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A spherical treadmill system to train head-fixed adult rats

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HIGHLIGHTS

- We present a novel spherical treadmill suitable to train adult rats.
- The sphere freely rotates supported by 3 bearings and its movement is video tracked.
- Head-fixation is used to facilitate acute electrophysiology and imaging recordings.
- We designed a training protocol that gradually introduces animals to head-fixation.
- Adult Long Evans rats were successfully trained in an auditory discrimination task.

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ABSTRACT

Background: While spherical treadmills are widely used in mouse models, there are only a few experimental setups suitable for adult rats, and none of them include head-fixation.
New method: We introduce a novel spherical treadmill apparatus for head-fixed rats that allows a wide repertory of natural responses. The rat is secured to a frame and placed on a freely rotating sphere. While being head-fixed, it can walk in any direction and perform different motor tasks.
Comparison with existing methods: Instead of being air-lifted, which is acceptable for light animals, the treadmill is sustained by three spherical bearings ensuring a smooth rotation in any direction. Movement detection is accomplished using a video camera that registers a dot pattern plotted on the sphere.
Results: Long Evans rats were trained to perform an auditory discrimination task in a Go/No-Go (walking/not-walking) paradigm. Animals were able to successfully discriminate between a 1 kHz and a 8 kHz auditory stimulus and execute the correct response, reaching the learning criterion (80% of correct responses) in approximately 20 training sessions.
Conclusions: Our system broadens the possibilities of head-fixation experiments in adult rats making them compatible with spatial navigation on a spherical treadmill.

1. Introduction

Head-fixation is a widely employed technique in neuroscience: it allows a combination of physiological, cellular and molecular manipulations along with behavioural observation of spontaneous or conditioned responses on a precise spatiotemporal scale. As the head is immobilized, it simplifies several recording techniques such as electrophysiology, two-photon microscopy or functional magnetic resonance imaging (fMRI) (Schwarz et al., 2010; Dolzani et al., 2014) while improving signal stability. Moreover, it offers greater experimental control over sensory inputs (tactile, visual, olfactory or auditory stimulation) and motor outputs (such as licking, lever pressing, walking and eye tracking) than freely moving designs (Schwarz et al., 2010; Guo et al., 2014). However, immobilization or restraint is one of the classical methods to induce acute and chronic stress (Buynitsky and Mostofsky, 2009; Gamaro et al., 1998) and it is known to affect learning (Buynitsky and Mostofsky, 2009; Sandi and Pinelo-Nava, 2007; Joëls et al., 2006). Therefore, in order to minimize stress responses, it is crucial to carefully select the duration, intensity and frequency of the restraint as well as to design a training protocol that gradually habituates the animal to the experimental apparatus.

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For many behavioural experiments a trade-off between animal fixation and a considerable degree of movement –including locomotion– is mandatory (Soltysik et al., 1996). In this sense, spherical treadmills are a useful tool. Moreover, spherical treadmills with head-fixation have been employed for physiological recordings during several motor tasks, always in mouse models (Dombeck et al., 2007; Keller et al., 2012; Ayaz et al., 2013; Sofroniew et al., 2014).

Though mice have been the preferred choice for perceptual studies, over the last few years this preference has changed (Zalocusky and Deisseroth, 2013; Carandini and Churchland, 2013). Rats have been proven to have better visual resolution than mice (Prusky et al., 2000), which could favor motor related tasks as spatial navigation. Also, they are bigger than mice and weight ten times more. As body weight strongly correlates with the number of trials performed *per* day (Busse et al., 2011), heavier animals need more trials to reach satiation, which results in longer training sessions and consequently, more collected data. In addition, different techniques as fMRI or multiple electrodes for single/multi cell recording (each one with its own spatial resolution) give greater specificity in bigger brains (Zalocusky and Deisseroth, 2013).

Multiple behavioural tasks related to sensory systems (Otazu et al., 2009), working memory (Jadhav et al., 2012) and decision making (Kepecs et al., 2008), have been studied and validated in rats, including more complex behaviours concerning empathy and cognitive tasks (Bartal et al., 2011; Schoenbaum et al., 2003). Moreover, rat models have contributed to the understanding of different pathologies such as obesity (Sclafani and Springer, 1976; Johnson and Kenny, 2010), addiction (Weeks, 1962; Vanderschuren and Everitt, 2004), Parkinson disease (Gradinaru et al., 2009), dopamine modulation in depressive behaviour (Tye et al., 2013), schizophrenia (Chambers et al., 1996) and autism (Umeda et al., 2010). Therefore, it would be useful to have a system that allows studying motor related tasks with head-fixation using rats. Rat navigation in a virtual reality environment has been previously reported (Hölscher et al., 2005), though animals were not head-fixed (and no physiological measurements were registered). Instead, they were held in a harness, and were able to freely move on the surface of the sphere.

In this work we addressed the need of an experimental apparatus suitable for adult rats that integrates both head-fixation and spherical treadmills by developing a training system where rats can perform a wide repertory of motor responses while being head-fixed. Using this system, we trained three adult Long Evans rats in a Go/No-Go auditory discrimination task.

2. Materials and methods

2.1. Spherical treadmill

The treadmill consists of a 46 cm diameter, 302.8 g expanded polystyrene foam ball (Fig. 1a) that rests on 3 smaller spherical bearings (Alwayse Euro 0) allowing a smooth rotation (Fig. 1b). A triangular base (65 cm side) of 20 mm square steel tube supports three articulated arms that hold the spherical bearings. This allows to adjust the height of the treadmill for each animal so that they can adopt an adequate and comfortable walking position.

The surface of the sphere is marked with uniformly distributed dots which are used to perform a camera-driven movement detection (PlayStation 3 Eye camera). The camera, which is placed at one corner of the structural base (Fig. 1c), is able to capture standard video (640 × 480 px) up to 60 frames per second. The sphere is covered with a thin layer of varnish in order to facilitate cleaning.

Liquid reward is administered using a stainless steel solenoid valve (K15, OCSA®, Buenos Aires, Argentina). The training software controls the valve opening and closing times therefore determining the volume of fluid delivered.

The position of the lickport (Fig. 1d) is adapted for each animal by means of a plastic holder (Fig. 1e) (Burman and Martínez Cáceres, 2017) that can be adjusted in three different axes. The holder is attached to the horizontal bar and it supports a video camera (Fig. 1f) to supervise water consumption and licking behaviour.

2.2. Head-fixation

Head-fixation is attained by surgical implantation of an H-shaped or X-shaped aluminium piece of 2 g of weight and 2 mm of thickness. Screwing the four ends of the head-fixation device to a plastic adapter and fastening the adapter to a steel frame allows to firmly secure the rats to the training apparatus (see Section 2.7.2).

Different head-fixation devices were developed depending on the brain areas of interest (e.g. H-shaped Fig. 2a– and X-shaped Fig. 2b–). They were designed with OpenSCAD according to a rat brain atlas (Paxinos and Watson, 2006) and then custom-built using a Roland MDX-15 milling machine. For the experiments here presented, all rats were implanted with the X-shaped (Burman and Martínez Cáceres, 2017) device.

The animal is fixed to the experimental apparatus by screwing the four ends of the head-fixation device to a plastic adapter (Figs. Figure 1g, Figure 3a) (Burman and Martínez Cáceres, 2017). This plastic adapter is designed to allow experimental manipulations on the animal head displacing the site of attachment to the frame (Fig. 3b).

A half polyvinyl chloride (PVC) tube (5.1 cm diameter) with two cemented screws is placed between the animal and the plastic adapter (Figs. Figure 1h, Figure 3b) (Burman and Martínez Cáceres, 2017). This tube is used to guide the animal position on the treadmill. A steel frame (Fig. 1i) sustains the animal on the treadmill. A horizontal T-profile bar is supported by two vertical columns –at each side of the sphere- soldered to a heavy squared steel base. Fastening the screws of the half PVC tube to the frame firmly secures the animal to the training apparatus.

2.3. Animals

Long Evans male rats (300–340 g) of two months of age were provided by the IBYME–CONICET and were individually housed in stainless-steel cages (40 cm × 25 cm × 18 cm, L × W × H) with sawdust as bedding and metal lids. All animals were kept in a well-ventilated, temperature-controlled room (22 ± 2 °C) with a 12/12 h light/dark cycle (lights on at 8 am).

2.4. Pre-surgery handling

Handling procedures start one week before surgery and are performed daily, to ensure that the animal becomes acquainted with the operator and is comfortable with being held for some minutes. The protocol is based in progressively increasing the amount of time the animals are held and releasing them only when they are calm and still. Progressing to the following step of the protocol depends on how well each rat responds to handling procedures. Thus, the first handling step consists in letting the rats explore the operator hands by placing them in their homecage. Next, without removing the rat from their cage, they are gently picked up by grabbing them with one hand over the shoulders and placing the other hand under the hindlimbs to provide support. Then, they are held in progressively increasing periods of time from 10 s to 2–3 min. Animals are not released when they try to escape from restraint. Instead, we wait a few seconds until they are calm and still before releasing them. At the end of the handling/pre-training sessions, up to 10 min of continuous restraint is attained (see Section 2.7.2).

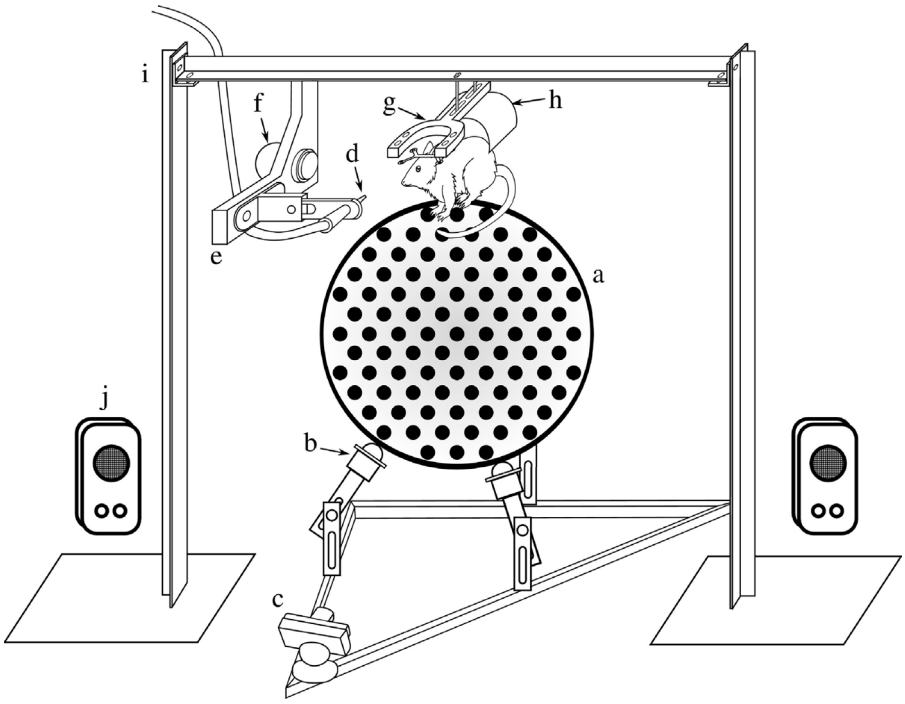

Fig. 1. Schematic representation of the training apparatus (not to scale). A polystyrene foam sphere (a) is mounted over three spherical bearings (b) allowing free rotation. Each bearing is held by an articulated arm for height adjustment. The sphere is marked with a uniform dot pattern and a PS3 eye video camera (c) is used for movement detection. The position of the lickport (d) is adapted for each animal by means of a plastic holder (e) that can be adjusted in three different axes. This holder supports a web-camera (f) to supervise water consumption during training. A plastic adapter (g) is used to secure the animal to the experimental apparatus. A half PVC tube (h) guides the animal position on the treadmill. A steel frame (i) sustains the animal on the treadmill: a horizontal T-profile bar is supported by two vertical columns soldered to a heavy squared steel base. Auditory stimuli are played with standard computer speakers (j).

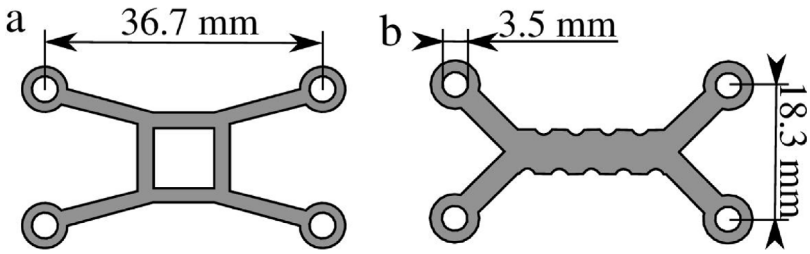

Fig. 2. (a) H-shaped device compatible with simultaneous recordings in prefrontal cortex and ventral tegmental area. (b) X-shaped device used when primary motor cortex was targeted.

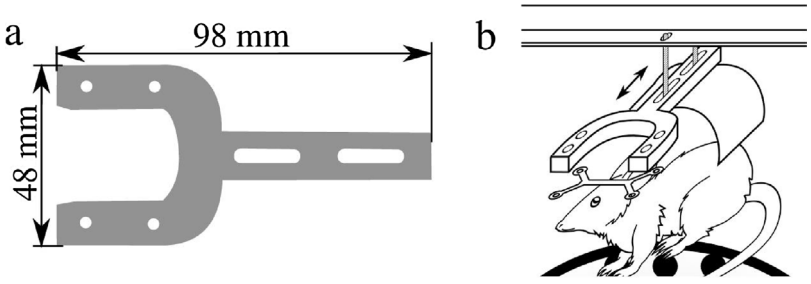

Fig. 3. Plastic adapter (a). Screws are used to secure the adapter to the head-fixation device (through the four holes on the left) and to the T-profile bar of the frame (through the elongated holes on the right). The position of the rat with respect to the frame can be adjusted in each case because of the elongated shape of the holes (b). A half PVC tube used to guide the animal position on the treadmill, bears the two screws that fix the plastic adapter to the experimental apparatus.

2.5. Surgery

Rats are anesthetized with a mixture of ketamine (75 mg/kg, i.p.) and xylazine (20 mg/kg, i.p.), and supplements of ketamine (10% of original dose) are administered as necessary to maintain the level of anaesthesia. Anesthetic depth is monitored along the procedure by testing the absence of the paw reflex and observing the breathing rate and colour of the paws. Animals are placed in the stereotaxic frame and their eyes are covered with ophthalmic gel (hydroxypropyl methylcellulose 0.3%).

After exposing the cranial surface, four small stainless-steel screws are implanted in the cranial vault to provide mechanical

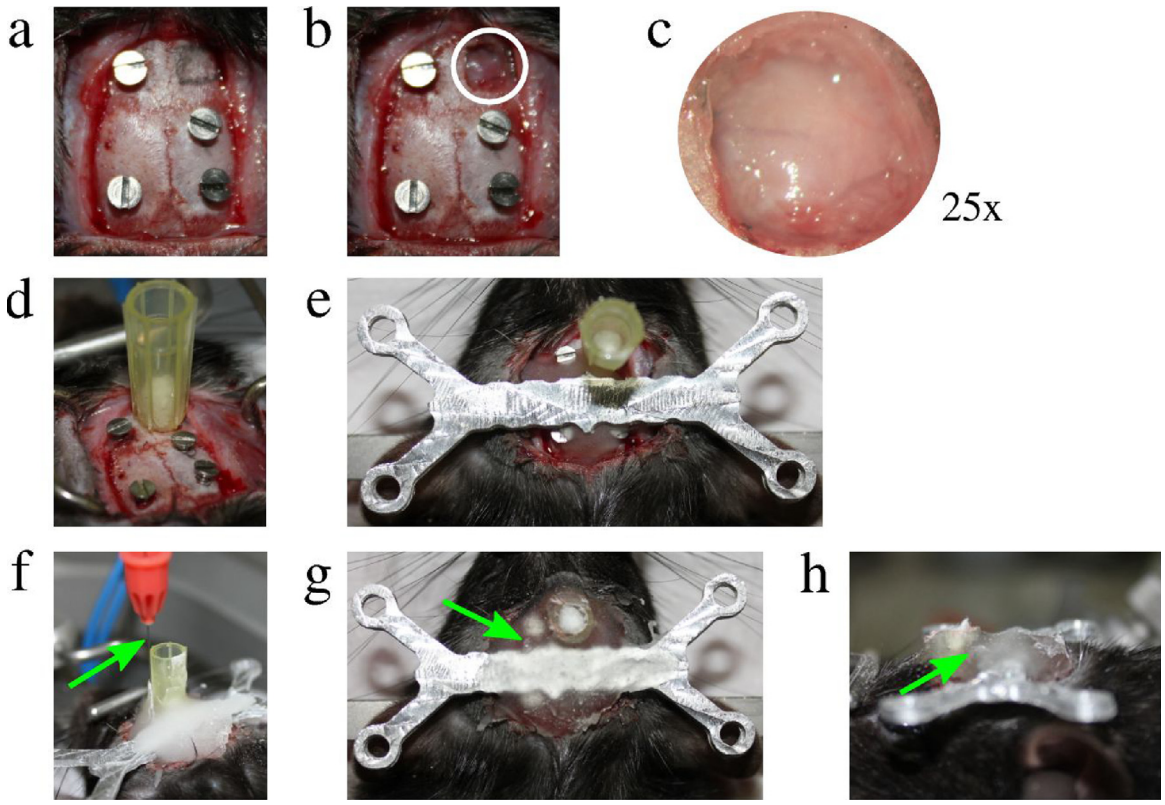

Fig. 4. Implant surgery: (a) Four small stainless-steel screws are used to secure the implant to the bone. (b) Craniotomy over the right primary motor cortex (M1) (white circle). (c) Brain surface under 25 x magnification (surgical microscope, Newton SRL). (d) A plastic tip surrounds the craniotomy in order to protect the brain. (e) The head-fixation device is placed over the screws behind the craniotomy. (f) Dental acrylic covers the device and fixes the implant to the screws and to the bone. A needle (arrow) is used as a mark for posterior reference. Finally, the plastic tip and the needle (arrow) are cut level with the acrylic: (g) dorsal view, (h) lateral view.

support, serving to secure the head-fixation device to the skull (Fig. 4a).

We made a craniotomy over the right primary motor cortex (M1) in one of the rats (Fig. 4b, c) that will enable electrophysiological recordings. The other rats had no craniotomy so the next steps were omitted. The position of the craniotomy is marked according to the coordinates indicated in a rat brain atlas (Paxinos and Watson, 2006) (for M1: 2.04 mm anterior and 2.6 mm lateral to Bregma). A 3 mm hole is drilled to expose the brain surface. A plastic cylinder (4 mm inner diameter) is glued to the skull to protect the craniotomy (Fig. 4d). The head fixation device is placed over the screws and posterior to the craniotomy (Fig. 4e). Next, the implant is secured with dental acrylic, sealing the entire region and binding the head fixation device to the screws and to the bone. Before the acrylic hardens, a needle (30G) is stereotaxically placed at a certain known distance from Bregma for subsequent reference in electrophysiological recordings (Fig. 4f). The plastic tip and the needle are cut level with the acrylic (Fig. 4g, h). The craniotomy is covered with antibiotic solution (neomycin, 3.5 mg/ml; polymyxin B, 5.000 UI/ml; gramicidin USP, 0.025 mg/m), and sealed with a cotton cap.

After surgery, yohimbine hydrochloride (2 mg/kg i.p.) is administered to reverse xylazine effects and promote animal recovery. Rats are given additional pain relief (meloxicam, 2 mg/kg, s.c.) and placed under heating until recovery. Analgesics (tramadol hydrochloride, 0.075 mg/ml) and antibiotics (enrofloxacin, 0.5 mg/ml) are administered orally in the tap water for the following 7 days. The cage is provided with a lid adapter that increases its height to 27 cm to facilitate animal circulation and food accessing. Allowing more space also reduces the risk of bumping the implant against the cage.

During the 7 days post-operative period the handling procedures continue as described, provided that the animal is recovering well and responds normally to the operator.

We have already employed the present surgical protocol to successfully implant over 12 animals with different head-fixation devices. None of them presented any illness or signs of distress, and carried a normal life with the implant firm on their heads, up to two years after surgery (unpublished data).

2.6. Training software

We developed an open source software program (using Python 2.7) to control stimuli, provide rewards, and record performance during training (Martínez Cáceres and Burman, 2017). The program allows to configure and execute different training protocols: classical conditioning, operant conditioning and discrimination learning. All parameters related to each training phase can be manually tuned. A graphical user interface provides feedback on current training parameters and animal performance, as well as the possibility of manually providing reward.

Auditory stimuli are generated with PyGame (Python custom library) and played with standard computer speakers (Fig. 1j). The program controls sound frequency, amplitude, and duration.

Our method for movement detection consists in comparing two consecutive frames and computing changes in position for every dot in the camera visual field. All differences are averaged and if this value surpasses a threshold, it is considered that the animal is moving in that time slot. The software retrieves frames from the camera as fast as it can, and the computation time between frames is 33 ms ± 2 ms (mean ± SD) (intel i5 2.6 GHz, 4 GB RAM, Ubuntu 14.04). The movement threshold is empirically selected

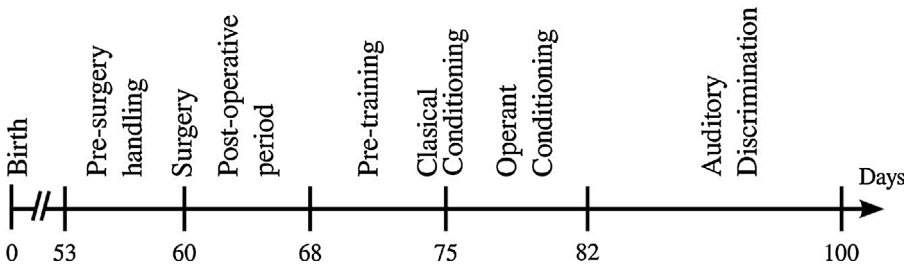

Fig. 5. Stages of experimental protocol. Surgery is carried out when rats are two months of age (60 postnatal days) and a 7 days post-operative period is allowed before training in all animals. The length of the following stages depends on animal performance and therefore is variable. In this scheme an average between subjects is shown.

during the pre-training sessions based on what the experimenter considers a proper movement. The threshold set in these experiments correspond to 8 cm/s. A correct Go response is achieved when the percentage of time slots surpassing the threshold during a 2 s opportunity window is at least 80%. Similarly, if at least 80% slots of a 2 s opportunity window had a not-moving result (does not surpass the threshold), the program considers the animal performed a correct No-Go response. Therefore, depending on the kind of trial (Go/No-Go) and the response executed by the animal (walking/not-walking) the training program delivers (or not) the reward. Further details on reward criteria for each training phase is described in Section 2.7.3.

2.7. Behavioural training

The different stages of the experimental protocol are depicted in Fig. 5 and referred to day of birth. Implant surgery is performed when the animals are 60 days of age, starting pre-surgery handling at least 1 week before. The length of each stage was variable depending on task performance.

2.7.1. Water restriction

Animal motivation is achieved by means of a water restriction regimen. Initially, animals are given free access to tap water. One week after the post-operative period the rats are water restricted to 10 ml of water *per* day, while food is available *ad libitum*. General health evaluation is conducted by daily monitoring animal weight, fur appearance, grooming behaviour and usual exploratory behaviour to prevent animal dehydration (Schwarz et al., 2010). Additional water is provided if animal dropped below 85% of its *ad libitum* body weight. However, from pre-training until the end of the behavioural procedures, we aim to provide the daily water supply exclusively during training.

2.7.2. Pre-training

The first step in behavioural training is to familiarize the animals with the head-fixation protocol and the experimental apparatus. Initially, it consists in daily incrementing the time the rat is held by the operator, providing small water rewards of 0.5–1 ml when the animal begins to move. In the end, 8–10 min of continuous restraint is attained. Every session is finished when animals present signs of stress such as agitation or teeth chattering.

In the following sessions, we proceed to gradually fasten the animal to the frame, giving water rewards at each new step. Progressively delaying the reward allows to eventually place the rat on the treadmill without any reward. The complete procedure consists in fastening the plastic adapter to the four ends of the implant, placing the half PVC tube between the animal and the plastic adapter and then fastening the tube to the horizontal T-profile bar. To facilitate the manoeuvre, the horizontal bar is previously removed from the setup and placed in a clear and spacious counter of the experimental room. First, one operator holds the rat with one hand over the shoulders and the other hand under the hindlimbs. Then, the second operator fastens the plastic adapter to the four ends of the implant. Special care to avoid any action that could stress or startle the animal is taken. For that reason, butterfly nuts that are quick and easily fastened (and unfastened) are used. Bubble wrap material is placed underneath the working space to absorb the impact in case any screw or nut fell. It is also necessary to always provide an underneath support to the animal, so that the implant never bears the animal weight. Finally, while the first operator still holds the animal, the second operator lifts the bar and they both approach to the setup. The first operator releases the animal when it is over the sphere. Once in place, the bar is screwed to the vertical columns and the lickport is positioned.

The following pre-training sessions consist in placing the animal on the treadmill and administering water with the fluid delivery system, first randomly and later only when the animal is walking.

Every effort is made to provide the daily water supply during pre-training sessions, sometimes performing a second session on one day to avoid giving water out of the training context.

2.7.3. Classical and operant conditioning training

Animals are subjected to 1 session of classical conditioning, pairing a tone (1 kHz or 8 kHz, 1 s duration) with a 20–30 μl water drop.

Next, operant conditioning training is conducted: presentation of the same auditory stimulus is followed by a 2 s window of opportunity during which the animal has to walk in any direction for at least 1.6 s to get the reward. The inter-trial interval is a uniformly distributed random number between 5 and 8 s. Trials start only after 1 s of no movement. Session length is defined by the willingness of the animal to retrieve reward. Therefore each session ended when rats were disengaged from the task or when they reached the volume of their daily water supply. Each session consisted of 650 trials in average.

In order to progress to the next training phase, the learning criterion was to perform over 80% of correct responses for three consecutive sessions.

2.7.4. Auditory discrimination task training

In the auditory discrimination task a second stimulus is introduced to complete a Go/No-Go paradigm. The new tone is either 8 kHz or 1 kHz (depending on the frequency used for classical and operant conditioning). Go trials are identical to operant conditioning trials. No-Go trials follow the same pattern, hence not walking (remaining still) for at least 1.6 s in the opportunity window is considered the correct response (Fig. 6). Go and No-Go trials are randomly presented in a 50:50 proportion.

Daily training sessions are carried out until learning criterion is reached (80% of correct responses). Each session consisted of 500–750 trials with an average duration of 2 h.

2.7.5. Tail immersion test

Acute exposure to stressors, such as restraint, attenuates reflex responses to nociceptive thermal stimulation (King et al., 2003),

Fig. 6. Scheme of a training trial. Presentation of the stimulus (a 1 s tone) is followed by a 2 s window of opportunity in which the animal has to perform the correct response for at least 1.6 s within this window in order to get the reward. The inter-trial interval is normally set to a random time between 5–8 s.

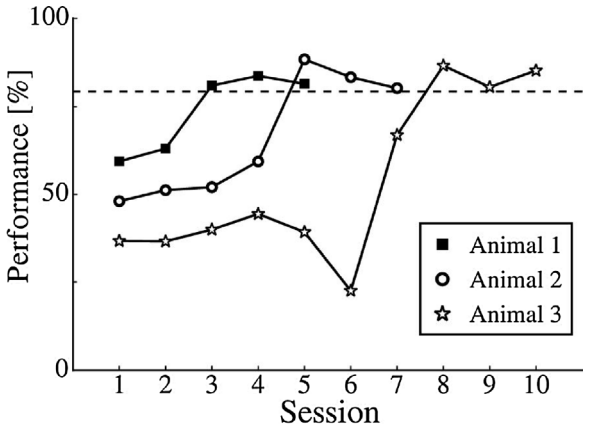

Fig. 7. Operant conditioning training performance. A learning criterion of 80% of correct responses (dotted line) for three consecutive sessions was established. All animals reached this criterion in 5–10 sessions.

an effect commonly referred to as stress-induced analgesia (Butler and Finn, 2009). Therefore to assess the stress of the animals during training, two tail immersion tests are daily performed (modified from Ben-Bassat et al., 1959) from the classical conditioning session to the end of the experiment. Every day, before carrying the animal to the experimental room, one operator picks up the rat as usual and the other immerses half the tail in water at 55 °C. The reaction time, i.e. the amount of time it takes the animal to remove the tail from the hot water, is measured with a stopwatch. The test is repeated at the end of the training session before removing the animal from the experimental setup.

2.8. Data analysis

Automatic data logging of training performances is accomplished by the training software. For all reported results, we discard the first and last 10% trials of each session in order to avoid trials where animals executed impulsive licks or were satiated (Berditchevskaia et al., 2016).

Wilcoxon signed rank test is used to compare latencies of the tail immersion test. A p-value <0.05 is considered statistically significant.

3. Results

Operant conditioning training performances are presented in Fig. 7. Animals 1, 2 and 3 started with a near 50% performance (49 ± 8, mean $\pm$ SD) and they reached the learning criterion in sessions 5, 7 and 10 respectively (7 ± 3, mean $\pm$ SD). Thereafter, auditory discrimination training began.

Performances of all animals in the auditory discrimination task are presented in Fig. 8a. Animal 1 exhibited a preference for walk-

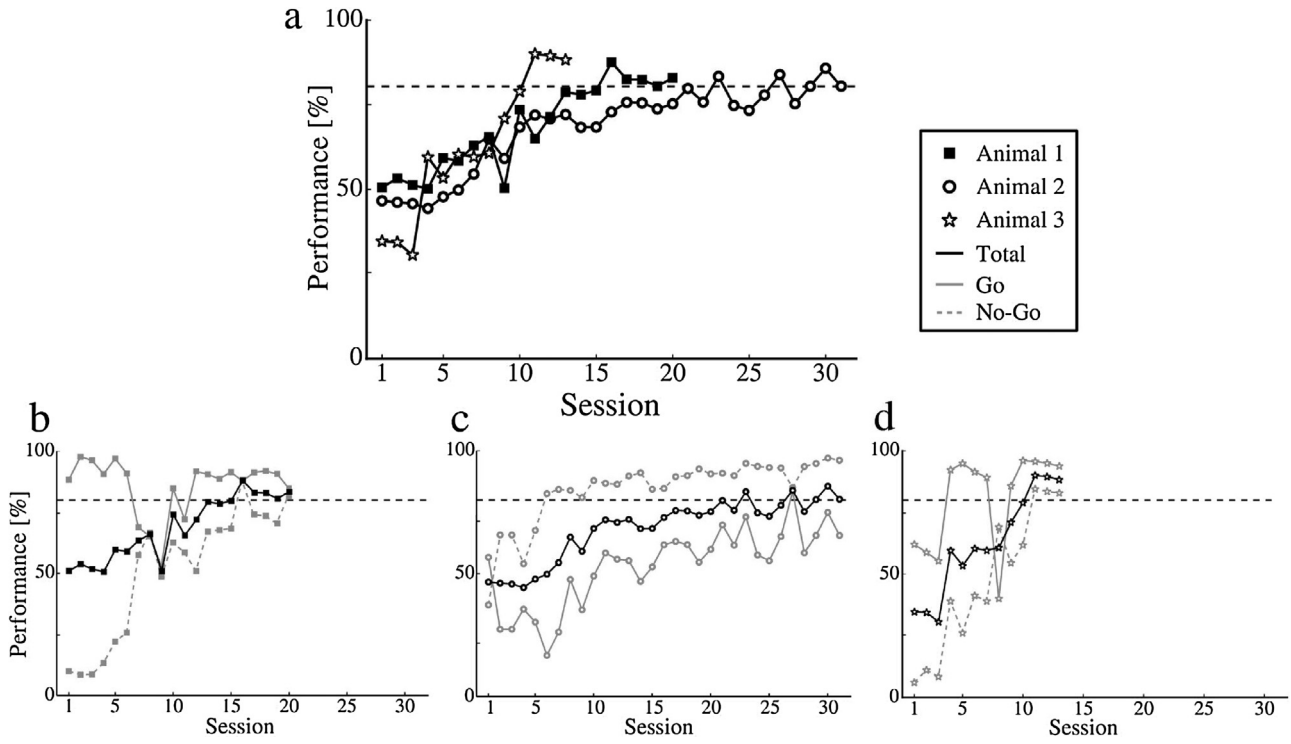

Fig. 8. Auditory discrimination training performance. A novel stimulus was introduced as the No-Go stimulus. Correct No-Go trials were those where the animal didn't walk for at least 1.6 s within the window of opportunity. Total performances for all animals (a) as well as performances for the Go (solid gray line) and No-Go (dotted gray line) trials separately are presented for animal 1 (b), 2 (c) and 3 (d).

ing, reaching 80% of correct responses in operant training in only 3 sessions (Fig. 7) and starting discriminatory task training with excellent performances in Go trials (Fig. 8b). Conversely, animal 2 displayed a preference for not-walking, taking more sessions to meet the operant conditioning learning criterion (Fig. 7). Also, performance in Go trials for this animal dropped during the first discrimination training sessions (Fig. 8c), probably because not-walking behaviour started to be rewarded and that was its initial preference. We did not observe any preference for walking or not-walking in animal 3. Although it took more sessions to reach the learning criterion in operant conditioning (Fig. 7), its performance in auditory discrimination was pronouncedly better after the third session, learning the task in 13 days (Fig. 8a,d).

Despite the variability in performance, all animals successfully learned to discriminate between two auditory stimuli according to our criteria. In average, rats learned the auditory discrimination task in 21 ± 9 sessions (mean $\pm$ SD). Supplementary file 1 shows part of a training session in the experimental apparatus where an animal performs correct Go and No-Go responses.

In order to ensure animal health and wellbeing, we daily evaluated its weight, fur appearance, grooming behaviour and usual exploratory behaviour (National Research Council (US), 2011). The water restriction regimen started one week after the post-operative period, and after a few days all rats presented a stable average of 88% of their *ad libitum* weight (Fig. 9a), drinking 9–12 ml of water per day (Fig. 9b). No unusual behaviour or appearance was observed.

When training sessions ended and rats returned to drink water *ad libitum,* they rapidly regained their initial weight as shown in Fig. 9a, regardless of how long they were in the regimen. Differences in the duration of water restriction are a consequence of differences in performance for each training stage. Pre-training sessions amounted to 13, 26 and 8 for animal 1, 2 and 3 respectively (16 ± 9 sessions, mean $\pm$ SD). Operant conditioning and auditory discrimination training session differences are visible in Figs. Figure 7 and Figure 8a.

We were also interested in evaluating the presence of stress. Recognition of stress in laboratory animals requires an understanding of species-specific normal behaviour and various parameters should be taken into consideration (National Research Council (US), 2008). Although glucocorticoid levels (usually corticosterone in rodents) are frequently used as indicators of the strength and impact of a stressor (National Research Council (US), 2008), most sampling procedures are invasive and stressful. Therefore, to avoid a direct association between pain and training sessions we chose a non-invasive method. In addition to the aforementioned clinical signs and behavioural parameters assessed, we performed a tail-immersion test before and after training sessions (Fig. 10).

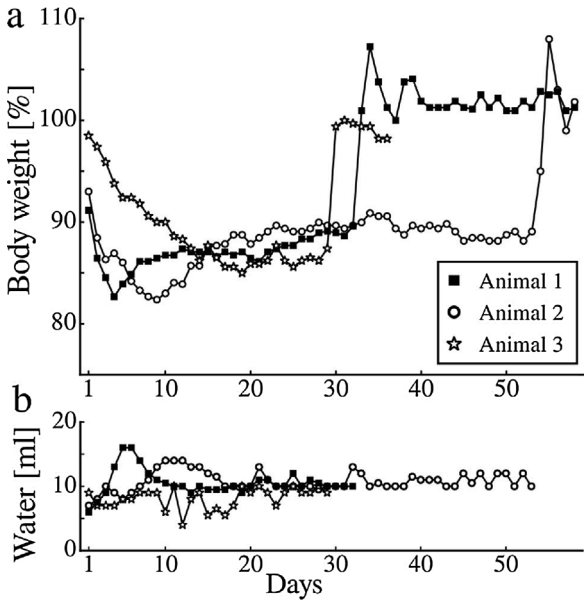

Fig. 9. Animal weight and water consumption. (a) Body weight is expressed as the percentage of the initial *ad libitum* weight. The horizontal axis represents the number of days from the beginning of the water restriction regimen. (b) Daily water consumption during the restriction regimen. Upon completion of behavioural procedures, animals returned to have *ad lilbitum* access to water (on days 31, 52 and 29 for animals 1, 2 and 3 respectively) and regained their initial weight.

Stress is known to result in analgesia (Butler and Finn, 2009), so longer times of tail-removing in the second test would be indicative of stress. According to this definition, all animals trained showed no evidence of stress as depicted in Fig. 10a. Moreover, animal 1 and 2 presented significantly shorter mean latencies after training than before ($p < 0.03$ for animal 1, and $p < 0.001$ for animal 2). Animal 3 did not show a significant difference between mean latencies before and after training sessions. This distinction is noticeable when inspecting the percentage change in tail-removing latencies before and after training for each session (Fig. 10b): for animals 1 and 2 this value is generally negative whereas in animal 3 is mostly around zero.

Taken together, these results show that the spherical treadmill system here presented it is suitable to train adult rats in operant conditioning and discrimination with no associated acute stress responses.

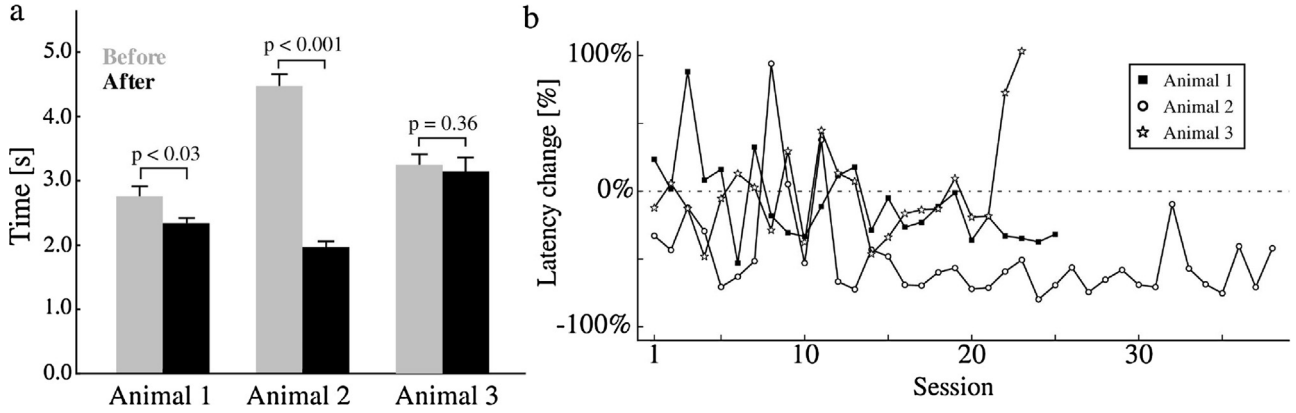

Fig. 10. Tail immersion test. a) Mean latency to withdraw the tail from 55 °C water. Latencies were measured in the homecage before training (gray bars) and at the end of every session (before removing the animal from the apparatus) (black bars). Bars represent mean $\pm$ SEM. b) Percentage change between before and after training tail-removing latencies for each session, normalized to before training latency for individual animals.

4. Discussion

In this work, we present a novel spherical treadmill apparatus for head-fixed animals along with a training protocol that gradually introduces them to the experimental setup, reducing possible stress responses. We successfully trained three adult Long Evans rats in an auditory discrimination task using a Go/No-Go (walking/not-walking) paradigm.

One of the biggest challenges of this system was to design an appropriate pre-training protocol that allowed to place the rats on the treadmill and fixed them to the frame with minimum discomfort and stress. We believe that both, gradually increasing the amount of time that animals were movement restrained and giving water rewards at each new step in the fixation and training protocol were crucial to avoid stress-induced responses.

All animals received one classical conditioning training session in order to create an association with auditory stimulus and to promote operant conditioning. Anticipatory licking behaviours were observed at the end of this session, therefore we always proceeded to the next training phase on the following session. Although we did not systematically record this phase of the training protocol, detection of anticipatory licking behaviour could be easily incorporated to the system through the web-camera placed next to the rat's mouth (Fig. 1f).

We did not observe any evidence of stress induced by the training procedure. This was expected because, in the first place, water restriction is a widely employed means of motivation and animals are well adapted to accommodate this homeostatic challenge (Schwarz et al., 2010; Toth and Gardiner, 2000). Secondly, although head-fixed and –during some procedures- shortly immobilized, the rats experienced a very small degree of restraint during experimental procedures (not comparable with the restraint used as a stressing method (Buynitsky and Mostofsky, 2009)). They were able to move all their limbs and, furthermore, they were able to perform behaviours of their natural repertoire like adopting a normal walking position or head grooming. Thirdly, we did not observe any of the clinical signs or behaviours related to a stress response (National Research Council (US), 2008) such as freezing, agitation, piloerection, teeth chattering, nor the presence chromodacryorrhea (porphyrin-filled tears). Moreover, we observed that animals were keen to participate in the training on the sphere, which is an enriched environment compared to their homecage. We also observed the "eye-boggling" behaviour while fastening the animals to the frame. This occurs when the animal is bruxing because the muscles that move the lower jaw cause a rapid vibration of the eyeball in and out of the socket, which is reported to be indicative of contentment and relaxation (Hanson and Berdoy, 2010).

The tail immersion test was aimed to measure the acute effect of training procedures by evaluating the tail-removing response immediately before and after the training sessions, from the classical conditioning session to the last discrimination session. Therefore, rats were already habituated to being handled and head-fixed. Since repeated exposure can lead to a process of adaptation, it would be reasonable to think of a chronic stress response. Chronic stress has been reported to produce hyperalgesia (Gamaro et al., 1998; Gameiro et al., 2006). We did not expressly evaluate this effect, but comparing the tail immersion test results between the first four sessions and the last four sessions (out of 27 ± 5 sessions, mean $\pm$ SD) revealed that final reaction times are either no different or significantly larger than the initial ones (Mann-Withney or Wilcoxon rank sum test, $p < 0.05$ in animal 1 and 2 and $p = 0.16$ in animal 3), contrary to what should be expected in chronically stressed animals.

As previously stated, spherical treadmills combined with head-fixation facilitate many recordings techniques in behavioural training protocols that require a considerable degree of movement. Considering the several advantages of using rat models previously mentioned, we addressed the need of a spherical treadmill suitable for adult rats. Previous works have used standard optical computer mice for movement tracking and pressurized air to lift the ball, which is an adaptation from studies made in insects (Dahmen, 1980). We evaluated the use of optic mice to perform movement detection but, due to irregularities in the sphere surface, we found difficulties to accurately record movement. We also noted that for standard expanded polystyrene foam balls, the bigger the size, the more irregular its surface. Using a video camera avoids problems caused by those irregularities allowing further possibilities of video processing for movement tracking. For instance, a virtual reality maze could be implemented by including flat screens and movable walls (Keller et al., 2012; Ayaz et al., 2013; Sofroniew et al., 2014). The use of three bearings is a simpler solution than air lifting, allowing to support heavier animal models and to adjust the sphere position for each individual. The apparatus here presented can easily accommodate different size of animals, designing in each case a suitable plastic adapter and head-fixation device. Although slight vibrations could appear when the animal is walking, they could be eliminated or further attenuated by increasing the weight of the base and/or the columns of the frame.

The spherical treadmill system here presented combines the advantages of head-fixation with the possibility of allowing adult animals a wide repertory of motor responses. The experimental apparatus can be easily adapted to other training tasks, different kind of stimuli, other animal species and many recording techniques.

Ethics approval and consent to participate

All experimental procedures were approved by the ethics committee of the IBYME–CONICET (CE 057-2/2012), and were conducted according to the Guide for Care and Use of Laboratory Animals (National Research Council (US), 2011).

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

BSZ and SEL conceived and designed the study. AIMC and AB built the sphere and developed the software. AMMMF and AB performed the experiments. AIMC and AB collected and processed data logs from the training software. AMMMF, AB, CJM and SEL analyzed and interpreted the data. AMMMF and AB wrote the first version of the manuscript. All authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jneumeth.2017.12.018.

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