# Amitriptyline reverses hyperalgesia and improves associated mood-like disorders in a model of experimental monoarthritis

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**Abstract**

Affective disorders are common comorbidities of chronic inflammatory pain that are often overlooked in primary care. As the impact of inflammatory pain upon mood-like disorders in animal models is not well known, our objective was to assess whether prolonged experimental monoarthritis (ARTH) induced the development of anxiety and depressive-like behaviours in rodents and if amitriptyline, an antidepressant commonly used in the treatment of chronic pain, could reverse both nociceptive and mood-like impairments. Experimental ARTH was induced through an injection of kaolin/carrageenan into the right knee joint with control (SHAM) animals injected with saline. Four weeks after induction, ARTH animals displayed mechanical hyperalgesia and a depressive-like phenotype as they showed a significant increase in immobility and a decrease in the latency to immobility in the forced-swimming test at the expense of the time spent climbing/swimming. ARTH animals also displayed a decreased sucrose preference, an index of anhedonia and anxiety-like behaviour as time spent exploring the open arms of the elevated-plus-maze was decreased when compared to controls. The anxiety-like phenotype was also supported by an increase in the number of fecal boli left in the open field. In ARTH animals, the administration of amitriptyline decreased mechanical hyperalgesia and increased sucrose preference and the time spent climbing, although it had a deleterious effect in the performance of control animals. Our data show that this model of ARTH can be useful for the study of chronic pain-mood disorders comorbidities and that amitriptyline is able to partly reverse the associated nociceptive and emotional impairments.

**Keywords:** Experimental monoarthritis, Pain-mood disorders comorbidity, Mechanical hyperalgesia, Amitriptyline.

## 1. Introduction

Joint pain is a condition that affects millions of people, especially the elderly, representing a huge burden on National Health Programs [1]. Pain is the most common complaint in arthritis but often patients exhibit clinical signs of comorbid depression and anxiety [2] that frequently go untreated [3].

Curiously, although many animal models of chronic inflammatory pain are available and extensively used, only a few studies addressed inflammatory pain and mood disorders comorbidity [4,5,6,7]. However, the animal models and time points chosen to perform the behavioural analysis are different among studies. For example, the time frame of osteoarthritis (OA) settlement is one feature of the behavioural experimental designs that could by itself limit the full development of neuropsychological impairments (anxiety- and depressive-like behaviour) in a slow developing disease such as OA. Accordingly, recent data obtained from studies on chronic neuropathy [8] demonstrated that the experimental time frame adopted is critical to model the affective pathologies associated to chronic pain. These authors showed that anxiety and depressive-like behaviours developed in a time-dependent manner, with the former present at three weeks of neuropathy while the latter was expressed only after 6 weeks of neuropathy.

In spite of all the evidence demonstrating the existence of pain-depression comorbidity, the actual mechanisms underlying this interaction are still mostly unknown [3]. While chronic pain patients are up to four times more prone to develop major depressive disorders than the general population [9], more than half of depressed patients present comorbid chronic pain [8], with the effects of having both chronic pain and depression being worse than suffering from chronic pain or depression alone [10]. Therefore, antidepressants, including tricyclic antidepressants (TCAs), are extensively used in the management of both chronic pain and depression [11]. TCAs are the first-line drugs for the treatment of chronic pain and

amitriptyline (AMI), the most commonly used TCA to treat depression in chronic pain patients [12], has also been associated with significant analgesia in different animal models of chronic neuropathic pain [13].

Accordingly, in the work herein, we used the kaolin/carrageenan model of experimental knee monoarthritis (MA) to establish a direct link between prolonged MA and the development of mood-like disorders in rodents. After four weeks of MA, we treated the animals with AMI to evaluate whether this antidepressant could reverse the nociceptive and mood-like changes induced by the model.

## 2. Materials and methods

### *2.1. Animals and ethical questions*

The experiments were performed in adult male Wistar rats (n=44, Charles Rivers, Barcelona, Spain) weighting 225–250g at the beginning of the experiment. The experimental protocol was approved by the Institutional Ethical Commission and followed the European Community Council Directive 86/609/EEC and 2010/63/EU concerning the use of animals for scientific purposes. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

Animals were housed in pairs under a 12h light cycle (starting at 8:00 a.m.) with food and water available *ad libitum*. General health parameters were surveyed twice per week by the resident veterinary and animal weight was recorded every week throughout the experimental period. Before the beginning of the experiment, all animals were handled daily by the experimenter for a week and on the day of the experimental sessions animals were left in the experimental room for an hour in order to habituate to the surroundings.

### *2.2. Induction of monoarthritis*

The induction of arthritis (ARTH) was performed as described in detail elsewhere [14]. Briefly, 3% kaolin and 3% carrageenan (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in distilled saline solution (0.9% NaCl) and injected into the synovial cavity of the right knee joint at a volume of 0.1 mL. This model produces mechanical hyperalgesia, which begins just in a few hours after surgery and lasts for several weeks [15]. Control animals (SHAM) were injected with 0.1 mL saline in the synovial cavity of the right knee joint.

### *2.3. Behavioural assessments*

#### *2.3.1. Nociceptive behaviour*

In each animal, the development of arthritis was verified 1–2 h prior to each experiment. Only those rats that vocalized every time after five flexion–extension movements of the knee joint were considered to have developed osteoarthritis, and were included in the ARTH group. SHAM animals did not vocalize to any of the five consecutive flexion–extension movements of the knee joint.

##### 2.3.1.1. Pressure Application Measurement

The application of noxious pressure to the primary site of injury is a classical approach to measure mechanical hyperalgesia [16], both in humans and animals [17]. Here, a new pressure application measurement (PAM) method was used. It allows an accurate behavioural measurement of primary mechanical hypersensitivity in rodents with chronic inflammatory joint pain [18] by the application of a force range of 0–1500g. To perform the test and with the animal securely held, the force transducer unit (fitted to the experimenter's thumb) is placed on one side of the animal's knee joint and the forefinger on the other and an increasingly force is gradually applied across the joint until a behavioural response is observed (paw-withdrawal, freezing of whisker movement, wriggling or vocalization) with a cut-off of 5s. The peak force

(in grams force (gf)) applied immediately prior to the behavioural response is recorded as the limb withdrawal threshold (LWT). LWT was measured twice in both the ipsilateral and contralateral limbs at 1 min intervals. The mean LWTs were calculated per animal. At the end of the session animals were returned to their home cage.

#### 2.3.2. *Locomotor activity and anxious-like behaviour*

##### 2.3.2.1. Open-field test

The open field (OF) test was used to evaluate differences in the locomotor ability between SHAM and ARTH animals and followed a protocol previously implemented in our lab [19]. In summary, locomotor behaviour was assessed by measuring the total distance travelled by the animal inside the arena and anxiety-like behaviour was evaluated through the analysis of the time spent in the centre of the arena and by counting the number of faeces (*fecal boli*) remaining in the arena at the end of each trial [20]. The OF test was performed in a square arena (43.2 cm wide with the central area of the arena corresponding to a square 21.6cm wide and equidistant from the borders) with transparent acrylic walls (Med Associates Inc., St. Albans, Vermont, USA) in a brightly illuminated room (240 lux in the centre of the arena). The test started when the animal was placed at the centre of the arena and its exploratory activity was automatically registered during 5 min. The arena was cleaned with 10% alcohol between each trial.

##### 2.3.2.2. Elevated-plus maze

The elevated-plus maze (EPM) was used to assess anxiety-like behaviour and followed a protocol previously implemented in our lab [21]. The EPM apparatus (ENV-560; MedAssociates, St. Albans, Vermont, USA) consisted of two opposite open arms (50.8 × 10.2 cm) and two closed arms (50.8 × 10.2 × 40.6 cm), which were elevated 72.4 cm above the floor. At the beginning of the test the animal was placed in the centre of the maze and allowed 5 min to freely explore the maze. During the duration of the trial, behaviour was recorded

through the use of an infra-red photobeam system connected to a computer with software (MedPCIV, MedAssociates) that allows for the quantification of the time spent in each arm. Between trials, the maze was cleaned with 10% ethanol. The percentage of time spent in the open arms was used as an index of anxiety-like behaviour.

### *2.3.3. Depressive-like behaviour*

#### 2.3.3.1. Sucrose preference test

Anhedonia, measured as a reduction in sucrose preference (SPT), was assessed through the application of a protocol adapted from Bessa *et al.* [21]. This protocol includes an initial exposure of the animals to the sucrose solution a week before the induction of ARTH. During this session, performed during their active period (8:00 p.m.), animals (without any previous water and food restriction) had free access to both water and the sucrose solution for 2h. In addition, the behaviour of the animals was monitored by the experimenter in order to certify that all animals tasted the sucrose solution. On the test day and after twenty-four hours of food/water deprivation, each animal was presented with two pre-weighted bottles [water and sucrose solution (2%)] for one hour (8:30-9:30p.m). Afterwards, the bottles were re-weighted and sucrose preference was calculated using the equation:

Sucrose_preference=[sucrose_intake/(sucrose+water)_intake]×100.

For experiment 1, the results are presented as the difference in sucrose preference between the data obtained at four weeks post-ARTH induction and the basal assessment that preceded the induction of monoarthritis. In experiment 2, the results are presented as the difference in sucrose preference between the data obtained during the fourth week of pharmacological treatment and the assessment performed at four weeks post-ARTH induction.

2.3.3.2. Forced swimming test

Learned helplessness was evaluated in the modified forced-swimming test (FST) [22] and followed a protocol previously implemented in our lab [21]. Animals were submitted to a pre-test session (10 min) in which they were individually placed in cylinders filled with water (25°C; depth 30 cm). Twenty-four hours later animals were again placed in the cylinders for a period of 5 min and the testing session was recorded with a video camera. The quantification of (i) latency to immobility, (ii) time spent immobile, (iii) time spent swimming and (iv) time spent climbing was performed by a blind observer that rated the videos using the Etholog software [23]. Note that the time spent swimming and climbing was discriminated as opposed to floating as previously described by Rénéric and colleagues (2002) [24]. Learned helplessness behaviour was defined as an increase in time of immobility at the expense of the time spent swimming /climbing and a decrease in the latency to immobility.

*2.4. Drug treatment*

Four weeks after arthritis induction, amitriptyline hydrochloride (AMI; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in saline (SAL) and administered intraperitoneally (i.p.) at a dose of 15mg/kg daily for three weeks, until the beginning of the behavioural tests. The pharmacological treatment with AMI or SAL was maintained during the duration of the behavioural evaluation with injections being administered daily at the end of the behavioural session.

*2.5. Experimental design*

The present study was divided in two main experiments (**Fig. 1A**), (i) the first one involving the assessment of the comorbid development of mood-like disorders in animals with experimental ARTH and (ii) the second determining the potential of amitriptyline in reversing both the nociceptive and the emotional-like components of chronic experimental ARTH.

In experiment 1, one week preceding the intrasynovial injection animals were presented to a sucrose solution and in the day before knee injection animals were submitted to a baseline evaluation in the sucrose preference test (SPT). After four weeks of the knee injection animals were tested in the SPT, PAM, OF, EPM and FST. At the end of the behavioural sessions animals were sacrificed with a lethal dose of pentobarbital and the knee joints were removed for further histological processing and analysis.

In experiment 2, one week preceding the intrasynovial injection animals were presented to a sucrose solution and in the day before the beginning of the treatment animals were submitted to a baseline evaluation in the sucrose preference test (SPT). After four weeks of the intrasynovial injection SHAM and ARTH animals were administered either saline (SHAM_SAL and ARTH_SAL) or amitriptyline (SHAM_AMY and ARTH_AMY). Three weeks after the beginning of the treatment, animals were behaviourally tested following the same experimental design as in experiment 1 (**Fig. 1A**).

### *2.6. Histology*

Right knees from SHAM and ARTH animals were excised and fixed in paraformaldehyde solution (4%). Knees were subsequently submerged in a decalcifying solution (BiodecR, Bio-Optica, Milan, Italy) for 48 hours, paraffin embedded, cut into 4-µm sections and stained with hematoxylin and eosin (**Fig. 1B**).

### 2.7. *Statistical analysis*

The GraphPad Prism® 6 software (GraphPad Software Inc, La Jolla, CA, USA) was used to perform the statistical analysis. Differences in weight gain between ARTH and SHAM groups were evaluated by applying a mixed-design two-way analysis of variance (ANOVA) followed by t-test with a Bonferroni correction for multiple comparisons. The comparisons of differences between groups in experiment 1 were performed using a Student's t-test for unpaired data. A

two-way ANOVA followed by t-test with a Bonferroni correction for multiple comparisons was used to compare results from the behavioural assessment at the end of experiment 2, except for the PAM test where a repeated measures two-way analysis of variance followed by t-test with a Bonferroni correction for multiple comparisons was performed. Statistical significance was accepted for $P<0.05$. Data in the results section are expressed as mean ± standard deviation (SD).

## 3. Results

### *3.1. Body weight*

In experiment 1, body weight of all animals increased significantly (main effect of elapsed time: $F_{4,40}=136.00$, $P<0.001$) but ARTH animals gained less body weight during the experimental period than SHAM animals (interaction between elapsed time and the experimental group: $F_{4,40}=3.16$, $P=0.024$). In experiment 2, the administration of AMI induced an overall 10% decrease in the body weight of SHAM and ARTH animals when compared with SAL-treated animals (interaction between weight gain and experimental group during AMI treatment: $F_{9,112}=20.68$, $P<0.001$).

### *3.2. Nociceptive Behaviour*

All animals in the ARTH group developed a clear swelling of the treated knee joint and all gave a vocalization response during a minor extension and flexion of the affected limb. SHAM animals displayed no obvious swelling of the knee joint and did not vocalize when the limb was flexed.

### *3.3. Histological analysis*

As shown in **figure 1-B1**, SHAM animals presented no surface abnormalities in the joint. In ARTH animals an overall tissue degeneration is evident with the medial femoral plateaux (FP) being more affected than the tibial plateaux (**Fig. 1-B2**). In the FP it is possible to observe focal damage of cartilage surface associated to a decrease in cartilage thickness in the central area, reduplication of the tidemark and some surface fibrillation. Additionally, there is an increase in subchondral bone volume concomitant with subchondral sclerosis, decreased bone marrow area and subchondral cyst formation.

### *3.4. Experiment 1*

#### 3.4.1. Mechanical hyperalgesia

LWT was significantly decreased in ARTH animals compared to SHAM ($t_{10}$=4.885, $P$<0.001) confirming their arthritic state (**Fig. 2A**).

#### 3.4.2. Locomotor activity

In the OF test, total distance travelled by the animals was not different between SHAM and ARTH animals ($t_{10}$ = 0.790, $P$=0.448) (**Fig. 2B**).

#### 3.4.3. Anxiety-like behaviour

In the OF, ARTH animals showed a strong tendency to spend less time in the centre of the arena ($t_{10}$=2.122, $P$=0.060) (**Fig. 2C**) and left a significantly higher number of *fecal boli* in the OF arena ($t_{10}$=3.078, $P$=0.012) (**Fig. 2D**) when compared to SHAM animals, indicating they developed an anxious-like phenotype. This was confirmed in the EPM test, as ARTH animals also spent less time in the open arms ($t_{10}$=2.665, $P$=0.024) (**Fig.2E**).

#### 3.4.4. Depressive-like behaviour

ARTH animals displayed a depressive-like phenotype as these animals showed a significantly decreased preference for sucrose solution (i.e., anhedonia-like behaviour) when compared with the SHAM group ($t_{10}$=2.319, $P$=0.043) (**Fig. 2F**). No differences in liquid consumption per body weight unit were found between the experimental groups ($t_{10}$=1.755, $P$=0.110). SPT results are further substantiated in the FST where ARTH animals showed a significant decrease in the latency to immobility ($t_{10}$=2.845, $P$=0.017) (**Fig. 3A**). This finding was accompanied by an increase in the immobility time ($t_{10}$=3.630, $P$=0.005) (**Fig. 3B**) and a corresponding decrease of the time spent swimming ($t_{10}$=2.955, $P$=0.014) (**Fig. 3C**) and climbing ($t_{10}$=3.267, $P$=0.009) (**Fig. 3D**) by ARTH animals.

### *3.5. Experiment 2*

#### 3.5.1. Mechanical hyperalgesia after treatment

LWT was significantly different between the experimental groups (main effect of the experimental group: $F_{1,28}$=12.310, $P$=0.002) with ARTH animals displaying a lower LWT when compared to SHAM animals (data not shown). Additionally, LWT also varied with the drug treatment (interaction between the experimental group and drug treatment: $F_{1,28}$=7.565, $P$=0.010) since ARTH animals treated with AMI displayed a higher LWT when compared to ARTH treated with SAL. *Post hoc* tests showed that AMI was able to reverse primary hyperalgesia as shown by the increase in LWT of ARTH_AMI animals to the level of SHAM animals (**Fig.4A**).

#### 3.5.2. Locomotor activity

In the OF test, total distance travelled by the animals was not different between experimental groups (main effect of test group: $F_{1,28}$=0.618, $P$=0.438) (**Fig. 4B**).

### 3.5.3. Anxiety-like behaviour after treatment

In the OF test, there were no differences in the time spent in the centre of the arena (main effect of test group: $F_{1,28}$=2.405, $P$=0.1322) (**Fig. 4C**). The number of *fecal boli* that remain in the OF arena varied with the drug treatment (interaction between the experimental group and drug treatment: $F_{1,28}$=7.488, $P$=0.011) (**Fig. 4D**). In the EPM there were no significant differences between the groups in the time spent in the open arms (main effect of test group: $F_{1,28}$=1.348, $P$=0.256) (**Fig. 4E**). Note that SHAM_AMI animals had behavioural performances similar to ARTH_SAL animals (**Fig. 4**).

### 3.5.4. Depressive-like behaviour after treatment

Sucrose preference was significantly different between the experimental groups (main effect of test group: $F_{1,28}$=5.536, $P$=0.026) with SHAM animals displaying more preference for the sugary beverage when compared to ARTH animals. Additionally, sucrose preference also varied with the drug treatment (interaction between group and drug: $F_{1,28}$=6.044, $P$=0.020) since ARTH animals treated with AMI consumed considerably more sucrose than ARTH animals treated with SAL. *Post hoc* tests confirm that ARTH_AMI animals increased sucrose preference when compared with ARTH_SAL animals (**Fig. 4F**). In the FST, the latency to immobility between the groups varied with the drug treatment (interaction between the group and drug: $F_{1,28}$=6.710, $P$=0.015) as SHAM and ARTH animals treated with AMI display a lower latency to immobility. *Post hoc* tests indicated that the latency to immobility was significantly shorter in the SAL-treated ARTH than SHAM group and also between SAL-treated SHAM and AMI-treated SHAM (**Fig. 5A**). The duration of the immobility time was significantly different between treatments (main effect of test drug: $F_{1,28}$=5.303, $P$=0.029) as SHAM animals treated with AMI increased the amount of immobility time. In parallel, the difference in the immobility time between the groups varied with the drug treatment (interaction between group and drug: $F_{1,28}$=6.801, $P$=0.015) as ARTH animals treated with AMI showed no difference in immobility

time while SHAM treated with AMI significant increased the time spent immobile. *Post hoc* tests confirm that the duration of immobility time was significantly longer in the SAL-treated ARTH than SHAM group (**Fig. 5B**). Concerning the duration of the active period, the time spent swimming was significantly different between the experimental groups (main effect of the group: $F_{1,28}$=5.658, $P$=0.024) since ARTH animals spent less time swimming when compared to SHAM. In addition this parameter also varied with the treatment (main effect of the drug: $F_{1,28}$=15.470, $P$<0.001) since the time spent swimming was decreased in SHAM animals after the treatment with AMI when compared with SAL-treated SHAM. Moreover, the difference in swimming time between the groups was dependent on the drug treatment (interaction between the group and drug: $F_{1,28}$=5.198, $P$=0.030) since the effects of AMI administration were restricted to SHAM animals. *Post hoc* tests confirm that the swimming time was shorter in the SAL-treated ARTH than SHAM group (**Fig. 5C**).

The difference in time spent climbing varied with the drug treatment and the experimental group (interaction between the group and drug: $F_{1,28}$=14.800, $P$<0.001) since ARTH animals treated with AMI increased the time spent climbing while AMI-treated SHAM animals decreased it. *Post hoc* tests confirm that the duration of the climbing time was significantly longer in the AMI-treated ARTH than SHAM group (**Fig. 5D**). Overall, SHAM animals treated with AMI had behavioural performances similar to ARTH animals treated with SAL (**Fig. 5**).

## 4. Discussion

In this study, we demonstrate that the K/C animal model can be used as a valid method to study the comorbidity between arthritis and mood disorders in the rat. In addition to sustained hyperalgesia (decreased withdrawal threshold to noxious mechanical stimulation of the inflamed knee), animals with four weeks of K/C–induced arthritis exhibited anxiety-like behaviour (low exploratory activity in the EPM and increased defecation in the OF arena),

anhedonic behaviour (reduced preference for sucrose) and learned helplessness (decreased mobility in the FST). We also demonstrated that after three weeks of antidepressant treatment, AMI is able to decrease mechanical hyperalgesia and partly reverse depressive-like behaviour, as the preference for sucrose is increased in ARTH animals and they spend more time climbing in the forced swimming test. Due to the importance of using animal models that mimic the greatest number of features of the human disorders, we anticipate that the concomitant study of pain and emotional-like behaviours in the experimental model of arthritis will significantly increase our understanding of the mechanisms underlying comorbidity of chronic pain and mood disorders.

In general K/C-induced arthritis has been established as model of acute monoarthritis that partly mimics the initial inflammatory stages of OA [25]. The advantage of also administering kaolin is the mechanical damage to intra-joint structures [26] triggered by this clay, which is absent in inflammatory models using carrageenan alone. Taking into account that inflammatory disorders are slowly progressing degenerative diseases it is most surprising that the majority of studies using this model use a restricted time frame (e.g. 4h [27], 5-6h [28] and 1 week [29]). Our data contrast with most studies, reporting marked cellular infiltration, as we observe a clear degeneration of the femoral plateaux (thinning of the cartilage, fibrillation and subchondral sclerosis). This degree of joint degeneration partly mimics features of later stages of chronic inflammatory diseases, in particular of osteoarthritis. Indeed and according to Pearson's [30] report, our animals display a knee joint degeneration level concomitant with stage three of human osteoarthritis that comprises the presence of deep fissures, loss of cartilage, remodelling of the bone plate and reduplication of the tidemark. Nonetheless, further studies involving a time dependent evaluation of the succession of events affecting knee joint structure are needed for this K/C-induced arthritis to be considered a valid osteoarthritis model.

Anxiety and depression [31] are important comorbidities that have been positively correlated with pain severity in arthritic patients. These mood disorders are 2 to 3 times more prevalent in OA patients than in patients with other chronic pathologies [3]. However, to date and to the best of our knowledge, only one experimental animal study [32] has addressed the comorbidity of pain and depression in OA, in spite of the availability of many animal models mimicking specific aspects of the OA pathology [33]. It should be emphasized that in our study anxiety- and depression-like behaviours were assessed after a sustained hyperalgesic state that had lasted 28 days, whereas the majority of the studies from other authors used more restricted time-frames (see above).

Our data shows that ARTH animals with 28 days of the K/C-induced arthritis display a profound mechanical hyperalgesia in the ipsilateral knee (PAM test), which parallels the hyperalgesic response of OA patients to noxious stimulation of the knee [34]. Interestingly, some reports have positively associated motor disability with the development of depression in OA patients [31]. However, although our ARTH rats present mechanical hyperalgesia, they did not display any locomotor impairments as the total distance travelled in the OF was not different from SHAM animals. Hence, in this work the development of mood-like disorders could not be associated to locomotor disability of rodents.

Clinical treatment of joint disorders focuses mainly on ameliorating pain and functional limitations [35], while less attention is paid to mood comorbidities [2]. However, several studies have shown that the prevalence of anxiety and depression e.g. in OA patients is very high [2,31], around 41% and 62%, respectively, although the recognition of both depression and anxiety in primary care settings is typically lower, less than 50% [2]. Similarly, in preclinical OA studies most authors have focused on acute changes in behaviour or molecular and cellular responses [4-7,27-29,36,37]. In experimental animal studies on neuropathic pain, comorbid anxiety- and depression-like behaviour has been studied more extensively. For example, Yalcin

*et al.*[8] assessing anxiety and depression-related behaviours in a mouse model of neuropathic pain demonstrated a time-dependent development of mood-like disorders. Their findings indicate that anxiety-related behaviour was developed 4 weeks after induction of the neuropathy, whereas depression-related behaviours were observed 2-4 weeks later.

In the currently used K/C model, the development of anxiety-like behaviour had already been shown by Ji *et al.* [28] as early as 24h after its induction. Using a similar set-up, we demonstrated that ARTH animals still displayed anxiety-like behaviour at four weeks post-induction as shown by the significant decrease in the time spent in the open arms of the EPM. In addition, ARTH animals also displayed an anxious-like phenotype in the open field test as this group left a significantly higher number of *fecal boli* in the OF arena when compared to SHAM animals. Despite its simplicity, defecation is considered an important indicator of emotional responses, particularly in anxiety-like behaviour [20].

While the results by Yalcin *et al.* [8] showed that the development of depression-like behaviour in neuropathic animals took 6-8 weeks, our results indicate that in the K/C model the depressive-like phenotype appeared earlier, at four weeks. This was shown by a decrease in the latency to immobility, an increase in the duration of immobility and a decrease in active behaviours (climbing and swimming) in the FST. This type of behavioural change in experimental animals is considered an index of learned helplessness that presumably represents human “resignation” [38]. Yet, based on studies on neuropathic pain by Wang *et al.* [39], it could be argued that the differences obtained between SHAM and ARTH animals in the FST could be explained by motor deficits of ARTH animals. However, ARTH animals of the present study failed to display any motor impairments in the OF. Nor could we detect any obvious difference in the use of the left and right hind paws during the FST. Moreover, the depressive-like phenotype obtained in the FST is further supported by the SPT data, a test that does not implicate/require motor coordination.

Although the analysis of anhedonia (lack of interest in pleasure) in rodents has to be approached with caution, a reduced preference for a sweet solution is considered to share some analogy with anhedonia and depressive behaviour in humans [40] and therefore, the SPT is widely used in stress studies [41]. Our ARTH animals showed a significant decrease in the preference for a sweet solution when compared to SHAM, hence displaying a clear hedonic deficit. It has been argued that sucrose intake can also be influenced by body weight [41], but comparing the ratio between animal body weight and liquid consumption between groups revealed no significant differences between ARTH and SHAM animals. This finding gives further support to the presence of an anhedonic state in ARTH animals.

In what concerns the performance of AMI-treated SHAM animals, there appears to be an anxiogenic and pro-depressive effect of AMI on these animals as shown in the OF and the FST tests. In clinical practice, antidepressants are known to have deleterious effects when administered to non-depressed patients [42] comprising, amongst others, sedation, lower reaction time in the choice reaction time task, sensory impairments, disruption of discrimination ability, dry mouth, dizziness and constipation [42,43]. In what concerns the effects of prolonged AMI administration to animals only a few works reported frequency-related depression of ventricular conduction in dogs [44,45]. Nonetheless, in a preliminary study (unpublished data) where we compared the motor performance of AMI- and SAL-treated SHAM animals in the Rotarod test (increasingly faster rotations) no differences could be found between experimental groups. These results are in accordance with a report from Bomholt *et al.* [46] also showing that the acute administration of AMI did not alter the motor performance of SHAM animals in the Rotarod test. Based on these results we considered that the detrimental effects of AMI administration to SHAM animals were not motor related.

Recurrent episodes of inflammation at the damaged joints in arthritic patients lead to the release of inflammatory mediators that activate voltage-gated sodium channels causing

peripheral/central sensitization and ultimately, persistent inflammatory pain [47]. Descending monoaminergic pathways play an important role in inhibiting pain transmission at the spinal cord level [48]. Tricyclic antidepressants (TCAs), which inhibit the reuptake of serotonin and noradrenalin, are essential components of the current pain management therapeutic strategies in chronic pain disorders, including rheumatoid arthritis [49]. Yet, Richards *et al.* [49] while studying the effect of AMI in controlling pain in a data base of controlled trials in adults with rheumatoid arthritis or ankylosing spondylitis could not confirm the efficacy of this TCA as an analgesic. No available data could be found concerning the use of AMI for pain management in monoarthritic/osteoarthritic patients. Nonetheless, our data shows that the administration of AMI to ARTH animals significantly decreased mechanical hyperalgesia after three weeks of treatment. Interestingly, this effect might result not only from increased availability of brain serotonin and noradrenaline and subsequent activation of descending pathways as demonstrated in other works [48,50], but also from a peripheral effect as this drug decreases the activity of sodium and calcium voltage dependent channels [51]. This hypothesis is supported by demonstration that AMI selectively inhibits ectopic discharge of low frequency and bursting discharge in animal models of neuropathic pain [52], and that the antidepressants' ability to block sodium channels could effectively suppress persistent peripheral signaling [53].

Although AMI had no effect upon anxiety-like behaviour, it efficiently reversed the hedonic-like behaviour as measured in the SPT. These results are consistent with data from other studies showing that the decrease in depressive-like behaviour results from the modulation of the activity of serotonin transporters [54] and of several receptors [54,55]. However taking into account our results from the FST it is unlikely that the effect of AMI reported herein is related to the modulation of serotonin receptors, as no differences were found for the swimming component of the test, but rather to an effect of this drug on noradrenergic receptors. In fact, similarly to what was observed by Detke *et al.* [56], in rats injected with specific noradrenaline

uptake inhibitors, the administration of AMI to ARTH animals selectively increased climbing behaviour without altering the time spent swimming.

## 5. Conclusions

So far, it is not clear which components of chronic inflammation are directly involved in the development of mood disorders in human patients with an inflammatory joint disorder such as OA [57]. The causes of pain-depression comorbidity in patients with arthritis are complex (involving sensory, affective and cognitive processes) and the abnormal peripheral and central mechanisms of pain processing involved in this comorbidity are far from being understood. Our present results support the proposal that the K/C model in the rat provides a good pre-clinical tool for studying the link between chronic inflammatory joint pain and mood disorders, as well as a model for testing novel drugs that may modulate pain intensity, mood disorders, or both. Importantly, AMI was able to reverse the nociceptive and hedonic components of chronic inflammatory pain with its influence likely due to a simultaneous combination of central and peripheral effects.

**Abbreviations**

AMI – Amitriptyline hydrocloride

ARTH – K/C induced monoarthritis

EPM - Elevated plus maze

FC - Femoral condyle

FP – Femoral plateaux

FST - Forced swimming test

gf – Gram force

K/C - Kaolin/carrageenan

LWT - Limb withdrawal threshold

M – Meniscus

OA - Osteoarthritis

OF - Open field test

PAM - Pressure application measurement

PL - Patellar ligament

SAL - Saline

SCB - Subchondral bone

SHAM – Control animals

SPT - Sucrose preference test

TCA – Tricyclic antidepressant

TP - Tibial plateau

**Competing interests**

The authors declare that they have no competing interests.

**Author contributions**

DA and ADP carried out the experimental work and scored the tests. DA analysed the data. DA and FPR designed the experiment, coordinated the tasks and wrote the manuscript. AP and AA revised the manuscript. All authors read and approved the final manuscript.

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**Figure Legends**

**Figure 1**. **(A)** Time course of the two main experiments, (i) the first one involving the assessment of the comorbid development of mood-like disorders in animals with experimental ARTH and (ii) the second that evaluated the potential of amitriptyline in reversing both the nociceptive and the emotional-like components of chronic experimental ARTH. One week preceding the intrasynovial injection animals were presented to a sucrose solution and in the day before the knee injection animals were submitted to a baseline evaluation in the sucrose preference test (SPT). Four weeks after the intrasynovial injection the first test performed was the SPT followed by 24h of rest to compensate for the fasting period. The next evaluation was the pressure application measurement (PAM), on day 3 followed by the open field test (OF) and elevated plus maze (EPM) on days 4 and 5, respectively. Finally, we opted to perform the forced swimming test (FST) in the last two days so that the physical effort associated to it would not bias the results of the other tests. In experiment 2, animals were divided into four experimental groups: SHAM animals that received saline (SHAM_SAL) or amitriptyline (SHAM_AMI) and ARTH animals that received saline (ARTH_SAL) or amitriptyline (ARTH_AMI). Four weeks after the intrasynovial injection animals were submitted to a baseline evaluation in the SPT followed by three weeks of daily injections of SAL or AMI. In the fourth week of the treatment, animals were behaviourally tested following the same experimental design as in experiment 1. **(B)** Sections of the femoro-tibial joint space stained with eosin-haematoxylin of SHAM and ARTH animals four weeks after the induction of arthritis. In SHAM animals (B1) the structure of the femoro-tibial joint is preserved (M: meniscus; PL: patellar ligament; FC: femoral condyle; TP: tibial plateau and SCB: subcondral bone). In ARTH animals (B2), cartilage damage (a) can be observed in addition to fibrillation and marked thinning of the central part of the articular surface. In the FC, subchondral sclerosis (c), cyst formation (d) and inflammation (infiltration of immune cells) (e) are present while in the TP only cyst formation (b) is observed at this time point.

**Figure 2**. Behavioural assessment four weeks after arthritis induction, in experiment 1. Evaluation of the right (ipsilateral) limb withdrawal latency (LWT) in the pressure application measurement (PAM). The LWT of arthritic (ARTH) group was significantly decreased when compared to results in control (SHAM) animals (mechanical hyperalgesia) **(A)**. No differences were found in locomotor behaviour between arthritic (ARTH) and control (SHAM) groups, as indicated by the total distance travelled in the open field (OF) test **(B)**. ARTH animals showed increased anxiety-like behaviour as indicated by: the tendency to avoid the centre of the OF arena **(C)**; the significantly higher number of *fecal boli* left in the arena at the end of the trial **(D)**; and the reduction in time spent in the open arms of the EPM test, when compared to SHAM animals **(E)**. ARTH animals displayed an anhedonic-like behaviour as showed by the significant decrease in the preference for sucrose solution in the sucrose-preference test (SPT) **(F)** (Δ sucrose preference is the variation in sucrose preference between the data obtained four weeks after and one day before monoarthritis induction). Results are expressed as Mean+SEM (n=6 per group). **P*<0.05, ***P*<0.01.

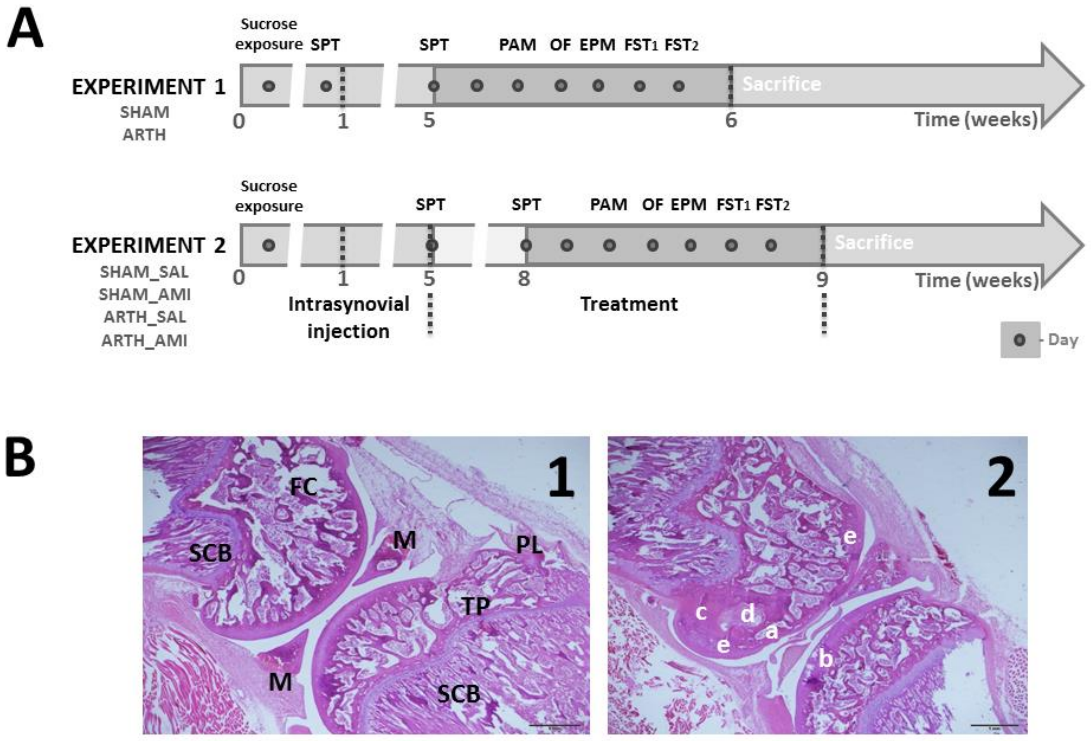
**Figure 3.** Behavioural assessment in the forced swimming test (FST), in experiment 1. Four weeks after the induction of experimental arthritis, ARTH animals become immobile significantly earlier than SHAM animals in the forced swimming test (FST) **(A)**. ARTH animals spent significantly more time immobile than SHAM animals **(B)**, at the expense of the time spent swimming **(C)** and climbing **(D)** in the FST test. Results are expressed as Mean+SEM (n=6 per group). **P*<0.05, ***P*<0.01.

**Figure 4**. Behavioural assessment after treatment with amitriptyline (AMI), in experiment 2. Evaluation of the right (ipsilateral) limb withdrawal latency (LWT) in the pressure application measurement (PAM). The LWT of arthritic (ARTH) animals treated with AMI increased when compared to ARTH animals treated with saline (SAL), reaching control (SHAM) levels (abolished mechanical hyperalgesia) **(A)**. No differences were found in locomotor activity between

arthritic (ARTH) and control (SHAM) groups, as indicated by the total distance travelled in the open-field (OF) test **(B)**. ARTH animal treated with AMI showed no difference in anxiety-like behaviour when compared to ARTH animals treated with SAL in both the time spend in the centre of the OF arena **(C)** and the number of *fecal boli* **(D)**. In the elevated plus maze (EPM) test no differences were observed in the time spent in open arms **(E)**. In the sucrose preference test (SPT), ARTH animals treated with AMI increased sucrose preference when compared with ARTH animals treated with SAL **(F)** (Δ sucrose preference is the variation in sucrose preference between the data obtained three weeks after and one day before the beginning of the treatment). Results are expressed as Mean+SEM (n=8 per group). $^{**}P<0.01$, $^{***}P<0.001$.

**Figure 5.** Behavioural assessment in the forced swimming test (FST) after treatment with amitriptyline (AMI), in experiment 2. AMI did not reverse the effect of arthritis on the latency to immobility **(A)**, the time spent immobile **(B)** or the time spent in swimming **(C)**. Interestingly, ARTH animals injected with AMI increased the time spent climbing **(D)** but only when compared with SHAM treated animals. Additionally, note that following AMI treatment of ARTH animals, no differences were observed with the SHAM group treated with SAL in the latency to immobility (A), total immobility (B) and time spent climbing (D). Results are expressed as Mean+SEM (n=8 per group). $^{*}P<0.05$, $^{**}P<0.01$.

**Figure 1**


A
Sucrose exposure
SPT
SPT
PAM
OF
EPM
FST1
FST2
EXPERIMENT 1
SHAM
ARTH
Sacrifice
0
1
5
6
Time (weeks)
Sucrose exposure
SPT
SPT
PAM
OF
EPM
FST1
FST2
EXPERIMENT 2
SHAM_SAL
SHAM_AMI
ARTH_SAL
ARTH_AMI
Sacrifice
0
1
5
8
9
Time (weeks)
Intrasynovial injection
Treatment
Day
B
1
FC
SCB
M
PL
TP
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SCB
2
e
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a
e
b

**Figure 2**

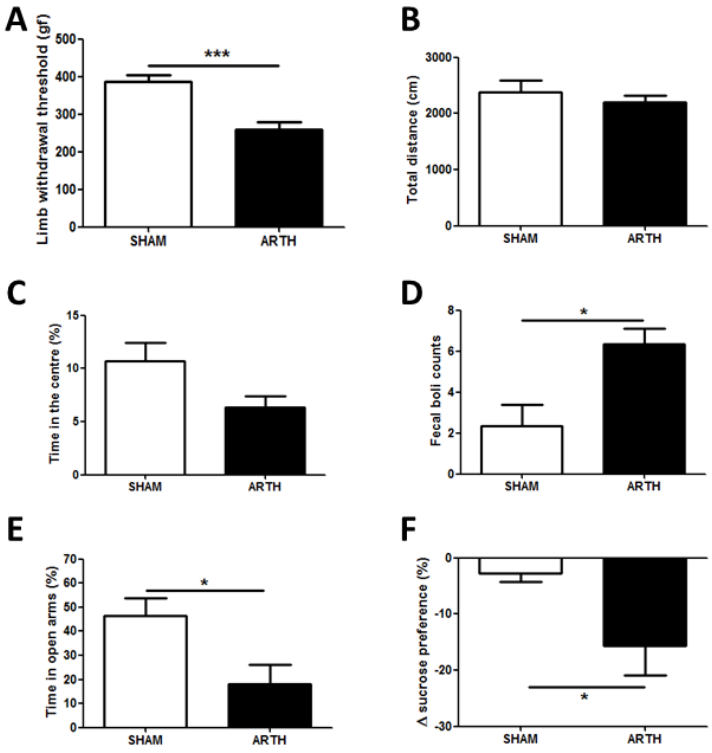

A
Limb withdrawal threshold (gf)
500
400
300
200
100
0
***
SHAM
ARTH
B
Total distance (cm)
3000
2000
1000
0
SHAM
ARTH
C
Time in the centre (%)
15
10
5
0
SHAM
ARTH
D
Fecal boli counts
8
6
4
2
0
*
SHAM
ARTH
E
Time in open arms (%)
70
60
50
40
30
20
10
0
*
SHAM
ARTH
F
Δ sucrose preference (%)
0
-10
-20
-30
*
SHAM
ARTH

**Figure 3**

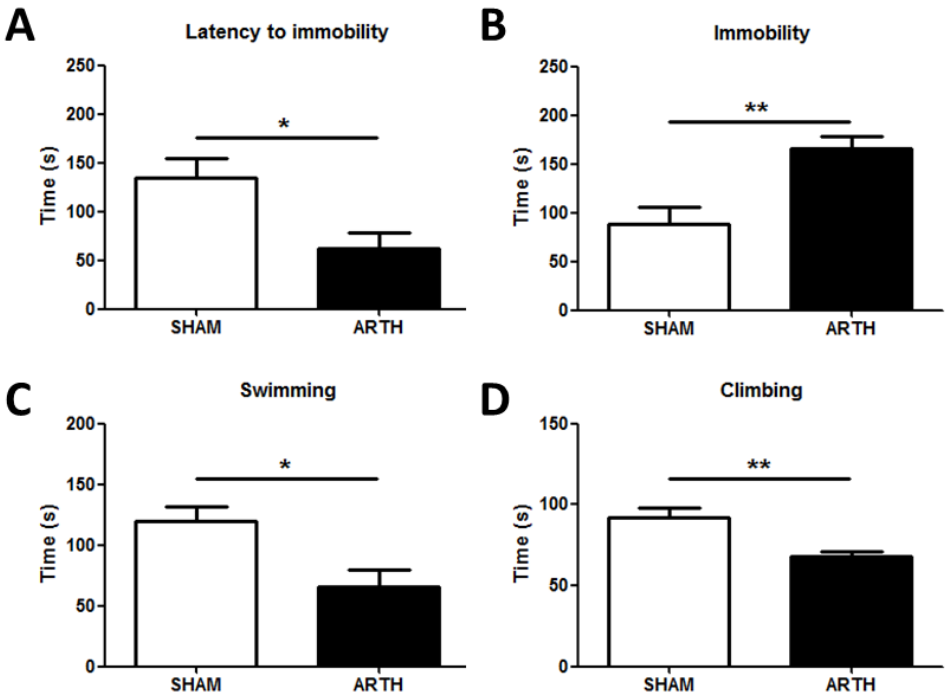

A
Latency to immobility
Time (s)
250
200
150
100
50
0
*
SHAM
ARTH
B
Immobility
Time (s)
250
200
150
100
50
0
**
SHAM
ARTH
C
Swimming
Time (s)
200
150
100
50
0
*
SHAM
ARTH
D
Climbing
Time (s)
150
100
50
0
**
SHAM
ARTH

**Figure 4**

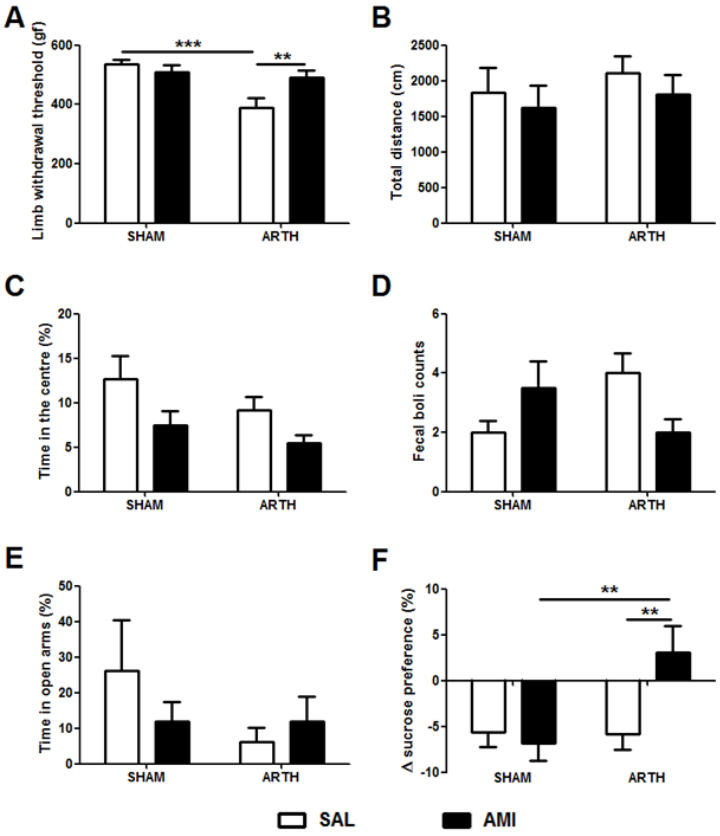
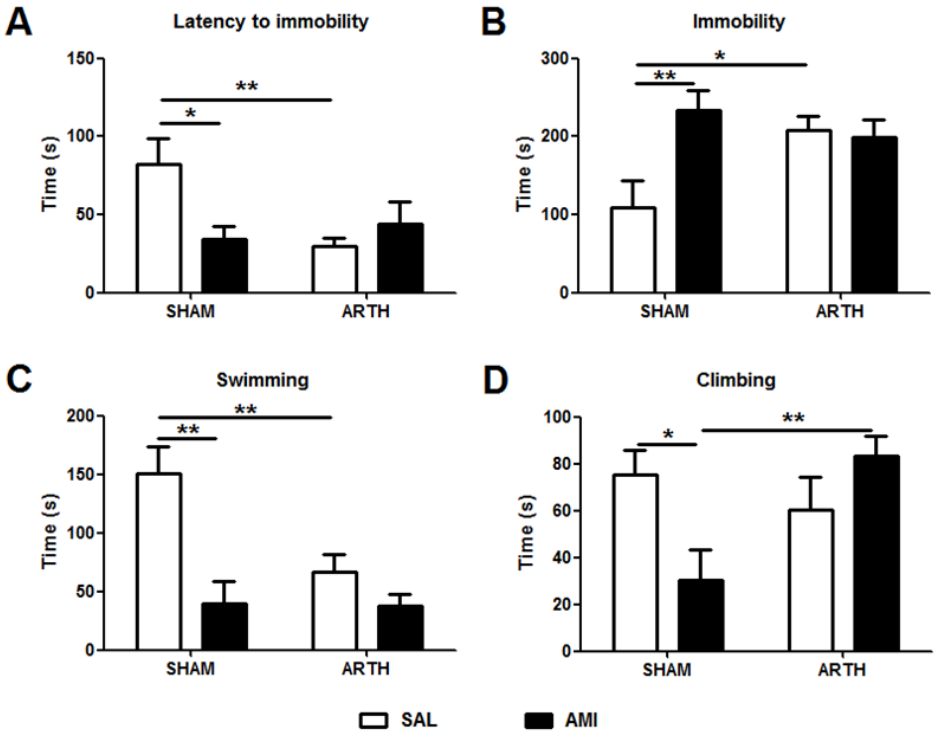

A
Limb withdrawal threshold (gf)
600
400
200
0
***
**
SHAM
ARTH
B
Total distance (cm)
2500
2000
1500
1000
500
0
SHAM
ARTH
C
Time in the centre (%)
20
15
10
5
0
SHAM
ARTH
D
Fecal boli counts
6
4
2
0
SHAM
ARTH
E
Time in open arms (%)
50
40
30
20
10
0
SHAM
ARTH
F
Δ sucrose preference (%)
10
5
0
-5
-10
**
**
SHAM
ARTH
SAL
AMI

**Figure 5**


A
Latency to immobility
Time (s)
150
100
50
0
**
*
SHAM
ARTH
B
Immobility
Time (s)
300
200
100
0
*
**
SHAM
ARTH
C
Swimming
Time (s)
200
150
100
50
0
**
**
SHAM
ARTH
D
Climbing
Time (s)
100
80
60
40
20
0
*
**
SHAM
ARTH
SAL
AMI