 9.60 ± 2.39 ; mean monoarthritic 0.14 ± 0.04 , Mann-Whitney $U = 0.00$, $P < 0.01$).

Radial Arm Maze - win-stay procedure 5 days after CFA or saline injection

In this procedure animals were trained only after the injection of CFA, and thus no prior knowledge of the task existed. A significant time effect was found ($F_{5,130} = 9.123$, $P = 0.01$). There was a group effect in the number of errors per session ($F_{1,130} = 7.61$, $P = 0.006$) and there was no interaction session \times group effect ($F_{5,130} = 0.39$, $P = 0.86$, n.s.). The average total number of errors was higher in the monoarthritic group (control 5.04 ± 0.33 , monoarthritic 6.76 ± 0.32 ; $t_{166} = 3.67$, $P = 0.0003$).

DISCUSSION

In this work, we studied the behaviour of control and monoarthritic animals in spatial memory tasks and compared tonic levels of biogenic monoamines in the HPC. Impaired performance in

several procedures was found as well as reduced hippocampal tonic levels of dopamine in monoarthritic animals. Specifically, monoarthritic rats presented impaired acquisition in a reference memory task as shown by longer latencies to find a platform in the water maze task at 5 days of monoarthritis; transitory impaired executive function at 5 days of monoarthritis, as shown by an increased number of visits to previously visited arms in the radial arm maze; and impaired short-term memory after 30 days of monoarthritis, as shown by an increased number of errors until finding a previously rewarded arm in the radial arm maze.

Reference memory differences were mainly found in the acquisition period of the water maze test as shown by the degraded learning curve of monoarthritic animals, but not when recall was tested. We did not compare individual values of sensory threshold with the performance of animals in any task, because a floor effect was observed in several monoarthritic animals (i.e. responded to the lowest von Frey filament). As previous results from our group have shown stable values of sensory threshold in this model of chronic pain (Neto et al., 1999;Schadrack et al., 1998) and the performances in the water maze were not day-dependent, but trial-dependent, it is unlikely that decreased performance might be due to a specific day decreased threshold for pain. With the exception of the water maze task after 15 days of chronic pain (see results for description of swim speeds), all the behavioural tests used here revealed adequate to study learning and memory processes in this animal model of chronic pain, since animals were able to perform all the tasks and no continuous floating in the water maze, or decreased arm entries in the radial arm maze were observed (see (Buccafusco, 2001) for a review of learning in water and radial arm mazes). In the water maze, the fact that there was no difference between groups in the total time spent in the platform quadrant in the probe trial, as well as similar latencies to find the platform in days 3, 4 and 5 of the protocol shows that monoarthritic animals were able to learn and recall the position of the platform in the pool after repeated training. These results, together with similar swim speeds, suggest that despite the presence of limb inflammation, the differences found between the two groups were not due to motor impairment. The presence of motor impairment is most likely also not the cause of the differences found in the radial arm maze, since there was no forced right or left orientation required in this task, a factor that could possibly lead to leftward or rightward bias in arm choice. As no animal needed the full six minutes to explore all the arms in the win shift procedure in any session, differences in the radial arm maze can also not be attributed to insufficient time to explore the maze in each trial.

In this work, our first hypothesis was that animals in chronic pain would present spatial learning and memory impairments and was confirmed in all tasks, although two of the deficits were only transitory (acquisition of the water maze and win-shift at 5 days of monoarthritis). Our second hypothesis was that animals in chronic pain would present altered levels of monoamines in the HPC and was also confirmed. Previous works from our group have shown a reduction of dopamine and serotonin in the orbitofrontal cortex of monoarthritic rats (Pais-Vieira et al., 2009). Here, we report that different learning and memory deficits appear when animals are tested either at 5 or 30 days of monoarthritis. Interestingly, the behavioural deficits observed somehow match the deficits that would have been expected if the dopamine reduction would have been observed at these timestamps (i.e. hippocampus related deficits – executive memory – would appear earlier, and orbitofrontal deficits – short term memory – would appear latter). When animals were tested at the beginning of the chronic pain process, mainly executive deficits were found. These were similar to those observed in rats with lesions of the dopaminergic enervation of the hippocampus (Gasbarri et al., 1996) and with the injection of a D2 antagonist in the hippocampus (Wilkerson and Levin, 1999) in similar tasks. The deficits found at 30 days match previous results showing that the magnitude of dopamine release in the mPFC predicts accuracy of memory (Phillips et al., 2004) and the injection of D1 and D2 antagonists in the mPFC of rats did not affect executive function in the radial arm maze, but impaired short term memory (Seamans et al., 1998), and data from monkeys showing that orbitofrontal neurons also play a role in working memory tasks (Ichihara-Takeda and Funahashi, 2007). Although our tasks were aimed at testing specific brain regions, we have not specifically tested the hypothesis that each one of the deficits found is related to a specific structure decrease in dopamine, and thus some of the results remain to be elucidated. It is not clear from our results why the deficits found at 5 days are not present at 30 days of monoarthritis. It is also not clear what was the cause for the acquisition deficits found in the water maze task at five days, since lesions of the prefrontal cortex (Mogensen et al., 1995) or the OFC (Vafaei and Rashidy-Pour, 2004) also lead to similar deficits in this task. More, we have recently shown that animals with 5 days of monoarthritis already present decision making deficits similar to OFC lesioned animals (Pais-Vieira et al., 2009).

Altogether, our results clearly show the presence of specific learning and memory impairments that were accompanied by monoaminergic changes. Despite a match in the time course of

these deficits and the reductions in dopamine, our data is not sufficient to link each deficit to a specific cause. Not only the PFC and HPC act closely to allow adequate use of executive and short term memory (Grace et al., 2007), but also other modulatory systems and areas that are involved in learning/memory and pain processing could be affected in this condition.

These results add to previous works in humans the fact that two main regions associated with spatial memory and with pain processing (the orbitofrontal cortex and the hippocampus) presented reduced levels of dopamine after the induction of chronic pain. Our results support a previous report which describes reduced presynaptic dopamine activity in the hippocampus of women with fibromyalgia (Wood et al., 2007) and several works that have described cognitive deficits in this same condition (Grace et al., 1999; Glass, 2006). According to this growing body of evidence it is plausible that the evolution of the chronic pain process leads to changes in dopamine levels in specific regions and consequently to cognitive deficits

Conclusions:

Our work shows that the development of chronic pain is accompanied by monoaminergic changes that match specific behavioral learning and memory deficits. These deficits are most likely related to decreases in dopamine in hippocampal and prefrontal regions, but other areas and neuromodulators might be involved.

Reference List

- Ali SF, David SN, Newport GD (1993) Age-related susceptibility to MPTP-induced neurotoxicity in mice. *Neurotoxicology* 14:29-34.
- Apkarian AV, Sosa Y, Krauss BR, Thomas PS, Fredrickson BE, Levy R, Harden RN, Chialvo DR (2004a) Chronic pain patients are impaired on an emotional decision-making task. *Pain* 108:129-136.

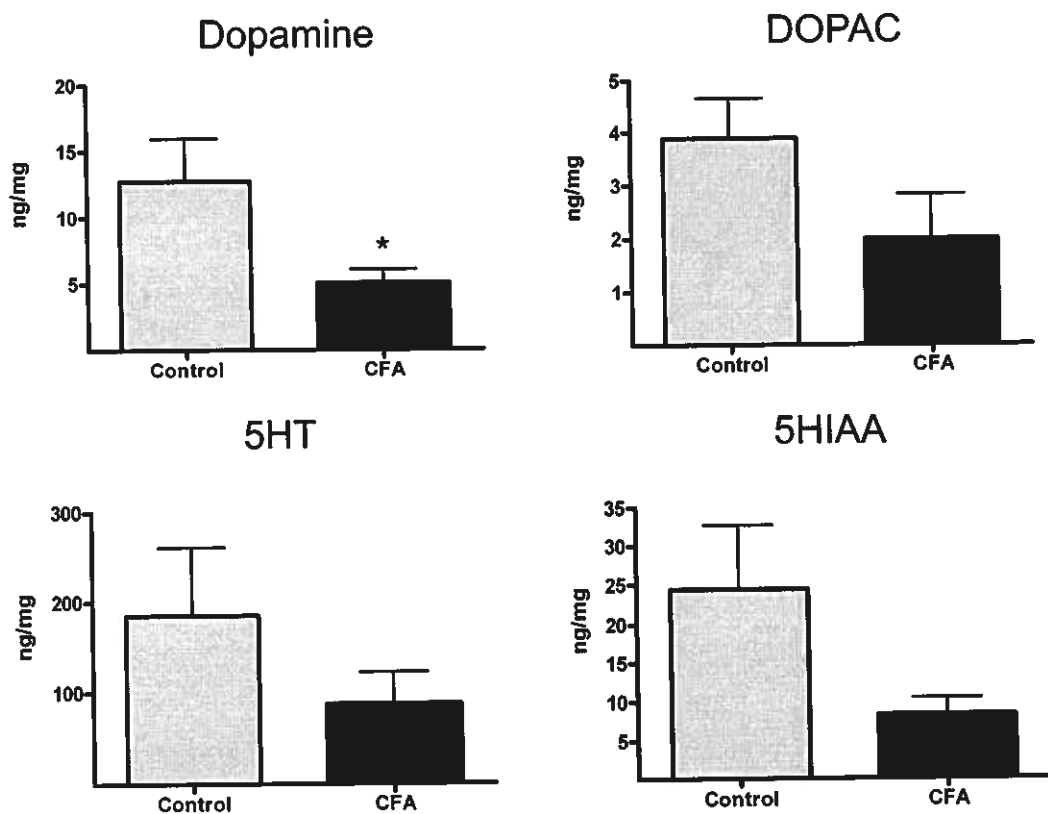
- Apkarian AV, Sosa Y, Sonty S, Levy RM, Harden RN, Parrish TB, Gitelman DR (2004b) Chronic back pain is associated with decreased prefrontal and thalamic gray matter density. *J Neurosci* 24:10410-10415.
- Baddeley A (1996) The fractionation of working memory. *Proc Natl Acad Sci U S A* 93:13468-13472.
- Brooks J, Tracey I (2005) From nociception to pain perception: imaging the spinal and supraspinal pathways. *J Anat* 207:19-33.
- Brown SC, Glass JM, Park DC (2002) The relationship of pain and depression to cognitive function in rheumatoid arthritis patients. *Pain* 96:279-284.
- Buccafusco JJ (2001) *Methods of Behavior Analysis in Neuroscience*. Boca Raton: CRC Press.
- Cain CK, Francis JM, Plone MA, Emerich DF, Lindner MD (1997) Pain-related disability and effects of chronic morphine in the adjuvant-induced arthritis model of chronic pain. *Physiol Behav* 62:199-205.
- Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL (1994) Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* 53:55-63.
- Crombez G, Hermans D, Adriaensen H (2000) The emotional stroop task and chronic pain: what is threatening for chronic pain sufferers? *Eur J Pain* 4:37-44.
- Dehghani M, Sharpe L, Nicholas MK (2003) Selective attention to pain-related information in chronic musculoskeletal pain patients. *Pain* 105:37-46.
- Dufton BD (1989) Cognitive failure and chronic pain. *Int J Psychiatry Med* 19:291-297.
- Floresco SB, Seamans JK, Phillips AG (1997) Selective roles for hippocampal, prefrontal cortical, and ventral striatal circuits in radial-arm maze tasks with or without a delay. *J Neurosci* 17:1880-1890.
- Gasbarri A, Sulli A, Innocenzi R, Pacitti C, Brioni JD (1996) Spatial memory impairment induced by lesion of the mesohippocampal dopaminergic system in the rat. *Neuroscience* 74:1037-1044.

- Glass JM (2006) Cognitive dysfunction in fibromyalgia and chronic fatigue syndrome: new trends and future directions. *Curr Rheumatol Rep* 8:425-429.
- Goldman-Rakic PS (1995) Architecture of the prefrontal cortex and the central executive. *Ann N Y Acad Sci* 769:71-83.
- Grace AA, Floresco SB, Goto Y, Lodge DJ (2007) Regulation of firing of dopaminergic neurons and control of goal-directed behaviors. *Trends Neurosci* 30:220-227.
- Grace GM, Nielson WR, Hopkins M, Berg MA (1999) Concentration and memory deficits in patients with fibromyalgia syndrome. *J Clin Exp Neuropsychol* 21:477-487.
- Grachev ID, Fredrickson BE, Apkarian AV (2000) Abnormal brain chemistry in chronic back pain: an in vivo proton magnetic resonance spectroscopy study. *Pain* 89:7-18.
- Grisart JM, Van Der LM (2001) Conscious and automatic uses of memory in chronic pain patients. *Pain* 94:305-313.
- Harris S, Morley S, Barton SB (2003) Role loss and emotional adjustment in chronic pain. *Pain* 105:363-370.
- Ichihara-Takeda S, Funahashi S (2007) Activity of primate orbitofrontal and dorsolateral prefrontal neurons: task-related activity during an oculomotor delayed-response task. *Exp Brain Res* 181:409-425.
- Iezzi T, Archibald Y, Barnett P, Klinck A, Duckworth M (1999) Neurocognitive performance and emotional status in chronic pain patients. *J Behav Med* 22:205-216.
- Kewman DG, Vaishampayan N, Zald D, Han B (1991) Cognitive impairment in musculoskeletal pain patients. *Int J Psychiatry Med* 21:253-262.
- McCracken LM, Eccleston C (2003) Coping or acceptance: what to do about chronic pain? *Pain* 105:197-204.
- McCracken LM, Iverson GL (2001) Predicting complaints of impaired cognitive functioning in patients with chronic pain. *J Pain Symptom Manage* 21:392-396.

- McDonald RJ, White NM (1993) A triple dissociation of memory systems: hippocampus, amygdala, and dorsal striatum. *Behav Neurosci* 107:3-22.
- Messaoudi M, Desor D, Grasmuck V, Joyeux M, Langlois A, Roman FJ (1999) Behavioral evaluation of visceral pain in a rat model of colonic inflammation. *NeuroReport* 10:1137-1141.
- Milicamps M, Etienne M, Jourdan D, Eschalier A, Ardid D (2004) Decrease in non-selective, non-sustained attention induced by a chronic visceral inflammatory state as a new pain evaluation in rats. *Pain* 109:214-224.
- Mogensen J, Pedersen TK, Holm S, Bang LE (1995) Prefrontal cortical mediation of rats' place learning in a modified water maze. *Brain Res Bull* 38:425-434.
- Morris RG, Garrud P, Rawlins JN, O'Keefe J (1982) Place navigation impaired in rats with hippocampal lesions. *Nature* 297:681-683.
- Neto FL, Schadrack J, Ableitner A, Castro-Lopes JM, Bartenstein P, Zieglgänsberger W, Tolle TR (1999) Supraspinal metabolic activity changes in the rat during adjuvant monoarthritis. *Neuroscience* 94:607-621.
- Olton DS, Samuelson RJ (1976) Remembrance of places passed: Spatial memory in rats. *Journal of Experimental Psychology: Animal Behavior Processes* 2:97-116.
- Phillips AG, Ahn S, Floresco SB (2004) Magnitude of dopamine release in medial prefrontal cortex predicts accuracy of memory on a delayed response task. *J Neurosci* 24:547-553.
- rias-Carrion O, Poppel E (2007) Dopamine, learning, and reward-seeking behavior. *Acta Neurobiol Exp (Wars)* 67:481-488.
- Schadrack J, Castro-Lopes JM, Avelino A, Zieglgänsberger W, Tolle TR (1998) Modulated expression of c-fos in the spinal cord following noxious thermal stimulation of monoarthritic rats. *J Neurosci Res* 53:203-213.
- Seamans JK, Floresco SB, Phillips AG (1998) D1 receptor modulation of hippocampal-prefrontal cortical circuits integrating spatial memory with executive functions in the rat. *J Neurosci* 18:1613-1621.

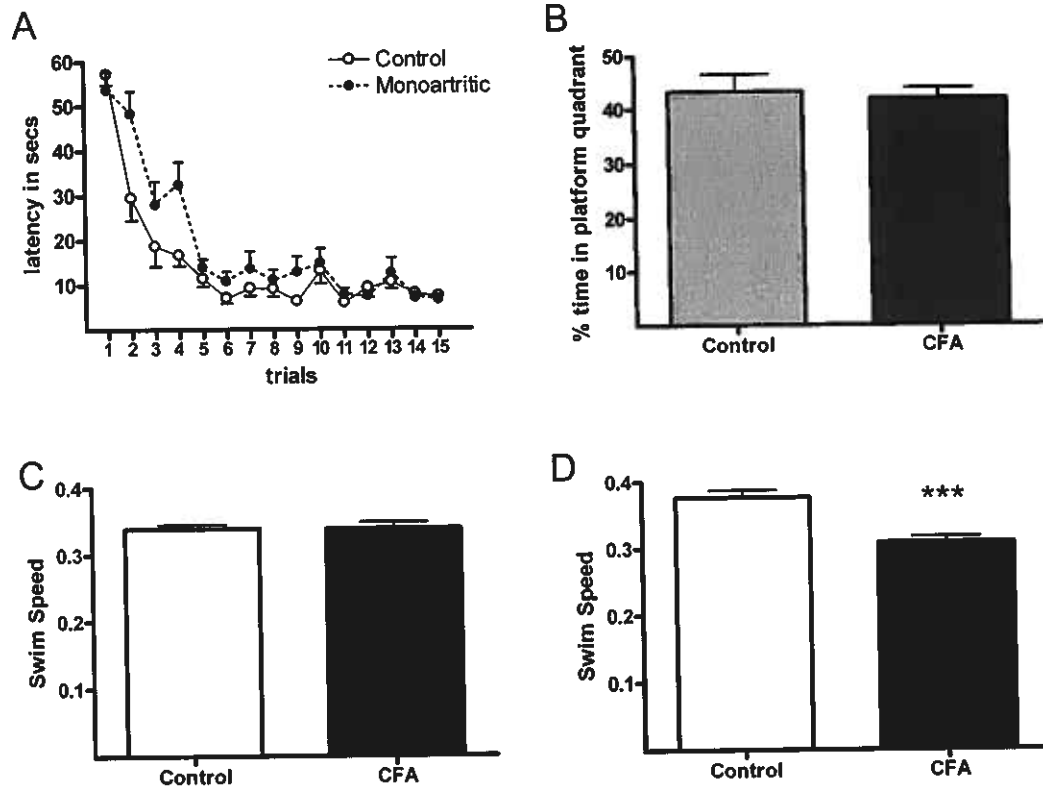
- Seamans JK, Phillips AG (1994) Selective memory impairments produced by transient lidocaine-induced lesions of the nucleus accumbens in rats. *Behav Neurosci* 108:456-468.
- Vafaei AA, Rashidy-Pour A (2004) Reversible lesion of the rat's orbitofrontal cortex interferes with hippocampus-dependent spatial memory. *Behav Brain Res* 149:61-68.
- Wang GW, Cai JX (2006) Disconnection of the hippocampal-prefrontal cortical circuits impairs spatial working memory performance in rats. *Behav Brain Res* 175:329-336.
- Wilkerson A, Levin ED (1999) Ventral hippocampal dopamine D1 and D2 systems and spatial working memory in rats. *Neuroscience* 89:743-749.
- Wolfer DP, Lipp HP (1992) A new computer program for detailed off-line analysis of swimming navigation in the Morris water maze. *J Neurosci Methods* 41:65-74.
- Wood PB, Patterson JC, Sunderland JJ, Tainter KH, Glabus MF, Lilien DL (2007) Reduced presynaptic dopamine activity in fibromyalgia syndrome demonstrated with positron emission tomography: a pilot study. *J Pain* 8:51-58.
- Zahrt J, Taylor JR, Mathew RG, Arnsten AF (1997) Supranormal stimulation of D1 dopamine receptors in the rodent prefrontal cortex impairs spatial working memory performance. *J Neurosci* 17:8528-8535.

Figure 1 – Tonic levels of monoamines in hippocampus



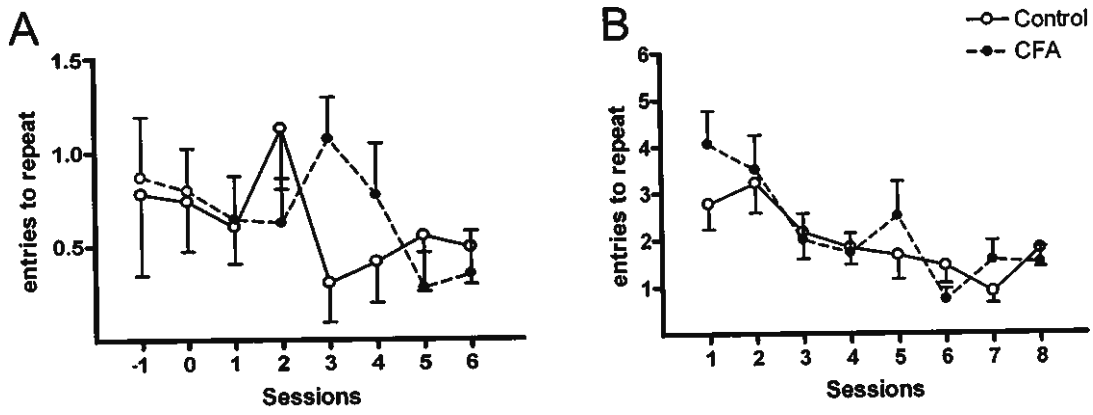
UN

Figure 2 - Water maze learning curves



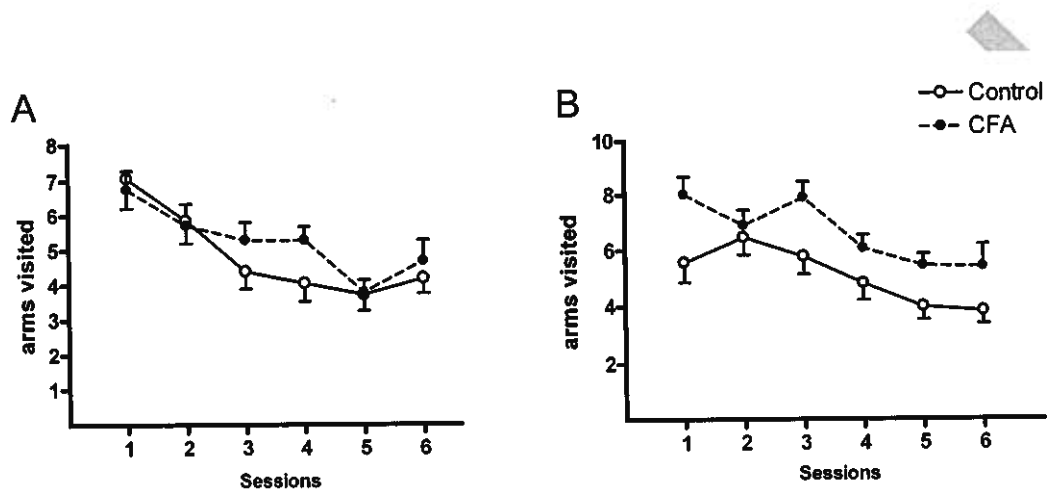
UNL

Figure 3 -Radial arm maze with a win shift protocol



UNDER REVIEW

Figure 4- Radial arm maze with a win stay protocol



UNDER REVIEW

Legends of figures:

figure 1: After 21 days of monoarthritis a reduction in all the monoamines in hippocampus is observed, although only dopamine levels were statistically significant. Symbol * represents significant results for 0.05.

figure 2: The learning curve in the water maze at 5 days of monoarthritis (A) shows that the major differences between groups are found in trials 2 and 4 although swim speeds were similar. The fact that each sessions is constituted by groups of three trials shows that the differences found between groups are not day dependent, and thus are most likely not due to daily changes in pain thresholds. When animals were tested after 15 days of chronic pain, swim speeds were significantly different among groups (B).

figure 3: In this protocol animals with normal foraging behaviours will perform better than animals which repeatedly visit the same arms. (A) Control group and experimental group with 5 days of monoarthritis. Sessions -1 and 0 represent the last two sessions before the injection of CFA in experimental group. The number of repeated entries in monoarthritic animals is higher in the third session after injection of CFA, which corresponds to day 6 of chronic pain. Although a significant interaction effect was found ($P=0.05$), no differences were found with the Bonferroni test. (B) Control group and experimental group with 30 days of monoarthritis. No differences were found in the performance of the two groups.

figure 4: In the first part of each trial animals were only allowed to visit one arm with all other arms blocked. At the end of this arm were two pellets. In the second part of the trial animals would be rewarded if they were able to inhibit the normal foraging behaviour and recall in which arm they had previously been. Monoarthritic animals show a trend to visit more arms before entering an arm previously visited. (A) Experiment with control and animals with 5 days of monoarthritis (B) Experiment with control and animals with 30 days of monoarthritis. Although a significant group effect was found ($P<0.01$), no differences were found with the Bonferroni test.