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TITLE PAGE

INTRACRANIAL SELF-STIMULATION ALSO FACILITATES LEARNING IN A VISUAL DISCRIMINATION TASK IN THE MORRIS WATER MAZE IN RATS.

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Running title: *Post-training ICSS improves visual discrimination*

Research Highlights

- Self-stimulation (ICSS) facilitates the learning of a visual discrimination task
- A direct, instead of a trial and error strategy is preferred by ICSS animals
- *Number of errors* is a more sensitive measure than *latency* in visual discrimination
- A strengthened implicit memory caused by ICSS, challenges reversal learning

ABSTRACT

Intracranial self-Stimulation (ICSS) of the medial forebrain bundle is a treatment capable of consistently facilitating acquisition of learning and memory in a wide array of experimental paradigms in rats. However, the evidence supporting this effect on implicit memory comes mainly from classical conditioning and avoidance tasks. The present work aims to determine whether ICSS would also improve the performance of rats in another type of implicit task such as cued simultaneous visual discrimination in the Morris Water Maze. The ICSS treatment was administered immediately after each of the five acquisition sessions and its effects on retention and reversal were evaluated 72h later. Results showed that ICSS subjects committed fewer errors than Sham subjects and adopted more accurate trajectories during the acquisition of the task. This improvement was maintained until the probe test at 72 h. However, ICSS animals experienced more difficulties than the Sham group during the reversal of the same learning, reflecting an impairment in cognitive flexibility. We conclude that post-training ICSS could also be an effective treatment for improving implicit visual discrimination learning and memory.

Keywords:
Intracranial self-stimulation
Memory enhancement
Simultaneous visual discrimination
Morris Water Maze
Medial Forebrain Bundle
Deep brain stimulation

1. INTRODUCTION

The electrical activation of the medial forebrain bundle (MFB) via Intracranial self-stimulation (ICSS) has been confirmed in our and other laboratories as a treatment capable of consistently facilitating the acquisition and retention in a wide array of experimental paradigms, for both implicit [1], [2], [3], [4] and explicit memory [5],[6], in rats. Several mechanisms of action have been proposed to explain these facilitating effects of ICSS on learning and memory. Stimulation of the MFB has been linked to activation of general arousal systems [7], [8] due to activity of dopaminergic, noradrenergic and serotoninergic ascendant fibers [9], [10]. Learning and memory facilitation has also been linked to structural plasticity induced by ICSS [11]. Recent work undertaken in our laboratory has shown an increase in the density of dendrite spines in the CA1 neurons of the hippocampus in rats that received ICSS after training in a spatial task [6]. These morphological modifications could be related to changes in the expression of several plasticity-related genes caused by the post-training ICSS treatment, with increased levels of Nurr1, c-Fos and Arc protein consistently being found in hippocampus, amygdala, dorsal striatum, lateral hypothalamus or retrosplenial cortex [12], [13], [14], [15].

While most evidence supporting the facilitating effect of the post-training ICSS on explicit memory comes mainly from spatial learning tasks in T-mazes and the Morris Water Maze (MWM), the type of implicit memory that has been subjected to ICSS treatment effects is an amygdala-dependent emotional memory. Thus, the most commonly used tasks have been aversive classical conditioning and avoidance learning. While some pioneering studies have looked into the effects of ICSS on other types of implicit tasks, such as appetitive classical conditioning [16], there are none that focus on tasks more related to perceptual learning and memory. Perceptual

abilities of recognition and discrimination between stimuli are the foundation of most of the learning processes both in animals and humans and, therefore, if ICSS were able to facilitate visual discrimination conditioning and memory it would extend the range of cognitive processes – involving stimuli perception – that are improved by ICSS or the stimulation of reward pathways. Furthermore, since a deficit in implicit learning and memory related to visual discrimination are observed in both Parkinson's disease [17] and the later stages of Alzheimer's disease [18], the possibility of positively affecting this type of memory could also be interesting in the field of neurodegenerative diseases.

In order to study the possible effect of post-training ICSS on a simultaneous visual discrimination task in the MWM (SVD), we modified the configuration of the MWM based on the model presented by Packard and McGaugh [19] of a two-platform task, in a non-spatial version of the MWM task, in which two visible white rubber balls were painted with black horizontal and vertical stripes and used as cues attached to the escape/non-escape platforms. As ICSS treatment demonstrates a higher effectiveness on high difficulty conditions [20], [21], [22] a SVD task would present the appropriate setup, given that the task involves the need to identify and compare two similar stimuli in order to solve it. Moreover, this task in the MWM does not require caloric restriction in order for the animal to learn to find the platform, thus reducing the possible interference of the motivational states on learning [23]. This task is considered to be a non-declarative memory task [24], which also requires the animal to establish an association between a specific stimulus and the location of the platform, generating an instrumental escape response; this associative nature would also involve the use of relatively inflexible memory processes [25] which could mean that reversing or changing a well-consolidated memory would be

extremely challenging. This suggests that, should the acquisition of the SVD task be facilitated by the ICSS treatment, the retention of the memory will be stronger while the reversal learning will be challenged.

2. MATERIALS AND METHODS

2.1. Subjects

A total of Forty-two Wistar male rats with mean age 90.35 days (SD=2.20), and a mean weight of 390.57 g (SD=20.83) from our laboratory's breeding stock were used. Three days before the stereotaxic procedure they were isolated and kept in individual cages (50x22x14-cm, plastic bottomed and sawdust-bedded). The animals were kept under conditions of controlled temperature and humidity, and subjected to an artificial 12-hour light/dark cycle (light on at 08:00). The experimental work was carried out during the first half of the light cycle. All subjects were in an ad libitum regime of food and water. All procedures were carried out in compliance with the European Community Council directives for care and use of laboratory animals and were approved by the institutional animal care committee.

2.2. Surgery

Previous to the surgery, two sessions of handling took place in order to diminish emotional reactivity of the animals towards experimental manipulation. Under general anesthesia (150 mg/kg Imalgène® ketamine chlorhydrate (Merial, Lyon, France) and 0.08 mg/kg Rompun® xylazine (Bayer, Barcelona, Spain); i.p.), all rats were chronically implanted with a monopolar stainless steel electrode (150 µm in diameter) aimed at the right lateral hypothalamus (LH) into the fibers of the MFB, according to coordinates from the stereotaxic atlas of Paxinos and Watson [26], anterior: -1.8 mm

from bregma, lateral: 2.0 mm (right hemisphere) and ventral: -8.5 mm with the cranium surface as the dorsal reference. In the post-surgery recovery period (7 days), the animals were weighed and handled daily.

2.3. Group designation and ICSS behavior shaping

The rats were randomly distributed into two groups, Sham and ICSS, according to the independent variable "ICSS-treatment". Subjects in the ICSS group were taught to self-stimulate by pressing a lever in a Skinner box (25x20x20cm). Electrical brain stimulation consisted of 0.3 s trains of 50 Hz sinusoidal waves at intensities ranging from 20 to 250 µA. The optimum intensity (OI), defined by the lowest intensity that led to a stable rate of about 250 responses in five minutes, was established.

2.4. Morris Water Maze Apparatus.

The MWM consisted of an elevated circular pool (2 m diameter; 60 cm above the pool floor) filled with water (45 cm height) maintained at 22±2 °C. The pool was in the middle of a semi-dark room and surrounded by black curtains reaching from a false ceiling to the base of the pool forming a circular enclosure 2.4 m in diameter. In an adapted version of the two-platform task of Packard and McGaugh [19], four imperceptible nylon threads hung from the false ceiling at equal distances from one another to provide suspension for the two mobile cues throughout the training. These cues rested in the middle of the virtual quadrant in the tank, 45 cm above the water level, and consisted of identical squares (40 cm^2) with a vertical or horizontal black and white stripes pattern of 1 cm wide stripes, as represented in Figure 1. For the escape task, a clear Plexiglas platform (11 cm diameter) was placed centrally in one of the four equal quadrants in which the tank was virtually divided, with its top 2 cm

below the surface of the water. All swim paths were recorded using a closed-circuit video camera (Smart Video Tracking System, Version 2.5, Panlab) with a wide-angle lens was mounted 1.75 m above the center of the pool inside the false ceiling.

2.5. Behavioral procedure

2.5.1. Acquisition sessions

Seventy-two hours after the ICSS shaping, all subjects were given six daily trials for five days (acquisition sessions). The average intertrial interval (ITI) was 120 s. Starting from one of four different cardinal points (N, E, S and W) in a pseudorandom schedule each water-maze trial consisted of one swim from the edge of the pool to the platform. The correct cue (1) was associated with the escape platform (*escape area*), while the incorrect cue (2) was associated with the area of no escape (*area of error*). When a rat failed to find the platform within 90 s, it was manually guided to the platform for 15 s and then removed from the tank. When a rat found the platform it was left on it for 15 s and then removed from the tank. The position of the two cues was manipulated so that every ten trials the correct cue was closer, farther or at the same distance than the incorrect cue in relation to the starting point. Thus, the correct cue changed quadrants every three trials (½ of each session), while the incorrect cue changed position after each trial among the remaining quadrants (see Figure S1 in the Supplementary Material).

2.5.2. ICSS treatment

Immediately after each SVD acquisition session, the ICSS rats were placed in the self-stimulation box and received the ICSS treatment, consisting of 2500 trains of stimulation at the OI established during the shaping phase for each rat. Rats in the

Sham group were instead placed in the self-stimulation box for 45 min after each training session without receiving any stimulation.

2.5.3. Probe test and reversal

The probe test took place seventy-two hours after the last acquisition session and consisted of removing the platform and placing the animal in the pool from the East (E) starting position.

Immediately after the 60 s, the reversal trials were initiated. The platform was placed in the quadrant associated to the incorrect cue and the animal was directed to mount the platform for 15 s and then removed. After 120 s ITI three reversal trials took place, which consisted of the exchange of the cues' associations. Animals were again placed in the tank from the three remaining starting points (N, W, S), and the cues changed quadrants anticlockwise for each trial, which had duration of 90 s and an ITI of 120 s.

2.6. Histology

The animals were transcardially perfused with 4% paraformaldehyde in phosphate buffer 0.1 M (PB; pH=7.4). The brains were removed and post fixed overnight in the same solution. They were then placed in a 30% sucrose solution before being cut into 40 µm sections on a freezing stage microtome (Cryocut 1800 with microtome 2020, Jung). The tissue was stained with Cresyl Violet and examined for electrode tip placement under a microscope for histological determination of the electrode location.

2.7. Statistical analysis

All statistical analysis was carried out with SPSS statistical package v. 23 (SPSS Inc., Chicago, IL, USA). Analysis was conducted with a 2 × 5 mixed

ANOVA (GROUP × SESSIONS) for the acquisition phase, and independent samples *t*-test analysis for the probe test and reversal (the average score of the three trials was analyzed). The main outcome variables for acquisition and reversal in the SVD were: 1) *Escape latency*: time (s) needed to find and climb onto the platform (the maximum value was 90 s), and 2) *Number of errors*: number of contacts with area associated to incorrect cue (no escape). In the probe test *Number of target crossings*, *Percentage of time spent in the target quadrant*, *Number of errors*, *Percentage of time spent in the error quadrant* were compared between groups. In addition, a one-sample *t*-test against a constant (50) was used for each group to determine whether the *Percentage of time spent in the target quadrant* was different from chance level (50%). Moreover, the control variables *Percentage of time spent near the walls* (measure of thigmotaxis), *Length* (total distance in cm) and *Speed* were also analysed. When the effect of SESSIONS factor was statistically significant, polynomial contrasts explored the presence of linear and/or quadratic trends in the performance. A Chi-square test for independence was performed to determine the relation between the group and the strategy used. In addition, a regression analysis was performed to examine the relationship between ICSS parameters and SVD performance. The α level for all tests was set at 0.05.

3. RESULTS

A total of five subjects were excluded from the analysis (two subjects lost the electrode in the middle of the treatment, and three did not continue to respond to the ICSS treatment). The final sample consisted of 37 subjects (Sham: n=19, ICSS: n=18). There was no statistical difference between groups in weight change.

3.1. Acquisition sessions

Mean *Escape latencies* are depicted in Figure 2A. The SESSIONS factor was significant [$F_{4,140}$=22.626, P<0.001], but since interaction GROUP × SESSIONS does not reach significance [$F_{4,140}$=2.034, P=0.09], a similar evolution between groups can be assumed. In addition, the GROUP factor was not significant [$F_{1,35}$=0.181, P=0.67]. Furthermore, both groups learnt the task in terms of the decrease of the *Escape latencies*, revealing a significant downward linear function (Polynomial contrast, Sham P<0.001; ICSS P<0.001).

Means of the *Number of errors* made are depicted in Figure 2B. The main effects of GROUP and SESSIONS are significant [$F_{1,35}$=18.024, P<0.001 and $F_{4,140}$=4.072, P=0.004, respectively], but there is no interaction GROUP × SESSIONS [$F_{4,140}$=1.224, P=0.30]. It is important to point out that no differences between groups were found in session 1 (P=0.267), before the ICSS administration, and the analysis of the sessions 2 to 5 confirms the lower *Number of errors* from the ICSS group [$F_{1,35}$=17.64, P<0.001]. Additionally, the decrease in the *Number of errors* in the ICSS group followed a linear evolution (P<0.001), while the Sham group did not (P=0.268). Furthermore, a simple effects analysis found that the differences between groups appeared in the third session (P=0.025), were maintained in the fourth session (P=0.018) and were stronger in the last acquisition session (P<0.001).

No differences were found for GROUP × SESSIONS for control variables: *Percentage of time spent near the walls*, *Length* or *Speed*.

3.2. Probe test

ICSS group had a higher *Number of target crossings* than the Sham group [*Welch's* $F_{1,23.95}$=4.974, P=0.035] (Figure 3A). Moreover, the ICSS group also showed a higher preference for the *target quadrant* [t_{35}=17.848, P<0.001] (Figure

3B), although neither group performed above chance level (Sham: t_{18}=-2.744, P=0.86; ICSS: t_{17}=-1.19, P=0.45).

Concerning the *Number of Errors* (Figure 3C), the ICSS group committed fewer errors than the Sham group during the first 30 seconds of the probe test [*Welch's* $F_{1,30.572}$=6.687, P=0.015]. Finally, means of *Percentage of time spent in the error quadrant* were not statistically different between groups, although Sham animals showed a tendency for higher preference [t_{35}=3.767, P=0.06].

3.3. Reversal trial

The ICSS group had significantly higher *Escape latencies* than the Sham group [t_{35}=4.532, P=0.04] (Figure 4A). Moreover, the ICSS group made significantly more *errors* than the Sham group [*Welch's* $F_{1,24.615}$=5.113, P=0.03] (Figure 4B).

No differences were found between groups for control variables (*Percentage of time spent near the walls*, *Length* or *Speed*) either in the probe test or in the reversal session.

3.4. Swimming trajectories

The qualitative analysis of swimming trajectories revealed that rats followed two defined strategies. While some animals performed the task in a "direct" manner (Figure 5A), others appeared to have applied a "trial and error" strategy (Figures 5B and 5C), which is exemplified by a frequent visit to the incorrect cue's associated area (error area) before choosing to approach the correct one. A chi-square test confirmed that the "trial and error" strategy was preferred significantly by the Sham group, while the ICSS-treated animals adopted a "direct" strategy ($\chi^2_{1,37}$ = 10.078, P = 0.006). A contingency table (Figure 5D) displays the frequency and percentages for each group and strategy.

At the same time, some animals that appeared to have chosen the correct cue failed to mount the platform due to their trajectory missing the target by a few millimeters. Figure 6 shows two examples (Figures 6A and 6B) of this behavior.

3.5. Histology

Evaluation of the location of the electrode tip under the microscope revealed that they were all between AP -1.80 mm and -3.14 mm from Bregma according to the stereotaxic atlas [26]. Additionally, a regression analysis showed that there is no relationship between the histological location of the electrode tip and the ICSS parameters or the performance in the SVD sessions.

4. Discussion

The present results indicate that post-training ICSS treatment facilitates the acquisition and retention of a visual discrimination task in the MWM. Although the escape latencies were equal for both groups, the ICSS subjects committed fewer errors than the Sham animals during the acquisition and the probe test. During the probe test, which assessed the memory retention after 72 h, the ICSS group spent more time in the target quadrant and achieved more target crossings than the Sham subjects, indicative of a higher level of memory in the ICSS subjects. Present results are in agreement with those obtained in our laboratory regarding ICSS facilitation of the acquisition and retention of implicit avoidance memory tasks [3], [4].

Furthermore, during the reversal phase ICSS treatment caused higher escape latencies and number of errors than the non-treated animals. Our results in the reversal test are also consistent with Hirsh's [25] consideration of a visual discrimination task as an inflexible and associative memory process and with previous work on the difficulty of achieving the reversal of a well-consolidated visual

discrimination task [27]. One aspect we believe to be of paramount importance is the fact that throughout the three phases of the experimental design, results consistently showed that the mean number of errors of each group was indicative of the facilitative effect of the ICSS treatment upon the visual discrimination task. Altogether, quantitative and qualitative results revealed that the ICSS rats applied a more efficient and direct strategy than Sham animals to learn the task and that ICSS treatment promotes a stronger and better-consolidated perceptual memory. As far as we know, this is the first time that the facilitation of the acquisition and retention of a visual discrimination task by post-training ICSS has been demonstrated.

On the other hand, and contrary to what we had expected, there were no differences between groups for the escape latencies in the acquisition sessions. This may seem contradictory with the idea of ICSS facilitation of the task, but these findings are similar to those of Packard and McGaugh [19], where escape latencies to mount the platform did not completely reflect the behavioral deficit observed in rats with lesion of the caudate nucleus. There are several possible explanations for this lack of differences. Looking at the trajectories of ICSS and Sham animals, some of them seem to experience difficulty finding the exact location of the escape platform as it is illustrated in Figures 6A and 6B, probably because the cue was too far from the platform. Therefore, even if rats accurately identify the correct cue, failing to find and mount the platform could cause the animal to retreat from the area in search of the alternative cue. This translates into valuable lost time, which would directly affect the escape latency values in the overall results. Thus, reducing the distance between the cues and the areas of *Escape* and *Error* would help avoid this type of error. In fact, a complementary experiment performed in our laboratory confirmed this hypothesis. As it is shown in Figure 7, when the distance between the edge of the tank and the cue

was reduced from 35cm to 25cm, the ICSS group solved the task showing significantly lower latencies than the Sham animals in the last acquisition session. Other studies performed in the MWM where the visual discrimination cues were separated from the platforms have prevented this effect by concomitantly using contextual cues, providing spatial information that facilitate the task [28].

Furthermore, the different strategies followed by the animals and the lower number of errors committed by ICSS group, suggests that ICSS treatment promotes a discriminatory perceptive learning process instead of a "trial and error" strategy. As in the aforementioned study by Packard and McGaugh [19], our present results also show that the *number of errors* could be a more sensitive measure than latencies to detect the degree of facilitation in the acquisition of visual discrimination tasks.

Moreover, this improvement could be related to increases in some excitatory neurotransmitters levels, such as dopamine (DA), acetylcholine (ACh) or glutamate (GLU), in the hippocampus and cortical regions [10],[29]. In that sense, it has been observed that the blockade of DA and ACh [30] or GLU [31] results in a higher number of errors to criterion in this kind of tasks, while an ACh blockade also impairs the strategy selection [32]. Additionally, lesions of the cholinergic nucleus basalis magnocellular have been shown to increase perseverative errors in a simple-stimulus response visual perceptual task [33], which could be linked to the modulatory effects of ACh on the attentional aspects of the task [34]. Furthermore, evidence from human studies shows that an enhanced efficacy of the cholinergic system's function facilitates the consolidation processes in a visual discrimination task [35] and promotes long-lasting improvements in perceptual learning [36]. All in all, an increased function of some excitatory neurotransmission systems activated by ICSS in memory-related regions, such as the hippocampus and cortex, could explain the lower

number of errors and the accurate trajectory portrayed by ICSS-treated subjects in the present experiment. The specific mechanisms through which the ICSS could promote the activity of these memory-modulatory neurotransmitters are yet to be defined. However, descendent fibers from the MFB could explain such an increase, since ICSS functionally activates monoaminergic and cholinergic brainstem regions, such as the locus coeruleus, the ventral tegmental area and the pedunculopontine area [37].

In summary, the main goal of the present research was to determine whether a visual discrimination task, in which the perceptive component is critical, could be facilitated by post-training administration of ICSS in the MFB. ICSS led to the animals not only committing fewer errors, but also to them using a more accurate strategy to solve the task. Moreover, cognitive flexibility assessed by a reversal test was compromised by a strengthened memory consolidation. An implication of some excitatory ICSS-related transmitters is suggested. As far as we know, this is the first time that ICSS has been able to facilitate this type of implicit-perceptual learning and its retention. These findings, together with previous research in our laboratory, contribute to the establishment of post-training ICSS in the MFB as a generic treatment useful for facilitating a wide range of learning tasks and procedures.

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TABLE AND FIGURE LEGENDS

Figure 1. **Representation of one of the configurations for MWM in the simultaneous visual discrimination task**. *Escape area* is associated to cue 1 and illustrated with a clear platform. *Area of error* represents the "no escape" associated to cue 2 and is signaled with a black X.

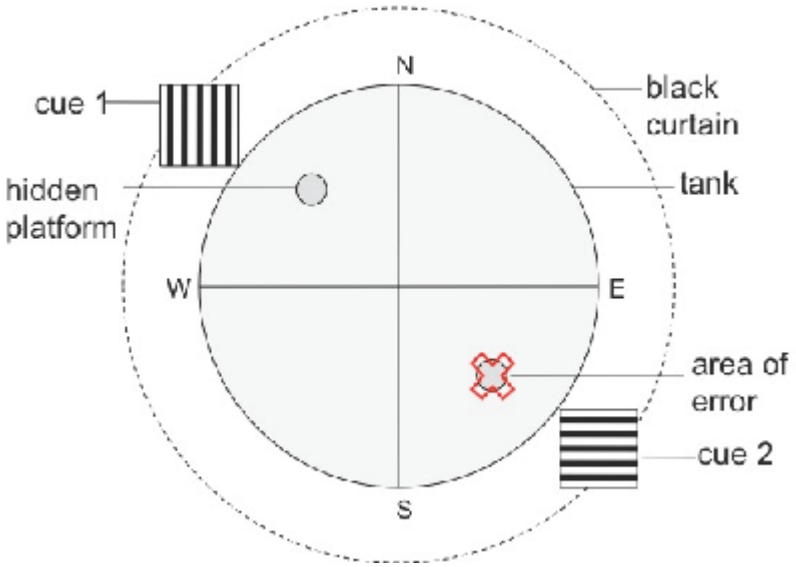


Figure 2. **Facilitative effects of ICSS on acquisition**. (A) Mean *Escape latencies* (±SE) for the five acquisition sessions. Arrow shows start of ICSS treatment; (B) Mean *Number of errors* (±SE) committed during the acquisition sessions by each group. Group factor significance is depicted with a horizontal bracket. * $P < 0.05$; ** $P < 0.001$

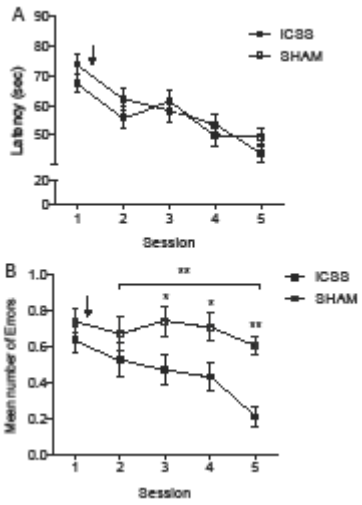


Figure 3. **Facilitative effects of ICSS on the probe test**. (A) Mean *Target crossings* (±SE); (B) Mean *Percentage of time spent in target quadrant* (±SE). The dotted line represents chance level; (C) Mean *Number of Errors* (±SE). * P < 0.05; ** P < 0.001

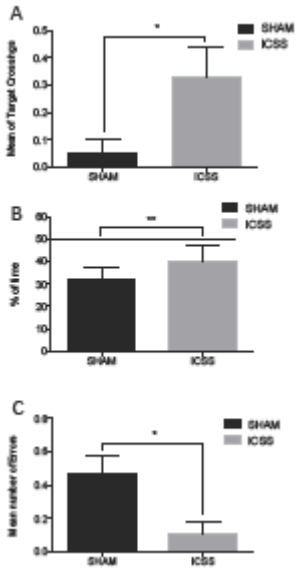


Figure 4. **Impairing effects of ICSS on reversal**. (A) Mean *Escape latencies* (±SE); (B) Mean *Number of errors* (±SE). An example of the trajectory of Sham and ICSS animals is presented above corresponding bars. * P < 0.05

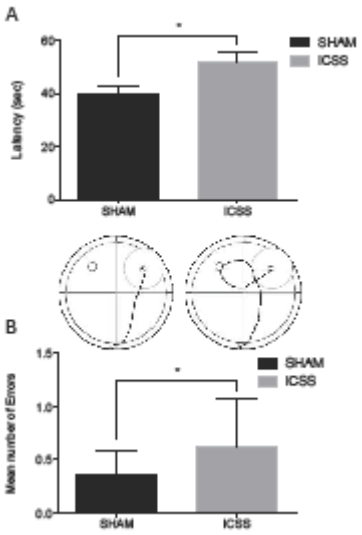
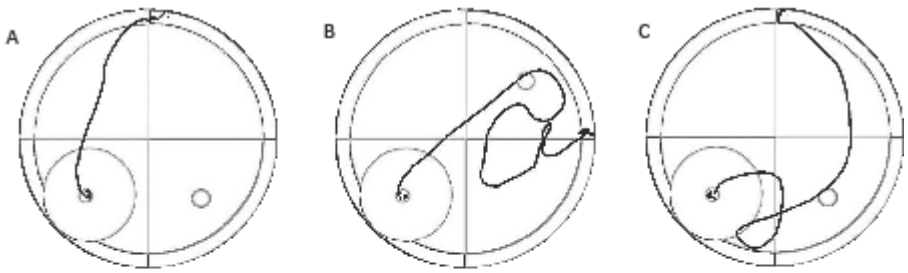


Figure 5. **Swimming trajectories in the acquisition**. Images of direct vs trial and error swimming trajectories that the rats used to solve the SVD task. In the lower-left quadrant, the inner concentric circle corresponds to the platform; outer concentric circle comprises the target zone; and in the lower-right quadrant, the circle represents the error area. (A) ICCS animal; (B) and (C) Sham animals; (D) Percentage of animals preferring "trial and error" or "direct" strategies, by group. N/a: non-applicable. Frequency is shown in brackets after the percentage.



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		STRATEGY			TOTAL
		Trial and Error	Direct	N/a	
GROUP	Sham	**68.4%** (13)	10.5% (2)	21.1% (4)	100% (19)
	ICSS	22.2% (4)	**55.6%** (10)	22.2% (4)	100% (18)

Figure 6. **Swimming trajectories of rats missing the platform**. The trajectory of some animals that approached the platform but failed to located it. This

behavior could explain the lack of differences in latency between groups. Both figures belong to ICSS subjects.

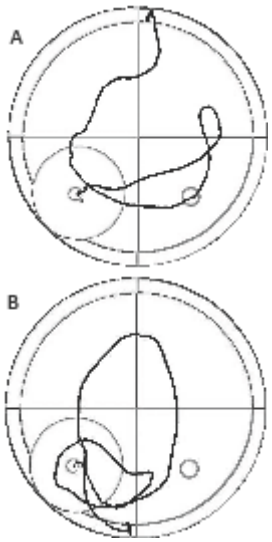

Figure 7. **Significant effects of ICSS on acquisition latencies when cues were closer to the MWM**. This figure depicts the Mean *Escape latencies* (±SE) for the acquisitions sessions of a complementary experiment where distance between cues and *Area of escape* and *Area of error* was reduced. Arrow shows start of treatment sessions. * $P < 0.05$